EFFECTS OF *TREPONEMA DENTICOLA* ON AN ORAL EPITHELIAL CELL MODEL

by

WAI KEUNG LEUNG

B.D.S., The University of Hong Kong, 1987

M.D.S., The University of Hong Kong, 1990

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Oral Biological and Medical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

0-612-34575-0

Canada

ABSTRACT

Periodontal diseases are destructive inflammatory conditions of the tissues supporting the tooth. The inflammatory responses are induced by mixed bacterial infection leading to eventual destruction of the periodontal tissue. Oral spirochetes of the genus *Treponema* are one of the bacterial groups that are closely related to these diseases. This study investigated the interaction of a periodontal pathogen, *T. denticola*, its whole cells and outer membrane (OM) proteins with cultured eukaryotic cells. Cultured porcine periodontal ligament epithelial cells (PLE, a junctional epithelium model), and various cell lines were challenged with *T. denticola* ATCC 35405 whole cells or with the major OM protein (Msp) complex, gel purified Msp, and chymotrypsin-like proteinase (CTLP). Msp complex was isolated from whole *T. denticola* by detergent extraction, autoproteolysis, ultrafiltration and detergent removal. The location of the Msp and CTLP on *T. denticola* OM was studied. Hexagonal array was observable on negative-stained *T. denticola* OM under electron microscope where Msp probably constituted the main building block of the regular pattern together with the previously reported OM located CTLP. Native *T. denticola* Msp is an oligomer containing 53 kDa protein subunits which had porin activity in an artificial lipid bilayer. CTLP was previously characterized. Microscopic and modified ELISA assays showed that spirochetes adhered to PLE cells whether prefixed with glutaraldhyde (fixed PLE-FPLE) or not. Pre-treatment of *T. denticola* with the protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone or phenylmethanesulfonyl fluoride blocked this binding and chymotrypsin-like activity. *T. denticola* intact or sonicated whole cells were cytotoxic to PLE cells and to several cell lines. *T. denticola* cytotoxicity was measured by microculture tetrazolium assay or by quantifying lactate dehydrogenase release upon cell lysis (LDH assay). *T. denticola* induced PLE cellular changes were observed by light microscopy,

image analysis and electron microscopy. These included: transient cell size increase of non-detached PLE cells leading to confluency maintenance, membrane disruption, vacuolation, loss of cell contacts, loss of cell size control, cytoskeletal rearrangement, and apoptosis of PLE cells. Msp complex, Msp and CTLP could attach to PLE/FPLE and were found to be cytotoxic to PLE cells. Adherence of Msp was partially blocked by specific antibodies. Adherence of CTLP was partially blocked by serine protease inhibitors and was further inhibited by specific antibodies. Cytotoxicity of Msp and CTLP was inhibited by the same treatments that inhibited adherence. Standard patch clamp recording methods were used to study the effects of Msp complex on HeLa cells. Msp bound to several HeLa cell proteins, including a 65 kDa surface protein and a 96 kDa cytoplasmic protein. The Msp complex depolarized and increased the conductance of the HeLa cell membrane in a manner that was not strongly selective for Na^+, K^+, Ca^{2+}, and Cl^- ions. Cell-attached patches of HeLa cell membrane exposed to Msp complex exhibited short-lived channels with a slope conductance of 0.4 nS in physiologically normal saline. These studies show that *T. denticola* ATCC 35405 and its major OM protein elements, namely Msp and CTLP, could attach to a cell model that resembles junctional epithelium and could produce cytopathic and cytotoxic effects. It was hypothesized that the strong proteolytic activities of CTLP and pore-forming activity of the Msp complex were responsible for the cytopathic and cytotoxic effects of the putative periodontopathogen *T. denticola* on PLE cells.

TABLE OF CONTENTS

Page

Abstract ii

Table of Contents iv

List of Tables viii

List of Figures x

Abbreviations, Nomenclature and Symbols xiii

Acknowledgements xviii

CHAPTER I Overview and Summary 1

1.1. Introduction 1
1.2. Human periodontal disease 1
1.2.1. Brief overview of periodontal disease 1
1.2.2. Periodontium in health 5
1.2.2.1. Human periodontium – a brief overview of the hard-soft tissue junction 5
1.2.2.2. A model system for junctional epithelium 9
1.3. *Treponema denticola* as one of the periodontopathogens 10
1.3.1. *Treponema denticola* 11
1.3.1.1. Physical appearance and habitat 11
1.3.1.2. Taxonomic history and classification 12
1.3.2. Association with periodontitis 13
1.3.3. Elimination of *T. denticola* with disease resolution 14
1.3.4. Host response to *T. denticola* 15
1.3.5. Animal model of *T. denticola* periodontal pathogenicity 16
1.3.6. Periodontopathogenic potential 17
1.3.6.1. Mobility 17
1.3.6.2. Adhesion 19
1.3.6.3. Invasion of host cell/tissue 25
1.3.6.4. Damage to host cell/tissue 25
1.3.6.5. Modulation of host defense 26
1.3.6.6. Synergism with other periodontopathogens 29
1.3.7. Mechanisms of pathogenicity 31
1.3.8. Summary 35
1.4. Statement of the problem 36
1.4.1. Hypothesis 36
1.4.2. Aim 36
1.5. Addendum 36

CHAPTER II Materials and Methods..... 46

2.1. Bacterial strains and culture condition..... 46
2.2. Cell culture/cell lines and culture conditions..... 47
2.3. Cytopathic assays..... 48
2.3.1. Morphometric study..... 48
2.3.2. Vacuolation assay..... 49
2.3.3. Others assays..... 50
2.4. Adhesion assays..... 51
2.4.1. Immunofluorescence microscopy..... 51
2.4.2. ELISA..... 52
2.5. Cytotoxicity assays..... 53
2.5.1. MTT..... 53
2.5.2. LDH..... 54
2.5.3. Trypan blue dye exclusion..... 54
2.5.4. Detachment assay..... 55
2.5.5. Thymidine uptake..... 56
2.5.6. Apoptosis assays..... 57
2.5.6.1. TUNEL..... 57
2.5.6.2. TEM..... 57
2.6. Screening for protease activities..... 58
2.6.1. Zymography..... 58
2.6.2. SAAPNA assay..... 59
2.7. Ultrastructural assays..... 59
2.7.1. Scanning electron microscopy..... 59
2.7.2. Electron microscopy..... 59
2.7.2.1. Negative staining..... 59
2.7.2.2. Ultra-thin sectioning..... 60
2.7.2.3. On-grid immunogold labeling..... 60
2.8. Preparation of *T. denticola* proteins..... 62
2.8.1. Detergent extraction..... 62
2.8.2. Preparation of Msp complex..... 63
2.9. Purification of native Msp and Msp monomer..... 64
2.9.1. FPLC..... 64
2.9.2. Preparative electrophoresis..... 65
2.10. Electrophoresis..... 66
2.10.1. SDS-PAGE..... 66
2.10.2. Isoelectric focusing..... 66
2.11. Preparation of antisera..... 66
2.12. Western blotting..... 67
2.13. Immunoprecipitation of HeLa cell proteins with the Msp complex..... 68
2.14. Autoradiography..... 69
2.15. Black lipid bilayer analysis..... 69
2.16. Patch clamp assays..... 71
2.17. Statistics..... 72

CHAPTER III Results..... 75

3.1. Attachment of *T. denticola* to eukaryotic cells..... 75
3.1.1. Attachment of *T. denticola* to PLE cells 75
3.1.2. Attachment of *T. denticola* to other eukaryotic cells... 76
3.1.3. Attachment of *T. denticola* to FPLE cells..... 76
3.1.4. Effects of pre-treatment of *T. denticola* on PLE attachment to cells..... 77
3.1.4.1. Physical pre-treatment..... 77
3.1.4.2. Chemical pre-treatment..... 78
3.1.4.3. Antibody pre-treatment..... 78
3.2. Cytopathic effects of *T. denticola* on PLE..... 79
3.2.1. Morphometric changes..... 79
3.2.2. Vacuolation..... 80
3.2.3. Blebbing..... 80
3.2.4. Cytoskeletal rearrangement..... 81
3.3. Cytotoxic effects of *T. denticola* on eukaryotic cells..... 81
3.3.1. Cytotoxic effects of *T. denticola* on PLE cells..... 81
3.3.1.1. PLE cells remaining on tissue culture plates. 81
3.3.1.2. PLE cells detached from the tissue culture plates..... 82
3.3.2. Cytotoxic effects of *T. denticola* on other eukaryotic cells..... 82
3.3.3. Effects of pre-treatment of *T. denticola* on cytotoxicity to PLE cells..... 83
3.3.3.1. Physical pre-treatment..... 83
3.3.3.2. Chemical pre-treatment..... 83
3.3.3.3. Antibody pre-treatment..... 83
3.4. Cytopathic and cytotoxic effects of *T. denticola* "putative virulence factors"..... 84
3.4.1. Purification and characterization of the "putative virulence factors"..... 84
3.4.1.1. Characterization of crude detergent extracts. 84
3.4.1.2. Purification of the 53 kDa protein or Msp... 86
3.4.2. Porin activity of purified Msp..... 87
3.4.3. Attachment of Msp complex, Msp and CTLP to PLE/FPLE cells..... 89
3.4.4. Cytotoxic effects of Msp complex, Msp and CTLP on PLE cells..... 89
3.4.5. Cytotoxic effects of Msp complex on eukaryotic Cells..... 90
3.4.6. Characterization of Msp complex induced cytotoxicity by patch clamping..... 91

CHAPTER IV Discussion..... 146

4.1. Methodology and limitations..... 146
4.2. Current results in comparison to information available in the literature and their implications..... 151
4.2.1. Attachment of *T. denticola* to eukaryotic cells..... 151
4.2.2. Cytotoxicity of *T. denticola* to eukaryotic cells..... 152
4.2.3. Major outer membrane elements of *T. denticola*..... 154
4.2.4. Biological activities of the major outer sheath elements of *T. denticola*..... 157
4.3. A model hypothesizing the way *T. denticola* induces cytotoxic and cytopathic effects on PLE cells..... 163

CHAPTER V Conclusions..... 207

Bibliography..... 210

LIST OF TABLES

Table		Page
1.1	Components of extracellular matrix in periodontium.....	38
1.2	Subgingival species associated with health, gingivitis and periodontitis.....	39
2.1	Cell lines or cell explants used.....	73
2.2	Compositions of saline solutions used in the patch clamp experiments.....	74
3.1	The number of *T. denticola* binding to individual PLE cells in sparse and dense cultures.....	94
3.2	The number of *T. denticola* binding to individual eukaryotic cells in sparse and dense cultures.....	95
3.3	Langmuir isotherm parameters of *T. denticola* - cell cultures adherence.....	96
3.4	The number of *T. denticola* binding to individual fixed eukaryotic cells.....	97
3.5	Effect of various treatments on binding and the chymotrypsin-like protease activity of *T. denticola*.....	98
3.6	Effect of serum proteins and anti-*T. denticola* antibodies on *T. denticola* binding to PLE cells.....	99
3.7	Inhibition of *T. denticola* – FPLE adherence by specific antibodies.....	100
3.8	Cytopathic effects of *T. denticola* on PLE cells.....	101
3.9	Effect of physical treatment on cytotoxic activity of *T. denticola*.....	102
3.10	Effect of various chemical treatments on cytotoxic activity of *T. denticola*.....	103
3.11	Inhibition of *T. denticola* cytotoxicity by specific antisera.....	104
3.12	Average single channel conductance of *T. denticola* 53 kDa protein.....	105
3.13	Adherence and cytotoxicity of Msp to FPLE and PLE cells respectively.....	106
3.14	Adherence and cytotoxicity of CTLP to FPLE and PLE cells respectively.....	107

Table		Page
4.1	*Treponema denticola* – extracellular matrix attachment studies.....	168
4.2	*Treponema denticola* – eukaryotic cell attachment studies.....	169
4.3	Studies investigating the *in vitro* host cell/tissue damage inducible by *Treponema denticola*.....	171
4.4	Studies investigating *Treponema denticola* modulation of host defense system.....	175
4.5	Studies on *Treponema denticola* interaction with periodontopathogens and other members of subgingival plaque flora.....	183
4.6	Studies investigating virulence or pathogenic factors of *Treponema denticola*...	186
4.7	Studies genetically identifying and characterizing virulence/pathogenic factors of *Treponema denticola*.....	200
4.8	A summary of the interactions that occurred between *T. denticola* and PLE cells with an attempt to relate and hypothesize the *T. denticola* induced cytopathic or cytotoxic effects.....	203
4.9	A summary of postulated interactions that might occur between *T. denticola* and human periodontal tissue *in vivo*.....	204

LIST OF FIGURES

Figure		Page
1.1	Periodontal disease manifestations	40
1.2	The local subgingival host response to microbial challenge	41
1.3	The periodontium in health	42
1.4	Schematic drawing of junctional epithelium	43
1.5	Schematic drawing of a treponeme	44
1.6	Diagrammatic representation of inter-bacterial relationships between *Treponema denticola/Treponema* species with other microbes in dental plaque	45
3.1	*T. denticola* binding to PLE cells	108
32	Time-dependent binding of *T. denticola* to PLE or FPLE cells	109
3.3	Scanning electron microscopy of *T. denticola* adhesion to monolayer of PLE cells	110
3.4	Percentage of PLE cells binding at least one bacterium after challenged by *T. denticola*	111
3.5	Effect of physical pre-treatment on *T. denticola* binding to FPLE cells	112
3.6	Effect of chemical pre-treatment on *T. denticola* binding to FPLE cells	113
3.7	*T. denticola* induced PLE cell morphological changes	114
3.8	PLE cells vacuolation induced by *T. denticola*	115
3.9	*T. denticola* induced blebbing of PLE cells	116
3.10	Confocal optical sections of blebbing and control PLE cells after treatment with *T. denticola*	117
3.11	Actin arrangement of *T. denticola* treated PLE cells	118

Figure		Page
3.12	Viability of *T. denticola* infected PLE cell monolayer.....	119
3.13	PLE cells proliferation after *T. denticola* infection.....	120
3.14	Trypan blue dye exclusion assay and TUNEL staining of PLE cells monolayer infected with *T. denticola*.....	121
3.15	Apoptotic change of PLE cells infected by *T. denticola*.....	122
3.16	Detachment assay of *T. denticola* infected monolayers of cells in culture.....	123
3.17	Morphology of detached PLE cellular materials post *T. denticola* challenge...	124
3.18	Viability of *T. denticola* infected monolayers of PLE, Saos-2, HGF, HeLa and CHO-K1 cell culture.....	125
3.19	SDS-PAGE and isoelectric focusing of *T. denticola* ATCC 35405 protein extracts.....	126
3.20	Effect of heat and proteolytic treatment on *T. denticola* ATCC 35405 protein.....	127
3.21	Immunoblotting of whole cell lysate of *T. denticola*.....	128
3.22	SDS-PAGE of *T. denticola* extract showing the 95 kDa CTLP.....	129
3.23	Electron micrographs of sonicated *T. denticola* ATCC 35405 cells.....	130
3.24	Transmission electron micrograph of outer membrane material released by mild sonication of *T. denticola* OTK.....	131
3.25	Immunogold labeling of *T. denticola* with specific anti-*T. denticola* surface protein antibodies.....	132
3.26	Immunogold labeling of *T. denticola* outer sheath preparation with anti-*T. denticola* 53 kDa protein antibody.....	133
3.27	FPLC purification of Msp from *T. denticola* ATCC 35405.....	134

Figure		Page
3.28	Analysis of single-channel conductance events due to incorporation of the 53 kDa porin into black lipid membranes.....	135
3.29	Histogram of single-channel conductance increases observed for the purified 53 kDa membrane protein.....	136
3.30	Binding of Msp complex and CTLP to PLE cells.....	137
3.31	*T. denticola* Msp complex.....	138
3.32	Reduction of viability of PLE cells due to challenge by Msp complex.....	139
3.33	Reduction of viability of cultured cells due to challenge by Msp complex.....	140
3.34	SDS-PAGE showing HeLa cell proteins immuno-precipitated by anti-Msp IgG after incubation with Msp complex.....	141
3.35	Effects of Msp complex on whole-cell currents in HeLa cell membranes recorded in potassium gluconate saline solutions.....	142
3.36	Effects of Msp complex on the current-voltage (*I-V*) relationship of the HeLa cell membrane.....	143
3.37	Effects of Msp complex on whole-cell currents in HeLa cells bathed in physiologically relevant solutions.....	144
3.38	Current consistent with the gating of a large conductance ion channel recorded from a cell-attachment patch of HeLa cell membrane.....	145
4.1	Bacterial risk assessment model for progressing periodontitis.....	206

ABBREVIATIONS, NOMENCLATURE AND SYMBOLS

2-NAs	*N*-aminoacyl-2-naphthylamines, 2-naphthylamides or nitroanilide, chromogenic substrates
AFPANA	L-Ala-L-Phe-L-Pro-L-Ala-2NA, elastase substrate
AMC	7-amido-4-methylcoumarin
ANOVA	analysis of variance
AP	adult periodontitis
AST	aspartate aminotransferase
ATCase	aspartate carbamoyltransferase, enzyme catalyzes the first committed step of de novo pyrimidine nucleotide biosynthesis
ATCC	American Type Culture Collection; Rockville, Md.
ATNA	*N*-acetyl-L-tyrosine-*p*-nitroanilide
BAEE	*N*-α-benzoyl-L-arginine ethyl ester
BAGFPMNA	*N*-α-benzoyl-L-arginyl-glycyl-L-phenylalanyl-L-proline-4-methoxy-β-naphthylamide
BAMC	benzoyl-DL-arginine-7-amido-4-methylcoumarin, trypsin substrate
BANA	*N*-benzoyl-DL-arginine-2-naphthylamide, trypsin, OPase substrate
BA*p*NA	α-*N*-benzoyl-L-arginine-*p*-nitroanilide hydrochloride, trypsin, OPase substrate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BFNA	*N*-benzoyl-D, L-phenylalaine-β–napthylamide
BHI-K	brain heart infusion broth containing Vitamin K (1 μg/mL)
BLNA	*N*-α-benzoyl-D, L-leucine-β-naphthylamide
BODIPY	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester
BTEE	*N*-benzoyl-L-tyrosine ethyl ester
BSA	bovine serum albumin
BVE	bovine (calf aorta) vascular endothelium explant
BVGAMNA	*N*-benzoyl-L-valyl-glycyl-L-arginine-4-methoxy-β-naphthylamide
Cbz-FAK	benzyloxycarbonyl-phenylalanine-alanine-diazomethyl ketone
Cbz-FCK	benzyloxycarbonyl-phenylalanine-cysteine-diazomethyl ketone
Cbz-FFK	benzyloxycarbonyl-phenylalanine-phenylalanine-diazomethyl ketone
Cbz-FNA	benzyloxycarbonyl-L-phenylalanine-2-naphthylamide, or PHENA, chymotrypsin substrate
Cbz-GAMNA	*N*-carbobenzoxy-glycyl-L-arginine-4-methoxy-β-naphthylamide
Cbz-GGANA	*N*-carbobenzoxy-glycyl-glycyl-L-arginine-β-naphthylamide
Cbz-GPNA	carbobenzoxy-Gly-Pro-*p*-nitroanilide
Cbz-PAGPMNA	*N*-carbobenzoxy-prolyl-L-alanylglycl-L-proline-4-methoxy-β-naphthylamide
Cbz -PNA	benzyloxycarbonyl-L-proline-2-napthylamide, or PRONA
CHAPS	3[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
CHO-K1	Chinese hamster ovary epitheloid line
CHX	chlorhexidine
CL	Chang Liver/human liver epithelium line
Con A	concanavalin A

CTLP	95 kDa chymotrypsin-like protease/proteinase of *T. denticola*, a 72 kDa subunit of which is encoded by the *prtP* gene
DAB	diamino-benzidine
DABCO	1,4-diazabicylo-[2,2]-octane
DEX	dexamethasone
DFP	diisopropyl fluorophosphate
D-Gal	D-galactose
D-GalNAc	*N*-acetyl-D-galactosamine
D-GluNAc	*N*-acetyl-D-glucosamine
Diazo-NDS	7-diazonium-1,3-naphthalene disulfonate, periplasmic enzymes inactivator
dLPP	delipidated lipo-protein fraction from *T. denticola* (particulate form)
D-Man	D-mannose
dmcA	gene encoding a methyl-accepting chemotaxis protein
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
E-64	*N*-*N*-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucylagmatine
ECBA	enriched cysteine blood agar
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride
EDTA	ethylene diamine tetra-acetic acid
EEDQ	1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline, a carboxyl-group reagent
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (β-amimoethyl ether)-*N,N,N',N'*-tetracetic acid
ELISA	enzyme-linked immunosorbant assay
FACITS	fibril-associated collagens with interrupted triple helices
FALGPA	2-furylacryloyl-L-leucylglycyl-L-prolyl-L-alanine
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
flgB, *C*; *fliE*, or *F*	genes encoding flagellar basal body
fliG	genes encoding a flagellar switch component
fliH, or *I*	genes encoding putative flagellar export proteins
fliJ	flagellar related gene with unknown function
FMLP	*N*-formyl methionyl-leucyl-phenylalanine, PMN chemotactic peptide and activator of O_2^- production
FPANA	L-Phe-L-Pro-L-Ala-2NA, elastase substrate
FPLC	fast-protein liquid chromatography
FPLE	glutaraldehyde fixed porcine periodontal ligament epithelium explant
GCF	gingival crevicular fluid
GC-MS	gas chromatography – mass spectrometry
GCT	gingival connective tissue
GGT	γ-glutamyltransferase
GJP	generalized juvenile periodontitis
GNA	*N*-α-L-glutamyl-4-nitroaniline
GPE	guinea pig ear epithelium line
HA	hydroxyapatite
HaCaT	spontaneous human keratinocyte line
Hase	hyaluronate lyase

HBE	human buccal epithelium explant
HBPs	human bioactive peptides
HBSS	Hanks balanced salts solution
HCE	human cornea epithelium explant
HeLa	human cervix adenocarcinoma (epitheloid) line
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HGase	hyaluronglucosaminidase
HGE	human gingival epithelium explant
HGF	human gingival fibroblast explant
HIAC	interaction affinity chromatography
hly	hemolysin or cystalysin gene
HSK	human skin (epidermal) keratinocyte explant
^{3}H-TdR	tritiated thymidine
HVE	human vascular endothelium (umbilical cord vein) explant
IDD	insulin-dependent diabetic
IEF	isoelectric focusing
IEF-PAGE	two-dimensional polyacrylamide gel electrophoresis
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1β	interleukin-1β
IP	inositol phosphate
KDO	2-keto-3-deoxyoctonic acid
KB	human oral epidermoid carcinoma line
KBM	keratinocyte base medium (modified MCDB153)
KSFM	keratinocyte serum free medium
L-929	murine fibroblast line
LDCL	luminol dependent chemiluminescence
LDH	lactate dehydrogenase
LJP	localized juvenile periodontitis
LPS	lipopolysaccharide
MCP	methyl-accepting chemotaxis protein
MDCK	canine kidney epithelium line
MEM	modified Eagle's medium
MMP	matrix metalloproteinase
MNA	L-methionine-*p*-nitroanilide
Msp	major outer sheath (surface) protein
msp	gene encoding a *T. denticola* major surface protein
MTT	3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or the microculture tetrazolium assay
NBT	nitroblue tetrazolium
NGAL	PMN gelatinase-associated lipocalin
NOS	new oral spirochetes medium
NP-40	Nonidet P-40
NPPC	*p*-nitrophenylphosphorylcholine
NSP	Phe-8(4-nitro)-substance P, synthetic substance P substitute for enzymatic assay
Octyl-POE	*n*-octyl-polyoxyethylene
OD	optical density

OM	outer membrane
OPase	endo-acting oligopeptidase, previously named trypsin-like or BANA-peptidase
PA	1,10-phenanthroline
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline, pH 7.3
PBS+	0.5 mM $CaCl_2$-0.5 mM $MgCl_2$-PBS, pH 7.3
*p*CMBA	*p*-chloromercuribenzoic acid
PDL	periodontal ligament
PG	prostaglandin
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PHAMC	glutaryl-phenylalanine-7-amido-4-methylcoumarin
*p*HMBA	*p*-hydroxymercuribenzoic acid
phoN	gene encoding a *T. denticola* neutral phosphatase
pI	isoelectric point
PIPase	proline iminopeptidase
PLC	phospholipase C or phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3.
PLE	porcine periodontal ligament epithelium explant
*p*MBS	*p*-mercuribenzensulfonic acid
PMN	polymorphonuclear neutrophils
PMSF	phenylmethanesulfonyl fluoride
PNA	L-proline-*p*-nitroanilide
pNPP	*p*-nitrophenyl phosphate
POPase	prolyl oligopeptidase
PRAMC	L-proline-7-amido-4-methylcoumarin hydrogen bromide, extracellular proline peptidase substrate
Pro-IL-1β	interleukin-1β precursor
prtA	incompletely characterized gene of *T. denticola* encoding a 36 kDa PZ-PLGPA peptidase and a 67 kDa protease
prtB	gene encoding a *T. denticola* 30.4 kDa protein with chymotrypsin-like activity
prtP	gene encoding a *T. denticola* 72 kDa protein subunit of the CTLP
PSPase	proline-specific peptidase
PWM	pokeweed mitogen
PY	basal medium/peptone yeast extract
Pyr-NA	*N*-α-L-pyrrolidonyl-2NA
PZ-PLGPA	phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine
RBC	red blood cell
RGDS	arginine-glycine-aspartate-serine (arg-gly-asp-ser) peptide or peptides with this amino acid sequence; intergrin adhering sequence
RIA	radioimmunoassay
RITC	rhodamine isothiocyanate
ROS 17/2.8	rat osteogenic sarcoma line
RPE	rat palatal epithelium line
RPP	rapidly progressive periodontitis
SAAANA	succinyl-alanyl-alanyl-alanine-*p*-nitroanilide, elastase substrate

SAAPLNA	succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucine-*p*-nitroanilide
SAAPNA	*N*-succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide hydrochloride, SAAPFNA or SAAPPNA, chymotrypsin substrate
SAAPVNA	*N*-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide hydrochloride, chymotrypsin substrate
SAAVNA	succinyl-alanyl-alanyl-valine-*p*-nitroanilide
Saos-2	human osteogenic sarcoma line
SD	standard deviation
SDS	sodium dodecyl sulphate or sodium lauryl sulphate
SEM	scanning electron microscopy, or standard error of the mean
SKSD	streptokinase – streptodarnase (recall antigen)
SOD	superoxide dismutase
SPLP	*N*-succinyl-L-phenylalanyl-L-leucyl-L-phenylalanine-thiobenzyl ester, chymotrypsin substrate
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TEM	transmission electron microscopy
TGPLNA	*N*-*p*-Tosyl-L-glycyl-L-prolyl-L-lysine-*p*-nitroanilide
TLCK	*N*-α-*p*-tosyl-L-lysine chloromethyl ketone
TMBZ	3, 3',5.5-tetramethylbenzidine
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone
Tris	tris[hydroxymethyl] aminomethane
TTBS	3% skim milk-0.05% Tween 20-TBS
TUNEL	TdT-mediated dUTP-biotin nick end labeling
UTHSC-SA	University of Texas Health Science Center at San Antonio
uv	ultra-violet light
Vero	African green monkey kidney epithelium line
VPI&SU	Virginia Polytechnic Institute and State University
v/v	volume/volume
WRK	Woodward's reagent K or *N*-ethyl-5-phenylisoxazolium 3'-sulphonate
w/v	weight/volume
αMEM	alpha-modified Eagle's medium
βG	beta-glucuronidase
βME	beta-mercaptoethanol
σ^{28}	Sigma factor binding motif
γGNA	L-γ-glutamyl-*p*-nitroanilide

ACKNOWLEDGEMENTS

I would like to specially thank Dr. B. C. McBride, my project supervisor, for his support and guidance during my studies. The financial support of the University of British Columbia, Canadian Bacterial Disease Network, Medical Research Council of Canada, and the University of Hong Kong Committee on Research and Conference Grants was much appreciated.

My gratitude goes to Drs. V.-J. Uitto, and D. A. Mathers for serving on my committee and their guidance throughout my project. I am also grateful to members of the University of British Columbia, Faculty of Dentistry, particularly Drs. J. D. Waterfield, D. M. Brunette, C. B. Wu, J. Tonzetich and various staff and student members for their help, support, and advise.

I would like to express my sincere thanks to current or previous members of the laboratory and staff of the Department of Microbiology and Immunology, the University of British Columbia, in particular Drs. P. M. Hannam, J. C. Fenno, K.-H. Müller, M. Haapasalo and fellow graduate students: A. Joe, Y. Park, B. Lu, Y.-M. Pan and E. Putnins for sharing their knowledge, experience, joy and happiness.

I am grateful to members of the Faculty of Dentistry, the University of Hong Kong particularly Drs. E. F. Corbet and L. P. Samaranayake for encouragement and support. Special thanks also goes to Miss Nerissa Chan for secretarial assistance, Mr. M. T. Lee and his team for laboratory assistance during the final part of the project, Miss Vicky Yip and Mr. Samuel Hui of Dental Illustration for their expert technical support.

I would like to convey my special thanks to a few of my best friends: A. S. K. Ng, A. K. S. Wong, H. Yan, L. J. Jin, P. L. Lim, J. and E. Theilade. They always offer help and support in various aspects and from whom I learned about the genuine meaning of friendship and care.

Finally, I would like to thank my family: Vivian, Jeffrey, Philip, my parents and mother-in-law, from whom I experienced the meaning of love.

CHAPTER I Overview and Summary

1.1. Introduction

The association of a microorganism with another living entity probably has existed for as long as the plant and animal kingdom. This occurred as a result of the struggle for existence by microbes and in some cases by the host itself. The human body undoubtedly provides various anatomic sites which different bacteria can take advantage of and find their refuge or habitat. In most situations, the microbe and its host establishes a balance that ensures the survival, growth, and propagation of both parties without any interference. However, in some cases, the host-parasite interaction might result in injuries to the host to such a degree that disease in the host might become apparent (Schuster 1990).

The human mouth is one of those sites that microorganisms would colonize, thrive, and live. Upset of the microbe-host balance in the oral cavity is by no means a rare event. Such a process actually gives rise to two of the most common diseases in human beings - dental caries and periodontal diseases. This thesis is dedicated to investigate, partially, the untoward effects that may be brought about by an oral microorganism-*Treponema denticola*-on mammalian cells in culture.

1.2. Human periodontal disease

1.2.1. Brief overview of periodontal disease.

The term periodontal disease describes a number of distinct clinical entities that affect the periodontium including the gingiva, gingival attachment, periodontal ligament, cementum and supporting alveolar bone (Genco 1990, Nisengard *et al.* 1988) (Figure 1.1). Periodontal disease is generally classified according to its clinical, host, and environmental characteristics. Different

classification schemes have been proposed by regional or international professional organizations (American Academy of Periodontology 1989, European Academy of Periodontology, Attström & van der Velden 1994). However, despite such dedicated attempts, the current classification of periodontal disease remains relatively inconclusive. It has been illustrated lately that there are overlaps between the different clinical entities/disease forms in the current classification systems and that there is a need for further development of evidence based descriptors for the diseases in order to more specifically identify cause and effect relationships (American Academy of Periodontology 1996).

The etiology of pathologic changes in the periodontal tissues is multifactorial. However, it is now well accepted that bacteria in dental plaque are the major etiological agents for human periodontal diseases despite the fact that the exact nature of the casual relationship of plaque bacteria in periodontal diseases remains unclear (Newman 1990). Two decades ago groups of specific bacterial species that may be related to human periodontitis were reported (Newman & Socransky 1977). Followed by many more similar reports, the concept of specific pathogenic periodontal microbiota evolved (Carlsson 1988). Periodontal diseases are now considered to be polymicrobial infections mediated by a group of periodontopathogenic microbiota. *Porphyromonas gingivalis, Bacteroides forsythus, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Campylobacter rectus*, and oral spirochetes such as *T. denticola* plus some other bacterial species strongly associated with periodontal disease (Genco & Loos 1991).

Periodontal disease manifestation reflects the interaction between microorganisms and host. The microbes can produce disease indirectly such as by toxic metabolites or by direct invasion and subsequent damage of the host tissues. On the other hand, the response from the host can be protective and/or destructive. Together, these interactions give rise to the spectrum

of disease manifestation observable (Figure 1.1). How the host's inflammatory response comes to be protective or destructive is far from being well understood. However, three basic factors seem to be important for the nature of the inflammatory response. These are: i) immunoregulatory competency and the related genetic make-up of the host; ii) the nature, quantity, location of invasion, and digestibility of the insulting agent, and iii) the duration of the insulting agent-host interaction (Synderman & McCarty 1982).

The pathogenesis of periodontal disease has been reviewed recently (Page & Kornman 1997). Kornman and coworkers (1997) summarized the local host response to the subgingival microbial challenge as occurring in 4 phases. These are: i) Acute bacterial challenge phase: the epithelial and vascular elements respond to the bacterial challenge (Nagatu *et al.* 1992, Stadnyk 1994, Meikle *et al.* 1994), ii) Acute inflammatory response phase: the tissues respond to the early signals (Bevilacqua *et al.* 1985, Osborn 1990, Bickel 1993, Adonogianaki *et al.* 1994), iii) Immune response phase: activation of mononuclear cells which shapes the local and systemic immune response (Garrison & Nichols 1989, Kinane *et al.* 1993, Manthey & Vogel 1994), iv) Regulation and resolution phase: determinants of protective components in the sulcus and collagen balance in the tissues (Meikle *et al.* 1994, Ingman *et al.* 1994, Tonetti *et al.* 1997) (Figure 1.2).

The intact epithelial barrier of the gingival, sulcular and junctional epithelia normally prevents bacterial invasion of the periodontal tissues and is an effective barrier against penetration by bacterial products and components (Marsh & Martin 1992). Saliva and GCF flush the oral cavity and subgingival area with a multiplicity of defense components against bacteria (Cimasoni 1983, Dolby 1986, Marsh & Martin 1992). Kornman and his co-workers recently (1997) comprehensively reviewed the subgingival host response to microbial challenge that is

summarized as follows. With colonizing subgingival bacteria, through release of secreted proteins and metabolic products, the junctional epithelial cells, the gingival connective tissues and associated unmyelinated nerve fibers are triggered to release inflammatory mediators and neuropeptides respectively, which affect the local vascular response (Byers *et al.* 1987, Socransky & Haffajee 1991, Birkedal-Hanson 1993, Tonetti 1997 Figure 1.2A). Then a defensive response to the bacterial products may follow, which includes enhanced recruitment of PMNs, proliferation of the epithelial cells and localized secretion of enzymes that can initiate extracellular matrix destruction such as MMPs from macrophages and junctional epithelial cells (Offenbacher 1996, Figure 1.2B). If irritation from subgingival bacteria persists, the bacterial products and cytokines from epithelial cells can activate the local tissue mononuclear cells that might shape the local immune response (Page 1991). In brief, there will be increases in tissue lymphocytes, plasma cells and macrophages (Meikle *et al.* 1994), and a shift in the metabolism of the local fibroblasts to favor a reduction in collagen synthesis (Irwin *et al.* 1994), and activation of the local and systemic specific immune response (Garrison & Nichols 1989), with production of antibody directed against highly immunogenic bacterial epitopes (Ishikawa *et al.* 1997, Figure 1.2C). Provided there are either i) a specific bacterial biomass that directly inhibits key components of the host defense mechanism (Darveau et al. 1997, and/or ii) host response modifiers, such as smoking, systemic disease and/or genetic variation (Hart & Kornman 1997, Salvi *et al.* 1997), a more destructive response to the bacterial challenge may be evidenced (Figure 1.2D). The net result will be initiation of loss of attachment and increased mononuclear cell activity in the periodontal tissue. The inflammatory mediators and the matrix enzymes load in the tissue will be increased, these being contributed by fibroblasts, PMNs, plasma cells, macrophages, junctional epithelial cells (Meikle *et al.* 1994). The impact of such changes is to

subtly shift the scene from one in which the host is controlling the bacterial challenge to one in which the challenge is less well controlled and the tissue-destructive phase is dominant (Komman *et al.* 1997).

1.2.2. Periodontium in health

The human periodontium is composed of tissues that surround and support the tooth in its natural and functional state. It consists of gingiva with the gingival fiber groups, cementum with embedded Sharpey's fibers, PDL fiber groups and alveolar bone with embedded Sharpey's fibers. The tooth is attached to the alveolar bone by a resilient suspensory fibrous attachment mechanism mediated principally by the PDL fibers. These are the elements that resist normal functional forces of mastication and enable the tooth to adapt to stress (van Rensburg 1995). At the soft-hard tissue junction, the gingiva provides a soft tissue collar around the cervical region of tooth and separates those parts of the tooth exposed to the oral environment from the underlying delicate connective tissues and tooth attachment apparatus (Williams *et al.* 1992).

1.2.2.1. Human periodontium - a brief overview of the hard-soft tissue junction

The components of the periodontium and the dentogingival junction are diagrammatically represented in Figure 1.3. The structures of the periodontium from outside towards the tooth comprise: gingiva, PDL, alveolar bone and cementum on the root surface of tooth. On the cellular or molecular level, human periodontium consists of various cellular elements and extracellular matrix molecules. Epithelial cells form the exterior border between where the hard tissue of the tooth meets the gingiva, while fibroblasts form the predominant cell type in the gingiva and the PDL. When compared with most connective tissues, the periodontal ligament is

highly cellular. Animal studies have shown 20 – 50% of the tissue in PDL specimens to be occupied by cells (Berkovitz and Shore 1995).

The epithelial cells of the human periodontium are highly differentiated, probably in response to various functional demands. Epithelial cells appear in the dentogingival junction in three presentations: i) oral gingival epithelium (orthokeratinized), ii) oral sulcular epithelium (non-keratinized), and iii) junctional epithelium (non-keratinized). The oral epithelia in general are stratified squamous epithelium and are composed of keratinocytes and non-keratinocytes or clear cells (Williams *et al.* 1992). Cell division normally occurs only in the basal cell layer, and the cells that arise from these divisions move gradually through the epithelium towards the surface and are shed into the oral cavity. Both oral gingival and oral sulcular epithelia synthesize membrane coating granules in the upper part of the prickle cell and granular cell layers. These granules discharge their lipid contents into the intercellular space between the upper cell layers of both keratinized and non-keratinized epithelia, forming a weak barrier to water and water soluble substances. Such activities are not observed in the junctional epithelium (Williams *et al.* 1992).

The junctional epithelium or the junctional epithelial cells, form an epithelial collar responsible for uniting the connective tissue of the gingiva and the tooth surface (Figure 1.3B). The most apical part of the junctional epithelium is only a few cells thick, whilst coronally there may be between 15 and 30 cell layers, with a thickness of 30-100 μm (Squier *et al.* 1975, Figure 1.4). The junctional epithelium consists of a basal layer of cells that are somewhat cuboidal or elongated in a cervical direction, and a stratum spinosum-like layer extending up to the tooth surface. The cells are progressively flatter the closer they are to the tooth surface. At its most apical termination the junctional epithelium probably consists entirely of basal cells. On the tooth side, an apparatus named the epithelial attachment mediates the attachment. This consists

of hemidesmosomal junctions lining the cell membrane adjacent to the tooth and a basement lamina (Figure 1.4B). The epithelial attachment is approximately 40 nm wide and the adhesive forces are molecular in nature (Berkovitz *et al.* 1995). The lamina between the junctional epithelium and the connective tissue is termed the external basement lamina, and those between the junctional epithelium and the tooth surface the internal basement lamina (Figure 1.4). Morphometric analysis has show the relative volume occupied by the cells to be approx. 80%, and that occupied by the intercellular space to be approx. 20% (Schreoder and Münzel-Pedrazzoli 1970). The intercellular spaces of junctional epithelium may allow varying degrees of PMN leukocyte and monocyte infiltration. The border between the junctional epithelium and the connective tissue is relatively straight, although slight ridges may project into the connective tissue from the coronal portion of the epithelium. The junctional epithelium is a tissue with a high rate of cellular turnover (Anderson and Stern 1966, Engler *et al.* 1965). In primates, the turnover time was calculated between 5 and 11 days and appears to be higher than that of oral gingival epithelium (Skogaard 1965).

Other than the specially differentiated epithelial cells at the gingival sulcus that possess a specific functional role, aggregations of epithelial cells are also found within the periodontal ligament. These are undifferentiated epithelial cell masses, completely surrounded by connective tissue cells (Brunette *et al.* 1979) and are named the cell rests of Malassez.

Beneath the junctional epithelium the periodontal ligament forms the structural component of the periodontium. Fibroblasts form the most important cellular element in that region. They lie between collagen fibers and their histological appearance is believed to be governed by the surrounding matrix. The role of the periodontal fibroblasts is to produce extracellular matrix for the periodontal ligament (Berkovitz and Shore 1995). Special cells for

particular functions in the human periodontium are also found, e.g. cementoblasts, osteoblasts and osteoclasts. Cementoblasts are the cells responsible for secreting the organic matrix of cementum. Located immediately adjacent to cementum, they possess growth hormone receptors on their membranes (Berkovitz and Shore 1995), indicating that cementogenesis is a controlled process. Osteoblasts within the confinement of periodontium are found on the surface of the alveolar bone, similar to osteoblasts from other regions of the body. They are responsible for new bone deposition. Osteoclasts are found within resorption lacunae of alveolar bone. They are responsible for resorption or remodeling of the alveolar bone. Morphologically and functionally similar clastic cells that take up specific responsibilities such as resorption of cementum and dentine have also been observed. They are sometimes referred to as cementoclasts and odontoclasts (Addison 1979). The junctional epithelium is constantly patrolled by PMN. They do not remain within the junctional epithelium. Rather, these defense cells pass through into the gingival crevice while new PMNs are supplied by the vasculature nourishing the periodontium. Like other fibrous connective tissues, the periodontal ligament contains defense cells too. Examples are macrophages, mast cells and eosinophils. These cells are responsible for defense against the invasion of dental plaque microbes (Berkovitz and Shore 1995, McCulloch *et al.* 1989).

The most important element of the periodontium extra-cellular matrix molecules or macro-molecules is the collagen arranged into fibers. Type I collagen forms the bulk of the collagenous element of periodontal ligament, with lesser amount of type III, IV, V, VI and XII collagens (Romanos *et al.* 1991 and 1992). Collagen types I, III and V are the fibril (or quarter staggered fibrils) collagens in the periodontal ligament. Collagen type IV forms the collagen sheets of the epithelial cell basement membrane. Collagen type VI is the beaded fibrils, together

with collagen type XII, the FACITS, are the short-chain collagens in the periodontal ligament. A fair proportion of extracellular matrix compounds identifiable in connective tissues is also reported to be isolated in the periodontal tissue (Embery *et al.* 1995, Table 1.1). Included in the list are non-collagenous proteins like i) laminin and entactin which are responsible for organization of basement membrane or cell adhesion; ii) vitronectin which is involved in cell regulation and attachment; iii) tenasin for cell migration, wound healing etc; and iv) fibronectin for maintaining integrity of connective tissue structure (Embery *et al.* 1995). Various extracellular matrix or cell surface proteoglycans are also reported to be present together with glycosaminoglycans such as hyaluronan, heparan sulphate, dermatan sulphate, chrondroitin sulphate and keratan sulphate (Bartold 1995, Table 1.1).

1.2.2.2. A model system for junctional epithelium.

As described in the previous section, junctional epithelium is an epithelial structure that is responsible for adhesion of gingiva to the tooth surface. The unique features of the junctional epithelium, such as lack of keratinization, limited differentiation and a relatively permeable nature, enables the innate defense system to act in its vicinity against foreign substances or organisms. The subgingival microbiota, including periodontopathogens, are localized in direct contact with the junctional epithelium. Modulation of the physiology of the junctional epithelium or direct damage to the junctional epithelium may favor the survival and proliferation of the periodontopathogens of the subgingival plaque bacteria. On the other hand, attempts by host defense mechanisms to limit the deleterious effect on junctional epithelium with the addition of defense cells will be constantly on display in the human subgingival environment. Recently, a model for junctional epithelium was developed using porcine periodontal ligament epithelial

(PLE) cells which are cells of the epithelial rests of Malassez (Pan 1993, Pan *et al.* 1995). *In vitro*, the multi-layer PLE cell culture morphologically resembles junctional epithelium, with spherical basal and suprabasal layers containing large intercellular spaces. The cytokeratin profile (k5, 6, 14, 16, 19) of these cells also resembles that of the junctional epithelium *in vivo* (Pan *et al.* 1995). While the model lacks the attachment structure resembling the external basal lamina, because no connective tissue is present in the culture system, the model, serves as a controlled organotypic cell culture system to study behavior of epithelial cells that resemble junctional or sulcular epithelial cells (Pan *et al.* 1995).

1.3. *Treponema denticola* as one of the periodontopathogens

Increasingly, more evidence has become available to demonstrate that periodontal disease is caused by specific pathogenic bacteria. A recent world workshop on periodontal disease has identified *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans* as causative agents of periodontal disease (American Association for Periodontology 1996). In fact, adult periodontitis is not associated with a single organism, and it is likely that a consortium of bacteria participate in the disease (Darveau *et al.* 1997, Table 1.2). The assigning of a subgingival microorganism to the list of periodontopathogens or agents associated with periodontitis is generally done in accordance with Socransky's modified Koch's postulates for dental infections (Socransky 1979). The unique nature of the periodontal opportunistic infection does not fit-in well with the original Koch's postulates, which were invented to describe the nature of a medical pathogen. In order to utilize the postulates in the dental or periodontal context, Socransky modified them and formulated the following version: i) association with disease, ii) elimination of the organism, iii) host response, iv) animal pathogenicity, and v) mechanisms of pathogenicity. A few of the

current well known periodontal or dental pathogens were identified based on the modified postulates such as *P. gingivalis* and *A. actinomycetemcomitans*. *T. denticola* has been suggested to be one of the subgingival species associated with periodontitis (Table 1.2). Part of the following sections (1.3.2. – 1.3.7.) is an account, based on the modified Koch's postulates, of why *T. denticola* is considered as a member of the periodontopathogens.

1.3.1. *Treponema denticola*

1.3.1.1. Physical appearance and habitat

T. denticola is slender, flexible, irregular helical shaped, unicellular, motile, gram-negative bacteria. The cell dimensions of the type strain ATCC 35405 are as follows: length, 7.74 ±0.94 μm; diameter, 0.20 ±0.02 μm; wave length, 1.23 ±0.15 μm; amplitude, 0.50 ±0.05 μm (Canale-Parole 1984, Chan *et al.* 1993, Ruby *et al.* 1997). *T. denticola*, one of the many spirochete species of the Order *Spirochaetales*, possess unique morphological features as outlined in the following. Enclosing the spirochete is an outer membrane (OM) sometimes referred to as the outer sheath or outer envelope (Johnson 1977). The peptidoglycan layer and the cytoplasmic membrane are located immediately beneath the OM (Canale-Parole 1984). The membrane-bound protoplasmic cylinder contains ribosomes, mesosomes, vacuoles, cytoplasmic tubules and nuclear areas. In between the OM and the peptidoglycan layer of the *T. denticola* type strain, 5 pairs of endoflagella or periplasmic flagella can be found (Chan *et al.* 1993). These attached bipolarly in a subterminal position and extend toward the opposite end of the cell (Johnson 1977). Ultrastructurally, the spirochete was found to have synthesized or acquired an extracellular polysaccharide or slime layer on the surface (Scott *et al.* 1997). Covered by the slime layer, the OM of *T. denticola* consists of 2 - 3 electron dense layers separated by an

electron transparent area which resembles the outer membrane of gram-negative bacteria (Cockayne *et al.* 1989). However, classical endotoxin was not associated with the OM (Johnson 1976); instead, only incomplete endotoxin like or lipopolysaccharide-like substance was reported (Akiyama 1977, Yotis *et al.* 1995, Dahle *et al.* 1996). The OM seems to be elastic and fragile in nature. When the spirochetes are exposed to adverse conditions, or during stationary phase of growth, the OM frequently separates from the protoplasmic cylinder and blebbing of the cell surface occurs, presumably in an attempt to reduce surface area (Wolf *et al.* 1993). The main structural features of *T. denticola* are outlined in Figure 1.5.

1.3.1.2. Taxonomic history and classification

Although Brumpt first described *Treponema denticola* in 1925, this spirochete was without nomenclatural standing until 1993. It was because no type strain was designated for the species. In 1993, Chan and coworkers proposed *T. denticola* as a distinct species of the genus *Treponema* and designated *T. denticola* type and reference strains for such purpose (Chan *et al.* 1993). *T. denticola* belongs to the family of *Spirochaetaceae* under the order *Spirochaetales*. *Spirochaetales* is a taxon that was proposed as a result of the tendency to attribute phylogenetic or taxonomic value to morphological features, i.e. spirochetes grouped together because of basic structural similarities (Canale-Parola 1984). However, it was confirmed later by 16S rRNA sequences analysis that the *Spirochaetaceae* members including *T. denticola* belong to a monophylectic bacterial phylum (Paster *et al.* 1991).

1.3.2. Association with periodontitis

Spirochetes were reported to be able to colonize the subgingival area of older children around the time of puberty (Socransky & Manganiello 1973). Relatively elevated proportions of spirochetes together with motile rods were frequently observed in periodontally diseased sites (Listgarten & Helldén 1978). Later, *T. denticola* as a member of the subgingival spirochete family was confirmed: To conceptualize the specific plaque hypothesis of periodontal disease causation by a consortium of periodontopathogens including *T. denticola*, researchers began to investigate the evidence for overgrowth of *T. denticola* in subgingival plaque bacteria in "diseased pockets". With approaches such as: i) direct immunohistochemical microscopy of periodontal pocket tissue block biopsies with subgingival plaque *in situ* (Kigure *et al.* 1995), ii) immunofluoresence or immunocytochemical microscopy of plaque samples (Loesche *et al.* 1992, Riviere *et al.* 1995), iii) ELISA detection of the spirochete from plaque samples (Simonson *et al.* 1988), iv) selective treponeme culture (Moore *et al.* 1982, Cheng & Chan 1983, Koseki *et al.* 1996), v) molecular biological methods such as use of a specific DNA probe, PCR, 16S rRNA sequencing etc. (DiRienzo *et al.* 1991, Söder *et al.* 1993, Choi *et al.* 1994, Dahle *et al.* 1995, Ashimoto *et al.* 1996, Haffajee *et al.* 1997), researchers have been able to document the association of *T. denticola* with diseased periodontal pockets.

Other than physical detection, isolation or cultivation of *T. denticola* from periodontal plaque samples, attempts have been made to imitate the subgingival plaque system *in vitro*. ter Steeg and coworkers (1988) modeled the cultivation or growth conditions of subgingival plaque in periodontal pockets. A slow-growing continuous culture enrichment of pooled subgingival plaque from periodontal pocket was established. They observed production of a wide variety of cell-bound hydrolytic enzymes in the system and production of acidic end-products in a ratio at

least partly resembling that of human gingival fluid samples. This led to their conclusion of having successfully established a preliminary *in vitro* subgingival culture model. When the components of the steady state culture were analyzed, they identified up to 24% of the total bacterial mass as having spirochetes, mainly *T. denticola*, together with bacteria of the species *Peptostreptococcus*, *Lactobacillus*, *Streptococcus*, *Prevotella*, *Eubacterium* and *Veillonella*. This study showed that *T. denticola* might be an important element of periodontal disease associated subgingival plaque.

A recent study by Gazi and co-workers (1996) collected and analyzed gingival tissue extract and/or GCF from untreated AP patients using a substrate-impregnated overlay technique on IEF gels. Sonicated periodontopathogens including *T. denticola* ATCC 33520 and ATCC 33521 were also analyzed in an attempt to identify the nature of the proteinases from the periodontally diseased human sample. They concluded that a contribution by *in vivo T. denticola* cysteine proteinase to the GCF sampled could not be ruled out. This report was among the first that documented probable *in vivo* subgingival effects of *T. denticola* in subjects suffering from periodontitis.

1.3.3. Elimination of *T. denticola* associated with disease resolution

It has been widely reported that subgingival spirochetes are among one of the bacterial morphotypes that respond most readily to periodontal therapy (Listgarten *et al.* 1978, Mousques *et al.* 1980, Slots *et al.* 1985). It was reported that *T. denticola* is among one of these spirochetes that respond to subgingival debriment (Haffajee *et al.* 1997). This was first documented 6 years ago by Simonson and coworkers (1992) who followed subgingival microbiology of AP patients before and shortly after non-surgical mechanical therapy using ELISA assays. Mean levels of *T.*

denticola were significantly reduced after therapy and the healing response in diseased sites, i.e. decrease in probing pocket depth, was associated with reduction in the immunologically detectable level of *T. denticola* in the same pocket. Conversely, non-responding sites had post-treatment *T. denticola* levels of greater than or equal to those of pre-treatment levels, suggesting that *T. denticola* had to be eradicated from periodontally diseased sites before resolution of disease could be realized.

1.3.4. Host response to *T. denticola*

Host systemic response upon the challenge or subgingival infection by *T. denticola* will be reviewed partly in section 1.3.6.5. The increased humoral immune response against *T. denticola* in AP subjects and the down regulated or minimal humoral immune response against the spirochete in severely periodontally affected individuals suggests that *T. denticola* plays a special role in the periodontopathogenic process (Steinberg & Gershoff 1968, Tew *et al.* 1985, Gunsolley *et al.* 1991). Local immune response of diseased subgingival sites of AP patients in general has been extensively reviewed (Figure 1.2, Kornman *et al.* 1997). From GCF samples, an array of parameters could be measured: i) increases in local PMN activity depicted by increased lysosomal βG, collagenase or elastase activities (Makela *et al.* 1994, McCulloch 1994, Nakashima *et al.* 1994), ii) increased production of inflammatory mediators such as PGE_2 and interleukins (Mendieta *et al.* 1985, Stashenko *et al.* 1991a & b); iii) liberation of acute tissue destruction indicators such as AST and/or LDH (Harper *et al.* 1989, Nakashima *et al.* 1994); iv) increased release of indicators of demineralization such as alkaline and acid phosphatases (Nakashima *et al.* 1994) etc. However, few investigators have attempted to correlate the relationship between subgingival local host responses with regards to the corresponding

microflora in the vicinity. Jin and coworkers (1998) recently studied such relationships. They observed colonization of untreated subgingival periodontitis sites with *T. denticola* plus at least 3 other periodontopathogens namely *P. gingivalis*, *B. forsythus* and *P. intermedia*. They observed significantly more prevalent colonization by the four pathogens in diseased sites than healthy or gingivitis sites from the same individuals. The local inflammatory response, in terms of GCF elastase and PGE_2 levels, was minimal in healthy or gingivitis site while at the periodontitis sites, diverse levels (low to very high) were observed. They postulated that such variation might be result of complex host-bacteria interactions and might be indicative of different stages of periodontal disease activity or progression.

1.3.5. Animal model of *T. denticola* periodontal pathogenicity

The ability of *T. denticola* to cause periodontal disease in an animal model has not been studied. So far, only laboratory transmission studies of necrotizing ulcerative gingivitis between beagle dogs have been reported. Weekly inoculation of dental debris from affected dogs to heavily steroid subdued unaffected dogs demonstrated such disease in these circumstances was transmissible. Spirochetes were one of the key components in such dental debris and they were found in intercellular spaces of the affected epithelium and were sometimes intra-cellularly located. (Maltha *et al.* 1985, Mikx & van Campen 1982, Mikx *et al.* 1990). However, no report is available illustrating whether *T. denticola* was among the invading spirochetes in such an animal disease condition. The difficulties of raising germ-free animals may be one major obstacle for studying whether *T. denticola* causes periodontitis in an animal model. Other problems may include choosing the right animal to test; whether periodontal disease induced in animals may resemble what is seen in humans; if it is appropriate to stimulate periodontal disease

in animals with systemic or local interference e.g. steroid injection, ligature placement etc. The ability of *T. denticola* to damage non-oral cells and tissues of laboratory animals was, however, quite intensively studied (Ebersole *et al.* 1995, Kesavalu *et al.* 1997). Details of these studies was reviewed in section 1.3.6.4..

1.3.6. Periodontopathogenic potential

The periodontopathogenic potential of *T. denticola* has been thoroughly reviewed recently (Fenno & McBride 1998). Being one of the most readily cultivable spirochetes from human subgingival plaque specimens (Chan *et al.* 1993), attention has been given to the *in vitro* investigation of this treponeme in terms of its character, physiology and pathological potentials. Given its close association with periodontal disease, certain characteristics or aspects of the bacterium that might be involved in periodontal disease pathogenesis were studied. Such characteristics include: i) motility of the organisms; ii) ability to adhere to host surfaces or host tissue; iii) ability to invade host tissues iv) ability to cause host cells damage; v) ability to evade, damage or undermine the host's defenses; and vi) ability to coexist or possibly augment the action by other periodontopathogens resulting in more severe host tissue damage.

1.3.6.1. Motility

Motility provides a microorganism with many ecological advantages. Motile microbes, including *T. denticola*, could quickly respond to alterations of selective pressure in the local environment (Chan *et al.* 1995). It had been documented recently that *T. denticola* present themselves at the surface layer (i.e. facing pocket epithelium and connective tissue) of subgingival plaque in deep periodontal pocket sites (Kigure *et al.* 1995). Based on the

knowledge that spirochetes and treponemes are not key elements of subgingival plaque biomass until that is matured (Löe *et al.* 1965, Listgarten *et al.* 1975), *T. denticola* very likely move in and colonize the subgingival area when the local conditions become favorable to them. They were also reported to be one of the bacterial species most sensitive to subgingival debriment or plaque control (Mousques *et al.* 1980, Haffajee *et al.* 1997). This was also the case when local antimicrobials were used as the subgingival plaque control agent. In ligature induced periodontitis in beagle dogs, Klinge and co-workers (1992) observed significant dramatic reduction in spirochetes in the metranidazole treated sites not observed in the placebo sites. Johnson and coworkers (1997) had shown re-population of subgingival areas of previously successfully treated periodontitis patients by spirochetes and motile rods 4 weeks after cessation of oral hygiene. Motility plus their ability to respond to chemotactic stimuli in the environment could enable *T. denticola* to colonize a particular subgingival area or site.

The locomotory abilities of treponemes in general and *T. denticola* in particular have been reviewed and studied *in vitro* (Berg 1976, Canale-Parola 1978, Pietrantonio *et al.* 1988, Charon *et al.* 1992a & b, Klitorinos *et al.* 1993, Ruby 1995). In general, spirochetes exhibit rotatory and flexing movements. The flexing movement includes lashing, looping, and bending. Vibrations, undulations, formation of helical waves traveling along the body and production of planar waves are also possible (Canale-Parola 1977). Berg in 1976 proposed that the periplasmic flagella rotate between the OM and the protoplasmic cylinder to create spirochetal locomotion. That was later confirmed by Charon *et al.* (1992a). Despite the fact that not many of the spirochetal motions other than the two distinct locomotion phenotypes (Chan *et al.* 1995) and the so called "jerky but rapid motion" were observed and described for *T. denticola* (Smibert 1984), attention has been drawn towards the study and characterization of motility control of the

spirochete (Pietrantonio *et al.* 1988, Klitorinos *et al.* 1993, Ruby 1995). Studies of *T. denticola* motility through viscous environments were carried out based on the hypothesis that this ability was considered very important for the establishment of the host-spirochete parasitic relation in the subgingival environment. Klitorinos and coworkers (1993) investigated velocity of spirochetes' movement in media of different viscocities. They showed that optimal migration of spirochetes was viscosity-dependent. For example, *T. denticola* exhibited the fastest migration speed of 18.7 ±4.4 μm/min at a viscosity of 30mPa•s while the spirochete was still able to move in media of viscosities up to 700 mPa•s, which was equivalent to an environment too viscous for any other non-spirochetal motile bacteria to move.

Research has been conducted to map out the flagellar genes of *T. denticola*. So far genes homologous to seven known flagellar genes organized as an operon under the probable control of a σ^{28} family promoter have been identified (Heinzerling *et al.* 1997). With the recent success in generation of a non-motile *T. denticola* mutant defective in *flgE* expression, (Li & Kuramitsu 1996, Li *et al.* 1996), researchers are hopeful that the assembly and function of spirochetal flagella in *T. denticola* and other spirochetes could be clarified in the not too distant future.

Furthermore, initial molecular biological studies of *T. denticola* chemotaxis also produced rewarding findings. A gene, named *dmcA*, encoding a homologue of a large family of methyl-accepting chemotaxis proteins, was identified (Kataoka *et al.* 1997). Mutation of this gene resulted in decreased chemotactic activity of *T. denticola* (Kataoka *et al.* 1997).

1.3.6.2. Adhesion

In vitro ability of *T. denticola* to adhere to various surfaces has been reported in many studies. Studies using *in vitro* attachment assays have reported that *T. denticola* bound to coated-

solid surface, extracellular matrix and cells in culture (Cimasoni & McBride 1987, Thomas 1996, Fenno & McBride 1998). The initial attachment of bacterial cells to niches in a host is one of the most important steps in the development of infection (Beachey 1981, Gibbons 1989, Höök *et al.* 1990). Such abilities or bacterial strategies that promote bacterial colonization of the host will undoubtedly augment the virulence of the related bacteria (Salyers & Whitt 1994). Therefore, the well recognized versatility of *T. denticola* host surfaces adhesion places this spirochete favorably among the periodontopathogens.

T. denticola was reported to be able to adhere to buffer coated hydroxyapatite (HA) beads as well as glass cover slips (Cimasoni & McBride 1987, Nakatani *et al.* 1992). In one study, it was shown that *T. denticola* could attach to HA beads equally well be it pre-treated with buffer, human saliva, human serum, or crevicular fluid from subjects with severe marginal inflammation as observed using SEM (Cimasoni & McBride 1987). Similar findings were also reported in another study when *T. denticola* attachment to glass cover slip was investigated (Nakatani *et al.* 1992). In this study it was observed through light and scanning electron microscopy that human saliva, human or animal sera, and bovine serum albumin had no major effect on *T. denticola*-glass adhesion. From the same report, inhibition of the spirochete-glass adhesion by i) pre-heating the *T. denticola* at 100°C, ii) fixing the spirochete using formaldehyde or glutaraldehyde, iii) pre-treating the spirochete with sodium metaperiodate, were observed. The Japanese group followed up with more investigations of spirochete pretreatment on *T. denticola*-glass adherence. Pretreatment by trypsin, proteinase K, lipase, and lysozyme significantly reduced the spirochete's ability to adhere to glass, however, βME pre-treatment of *T. denticola* doubled the spirochete attachment. The importance of motility of *T. denticola* on HA attachment was also studied. Spirochetes rendered immotile by chloroform treatment attached to the HA in a fashion similar to

motile spirochete (Cimasoni & McBride 1987). One interesting finding from the same report was that pretreatment of HA with PMN lysosomal enzymes clearly reduced *T. denticola* adhesion. However, no further experiments were conducted on the effects of lysosomal enzymes on *T. denticola* and subsequently its ability to adhere, so the role of PMN lysosomal enzymes in *T. denticola*-HA attachment remains to be more exhaustively elucidated.

Studying the ability of *T. denticola* to adhere to extracellular matrix was also one major aspect that investigators wanted to pursue. Extracellular matrix components pre-coated onto plastic, glass, nitrocellulose, latex spheres or microbeads surfaces were employed to study the *T. denticola*-extracellular matrix adherence (Table 4.1, Dawson & Ellen 1990 and 1994, Haapasalo *et al.* 1991 and 1996, Nakatani *et al.* 1992, Ellen *et al.* 1994a). Nakatani and coworkers (1992) reported collagen VI or fibronectin pre-coated glass cover slips bound significantly more *T. denticola* than BSA control. Similar preferred adherence to fibronectin by the spirochete was also reported in other studies (Dawson & Ellen 1990, Haapasalo *et al.* 1991). Knowing that pathogenic treponemes such as *T. pallidum* attach to eukaryotic cells binding domain on fibronectin of amino acids sequence: arginine-glycine-aspartic acid-serine, RGDS, (Thomas *et al.* 1985), Dawson and Ellen (1990) tested the adherence of *T. denticola* to such peptides. The spirochetes were found to attach to the RGDS peptides as efficiently as to fibronectin molecules. However, a later study which detected attached *T. denticola* using ELISA technique could not repeat similar attachment behavior of the spirochete to GRGDS peptide (Table 4.1, Haapasalo *et al.* 1991). Binding of *T. denticola* to extracellular matrix proteins other than fibronectin was also studied using microscopic or ELISA methods (Dawson & Ellen 1990, Haapasalo *et al.* 1991). Dawson and Ellen (1990) observed microscopically significantly increased adherence of *T. denticola* strains ATCC 33520, e and e' to laminin coated plastic, however, type IV collagen

coated plastic surface did not stimulate more adherence. Haapasalo and coworkers (1991) later also reported similar binding behavior of *T. denticola* strain ATCC 35405 to laminin. They demonstrated that among the 140 kDa eukaryotic cell binding fragment and the 50 kDa heparin binding fragment of laminin, the 140 kDa fragment bound better to *T denticola*. Also reported were the abilities of *T. denticola* adhesion to fibrinogen, gelatin, type I and type IV collagens. The result about the *T. denticola* attachment to type IV collagen was different from that of the previous report (Dawson & Ellen 1990). Another area of difference between the two reports was that while Dawson and Ellen (1990) observed increased adherence of fibronectin pre-treated *T. denticola* to fibronectin, Haapasalo and coworkers (1991) could not see such an effect, and suggested that differences in experimental design may have accounted for the differences observed. In addition, the different *T. denticola* strains used in these studies may also have contributed to some extent to the discrepancies (Thomas 1996).

The effects of various pre-treatments of *T. denticola* on its ability to adhere to immobilized extracellular matrix protein were also studied (Haapasalo *et al.* 1991). Heat treatment ($\geq$ 70°C, 10 min) reduced the spirochete attachment to laminin, gelatin, and fibrinogen. Mixed glycosidases treatment of the spirochetes demonstrated attachment inhibition to varying extents. Pre-treatment of *T. denticola* with soluble substrate proteins such as laminin, gelatin, BSA, or fibrinogen had no effect on the spirochete – coated protein binding. However, if sulfhydryl reagents such as *p*CMBA or oxidized glutathione were used to pre-treat *T. denticola* before attachment experiments on extracellular matrix proteins, significant reductions in the spirochete attachment were observed (Haapasalo *et al.* 1991). The authors concluded that the binding of *T. denticola* to extracellular matrix proteins appeared to involve protein sulfhydryl groups and/or carbohydrate residues on the surface of the spirochete.

Observing a proportion of the *T. denticola* cells bound to immobilized fibronectin by their ends, Dawson and Ellen (1990) designed more experiments to investigate this observation. In their later report (Dawson & Ellen 1994), using a 2% methylcellulose migration column plus SEM and TEM techniques, demonstrated *T. denticola* had patchy fibronectin binding regions on its cell surface. While the fibronectin contact *per se* might stimulate a re-arrangement of such fibronectin binding regions so that they concentrated towards the tip of the spirochete, thus explaining the tip attachment behavior of *T. denticola.*

Study of *T. denticola* adhesion to extracellular matrix was not limited to matrix proteins only. Recently, Haapasalo and coworkers (1996) investigated the adhesion of *T. denticola* to hyaluronan coated plastic or microbeads. They reported that pre-treatment of the bacteria with hyaluronan or fibrinogen inhibited its binding to the hyaluronan, while gelatin, BSA, chrondroitin-4-sulfate, chrondroitin-6-sulfate, heparin, dermatan sulfate, glucuronic acid, D-GluNAc and D-GalNAc did not inhibit binding. Physical or chemical treatments that inhibited the *T. denticola* CTLP activity, such as heating to $\geq 80°C$, pre-treatment in $pH \leq 3$, pre-treatment with sodium periodate, PMSF or *p*CMBA, inhibited the binding of the spirochete to hyaluronan. They postulated that *T. denticola* bound to hyaluronan by a mechanism involving the CTLP of the spirochete.

T. denticola strains were also reported to be able to attach to eukaryotic cells in culture. Studies of at least 19 strains of *T. denticola* and 11 cell lines or explant cultures have been reported (Table 4.2). Basically, *T. denticola* attached to all cells tested, whether epitheloid or fibroblast-like. Not all cell lines/explants were equally attractive to *T. denticola* binding: HCE, GPE and MDCK were three of the cells to which *T. denticola* attached least well. Different *T. denticola* strains also behaved differently (Table 4.2). In many studies, there was great variation

in the number of spirochetes attached to individual cells on a culture. Cells in a particular stage of their cell cycle might be more attractive to *T. denticola*, which may account for observed increased spirochete binding to dividing cells or cells of low confluency. Attachment of *T. denticola* to fibroblasts was shown occurring together with degradation of fibronectin on the HGF surface (Ellen *et al.* 1994). Hyaluronan, as was shown in two separate studies (Olsen 1984, Haapasalo *et al.* 1996), might play a part in the *T. denticola* – epithelial cell adhesion process (Table 4.2). *T. denticola* surface lectins as well as glycoproteins, proteins or carbohydrate molecules might mediate attachment of the spirochete to animal or human cell surfaces (Weinberg & Holt 1990, Keulers *et al.* 1993a, 1993b, 1993c, Haapasalo *et al.* 1996, Table 4.2).

In addition to the ability to adhere to different cell surfaces, the ability of *T. denticola* to agglutinate erythrocytes was also studied (Grenier 1991, Mikx & Keulers 1992). *T. denticola* was shown to agglutinate human (A, B, or O blood group), rabbit, horse or bovine red blood cells. Both reports indicated the hemagglutination activities of *T. denticola* to be cell bound. Mikx and Keulers (1992) reported that the hemagglutination activity was not related to cell appendages, such as fimbriae. They also observed that growth phase related hemagglutination activity was abolished by heating, alkylation, or proteolytic enzyme digestion of *T. denticola*, leading to their postulation that the agglutinin might be proteinaceous. Grenier (1991) also observed reduction of hemagglutination activity in the presence of D-glucosamine, EDTA or sodium salicylate. Mikx and Keulers (1992) observed a reduction of hemagglutination with sialic acid and postulated that the agglutinin might be a glycoprotein that recognized sialic acid as a receptor.

1.3.6.3. Invasion of host cell/tissue

T. denticola was actually infrequently found to be intracellular in *in vitro* experiments (Olsen 1984, Ellen *et al.* 1994a, de Filippo *et al.* 1995). Pan (1993) reported occasional observation of spirochete fragments intracellularly after PLE cells were infected with *T. denticola*. Most *T. denticola*, once internalized in PLE cells, were found to be without both PLE endosomal membrane and the treponemal outer membrane. He postulated that the PLE cells might have digested the treponeme outer membrane of the internalized *T. denticola* (Pan 1994). Another Canadian group (Ellen *et al.* 1994a, de Filippo *et al.* 1995) observed internalization of *T. denticola* in HGF. They also observed no HGF endosomal membrane around internalized spirochete. No follow up study, was carried out to describe how the internalization came about or why the internalized spirochetes were without their outer-membrane. One possible explanation might be that the host digested the outer-membrane, or that the treponeme shedded its outer-membrane before invading into the host cell. Later, Keulers (1995), reported an attempt to study *T. denticola* invasion in an *in vitro* epidermis tissue culture model using HSK on de-epidermized human dermis. He could not demonstrate invasion of spirochetes into the cornified epithelial cell layer. Keulers commented that the wrong epithelial cell might have been chosen for such an assay. The abundant cornification might simply have protected the epithelial cell culture from invasion or infection by *T. denticola*. Although it has been widely postulated that *T. denticola* can invade periodontal tissues of humans, this postulate has yet to be clearly demonstrated.

1.3.6.4. Damage to host cell/tissue

T. denticola's ability to damage eukaryotic cells or tissues of laboratory animals has been

reported (Reijntjens *et al.* 1986, Ebersole *et al.* 1995). *T. denticola* can damage a variety of host cells, from hemolysis of red blood cells to abscess formation in an animal model (Grenier 1991, Ebersole *et al.* 1995). The effects of *T. denticola* on animal cells and tissue are summarized in Table 4.3. In brief, the treponeme can lyse red blood cells, detach epithelial cells or fibroblasts from their substratum in culture, cause cytoplasmic vacuolation, membrane blebs, rounding up of cells or failure in cell volume regulation, actin or cytoskeletal rearrangement, intercellular junctions breakdown etc. *in vitro*. It can also retard proliferation of cells and inhibit new protein synthesis (Boehringer *et al.* 1984, Hanks *et al.* 1991, Table 4.3). *T. denticola* outer-membrane preparation was shown to be able to induce bone resorption of rat long-bone organ culture (Gopalsami *et al.* 1993). In animal models, increased vascular exudation and spreading of the spirochetes to other tissue compartments were also observed upon inoculation of *T. denticola* (Lindhe & Socransky 1979, Ebersole *et al.* 1995, Kesavalu *et al.* 1997, Table 4.3). The ability to induce cellular detachment by *T. denticola* appears to be related to proteolytic activities and viability of the spirochete. Heating and pre-treatment of the spirochete with protease inhibitors such as PMSF or antibiotics such as metronidazole reduced the number of cells detached (Baehni *et al.* 1992). *T. denticola* was also reported to retard the proliferative response of fibroblasts and endothelial cells in culture both in the levels of DNA and protein synthesis (Taichman *et al.* 1982, 1984, Hanks *et al.* 1991). Heating the *T. denticola* sonic extract attenuated the effect on fibroblasts but not on endothelial cells (Taichman *et al.* 1982, 1984, Table 4.3).

1.3.6.5. Modulation of host defense

Host defense against colonization by and deleterious effects of periodontopathogens begins even before microorganisms attach to any oral surfaces (Marsh & Martin 1992).

Examples are saliva and the various factors or molecules that can lyse, kill, prevent bacterial adherence or create an environment not favorable for bacterial survival. The observation of a *T. denticola* protein binding lactoferrin (Staggs *et al.* 1994) might be a strategy used by the spirochete to interfere with the host non-immunological salivary defense. They reported the identification of two distinct lactoferrin-binding proteins of 50 kDa and 35 kDa from the *T. denticola* strain GM-1. The spirochete, however, did not associate with human transferrin, another host defense protein (Staggs *et al.* 1994). Recently, *T. denticola* was reported to be able to interfere with host inflammatory response through activation or cleavage of the pro-1L-1β (31 kDa) into the 17.5 kDa biological active IL-1β (Beauséjour *et al.* 1997). This was the first report demonstrating that *T. denticola* could potentially activate inflammation cascade in addition to the widely reported direct deleterious effect on host tissues or cells.

Regarding the effects of *T. denticola* on specific and non-specific cellular and humoral immune response, a number of investigations have been carried out (summarized in Table 4.4). *T. denticola* has been reported to be able to activate classical or alternative complement pathways (Schenkein & Berry 1991). Activated C3 appeared to be resistant to proteolytic degradation by the spirochete (Schenkein & Berry 1991). Regarding the interaction of the spirochete to innate immunity of the host, *T. denticola* was shown to be able to activate strong PMN chemotaxis in a laboratory animal infection model (Lindhe & Socransky 1979). Secondly, the spirochete, once opsonized, could be engulfed by PMN's in aerobic and anaerobic environments (Taichman *et al.* 1982, Hurlen *et al.* 1984, Olsen *et al.* 1984). Many reports observed however, *in vitro*, that there were not much degranulation activities by the *T. denticola* engulfed PMN's and the digestion of internalized spirochetes appeared to be a slow process (Taichman *et al.* 1982, Hurlen *et al.* 1984, Boehringer *et al.* 1986) despite many such PMNs remaining viable and expressing increased

oxygen consumption (Hurlen *et al.* 1984, Boehringer *et al.* 1986, Sela *et al.* 1988). It was also shown in one report that unpurified soluble *T. denticola* extract inhibited PMN response (Hanks *et al.* 1991, Table 4.4).

The host adaptive immune responses are also modulated by *T. denticola* infection (Table 4.4). It was shown that lymphocytes isolated from periodontally healthy or edentulous individuals did not react with strong blastogenic responses to *T. denticola* sonic extract (Patters *et al.* 1980, Mangan *et al.* 1982, Suzuki *et al.* 1984). When the human lymphocytes were stimulated (e.g. by mitogens) a clear suppression of the blastogenic response by *T. denticola* sonic extract was observed, which was probably related to prostaglandin and H_2O_2 in the local environment (Taichman *et al.* 1982, Shenker *et al.* 1984). When mouse lymphocytes were used, similar suppressive responses was observed when a sonic extract of *T. denticola* ATCC 33520 was applied (Ishihara 1988, Ishihara *et al.* 1992). A stimulatory response, on the contrary, was observed when a sonic extract of the spirochete strain ATCC 35405 was used (Ishihara 1988, Ishihara *et al.* 1992, Takahashi *et al.* 1996). Other *T. denticola* strains, however, did not produce marked suppression or stimulatory response in murine lymphocytes (Ishihara 1988, Ishihara *et al.* 1992, Takahashi *et al.* 1996).

Investigators went on to study the *in vitro* reaction of lymphocytes isolated from subjects suffering from different severities of periodontal diseases. A few studies reported that stimulated lymphocytes from LJP, GJP and AP subjects reacted similarly to that of normal individuals upon *T. denticola* challenge (Patters *et al.* 1980, Mangan *et al.* 1982, Suzuki *et al.* 1984). When stimulated lymphocytes from severely periodontally affected individuals were studied, increased blastogenic response was observed after treatment with a sonic *T. denticola* extract (Patters *et al.* 1980). Most investigations reported viability of lymphocytes to be maintained throughout the

study. One other report also observed polyclonal B cell activation by an unpurified *T. denticola* outer membrane preparation (Takahashi *et al.* 1996, Table 4.4).

Adaptive humoral immune responses against *T. denticola* in human subjects were also among the areas that have been widely studied. Different investigators seemed to come up to more or less similar observations (Table 4.4). In general, healthy and edentulous individuals had low anti-*T. denticola* antibody titers in their peripheral blood (Steinberg *et al.* 1966, Steinberg & Gershoff 1968, Steinberg 1970, Jacob *et al.* 1982, Tew *et al.* 1985, Lai *et al.* 1986, Yotis *et al.* 1995). Moderate to advanced adult periodontitis patients had high serum IgG and IgA titers against *T. denticola* (Jacob *et al.* 1982, Mangan *et al.* 1982, Tew *et al.* 1985, Lai *et al.* 1986). However, severely affected periodontitis patients were repeatedly reported to possess low/no immune titers against *T. denticola* (Steinberg & Gershoff 1968, Steinberg 1970, Tew *et al.* 1985, Gunsolley *et al.* 1991, Table 4.4). It was only the serum samples from LJP patients where conflicting immune responses have been observed (Tew *et al.* 1985, Lai *et al.* 1986, Sasaki *et al.* 1989).

1.3.6.6. Synergism with other periodontopathogens

The co-existence between *T. denticola* and other periodontal organisms, and their relationship with clinical disease status had been another focus for investigations (Loesche 1993, Söder *et al.* 1993). Simonson and coworkers (1992) reported association between *T. denticola* and *Porphyromonas gingivalis* in human subgingival plaque samples in a multinational survey using ELISA techniques for bacterial detection. They postulated that the *in vivo* association between *T. denticola* and *P. gingivalis* might be synergistic. Later, the same relationship or association of both bacteria was also observed from subjects with periodontal disease (Pederson

et al. 1994, Riviere *et al.* 1996).

These observations stimulated investigators to look for signs of synergism between *T. denticola* and other periodontopathogens *in vitro*. Table 4.5 summarizes such investigations. *Bacteroides forsythus* and *P. gingivalis* formed the best coaggregation partners with *T. denticola* (Grenier 1992a, Onagawa *et al.* 1994). Oral *Fusobacterium* strains were also reported to coaggregate with *T. denticola* (Kolenbrander *et al.* 1995). The spirochete fusiform coaggregation could be reversed by sugars like lactose or galactosamine, while the *B. forsythus-T. denticola* and *P. gingivalis* FDC 381-*T. denticola* ATCC 33520 association could not be reversed by sugars, serum, saliva, amino acids or EDTA (Yao *et al.* 1996, Onagawa *et al.* 1994). The *P. gingivalis-T. denticola* binding or coaggregation, however, appears to be strain-specific because *P. gingivalis* ATCC 33277-*T. denticola* GM-1 binding was shown to be on the contrary affected by co-incubation with diluted serum or saliva and arginine (Yao *et al.* 1996). Kolenbrander and London (1993) in their review article summarized the relationship of *T. denticola* and other treponemes to their coaggregation partners in dental plaque (Figure 1.6).

Periodontopathogens most likely do not adhere with each other without good reason. Several studies have shown that these organisms produce factors that stimulate synergistic growth of each other. For example, *P. gingivalis* was reported to produce isobutyric acid and proteinaceous factor (> 50 kDa) which promoted *T. denticola* growth (Grenier 1992b, Nilius *et al.* 1993). In return, *T. denticola* was reported to produce succinic acid which was required by *P. gingivalis* (Grenier 1992b). The possibility of spirochetes such as *T. denticola* acting as a carrier of non-motile bacteria forms (To *et* al. 1978) including the periodontopathogenic co-aggregation partners such as *P. gingivalis* or *B. forsythus* might be an add-on benefit for their synergistic relationship. Other late colonizers of dental plaque (Figure 1.6), were also found to be able to act

synergistically with *T. denticola* in terms of growth stimulation. These include *Prevotella intermedia*, *Eubacterium nodatum*, *Veillonella parvula* and *Fusobacterium nucleatum* (ter Steeg & Hoeven 1990). On the other hand, *Staphylococcus aureus* and *Streptococcus mutans* inhibited the growth of *T. denticola* have also been reported (Grenier 1996a, Table 4.5).

In summary, *T. denticola* possesses the periodontopathogenic potential as per *in vitro* experiments, including colonization, invasion and host tissue destructive capabilities. Serum samples from individuals with different clinical periodontal disease presentation also demonstrated antibody reactivity depicting a previous *T. denticola* challenge. Together with the fact that the spirochete thrives synergistically with other major periodontopathogens *in vitro*, the concept that *T. denticola* could act as a periodontopathogen *in vivo* is highly probable.

1.3.7. Mechanisms of pathogenicity

The periodontopathogenic potentials of *T. denticola* in terms of motility; ability of the whole spirochete to adhere; to invade or damage host cell/tissue; and modulation of host defense systems were reviewed and analyzed in the previous section (1.3.6.). In this section, attention will be given to summarize relevant individual *T. denticola* element(s) tested *in vitro*, which are postulated to be potentially of high virulence *in vivo* (Table 4.6).

Much attention had been given to the detection, isolation and identification of enzymatic or biologically active components of *T. denticola* at biochemical or molecular levels (Table 4.7). *T. denticola* possess hydrolytic enzymes such as hyaluronidase and proteases, that could be used by the bacteria in degradation of extracellular matrix components and disruption of host tissue structure as many other pathogenic bacteria (Salyers & Whitt 1994). Proteases also provide

bacteria with source of carbon and energy by breaking host polymers into usable amino acids etc. Proteolytic enzymes of *T. denticola* were widely studied (reviewed in Mäkinen & Mäkinen 1996). In summary, *T. denticola* possess a well characterized 95 kDa CTLP, sometimes reported as 98-100 kDa doublet, which is the main true proteinase that is expressed on the *T. denticola* OM (Table 4.6). Other than native collagen type I or rhemazo brilliant blue elastin, the 95 kDa CTLP digested totally or partly all native proteins or peptides tested: i) serum or interstitial proteins – serum albumin, fibrinogen, α_1-antitrypsin, antichymotrypsin, α_2-macroglobulin, antithrombin III, antiplasmin, cystatin, transferrin, IgG and IgA (Uitto *et al.* 1988b, Grenier 1990, 1996b), and ii) extracellular matrix proteins – fibronectin (cell associated or not), laminin, type IV collagen (Uitto *et al.* 1988b, 1992, 1995, Grenier 1990). CTLP could also activate latent PMN, HGF or dental plaque collagenase (Sorsa *et al.* 1992, 1995) or induce PLE cells to secrete collagenase *in vitro* (Uitto *et al.* 1992). A pro-inflammation role of CTLP was also reported including hydrolysis of inactivated substance P and conversion of angiotensin I to angiotensin II (Mäkinen *et al.* 1995b). Such proteinase could contribute, *in vivo*, significantly towards *T. denticola* survival and maintenance of the inflammation status in human diseased periodontal pockets.

Another 30.4 kDa chymotrypsin-like enzyme of *T. denticola* had been studied (Arakawa & Kuramitsu 1995). The corresponding gene, *prtB* from *T. denticola* Type strain (ATCC 35405) has been cloned and characterized (Table 4.7). The enzyme inhibition profile of such 30.4 kDa protein (PrtB) is slightly different from that of the 95 kDa CTLP. However, the protein degradation spectrum was shown to be much less for PrtB (Arakawa & Kuramitsu 1995). There is no evidence that PrtB is surface-exposed and there are no published reports that it has any role in periodontal disease pathogenicity.

Other non-proteinase peptidase activities were also identified from *T. denticola*. They included the arginyl/lysyl oligopeptidase (OPase), e.g. 50-65 kDa protein in *T. denticola* strain ASLM or 78 kDa protein in *T. denticola* Type strain (Mäkinen *et al.* 1990b); FALGPA-peptidase (a 62 kDa metallopeptidase, Mäkinen *et al.* 1992); endo-acting proline-specific endopeptidase (POPase, a 76 kDa protein, Mäkinen *et al.* 1994); proline iminopeptidase (PIPase, a 30.4 kDa protein, Mäkinen *et al.* 1996); and γ-glutamyl transpeptidase (GGT, a 26 kDa protein, native oligomeric form of 213 kDa; suggested to be a metabolite transporting agent, Mäkinen & Mäkinen 1997). Partially characterized peptidases were also reported, such as PZ-PLGPA peptidase (Mäkinen *et al.* 1986); BANA/B*ap*NA peptidases of 45 kDa, and 69 kDa (Mäkinen *et al.* 1986, Ohta et. Al. 1986); fibrinolytic enzyme of 91-228 kDa (Rosen *et al.* 1994, 1995), or up to 1000 kDa (Nitzan *et al.* 1978), plus various other unspecified proteinases or peptidases from crude *T. denticola* extract (Uitto *et al.* 1986, 1988a, Mäkinen *et al.* 1990a, Yoshida *et al.* 1990,. Alternatively the *T. denticola* peptidases were classified as proline-specific peptidase (PSPase) including the FALGPA-peptidase, POPase, PZ-PLGPA peptidase and the PIPase (Mäkinen & Mäkinen 1996). The main function of these peptidases *in vivo* was suggested to be digestion of collagen fragments (by PSPase), or other host tissue peptides and the transportation of breakdown products into *T. denticola* cells (by GGT) (Mäkinen & Mäkinen 1996).

Mäkinen and co-workers (1995b) also reported that the 95 kDa CTLP, together with POPase, and FALGPA-peptidase could degrade human bioactive peptides such as substance P, bradykinin, etc. This suggested that even though the non-proteinase peptidases could not digest host connective tissue proteins, they could contribute to the initiation and/or substantiation of the chronic inflammation in the periodontal tissues *in vivo*. The 95 kDa CTLP, although it could not degrade collagen type I (Uitto *et al.* 1988a, 1992), was found to be able to activate latent HGF,

PLE and dental plaque collagenases (Sorsa *et al.* 1992, 1995, Table 4.6) leading to collagen degradation *in vitro*. The ability of 95 kDa CTLP to degrade host protease inhibitors was also reported (Uitto *et al.* 1988b, Grenier 1996b, Table 4.6).

Other potentially pathogenic enzymes, isolated or detected from *T. denticola*, have also been reported. Included in the list are a 60 kDa phospholipase C (Chan *et al.* 1991), acid or neutral phosphatase (Norton Hughes & Yotis 1990), cysteine desulfhydrase (a 46 kDa protein demonstrating hemolysis, hemoxidation activities, H_2S production ability; also named hemolysin or cystalysin, Chu & Holt 1994, Chu *et al.* 1997), and hyaluronglucosaminidase (HGase, 59 kDa protein, Scott *et al.* 1996, Table 4.6).

Non-proteolytic or enzymatic *T. denticola* outer membrane proteins characterized so far have been mainly the major surface protein or Msp of 53/64 kDa, depending from what spirochete strain this was isolated (Weinberg & Holt 1991, Haapasalo *et al.* 1992, Kokeguchi *et al.* 1994). The native Msp is an oligomeric form. Reports have indicted that the protein may be glycosylated or lipoprotein in nature (Weinberg & Holt 1991, Sela *et al.* 1997, Table 4.6). The biological activities of the Msp suggest its involvement in attachment of the spirochete to eukaryotic cells or extracellular matrix macromolecules (Tables 4.6, 4.7). A recent study has shown that partially purified 53 kDa protein (contaminated with a small amount of CTLP) induced MMP-8, MMP-9, NGAL, elastase and cathepsin G release from PMN's (Ding *et al.* 1996). Some AP patients possess IgG against the 53 kDa protein (Kokeguchi *et al.* 1994, Table 4.6), indicating that this *T. denticola* protein might be immunogenic and might modulate host defense system. Intriguingly, *T. denticola* Msp was reported to be homologous to predicted products of a number of repetitive sequences present in the *T. pallidum* genome (*Treponema pallidum* server, 16 June 1997 [http://utmmg.med.uth.tmc.edu/treponema/tpall.html], Fenno *et*

al. 1998a). The Msp and its related peptide family might play a key role in induction and/or maintenance of the treponeme-host chronic inflammatory process. Fenno and coworkers had recently shown cytotoxic activity of *T. denticola* CTLP and Msp (Fenno *et al.* 1998a). A later study by the same group reported possible structural interactions possibly in terms of transport and assembly of the two protiens on *T. denticola* OM (Fenno et al. 1998b). Other OM protein species of *T. denticola* studied include the 44 kDa hemin binding protein (Chu *et al.* 1994), which might play important role in enabling the spirochete to survive in the harsh and competitive subgingival environment. Miscellaneous *T. denticola* proteins or biological activities studied so far have included the β-D-galactosidase activities from sonicated *T. denticola* extract, and probably an aspartate carbamoyltransferase (Suzuki & Watanabe 1988).

The pathogenic potential of *T. denticola* LPS or LPS-like materials have also been studied. The LPS-like material could induce superoxide or H_2O_2 production (Sela *et al.* 1988), release of MMP-9 and NGAL from normal human PMNs (Ding *et al.* 1996). However, the LPS-like material was found to be minimally cytotoxic to PLE cell *in vitro* (Grenier & Uitto 1993). Grenier and Uitto (1993) studied the cytotoxic effect of *T. denticola* peptidoglycan and reported that this material readily killed the PLE cells tested. Later, the same group reported that *T. denticola* peptidoglycan triggered release of MMP-9 and NGAL from PMNs (Table 4.6).

1.3.8. Summary

The oral spirochete *T. denticola* has been shown to have characteristics that could satisfy four out of the five criteria of the modified Koch's postulates. The induction of periodontitis in animal models may, for the time being, be hard to determine due to various limitations. However, based on the accumulated evidence gathered so far, most researchers will identify *T.*

denticola be one of the periodontopathogens (Dahle *et al.* 1993, Fenno and McBride 1998).

1.4. Statement of the problem

1.4.1. Hypothesis

Treponema denticola, a putative periodontopathogen, possesses virulence factor(s) or determinant(s) that could mediate its host-parasitic/pathogenic relationship in human periodontal disease.

1.4.2. Aim

i) To investigate the cytopathic and/or cytotoxic effects of *Treponema denticola* on eukaryotic cells using a porcine junctional epithelial cell (PLE) model,

ii) To identify the "putative virulence factor(s)" in whole *T. denticola* that may contribute to the most readily observable event(s) in the bacterial-PLE cell interaction,

iii) To characterize the "putative virulence factor(s)" of *T. denticola* by studying the corresponding effects of those semi-purified, or purified native factor(s) on PLE cells,

iv) To determine the probable ways by which the "virulent factor(s)" might cause such cytotoxic/cytopathic effect on PLE cells.

1.5. Addendum

A portion of the present investigation was published (Haapasalo *et al.* 1992, Egli *et al.* 1993, Uitto *et al.* 1995, Leung *et al.* 1996, Mathers *et al.* 1996, Fenno *et al.* 1997, 1998). In order to put the results of this investigation in context with current available information, summary tables were made up (Tables 4.1 – 4.7) and were referred to as appropriate. Also note

that a proportion of the Results, Discussion, Tables and Figures are taken directly (verbatim) from the work published during the course of this investigation.

Table 1.1 Components of extracellular matrix in periodontium (modified from Bartold 1995).

Fibrous:	Collagen Elastin
Ground substances:	Glycoproteins and non-collagenous proteins fibronectin laminin vitronectin thrombospondin tenascin or cytotactin entactin (nidogen) Proteoglycans Extracelluar matrix proteoglycans versican decorin Cell surface proteoglycans syndecan glypican betaglycan CD-44 Glycosaminoglycans hyaluronan heparan sulphate (GCT) dermatan sulphate (PDL) chrondroitin sulphate keratan sulphate Lipids Mineral Water
Growth factors:	Fibroblast growth factors Transforming growth factors Platelet-derived growth factors

Table 1.2 Subgingival species associated with health, gingivitis and periodontitis (From Darveau *et al.* 1997).

Health	**Gingivitis**	**Periodontitis**
Streptococcus oralis[g]	*Streptococcus oralis*	*Porphyromonas gingivalis*[g]
Streptococcus sanguis[g]	*Streptococcus sanguis*	*Actinbacillus actinomycetemcomitans* serotype b
Streptococcus mitis[g]	*Streptococcus mitis*	*Bacteroides forsythus*[g]
Streptococcus gordonii	*Streptococcus intermedius*	*PRO spirochete*
Streptococcus mutans	*Capnocytophaga ochracea*	*Treponema denticola*
Streptococcus anignosus	*Capnocytophaga gingivalis*	*Prevotella intermedia*[g]
Streptococcus intermedius	*Campylobacter gracilis*	*Prevotella nigrescens*[g]
Gemella morbillorum	*Prevotella loescheii*	*Campylobacter rectus*[g]
Rothia dentocariosa	*Peptostreptococcus micros*	*Peptostreptococcus micros*
Actinomyces naeslundii	*Eubacterium nodatum*	*Fusobacterium nucleatum* subsp. *vincentii*
Actinomyces gerencseriae	*Actinomyces naeslundii*	*Fusobacterium nucleatum* subsp. *nucleatum*
Actinomyces odontolyticus	*Actinomyces israelii*	*Selenomonas noxia*
Peptostreptococcus micros	*Campylobacter concisus*	*Selenomonas flueggei*[g]
Eubacterium nodatum	*Actinomyces odontolyticus*	*Enteric species*
Capnocytophaga ochracea	*Fusobacterium nucleatum* subsp. *nucleatum*	*Fusobacterium alocis*
Capnocytophaga gingivalis	*Eubacterium brachy*	*Lactobacillus uli*[g]
Campylobacter gracilis	*Eikenella corrodens*	*Veillonella parvula*[g]
Fusobacterium nucleatum subsp. *polymorphum*	*Actinobacillus actinomycetemcomitans* serotype a	

[g] Species also associated with gingivitis. Any subject can at any time be colonized by species from other clinical categories, but usually these additional species constitute a minor segment of the subgingival microbiota. Species in the Table are ordered with those being more likely to be found at the top and less likely at the bottom.

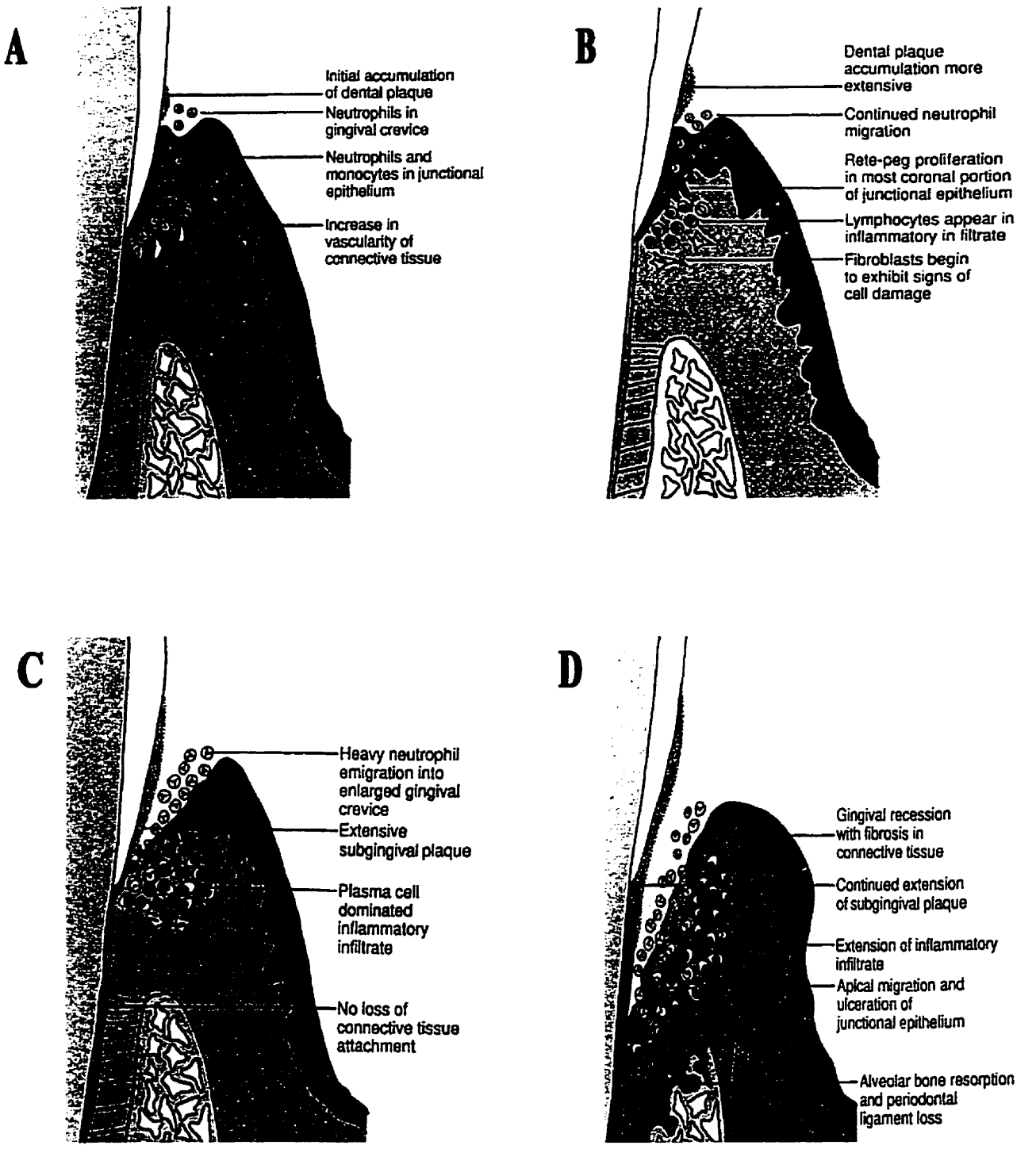


Figure 1.1 Periodontal disease manifestations. The components of the periodontium at the dentogingival junction with A) Early gingivitis: 1 week of plaque accumulation, B) Gingivitis: 2 weeks of plaque accumulation, C) Chronic marginal gingivitis, D) Periodontitis. (From Williams et al. 1992)

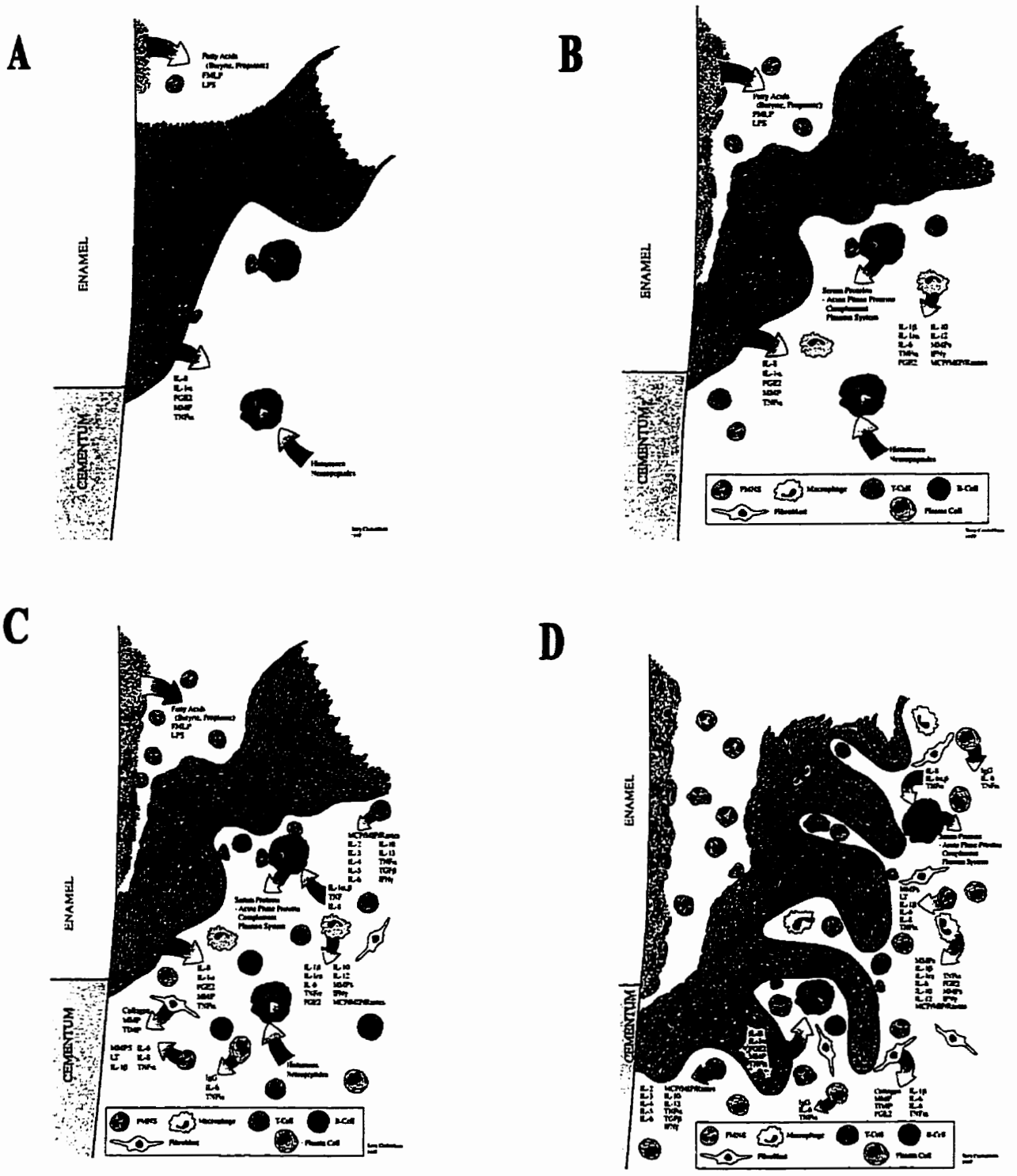
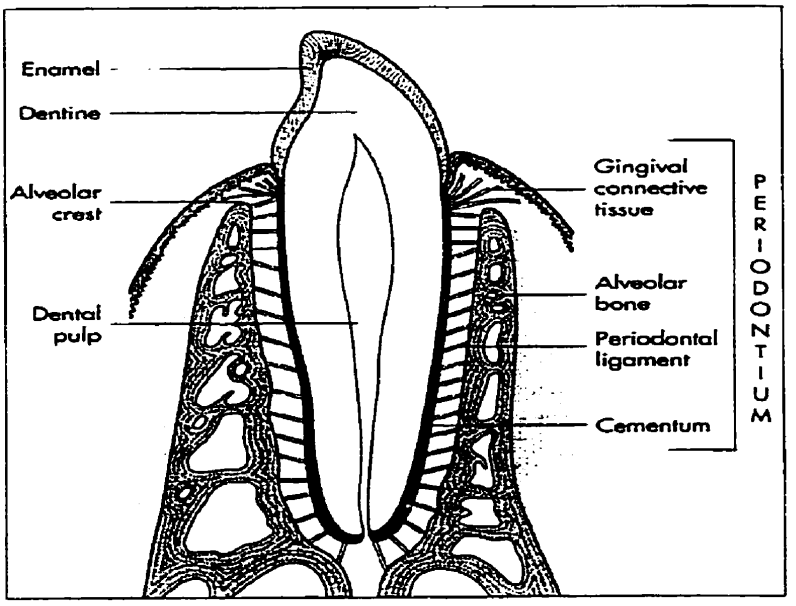


Figure 1.2 The local subgingival host response to microbial challenge. A) Acute bacterial challenge phase, B) Acute inflammatory response phase, C) Immune response phase, D) Regulation and resolution phase. (From Kornman et al. 1997).

A



B

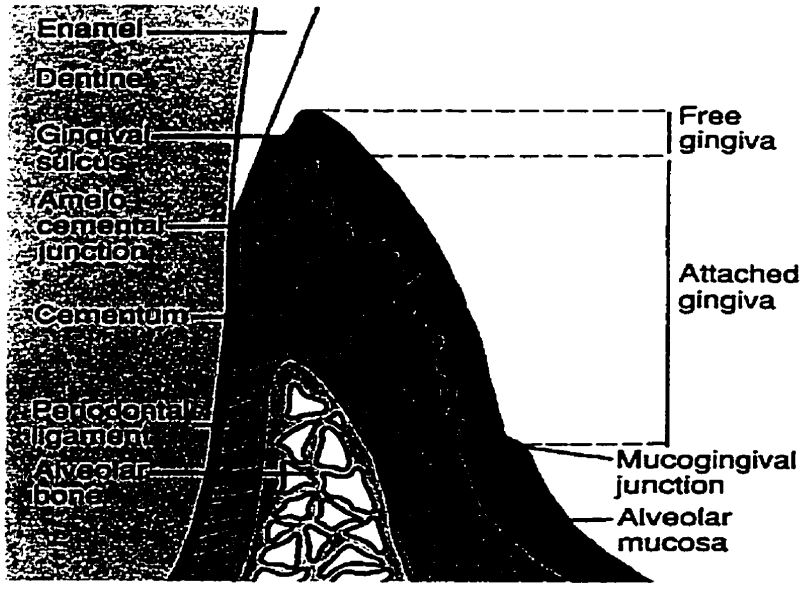
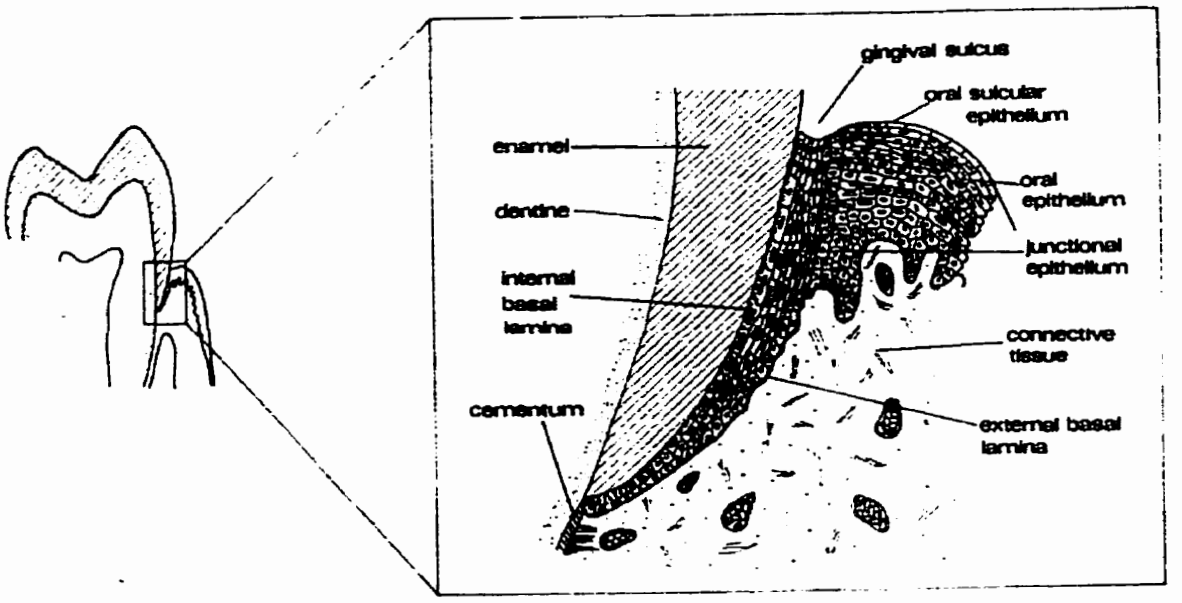


Figure 1.3 The periodontium in health. A) Components of the periodontal ligament (From Berkovitz et al. 1995); B) Components of the periodontium at the dentogingival junction (From Williams et al. 1992).

A



B

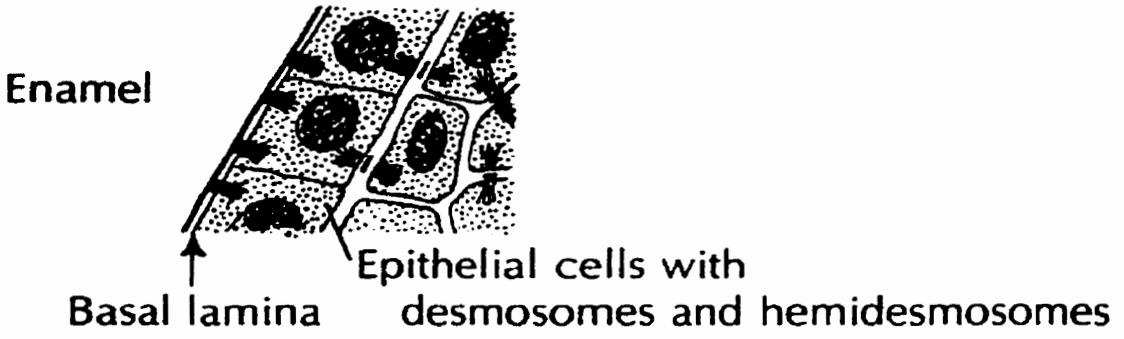


Figure 1.4 Schematic drawing of junctional epithelium. A) Organization of epithelium at the dentogingival junction (From Squier et al. 1975); B) Nature of the attachment between junctional epithelium and tooth surface (From Moss-Salentijn & Hendricks-Klyvert 1990).

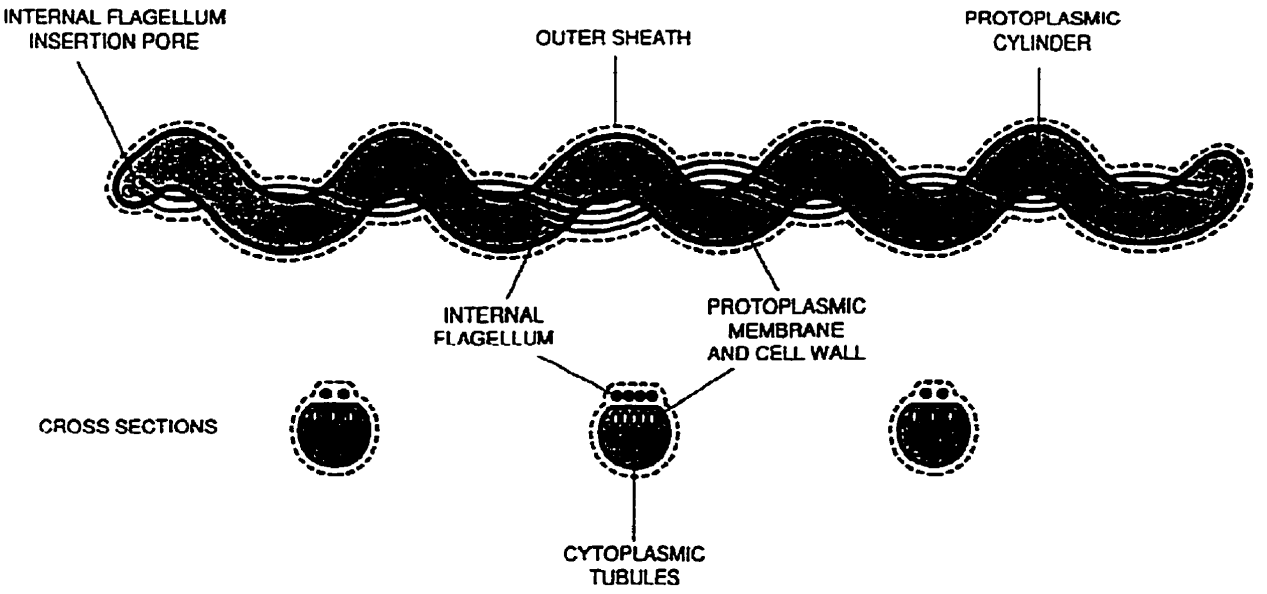


Figure 1.5 Schematic drawing of a treponeme. (From Hudson 1991)

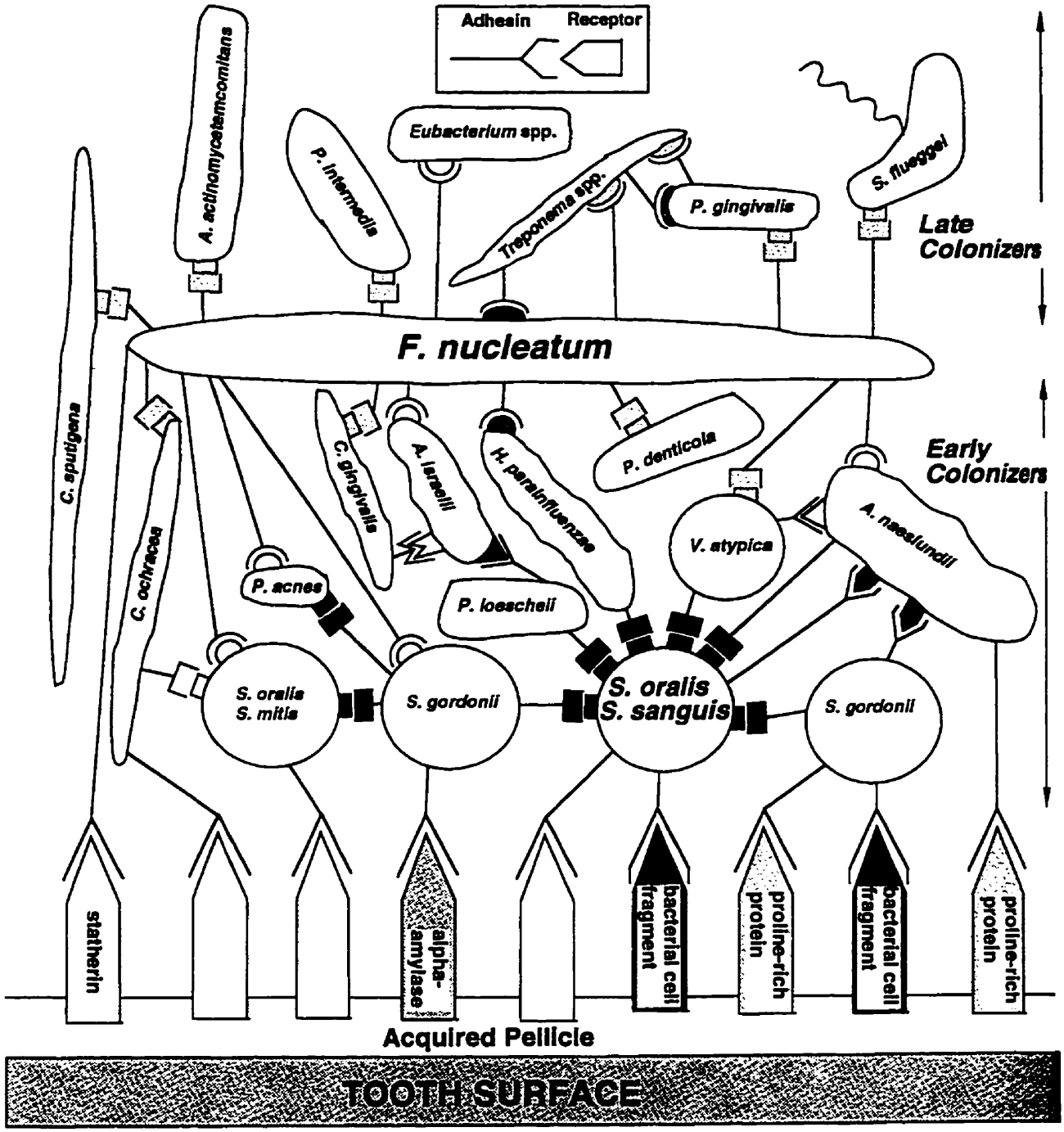


Figure 1.6 Diagrammatic representation of inter-bacterial relationships between *Treponema denticola/Treponema* species with other microbes in dental plaque. (From Kolenbrander & London, 1993).

CHAPTER II Material and Methods

2.1. Bacterial strains and culture conditions

T. denticola ATCC 33520, ATCC 35404, ATCC 35405 and *T. pectinovorum* ATCC 33768 were obtained from the American Type Culture Collection. *T. denticola* OTK was a gift from Dr. Russell Johnson of the University of Minnesota. All strains were grown anaerobically in NOS broth (Leschine & Canale-Parola, 1980) or NOS broth supplemented with 0.3% pectin (Weber & Canale-Parola 1984). Purity of the cultures was checked regularly during weekly transfer by phase-contrast microscopy. Spirochetes of 3-5 days-old were used for experiments. If indicated, *T. denticola* cultures were subjected to various physical, chemical or antibody pretreatment before the experiments. For the pretreatment experiments and the inhibition studies, washed *T. denticola* samples were pretreated with one of the following for 1 h, 4°C before addition to PLE/FPLE: 0.1-2mM PMSF; 0.3-2mM TPCK; 0.1-2mM TLCK; FBS; BSA; fibrinogen; fibronectin; anti-*T. denticola* serum/IgG; anti-Msp serum/IgG; anti-CTLP serum/IgG; anti-rMsp IgG; control/pre-immune rabbit serum/IgG. Protease inhibitors were removed before adherence assays by repeated washing of the treated spirochetes in PBS. Parallel untreated *T. denticola* samples were subjected to identical incubation and washing condition. SAAPNA activities of the samples, were monitored before and after treatment.

In some occasions, the particulate fraction of *T. denticola* was used and this was obtained as follows. The spirochete cells were disrupted in 20mM Tris buffer (pH 8.0) by ultrasonication (3-min pulse sonication at 20% duty cycle, output of 20 using a Sonifier cell Disrupter 350; Branson Sonic Power Co., Danbury, CT). Unbroken cells were removed by centrifugation (8,000 × *g*, 10 min). The supernatant was centrifuged again at 16,000 × *g* for 10 min, and the

upper, lighter-colored, area of the pellet was carefully collected, washed twice, and used for the experiments as the particulate fraction.

2.2. Cell culture/cell lines and culture conditions

Cell cultures used and their corresponding growth conditions were as listed in Table 2.1. All cell cultures were maintained in a humidified, 5% CO_2 atmosphere at 37°C. HGF and PLE cells between the fifth and tenth passages were used for experiments. The cell cultures were plated and maintained in wells of 8-well chamber slides (Nunc, Naperville, IL) to predetermined confluence level (30% or 95%) for morphometric, immunofluorescence and microscopic studies. For ELISA experiments, LDH, MTT, thymidine uptake, vacuolation and trypan blue uptake assays, the cells were plated onto wells of 96-well flat bottom tissue culture plates (Falcon NJ). The cells were plated at approximately 10,000 cells per well (counting performed using Coulter Counter, Coulter Electronic Inc., Hialeah, FL). When the cells were at an early confluent stage, they were washed once with plain culture medium before assays. For some *T. denticola* or *T. denticola* outer membrane protein attachment experiments, the cells were fixed with glutaraldehyde. The cells on chamber slides or 96 well plates were fixed with glutaraldehyde as described by Stanislawski and co-workers (1985). In brief, monolayers of cell cultures were washed twice in PBS+ and fixed with 0.25% glutaraldehyde in PBS+, 10 min, room temperature followed by 3 times wash with PBS+. The free aldehyde groups on the fixed cells were blocked with 0.1% BSA in 0.2M glycine 30 min, room temperature, then 1% BSA PBS+, 30 min, room temperature. The fixed cells were then ready for attachment assays to be conducted within 4 hours post-fixation.

2.3. Cytopathic assays

In general, *T. denticola* induced PLE cytopathic changes were assayed using PLE cells on chamber slides. *T. denticola* suspensions of various concentrations, were used to challenge the PLE cells for a predetermined period of time. This protocol was also applied for some of the cytotoxic assays.

2.3.1. Morphometric study

T. denticola treated PLE cells were washed (PBS, 37°C) fixed (3.3% formaldehyde in 0.9% w/v NaCl, pH 7.3, at 37°C, 20 min) and stained with May-Grünwald and Giemsa stains as described by Sedgley *et al.* (1996). In brief, the fixed PLE cells were stained with May-Grünwald stain (E. Merck, Darmstadt, Germany) diluted 1:2 in PBS, 5 min, then Giemsa stain (Merck) diluted 1:3 in PBS for 20 min followed by soaking in PBS for 10 min. After staining, the preparations were air dried and then mounted with Permount (Fisher Scientific, NJ). The May-Grünwald and Giemsa stained slides were examined microscopically under × 40 magnification (Axioplan, Zeiss, Germany) with a scanning stage, a video camera (DXC-930P, Sony 3CCD, Japan) and image analyzer system (IBAS, Kontron, Germany). The image displayed on the monitor represents a field of 165 × 247.5 μm^2. The settings of the image analysis software were designed to perform a systematic sampling of the specimen on the chamber slide. The first sample site was randomly selected at the upper left hand corner of the specimen, approximately 1.5 mm away from the margins. Once the first sample site was determined, the whole preparation was scanned/sampled systematically using pre-determined regular spacing (in the present setting, 4 × 5 fields per well of the chamber-side). In brief, after the first sample site was measured, the microscope stage automatically moved to the next

predetermined position until 20 fields were measured. The stage was programmed to move along the x-axis at an interval of 6 fields (1.5 mm) and along the y-axis at an interval of 7 fields (1.2 mm). Four wells on the chamber slide were prepared for the same test, i.e. 80 systematic fields were measured per experimental condition. Each experiment was repeated at least 3 times. The morphometric analysis was carried out as the followings. The system made use of differences in greyness of the images captured so that various parameters could be measured. In brief, at the start of the experiment, after lighting adjustment and then proper greyness cut-off selected, the machine was ready for measurement automatically. Due to the differential up-take of stains for the cell nucleus versus cytoplasm, both cell number and cell area could be measured simultaneously, using a higher grey level cut-off for cell count (equals to number of nuclei stained) and a lower grey level cut-off for cell area or confluence (area on substratum covered by cells). Mean cell size was also measured. For the sake of objectivity, the total count of the nuclei was taken as the exact cell number without taking into consideration some cells which might be only partially included in the selected field. All measurements were carried out automatically by the IBAS system as pre-set; with only supervision of smooth running of the recording.

2.3.2. Vacuolation assay

After the spirochetes' challenge to PLE, the vacuolation induced, if any, was detected as described by Cover *et al.* (1991). The treated PLE were washed once with warm (37°C) sterile PBS and then stained with neutral red: A stock solution of 0.5% purified grade neutral red (Sigma, St. Louis, MO) was prepared in 0.9% saline and filtered. Staining solutions were prepared before each experiment by diluting the stock solution 1:10 in 10% FBS-αMEM. 200 μl

of staining solution was added into each well of the chamber slide for 4 min. The cells were washed twice with 300 µl of 0.9% saline per well and then fixed, air dried and mounted for differential counting under an IBAS image analysis system as described previously in section 2.3.1. The only variation to the methodology was that the quantification of vacuolation had to be performed semi-automatically with a recorder reading from the monitor. PLE cell specimens treated with *T. denticola* under identical conditions were stained with May-Grünwald and Giemsa stains for mean count per microscopic field measurement.

2.3.3. Other assays

Other *T. denticola* induced effects on cell cultures, including blebbing and cytoskeletal re-arrangements, were investigated using immunofluorescence microscopy, SEM, May-Grünwald and Giemsa stains followed by quantifying image analysis. Immunofluoresence microscopic study of *T. denticola* whole cell or Msp complex induced actin rearrangement in cell culture was carried out as described in section 2.4.1. SEM ultrastructural assay on the effect of *T. denticola* on cell culture was studied as described in the later section 2.7.1. The number of attached cells showing blebbing was measured either by differential microscopic counting of fluorescence labeled cell preparations or by quantifying image analysis using the IBAS system. May-Grünwald and Giemsa stains, stained the blebs on the eukaryotic cells more intensely. Basically, the same procedures described in section 2.3.1. were used to quantify blebbing. The amount of cells with blebbing was measured semi-automatically with a recorder reading the blebbed cell count from the IBAS system monitor, while counts of cells per microscopic field was performed as described previously in section 2.3.1.

2.4. Adhesion assays

2.4.1. Immunofluoresence microscopy

The cells grown in 8-well chamber slides, viable or fixed, were washed once with plain growth medium and mixed with 0.2 mL of *T. denticola* suspended in the same medium. The mixture was incubated at 37°C in an atmosphere of air and 5% CO_2. Controls consisted of PLE exposed to the same medium that was used to suspend the spirochetes.

Preparation of samples for fluorescence microscopy was done as follows: after a predetermined time-period of challenge by *T. denticola*, the cell cultures were washed twice with culture medium without serum and antibiotic, fixed with 2% formaldehyde - 0.05% glutaraldehyde - PBS, pH 7.3 for 30 min and washed twice with PBS. The cells were then permeablized by 0.2% saponin (Sigma, St. Louis, MO) in PBS for 20 min, blocked by 0.5 mg/mL sodium borohydride (Fisher Scientific, NJ) in 0.2% saponin-PBS for 20 min, followed by blocking with 3% BSA (Sigma, St. Louis, MO). Double-labeling for *T. denticola* and actin was performed by treating the samples first with anti-whole *T. denticola* antibody (1:3000) and then with goat anti-rabbit IgG-FITC (1:200) mixed with 5 units/mL of rhodamine phalloidin (Molecular Probes, Eugene, OR). The specimens were rinsed in PBS, then mounted in 10% (w/v) DABCO (Sigma, St. Louis, MO)-0.02% (w/v) sodium azide (Fisher, Scientific NJ) in 1:1 PBS-glycerol, and then examined with a confocal laser scanning microscope (LSM, Zeiss). Experiments were carried out in quadriplicate and were repeated in 3-4 independent occasions.

The *T. denticola* – PLE cells adhesion data obtained were analyzed with Langmuir isotherms, Scatchard plots and Hill plots as described previously (Gibbons *et al.* 1976, Dahlquist 1978, Morris & McBride 1984). In brief, the model assumes that there are a finite number of identical sites for attachment per unit PLE cell area and the adhesion process is reversible. Then

the following equation could be drawn at attachment equilibrium:

$$\frac{C}{Q} = \frac{1}{KN} + \frac{C}{N}$$

Where C = concentration of free cells at equilibrium, N = are the maximum number of receptor or binding sites per PLE cell, and Q = the number of *T. denticola* adsorbed on to the PLE monolayer, K = proportional constant. Hill coefficients for the attachment experiments were then calculated as per Morris and McBride (1984). In case of a single non-interacting site, the Hill coefficient is unity. For positive or negative cooperativity in ligand binding, the Hill coefficient will be >1 or <1 respectively (Dahlquist 1978).

2.4.2. ELISA

Alternatively, attachment of *T. denticola* or *T. denticola* proteins to cell monolayers was assayed by an ELISA method. The bacteria/material with or without various physical, chemical or antibody pretreatment were appropriately suspended at various concentrations before being added to the cell culture. 200 μL of *T. denticola* cells or outer membrane protein suspensions in PBS were added to the wells of the microtiter plate. Following a predetermined duration of incubation at 37°C, room temperature or 4°C, the wells were rinsed twice with 0.05% Tween 20 PBS and once with PBS. *T. denticola* whole cell antiserum (1:3000 in 1% BSA-PBS) was added to the wells then incubated for 1 h, room temperature, and rinsed as above. Goat-anti-rabbit antibody conjugated to alkaline phosphatase (1:3000; Bethesda Research Laboratories) in 1% BSA-PBS was added to the wells and let incubate for 1 h at room temperature. Color formation of the chromogenic substrate for alkaline phosphatase (pNPP, Sigma *Fast*™ kit, Sigma, St.

Louis, MO) was monitored at 405 nm using a microplate reader (BioRad, Model 3550). Attachment of PLE to the well after the treatment was assayed using an Olympics CK2 inverted microscope.

2.5. Cytotoxicity assays

2.5.1. MTT

Viability, or the microculture tetrazolium assay (MTT assay), on the effect of *T. denticola* towards various eukaryotic cells were carried out as described by Mosmann (1983) using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO). In brief, cell lines or explants on 96-well plates were challenged with 100 μL *T. denticola*, pre-treated or not, in appropriate dilutions (in culture media free of phenol red and antibiotics but with 1% FBS; in the case of HGF, also without ascorbic acid) were added onto designated wells and let incubate for 24 h, at 37°C, 5% CO_2 in air. Afterwards, MTT freshly dissolved in PBS at 5 mg/mL was filter-sterilized and then 10 μL was added to the wells and incubated at 37°C for 2 - 4 h. 150 μL of acidified sodium dodecyl sulphate (SDS) dimethyl formamide solution(20% SDS in 50% dimethyl formamide pH to 4.7 with acetic acid) was added to all wells and mixed thoroughly to dissolve any dark blue formazan crystals formed. The plate was then read using a microplate reader (BioRad Model 3550) at 595 nm. The percentage viability of the cells after *T. denticola* challenge was computed using the following equation:

$$\text{Viability} = \frac{E - CB - EC}{CS - CB - MB} \times 100\ \%$$

with CB = cell blank; CS = cell spontaneous MTT reduction; E = experimental MTT reduction;

EC = effector (*T. denticola*) MTT reduction control; MB = medium blank.

2.5.2. LDH

Cytotoxicity of *T. denticola* and *T. denticola* components toward cultured cells were quantified by measuring release of the cytosolic enzyme lactate dehydrogenase (LDH) in culture supernatants (Korzeniewski and Callewaert 1983) using the CytoTox 96^{TM} kit (Promega, Madison, WI). Confluent cell cultures in 96-well plates were washed in growth media lacking FBS and antibiotics, bathed in growth media lacking FBS, antibiotics, and phenol red, then challenged with *T. denticola* preparations in tissue culture media or PBS. After challenge, 50 µL of the supernatant was transferred to a fresh 96-well plate, followed by detection of the LDH activity according to manufacturer's protocol. Absorbance at 490 nm of each well was determined using a BioRad Model 3550 microplate reader, and compared to positive lysis control (supplied by the manufacturer) and a negative lysis control (saline or growth media lacking FBS, antibiotics, and phenol red). Cultures were also inspected microscopically before and after challenge. The ability of various compounds and antibody pre-treatments in inhibiting cytotoxic effects of *T. denticola* and *T. denticola* proteins was tested as described in the previous section (2.1.).

2.5.3. Trypan blue dye exclusion

Viability of PLE cells under the influence of *T. denticola* was measured by trypan blue exclusion as described by Martin and Clynes (1993). After the spirochetes' challenge, the detached PLE cells in the 20 µL *T. denticola*-PLE interaction supernatant was mixed with 40 µL of filtered trypan blue solution in PBS (0.4%, w/v, Gibco) in a microcentrifuge tube. After 5 - 15

minutes, the detached PLE cells were differentially counted under wet mount using a hemocytometer (Neubauer Improved, Germany) for colored ("non-vital") and dye-excluding ("viable") cells. 300 - 500 cells per preparation were counted. The PLE cells remaining attached to the chamber slide were stained as follows: the preparation was washed once with warm (37°C) PBS pH 7.3, stained with filtered 0.1% trypan blue solution in PBS 5 - 15 min at room temperature, briefly rinsed with distilled water and then air dried and permanently mounted for differential counting under the IBAS image analyzing system (Kontron, Germany). PLE cell specimens treated with *T. denticola* under identical conditions were stained with May-Grünwald and Giemsa stains for mean count per microscopic field measurement.

2.5.4. Detachment assay

Detachment of PLE cells or other cell cultures was studied indirectly by morphometric image analysis as per section 2.3.1, or crystal violet dye elution (Kueng *et al.* 1989, Scragg & Ferreira 1991). In brief, cell lines or explants were plated onto 96-well plates (Falcon) at approximately 10,000 cells per well. When the cells were confluent, they were washed once with plain culture medium. Next, 100 µL *T. denticola* in appropriate dilutions (in full strength culture media free of phenol red and antibiotics) were added onto designated wells and incubated for 24 h at 37°C, 5% CO_2 in air. Afterwards, the culture supernatant (*T. denticola* and detached cells) were removed, the wells were rinsed with 200 µL warm (37°C) PBS/well, fixed with 2% glutaraldehyde in PBS, 20 min, air-dried, and stained with 100 µL 0.25% (w/v) filtered aqueous crystal violet in 0.2 M 2-[N-Morpholino]ethanesulfonic acid, pH 6.0 for 20 minutes. Plates were then rinsed 4 times in distilled water and air-dried at 37°C overnight. When dry, 100 µL/well of 10% glacial acetic acid (v/v) was added with repeated pipetting to dissolve the contents of each

well. The percentage proportion of PLE cells detachment was then estimated by quantification of the amount of crystal violet dye eluted from test versus control wells using a microplate reader (BioRad, Model 3550) at 590 nm. The percentage detachment of the cells after *T. denticola* challenge was computed using the following equation:

$$\text{detachment} = \frac{C-E}{C-CB-MB} \times 100\%$$

When C = untreated control; CB = cell blank; E = experimental; MB = medium blank.

2.5.5. Thymidine uptake

T. denticola induced PLE cell proliferation was studied as per Putnins (1995). The PLE cells were seeded at a density of 7.5×10^3 cell/cm^2 on 96-well tissue culture plate. The cells were cultured in 15% FBS-αMEMS for 1 - 3 days until 50% confluent and sequentially cultured for 2 days in 1% FBS-αMEMS to bring the cells to quiescence. *T. denticola* at varying concentrations in 1% FBS-αMEMS was added to cultures. *Escherichia coli* lipopolysaccharide (L-2880; Sigma, St. Louis, MO) was included in the experiment as a positive control (10 μg/ml). After 15 hours incubation with *T. denticola*, 10 μCi/ml of $^3_1$H - thymidine (specific activity - 2.0 Ci/mmol, ICN Biomedicals, Mississauga, Ontario) was added for 24 h. The cells were washed twice with PBS, detached from culture plated with 10 x trypsin-EDTA (Gibco Laboratories, Grand Island, NY), lysed with distilled water and then the DNA collected with a cell harvester (Mini-MASH II; Whittaker Bioproducts Inc., Walkerville, MD) onto glass microfibre filters 934/AH (Whatman, Maidstone, England). The filters were dried and tritiated-thymidine incorporation was subsequently measured with a liquid scintillation counter (MacNeil *et al.* 1986).

The PLE cell proliferation was computed as follows:

$$^{3}_{1}H \text{ - thymidine uptake} = E - EC - MB$$

when E = experimental $^{3}_{1}H$ - thymidine uptake; EC = effector (*T. denticola*) $^{3}_{1}H$ - thymidine uptake control; MB = medium blank.

2.5.6. Apoptosis assays

2.5.6.1. TUNEL

T. denticola induced PLE cells apoptosis was studied using an *in situ* cell death detection kit (Boehringer Mannheim Biochemicals, IN). The recommended protocol for adherent cells was followed. In brief, the spirochete treated PLE cells were washed, fixed and then blocked by 0.3% H_2O_2 in methanol against endogenous peroxidase, 30 min, room temperature. After rinsing, the preparations were trypsinized by warm (37°C) 0.05% trypsin (Sigma, St. Louis, MO) in 0.7 mM $CaCl_2$ - 50 mM Tris buffered saline (pH 7.4) 20 min 37°C. The preparations were washed with PBS and TUNEL reaction mixture was added for 60 min, 37°C and then washed again. Anti-fluorescein antibody conjugated with horse-radish peroxidase was added, incubated at 37°C, 30 min, followed by DAB substrate solution for 5 - 20 min, room temperature. When optimum color was developed, the samples were lightly counter-stained with haematoxilin, before dehydration and mounting. The apoptotic cells showing positive TUNEL (darker stained) were quantified using the IBAS image analysis system.

2.5.6.2. TEM

Apoptosis of PLE cells was also studied using TEM. PLE cells challenged by *T.*

denticola cells or proteins were processed and examined as described in section 2.7.2.2. Special attention was paid to observing presence or absence of PLE cellular changes that are suggestive of apoptosis such as described in Figure 3.15.

2.6. Screening for protease activities

Proteolytic activities of *T. denticola* strains or *T. denticola* protein extracts were examined using substrate gel zymography or chromogenic substrate microplate assay.

2.6.1. Zymography

Proteinase substrate zymography was carried out as described previously (Heussen & Dowdle 1980, Kelleher & Juliano 1984). BSA or gelatin were either covalently bound to linear polyacrylamide (Kelleher & Juliano 1984, Uitto *et al.* 1988) or simply added into the polyacrylamide mixture (Heussen & Dowdle 1980). Slab gel electrophoresis was carried out as described in the following section 2.10.1. The slab gel was cast with protein-conjugated acrylamide or with protein in acrylamide to the final concentration of 0.1 mg/ml. Before loading the protein samples onto gel, the samples in SDS-sample buffer were incubated for 30 min at 37°C. After electrophoresis, the gels were gently shaken for 30 – 60 min in 2% Triton X-100-0.1 M Tris buffer, pH 7.0 to remove the SDS. Gels were then incubated at 37°C for 2 h in 50 mM L-cysteine-0.1 M Tris, pH 7.0 to allow the enzymatic degradation of the conjugated protein substrates. The gel was then stained with Coomassie brilliant blue. After the gel was destained, the presence of proteolytic activity was visualized as a clear band against a blue background. The percentage of acrylamide used was dependent on the molecular weight of the protease investigated.

2.6.2. SAAPNA assay

Hydrolysis of SAAPNA was determined by incubating samples at 37°C with the chromogenic substrate (final concentration, 2 mM) in 2 mM DTT-50 mM Tris, pH 7.2. Enzyme activity was determined by measuring absorbance at 405 nm using a microplate reader (BioRad, Model 3550) after incubation (Grenier *et al.* 1990).

2.7. Ultrastructural assays

Ultrastructural microscopic assays were carried out to study the ultrastructure of *T. denticola* whole cell and outer membrane preparations. Similar protocols were also employed to study the effects of *T. denticola* whole cell or proteins on eukaryotic cells in culture.

2.7.1. Scanning electron microscopy

PLE cells grown on coverslips or polycarbonate membranes were used for the study. After bacterial/bacterial protein challenge, the cells were rinsed in PBS, 37°C and then fixed with 2.5% glutaraldehyde in PBS at 4°C for 16 h. and processed by the tannic acid technique of Katsumoto *et al.* (1981). Specimens were DC-sputtered with 15-20 nm gold in an Edwards coating unit (Gibco, BRL), and examined using a Cambridge Stereoscan microscope (Cambridge, U.K.).

2.7.2. Electron microscopy

2.7.2.1. Negative staining

A negative staining technique was used to study the ultrastructure of *T. denticola* outer sheath. Twenty milliliters of 3-day-old cultures of *T. denticola* ATCC 35405 was harvested and

washed 2 times by PBS, 4°C. The washed cell pellet was suspended in PBS and subjected to mild ultrasonication (1-min pulse sonication at 20% duty cycle and output of 2 with a Sonifier cell Disrupter 350 (Branson Sonic Power Co., Danbury, Conn.). After sonication, the suspension was diluted with PBS to an OD at 660 nm of 0.2 and applied to a Parlodion film-covered, carbon-stabilized copper grid. Routine negative staining was carried out with 4% uranyl acetate, pH 4.5, 2 minutes (Masuda & Kawata 1982). Specimens were examined using a Philips 300 electron microscope operating at 60 or 80 kV.

2.7.2.2. Ultra-thin sectioning

PLE cells grown on polycarbonate filters (Falcon) for 5 – 7 days in 15% FBS-αMEMS were challenged by *T. denticola* whole cells or Msp complex in 1% FBS-αMEM for predetermined periods. The specimens were processed as outlined by Reynolds (1963). In brief, the preparation was rinsed with warm (37°C) PBS and then fixed with 2.5% glutaraldehyde in Sorensen's phosphate buffer, pH 7.2, 4°C for 16 h and post-fixed sequentially in alternate 1% osmium tetroxide and 2% thiocarbohydrazide in Sorensen's buffer, 20 min each, stained in a block by 1% aqueous uranyl acetate, 4 min, and then dehydrated. The dehydrated specimens were infiltrated with Epon-Araldite resin (Polyscience) polymerized at 55°C for 3 days. Ultra-thin sections were then cut and put onto 200 mesh grids, stained sequentially with saturated uranyl acetate and lead citrate. The samples were examined in a JEOL JEM 100 SX electron microscope (Japan) at 60 kV.

2.7.2.3. On-grid immunogold labeling

On-grid immunogold labeling was carried out for *T. denticola* whole cell or outer sheath

preparations or PLE cell sections after treatment by the spirochete. Fixed *T. denticola* whole cells or PLE cells on polycarbonate filters were embedded into LR-Gold resin (Ted Pella, CA) according to the manufacturer's instructions. In brief, the dehydrated specimen was infiltrated with increasing concentrations of LR-Gold preparation and then the specimens in LR-Gold-catalyst mixture were polymerized by *uv* as per manufacturer's instructions at –25°C. The sections were placed on Parlodion-coated nickel grids and dried at room temperature. After quenching the residual aldehyde molecules with 0.5 M NH_4Cl, the samples were blocked with 0.05% Tween 20-1% goat serum-50 mM Tris (pH 7.2). Serial dilutions at 1:20 to 1:500 of primary antibody, i.e. either anti-whole *T. denticola* IgG or anti-*T. denticola* 53 kDa protein IgG, were added overnight at 4°C, followed by gold conjugated goat anti-rabbit antiserum at 1:50 for 8 h. The specimens were counter-stained by uranyl acetate-lead citrate method (Newman 1989).

Single or double immunostaining techniques were used to study the location of the surface proteins on *T. denticola*. In brief, whole or sonicated *T. denticola* preparations were applied to Parlodion film covered, carbon-stabilized copper grid. The location of target protein on whole *T. denticola* or isolated sheaths of *T. denticola* was studied by immunoelectron microscopy. Bacterial preparations were washed, incubated for 1 h, 20°C with primary antibody (1:20 or 1:50), followed by secondary antibody (1:50) for 1 h, 20°C. Bound antibodies were detected with goat anti-rabbit antibody and/or goat anti-mouse antibody conjugated to gold particles (5 nm or 10 nm respectively, Sigma). After being washed, the grids were negatively stained with 4% uranyl acetate and studied by transmission electron microscopy (Philips 300) at 60 kV.

2.8. Preparation of *T. denticola* proteins

2.8.1. Detergent extraction

T. denticola protein detergent extraction was carried out as follows. Freshly cultured spirochete cells were incubated in 20 mM Tris buffer (pH 8.0) in the presence of a variety of anionic and nonionic detergents: 1% NP-40 (Sigma), 0.1% Triton X-100 (Sigma), 0.001 to 0.5% SDS (Sigma), 0.1 M *n*-octyl-β-D-glucopyranoside (Sigma), 0.5% CHAPS (sigma), 0.5% Zwittergent 3-12 and 3-16 (Calbiochem, CA), 10 mM deoxycholate (Sigma). The cells were gently shaken with the detergents at 20°C, 30 min and centrifuged (16,000 x *g*, 10 min.). The supernatant and the pellet were analyzed by SDS-PAGE.

Large scale detergent extraction of *T. denticola* protein was carried out as follows. Up to 60-liter cultures (approx. 1.5 – 2 g wet weight spirochete per liter) of *T. denticola* cells from the late-logarithmic-phase growth were harvested by centrifugation at 5,000 x *g*, 1 h. at 4°C. The cells were washed twice with PBS and then once with ddH_2O. NP-40 was added to the spirochete suspension to a final concentration of 0.1%. The mixture was stirred gently overnight at 4°C. The undissolved material was pelleted by centrifugation at 10,000 x *g*, 45 min. at 4°C. Tris-NaCl-SDS buffer was added to the supernatant to a final concentration of 50 mM Tris-0.2 M NaCl-3.5 mM SDS, pH 7.5. The mixture was incubated in a 37°C water bath for 2 h. for autoproteolysis. After autoproteolysis, the solution was centrifuged at 10,000 x *g* for 45 min at 4°C. The supernatant obtained was concentrated at 4°C to approx. 5 mL by Amicon ultrafiltration with an XM 50 filter. The specimen was then dialyzed against 10 mM Tris (pH 7.5). The protein without the detergent precipitated inside the dialysis bag. The precipitate was pelleted by centrifugation at 10,000 x *g* for 45 min at 4°C, washed once with ddH_2O, and then redissolved in detergent and subjected to FPLC.

Extraction of Msp and CTLP was also carried out using Triton X-114 extraction and phase partitioning as described for *T. pallidum* (Cunningham *et al.* 1988) with slight modifications. Treponemes were harvested by centrifuge at 10,000 x *g* (10 min, 4°C), washed in Tris-buffered saline, then suspended in 1/40 volume of 1% Triton X-114-2 mM EDTA-1 mM DTT-20 mM Tris (pH 7.5), and stirred gently overnight at 4°C. Detergent-extracted cells were centrifuged at 17,000 x *g* for 10 min at 4°C. The supernatant, enriched for outer membrane components, was warmed to 37°C and incubated for 10 min, then centrifuged at 2,000 x *g* for 10 min to separate the detergent and aqueous phases. Aqueous and detergent phases were chilled to 4°C and re-extracted by adding 10% Triton X-114 or detergent-free buffer, respectively, followed by phase separation as described above.

2.8.2. Preparation of Msp complex

Extraction of Msp complex from *T. denticola* was performed partly according to steps laid out in section 2.8.1. with minor modifications. All procedures were carried out at 4°C unless otherwise specified. The cells, after harvesting and washing, were resuspended in 0.1% deoxycholate-1 mM DTT-2 mM EDTA-20 mM Tris (pH 7.5) at 0.2-0.3 g spirochete per mL. The mixture was stirred gently overnight, and undissolved material was collected by centrifugation at 200,000 x *g*, 45 min. The deoxycholate extraction was repeated twice. The pellet was then suspended in the same volume of 1% Octyl-POE (Bachem, PA)-10 mM Tris, pH 8.0 and stirred gently overnight. The Octyl-POE extraction supernatant enriched for Msp was collected after centrifugation at 200,000 × *g*, 45 min at 25°C. The solution was then filter-sterilized followed by incubation at 37°C for 24-48 h until proteinase activity could no longer be detected with anti-95 kDa CTLP IgG and SAAPNA assay. The protein solution was then

concentrated approximately 30-fold by Amicon ultrafiltration with an XM 50 filter. Detergent was removed from the concentrated protein solution by passing 3.5 L of 10 mM Tris (pH 8.0) through the Amicon unit. The solubilized Msp with the detergent washed out precipitated inside the Amicon unit. The precipitate was then washed three times with ddH_2O and twice with Ringer's solution (pH 7.4) or 10 mM Tris (pH 8.0) and centrifuged at 200,000 × *g* for 2 h. at 25°C to remove any possible traces of detergent. The washed Msp complex, in a small volume of buffer, was mildly ultrasonicated with a ultrasonic processor (XLC; 5-min pulse sonication at 20% duty cycle and output of 2; Heat Systems Inc., NY) before use in assays.

2.9. Purification of native Msp and Msp monomer

2.9.1. FPLC

Native *T. denticola* Msp was prepared as described in the following. An NP-40 extract of *T. denticola* protein was passed through a 0.45 μm filter and then purified by anion-exchange chromatography on a Mono Q (HR 6) column equilibrated with 1% NP-40 in 20 mM Tris (pH 7.4). The bound proteins were eluted with a 0-2 M NaCl linear gradient in the buffer described above. The eluted fractions were further analyzed by SDS-PAGE. Alternatively, the Msp was purified as described in the following protocol. The precipitated *T. denticola* protein extract (Section 2.8.1., paragraph 2) was dissolved in 1% Octyl-POE-10 mM Tris, pH 8.0, concentrated by Amicon ultrafiltration and resuspended in the same buffer. The protein solution was filtered and applied onto a Mono Q (HR 5/5) column. The bound protein was eluted from the Mono Q column by applying a gradient of 0-0.4 M NaCl in 1% Octyl-POE-10 mM Tris, pH 8.0 at a flow rate of 1 mL/min. Fractions (1 mL) containing the Msp oligomer were rechromatographed under the same conditions. The peak contained pure Msp oligomer. Analysis of all fractions was

performed by SDS-PAGE.

2.9.2. Preparative electrophoresis

Preparative electrophoresis purification of Msp monomer was carried out using a Mini-Protean II electrophoresis apparatus (BioRad), as will be described in the following section 2.10.1. *T. denticola* particulate fraction (as defined in section 2.1.), separated by SDS-PAGE was electroblotted onto nitrocellulose membrane, according to a standard protocol (as will be described in the following section 2.12.). The 53 kDa Msp monomer was found to be transferred poorly (approx. 20%), whereas contaminating proteins transferred with high efficiency. After blotting, the gel was sliced to remove the 53 kDa band. This method was used to prepare 53 kDa protein for antibody generation in rabbits.

Preparative electrophoresis using a Model 491 Prep Cell (BioRad Laboratories, CA) was utilized to purify Msp and CTLP as follows. Aqueous and detergent phases of Triton X-114 extracts of 3 L *T. denticola* cultures were concentrated to approx. 5 mL each in a CentriPrep 30 ultrafiltration unit (Amicon, MT), mixed with an appropriate volume of standard sample buffer containing reducing agent, and layered on the stacking gel matrices. Samples were electrophoresed at 60 mA, 4°C, through the 4% acrylamide stacking gel and the 7.5% acrylamide separation gel. The running and elution buffers were 0.1% SDS-192 mM glycine-25 mM Tris (pH 8.3). The eluate was collected in fractions of 2.5 mL at a flow rate of 1 mL per min. Fractions containing proteins of interest were concentrated by ultrafiltration, precipitated in acetone to remove detergent (Hager and Burgess 1980) and stored in aliquots at –70°C. Analysis of all fractions was performed by SDS-PAGE.

2.10. Electrophoresis

2.10.1. SDS-PAGE

SDS-PAGE was carried out by the Laemmli method (1970), using a mini Protean II cell (BioRad, CA). Protein samples were prepared as follows. The specimens to be separated were solubilized in sample buffer (10% βME-4%-SDS-20% glycerol-125 mM Tris, pH 6.8) for 30 min at 37°C for un-heated samples or 5 min at 100°C for regular samples. Electrophoresis was conducted at 200 V.

2.10.2. Isoelectric focusing

IEF of detergent (NP-40) extracts and the particulate fraction of *T. denticola* was done in the presence of 9.5 M urea and 20% NP-40 using a mini-Protean II two-dimensional electrophoresis apparatus (BioRad, CA). The first-dimension capillary gels contained 1.6% 5-8 Bio-Lyte (BioRad, CA) and 0.4% 3-10 Bio-Lyte (BioRad, CA) and were subsequently analyzed in the second dimension by SDS-PAGE as described in the above section.

2.11. Preparation of antisera

New Zealand white rabbits were immunized with whole cells of *T. denticola* ATCC 35405 or gel purified 53 kDa *T. denticola* protein for polyclonal antibodies generation. For anti-*T. denticola* antibodies, 1 mg of cells with complete Freund's adjuvant were injected intramuscularly into a rabbit. Subsequent intramuscular injections without adjuvant were performed after 1, 2, 3, 5, and 7 weeks. The rabbits were bled one week after the last injection. The specificity of the antiserum was determined by ELISA using alkaline phosphatase-conjugated goat anti-rabbit antibody as per section 2.4.2.

Antibodies to the *T. denticola* 53 kDa protein were prepared as follows. The 53 kDa protein in acrylamide gel was washed with ddH_2O, equilibrated with PBS, minced, emulsified with Freund's complete adjuvant and injected into a New Zealand white rabbit. Subsequent injections of the same amount of protein were administrated with Freund's incomplete adjuvant at weeks 3 and 6; injections without any adjuvant were administrated at weeks 9 and 12.

Antibody against the 53 kDa protein was also prepared by an alternate protocol by using affinity purification from the whole-cell antiserum. In the latter procedure, a partially purified preparation of the 53 kDa protein was subjected to SDS-PAGE and transferred to nitrocellulose membranes. Two reference lanes were stained with Coomassie brilliant blue to localize the proteins. The membrane piece containing the 53 kDa protein was excised, blocked with 3% BSA, and incubated at 4°C for 3 h with 1 ml of a 1:10 dilution of *T. denticola* whole-cell antiserum. After being washed once with PBS and twice with TTBS (below), the antibody attached to the 53 kDa protein was eluted by treatment of the membranes with 0.5% Tween 20-0.02% BSA-0.5 M NaCl-50 mM glycine, pH 2.3. The eluted antibody was immediately neutralized with 10 mM Na_2HPO_4 and stored at –20°C. The final dilution in Western blots was 1:1,000, calculated from the original amount of whole-cell serum (100 μL) used.

2.12. Western blotting

Western blot immunoassays were done as per Renart and Sandoval (1984). Proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose. The blots were incubated for 1 h with blocking buffer (3% BSA-0.5 M NaCl-20 mM Tris, pH 7.5), and then incubated for 2 h at room temperature or overnight at 4°C with the primary antibody diluted in 1% BSA-TBS. Following two 10 min washes with 0.05%-Tween 20-TBS, the blots were

incubated for 1 h at room temperature with the secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (human adsorbed, BRL, Md.), diluted in 1% BSA-TBS. Immunoreactive bands were developed with solution containing BCIP and NBT.

2.13. Immunoprecipitation of HeLa cell proteins with the Msp complex

HeLa cells incubated with 10 μCi of ^{35}S-methionine (Translabel, ICN) per mL of culture medium, 24 h, were used for the study (Roberts *et al.* 1988). Alternatively, HeLa cell surface proteins were labeled with Sulfo-NHS-LC-biotin (Pierce, IL) by the method of Lisanti *et al.* (1988). After labeling, the cell layer was washed extensively with PBS. Cell membrane and cytoplasmic proteins were extracted at 4°C with 2% Triton X-100-2mM PMSF-0.0015% leupeptin-PBS (Roberts *et al.* 1988). After centrifugation at 10,000 X *g*, 5 min, the supernatant containing solubilized protein was mixed with rabbit IgG conjugated to protein A Sepharose (Sigma) to remove non-specifically binding material. Following another centrifugation, the supernatant was mixed with Msp complex for 1 h at room temperature. Anti-Msp IgG conjugated to protein A Sepharose that had been preincubated with unlabeled HeLa cell proteins (Firestone and Winguth 1990) were added to the labeled HeLa protein-Msp complex mixture. For additional controls, immunoprecipitation of ^{35}S-methionine-labeled HeLa cell proteins was carried out under conditions in which Msp complex was omitted or in which anti-*P. gingivalis* IgG (Joe *et al.* 1993) was substituted for anti-Msp IgG. The resulting Msp-HeLa protein aggregates were collected by centrifugation and analyzed by SDS-PAGE to detect radiolabeled proteins or by SDS-PAGE and transferred to nitrocellulose membranes followed by biotinylated protein detection.

2.14. Autoradiography

Radiolabeled samples separated by SDS-PAGE, were first stained with Coomassie brilliant blue stain and treated with fluorography amplification reagent and dried at 80°C under vacuum. Autoradiography was done at –70°C with Kodak X-OMAT film. For biotinylated surface proteins, proteins were transferred electrophoretically from SDS-polyacrylamide gels to nitrocellulose membranes as described by Renart and Sandoval (1984). Following transfer, the membranes were blocked with 3% BSA in TBS and then incubated with streptavidin-conjugated alkaline phosphatase (Bethesda , Md.). The membranes were developed with BCIP and NBT (Sigma, St. Louis, MO).

2.15. Black lipid bilayer analysis

Single-channel conductance (Benz *et al.* 1985), macroscopic conductance inhibition (Hancock & Benz 1986), and zero-current membrane potential experiments (Benz *et al.* 1985) were performed as described previously. The instrument for black lipid bilayer study consisted of a Teflon chamber with two compartments separated by a thin wall. The circular hole in the wall connecting the two compartments had an area of either 0.1 mm^2 or 2 mm^2. The membranes were formed across the hole by painting on a 1% (w/v) solution of lipids (diphytanol phosphatidylcholine, or oxidized cholesterol, the results of lipid bilayer experiments were not influenced by the composition of the membranes, Hancock & Benz 1986) dissolved in *n*-decane. Bilayer formation was indicated when the membrane turned optically black to reflected light. The experimental conditions were kept at 25°C. The protein to be tested was added to the aqueous phase from the stock solutions either immediately before membrane formation or after the membrane had completely turned black. The single-channel conductance of the pores was

measured after application of a fixed transmembrane potential with a pair of calomel electrodes with salt bridges (Metrohm, Switzerland) inserted into the two aqueous salt solutions on both sides of the membrane. The current through the pores was boosted by a current amplifier (Keithley 427) monitored on a storage oscilloscope (Tektronic 5115) and recorded on a strip chart or tape recorder.

Macroscopic conductance inhibition experiments were initiated by adding detergent-purified Msp to the bathing solutions - 40 mM KCl-1 mM Tris, pH 7.0 - on either side of the lipid bilayer membrane. The increase in conductance was followed for 20 – 30 min or until the rate of increase had slowed down considerably. At this time the membrane conductance had generally increased 2 – 4 orders of magnitude. The bathing solutions in both compartments of the chamber were stirred gently with a magnetic stir bar and aliquots of inhibitors (human fibrinogen, final concentration 0.2 mg/mL; or bovine fibronectin, final concentration 0.1 mg/mL) were pipetted carefully to both compartments. Sufficient time was allowed for the new current level to be established before addition of the next aliquots.

The zero-current membrane potential measurements were made as previously described (Benz *et al.* 1979). The membranes were formed in a 10 mM salt solution containing a predetermined concentrated protein stock so that the conductance of the membrane increased about 100- to 1000-fold within 20 to 30 min. The voltage was then switched off and the instrumentation was switched to allow measurement of zero-current membrane potentials. The salt concentration on one side of the membrane was raised by adding small amounts of concentrated solutions while stirring. The zero-current membrane potential reached its final value within 5 – 10 min. For macroscopic conductance inhibition experiments and zero-current potential measurements, one electrode was again connected to a voltage source while the other

was connected to a Keithley 610B electrometer. In all cases the circuits were completed through earth.

2.16. Patch clamp assays

Patch clamp recordings from HeLa cells were carried out at room temperature (21-23°C) using a List EPC-7 amplifier with electrodes having a resistance of 5-10 MΩ. Membrane currents were filtered at a direct current band width of 2 kHz. For single-channel analysis of data obtained in the cell-attached mode (Hamill *et al.* 1981), membrane current and voltage were digitized at 8 kHz and analyzed on an Atari Mega 4 computer using commercial software (Instrutech, NY). Channel conductance was estimated from the single-channel current-voltage relationship.

For analysis of whole-cell, voltage clamp currents (Hamill *et al.* 1981), 400-ms-duration voltage clamp command pulses were applied from a holding potential of –57 or 0 mV, and corresponding whole-cell currents were stored and analyzed using the computer protocol as mentioned above. Fast and slow capacitance neutralization and series resistance compensation were employed to minimize artifacts associated with whole-cell voltage clamp. These recordings utilized isolated, spherical cells to avoid artifacts associated with cell-cell coupling and complex cell geometries.

The composition of bath and pipette saline solutions used are given in Table 2.2. For whole-cell, current clamp determination of resting membrane potentials, pipettes containing solution *c* and cells were bathed in solution *a*. During whole-cell, voltage clamp recordings, intrinsic membrane currents of HeLa cells were normally suppressed by using pipettes filled with solution *d* while cells were bathed in solution *b* (Takahashi *et al.* 1993). In cell-attached

recordings, both the bath and pipette contained solution *a*, and Msp complex was applied by adding the complex to the patch electrode solution.

2.17. Statistics

Raw data were used for statistical analysis if possible. However for semi-quantitative assays or assays leading to processed readings, the data will be normalized and presented as percentages to facilitate comparison. The corresponding standard deviation was deduced according to the method of standard deviation measurement in functional relationships (Parratt 1961). When applicable, difference between means were evaluated by non-parametric multiple comparisons (Bonferroni test, Zar 1996).

Table 2.1 Cell lines or cell explants used.

Abbreviation/line code	Cell types	Source[a]	Medium	Supplement[b]	Remark
CHO-K1	Chinese hamster ovary	F. Tufaro	RPMI 1640	10% FBS	
Vero (ATCC CRL 6318)	African green monkey kidney	F. Tufaro	DMEM	10% FBS	
HaCaT	Spontaneous human keratinocyte	H. Larjava	DMEM	10% FBS	
HeLa	Human cervix epitheloid carcinoma	D.A. Mathers	MEM	10% FBS, non-essential amino acids	
HGF	Human gingival fibroblast	J. Tonzetich	DMEM	10% FBS, non-essential amino acids, L-ascorbic acid	5 - 10th passage used
PLE	Porcine periodontal ligament epithelium	D.M. Brunette	αMEM	15% FBS	5 - 10th passage used
ROS 17/2.8	Rat osteogenic sarcoma	C.B. Wu	F-12	5% FBS	
Saos-2 (ATCC HTB85)	Human osteogenic sarcoma	C.B. Wu	F-12	5% FBS	
HEK	Human epidermal keratinocytes	Clonetic Corp. San Diego, CA	KBM, bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone.	Gentamicin sulphate, amphotericin B	
HGE	Human gingival epithelium	D. Oda U. Washington	KSFM, recombinant epidermal growth factor, bovine pituitary extract.		

[a] All lines were obtained from the person listed at the University of British Columbia unless indicated.
[b] All lines were maintained in media containing the following antibiotics or antifungal agent per mL unless indicated: 10,000 U of penicillin G, 10 mg streptomycin, 1.2% (v/v) fungizone.

Table 2.2 Compositions of saline solutions used in patch clamp experiments[a]

Solution	Concentration (mM) of component										
	KCl	NaCl	$CaCl_2$	$MgCl_2$	K Gluconate	Ca Gluconate	Mg Gluconate	K-ATP	EGTA	HEPES	Glucose
a	4	135	1.8	1						10	5
b					150	1.8	1			15	20
c	135	5	2.2	1					3	10	
d					150	0.005	1	2	1	5	5

[a] All saline solutions were buffered to a pH of 7.2 to 7.4.

CHAPTER III Results

3.1. Attachment of *T. denticola* to eukaryotic cells

3.1.1. Attachment of *T. denticola* to PLE cells

T. denticola bound to epithelial cells (Figure 3.1, Tables 3.1 & 3.2). In general, the binding of *T. denticola* to PLE cells was relatively quick. At 15 min, near maximum attachment was observed, and after 2 h the number of attached bacteria declined (Figure 3.2). SEM examination also showed binding of *T. denticola* to the epithelial cells and in some areas, contacts of the spirochetes concentrated at cell envelope blebs could be observed (Figure 3.3). No other specific sites of *T. denticola* or PLE cells favoring the adhesion were observed.

Larger numbers of epithelial cells had *T. denticola* adherent to them as the spirochete concentration in the incubation increased (Figure 3.4). The density of the epithelial cell culture affected the bacterial binding. In 30% confluent cultures the maximum number of *T. denticola*-binding epithelial cells (90% of the cells) was obtained at a concentration of 4×10^9 bacteria/mL (Figure 3.4B), while in near confluent cultures the maximum proportion of bacteria-binding cells was lower (approx. 65% of the cells, Figure 3.4A). This maximum value was obtained at much higher *T. denticola* concentration (2×10^{10} spirochetes/mL) than in the more sparse PLE culture.

The number of bacteria bound per epithelial cell was highly variable (Table 3.1). Even at high bacterial concentrations a considerable proportion of cells bound no or only a few bacteria. On other cells of the same cultures up to 80 spirochetes could be counted. Quantitative analysis of the adhesion data with Langmuir isotherms, Scatchard plots etc. (Gibbons *et al.* 1976, Morris & McBride 1984) arrived at an estimation of 2.5 attachment sites for *T. denticola* per PLE cell in 30% confluent (in full culture medium) versus the 1 site per PLE cell in 90% confluent culture

(Table 3.3) with Hill coefficient as low as 0.703 suggesting that some negative factor(s) was(were) acting against the *T. denticola*-PLE cells adhesion (Dahlquist 1978). Although maximum *T. denticola* attachment to PLE confluent monolayer was observed as early at 15 min, a time course microscopic assay revealed that not all PLE cells had attached spirochetes at the same moment. In contrast to the rapid maximum attachment of spirochetes to confluent PLE monolayers, the proportion of semi-confluent PLE cells binding *T. denticola* increased with time until a peak at 4 h and then the proportion decreased to a low level at 25 h. Variability of attachment data was later found to be partly due to detachment of PLE cells after *T. denticola* challenge (Section 3.3.1.2.).

3.1.2. Attachment of *T. denticola* to other eukaryotic cells

T. denticola binding to other eukaryotic cells was also studied (Table 3.2). *T. denticola* did not attach to different cell lines/explants equally. HSK and HGE cells bound the spirochetes less well, whereas almost all Vero or HGF cells bound some *T. denticola* while Saos-2 behaved similar to PLE cells (Tables 3.2 & 3.3). On those cell type where *T. denticola* was more readily attached, variation in the numbers of spirochetes binding per cell was also evident.

3.1.3. Attachment of *T. denticola* to FPLE cells

Attachment of *T. denticola* onto FPLE cells was studied in an attempt to look for ways that could reduce the variability observed in attachment studies, which might have been due to detachment of the PLE monolayer substrate (see also Figure 3.7). This could complicate the interpretation of the results of the attachment assays. In general, *T. denticola* bound to FPLE and PLE similarly (Table 3.1). The high variability in the number of bacteria bound per PLE could

also be observed when FPLE cells were used (Table 3.1). At high *T. denticola* loading, about half of the FPLE bound no spirochetes, while a small proportion of the cells bound up to 45 spirochetes. Similarly, a relatively quick rate of *T. denticola* attachment to FPLE cells was also observable (Figure 3.2B).

Quantitative analysis of attachment data revealed FPLE cells provided a simpler as well as a more convenient model for studying *T. denticola* attachment. An estimated approximate 3.5 attachment sites for *T. denticola* per FPLE cell (Table 3.3), corresponding to a Hill coefficient of 0.97 for *T. denticola* - early confluent FPLE attachment . This approximates that of a simple unipair binding system (when Hill coefficient = 1.0; Dahlquist 1978). These readings suggest that the FPLE system was more stable, and thus, illustrate an advantage to using FPLE cells.

Attachment of *T. denticola* to fixed monolayers of other cell lines or explants was also studied (Table 3.4). The attachment of *T. denticola* to fixed PLE, Vero, and HGF cells was slightly affected (Tables 3.1, 3.2 & 3.4). At least ten times more concentrated *T. denticola* suspensions were needed to create similar binding patterns in fixed Vero and HGF cells versus their unfixed counter-parts (Tables 3.2 & 3.4). Despite the fact that the experimental conditions during the spirochete-cell interaction between fixed and unfixed cells were not exactly the same, the glutaraldehyde fixation was likely the prime cause of the differences. This was, however, not further investigated.

3.1.4. Effects of pre-treatment of *T. denticola* on attachment to PLE cells

3.1.4.1. Physical pre-treatment

To characterize the nature of *T. denticola* binding to epithelial cells further, the spirochetes were subjected to various treatment protocols before PLE/FPLE cell binding assays.

The effects of heating and pre-incubation with buffers of varying pH before attachment assays, were tested and results are shown in Table 3.5. Pre-treatment at pH 3.8 had no effect, but exposure to pH 3.2 inhibited binding by 89%. Inhibition of attachment of 80% was obtained by heating the bacteria at 60°C. Similar results were also obtained when FPLE cells were used (Figure 3.5).

3.1.4.2. Chemical pre-treatment

Serum inhibited the attachment of the treponemes. In the absence of serum or in the presence of 1% serum, *T. denticola* binding took place at much lower bacterial concentrations than in the presence of 15% serum (Figure 3.4B). Some major serum proteins were tested for their effect on binding. Fibrinogen strongly inhibited the binding, while serum albumin and fibronectin had no effect at the concentrations tested (Table 3.6).

Effects of other agents on *T. denticola* and its binding to PLE cells were also tested. Proteinase K treatment of *T. denticola* inhibited its binding by 30%, but mixed glycosidase had no effect. PMSF, a serine proteinase inhibitor and *p*CMBA, an inhibitor of thiol proteases inhibited the attachment by 62% and 78%, respectively (Table 3.5). When FPLE cells were used for attachment assays, similar observations could be registered (Figure 3.6). All treatments that inhibited *T. denticola* binding, i.e. heating, acidic pH, PMSF and *p*CMBA also inhibited the activity of the CTLP enzyme of *T. denticola* (Table 3.5, Figs. 3.5, 3.6), while TPCK, a cysteine protease inhibitor, had no significant effect on binding nor on CTLP enzyme activity.

3.1.4.3. Antibody pre-treatment

As implied from results presented in sections 3.1.4.1. and 3.1.4.2. regarding the

possibilities of CTLP mediating the spirochete attachment to PLE cells, the effect of antibodies against *T. denticola* CTLP on binding to epithelial cells was studied. Anti-CTLP IgG and anti-whole *T. denticola* IgG significantly inhibited the binding (Tables 3.6, 3.7). Anti-Msp or Anti-rMsp IgGs, on the other hand, were also observed to be able to inhibit the binding of *T. denticola* to FPLE. However, there existed different aspects, which affect the results observable from the two different set of experiments. Greater inhibition was observable when anti-*T. denticola* or anti-95 kDa protease IgGs were used to block *T. denticola* attachment of PLE cells (Table 3.6). When FPLE was used instead, the inhibition of binding by such pre-treatment was less dramatic (Table 3.7). That might be explained by the fact that PLE cells, if unfixed, upon the influence by *T. denticola* could be detached from the substratum, probably bringing along with them certain attached spirochetes.

3.2. Cytopathic effects of *T. denticola* on PLE

3.2.1. Morphometric changes.

Microscopic and morphometric examination showed loss of cell contacts, retraction of epithelial cells and detachment leading to large cell-free areas in the cultures (Figure 3.7, see also Figure 3.3B). The reduction in mean attached PLE cell size plateaued at *T. denticola* challenging concentrations of $2 - 9 \times 10^{10}$ spirochete/mL before it continuously fell down to 90 μm^2 which was about 30% of the original cell size (Figure 3.7A). When *T. denticola* induced PLE cell morphometric changes was followed against time, the following were observed. Despite the rapid cell detachment with time, the confluency of the monolayer was maintained at around a 90% level with a dramatic increase in cell size of the remaining PLE cells (Figure 3.7B). The mean PLE size increased from about 300 μm^2 at time 0 to more than 600 μm^2 at 5 h. However,

with the continuous insult from *T. denticola*, the number of cells remaining attached, the mean cell size, and the confluency of the culture reduced with time until at 13 h when virtually no PLE cells were left behind.

3.2.2. Vacuolation

Vacuolation of *T. denticola* treated PLE cells was studied as per Cover *et al.* (1991), and a micrograph of a typical field is shown in Figure 3.8. Vacuolation induced by *T. denticola* was not dramatic in PLE cells remaining on the tissue culture wells but was statistically significant (Table 3.8). The presence and location of intracellular vacuoles were also observed from TEM sections of PLE infected with *T. denticola* (please refer to Figure 3.15D, Section 3.3.1.1.). Vacuoles of different sizes were observable. The effect of *T. denticola* in causing vacuolation of other cell lines was tested. The extent of vacuolation induced appeared similar to what was observed in PLE cells (data not shown).

3.2.3. Blebbing

The addition of *T. denticola* to sparse PLE cell cultures rapidly led to extensive membrane blebbing of some solitary cells (Figure 3.9). Less intensive transient membrane ruffling was also observable in control cultures (data not shown). However, the addition of *T. denticola* increased the number of blebbing cells in cultures by 3 – 6 fold. In 30% confluent cultures, the proportion of blebbing cells increased from 1.6 ±0.9% to 6.5 ±1.8% upon *T. denticola* treatment. Similar observations were recorded when early confluent PLE was used (Table 3.8). The blebbing phenomenon was observed irrespective of whether FBS was present or absent.

3.2.4. Cytoskeletal rearrangement.

The cytoskeleton rearrangement of PLE cells in relation to blebbing, and loss of cell contacts resulting from *T. denticola in vitro* infection was investigated. In blebbing cells, the actin network was grossly disorganized. While the actin arrangement was relatively unaffected in the basal sections of the cell, marked disarray was observed in the cortical actin. The normal circular arrangement was replaced by a disorganized peripheral meshwork. Part of the actin filament appeared to end in the peripheral membrane blebs (Figure 3.10). The cytoskeletal arrangement in the cell colonies that did not show any signs of blebbing were not markedly changed (Figure 3.11B). Blebbed cells often became elongated, and the actin filaments were arranged as long filament bundles at the lateral borders of the cell (Figure 3.11A). No specific arrangement of actin was observed in the area where the spirochetes had adhered to or associated with the basal third of the epithelial cells (Figure 3.11B).

3.3. Cytotoxic effects of *T. denticola* on eukaryotic cells.

3.3.1. Cytotoxic effects of *T. denticola* on PLE cells.

3.3.1.1. PLE cells remaining on tissue culture plates.

Concentration dependent toxicity of *T. denticola* ATCC 35405 to confluent monolayer cultures of PLE cells (including detached and attached cells) was assayed by MTT assay (Figure 3.12). Alternatively the ability of PLE cells to incorporate radioactive thymidine upon the influent of *T. denticola* was measured (Figure 3.13). The viability of PLE cells remaining attached in tissue culture wells was measured utilizing trypan blue dye exclusion assay (Figure 3.14). *T. denticola* sonicate and whole cells were cytotoxic to PLE cells. At a spirochete concentration of 1×10^{11} per mL, approx. 50% of the cells could not degrade the MTT salt

(Figure 3.12). The proliferation experiment, however, identified PLE cells responding to lower concentration of *T. denticola* challenge (Figure 3.13). The spirochete at concentration of 1×10^8 per mL induced mild proliferation response of PLE cells. Most remaining PLE cells retained their ability to exclude Trypan blue dye (Figure 3.14). Some *T. denticola* treated PLE cells, when subjected to TUNEL staining, were shown to be undergoing apoptotic changes (Figure 3.14). The portion of apoptotic attached PLE cells increased when concentration of *T. denticola* in the system increased. The same cell death process was also witnessed in TEM preparation (Figure 3.15).

3.3.1.2. PLE cells detached from the tissue culture plates.

T. denticola induced detachment of culture cells was measured by crystal violet dye elution (Figure 3.16) and morphometric analysis (Figure 3.7). Viability of detached PLE cells was also measured (Figure 3.14). In general, the more and/or the longer *T. denticola* challenge of PLE cells, the larger the amount of PLE cells were detached. Most of the detached cells were dead, with loss of regular cytoplasmic or cellular structure (Figure 3.17).

3.3.2. Cytotoxic effects of *T. denticola* on other eukaryotic cells.

Concentration dependent toxicity of *T. denticola* to various cell lines/cell explants was studied by MTT (Figure 3.18). *T. denticola* could also induce detachment of various cells (Figure 3.16). Under the current experimental conditions, PLE was found to be the most resistant cell to the insult by *T. denticola*, followed by Saos-2, HGF, HeLa and CHO-K1.

3.3.3. Effects of pre-treatment of *T. denticola* on cytotoxicity to PLE cells.

3.3.3.1. Physical pre-treatment

Similar cytotoxic effects were seen when PLE monolayers were challenged with intact *T. denticola* or sonicated *T. denticola* (Figure 3.12). Heating the spirochetes at 80°C or above for 60 min markedly prevented the cytotoxic effect (Table 3.9). Other physical pre-treatment of *T. denticola*, such as pre-treating the *T. denticola* with buffers of different pH, did not affect the cytotoxic activities of *T. denticola* as measured by MTT (Table 3.9).

3.3.3.2. Chemical pre-treatment

Pre-treatment of *T. denticola* with protease inhibitors or agents that enhance the CTLP activities of the spirochete did not affect the cytotoxicity (Table 3.10). Similarly, BSA, FBS, fibronectin, fibrinogen pre-treatment of *T. denticola* did not affect its cytotoxic activity on PLE cells monolayer cultures (Table 3.10). Enzyme pre-treatment by mixed glycosidase did not affect the cytotoxic effect of *T. denticola* cytotoxicity of *T. denticola* on PLE. Pre-treatment of the spirochete by proteinase K, however, did reduce the cytotoxicity of the spirochete on PLE (Table 3.10).

3.3.3.3. Antibody pre-treatment

The effects of *T. denticola* whole cells or surface proteins antibody pre-treatment on the cytotoxic action of the spirochetes was studied. Anti-*T. denticola* whole cell serum, anti-*T. denticola* CTLP or anti-*T. denticola* Msp pre-treatment of the spirochetes were not only able to inhibit the bacteria binding (Tables 3.6, 3.7) but also reduced the cytotoxicity of the spirochete on the PLE cells (Table 3.11).

3.4. Cytopathic and cytotoxic effects of *T. denticola* "putative virulence factors"

3.4.1. Purification and characterization of the "putative virulence factors"

3.4.1.1. Characterization of crude detergent extracts

NP-40 detergent extracts of both whole cells and the particulate fraction of sonicated *T. denticola* cells were analyzed by SDS-PAGE (Figure 3.19, lanes A and B). In both samples, most of the protein was found in a 53 kDa band. Similar results were obtained when whole cells of *T. denticola* strain ATCC 33520, ATCC 35404, ATCC 35405 were extracted with Triton X-100, SDS, octylglucoside, CHAPS, Zwittergents 3-12, Zwittergents 3-16, and deoxycholate (data not shown). The protein profile remained the same regardless of whether reducing or non-reducing sample buffers were used. The 53 kDa protein band was absent from samples which had not been heated prior to electrophoresis, and instead, a number of protein bands appeared in the 130-200 kDa region. These high molecular mass complexes were dissociated, and the 53 kDa protein appeared to be the major component when samples were incubated at 70-80° or above (Figure 3.20, lanes C and D). The high molecular mass complexes were highly resistant to treatment with a wide range of proteases, including the intrinsic CTLP (Figure 3.20, lane E). In subunit form, the proteins were readily degraded by these proteases. This can be observed when the complexes were dissociated by heating prior to the protease treatment (Figure 3.20, lane F).

IEF-PAGE suggested that the 53 kDa protein existed in several forms having isoelectric points ranging from 8.0 to 5.5 (Figure 3.19, C). These may be true subunit isoforms, or they may have had similar pIs. Some lower molecular mass proteins also showed forms with different pI values. These lower molecular mass proteins were detected with the anti-53 kDa protein antibody and are probably degradation products of this protein (see also Figure 3.21).

The relationship between the 53 kDa protein and the high molecular mass proteins was

investigated immunologically (Figure 3.21). Proteins from whole cell lysates separated by SDS-PAGE and transferred to nitrocellulose were reacted with antisera against *T. denticola* ATCC 35405 cells (Figure 3.21, lanes A and B), or the *T. denticola* 53 kDa protein. The whole cell antiserum reacted with the 53 and 72 kDa proteins in heated samples and with the > 130 kDa complexes in the unheated sample. The antiserum also detected several low molecular mass proteins; the number and size of these polypeptides varied from preparation to preparation (Figure 3.21, lanes A and B). The anti-53 kDa protein antibody reacted with a 53 kDa band from all three strains tested (Figure 3.21, lanes C to E). Weak reactions at > 130 kDa region were detected with heated strains of ATCC 35404 and ATCC 35405. Such > 130 kDa proteins were probably trace amounts of the undissociated high molecular mass proteins left behind even after the 5 min 100°C heating. The anti-53 kDa protein antibody also reacted with several low molecular mass bands of the strains; the strongest reaction was with strain ATCC 35405. The 72 kDa protein showed no cross-reactivity with the anti-53 kDa protein antiserum.

CTLP was observable as a doublet at 95 kDa in SDS-PAGE of *T. denticola* detergent extract, and was particularly evident in sodium deoxycholate extracts (Figure 3.22). The isolation, purification and characterization of the CTLP were described by Uitto *et al.* (1988b) and Grenier *et al.* (1990).

The morphological features formed by the major surface proteins of *T. denticola* were visualized with negative staining (Figure 3.23). In intact *T. denticola* cells, the cell body obscured observation of potential arrays. Various techniques to separate the outer sheaths from the rest of the spirochetes were attempted. These included vortexing for 5 min with 1- to 5-μm-diameter glass beads, freezing and thawing 40 times, and mild ultrasonication of cultures of various ages. Sonication of 3-day-old cultures produced the most adequate result. Such

preparations demonstrated isolated sheaths, small vesicles that derived from these sheaths, and darkly staining bacterial bodies that were sometimes surrounded by sheath material (Figure 3.23A). The sheath material demonstrated an apparent regular distribution of darker staining regions with an approximate center-to-center distance of 20 nm and darker stained areas approx. 4 – 5 nm in diameter. In some micrographs, areas with clear hexagonal arrays were observed (Figure 3.23B). Other *T. denticola* strains, apparently containing major surface protein (Msp) of a different size, were also found to be having hexagonal array which was similar in pattern and size to that of *T. denticola* (Figure 3.24).

Immunogold bead labeling with anti-53 kDa protein IgG and anti-95 kDa CTLP protein IgG showed that the 53 kDa and the 95 kDa proteins were located on the *T. denticola* cell surface (Figure 3.25). Gold beads can be seen on the cell surface and on material dissociated from the cell. The immunogold electron micrograph in Figure 3.25B shows the alignment of 53 kDa protein (10 nm gold beads) with the centers of the units of a regular hexagonal array of *T. denticola* outer membrane materials, and suggests that CTLP (5 nm gold beads) is closely associated with the 53 kDa protein in the outer membrane.

The abundance of the 53 kDa protein in the *T. denticola* cell and its localization to the OM were more easily observable by immunogold labeling of the protein alone (Figure 3.26). The evidence available implied that the hexagonal array consisted of 53 kDa oligomers of the Msp.

3.4.1.2. Purification of the 53 kDa protein or Msp

In order to partially purify the 53 kDa protein, detergent solubilized whole cell extracts and particulate fraction of sonicated *T. denticola* cells were subjected to gel filtration and anion-

exchange chromatography using FPLC. Adding SDS (SDS was used only in gel filtration), NP-40, Triton X-100, or urea did not change the chromatographic behavior of the proteins. When the fractions collected from gel filtration were analyzed by SDS-PAGE, the 53 and 72 kDa proteins were present in several fractions covering a wide molecular mass range. Figure 3.27A shows separation of proteins from the particulate fraction of *T. denticola* ATCC 35405 by anion-exchange chromatography on a Mono Q column. A sharp peak containing the 53 kDa protein and the 72 kDa protein could be obtained.

The detergent soluble 53 kDa protein was then purified to homogeneity after modification of the above FPLC protocol (Figure 3.27B). A detergent exchange step prior to ion-exchange FPLC was carried out. The protein ran on SDS-PAGE as a single oligomer band with an apparent molecular weight of 160,000 when it was not heated prior to electrophoresis and with an apparent molecular weight of 53 kDa when it was heated in SDS-PAGE sample buffer for 10 min at 100°C prior to electrophoresis.

3.4.2. Porin activity of purified Msp

Addition of the purified 53 kDa protein to the salt solution bathing a lipid bilayer membrane led to step increases in membrane conductance (Figure 3.28). By analogy with other model membrane studies (Benz *et al.* 1985, Hancock 1987), these step increases were considered single-channel events because of the progressive incorporation of pore-forming units into the planar lipid bilayer membrane separating two aqueous compartments. Consistent with this, the sizes of the step increases in, e.g., 1 M KCl showed a rather narrow range, with few steps larger than 12 nS or smaller than 10 nS (Figure 3.29). Other salts demonstrated similar histograms of channel size distribution.

The channel formed by the 53 kDa protein was very large, as indicated by the large single channel conductance. Although the single channel conductance was influenced by the specific conductance properties of the salt used, the ratio of single channel conductance (G) to specific conductance (σ) was little affected by salt or ion composition (Table 3.12). This means that these ions moved through the channel in a fashion similar to their movement through a similar salt solution that separated the two electrodes across which voltage had been applied. Thus, this result was consistent with the 53 kDa protein forming a large, relatively non-selective channel. When both the anion and the cation were replaced by the very large ions $Tris^+$ (0.67 nm diameter)and $Hepes^-$ (an ellipsoid molecule 1.4 by 0.6 by 0.5 nm), there was a small decrease in the G/σ ratio observed. Thus, only in this case did interaction of the interior of the channel with these large ions (which are even bulkier because of the hydration shell they carry) restrict ion movement. Even in this case the effect was smaller than that observed for other porins. This is consistent with the 53 kDa porin forming a very large porin channel.

The single channel conductance experiments did not indicate a strong preference for any ions. In zero-current membrane potential experiments, a KCl concentration gradient was imposed between the two chambers separated by a membrane containing 100 channels of the 53 kDa porin. Preferential movement of Cl^- was indicated by the formation of a negative potential which balanced the osmotic potential caused by the concentration gradient. Application of the Goldman-Hodgkin-Katz equation to these data indicated that the channel was weakly anion selective, with a ratio of permeability to Cl^- over permeability to K^+ of 1.6. Decreasing the salt concentration resulted in an approximately linear decrease in conductance (Table 3.12). Addition of human fibrinogen or bovine fibronectin to the solutions bathing a membrane loaded with approx. 500 channel forming units of the 53 kDa protein had no effect on conductance of

KCl through these channels. The inability to block KCl movement suggested either that the binding affinity of the putative binding site is low or that the binding site is so located that it does not influence the channel properties.

3.4.3. Attachment of Msp complex, Msp and CTLP to PLE/FPLE cells

T. denticola outer membrane components such as Msp complex, Msp and CTLP adhered to PLE (Figure 3.30). Adherence was, in most instances, inhibited by the same treatments that inhibited adherence of whole cells. Pre-treatment of Msp with anti-rMsp IgG significantly inhibited adherence (Table 3.13). As shown in Table 3.14, adherence of CTLP was partially blocked by inhibitors of serine proteases. Pre-treatment with anti-CTLP IgG alone had no adherence blocking activity, but significantly inhibited SAAPFNA activity. Adherence of PMSF-treated CTLP to FPLE was inhibited to a much greater degree by anti-CTLP IgG than was adherence of untreated CTLP by either PMSF or anti-CTLP IgG alone. CTLP bound rapidly to PLE cells (Figure 3.30B). The adherence of CTLP to FPLE was also rapid. The CTLP-PLE cell binding was somewhat better at 4°C than at 20°C (Figure 3.30B). Adherence of Msp to FPLE appeared similar at room temperature and at 4°C.

3.4.4. Cytotoxic effects of Msp complex, Msp and CTLP on PLE cells.

Msp complex was electrophoretically similar to Msp purified by FPLC and contained neither proteolytic activity nor detectable CTLP (Figure 3.31). The effect of Msp complex on PLE cells was assayed using LDH release and MTT reduction assays, and the results are shown in Figure 3.32. Both assays gave similar results for Msp complex at final concentrations between 6 and 50 μg/mL, suggesting that there were intracellular effects of Msp complex on PLE cells

that preceded cytolysis.

Purified Msp and CTLP induced high levels of LDH release in PLE cell cultures, indicating severe cytotoxic effects. Cytotoxicity data shown in Tables 3.13 and 3.14 represent experiments using the lowest concentrations of protein that caused complete lysis of PLE within 1 h. Msp was cytotoxic at concentrations of 40 – 50 nM, while CTLP was cytotoxic at approx. 1.5 nM. Inhibition of cytotoxicity followed the same pattern as inhibition of adherence for Msp and CTLP, suggesting that cytotoxic effects resulted from adherence of the treponemal protein to the epithelial cell surface. Anti-rMsp IgG inhibited the cytotoxic effects of Msp and rMsp by 40 – 50%. Partial inhibition of cytotoxic effects (15 – 30%) was attained by pre-treatment of CTLP with PMSF. Anti-CTLP IgG alone had no significant effect on cytotoxicity of CTLP, but when CTLP was pre-treated with PMSF, anti-CTLP IgG inhibited cytotoxicity by > 50%.

3.4.5. Cytotoxic effects of Msp complex on eukaryotic cells.

The Msp complex had cytotoxic effects on other cell types, as measured by MTT (Figure 3.33). Msp complex was cytotoxic to all cell types tested, including several epitheloid cells: HeLa, ROS, HSK, CHOK1, ROS; and HGF. In this assay, HSK cells were found to be the most sensitive to Msp complex, while ROS, HeLa, PLE, CHO-KI and HGF cell cultures were progressively more tolerant which differed to some extend to the results of cytotoxic assays when whole *T. denticola* was used (Figure 3.18). All the cells were killed within 24 h by Msp complex at a concentration of 15 μg/mL.

3.4.6. Characterization of Msp complex induced cytotoxicity by patch clamping

The characterization of Msp complex induced cytotoxicity was studied using HeLa cells, a human epitheloid cell line. HeLa cell was chosen because its membrane electrophysiology and membrane channels had been well studied (Sauve *et al.* 1986). First, the attachment or binding of Msp complex to HeLa cell proteins were studied, and then the effects of Msp complex on membrane conductance in HeLa cells was examined.

SDS-PAGE of HeLa cell proteins showed ^{35}S-methionine labeling at many molecular masses over the range form 30 – 200 kDa (Figure 3.34A, lane 1). A ^{35}S-methionine labeled HeLa protein of 96 kDa was immunoprecipitated by anti-Msp IgG after exposure to the Msp complex, with additional reactivity at several other bands including bands of 50 – 53, 65, and 75 kDa (Figure 3.34A, lane 2). Control gels in which the Msp complex was omitted or in which anti-*P. gingivalis* IgG replaced anti-Msp IgG showed negligible labeling (Figure 3.34A, lanes 3 and 4). A biotinylated HeLa cell surface protein of 65 kDa was immunoprecipitated by anti-Msp IgG after exposure to the Msp complex (Figure 3.34B). The biotinylated protein recognized by Msp had the same relative molecular weight as one of the ^{35}S-methionine-labeled HeLa proteins recognized by Msp.

HeLa cells incubated for 24 h, 37°C with the Msp complex (39 μg/mL) in 10% FBS-MEM were found to have a significantly lower mean membrane potential than was observed in cells incubated in 10% FBS-MEM alone. The mean resting potential was –51 ± 2.5 mV for untreated cells (n= 23) and –24 ± 2.9 mV for Msp-treated cells (n=11) (p<0.001, student's t test).

To determine if the Msp complex induced a conductance change in the HeLa cell membrane, whole-cell, voltage clamp recordings were performed. In the first series of these experiments, patch electrodes contained solution *d* (Table 2.2), while cells were bathed in

solution *b*. In these solutions, Na^+ was absent, K^+ was the predominant cation, and Cl^- was replaced by gluconate, which cannot pass through most anion channels in eukaryotic cell membranes. Sample recordings obtained are shown in Figure 3.35. Under these conditions, cells incubated with 10% FBS-MEM alone had current-voltage (*I-V*) curves showing slight inward rectification (Figure 3.36A). In cells incubated for 30 min, 37°C in the presence of 39 μg Msp complex/mL, the *I-V* curve became almost linear, and the mean slope conductance measured at positive membrane potentials was significantly higher than in the control ($p < 0.05$, ANOVA, Figure 3.36A). These experiments were then repeated in physiologically relevant saline solutions. Cells were bathed in solution *a*, while patch pipettes contained solution *c* (Table 2.2). Sample recordings are shown in Figure 3.37. Under these conditions, the *I-V* curves of untreated cells were almost linear (Figure 3.36B). In contrast, the *I-V* graph for cells incubated with 39 μg Msp complex/mL showed marked outward rectification and displayed increased slope conductance for both inward and outward membrane currents ($p < 0.05$, ANOVA; Figure 3.36B). The *I-V* curves of control and Msp-treated cells intercepted at a membrane potential of −18 mV (Figure 3.36B). This voltage presumably represents the equilibrium potential for Msp-induced membrane currents (Eriksen *et al.* 1994).

The depolarization and conductance increase caused by Msp complex could reflect incorporation or activation of ion channels in the HeLa cell membrane. To test this possibility, we recorded current from cell-attached patches of HeLa membrane with electrodes containing 39 μg Msp complex/mL dissolved in solution *a* (Table 2.2). These patch pipettes also contained 0.03% Octyl-POE as the vehicle and 5% BSA to minimize binding of the Msp complex to glass. The remainder of the cell membrane was bathed in normal solution *a*. No ionic channels with a slope conductance of 200 pS or greater were observed when patch electrodes containing Octyl-

POE and BSA but lacking Msp complex were applied to the cell membrane (0 of 14 patches on 14 cells). In contrast, abrupt, square-wave changes in current consistent with the gating of large conductance ion channels were seen in 18 of 51 patches exposed to Msp complex, Octyl POE, and BSA ($p < 0.001$, Fisher exact test, Figure 3.38). Msp-induced channels had a mean slope conductance of 386 ± 49 pS (17 patches). When recorded at the normal resting potential of the HeLa cell, current through open channels was inwardly directed. Channels remained closed most of the time, occasionally undergoing transitions to a short-lived open state.

Table 3.1 The number of *T. denticola* binding to individual epithelial cells in sparse and dense culutres[a].

Number of *T. denticola* bound	30% confluent PLE	95% confluent cells			
		10[b]		100[b]	
	3.5[b]	PLE	FPLE	PLE	FPLE
0	7	79	67	31	49
1 – 5	50	14	26	45	37
6 – 10	28	2	5	5	7
11 - 15	11	1	2	2	4
16 - 20	2	1	<1	3	2
21 - 25	1	<1	1	1	<1
26 - 35	1	<1	1	5	1
36 - 45	0	<1	0	3	<1
>46	0	<1	0	4	0

[a]PLE/FPLE cells were incubated with *T. denticola* in 15% FBS-αMEM for 2 h at 37°C. The readings showing percentage proportion of individual PLE/FPLE cell binding different number of spirochetes (left-hand column).
[b]Concentration of *T. denticola* in the incubation x 10^9 bacteria/mL.

Table 3.2 The number of *T. denticola* binding to individual eukaryotic cells in sparse and dense cultures[a].

Number of *T. denticola* bound	HSK		HGE		Saos-2		PLE		Vero		HGF	
	L	H	L	H	L	H	L	H	L	H	L	H
0	61	46	67	50	25	25	7	17	5	2	0	<1
1 – 5	38	45	30	39	30	27	36	20	71	57	6	1
6 – 10	<1	8	3	9	21	25	29	15	22	29	22	15
11 – 15	0	1	<1	2	13	5	14	15	1	8	19	28
16 – 20	1	0	0	1	10	6	7	10	0	2	21	15
21 – 25	0	0	<1	0	1	6	4	7	0	1	13	11
26 - 35	0	0	0	0	0	5	2	8	<1	<1	15	18
36 – 45	0	0	0	0	0	1	<1	5	<1	0	3	10
> 46	0	0	0	0	0	1	0	3	0	0	1	1

[a] Cell lines in low (L: approx. 50%) or high (H: approx. 80%) confluence were incubated with 5 x 10^8 (L) or 1 x 10^9 (H) *T. denticola*/mL medium without serum, respectively, 2 h at 37°C. PLE cells were also included as a reference. The readings showing percentage proportion of individual eukaryotic cell binding different number of spirochetes (left-hand column).

Table 3.3 Langmuir isotherm parameters of *T. denticola* – cell cultures adherence[a].

Cell type	Serum content in medium (%)	Confluence (%)	N (x 10^4)	Approx. sites/cell	K (x 10^{-10})	r
PLE	15	95	1.20	1	4.51	0.99
FPLE	0	95	7.09	3.5	1.37	0.99
PLE	0	30	2.91	6	7.58	0.99
PLE	1	30	2.24	5	6.11	0.97
PLE	15	30	1.27	2.5	-0.81	0.99
Saos-2	0	50	2.23	2.5	4.41	0.99

[a] *T. denticola* were incubated with the cell cultures for 2 h at 37°C. N, estimated number of binding sites; K, association constant; r, correlation coefficient.

Table 3.4 The number of *T. denticola* binding to individual fixed eukaryotic cells[a].

Number of *T. denticola* bound	HeLa	HaCaT	Saos-2	FPLE	Vero	HGF
0	90	75	70	65	37	7
1 - 5	7	20	21	25	36	44
6 – 10	3	2	8	6	14	23
11 – 15	0	1	0	2	6	12
16 – 20	<1	1	1	1	4	5
21 – 25	0	0	<1	<1	2	3
26 – 35	0	<1	0	0	<1	3
36 – 45	0	<1	0	<1	<1	1
>46	0	0	0	0	0	2

[a] Cell lines in early confluence are treated with 1 x 10^{10} *T. denticola*/mL medium without serum, respectively, 2 h at 37°C. FPLE cells were also included as a reference. The readings showing percentage proportion of individual eukaryotic cell binding different number of spirochetes (left-hand column).

Table 3.5 Effect of various treatments on binding and the chymotrypsin-like protease activity of *T. denticola.*

T. denticola treatment	Enzyme activity[a] (mean % ±SD)	*T. denticola* attachment to epithelial cells[b] (mean % ±SD)
None	100.0±5.4	100.0±8.9
PMSF, 1 mM	5.8±1.1*	38.0±3.3*
PCMBA, 2 mM	1.7±0.4*	22.3±2.1*
Cysteine, 50 mM	ND[c]	86.3±5.6
Mixed glycosidase, 100 μg/mL	ND	92.7±6.9
Proteinase K, 100 μg/mL	ND	69.7±5.5*
pH		
3.2	5.6±0.3*	11.0±1.9*
3.8	76.7±0.8*	87.0±2.3
Heating at 60°C	2.5±0.4*	22.2±7.8*

[a] Following 1 h treatment, the spirochetes (1.4×10^{10}) were washed in PBS and assayed for SAAPFNA degradation.
[b] *T. denticola* attachment measured by ELISA.
[c] ND = not determined.
* $p<0.05$, Bonferroni test.

Table 3.6 Effect of serum proteins and anti-*T. denticola* antibodies on *T. denticola* binding to PLE cells.

Protein	Amount (mg/mL)	*T. denticola* attachment to epithelial cells (% of control, mean ±SD)
Experiment 1[a]		
Control		100.0±11.1
Fetal bovine serum	1.0	75.5±12.4*
	10.0	1.6±0.2*
Bovine serum albumin	1.0	92.5±7.1
	10.0	89.7±4.4
Fibrinogen	1.0	0.7±0.5*
	10.0	11.1±2.0*
Fibronectin	0.1	99.8±9.9
	1.0	98.0±11.6
Anti-*T. denticola* IgG	0.0005	61.8±6.0*
Anti-95 kDa protease IgG	0.0005	61.3±8.6*
Experiment 2[b]		
Control		100.0±6.8
Anti-*T. denticola* IgG	0.0005	34.7±12.8*
Anti-95 kDa protease IgG	0.0005	30.4±15.0*

[a] *T. denticola* (2.5×10^8 spirochetes/mL) were preincubated with the protein prior to adding to 30% confluent cultures of periodontal ligament epithelial cells and incubating for 2 h at 37°C. The values are calculated from number of epithelial cells binding one or more *T. denticola* compared to epithelial cells with untreated *T. denticola*.

[b] *T. denticola* (1.4×10^{10} spirochetes/mL) preincubated with antibodies were added to confluent epithelial cultures for 2 h at 20°C. The attachment was measured with ELISA.

* $p<0.05$, Bonferroni test.

Table 3.7 Inhibition of *T. denticola*-FPLE adherence by specific antibodies.

Pre-treatment[a]	Adherence[b]
PBS	100 ± 4
Rabbit (pre-immune)	
serum	100 ± 8
IgG	100 ± 7
anti-*T. denticola*	
IgG	62 ± 6*
anti-Msp	
serum	68 ± 5*
IgG	62 ± 3*
anti-rMsp	
serum	64 ± 3*
IgG	71 ± 10*
anti-CTLP	
serum	49 ± 7*
IgG	50 ± 1*
anti-rMsp IgG + anti-CTLP IgG	46 ± 5*

[a] *T. denticola* cells (5 x 10^9 spirochetes/mL) were incubated in specific antisera (5 mg/mL) or IgG (20 μg/mL) for 1 h, then washed in PBS before adherence assay.
[b] Adherence of *T. denticola* to FPLE cells is expressed as % of adherence of PBS-treated control.
* $p<0.05$, Bonferroni test.

Table 3.8 Cytopathic effects of *T. denticola* on PLE cells[a].

Treponema denticola concentration (Spirochete/ml)	Number of PLE remaining attached[b,c]	Number of PLE taking up trypan blue[b,d]	Number of PLE vacuolated[b,e]	Number of PLE blebbed[b,c]
0	130.8 ± 13.9	3.4 ± 1.2	8.0 ± 2.4	0.4 ± 0.8
7×10^9	115.5 ± 13.1	14.8 ± 1.2*	16.3 ± 2.8*	4.0 ± 4.0
1.5×10^{10}	103.1 ± 12.8*	18.8 ± 7.0*	14.6 ± 3.4*	9.7 ± 8.0*

[a] Early confluent PLE incubated with *T. denticola* in 15% FBS - αMEM, 37°C, 5% CO_2, 2 h.
[b] Mean ± SD values per field; x 40 mag, field area = 0.04 mm^2.
[c] PLE stained with May-Grünwald and Giemsa stains (Sedgley et al. 1996).
[d] PLE stained with Trypan blue (Martin & Clynes 1993).
[e] PLE stained with Neutral red stain (Cover et al. 1991).
* $p<0.05$, Bonferroni test.

Table 3.9 Effect of physical treatment on cytotoxic activity of *T. denticola*.

T. denticola treatment[a]	Concentration (x 10^{10} spirochetes/mL)	Percentage PLE cell viability (mean ± SD)
None	8.5	62.2 ± 11.4
	17.0	42.8 ± 10.4
	35.0	8.4 ± 7.2
Heating		
37°C	8.5	61.4 ± 4.1
	17.0	42.3 ± 6.0
	35.0	0 ± 7.2
60°C	8.5	74.5 ± 13.4
	17.0	59.1 ± 12.1
	35.0	26.7 ± 8.4
80°C	8.5	97.9 ± 20.1*
	17.0	96.7 ± 17.1*
	35.0	74.1 ± 19.2*
100°C	8.5	87.8 ± 17.5
	17.0	98.6 ± 16.4*
	35.0	71.7 ± 13.4*
pH		
2.0	8.5	41.9 ± 10.3
	17.0	36.4 ± 9.8
	35.0	0 ± 17.5
4.0	8.5	43.9 ± 11.6
	17.0	39.1 ± 11.8
	35.0	5.3 ± 8.9
6.0	8.5	60.0 ± 16.8
	17.0	44.3 ± 11.6
	35.0	8.0 ± 8.1
8.0	8.5	64.7 ± 14.0
	17.0	51.0 ± 11.5
	35.0	13.1 ± 6.9
10.0	8.5	60.8 ± 12.5
	17.0	48.0± 13.1
	35.0	14.4 ± 10.3
12.0	8.5	61.5 ± 13.0
	17.0	47.9 ± 18.4
	35.0	2.6 ± 21.3

[a] Following 1 h treatment at specified temperature or at 4°C, the spirochetes were washed 2 × in sterile PBS before being resuspended in 1% FBS-αMEM for viability assay.

* $p < 0.05$, Bonferroni test.

Table 3.10 Effect of physical treatment on cytotoxic activity of *T. denticola*.

T. denticola treatment[a]	Concentration (x 10^{10} spirochetes/mL)	Percentage PLE cell viability (mean ± SD)
None	8.5	62.7 ± 10.3
	17.0	45.1 ± 12.1
	35.0	0 ± 7.2
Protease inhibitors		
PMSF, 2 mM	8.5	74.3 ± 11.6
	17.0	49.2 ± 9.8
	35.0	2.0 ± 8.1
TLCK, 2 mM	8.5	61.0 ± 13.5
	17.0	43.3 ± 11.0
	35.0	13.1 ± 2.9
TPCK, 2 mM	8.5	58.0 ± 12.1
	17.0	47.8 ± 11.5
	35.0	14.4 ± 7.3
Cysteine, 50 mM	8.5	64.3 ± 18.1
	17.0	41.3 ± 11.6
	35.0	0 ± 6.7
Enzymes		
mixed glycosidase, (100 μg/mL)	8.5	59.7 ± 8.7
proteinase K, (100 μg/mL)	8.5	83.6 ± 6.5
Serum proteins etc.		
BSA (100 mg/mL)	8.5	63.7 ± 14.1
	17.0	45.6 ± 10.3
	35.0	0 ± 9.2
FBS (100 mg/mL)	8.5	71.3 ± 9.0
	17.0	41.5 ± 16.4
	35.0	0 ± 7.2
Fibrinogen (100 mg/mL)	8.5	56.4± 9.8
	17.0	39.8 ± 10.3
	35.0	0 ± 17.5
Fibronectin (1 mg/mL)	8.5	60.5 ± 11.2

[a] Following 1 h treatment at 4°C, the spirochetes were washed 2 x in sterile PBS, resuspended in 1% FBS-αMEM counted/O.D. before viability assay.

Table 3.11 Inhibition of *Treponema denticola* cytotoxicity by specific antisera.

Pretreatment[a]	% Viability[b]
PBS	25 ± 6
Rabbit serum	26 ± 5
anti-*T. denticola* whole cell serum	62 ± 3*
anti-CTLP serum	66 ± 4*
anti-Msp serum	62 ± 12*

[a] *T. denticola* (2 x 10^{11} spirochetes/mL) cells were incubated in specific antiserum for 2 h, 4°C, then washed 2 x in sterile PBS before cytotoxic assay (MTT). Antisera concentration was at 5 mg protein/mL

* $p < 0.05$, Bonferroni test

Table 3.12 Average single-channel conductance of *T. denticola* 53 kDa porin[a].

Salt	Concentration (M)	G (nS)	G/σ (10^{-8} cm)	n
KCl	1	10.9	10.6	318
	0.1	1.8	15	145
	0.05	1.4	20	77
	0.01	0.2	14	108
LiCl	1	9.2	13	162
NaCl	1	11.8	14	129
$K^+CH_3COO^-$	1	9.8	14	165
KI	1	18.8	15	195
KBr	1	13.3	12	112
KF	1	15.0	19	149
$Tris^+Cl^-$	1	7.1	23	224
$Tris^+Hepes^-$ (pH 8)	1	0.5	7.3	200
Na^+Hepes^- (pH 9)	1	2.8	15	215

[a] The pH of the salt solutions was 7 if not otherwise indicated. A constant voltage of 10 mV was applied to the diphytanoyl phosphatidylcholine membrane. Average conductance increments (G) were determined by recording a large number (n) of conductance steps and averaging them. σ is the specific conductance of the aqueous salt solution.

Table 3.13 Adherence and cytotoxicity of Msp to FPLE and PLE cells respectively.

Pre-treatment	Adherence[a,b]	Cytotoxicity[c]
PBS	100 ± 0.5	100 ± 1.2
anti-rMsp IgG	43 ± 2.7*	54 ± 4.3*
Rabbit IgG	100 ± 12.4	97 ± 6.7

[a] Adherence to FPLE cells expressed as per cent of untreated control after 1 h challenge.
[b] Native Msp was used at 1.75 μg per ml. Assays were conducted at room temperature.
[c] Cytotoxicity to PLE cells as measured by release of cytoplasmic LDH is expressed as per cent of positive lysis control after 1 h challenge.
* $p < 0.05$, Bonferroni test

Table 3.14 Adherence and cytotoxicity of CTLP[a] to FPLE and PLE cells respectively.

Pre-treatment[b]	Adherence[c]	SAAPNA[d]	Cytotoxicity[e]
PBS	100 ± 9.6	100 ± 2.0	100 ± 1.6
anti-CTLP IgG	92 ± 11.2	38 ± 3.9*	87 ● 6.3
Rabbit IgG	98 ● 14.1	100 ± 0.3	97 ± 5.1
PMSF	73 ± 1.3*	6.2 ± 1.5*	71 ± 3.2*
TLCK	100 ± 3.2	88 ± 11.6	96 ± 0.4
TPCK	85 ± 1.6	12.8 ± 3.3*	83 ± 5.0*
PMSF + anti-CTLP	46 ± 7.3*	8.4 ± 1.0*	42 ± 4.0*

[a] CTLP was used at 0.15 μg per ml. Assays were conducted at room temperature.
[b] Samples were pre-treated by incubation for 1 h and washed by ultrafiltration.
[c] Adherence to FPLE cells after 1 h challenge, detected by anti-CTLP IgG, is expressed as per cent of PBS-treated control.
[d] Chymotrypsin-like activity of pre-treated CTLP before PLE challenge, as measured by cleavage of chromogenic substrate.
[e] Cytotoxicity of PLE cells after 1 h challenge, as measured by release of cytoplasmic LDH, is expressed as per cent of positive lysis control.
* $p < 0.05$, Bonferroni test

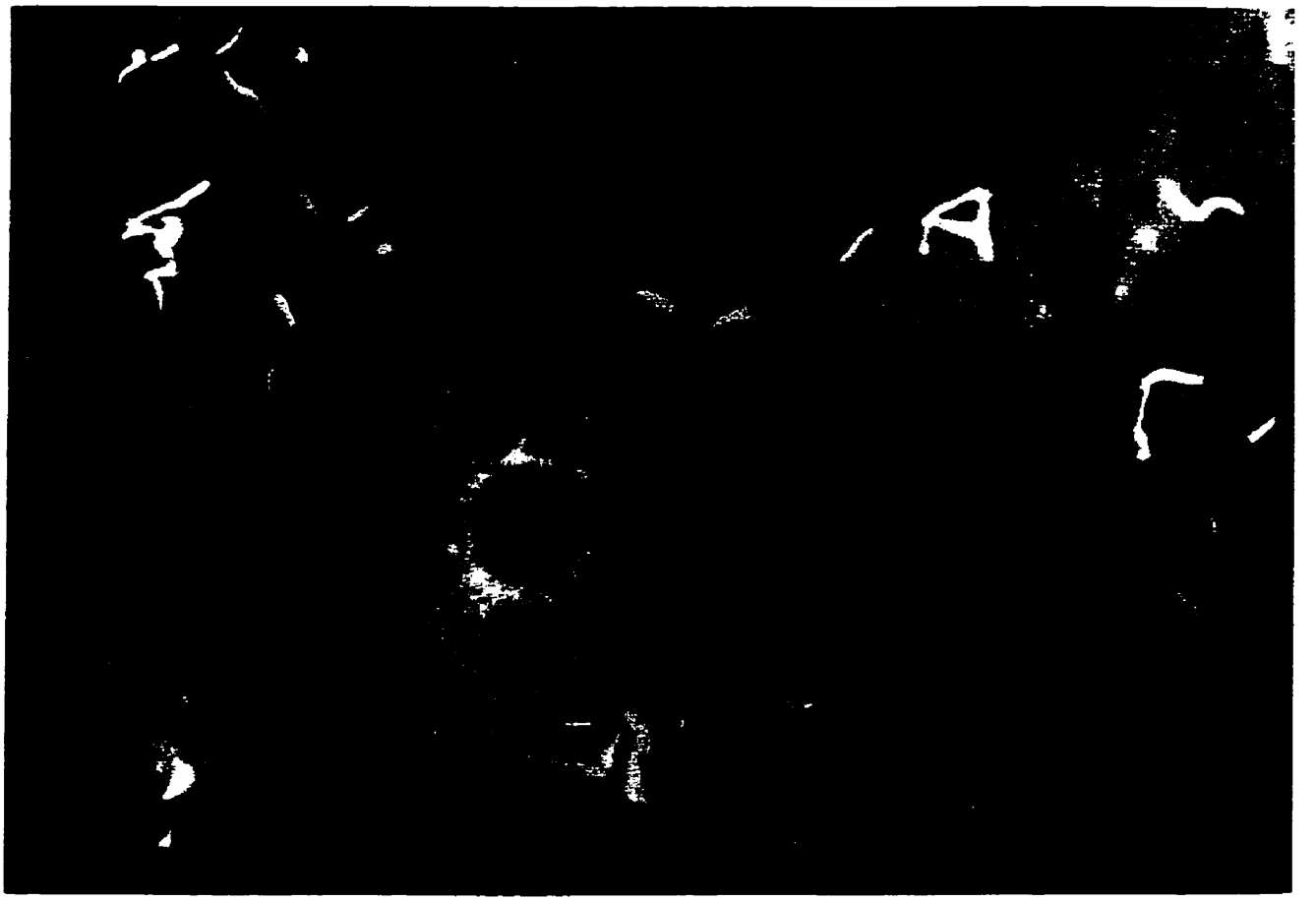


Figure 3.1 *T. denticola* binding to PLE cells. The interaction was observed under fluorescence microscopy using FITC-labeled antibody for *T. denticola* immunostaining and rhodamine-phalloidin for actin detection. A 30% confluent epithelial culture incubated with 2.5×10^8 spirochete / mL for 2 h at 37°C.

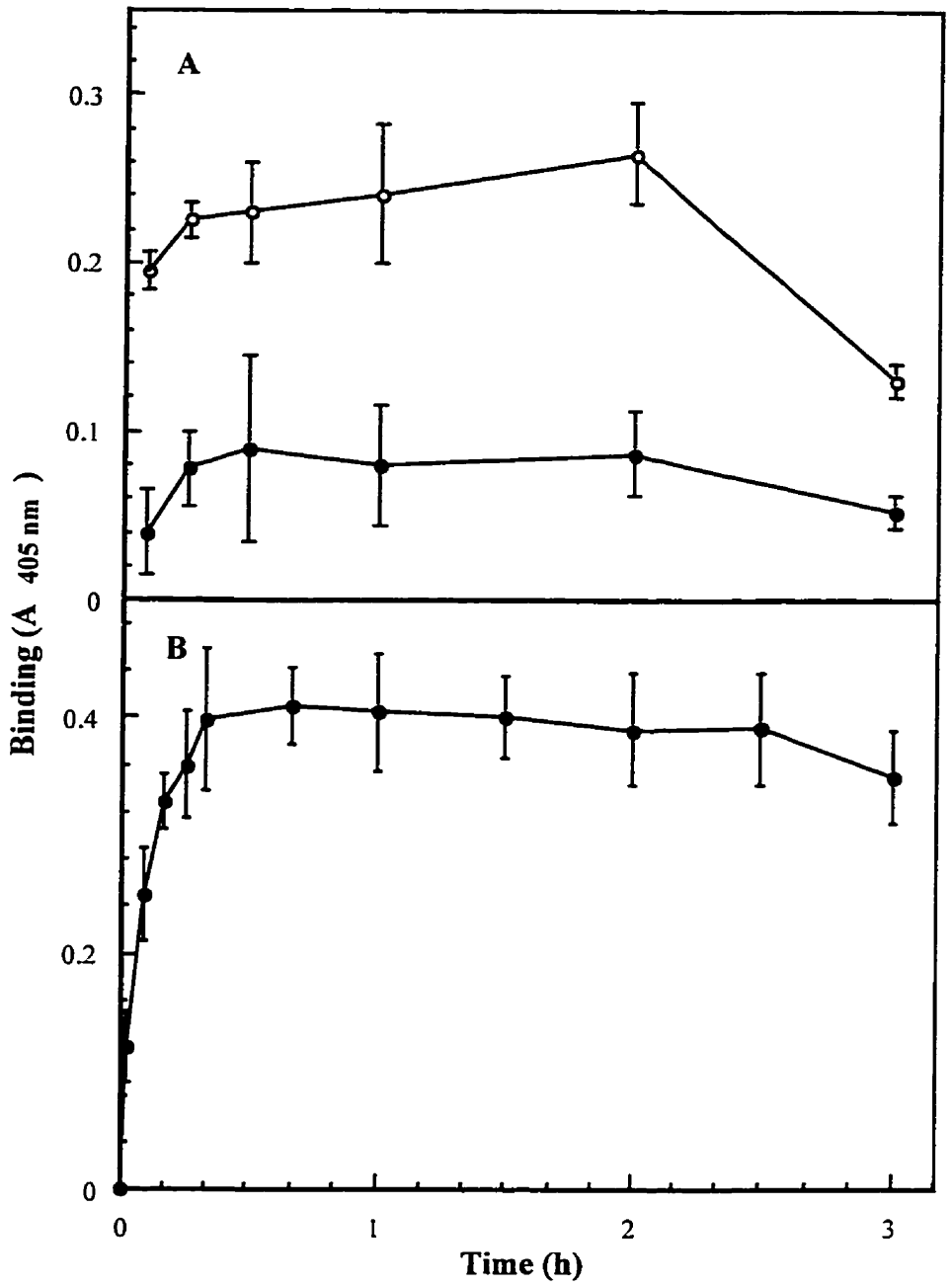


Figure 3.2 Time dependent binding of *T. denticola* to PLE/FPLE cells. *T. denticola* (1.4 x 10^{10} spirochetes / mL) was added to epithelial confluent cultures: A) PLE cells, B) FPLE cells; open circles, 4°C; solid circles, 20°C. The binding was assayed with ELISA using anti-whole *T. denticola* antibodies. Values are mean ± SD of four samples.

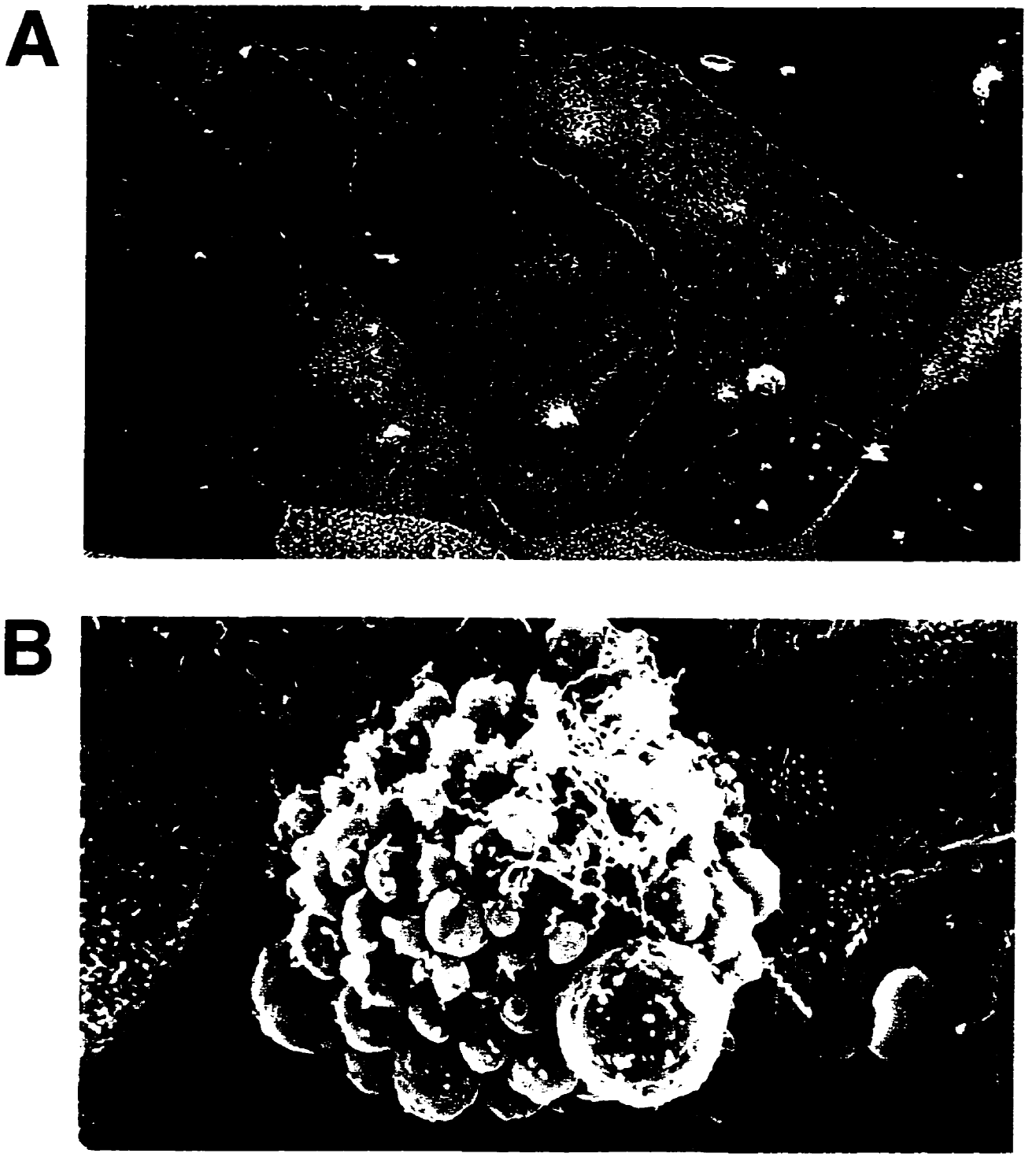


Figure 3.3 Scanning electron microscopy of *T. denticola* adhesion to monolayer of PLE cells. A) Control PLE cells culture (× 720 magnification). B) *T. denticola* challenged PLE cell culture. Retraction of PLE cells (lower portion of micrograph) and cell surface blebbing is evident. *T. denticola* also attached to PLE cell blebs (× 2500 magnification).

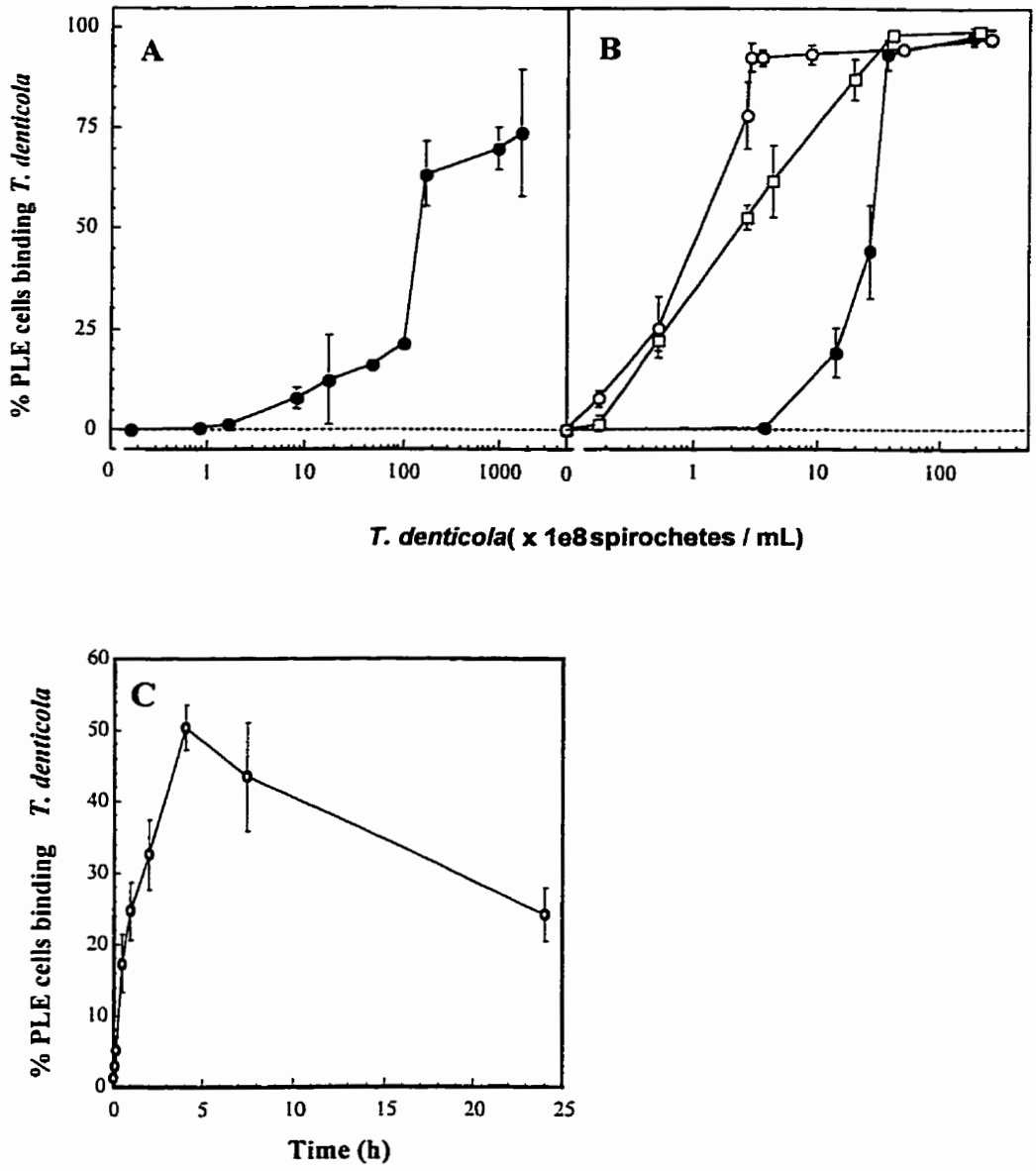


Figure 3.4 Percentage of PLE cells binding at least one bacterium after challenge by *T. denticola*. High (A) or low (B) density epithelial cultures were incubated for 2 h at 37°C with *T. denticola* in αMEM containing different FBS concentrations: solid circle, 15% serum, open square, 1% serum; open circle, no serum. C) 50% confluent PLE cells culture was incubated at 37°C with 1.3×10^8 *T. denticola* in αMEM. Values are mean ± SD of four samples.

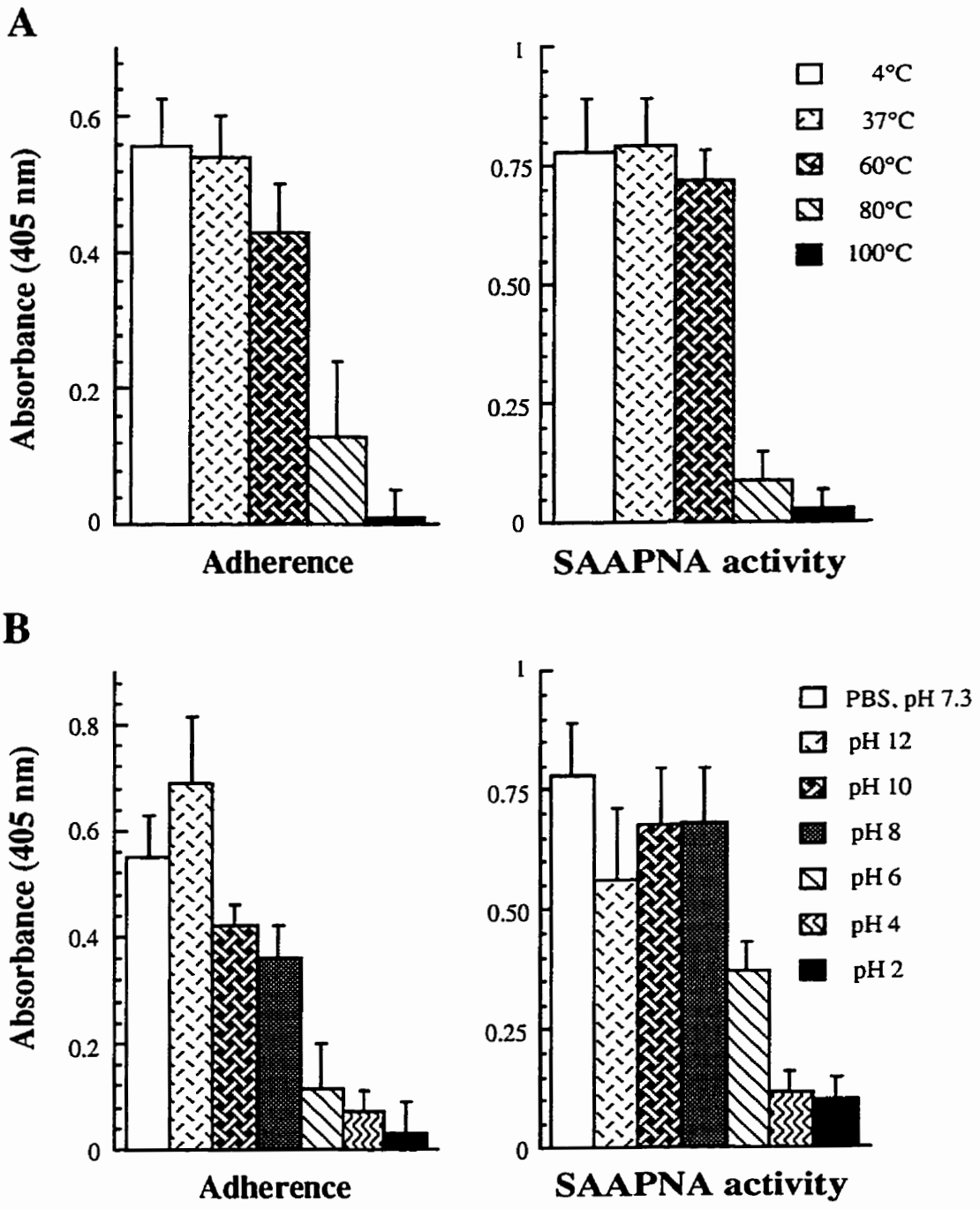


Figure 3.5 Effect of physical pretreatment on *T. denticola* binding to FPLE cells. 5×10^9 spirochetes/mL in plain αMEM, 2h, 37°C. Attachment measured by ELISA. Values are mean ± SD of four samples.

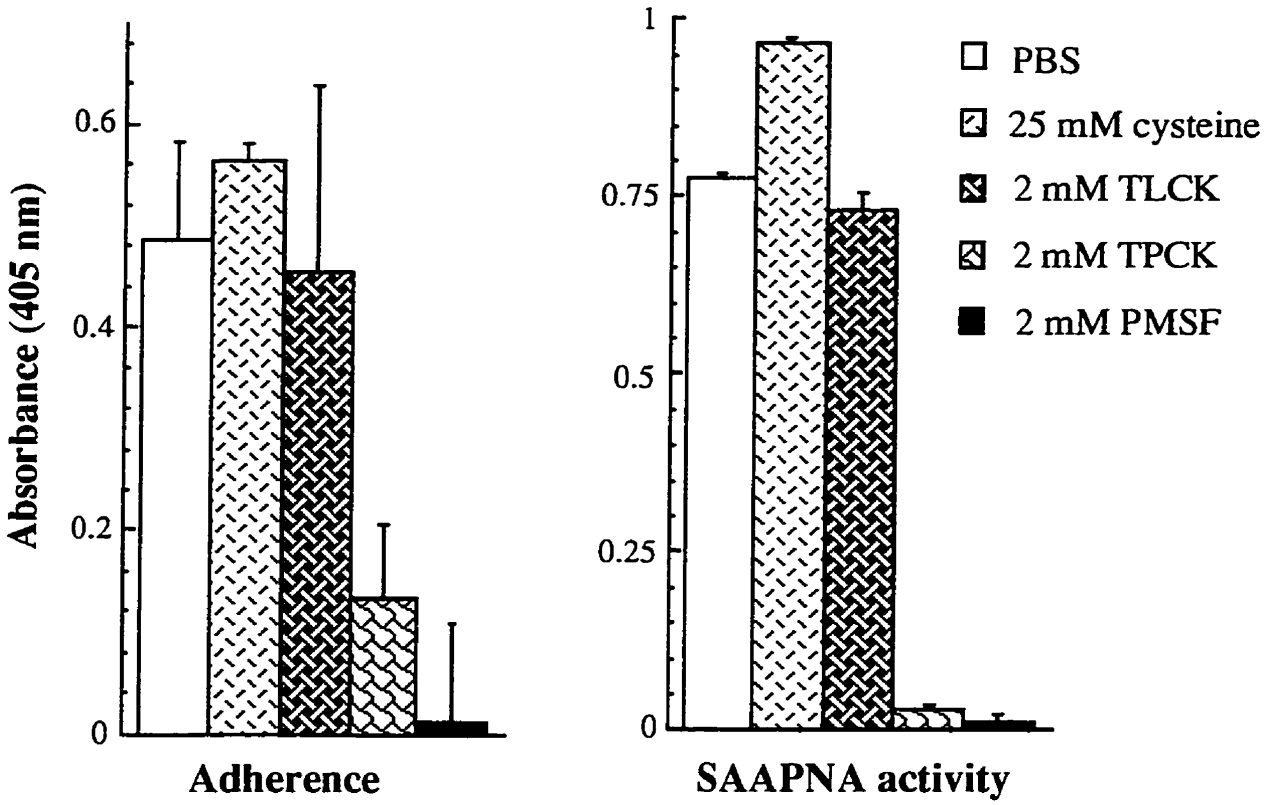


Figure 3.6 Effect of chemical pretreatment on *T. denticola* binding to FPLE cells. 5×10^9 spirochetes/mL in plan αMEM, 2h, 37°C. Attachment measured by ELISA. Values are mean ± SD of four samples.

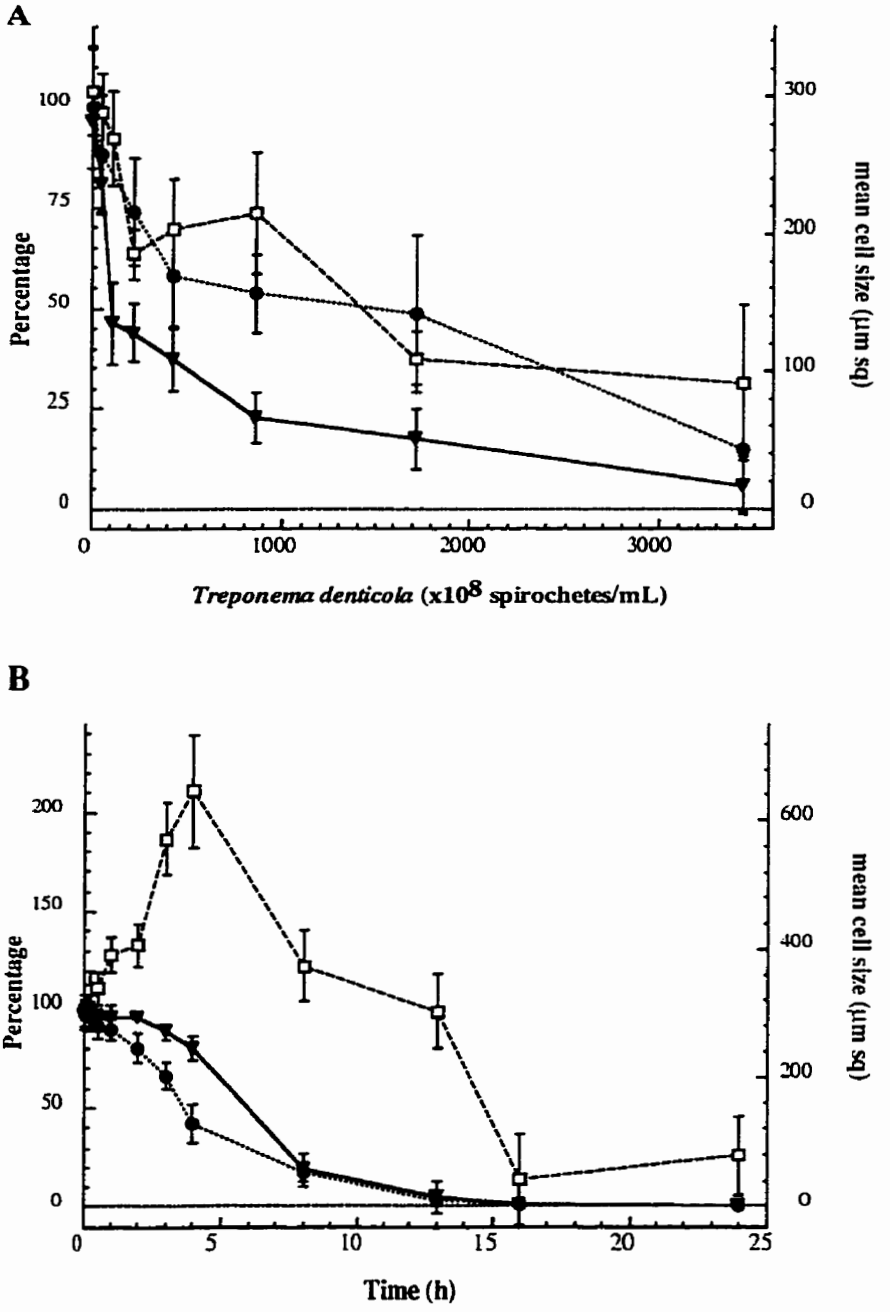


Figure 3.7 *T. denticola* induced PLE cell morphological changes. A) Morphometric assay of early confluent PLE monolayers infected with *T. denticola*, 24 h; B) Morphometric assay of early confluent PLE monolayers infected with 3×10^{11} *T. denticola*/mL. *T. denticola* was suspended in 15% FBS-αMEM, 5% CO_2, 37°C. Data showing mean ± SD of results from 6 independent experiments. Morphometric recording carried out by an IBAS image analyzing system. Open square: mean cell size; solid circle: cell count; solid inverted triangle: confluence.

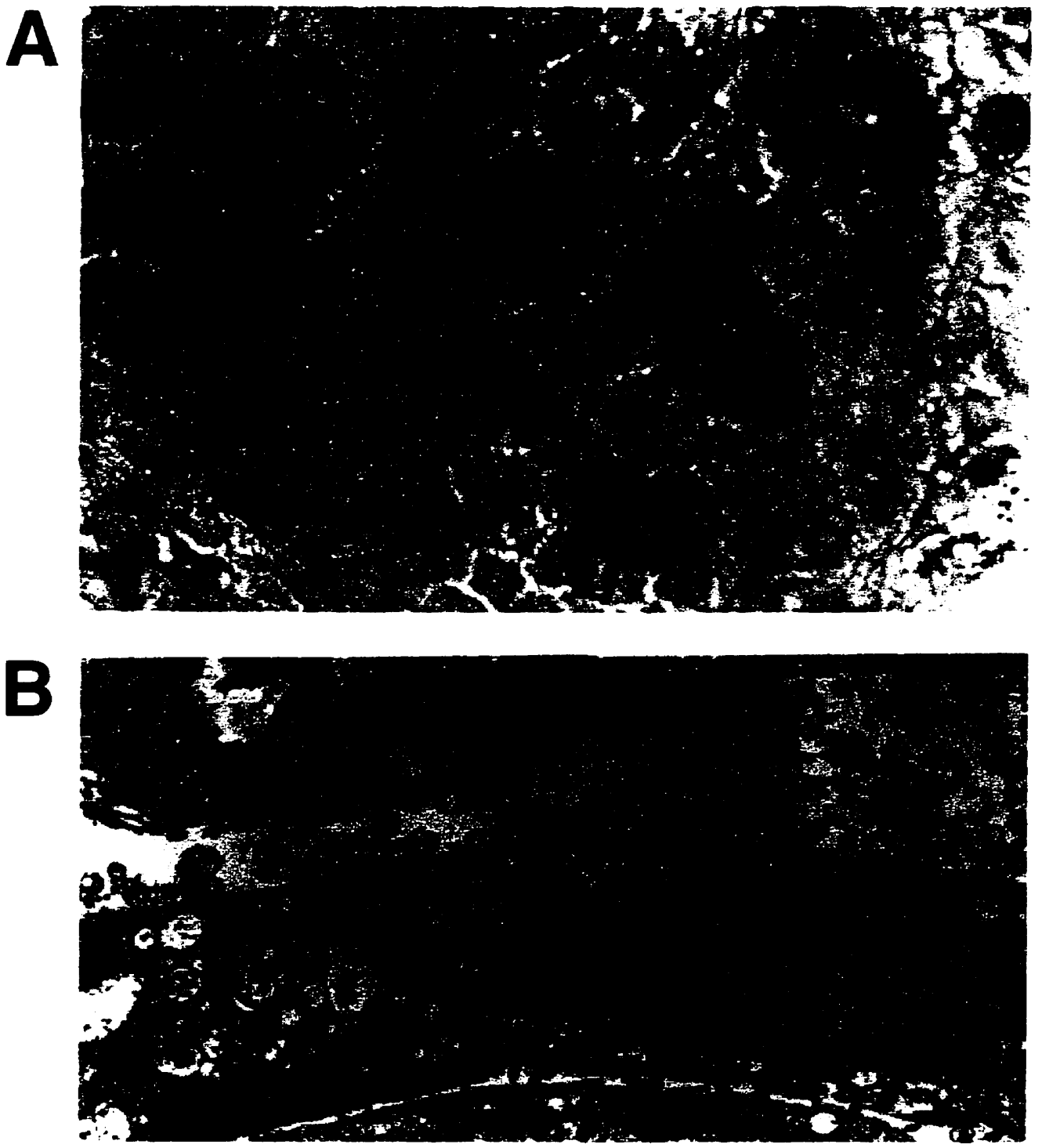


Figure 3.8 PLE cells vacuolation induced by *T. denticola*. A) Confluent PLE cell culture was challenged by *T. denticola* and stained with neutral red as per Cover et al. (1991). Note groups of vacuolated cells taking up the neutral red stain (×200 magnification). B) TEM section of multi-layer PLE cell culture infected with *T. denticola* showing cytoplasmic vacuolation (arrowheads). Bar = 1 μm.

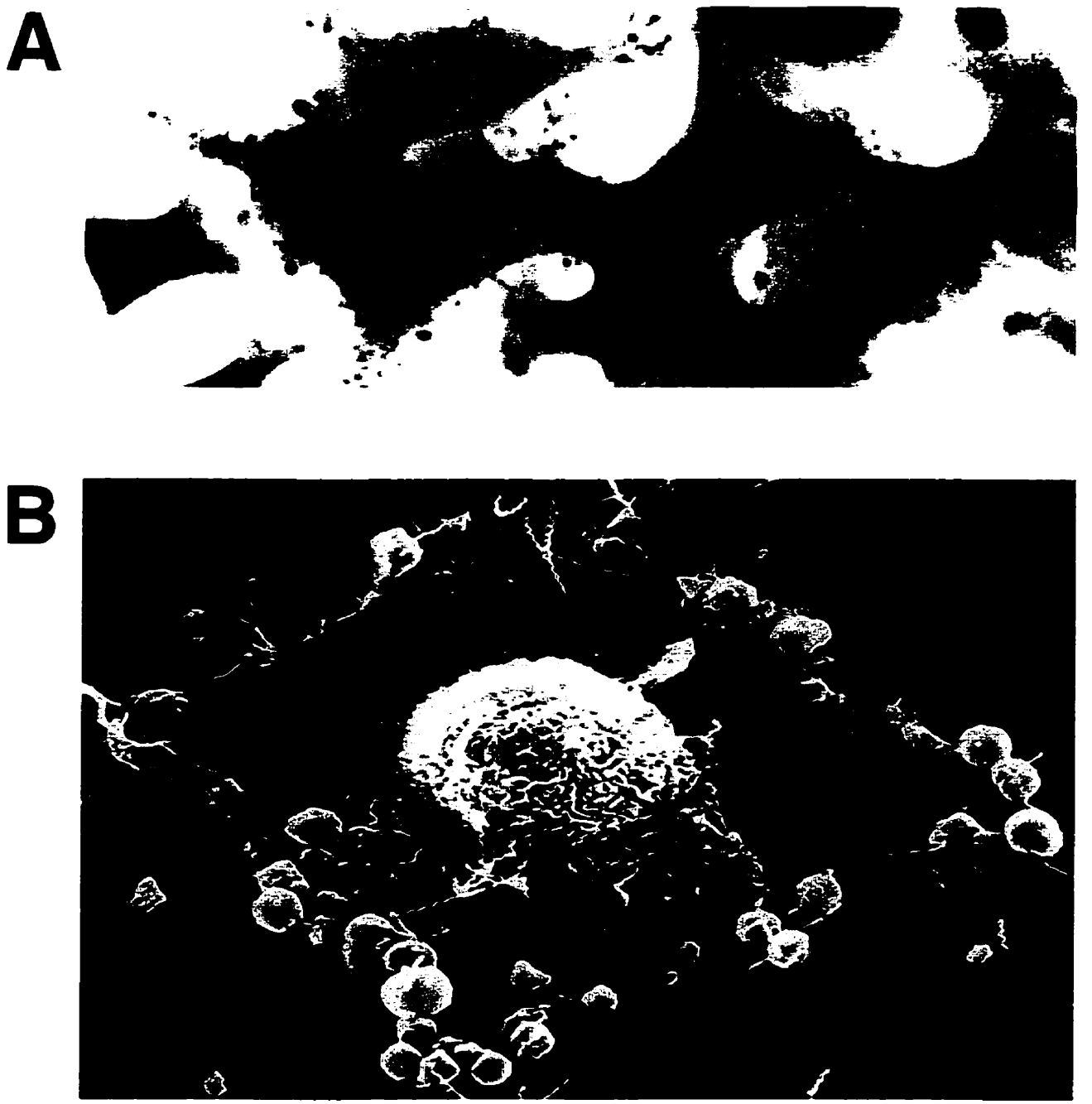


Figure 3.9 *T. denticola* induced blebbing of PLE cells. A) PLE cell monolayer infected with 1.5 × 10^{10} *T. denticola*/mL. Close up of a colony of blebbed PLE cells. PLE cells stained with May-Grünwald and Giemsa stains, × 500 magnification. B) Sparse PLE cell culture challenged with 1 × 10^{8} *T. denticola*/mL. Observed under SEM, × 2500 magnification

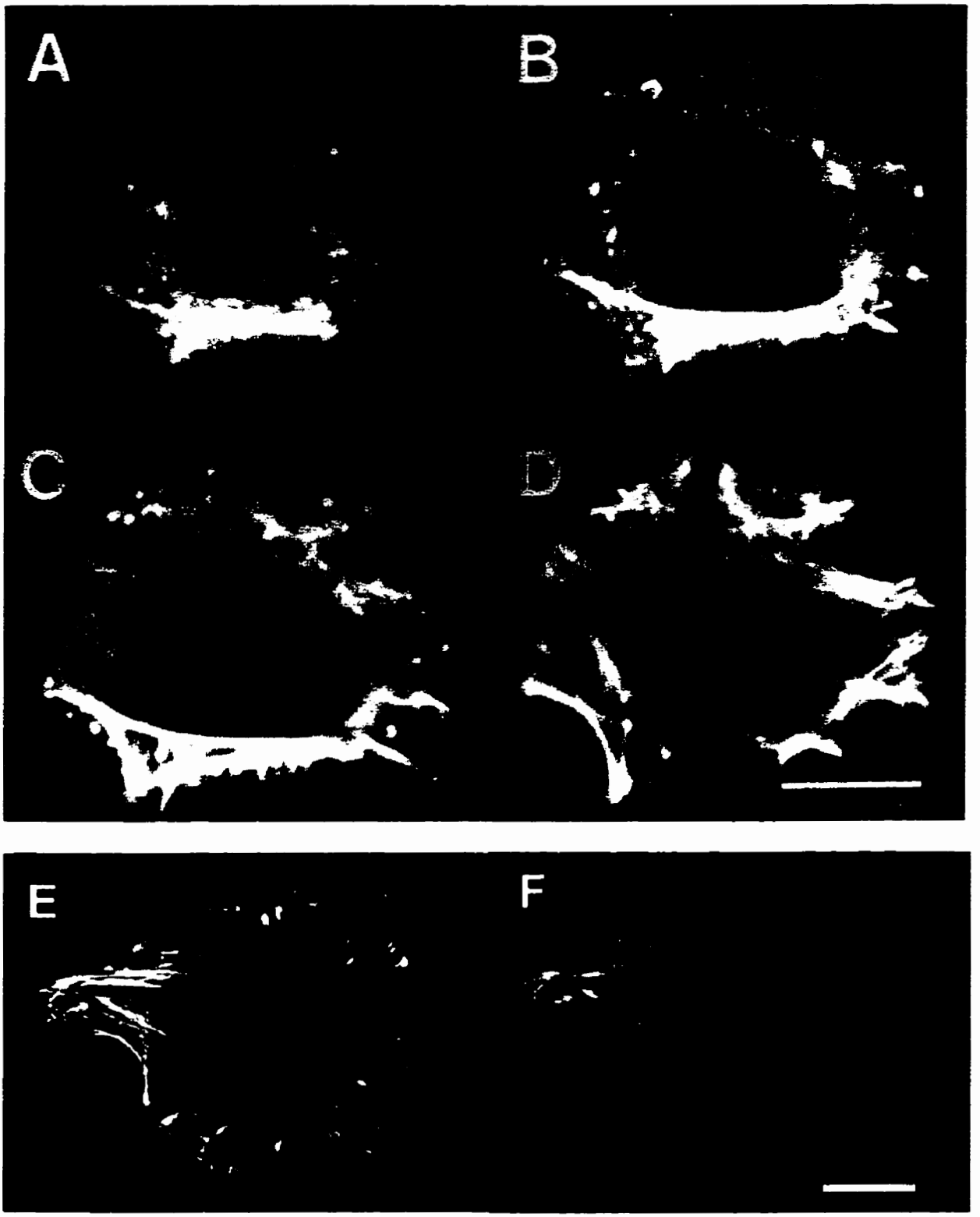


Figure 3.10 Confocal optical sections of blebbing (A-D) and control (E-F) PLE cells after treatment with *T. denticola*. PLE cells were treated with *T. denticola* for 2 h and then processed for immunofluorescence staining of actin. Confocal microscopy of rhodamine-phalloidin-stained F-actin examined at 3 μm increments from the apical to the basal cell surface showing disruption of the peripheral actin network in test cell (A-D versus E-F). Note that blebs were only observable at the apical sections (A, B and C). Bars = 20 μm.

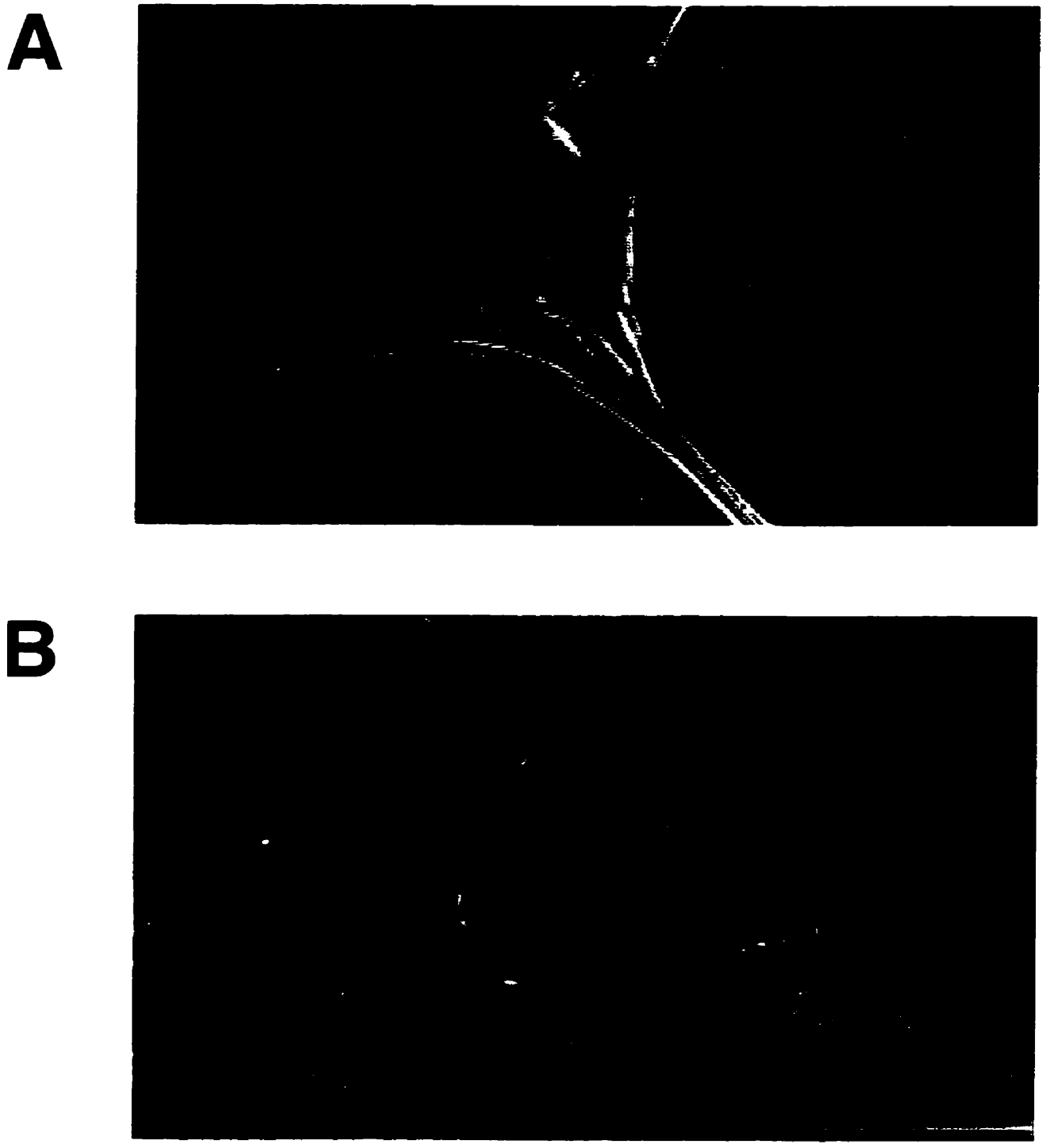


Figure 3.11 Actin arrangement of *T. denticola* treated PLE cells. Confocal micrograph of rhodamine-phalloidin-stained F-actin. A) A blebbed PLE cell showing marked cytoplasmic contraction and/or elongation; B) A non-blebbing PLE cell colony infected with *T. denticola*: confocal optical section 5 µm from the basal cell surface, preparation double-stained for *T. denticola* (FITC). Note that no specific arrangement of actin was observed in the area where the spirochetes (more brightly stained) were in close contact with the PLE cells. PLE cell treatment conditions similar to Figure 3.10.

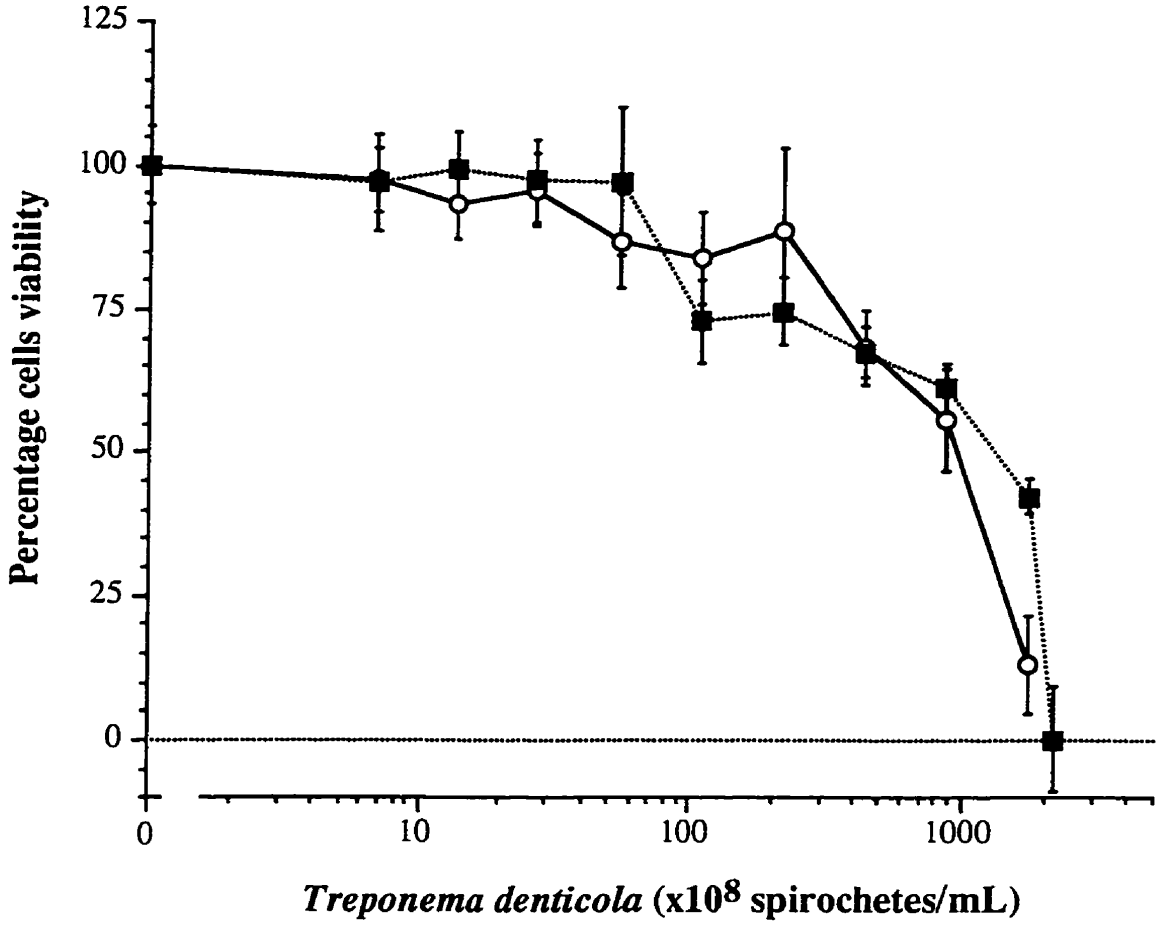


Figure 3.12 Viability of *T. denticola* infected PLE cell monolayer. Early confluent PLE cells challenged with *T. denticola* whole cell (solid square) or sonicate (open circle) in 1% FBS-αMEM, 5% CO_2 in air, 37°C, 24 h. MTT assay for PLE cells viability measurement. Data showing mean ± SD (n=6).

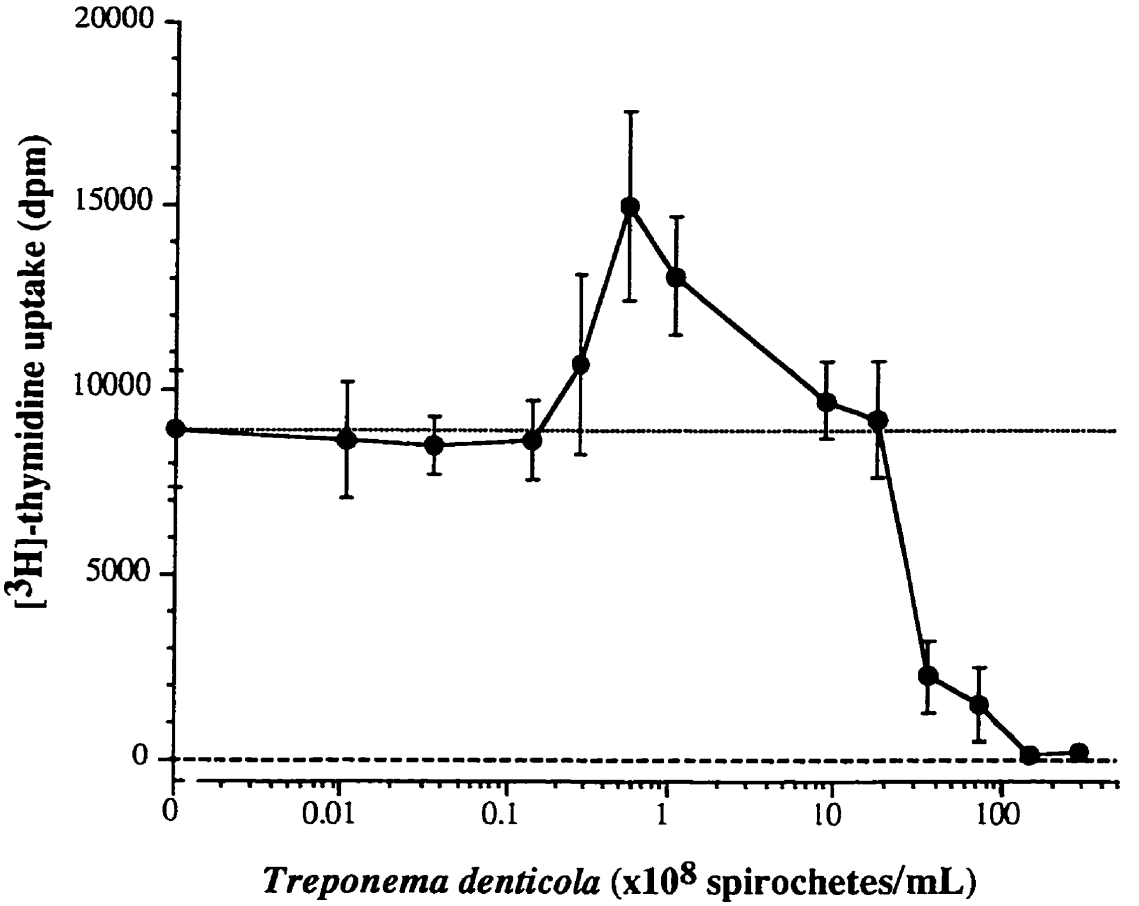


Figure 3.13 PLE cells proliferation after *T. denticola* infection. Semi-confluent (50%) quiescent PLE cells were challenged with *T. denticola* in 1% FBS-αMEM, 5% CO_2 in air, 37°C, 30 h, ^{3}H-TdR uptake was recorded. Data showing mean ± SD (n=6). Positive controls: 83 μg/mL *E. coli* LPS in 1% FBS-αMEM, or 15% FBS-αMEM triggered PLE cells ^{3}H-TdR uptake at or above 30000 dpm level.

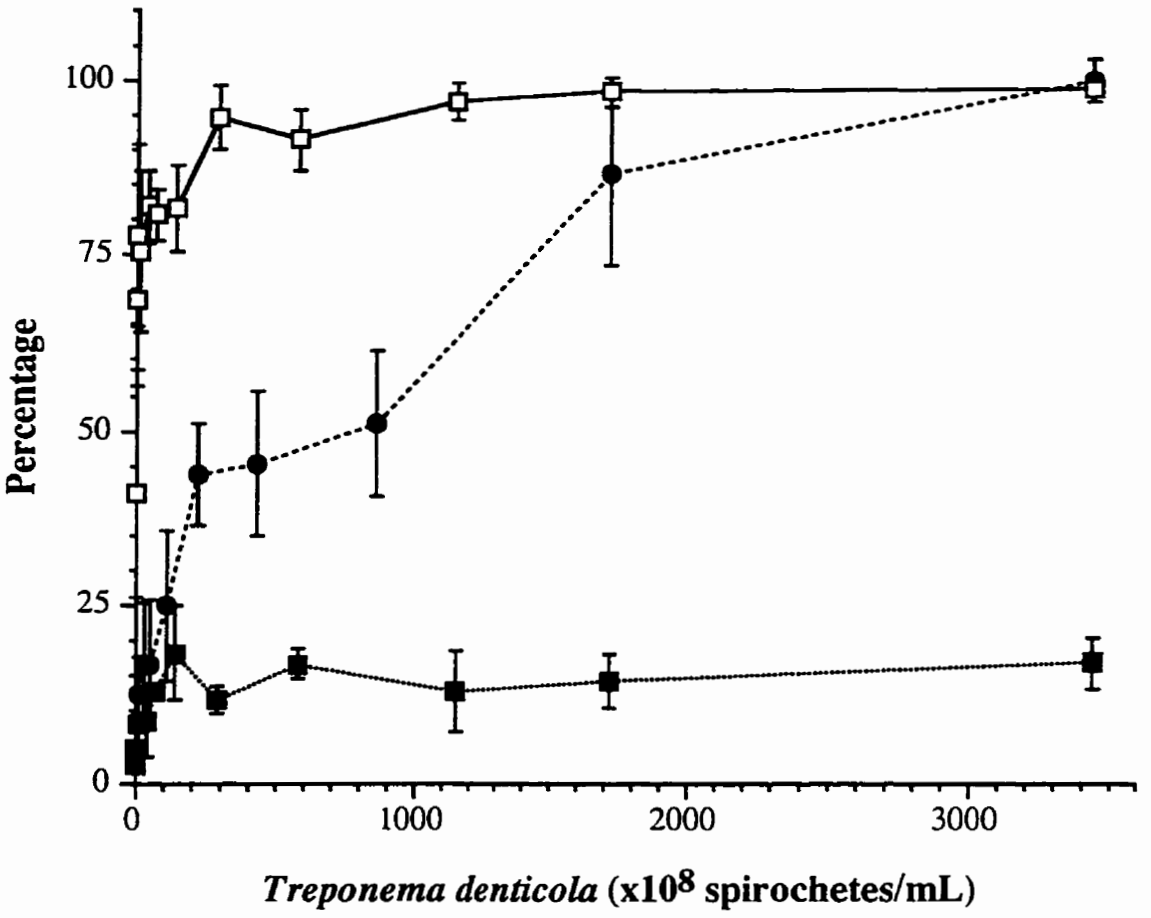


Figure 3.14 Trypan blue dye exclusion assay and TUNEL staining of PLE cells monolayer infected with *T. denticola*. PLE cells challenged with *T. denticola* in 15% FBS-αMEM, 5% CO_2 in air, 37°C, 24h. Open square: trypan blue uptake of detached PLE; solid square: trypan blue uptake of attached PLE cells; solid circle: positive TUNEL stained PLE cells. Data showing mean ± SD (n=6).

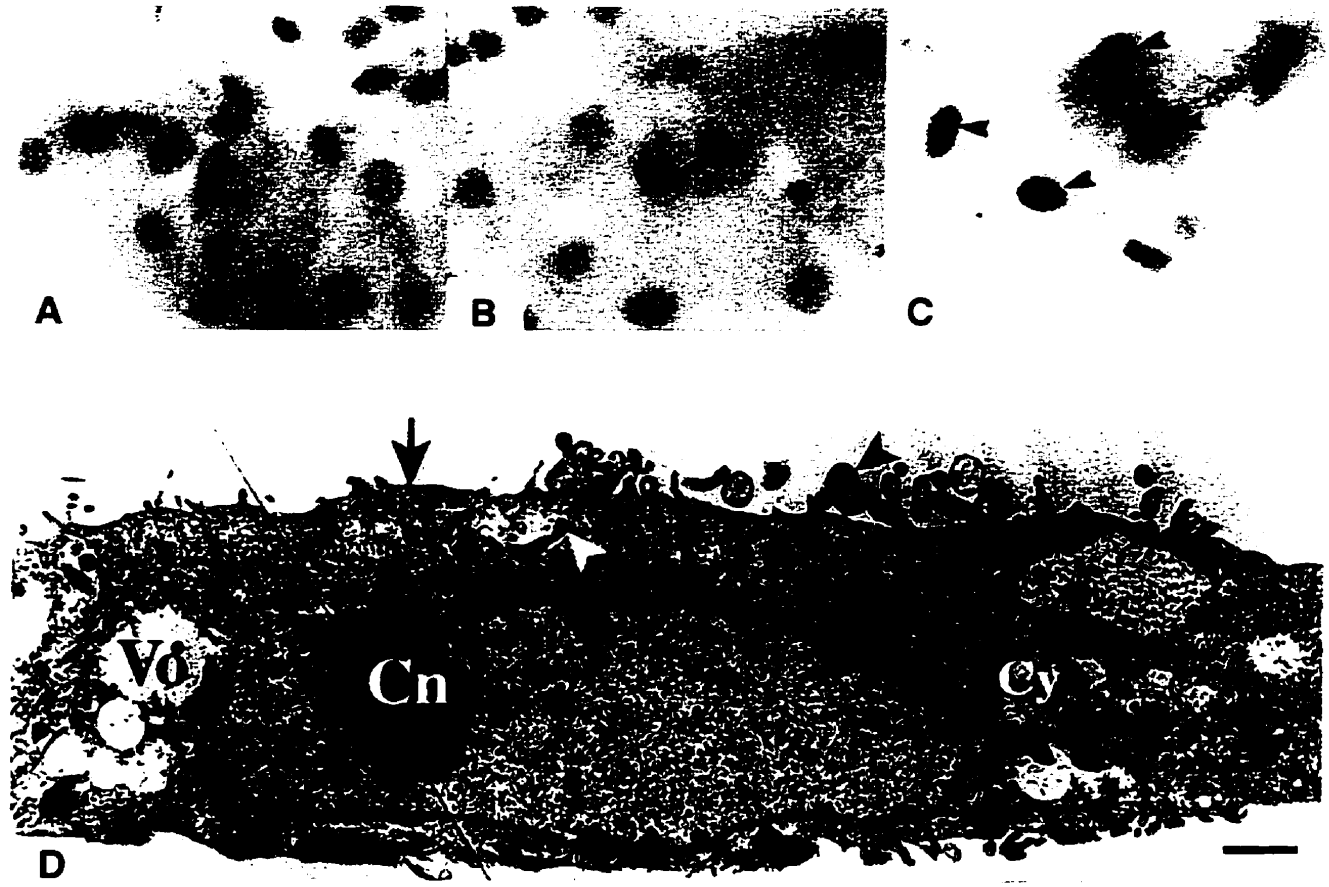


Figure 3.15 Apoptotic changes of *T. denticola* infected PLE cells. PLE cells subjected to TUNEL staining after challenge by *T. denticola* in 15% FBS-αMEM, 5% CO_2 in air, 37°C, 24 h (A-C). Nucleus of apoptotic cells were TUNEL positive (darkly stained, arrow heads). A) Control PLE cells; B) PLE cell monolayer infected with 5×10^9 spirochetes/mL; C) PLE cell monolayer infected with 1.7×10^{11} spirochetes/mL. (× 150 magnification); D) TEM section of PLE cells infected with *T. denticola*. Cytopathic changes diagnostic of apoptosis (Kerr et al. 1995) are evident from the preparation, including: i) protrusion / blebbing of the cell surface (arrow head), ii) reduction / disappearance of cell surface microvilli (arrow), iii) vacuoles in cytoplasm (Vo), iv) cytoplasmic condensations (Cy), v) condensation of nuclear chromatin (Cn). Note also binding of *T. denticola* on PLE cell surface and internalization of *T. denticola* (white arrow head) at site where the PLE cell membrane ruptured. Bar = 1μm.

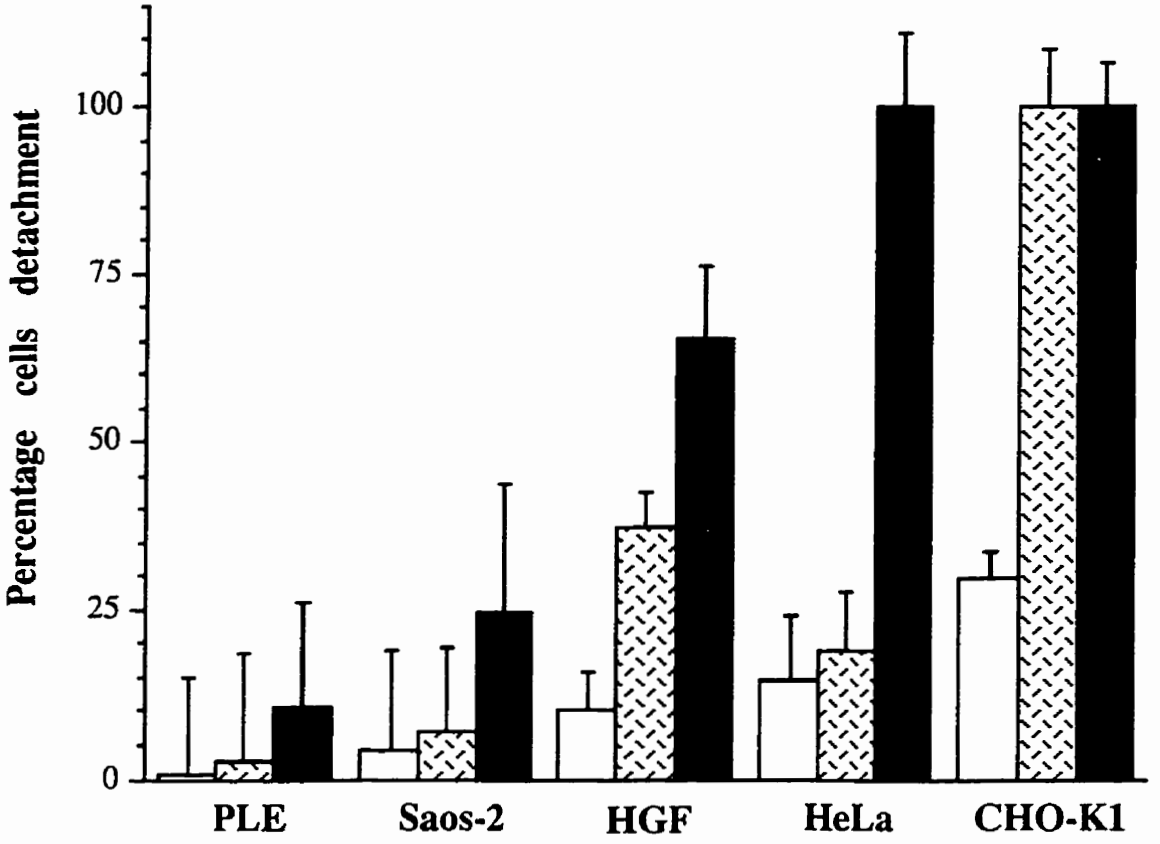


Figure 3.16 Detachment assay of *T. denticola* infected monolayers of cells in culture. All cell incubated with *T. denticola* at (columns l-r): 1×10^8, 1×10^9 and 5×10^9 spirochetes/mL in full strength culture medium without antibiotics, 5% CO_2 in air, 37°C, 24 h. Detachment was measured indirectly with crystal violet dye elution assay. Data showing mean ± SD (n=6).

Figure 3.17 Morphology of detached PLE cellular materials post *T. denticola* challenge. PLE cells infected with *T. denticola* for 2 h and then detached PLE cells were processed for fluorescence staining of actin. Detached cells stained with rhodamine-phalloidin. Note loss of cellular and cytoskeletal structures (× 800 magnification).

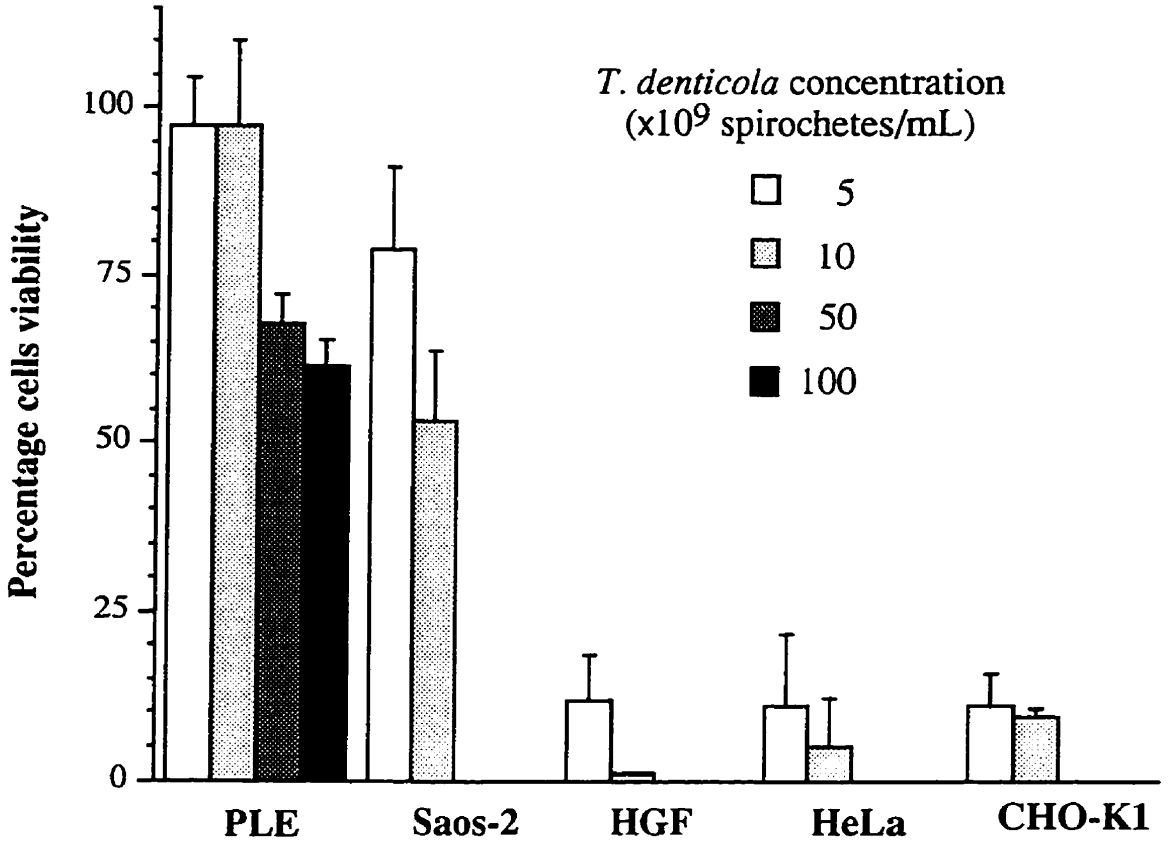


Figure 3.18 Viability of *T. denticola* infected monolayers of PLE, Saos-2, HGF, HeLa and CHO-K1 cell cultures. All cells incubated with *T. denticola* in 1% FBS-αMEM, 5% CO_2 in air, 37°C, 24 h. Viability measured by MTA. Data showing mean ± SD (n=6).

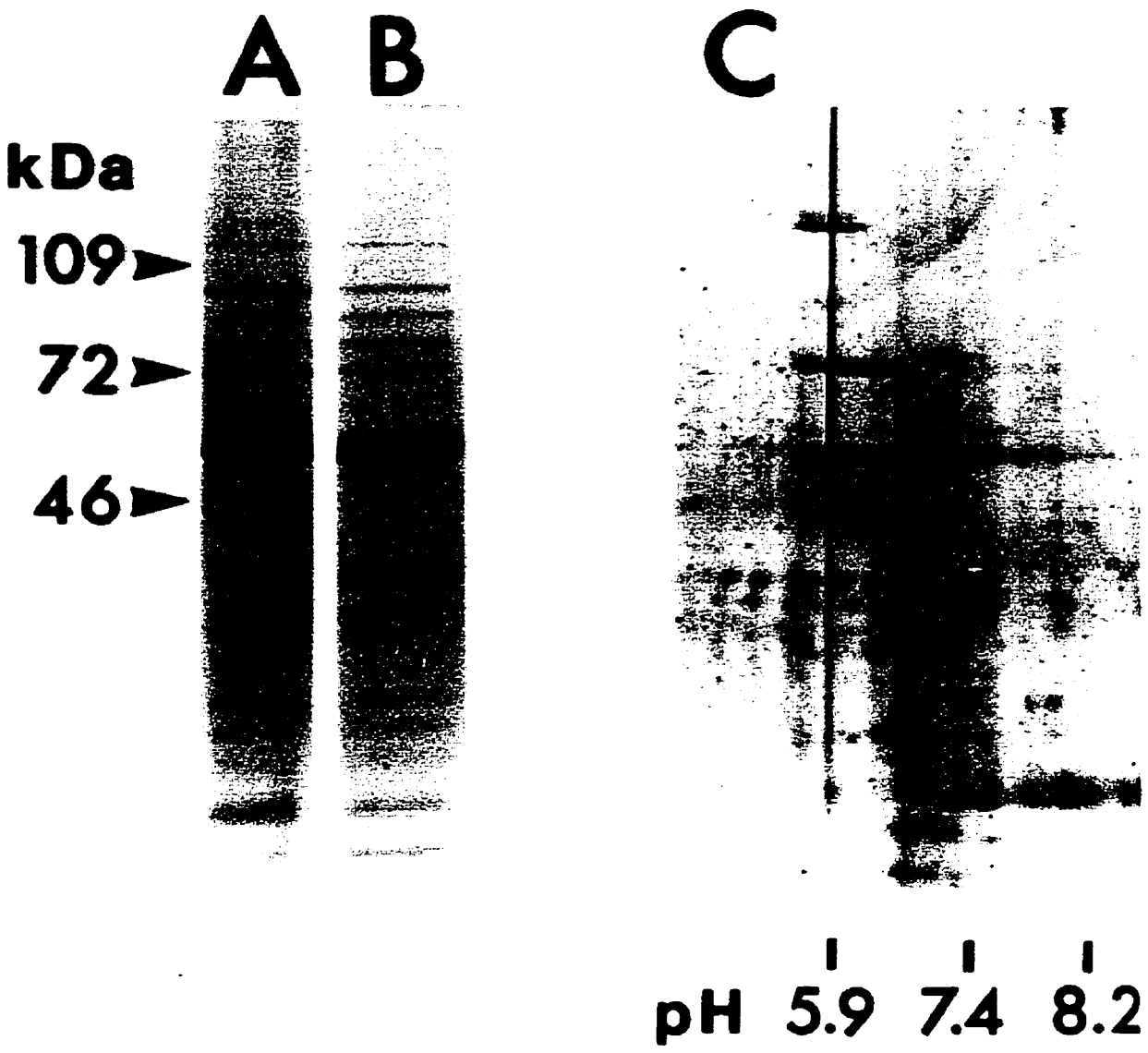


Figure 3.19 SDS-PAGE and IEF of *T. denticola* ATCC 35405 protein extracts. Lanes: A) NP-40 extract of *T. denticola* whole cells: B) NP-40 extract of the particulate fraction of sonicated *T. dentcola*: C) IEF of proteins of the *T. dentcola* particulate fraction.

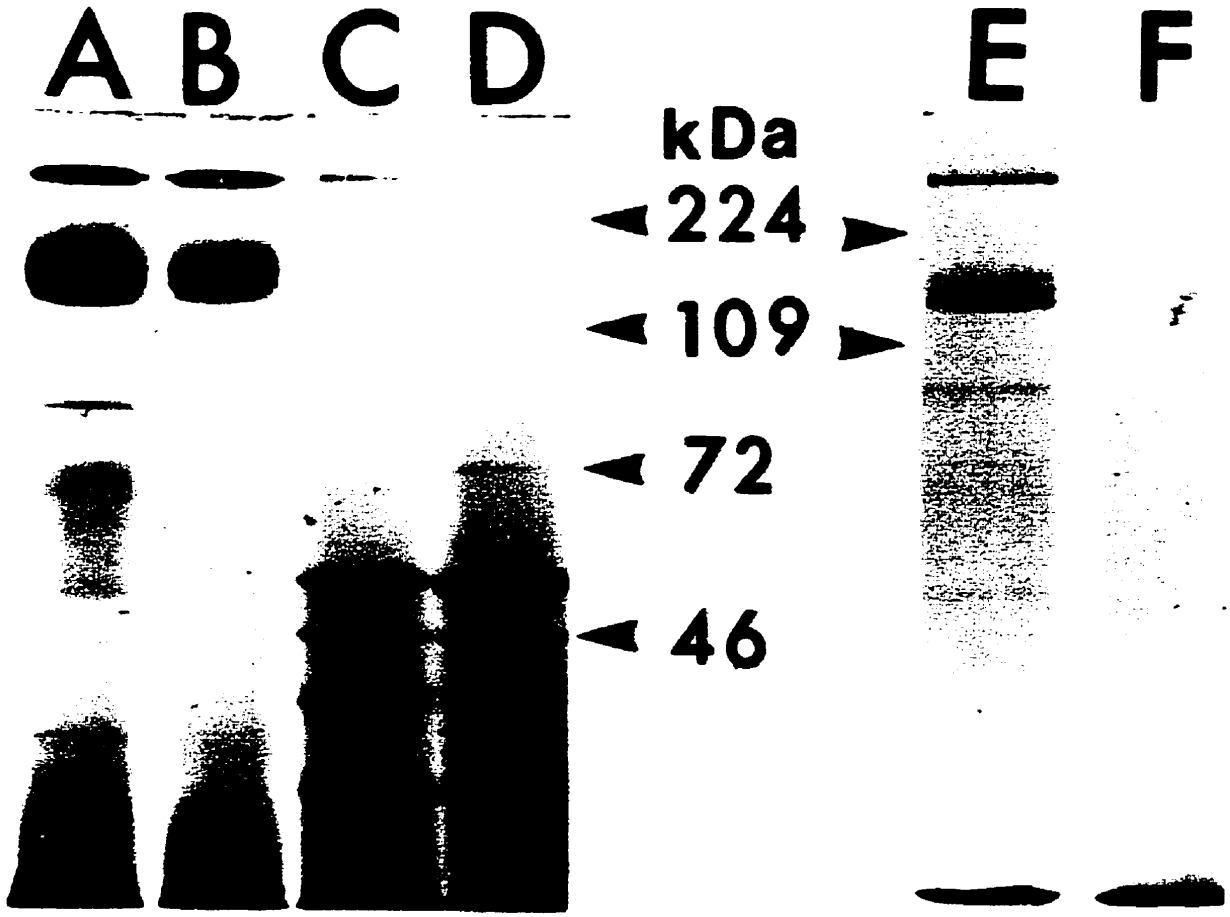


Figure 3.20 Effect of heat and proteolytic treatment on *T. denticola* ATCC 35405 protein. SDS-PAGE protein profiles. Lane: A, unheated whole-cell extract; B-D, whole-cell extract incubated at 60, 70 and 80°C respectively, prior to electrophoresis; E and F, proteinase K treatment of the particulate fraction of *T. denticola* before (E) and after (F) heating at 100°C for 5 min.

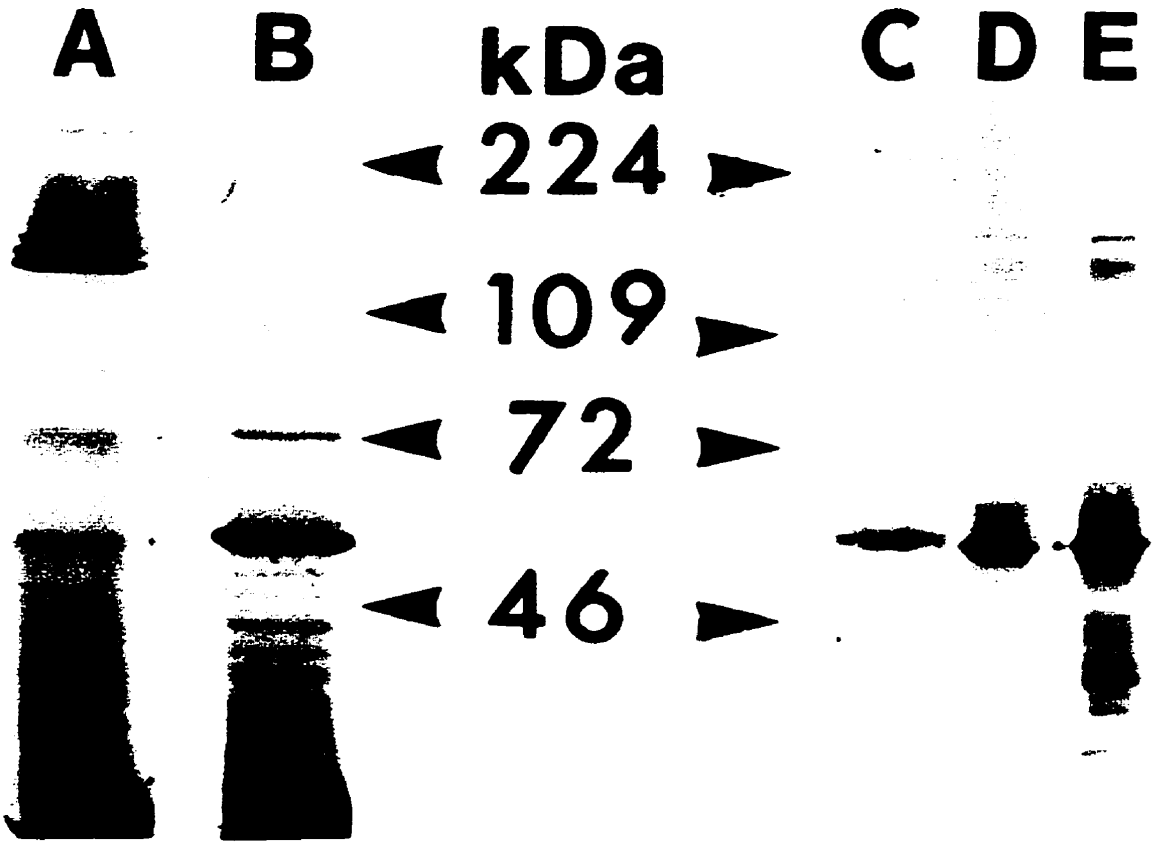


Figure 3.21 Immunoblotting of whole cell lysate of *T. denticola*. Anti-*T. denticola* whole cell antiserum (lanes A and B) and anti-*T. denticola* 53 kDa protein antiserum (lanes C to E) were used. Lanes: A) *T. denticola* ATCC 35405, sample not heated; B) *T. denticola* ATCC 35405, sample heated; C)) *T. denticola* ATCC 33520, sample heated; D)) *T. denticola* ATCC 35404, sample heated; E)) *T. denticola* ATCC 35405, sample heated.

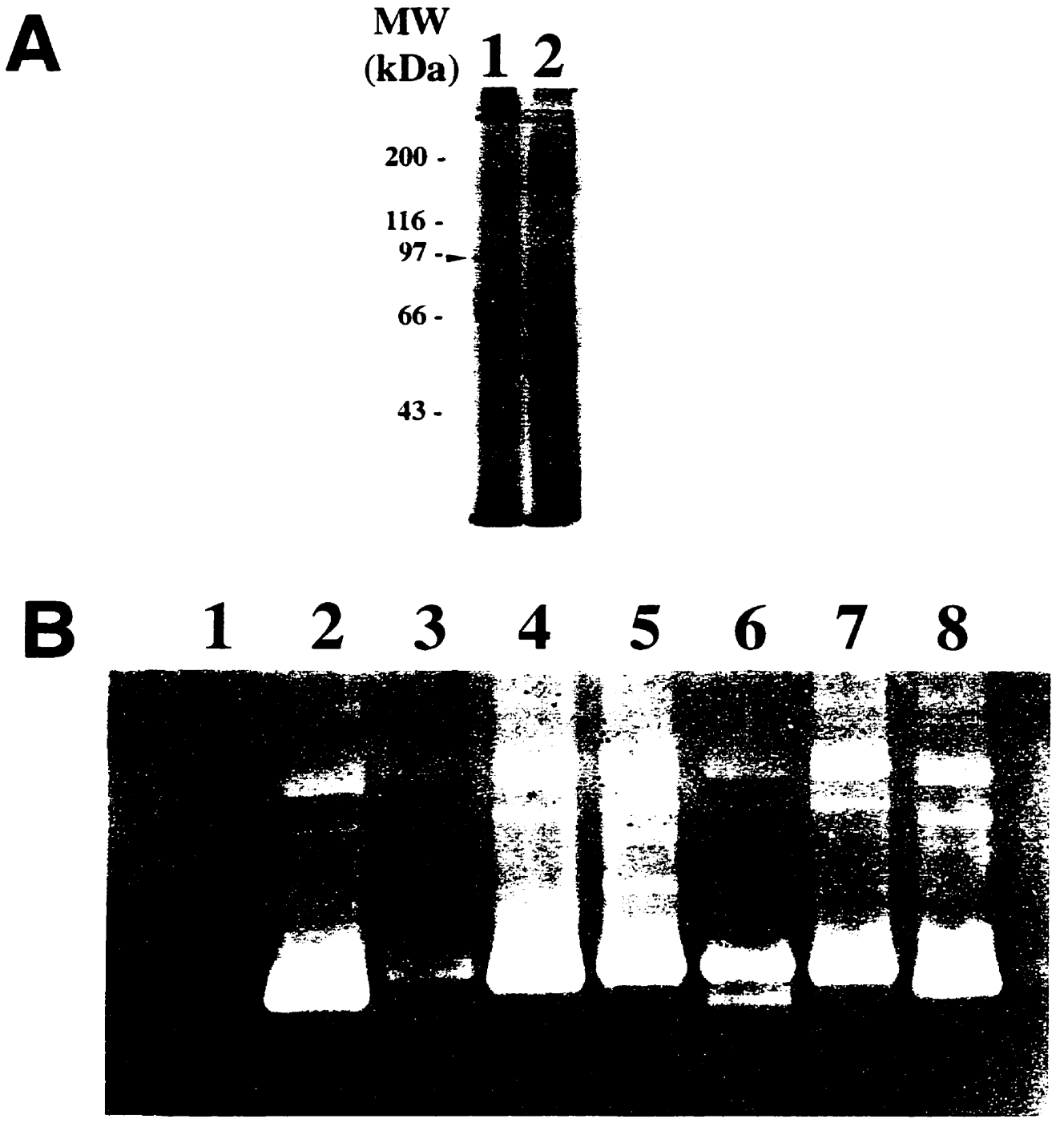


Figure 3.22 SDS-PAGE of *T. denticola* extract showing the 95 kDa CTLP. A) *T. denticola* ATCC 35405 detergent extracts: lane 1 – enhanced extraction of 95 kDa CTLP with 0.1% sodium deoxycholate (arrowhead); lane 2 – protein profile of 0.1% NP-40 extract. B) 0.02% gelatin zymogram (7.5% acrylamide slab gel). Lanes: 1) MW standards (high to low, kDa): 200, 116, 97, 2) *T. denticola* ATCC 35405, 3) *T. denticola* ATCC 33520, 4) *T. vincentii* LA-1, 5) *T. denticola* S-2, 6) *T. denticola* OTK, 7) *T. denticola* e', 8) *T. denticola* e.

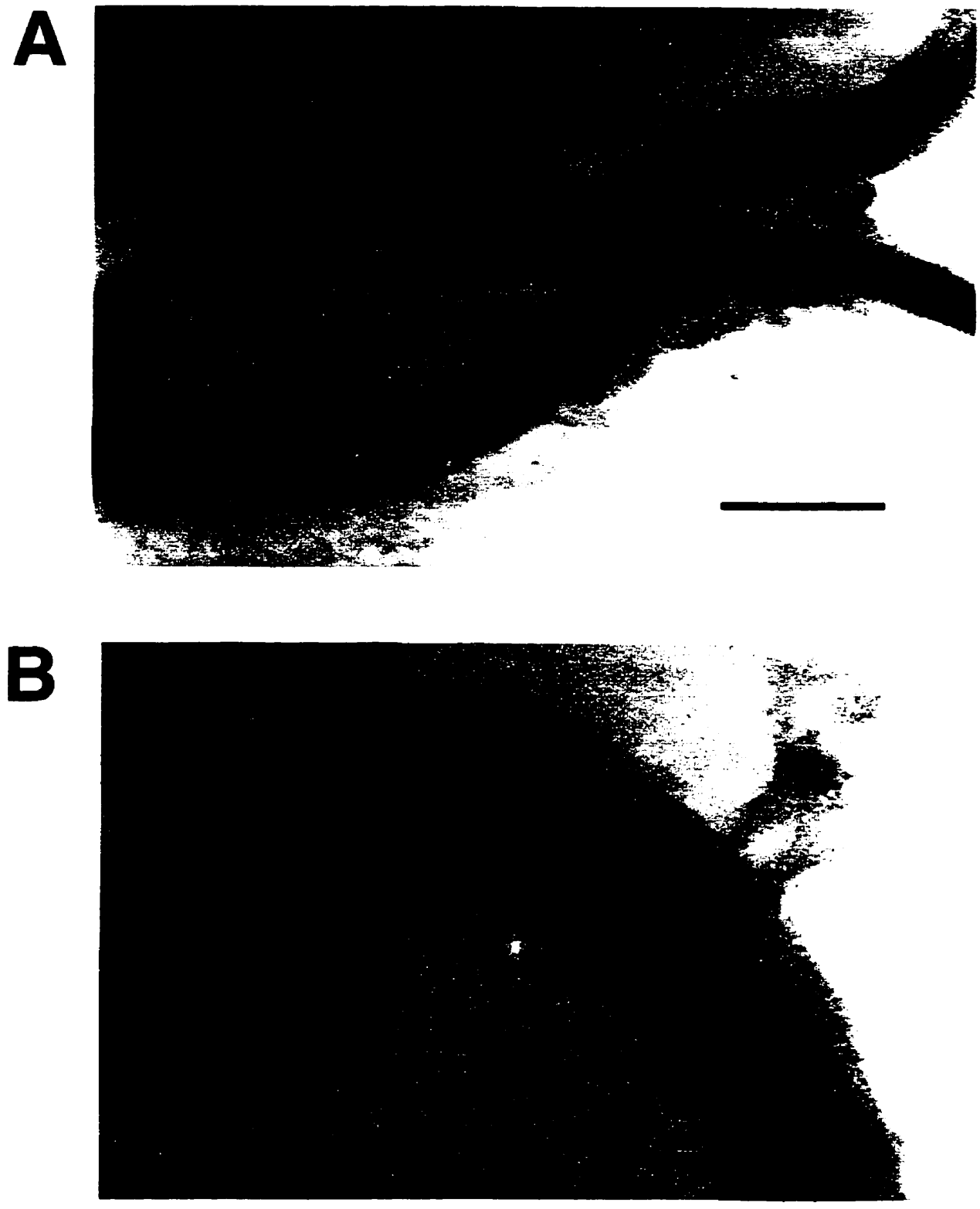


Figure 3.23 Electron micrographs of sonicated *T. denticola* ATCC 35405. A) Darkly staining cell bodies, periplasmic flagella, and outer sheaths in the form of both vesicles and extended sheaths are evident. B) outer sheath of *T. denticola* ATCC 35405, showing a definite hexagonal array. Bars = 0.3 μm.

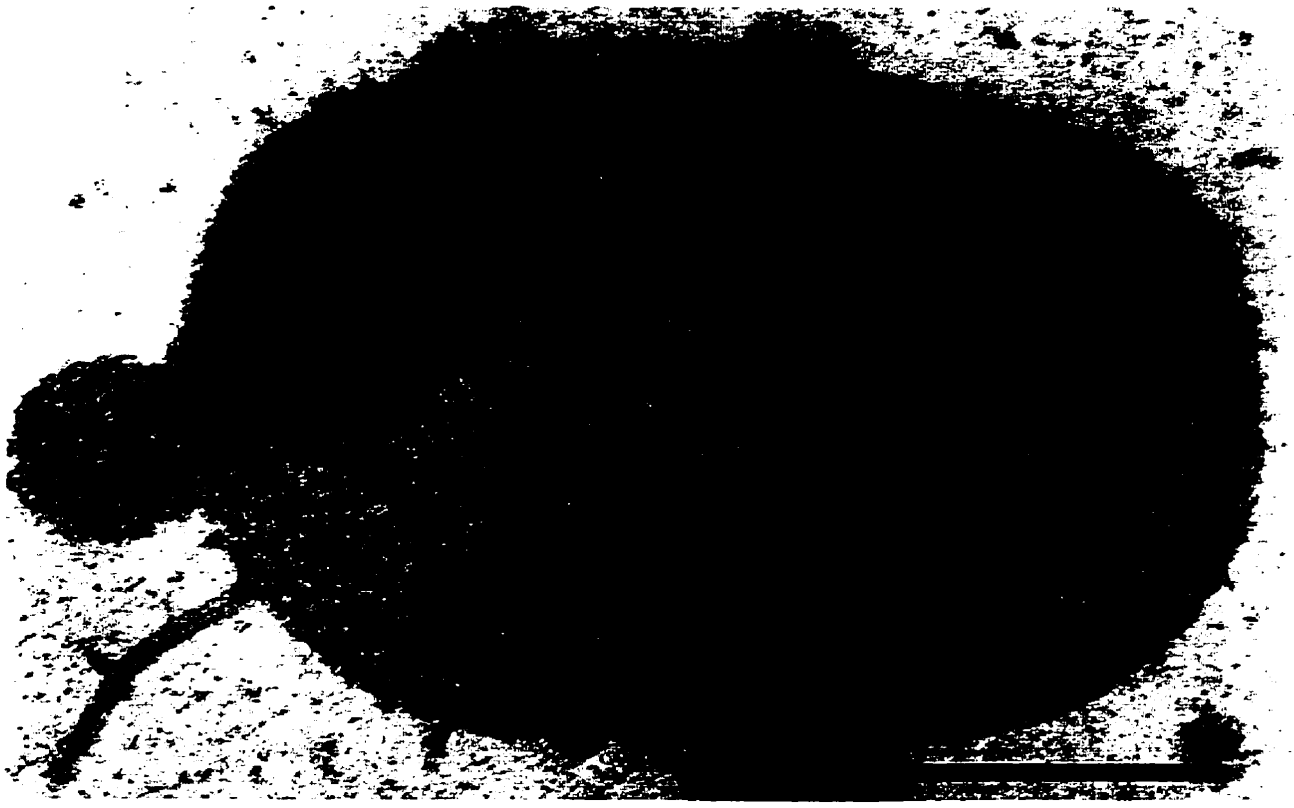


Figure 3.24 Transmission electron micrograph of outer membrane released by mild sonication of *T. denticola* OTK. The hexagonal array was similar in pattern and size to those reported for other oral treponemes. Bar = 0.3 μm.

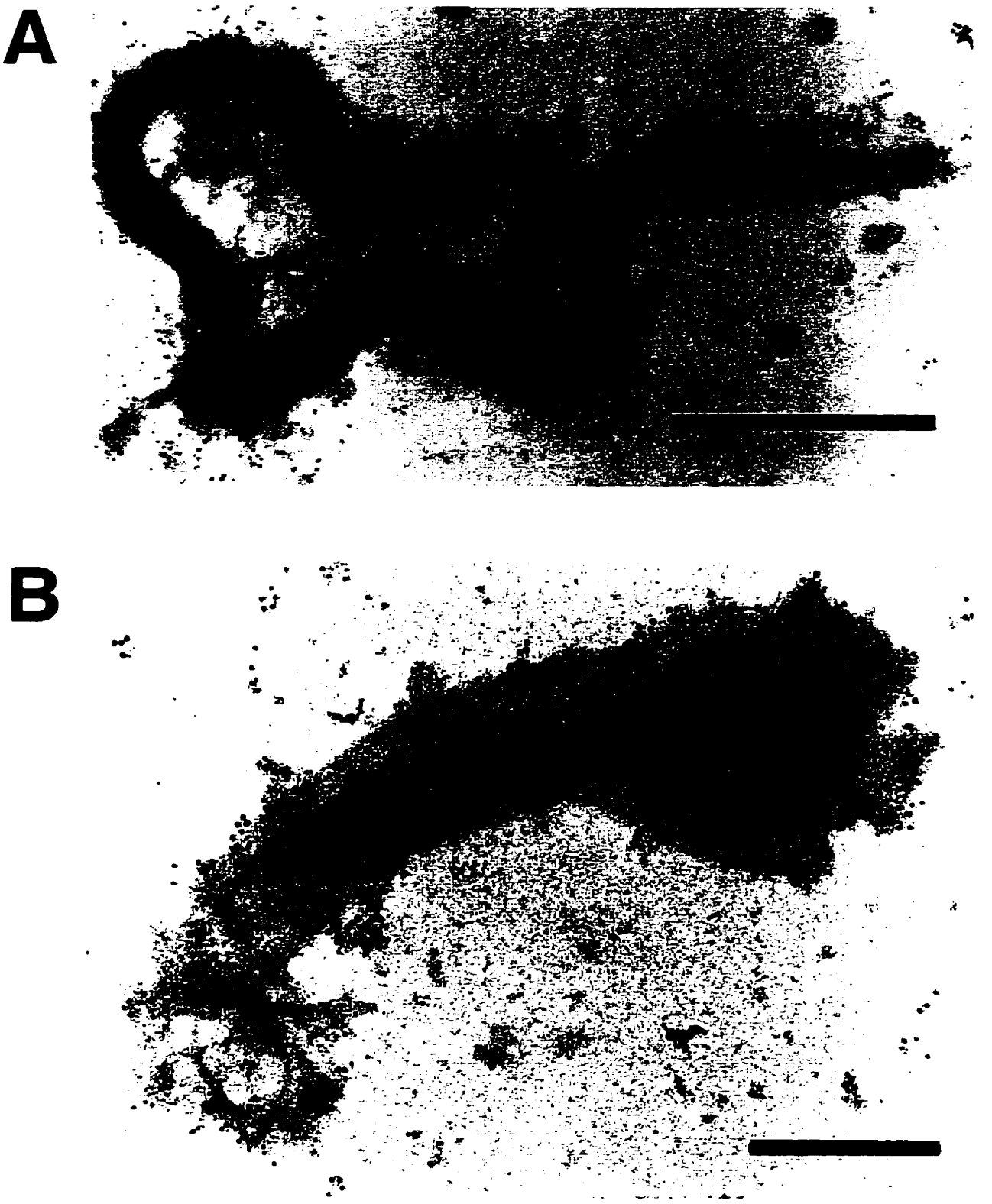


Figure 3.25 Immunogold labeling of *T. denticola* with specific anti-*T. denticola* surface antibodies. *T. denticola* whole cell (A) or sonic extract (B) double labeled with IgGs against 95 kDa CTLP (5 nm gold beads), and 53 kDa protein (10 nm gold beads). Panel A, bar = 1 μm; panel B bar = 0.3 μm.

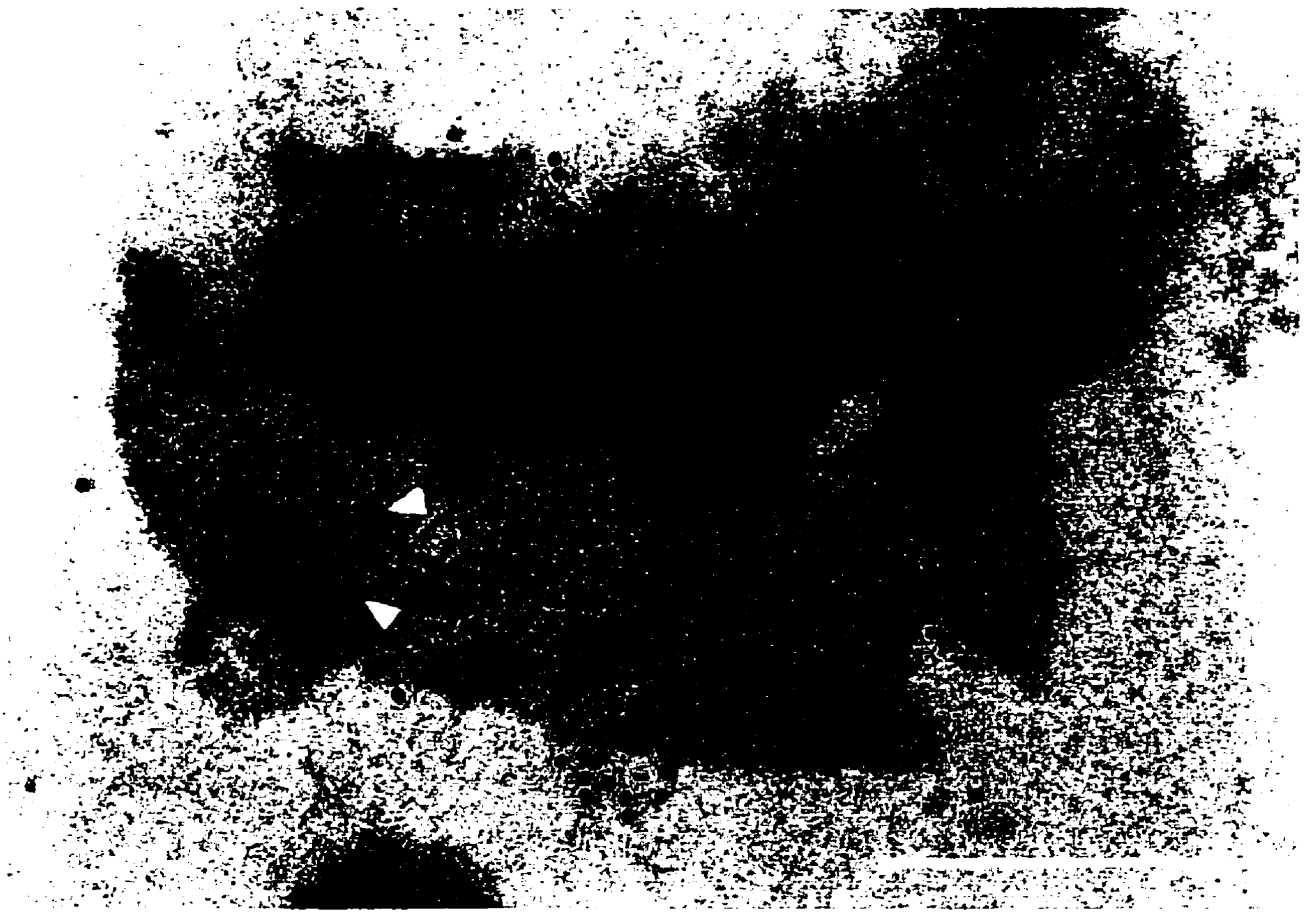


Figure 3.26 Immunogold labelling of *T. denticola* outer sheath with specific anti-*T. denticola* 53 kDa protein IgG. Note that alignment of the gold beads with the outer sheath hexagonal array (arrowheads). Bar = 0.3 μm. 5 nm gold beads used.

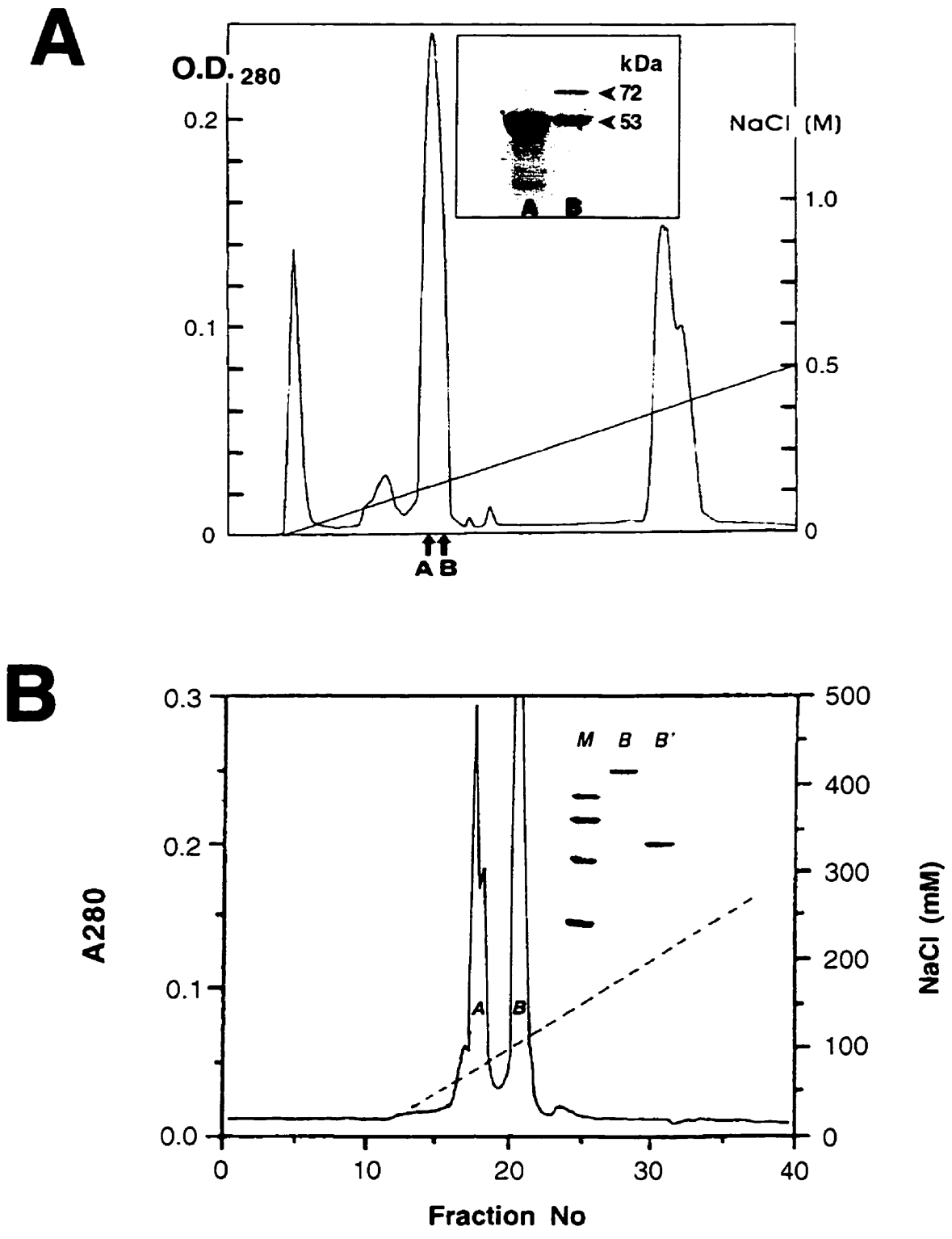


Figure 3.27 FPLC purification of Msp from *T. denticola* ATCC 35405. Panel A: Initial purification of the *T. denticola* NP-40 extract, SDS-PAGE analysis of fractions A and B (heated) is shown in the insert; panel B: Final purification of the Msp or the 53 kDa porin from reconstituted *T. denticola* Octyl-POE extract. The purified protein was eluted from the column with 0.11 M NaCl (peak B). The insert represents SDS-PAGE of unheated (lane B) and heated (lane B') samples of peak B. The molecular mass markers (lane M) used were 94, 67, 43, and 30 kDa.

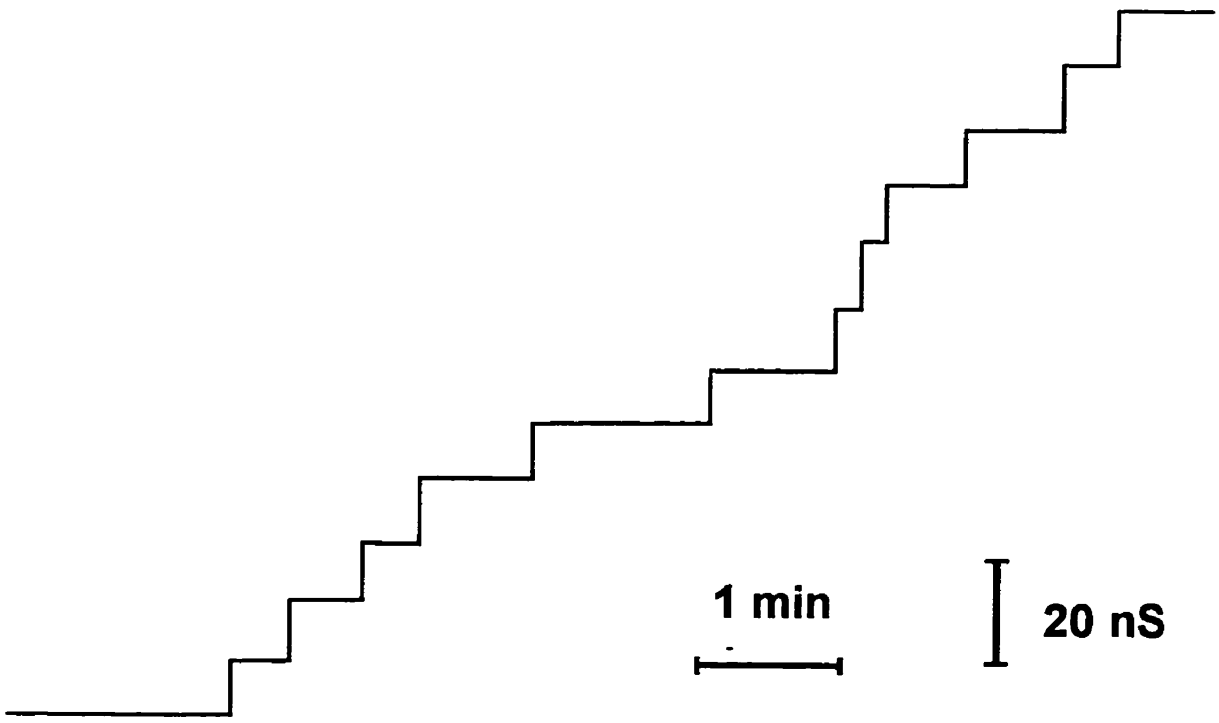


Figure 3.28 Analysis of single-channel conductance events due to incorporation of the 53 kDa porin into black lipid membranes.

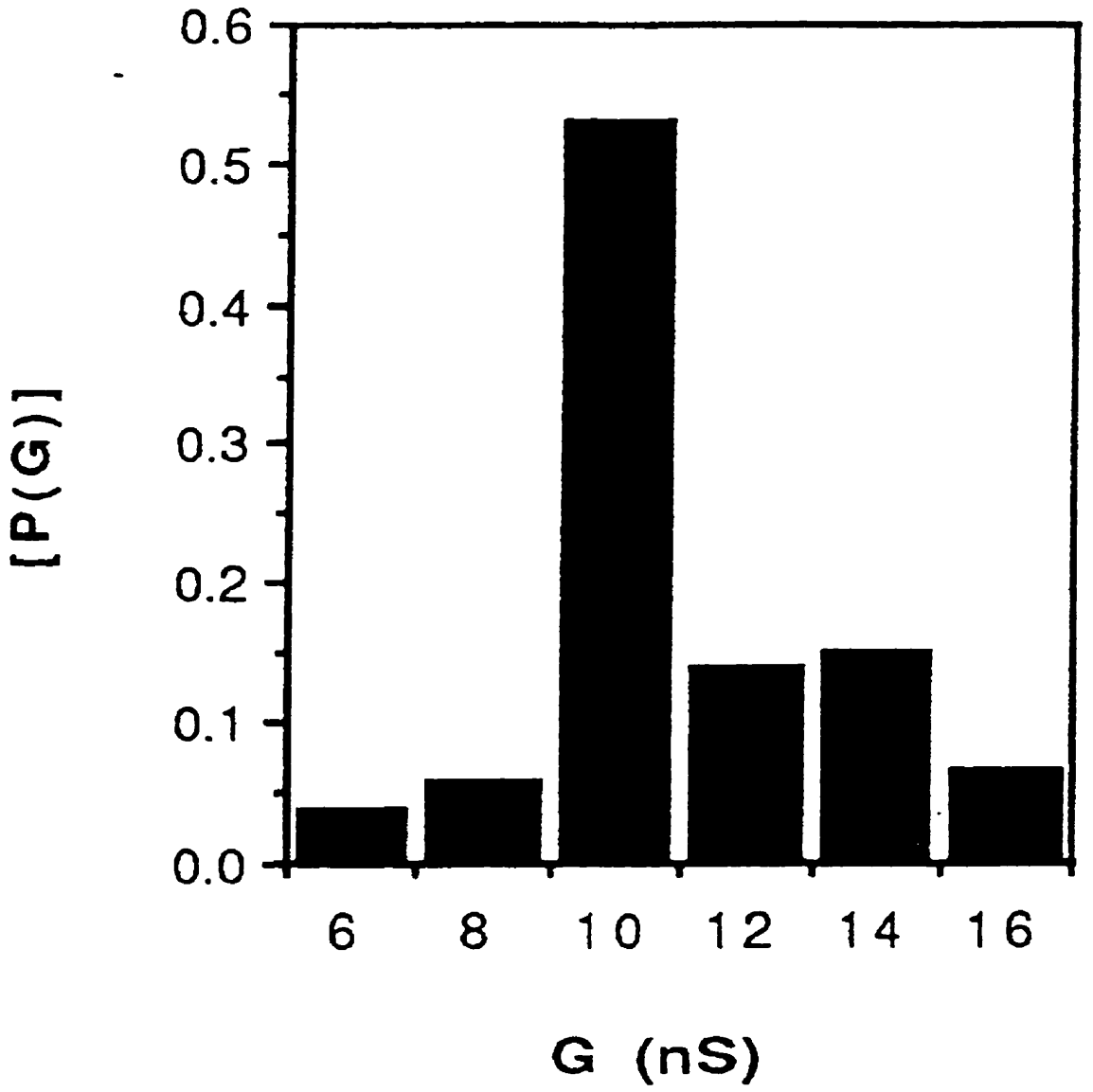


Figure 3.29 Histogram of single-channel conductance increases observed for the purified 53 kDa membrane protein. The 53 kDa protein was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. A total of 216 events was recorded for this histogram, which shows the probability of a given single-channel conductance increment [P(G)] as a function of single-channel conductance (G). The single-channel conductance was determined to be 10.9nS.

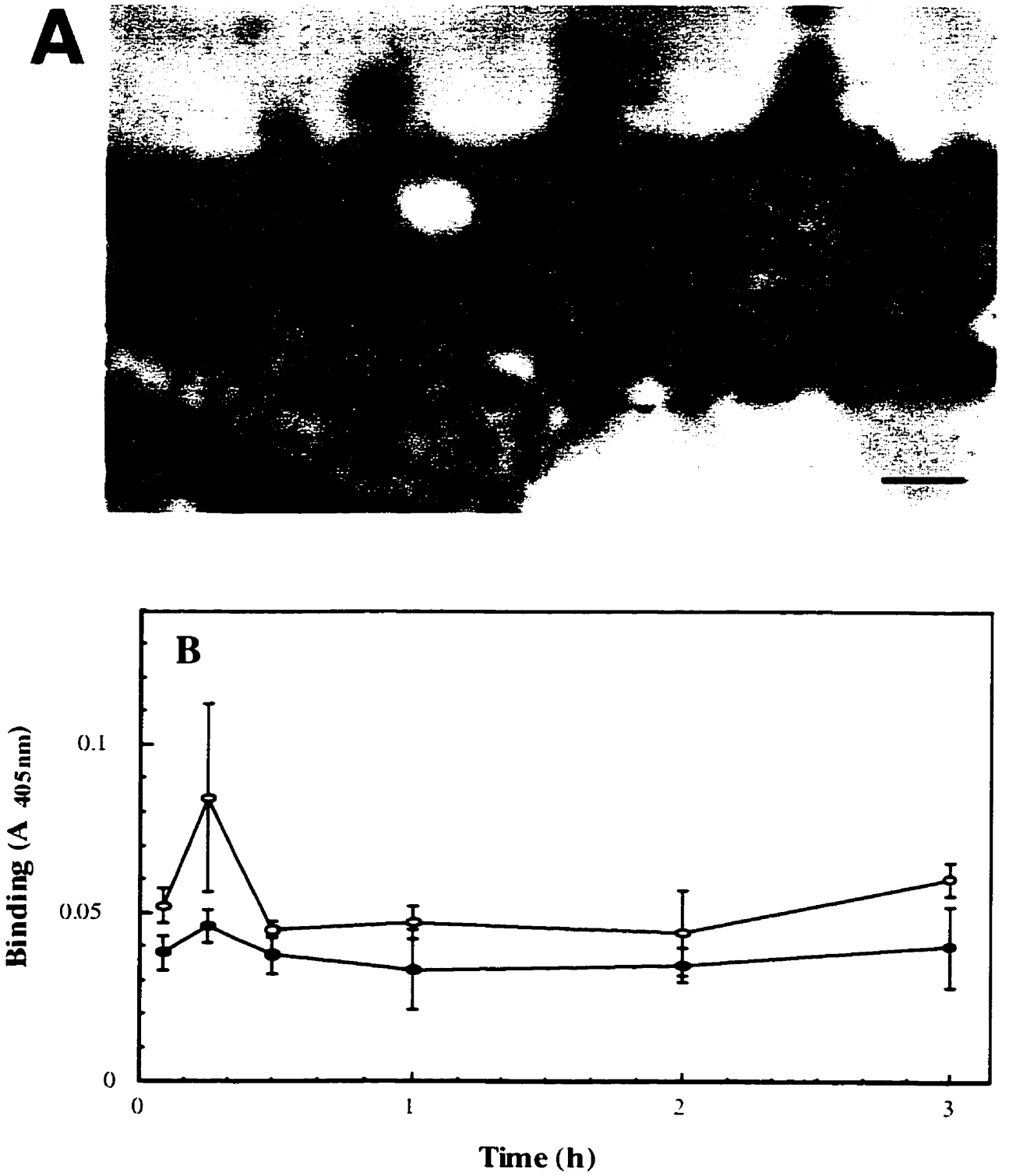


Figure 3.30 Binding of Msp complex and CTLP to PLE cells. A) TEM section showing attachment of Msp complex on to multi-layer PLE cell culture. Msp complex immunolabeled with anti-*T.denticola* 53 kDa protein IgG (5 nm gold beads). B) Time dependent binding of *T. denticola* CTLP to PLE cells. CTLP (20 µg/mL) was added to epithelial confluent cultures: open circles, 4°C: solid circles, 20°C. The binding was assayed with ELISA using anti-whole *T. denticola* antibodies.

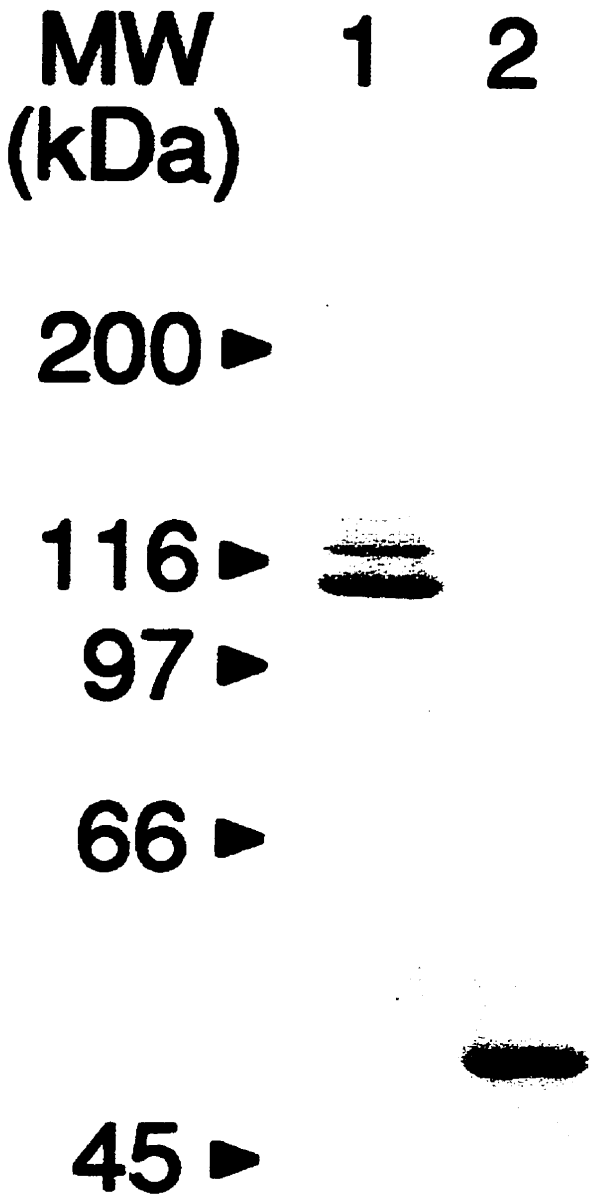


Figure 3.31 *T. denticola* Msp complex. The *T. denticola* Msp complex was prepared as described in the text, subjected to SDS-PAGE and stained with Coomassie blue. Lanes: 1. Msp complex that was not heated; 2. Msp complex heated to 100°C, 10 min.

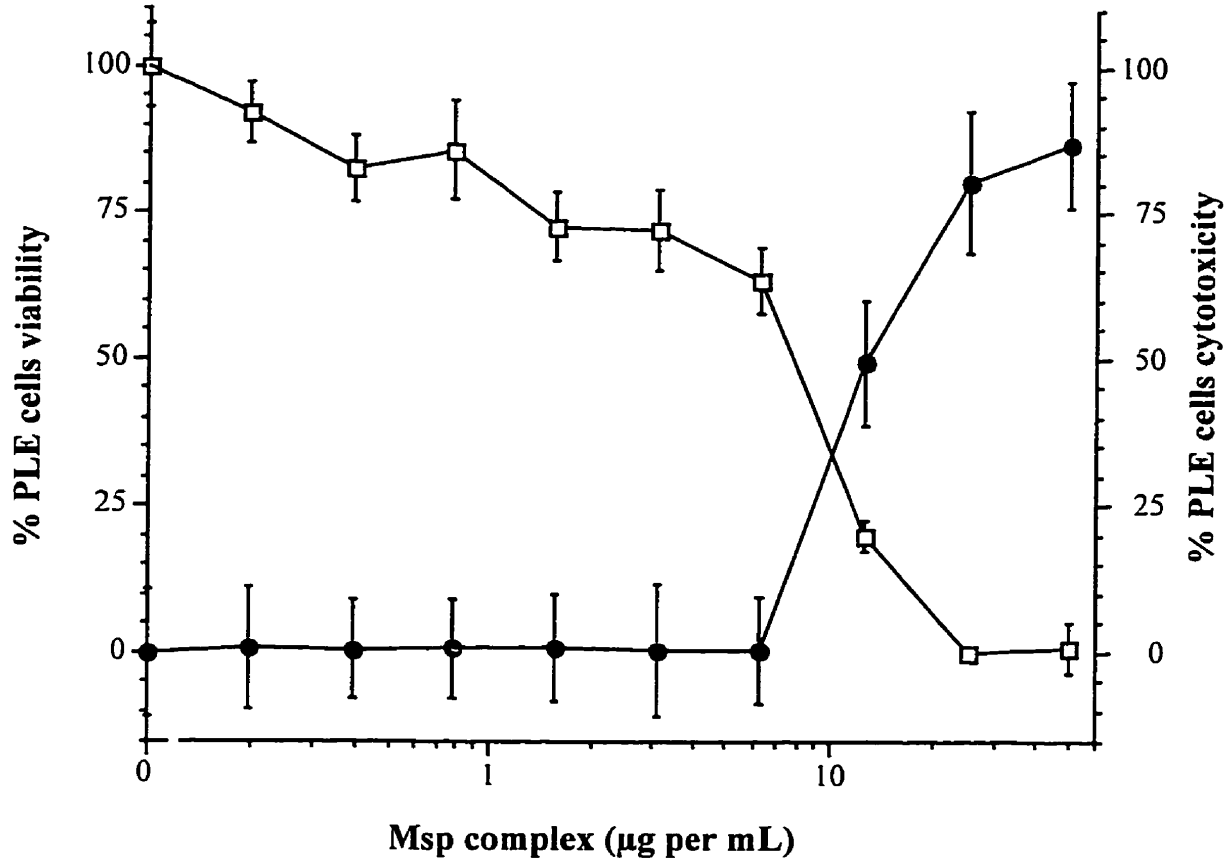


Figure 3.32 Reduction of viability of PLE cells due to challenge by Msp complex. Effects of Msp complex on cells were detected by LDH release (open square, % cytotoxicity) and MTT reduction (solid circle, % viability). Values are mean ± SD of six samples.

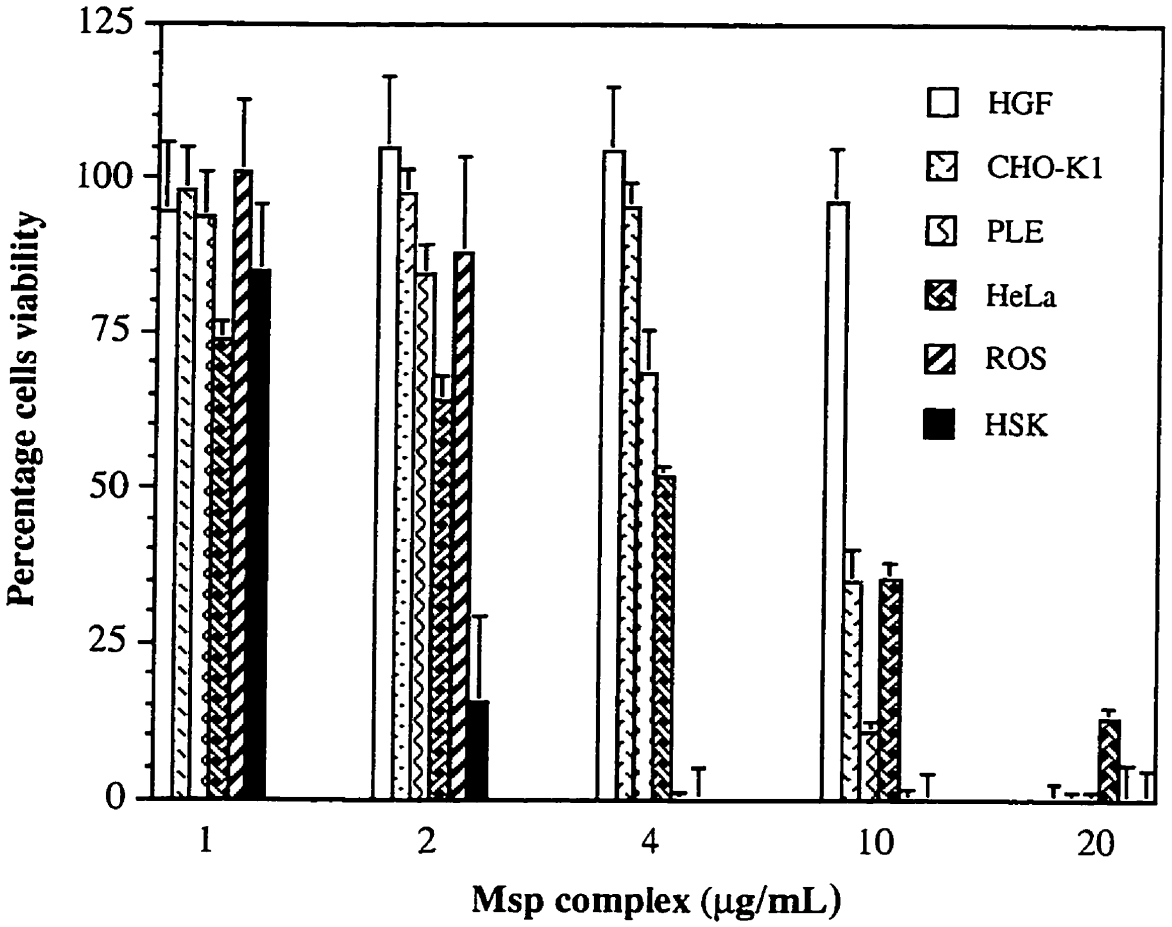


Figure 3.33 Reduction of viability of cultured cells due to challenge by Msp complex. Effects of Msp complex on cultured eukaryotic cells as detected by MTA. Data showing mean ± SD (n=6).

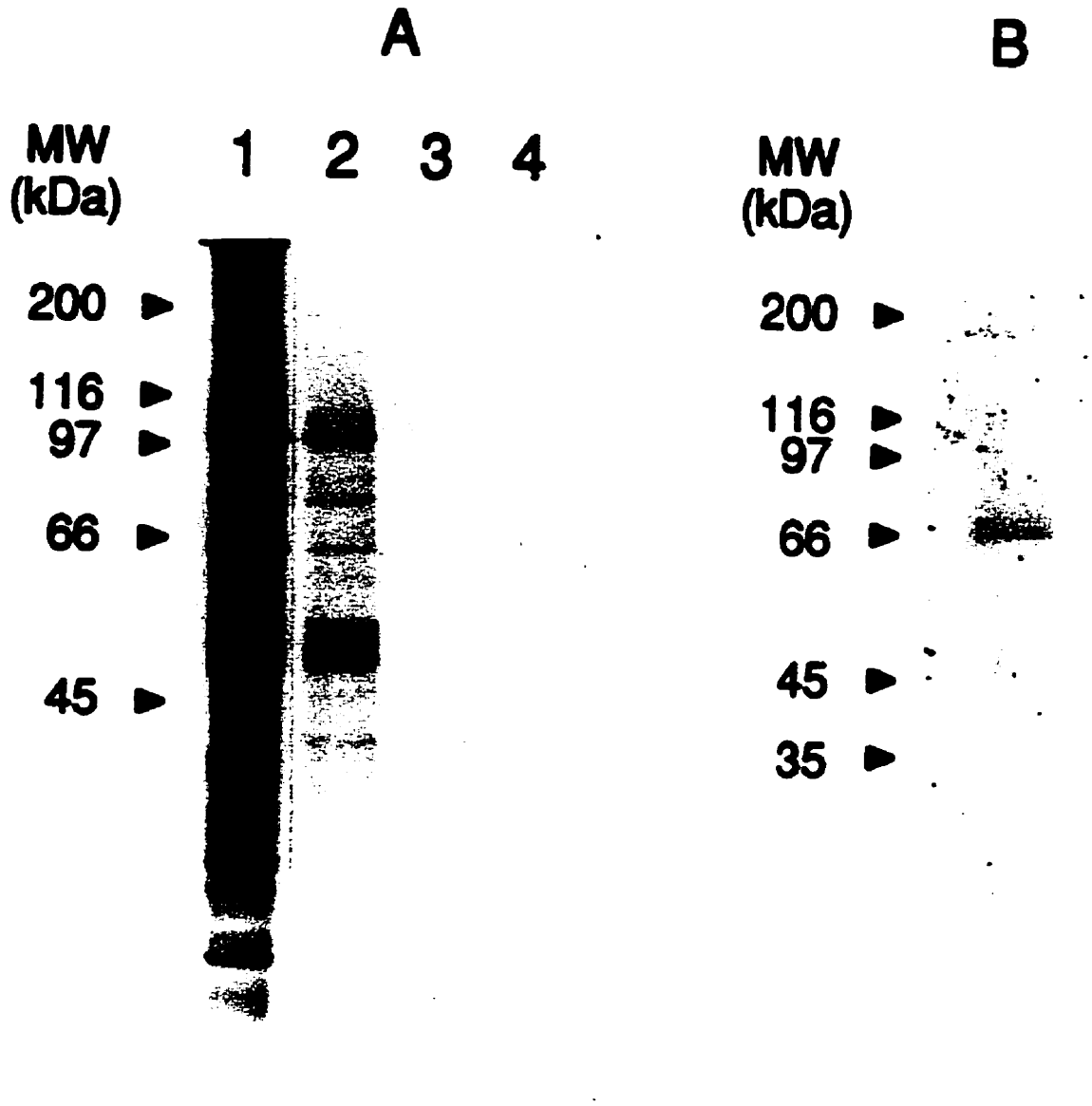


Figure 3.34 SDS-PAGE showing HeLa cell proteins immunoprecipitated by anti-Msp IgG after incubation with Msp complex. A) ^{35}S-methionine-labeled HeLa cell proteins. Lanes: 1, total HeLa cell proteins showing incorporation of ^{35}S-methionine label; 2, ^{35}S-methionine-labeled HeLa cell proteins binding Msp complex and immunoprecipitated by anti-Msp IgG; 3, as in lane 2 except anti-*P.gingivalis* IgG replaced anti-Msp IgG; 4, as in lane 2 except the cells were not exposed to Msp complex. B) Biotin-labeled HeLa cell proteins binding Msp complex and immunoprecipitated by anti-Msp IgG.

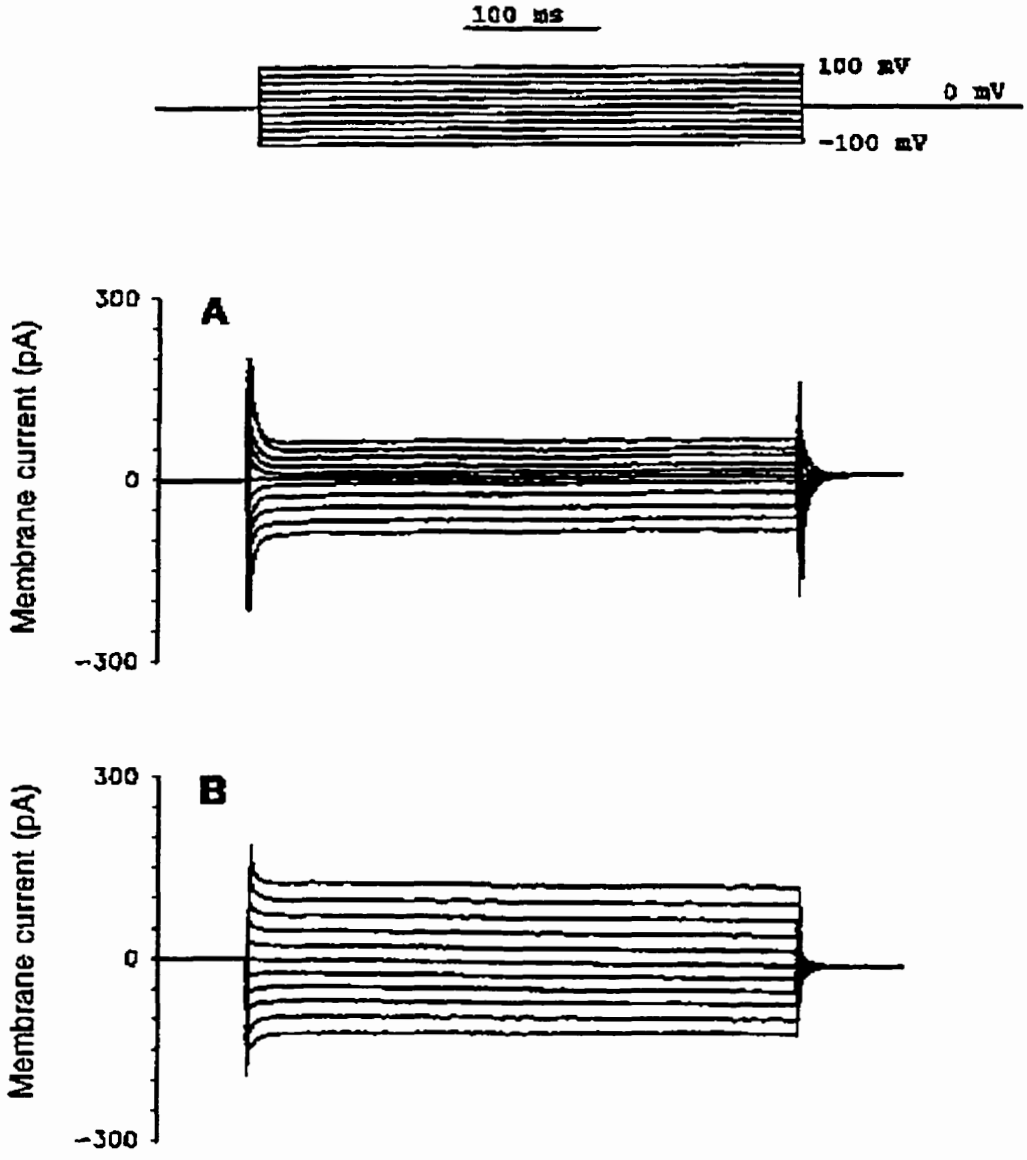


Figure 3.35 Effects of Msp complex on whole-cell currents in HeLa cell membranes recorded in potassium gluconate saline solutions. Bathing and pipette solutions contained 150 mM potassium gluconate (solutions *b* and *d*, respectively; Table 2.2) with a free intracellular Ca^{2+} concentration of 1 nM. Currents were generated in response to 400 ms – duration voltage commands applied from a holding potential of 0 mV. A) Currents obtained from a representative cell incubated in 10% FBS-MEM without Msp complex, B) currents obtained from a representative cell incubated for 30 min at 37°C in 10% FBS-MEM containing 39 μg of Msp complex / mL.

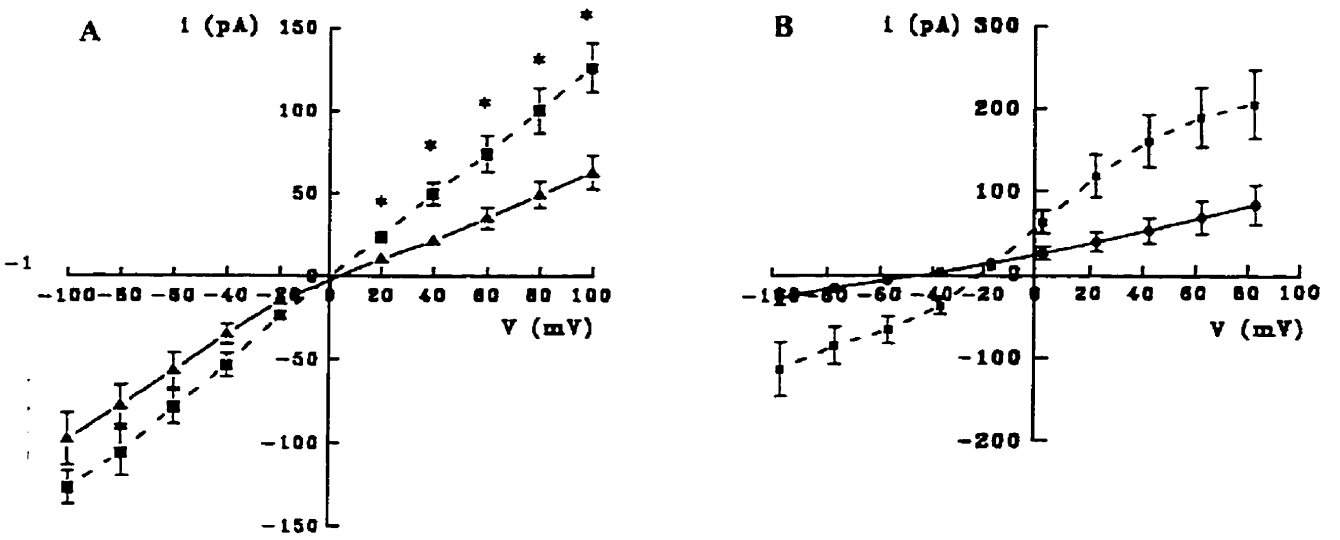


Figure 3.36 Effects of Msp complex on the current-voltage (*I-V*) relationship of the HeLa cell membrane. Data were obtained as described in the legends to Figures 3.35 and 3.37. A) Data from cells exposed to solution *b* extracellularly and solution *d* intracellularly (Table 2.2). Datum points indicate the values (mean ± SEM) for currents for 8 cells incubated in 10% FBS-MEM only (triangles) and for 12 cells incubated in 10% FBS-MEM with 39 μg Msp complex / mL (squares). Asterisks indicate pairs of datum points which were significantly different ($p < 0.05$, ANOVA). Data from cells exposed to solution *a* extrcellularly and solution *c* intracellularly (Table 2.2). Datum points indicate the values (mean ± SEM) for currents obtained from 14 cells incubated in 10% FBS-MEM only (circle) and from 11 cells incubated in 10% FBS-MEM with 39 μg Msp complex / mL (squares). All pairs of datum points were significantly different except those obtained at a membrane potential of –20 mV ($p < 0.05$, ANOVA).

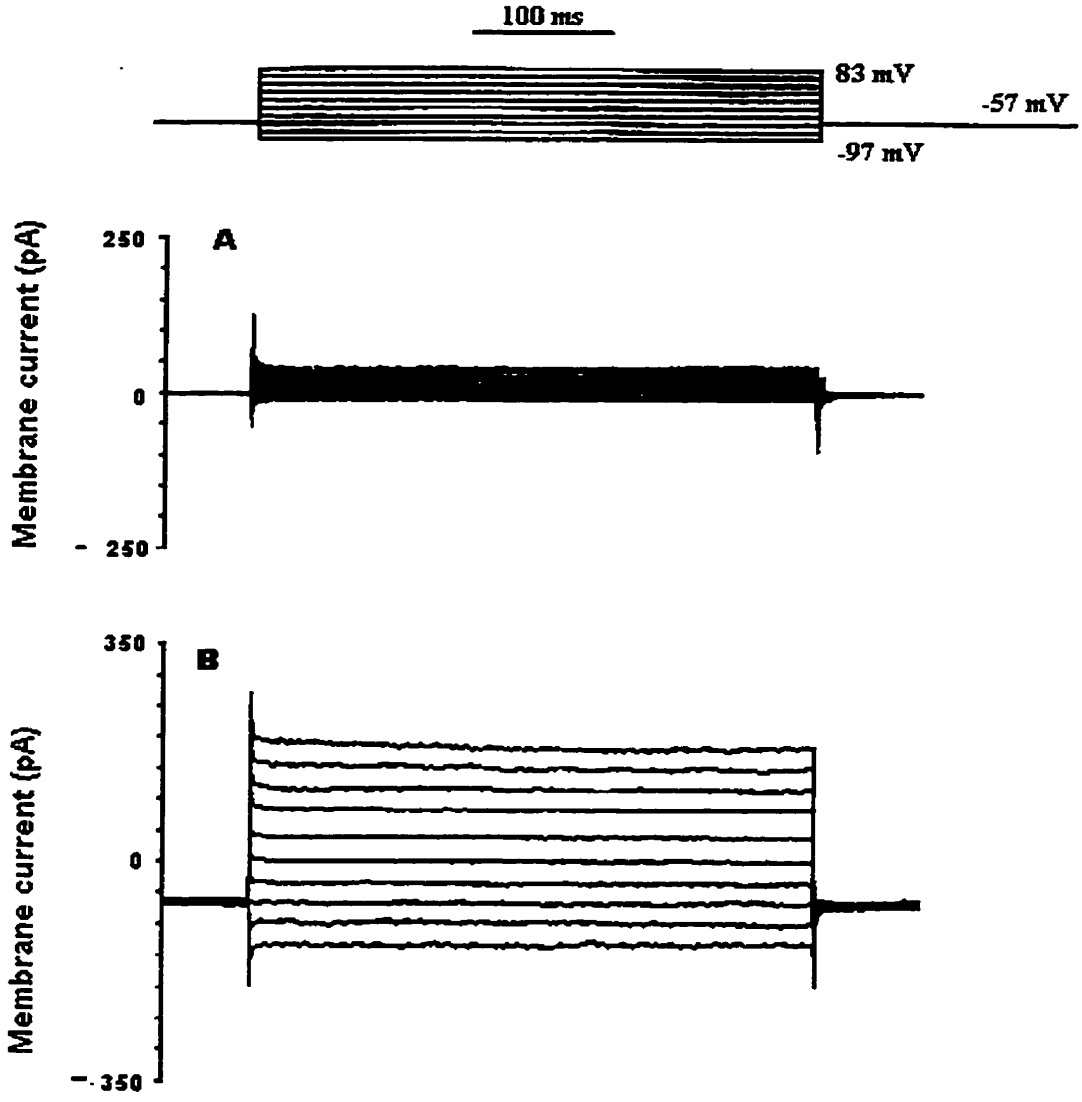


Figure 3.37 Effects of Msp complex on whole-cell currents in HeLa cells bathed in physiologically relevant solutions. Patch pipettes contained 135 mM KCl (solution *c*, Table 2.2), while cells were bathed in saline containing 135 mN NaCl (solution *a*, Table 2.2). The intracellular free Ca^{2+} concentration was 140 nM. Currents were generated in response to 400-ms-duration voltage commands delivered from a holding potential of –57 mV. A) Currents obtained from a representative cell incubated in 10% FBS-MEM without Msp complex. B) Currents obtained from a representative cell incubated for 30 min at 37°C in 10% FBS-MEM containing 39 μg Msp complex/mL.

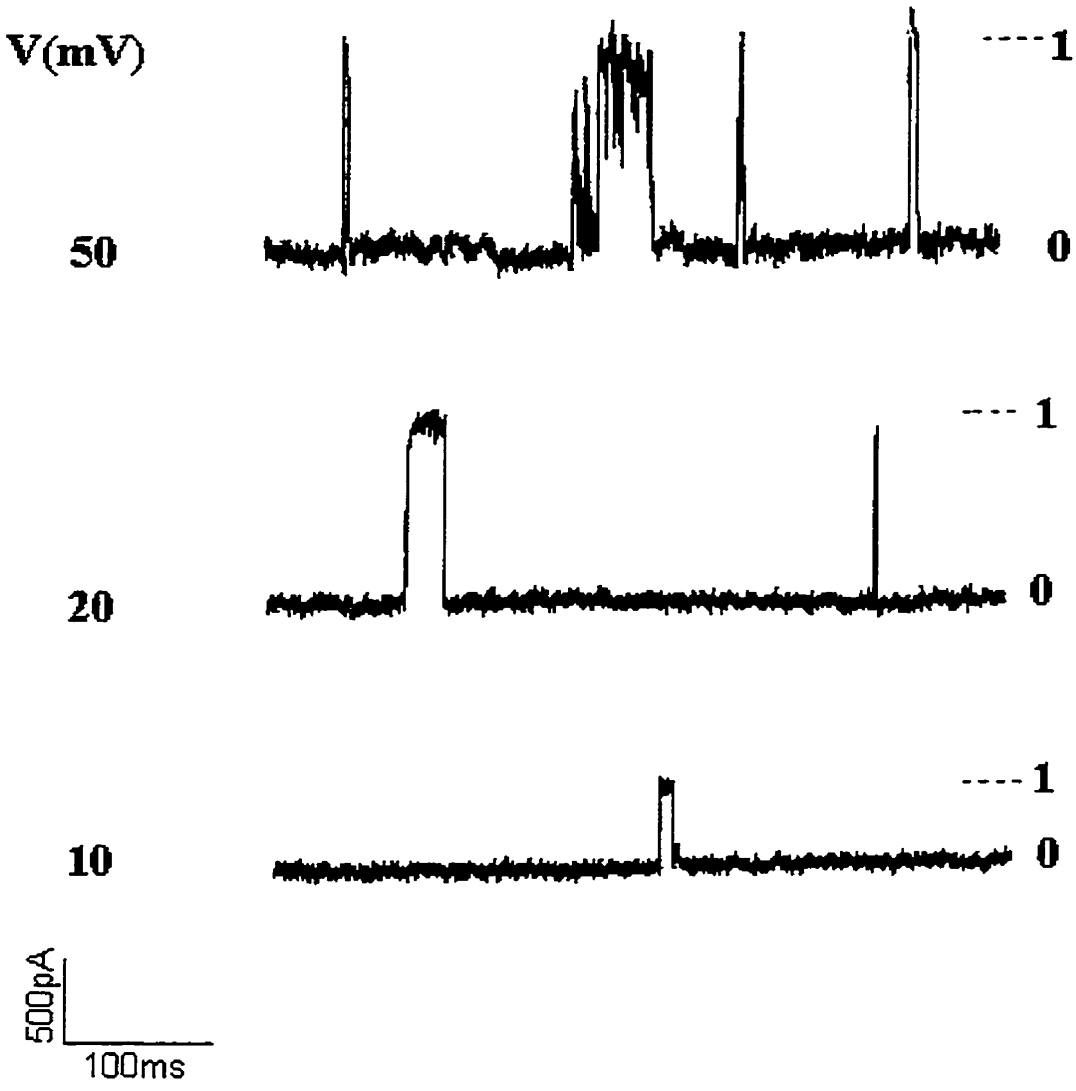


Figure 3.38 Currents consistent with the gating of a large conductance ion channel recorded from a cell-attached patch of HeLa cell membrane. The patch electrode contained 39 μg Msp complex/mL of 5% BSA-).03% Octyl-POE-solution *a* (Table 2.2). Solution *a* alone bathed the remainder of the cell surface. The traces show currents obtained at various membrane potentials (V). V was calculated by substracting the pipette potential from the cell resting potential, -51 mV, obtained by whole-cell recording at the end of the experiment. The closed-channel current level is denoted by 0, while 1 indicates the open-channel current level. Downward deflection from baseline indicates inward membrane current. The direct current bandwidth of recording is 800 Hz.

CHAPTER IV Discussion

4.1 Methodology and limitations

Epithelial cells of different mammalian tissue origin and at varying stages of differentiation exhibit quite specific characteristics. In particular these include organization of membrane bound and pericellular molecules that serve as receptors for bacterial adhesion (Uitto & Larjava 1991, Finlay 1990, Patti & Höök, 1994). For these reasons it is important to use an appropriate cell type when studying binding of periodontal microbes. In the present investigation, PLE cells were chosen as the model cell in attachment and cytotoxic studies because they resemble junctional epithelial cells in many respects. Both PLE and junctional epithelial cells have the same developmental origin, this being cells of the embryonic primary epithelial band. The cultured PLE cell lines do not keratinize, and they express a similar cytokeratin pattern to human junctional epithelial cells *in vivo* (Pan *et al.* 1994). Cultured PLE cells express a number of matrix molecules and their receptors. Fibronectin, tenascin, laminin, kalinin and several β_1-integrins have been detected in PLE cultures (Uitto *et al.* 1992). The PLE cells in culture also produce hyaluronan and various other glycosaminoglycans (Merrilees *et al.* 1983).

Other cell types/cell lines have been used by different groups studying the adhesion of spirochetes. *T. denticola*, however, did not bind equally well to the various cells tested (Keulers *et al.* 1993a, Carranza Jr. *et al.* 1997, to be discussed in Section 4.3). Such an observation was also noticeable in the present investigation (Table 2.1). The PLE cell culture was chosen as the cell model to be tested based on its oral origin, its resemblance to human junctional epithelium, and its ability to bind *T. denticola*. Nevertheless, the interaction between *T. denticola* and PLE

cells was found to be not at all simple. Several events were observed during the bacterial-PLE cell interaction. One event that might affect the attachment assay was that the spirochete could induce PLE cell detachment from the substratum (Figures 3.7, 3.17). To simplify the model and to focus on *T. denticola*-PLE cell attachment, PLE cells were prefixed as per Stanislawski *et al.* (1985). The compromises which must be accepted when using FPLE cells were: i) the possibility of modification of PLE cell receptors after the glutaraldehyde treatment, and ii) the fact that any cell responses or *T. denticola* inducible PLE-spirochete binding behavior will not be observed when FPLE cells are used. In fact, differences were observed between *T. denticola* binding to unfixed and fixed cells that may be attributed to one of these possible effects of fixation (Tables 3.2 and 3.4). The adhesion of *T. denticola* to Saos-2 and PLE cells was clearly affected by fixation, as a higher density suspension of *T. denticola* had to be used for attachment studies when the eukaryotic cells were prefixed. With respect to FPLE cells, while the glutaraldehyde fixation did affect to certain extent the *T. denticola* binding, similar results were obtained in adhesion inhibitor studies using either fixed or unfixed PLE (Sections 3.13 and 3.14). However, an obvious benefit of using FPLE was noted as no PLE will be detached from the substratum once they were fixed with glutaraldehyde which enable the interpretation of results from adhesion inhibition assays easier (Tables 3.6 and 3.7). For the purposes of the present study, the FPLE cell model was deemed appropriate for adherence studies.

Another source of intrinsic limitation was the strong proteolytic activity of purified CTLP. When anti-CTLP IgG alone was used in CTLP-FPLE adherence inhibition assay, no significant inhibition effect could be observable (Table 3.14). Immunoglobulins are substrate for CTLP (Uitto *et al.* 1988) and the specific IgG could have been degraded by the highly active purified enzyme, thus preventing effective antibody-antigen recognition. When the antibody

inhibition assay was done with PMSF-treated CTLP, inhibition of binding was significantly greater than that due to protease inhibitor alone (Table 3.14). Similar limitation probably applied to study of CTLP adherence to unfixed PLE (Figure 3.30B). The strong proteolytic activity of CTLP is probably one reason for the relatively weak adherence even when the assay protocol was done relatively quickly and/or carried out at 4°C. Some PLE bound CTLP could release itself into the aqueous phase simply by its active proteolytic activities. Alternatively, the protease could digest the anti-*T. denticola* antibodies leading to reduction of the signal.

PLE cells were also chosen to be the cell model for cytotoxicity studies of *T. denticola*. It was shown that PLE cells were relatively resistant to the challenge from *T. denticola* (Figures 3.16 and 3.18). A high density suspension of *T. denticola* was needed to kill PLE cells under the experimental conditions tested. However, other cell types tested e.g. HGF, HeLa, CHO-K1 appeared relatively sensitive to the spirochete challenge. Superficially, it might seem more logical to use sensitive cell lines such as HGF, HeLa or CHO-K1, rather than PLE cells for *T. denticola* cytotoxicity assays. However in chronic inflammatory periodontal disease, wide-spread acute tissue destruction including the JE or pocket epithelial cells is rarely observed (Page & Schroeder 1990). It seems, in nature, that the human JE and pocket epithelial cells are somewhat adapted to the challenge of subgingival microflora, (including certain levels of periodontopathogens). PLE cells were relatively resistant to *T. denticola* challenge *in vitro*, suggesting that the PLE cell was a realistic model to study.

Considering the same issue from another point of view, one might question whether the concentration of *T. denticola* used in the assays was excessive. This issue can be analyzed from two perspectives. Firstly, microscopic enumeration of spirochetes is quite difficult due to their size and morphology. Furthermore, it is difficult to standardize microscopic counts with colony

forming units, due to the low and highly variable viability of oral treponemes grown on solid media. As a consequence, the literature contains a range of more than 2 logs in the reported number of spirochetes per mL at a standard optical density (Haapasalo *et al.* 1991, Ellen *et al.* 1994b, Rosen *et al.* 1994). The variation in numbers per mL appears to be due to counting methods of individual investigators. In our studies, the number of spirochetes per mL at standard optical density was at the high end of the range reported in the literature. Therefore, we could in fact be overestimating the actual numbers of *T. denticola* we used in our challenge assays. Secondly, in subgingival plaque from advanced periodontal lesions, up to 50% of observable organisms are spirochetes (Armitage *et al.* 1982, Listgarten 1982). A more recent study showed a mean of 1.56×10^7 spirochetes per paper point sample from periodontal lesions (Qiu *et al.* 1994). Similar recovery rates were also reported using the same, as well as different, techniques (Koseki *et al.* 1996). From a case report by Choi *et al.* (1994), eight major groups of treponemes were identified by partial 16S rRNA gene cloning of PCR-amplified products of DNA from a 1 mg (wet weight) subgingival plaque sample of a single periodontal pocket. Only *T. denticola* and *T. vincentii* were identified, while the remaining 6 groups were unclassified treponemes. Using a culture method, Chan *et al.* (1993) reported that *T. denticola* appears to be the treponemal species that is most frequently isolated from periodontal pockets, reconfirming that *T. denticola* is a dominant member of the subgingival spirochete population. Assuming a 1 mg subgingival plaque sample consists of 50% spirochetes, this is equivalent to approximately 0.3 mL of *T. denticola* in late-logarithmic culture (Section 2.8.1). This means that the pocket epithelium might be casually facing spirochetes at the magnitude of $10^{10} - 10^{11}$. Taking the small volume of the periodontal pocket, the concentration or density of *T. denticola* with which the pocket epithelium could be challenged would be comparable or greater than the concentration

used in the present investigation. In addition, one would have to be mindful of the fact that *T. denticola* and the other subgingival spirochetes are motile and capable of chemotaxis. Therefore, the JE cells of the periodontal pocket are exposed to dense colonies of spirochetes, including *T. denticola* which are located at the epithelial surface of subgingival plaque. This was reported in early EM studies on periodontally diseased humans and animals (Listgarten 1976, Theilade & Attström 1985) and was recently characterized more thoroughly using immunofluorescence microscopy techniques (Kigure *et al.* 1995).

Msp complex or semi-purified Msp was used for cytotoxicity and patch clamp experiments. Great care was exercised during the Msp complex extraction procedures to minimize the chances of co-purifying contaminants. Steps including filter sterilization, autoproteolysis, ultracentrifugation, ultrafiltration to remove low molecular weight proteins and regular monitoring of the extract by SDS-PAGE (Figure 3.31) were carried out. It is likely that the insoluble cytotoxic *T. denticola* peptidoglycan would have been excluded during the purification. The outer membrane lipid of *T. denticola* including the incomplete LPS-like substance, however, could be retained after the extraction steps. The study by Grenier and Uitto (1993) reported that no PLE cell cytotoxic activities could be detected from the purified LPS-like materials of *T. denticola*. Taking this fact into consideration, no attempt was made to remove the LPS-like material, if any, from the Msp complex we prepared. However, despite the apparent similarities between the cytotoxic effects of Msp and Msp complex on PLE cells including the cytotoxicity reduction by specific anti-rMsp IgG or anti-53 kDa protein IgG pre-treatment (data not shown), and the fact that Msp complex did create large conductance channels on HeLa cell membrane, we could not rule out completely the possibility of effects of contaminant co-purified with Msp complex at this point. We are hopeful that with the aid of specific molecular

approaches specifically designed for treponemes (as reported recently from our laboratory, Fenno *et al.* 1998b), the full cytotoxic nature of Msp can be established.

4.2 Current results in comparison to information available in the literature and their implications

The interaction between *T. denticola* and periodontal tissues is a complex process. Initial adhesion events may involve carbohydrate receptors on the host cell surface (Weinberg & Holt 1990) and as well as Msp-mediated adhesion to ECM components including laminin, fibronectin, fibrinogen, collagen, and hyaluronan (Fenno *et al.* 1996, Haapasalo *et al.* 1991, 1992 and 1996). Later events include induction of F-actin rearrangement and induction of membrane blebbing adjacent to adherent bacteria (Sections 3.2.3, 3.2.4, Baehni *et al.* 1992) followed by cytopathic or cytotoxic events in the affected bacteria.

4.2.1. Attachment of *T. denticola* to eukaryotic host cells

Based on the previous observation that *T. denticola* could bind to ECM components and serum proteins, it was surprising to find relatively limited spirochete attachment to epithelial cells carrying an array of different proteins and proteoglycans on their surface. Scarce *T. denticola* attachment has been reported to other epithelial cells (Keulers *et al.* 1993a, Carranza Jr., *et al.* 1997). PLE and HGF cells were two of the cell explants to which attachment of *T. denticola* was reported to be at relatively high levels (Table 4.2). The present investigation confirmed this observation (Table 3.2). HSK and HGE did not bind *T. denticola* at comparable levels.

From the studies conducted with PLE cells, variations in cellular response to the *T.*

denticola challenge were observed. Some cells bound no or only a few treponemes while more than 80 bacteria could be counted on other cells. Increasing the concentration of *T. denticola* did not cause a major shift in the binding patterns. Such preferential *T. denticola* attachment was also reported by others (Reijntjens *et al.* 1986, Keulers *et al.* 1993a, Carranza Jr. *et al.* 1997). The different amounts of *T. denticola* binding appeared to be associated with physiological status of the epithelial cells. The proportion of PLE cells binding *T. denticola* and the number of attached *T. denticola* per PLE cells were higher in young, sparse epithelial cell cultures than in older, near confluent cultures (Table 3.1). Other research groups using different cell lines (Olsen 1984, Carranza Jr. *et al.* 1997), although not as well quantified, reported similar observations. Migrating epithelial cells with free edges and active cytoplasmic protrusions are able to form effective attachments with other cells, substrate or particles. The cells of epithelial sheets with stabilized cell contacts lose their adhesiveness and ability to phagocytose (DiPasquale & Bell 1974, Vasiliev *et al.* 1975). This is probably the result of reorganization of the supramembranous microfilament network (Vasiliev *et al.* 1975) and loss or relocation of cell surface proteins e.g. integrins (Finlay 1990, Uitto *et al.* 1992).

4.2.2. Cytotoxicity of *T. denticola* to eukaryotic cells

In addition to attachment to PLE cells, *T. denticola* could also induce cytopathic and cytotoxic changes in PLE cells including detachment of the cells from substratum (Sections 3.2 and 3.3). Because of this, *T. denticola* attachment was also studied using fixed PLE cells to prevent variability due to detachment of cells from the substratum. As mentioned in section 4.1, any induced modification of receptiveness of PLE cells by *T. denticola* could not be studied under these conditions. Despite that limitation, similar adhesion inhibition assay results were

observed with both PLE and FPLE cells (Sections 3.1.3 and 3.1.4).

The current investigation showed *T. denticola* to be cytotoxic and cytopathic to eukaryotic cells, including PLE. Affected PLE cells were detached, were killed, and cytopathic changes were induced. Most detached cells were killed and at least some of the attached cells were non-viable and appeared to be undergoing apoptosis (Sections 3.2 and 3.3). Some previous investigations have focused on investigating the attachment behavior of *T. denticola* to host cells, while many more have focused on studying the spirochete's ability to modulate host defense or defense cells (Tables 4.2, 4.3, 4.4). Few research groups have studied the cytotoxic or cytopathic activities of *T. denticola* on cell lines. Boehringer *et al.* (1984) reported retardation of proliferation of fibroblastic cells upon challenge from *T. denticola.* Ellen's group reported cytopathic changes in HGF and KB cells under the effects of *T. denticola* challenge (Baehni *et al.* 1992, de Filippo *et al.* 1995, Ko *et al.* 1998, Yang *et al.* 1998). Similar cytopathic and cytotoxic effects were observable in the current study (Figure 3.3, 3.10 and 3.16). In-depth investigations were carried out in the present study to enable the cytotoxic and cytopathic events induced in PLE cells to be more comprehensively described. Attempts were also made to identify and characterize cytopathic/cytotoxic factors from *T. denticola.* The fact that pre-heating *T. denticola* retarded the cytotoxic effect of this spirochete to PLE cells suggested that *T. denticola* proteinaceous substances might be involved, rather than LPS. The ability of anti-*T. denticola* Msp and anti-*T. denticola* CTLP sera to inhibit *T. denticola* cytotoxicity indicated that Msp and CTLP are involved in cytotoxicity. The similarities of cytotoxic effects from intact or sonicated *T. denticola* indicated that the cytotoxic elements(s) is/are readily available on *T. denticola,* and that induction of new protein expression upon interaction with a PLE cell was not necessary. The focus for investigating cytotoxic activities was therefore directed towards the

major OM proteins of *T. denticola*.

4.2.3. Major outer membrane elements of *T. denticola*

Two major outer sheath elements were investigated in the current study, i.e. CTLP and Msp. The OM elements, including the two major proteins, are released to the environment under normal growth conditions and during cell lysis. The Msp, similar to other bacterial porins, was resistant to proteolytic degradation. These conditions, i.e. normal growth and cell lysis, resemble those found in the gingival sulcus or periodontal pocket during inflammatory periodontal disease. The location and occurrence of the 95 kDa CTLP of *T. denticola* had been well described and characterized (Uitto *et al.* 1988b Grenier *et al.* 1990). Recently, treponemal DNA carrying the gene or a fragment of the gene encoding the CTLP has been identified and sequenced (Ishihara *et al.* 1996, Table 4.7).

With respect to investigations of *T. denticola* Msp, a number of studies have been conducted. Umemoto and coworkers (1988) were among the first to report the identification of a 53 kDa protein from *T. denticola* ATCC 33520. They later demonstrated the outer sheath location of the protein (Umemoto *et al.* 1989). In the same year, Cockayne *et al.* (1989) also reported identification of a major protein of 54 kDa from *T. denticola* ATCC 33520 detergent extract. They assumed this protein to be a breakdown product of a large, heat modifiable polypeptide. This was confirmed in the present study by the dissociation of purified *T. denticola* Msp oligomers into a 53 kDa protein upon heating. The high molecular mass aggregate oligomer of *T. denticola* Msp was highly resistant to hydrolysis by proteases, whereas the 53 kDa monomeric form was readily degraded. The native conformation of *T. denticola* surface proteins might protect the spirochete against proteolytic enzymes produced by the bacterial cells

themselves as well as those produced by the host or other bacteria.

The Umemoto group (1989) proposed that the 53 kDa protein was a major antigen of the cell envelope of *T. denticola* ATCC 33520. Weinberg and Holt (1991) also reported similar findings on outer sheath preparation from *T. denticola* ATCC 35404. They estimated the molecular weight of the major antigen to be 58 kDa. In the same report, a similar protein band from different *T. denticola* strains was also identified, the molecular mass was reported to be 60 - 64 kDa. In later report by Tall and Nauman (1994), a 56 – 58 kDa major protein from *T. denticola* ATCC 33520 was identified. In a later report by the Umemoto group (Nakamura *et al.* 1993), anti-53 kDa protein antibody against ATCC 35404 cross-reacted weakly with the 53 kDa protein from strain ATCC 33520. This was similar to the current findings. The present study suggested at least some common antigenic determinants between the *T. denticola* strains tested. The antibody against the 53 kDa protein of *T. denticola* ATCC 35404 cross-reacted strongly with the 53 kDa band of strain ATCC 35405 and a clear, although weaker, reaction was observed also with the 53 kDa protein of strain ATCC 33520. The present results suggest that the 53 kDa protein described is similar to the proteins studied by Umemoto *et al.* (1988, 1989), Cockayne *et al.* (1989), Weinberg and Holt (1991) and Tall and Nauman (1994).

Initial characterization of the *T. denticola* Msp in the current study clearly indicated that this 53 kDa protein is a porin. Taken together with its previous proposed function as an adhesin for cells and/or certain matrix proteins (see Section 4.3.2 Haapasalo *et al.* 1992), the 53 kDa protein joins a select group of porins, including the *Chlamydia trachomatis* (Su *et al.* 1988) and *Legionella pneumophila* (Payne & Horowitz 1987) major outer membrane proteins, which have dual adhesion-porin functions. The single-channel conductance of the 53 kDa protein was 1.8 nS in 0.1 M KCl. The estimated channel diameter from this measurement was 3.4 nm, calculated as

per Kropinski *et al.* (1987). This represents a porin of very large channel size. Interestingly, another reported huge porin channel was of spirochetal origin, the *Spirochaeta aurantia*, which had porin molecules of 2.3 nm channel diameter (Kropinski *et al.* 1987). This is consistent with the concepts that the spirochete may be a primitive filter feeder and that the outer sheath serves as a crude controlled filtration device (Delcour *et al.* 1992) permitting free flow of nutrients through the periplasm during the movement of spirochetes through their environment. Interestingly, despite the rather exotic appearance of spirochetes, the properties of the 53 kDa porin, including resistance to protease degradation and detergent denaturation, formation of native oligomers, and apparent acidic pI, match those of other members of the porin family.

Examination of isolated outer sheaths revealed a hexagonal array. Evidence was consistent with this hexagonal array being due to the 53 kDa protein (Figure 3.26). Indeed, the approximate magnitude of the regularly arrayed, more highly stained areas (approx. 5 nm) was consistent with the estimated channel diameter for this protein. Some other porins are also arranged in hexagonal arrays, e.g. OmpF from *E. coli* (Steven *et al.* 1977), and the major porins of *Thermatoga maritima* (Rachel *et al.* 1990) and *Bordetella pertusis* (Kessel *et al.* 1988). In the latter two cases, the hexagonal arrays can be visualized in the native outer membrane.

Recently, the gene encoding the Msp of *T. denticola* ATCC 35405 was cloned, sequenced and expressed in *E. coli* (Fenno *et al.* 1996). Biological activities of the rMsp were studied (to be discussed in Section 4.3.4). Conservation of the *msp* gene in a *T. denticola* strain with a 62 kDa hexagonal Msp (OTK) was demonstrated (Fenno *et al.* 1997), and this further illustrated that possession of Msp on the outer sheath is common to *T. denticola* strains.

4.2.4. Biological activities of the major outer membrane elements of *T. denticola*

The biological activities of the *T. denticola* major outer membrane elements were studied concurrently with their isolation and characterization. In addition to the well established proteolytic activities of the CTLP (Uitto *et al.* 1988b, Grenier et la. 1990), the results of the current study imply that the CTLP might be responsible for *T. denticola* adhesion to eukaryotic cells. From the findings of the present study (Section 3.1.4.2), it seems that *T. denticola* binding to epithelial cells is specific and may be mediated by an adhesin that also recognizes the serum protein fibrinogen. The fact that treatment of *T. denticola* with protease inhibitors such as PMSF and *p*CMBA inhibited both cell binding and CTLP activity (Section 3.1.4.2) suggested that the outer membrane associated CTLP played a role in the *T. denticola* attachment. The strongest evidence illustrating CTLP-mediated attachment was that the antibodies against the purified proteinase inhibited the attachment of both the spirochete and the purified proteinase to PLE cells. Interestingly, other investigators reported that, pre-treatment of *T. denticola* with PMSF did not influence attachment to HGF (Ellen *et al.* 1994b) and increased attachment to Hep-2 epithelial cells up to 9-fold (Wang and Holt 1993). Probably, the contradictions between these findings and those of the present study may be due to: i) presence of other *T. denticola* outer sheath element(s) that could mediate binding, and/or ii) differences in the substrate cell surfaces. This further emphasizes the importance of the proper selection of the cell model for bacterial attachment experiments (Section 4.2.1). Information is available in the literature documenting that HCE, RPE (Keulers *et al.* 1993a) and MDCK cells (Carranza Jr. *et al.* 1997) bind minimally to *T. denticola.*

Other biological activities of CTLP, including its cytotoxic activities on PLE cells, were also studied. Specific protease inhibitors against CTLP could reduce cytotoxicity of the protease

to PLE cells. However, the most significant inhibition of cytotoxicity was obtained by pre-treating CTLP first with PMSF and then with polyclonal anti-CTLP IgG, suggesting that the enzyme activity of the protease was partially responsible for the PLE cell killing. The current investigation hypothesized that the 95 kDa CTLP most likely possesses some other determinants that can create damage in host cells. Attempts to isolate and characterize the *T. denticola* CTLP gene were commenced as early as in the late 1980s (Que & Kuramitsu 1990, Table 4.7). However, it was not until recently (Ishihara *et al.* 1996) that this gene was successfully isolated and characterized. The labile nature of native CTLP and the toxicity of expression of exogenous outer membrane protein in *Escherichia coli* may have contributed to the long delay in identification of the genes encoding CTLP. Future studies using the native and recombinant CTLP peptides may clarify the different activities of this molecule.

Biological activities of *T. denticola* Msp was also studied (Sections 3.4.3 – 3.4.5). Msp was found to possess both attachment to and cytotoxic activities on PLE cells. With respect to the *T. denticola* Msp, not much information is available regarding its adherence to junctional epithelium or epitheloid cells. The ability of recombinant Msp to adhere to immobilized laminin and fibronectin was reported recently (Fenno *et al.* 1996). That was complementary to a previous report identifying the potential role of native *T. denticola* Msp in mediating attachment to extracellular matrix macromolecules (Haapasalo *et al.* 1992). With the knowledge of the abundance of fibronectin expression on PLE cells, particularly the migrating phenotypes, it can be inferred that the *T. denticola* Msp could bind to PLE cells at least in part through interaction with the cell bound fibronectins. Attachment of the *T. denticola* OM protein or semi-purified Msp to HGF was reported by Weinberg and Holt (1991, Table 4.6). Specific antibody against a Msp (64 kDa) of *T. denticola* strain GM-1 inhibited attachment of the bacteria to HGF. Amino

acid analysis of cyanogen bromide cleavage fragments of purified Msp from *T. denticola* strain GM-1 revealed a fragment of the protein with a seven residue amino acid sequence 100% homologous to the integrin alpha subunit of a human leukocyte adhesion glycoprotein. This suggested the potential of *T. denticola* Msp perturbing eukaryotic intercellular attachment systems. Msp has also been proposed to interact with PMN's. Ding *et al.* (1996) reported that partially-purified *T. denticola* 53 kDa protein induced MMP-8, elastase and cathepsin G release from PMN's, while the challenged PMN's remained viable throughout the 2 h study period. A later investigation (Sela *et al.* 1997) also reported PMN stimulatory activities of a *T. denticola* dLPP extract resembling Msp (Table 4.6).

Other major biological activities of *T. denticola* Msp reported in the present investigation, i.e. the cytopathic and/or cytotoxic activities, have not been widely investigated in the literature. The present studies are the first to investigate the cytotoxic effects of outer membrane porin-like molecules of spirochete bacteria. *T. denticola* pre-treated with buffers at pH 2.0 was found to retain its cytotoxic activities (Table 3.9). Under such harsh pre-treatment condition, the *T. denticola* CTLP enzyme activities and the CTLP mediated PLE adhesion would be abolished (Table 3.5), suggesting *T. denticola* might possess some other factor(s) with cytotoxic activities. Msp complex, however, was found to be resistant to such harsh pH treatment and remained cytotoxic to PLE (data not shown). Both Msp and Msp complex were found to be cytotoxic to PLE cells and to other cell lines. Cytopathic effects on eukaryotic cells due to translocation of bacterial porin-like molecules to the cell membrane have been reported for *Neisseria gonorrhoeae* (Haines *et al.* 1991), *Salmonella typhimurium* (Galdiero *et al.* 1993), *Porphyromonas gingivalis* (Novak and Cohen 1991), and *Eikenella corrodens* (Tufano *et al.* 1986). A recent study implicated bacterial porins in bone resorption (Meghi *et al.* 1997),

presumably through modulation of pro-inflammatory cytokines (Henderson *et al.* 1996). The mechanism of Msp-induced cytotoxicity has not yet been identified, but the current data suggest that Msp pore-forming activity is involved. Msp was cytotoxic at monomer concentrations of less than 50 nM. Higher concentrations of the Msp complex, however, were required for cytotoxicity. This could be due to insolubility of the complex in the regular assay medium and/or formation of inactive Msp aggregates or artifact during the preparation procedure. Discrepancies between the patterns of cytotoxicity of *T. denticola* to various cell types versus that of Msp complex, however, was observed (Figures 3.18 and 3.33). PLE cells appeared to be the most resistant cells to be tested when whole *T. denticola* (Figure 3.18) was used while HGF and HeLa cells were more resistant against Msp complex (Figure 3.33). One possible explanation is that, during the challenge by *T. denticola* whole cells, probably some cytotoxic agent/ factor other than the Msp might also be actively involved. A likely candidate is *T. denticola* CTLP.

The present investigation showed that Msp binds certain epithelial cell surface and cytoplasmic proteins and depolarizes the epithelial cell membrane. Modified immunoprecipitation experiments were done to identify potential Msp receptors(s) on HeLa cells. Msp present in the Msp complex preparation bound to at least one biotin-labeled HeLa cell surface protein of 65 kDa, as well as to several other presumably cytoplasmic proteins including a strongly reactive protein of 96 kDa. It is likely that specific interaction between bacterial outer membrane components and epithelial cell surface components are important for the insertion of a bacterial porin into an epithelial cell membrane. The 65 kDa protein, a likely Msp receptor, remains to be further characterized. The strong signal due to binding of Msp to the ^{35}S-methionine-labeled 96 kDa protein is intriguing and suggests that Msp may also have specific

interactions with cytoplasmic proteins.

Msp is a multifunctional outer membrane protein, with pore-forming ability and adhesion activity toward a number of ECM components. A specific role for the Msp porin activity in *T. denticola* metabolism has not yet been determined. The Msp complex depolarized and increased the conductance of the HeLa cell membrane. The increase was observed even after substitution of Cl^- by gluconate, an anion that cannot pass through HeLa cell chloride channels (Takahashi *et al.* 1993). The effect of Msp complex is therefore unlikely to result from the activation of intrinsic chloride channels in the HeLa cell membrane. Complete replacement of Na^+ by K^+ in the extracellular solution also failed to block the Msp-induced conductance increase. In physiologically normal solutions, the conductance induced by Msp complex showed a reversal potential of −18 mV, a value that did not correspond to the Nernstian potential of any single ionic species present.

These observations indicated that the Msp complex increased membrane conductance in a relatively non-specific manner. The current-voltage relationship of Msp-treated cells showed a greater enhancement in outward current than was seen for inward membrane current, indicating a moderate voltage dependency for Msp-induced conductance.

The relatively non-specific, weakly voltage-dependent nature of Msp-induced conductance is consistent with the Msp complex inducing porin-like channels in the HeLa cell membrane (Barrier *et al.* 1993, Lakey & Pattas 1989, Morgan *et al.* 1990). This hypothesis was strengthened by direct observation of channel-like currents in membrane patches exposed to the complex in the patch pipette solution. The slope conductance of these channels was about 0.4 nS in a total salt concentration of 0.3 M. This value is lower than the 1.8 nS for Msp reconstituted in black lipid bilayer but remains within the range typical of bacterial porins (Benz 1988, Benz

and Bauer 1988, Berrier *et al.* 1992). The smaller conductance value obtained in the present study may reflect a dependence of channel conductance on the reconstitution medium employed, as previously reported for the OmpC porin of *E. coli* (Delcour *et al.* 1991) and for channels formed by diphtheria toxin (Eriksen *et al.* 1994).

Channels induced by the Msp complex in HeLa cell membranes exhibited only brief excursions into a short-lived open state. In contrast, Msp reconstituted in black lipid bilayers formed channels, showing very long duration openings with no evidence of closing transitions (Figure 3.28). Porins reconstituted in artificial membranes exist mainly in the open state, leading to the suggestion that these channels import sieve-like properties to the bacterial outer membrane (Benz 1988, Xu *et al.* 1986). However, more recent cell-attached recordings from intact bacteria suggest that the majority of native porin channels are normally closed, perhaps as a result of the presence of intracellular modulators of channel function (Baechner *et al.* 1990, Delcour *et al.* 1992). The current results support the view that porin channels open infrequently when studied in intact biological membranes. However, determination of the kinetic and conductance properties of native Msp channels in *T. denticola* awaits patch clamp recordings from the intact outer membrane of *T. denticola*.

The present investigation showed clearly that tested factors which were inhibitory to *T. denticola* adherence to PLE/FPLE were also able to affect the cytotoxic activities of CTLP or Msp (Tables 3.13 and 3.14). Apparently, the adherence and cytotoxic activities of CTLP and Msp are closely related. Study of the inter-relationship of CTLP adherence and cytotoxicity are somewhat difficult because of the strong proteolytic activity of the protease. Probably, the exact molecular nature of the CTLP-PLE cell attachment must be clarified before the relationship of adherence and cytotoxicity can be fully elucidated. On the other hand, provided pore/channel-

formation on eukaryotic cell membrane is one key cytotoxic mechanism of Msp/Msp complex, attachment/adherence would be a key process of the Msp-mediated cell cytotoxicity. To further investigate this aspect, one could design experiments to test if cytotoxic effects of purified Msp would be less when cell lines that do not adhere well to Msp were used.

4.3 A model hypothesizing the way *T. denticola* induces cytopathic and cytotoxic effects on PLE cells.

The following is an attempt at formulating a model to summarize *T. denticola* induced cytopathic and cytotoxic effects on epithelial cells. The model was designed based on available information in the literature, the findings from the present investigation, and deductive reasoning. Because the specific molecular interactions between *T. denticola* and PLE cells have not yet been established, what is presented here is speculative and is by no means definitive.

The present knowledge of the nature of the periodontal disease and its chronic course suggests that most periodontopathogens, unlike many acute medical pathogens, are opportunists and produce their destructive effect as a result of interaction between the pathogen or its metabolites and an unfavorable host response. Thus in understanding how an organism, such as *T. denticola* acts as a periodontopathogen, the critical area of knowledge may not be how it might induce bone resorption but rather how it may establish a favorable environment in the subgingival ecological niche, be sustained there and thrive therein. The complexity of the subgingival flora and its high variability (Moore & Moore 1994) together with perhaps coincidental or consequential alterations of local defense over time must act in the same unfavorable direction before periodontal tissue destruction could occur (Figure 4.1).

From the current investigations plus information available in the literature, what we learn

is that *T. denticola* can resist to a great extent the host defense and immune response (Tables 1.4, 1.8). *T. denticola* can also modulate local inflammatory responses by degrading human bioactive peptides (HBPs). From what could be observed *in vitro*, *T. denticola* may be capable of destroying the tight junctional epithelium (JE) barrier so that nutrients from the host can be readily supplied from the connective tissue layer to the spirochete in the subgingival environment, thereby allowing *T. denticola* to thrive and flourish. The study of the interaction between *T. denticola* and JE, would therefore, be crucial to the understanding of *T. denticola* colonization and subsequent establishment in the periodontal pocket. Porcine PLE cell culture was used because it was shown to be resemble JE *in vitro* (Pan *et al.* 1995).

The interaction between *T. denticola* and PLE cells could roughly be conceptualized as occurring in 3 phases: (1) initial PLE cell encounter/attachment, (2) induction of cytopathic effects on PLE cells and preparation for more *T. denticola* attachment, (3) induction of cytotoxic effects and killing of PLE cells. Based on the existing knowledge and the current findings, a timetable of spirochete-PLE cells interactive events is postulated (Table 4.8).

It has been established from the current study that *T. denticola* possess a regular hexagonal protein array containing Msp and CTLP in its outer surface. These two proteinaceous elements bind to extracellular matrix macromolecules and eukaryotic cells (Chapters I and III). It was shown that factors that could inhibit the enzyme activity of the *T. denticola* CTLP could also reduce the attachment of the spirochete to PLE cells (Section 3.14), which indicates that the CTLP plays a role in the *T. denticola* attachment. The observation that the cell binding of *T. denticola* increased quickly and then declined indicated that the attachment mechanism is a dynamic process. The adhesin and the proteinase domains appear to be located in close proximity on the *T. denticola* surface. The *T. denticola* CTLP may expose cryptic epitopes on

host cells for spirochete binding through CTLP, Msp or other factors. Msp was also identified to be able to bind to PLE/FPLE cells. The blocking of either whole *T. denticola* or Msp attachment to PLE cells with anti-Msp antibodies clearly illustrates the role that Msp plays in adhesion of *T. denticola* to living cells. It is likely that motility and chemotactic ability of the *T. denticola* would also be very important for the attachment process.

Once attachment to host cells has been secured, the spirochetes exert effects on the cells. Through as yet unknown mechanisms, the cellular cytoskeleton was affected; namely a disorganization and reduction of the cytoskeleton of the affected cells took place. This was associated with cytoplasmic changes such as blebbing, cell contraction and vacuolation. Such effects were compatible with signs of cellular apoptotic changes. The blebbing and contraction may have resulted from effects of the *T. denticola* CTLP that degraded the cell anchorage protein and/or fibers. On the other hand, cellular contraction may be a result of loss of volume and electrolyte balance control when the *T. denticola* Msp becomes incorporated onto the PLE cells and creates very large channels. Recent studies from the Ellen group (Ko *et al.* 1998, Yang *et al.* 1998) showed that cytoskeletal disruption, loss of volume control and disruption of calcium responses were due to proteinaceous OM components of *T. denticola*, but were not due to CTLP. The Msp and/or the CTLP could perhaps also activate latent collagenases or MMPs (Sorsa *et al.* 1992, 1995, Ding *et al.* 1996) into active forms so that the regular host regulated cell homeostasis will be upset resulting in cell detachment and breakdown not just within the epithelial barrier.

Although it was observed that the PLE cells initially respond to *T. denticola* by expanding and maintaining the confluence of the monolayer, such spreading of PLE cells could lead to expression of more epitope/sites that favor *T. denticola* adhesion (Figure 3.4C). *T. denticola* Msp, on the other hand, may continue to exert its cytotoxic effects on PLE cells. One

possible mechanism is disturbance of the PLE cell membrane integrity by creating mega-channels on the host cell surface. Whether the native Msp on *T. denticola* surface can create a similar effect is not yet clear.

Some other potential pathogenic factors of *T. denticola* which might be of importance *in vivo* have been identified (Tables 4.6 & 4.7). These included the 46 kDa cystalysin or the hemolysin (Chu *et al.* 1995, 1997); the 44 kDa hemin binding protein (Chu *et al.* 1994) and several peptidases (Mäkinen *et al.* 1994) that can hydrolyze HBPs such as substance P. The *T. denticola* 60 kDa PLC may also play a part in affecting the stability of PLE cell membrane (Chan *et al.* 1991). The 59 kDa HGase of the spirochete could also help in affecting the integrity of the monolayer cell culture (Scott *et al.* 1996). The 46 kDa cystalysin can generate large amount of H_2S, which will be toxic to the host cells (Chu *et al.* 1995, 1997). Such factors together with the CTLP and HGase could modulate the local host inflammatory response, and the local initial healing response (e.g. lysis of fibrin etc., Table 4.9). The local defenses, be it innate, adaptive, humoral or cellular, could also be affected by *T. denticola* as demonstrated by various previous studies (Table 4.4). These factors can all contribute to the success of *T. denticola* in establishing itself in the subgingival environment and establishing, perpetuating or modulating periodontal disease conditions.

The current investigation studied and summarized the pathogenic role of the human periodontopathogen – *T. denticola*. However, this study is by no means going to minimize the importance of other known/unknown periodontal pathogens. The knowledge we gained about the pathogenecity and virulence of *T. denticola* makes a small addition to the complex field of polymicrobial host-parasitic interaction involved in human periodontal diseases. Many challenges still remain ahead before dental researchers can full understand human periodontal

disease causation.

Table 4.1 *Treponema denticola* – extracellular matrix attachment studies.

Author/Year	*Treponema denticola* strain	Extracellular matrix studied	Attachment Assay	Attachment behavior observed
Dawson & Ellen 1990	ATCC 33520, ATCC 35404, ATCC 35405; b, d, e, e', (McGill University)	Fibronectin, RGDS peptides, collagen IV, laminin	Matrix protein coated cover slips for *T. denticola* (in PBS) adhesion, 2 h, 37°C. Microscopic quantitative assay. BSA used as control.	*T. denticola* adhered to fibronectin, RGDS peptides and laminin but not to collagen IV. Reported tip binding of *T. denticola*.
Haapasalo *et al.* 1991	ATCC 35405	Laminin, laminin fragments, fibronectin, GRGDS peptide, collagen I & IV	Binding to fibrinogen and gelatin were also tested. Matrix protein coated microwells for *T. denticola* (in PBS) adhesion, 1 h, 20°C. ELISA-semi-quantitative assay. BSA used as control. Effect of pre-treatment on spirochete attachment was also tested.	*T. denticola* bound well to all proteins or peptides tested except 50 kDa laminin fragment, BSA and GRGDS peptide. *p*CMBA, oxidized glutathione, mixed glycosidase and heating inhibited the *T. denticola* attachment.
Nakatani *et al.* 1992	ATCC 33520	Fibronectin, collagen VI, laminin	Matrix protein coated cover-slips for *T. denticola* (in plain DMEM) adhesion, 1 h. Microscopic quantitative assay. BSA as control.	*T. denticola* adhered to fibronectin, collagen VI, laminin, and Con A.
Dawson & Ellen 1994	ATCC 33520	Fibronectin, laminin	*T. denticola* methylcellulose migration column. Colloidal gold-EM study.	Fibronectin-gold probes clustered toward a particular end of *T. denticola* when the spirochete was in contact with the fibronectin coated nitrocellulose.
Haapasalo *et al.* 1996	ATCC 35405	Hyaluronan, collagen I	Binding to fibrinogen and BSA were also tested. *T. denticola* (in PBS) adhesion, 1 h, 20°C. ELISA assay.	*T. denticola* bound to hyaluronan. *T. denticola* CTLP might be involved.

Table 4.2 *Treponema denticola* – eukaryotic cell attachment studies.

Author/Year	*Treponema denticola* Strain	Host Cell Used	Assay Conditions	Attachment Behaviour Observed
Olsen 1984	B2, T1, (Forsyth Dental Centre)	HEK, NCTC 2544, Norway	Anaerobic incubation. *T. denticola* in culture medium, 1 h. Microscopic descriptive study.	Hyaluronidase treated cells bind fewer treponemes; dividing cells bind more spirochetes.
Reijntjens *et al.* 1986	$L_{12}D$, (University of Pennsylvania)	GPE 23952, UK	Aerobic incubation. *T. denticola* in cell culture medium, 0-48 h. Microscopic quantitative assay.	Attachment plateau at approx. 4-6 hr., 0-40 spirochetes per cell.
Weinberg & Holt 1990	ATCC 35404; GM-1, MS25, (UTHSC-SA)	HGF: Gin 1 – ATCC Gin 3 – explant	Aerobic or anaerobic incubation. *T. denticola* mainly in DMEM, 1 h. Radiolabeled (^{125}I) treponemes quantitative study.	Strain ATCC 35404 adhered least. Divalent cations had no effect on the binding. *T. denticola* surface lectin and serum host factor(s) might be involved in the binding, 54±6 spirochetes per cell. D-Gal, D-Man, or D-GalNAc at 50 mM reduced spirochetal adherence to 40%.
Baehni *et al.* 1992	ATCC 35405; e, e', (McGill University)	HGF	Aerobic incubation in plain αMEM 1-17 h. Microscopic descriptive assay.	*T. denticola* adhered to HGF.
Keulers *et al.* 1993 a, b, c	ATCC 33520, ATCC 35404, ATCC 35405; B11, B12, Ny535, Ny541, Ny572, Ny573, (University of Nijmegen); $L_{11}D$, (University of Pennsylvania)	HCE, HBE, Netherlands; RPE, Denmark; GPE 23952, U.K.	Aerobic incubation. *T. denticola* in plain MEM, 6 h. Microscopic semi-quantitative assay.	Strain ATCC 35404 binding minimally to HCE and RPE. 0.1 spirochete/RPE cell, 0.3 spirochete/HBE cell. Spirochetes preferably attached to rounded RPE cells. Attached best at 37°C, 6 h. D-Man, D-Gal NAc, (not D-Gal), Sialic acid, Na metaperiodate, L-phenylalanine, proteinase K, heat, fixation and serum pre-treatment reduced *T. denticola* adhesion.
Pan 1993	ATCC 33520	PLE	Aerobic incubation. *T. denticola* in plain αMEM, 2 h. Microscopic descriptive assay.	Cell explant resembling JE used.

Table 4.2 cont'd *Treponema denticola* – eukaryotic cell attachment studies.

Author/Year	*Treponema denticola* Strain	Host Cell Used	Assay Conditions	Attachment Behaviour Observed
Ellen *et al.* 1994b	ATCC 35405	HGF	Aerobic incubation. *T. denticola* in plain αMEM, 2h. ELISA assay.	The number of adherent treponemes increased together with concomitant reduction of fibronectin in the microwell. PMSF inhibited fibronectin degradation but not *T. denticola* adhesion.
de Filippo *et al.* 1995	ATCC 35405	KB, ATCC CCL17	Aerobic incubation. *T. denticola* in plain αMEM, 1-4 h. Microscopic descriptive assay.	*T. denticola* adhered to KB cells
Haapasalo *et al.* 1996	ATCC 35405	PLE	Aerobic incubation. *T. denticola* in PBS at 20°C, 1 h. ELISA assay.	Pre-treatment of spirochete with hyaluronan inhibited binding of *T. denticola* to PLE. Gelatin, BSA, chrondroitin-4-sulfate, chrondroitin-6-sulfate, heparin, dermatan sulphate, glucuronic acid, D-GalNAc, D-GluNAc did not inhibit spirochete binding. *T. denticola* CTLP might be involved.
Carranza Jr. *et al.* 1997	ATCC 33520, ATCC 33521, ATCC 35404, ATCC 35405; ST-10, (Naval Dental Research Institute)	CL, ATCC CCL13; MDCK, ATCC CCL14	Aerobic incubation. *T. denticola* in plain DMEM, 37°C, 7 h. Microscopic semi-quantitative assay.	*T. denticola* did not bind well to MDCK cells. Spirochetes attached better to low confluency cells. Variation in amount of spirochetes attached per cell.

Table 4.3 Studies investigating the *in vitro* host cell/tissue damage inducible by *Treponema denticola*.

Author/Year	*Treponema denticola* Strain	Cell/Animal Used	Assay Conditions	Cell/Tissue Damage Observed
Lindhe & Socransky 1979	K1, (Forsyth Dental Center)	Spraque-Dawley rats, Indianapolis	Wound chambers.	++ vascular exudation.
Taichman *et al.* 1982	LL 2513, LL 2516, LL 2519, (University of Pennsylvania); CD-1, 51B2, (University of Michigan)	L-929, ATCC CCL1	*T. denticola* sonic extract, aerobic incubation, 48 h. ^{3}H-TdR incorporation.	Cell vacuolation. Inhibition of DNA synthesis. Heating the *T. denticola* sonic extract abolished its DNA inhibition effect on murine fibroblast.
Boehringer *et al.* 1984	LL 2513, LL 2516, LL 2519, LL 2533, LL 2535, (University of Pennsylvania); CD-1, (University of Michigan)	L-929 ATCC CCL1; Human skin fibroblast, University of Pennsylvania	*T. denticola* sonic extract, aerobic incubation in culture medium, 72 h. ^{3}H-labelled thymidine, uridine, and leucine incorporation.	Murine and human fibroblast proliferation inhibited by *T. denticola* sonic extract.
Taichman *et al.* 1984	LL 2513, LL 2516, LL 2519, LL 2533, (University of Pennsylvania); CD-1, (University of Michigan)	HVE BVE	*T. denticola* sonic extract.	Inhibition of DNA synthesis. Heating did not reduce the biologic activity of the *T. denticola* sonic extract on endothelial cells.
Reijntjens *et al.* 1986	$L_{12}D$, (University of Pennsylvania)	GPE 23952, U.K.	Aerobic incubation. *T. denticola* in cell culture medium, 0-48 h.	Vacuolation, rounding up of GPE. 20% GPE cell detachment.
Grenier 1991	ATCC 35405	Human AB$^+$ erythrocytes	Aerobic incubation. *T. denticola* in 2% saline 37°C, 4 h. Quantification of hemolysis.	Hemolysed human red blood cells. Hemolytic activity was heat labile. $CaCl_2$, $MgCl_2$, Na salicylate and EDTA reduced hemolysis.
Hanks *et al.* 1991	ATCC 35405	L-929, ATCC CCL1	Aerobic incubation. Sonicated *T. denticola* in culture medium in micro-wells or *in vitro* pulp chamber. ^{3}H-leucine incorporation.	New protein synthesis by L-929 cells was affected.

Table 4.3 cont'd. Studies investigating the *in vitro* host cell/tissue damage inducible by *Treponema denticola*.

Author/Year	*Treponema denticola* Strain	Cell/Animal Used	Assay Conditions	Cell/Tissue Damage Observed
Baehni *et al.* 1992	ATCC 35405; e, e', (McGill University)	HGF	Aerobic incubation. *T. denticola* in plain αMEM, 1-5 h. Microscopic descriptive study.	Cell rounded up, blebbing, actin re-arrangement and cell detachment. *uv*, heat, metronidazole, or PMSF pre-treatment of *T. denticola* reduced detachment of HGF.
Pan 1993	ATCC 33520	PLE	Aerobic incubation. *T. denticola* in plain αMEM, up to 20 h. Microscopic study and epithelial multi-layer permeability assay.	Collapsed intercellular spaces and disruption of intercellular junction. Increase in epithelial permeability. Denuded surface of the multilayer PLE culture, blebbing, rounding up, vacuolation, disorganization of cellular intermediate filaments, loss of cytoplasmic details and definition of cellular organelles.
Gopalsami *et al.* 1993	ATCC 33521, ATCC 35404, ATCC 35405	$^{45}CaCl_2$ labeled fetal rat radii and ulnal organ culture	Detergent extracted *T. denticola* outer membrane preparation in tissue culture medium incubated with fetal rat bone organ culture, 37°C, 48 h., then 120 h. in medium. Assay of ^{45}Ca release over time.	*T. denticola* outer membrane preparation appeared to induce bone resorption. LPS like substance from the extract was suggested to be responsible.
Ebersole *et al.* 1995	GM-1, (UTHSC-SA)	BALB/cJ and BALB/cN mice	Subcutaneous challenge with spirochetes up to 15 days.	Multi-tissue involvement after subcutaneous spirochete inoculation
de Filippo *et al.* 1995	ATCC 35405	KB, ATCC CCL 17	Aerobic incubation. *T. denticola* in plain αMEM, 1-4 h., 37°C. Detachment: trypsinized cell counting; Viability: Propidium iodine exclusion; Cell size and volume regulation: Coulter channelyzer to measure trypsinized cells and standard; Cytoskeleton changes: RITC– phalloidin, keratin and desmoplakins expression.	*T. denticola* induced KB cell detachment from substratum. Detached cells were non-viable, smaller in size, while cells on wells were viable despite showed decreased F-actin, cell diameter, and expression of desmoplakin II and slight reduction in keratin expression. Treponeme induced volume change were irreversible.

Table 4.3 cont'd. Studies investigating the *in vitro* host cell/tissue damage inducible by *Treponema denticola*.

Author/Year	*Treponema denticola* Strain	Cell/Animal Used	Assay Conditions	Cell/Tissue Damage Observed
Uitto *et al.* 1995	ATCC 35405	PLE, HGF	PLE cells in sparse, confluent and multilayer were used. Time-lapse cinemicrography to document the *T. denticola* cytopathic effects on PLE cells. Immuno-detection of HGF and PLE cells related fibronectin after spirochete treatment. PLE cells multilayer permeability assay.	*T. denticola* induced blebbing and disorganization of actin and α-actinin of PLE cells. Peri-PLE/HGF cell fibronectin was degraded by *T. denticola*. Confluent PLE cells were more resistant to *T. denticola* than isolated cells. *T. denticola* did not seem to invade PLE multilayer, however, the spirochete treated multilayer showed increased permeability. Immunogold EM showed rapid internalization of CTLP containing *T. denticola* materials into PLE cells vacuoles.
Kesavalu *et al.* 1997	ATCC 35404; GM-1, MS 25, (UTHSC-SA)	Normal mice, DEX mice, neutropenic mice; ICR (out bred) and BALB/c (in bred) mice used.	Subcutaneous injection of *T. denticola*, then observed for abscess, death. Histopathology of injection site, liver, spleen and mesenteric lymph nodes.	*T. denticola* produced abscess at injection site with increased PMNs and macrophages infiltration. Pre-treating the spirochete with TLCK or DTT could not affect size or severity of the lesion induced by *T. denticola*. Systemic treatment of mice that either reduce PMN infiltration (DEX) or deplete the PMN in the system did not modify the progression or size of the abscesses induced by the spirochete.
Ko *et al.* 1998	ATCC 35405	HGF	Triton X-100 extracted *T. denticola* OM preparation. To determine the potential mechanisms for the *T. denticola* detergent extract induced cytoskeleton perturbance. Test of the effect of detergent OM extract released of internal calcium stimulated by ATP and thapsigargin.	Reconstituted *T. denticola* detergent extract did not affect baseline intracellular [Ca^{2+}] of HGF, however regular spontaneous Ca^{2+} oscillations in HGF were increased in the initial 20 – 30 min post-detergent extract challenge followed by dramatic reduction after 70 min. Detergent OM extract reduced action of ATP and thapsigargin on HGF by up to 40% (internal release). Detergent OM extract impaired the Ca^{2+} replenishment of Ca^{2+} depleted HGF by 60% (uptake from exterior). Action of reconstituted *T. denticola* detergent extract was sensitive to heat (60°C) but not to PMSF.

Table 4.3 cont'd. Studies investigating the *in vitro* host cell/tissue damage inducible by *Treponema denticola*.

Author/Year	*Treponema denticola* Strain	Cell/Animal Used	Assay Conditions	Cell/Tissue Damage Observed
Yang *et al.* 1998	ATCC 33520, ATCC 35404, ATCC 35405; e, e', (McGill University)	HGF	Whole *T. denticola* and Triton X-100 extracted *T. denticola* ATCC 35405 OM preparation effects on HGF IPs accumulation, and F-actin depolymerization, up to 140 min assay.	*T. denticola* caused significant reduction of HGF F-actin within the first h., especially at the ventral 1/3. *T. denticola* and reconstituted detergent extract caused proportional increased of HGF cells with disorganize actin fibres with time. *T. denticola* challenged HGF showed diminished IPs accumulation corresponded time-wise with reduction of HGF F-actin quantity. Pre-treatment of *T. denticola* whole cells or reconstituted detergent extract with TLCK or PMSF did not affect the actin disorganization or IPs accumulation effect.
Fenno *et al.* 1998	ATCC 35405	Human erythrocytes	Hemolysis assay.	*T. denticola* was hemolytic. Hemolytic activity could be blocked by anti-rMsp IgG.

Table 4.4 Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Steinberg *et al.* 1966	Unspecified, (Forsyth Dental Center)			Spirochetes on freshly tanned sheep erythrocytes reacted with complement inactivated patient sera (Healthy, n = 5; Periodontitis, n = 9).	Suggested higher titers of circulating anti-*T. denticola* antibodies in advanced periodontitis patients (8 of 9).
Ghosh & Stevens 1967	Unspecified, (Harvard School of Dental Medicine)			Investigation of anti-*T. denticola* titer in human sera (n = 77, periodontal status unknown) by hemagglutination and complement-fixing techniques.	5% (detected by complement fixing) or 12% (detected by hemagglutination) had increased anti-*T. denticola* titer.
Steinberg & Gershoff 1968 and Steinberg 1970	Unspecified, (Harvard School of Dental Medicine)			Spirochetes on freshly tanned sheep erythrocytes with complement inactivated patient sera (Healthy, n = 9, Periodontitis, n = 17).	Subjects with moderate periodontal involvement had higher levels of anti-*T. denticola* reaction; healthy subjects had weak reaction, subjects with severe periodontitis had no reaction.
Lindhe & Socransky 1979	K1, (Forsyth Dental Centre)	Albino rabbits: glycogen induced peritoneal exudates. Chemotaxis: sonicated *T. denticola* in Boyden chamber, 180 min.			Strong chemotactic activity induced by spirochetes.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Patters *et al.* 1980	K-1, (Forsyth Dental Center)		Human lymphocyte (from normal, n = 5; edentulous, n = 10; gingivitis, n = 24; moderate periodontitis, n = 11; severe periodontitis, n = 12) blastogenic response versus sonic extract of *T. denticola* by ^{3}H-TdR incorporation.		Significantly greater stimulation of blastogenesis by *T. denticola* sonic extract in the (esp. severe) periodontitis group than normal or edentulous group (relatively low stimulation activities).
Jacob *et al.* 1982	ATCC 33520, ATCC 33521; TT, (University of Maryland, deoxycholate-ethanol extract)			ELISA detection of serum antibodies (IgG) in: periodontitis, n=11 (mean 48.3 y.), healthy, n=13 (dental students, unknown age), and edentulous, n=13 (61.5 y.).	Sera from periodontitis individuals had strong anti-*T. denticola* response. Weak or no antibody response in healthy or edentulous individuals.
Mangan *et al.* 1982	FM, (VPI&SU); C-45, 60A-1, CA-1 HA-1, SI-4, 75B2 CD-1, (University of Michigan)		Human lymphocyte blastogenic response versus sonic extract of *T. denticola* by ^{3}H-TdR incorporation.	ELISA detection of serum antibodies (IgG, IgM, IgA) in: periodontitis, n=26 (mean 46 y.); healthy, n=16 (25 y.).	No difference in lymphocyte blastogenesis effect among groups as per challenge by treponemes. Patients had significantly higher IgA titer than controls.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Taichman *et al.* 1982	LL 2513, LL 2516, LL 2519, (University of Pennsylvania); CD-1, 51B2, (University of Michigan)	Normal human PMN lysosomal enzymes release. 60 min, 37°C incubation with spirochetes. Chemotaxis induced by sonic extract of *T. denticola*, 90 min.	Human lymphocytes blastogenic response vs. sonic extract of *T. denticola* by ^{3}H-TdR incorporation.		Without serum, sonic extract from *T. denticola* did not trigger significant chemotaxis of PMN. PMN up take spirochetes but without too much degranulative action. Minimal to small amount of lysosomal enzyme release from PMN. PMN response to other stimulus was inhibited by pre-incubation with spirochetes. Sonic treponeme extract had no effect on lymphocyte DNA synthesis, however, it suppressed blastogenesis in response to mitogens.
Hurlen *et al.* 1984	B2 (Forsyth Dental Center)	Healthy human PMN. Incubation with spirochetes in 10% serum, 5-120 min, 37°C. LDH and lactoferrin detection.			PMN viability maintained. PMN challenged with serum opsonized *T. denticola* released lactoferrin indicating PMN degranulation.
Olsen *et al.* 1984	B2, (Forsyth Dental Center)	Normal human PMN. Aerobic and anaerobic incubation with spirochete in 10% rabbit serum, 5 – 120 min. SEM + TEM study.			PMN engulfed *T. denticola* equally well in aerobic and anaerobic conditions. Postulated that PMN digestion of internalized treponeme could be slow.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied: PMN	Cellular Defense Studied: Lymphocytes	Humoral Defense Studied	Results Observed
Shenker *et al.* 1984	CD-1 (University of Michigan); LL 2513, LL 2516, LL 2519, (University of Pennsylvania)		Effect of sonic extract of *T. denticola* on human lymphocytes (followed by Con A treatment) blastogenesis assayed by ^{3}H-TdR incorporation.		Heat liable sonic extract (mainly 100 kDa protein) of *T. denticola* strains (except LL 2519) inhibited human lymphocyte proliferation response to Con A, PHA , PWM mitogens, and SKSD in terms of DNA, RNA and protein synthesis. Lymphocyte viability maintained through out study period. Prostaglandins and H_2O_2 were needed for the *T. denticola* induced lymphocyte inhibition.
Suzuki *et al.* 1984	ATCC 33520, ATCC 33521; 14, TT, and unspecified strains, (University of Maryland, deoxycholate-ethanol extract)		Normal (n = 24, 20.6±4.1 y.), LJP (n = 29, 17.2±3.0 y.), and GJP (n = 24, 23.3±4.8 y.) human lymphocytes. Blastogenesis response of stimulated lymphocytes by *T. denticola* detergent extract assayed by ^{3}H-TdR incorporation.		No significant differences in the blastogenesis response of the stimulated lymphocytes from the 3 clinical groups as induced by *T. denticola* detergent extract.
Tew *et al.* 1985	D3A1, D3A9, D75APP3, (VPI&SU)			RIA detection of serum anti-*T. denticola* IgG in patients with severe periodontitis, n = 52 (mean 25 y.); LJP, n = 47 (19 y.); Healthy, n = 52 (25 y.).	Highest treponeme reaction titer of serum from LJP subjects. Low level or lack of antibody in severe periodontitis sera.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Boehringer *et al.* 1986	ATCC 33520; LL 2513, LL 2516, LL 2519, LL 2533, LL 2535, (University of Pennsylvania); CD-1, 51B2, (University of Michigan); A-16, F-2, (University of Nijmegen); B-1, (Forsyth Dental Center)	Normal human PMN-oxygen consumption, chemiluminescence, degranulation, cell viability, TEM study.			Treponemes incubated with human serum induced more oxygen consumption, chemiluminescence and phagocytosis of PMN. Limited degranulation observed even after PMN engulfed treponemes. Soluble spirochete extract inhibited PMN response. PMN viability maintained during spirochete challenge.
Lai *et al.* 1986	CD-1, (University of Michigan); B1, (Forsyth Dental Center)			ELISA detection of serum anti-*T. denticola* sonicate (supernatant) IgG, or IgA in patients with untreated periodontitis, n = 13 (mean 42 y.); healthy, n = 10, (young adults and children); umbilical cord blood, n = 1.	IgA and IgG antibody levels to *T. denticola* significantly elevated in AP subjects than LJP or healthy individuals.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied: PMN	Cellular Defense Studied: Lymphocytes	Humoral Defense Studied	Results Observed
Ishihara 1988, and Ishihara *et al.* 1992	ATCC 33520, ATCC 33521, ATCC 35404, ATCC 35405		Effect of *T. denticola* sonic extract on BALB/c mouse splenocyte proliferation with or without mitogen stimulation by ^{3}H-TdR assay, 37°C, 72h.		Sonicate of ATCC 35405 was mitogenic while that of other strains were not. Heat labile (60°C, 10 min) sonicates of ATCC 33520 (particularly a 90 kDa protein) caused suppression of Con A, PWM, or LPS induced lymphocyte proliferation (not observed with sonic extracts from other strains). Suppression might be PG related. Lymphocyte remained viable.
Sela *et al.* 1988	ATCC 305405	Normal human PMN. Spirochete culture supernatant and treponeme sonic extract induced superoxide (O_2^-) or H_2O_2 production after 10 min, 37°C incubation.			PMN remain viable through the incubation with *T. denticola*. Inhibition of superoxide production was not evident when either opsonized or un-opsonized whole spirochetes were reacted with PMNs.
Morinushi *et al.* 1989	ASLM, (University of Michigan)			ELISA detection of serum anti-*T. denticola* (fixed) IgG in IDD children or adolescence. n = 35 (7 – 18 y.).	No relationship between anti-*T. denticola* IgG titer with age.
Sasaki *et al.* 1989	ATCC 33520			ELISA detection of serum anti-*T. denticola* IgG in periodontitis patients.	Case report of 2 patients, 18 and 20 y. No significantly increased anti-*T. denticola* IgG titer.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Gunsolley *et al.* 1991	D3A1, D3A9, D75APPD, (VPI & SU)			RIA detection of serum anti-*T. denticola* IgG in patients with severe periodontitis, n = 50, ≤ 35 y.; AP, n = 40, > 35 y.; non-periodontitis, n = 59.	Serum antibody reactivity of severe periodontitis group to treponeme was low. No data for the other two groups.
Hanks *et al.* 1991	ATCC 35405	Effect of *T. denticola* sonic extract on healthy human peripheral neutrophil chemotaxis in serum free medium.			*T. denticola* extract was not chemo-attractive to human PMN
Schenkein & Berry 1991	TD2, FM, (VPI & SU)			*T. denticola* binding to ^{125}I-C3 in diluted pool serum (1:3) in Mg-EGTA, 37°C, 30 min.	Both classical and alternative complement activated by *T. denticola*. *T. denticola* could not degrade hemolytically active C3 in 15 min, 37°C.
Yotis *et al.* 1995	ATCC 33521, ATCC 35404, ATCC 35405; 11, (University of Chicago)			Western blot detection of human serum antibodies against *T. denticola* LPS-like antigen: Periodontally healthy children, 1-11 yr., n=7; 3-6 month old infant, n=6; gingivitis adult, 21-27 y., n=8; adult periodontitis, n=13.	No antibody reaction against *T. denticola* LPS-like antigen in healthy subjects. Sera of periodontitis adults had antibody reaction against two 8-14 kDa LPS-like bands of *T. denticola*. Sera of gingivitis patients were weakly reactive.

Table 4.4 cont'd. Studies investigating *Treponema denticola* modulation of host defense system.

Author/Year	*Treponema denticola* Strain	Cellular Defense Studied		Humoral Defense Studied	Results Observed
		PMN	Lymphocytes		
Takahashi *et al.* 1996	ATCC 33521, ATCC 35405		*T. denticola* outer membrane preparation on BALB/c mouse splenocytes mitogenesis, or polyclonal B-cell activation at 37°C, 72 h., measured by MTT assay and hemolytic plaque-forming cell technique respectively.		*T. denticola* outer membrane preparation induced mouse splenocyte mitogenesis, polyclonal B-cell activation and adjuvant activities.

Table 4.5 Studies on *Treponema denticola* interaction with periodontopathogens and other members of subgingival plaque flora.

Author/Year	*Treponema denticola* strain used	Interacting bacterial species	Assay	Results Observed
ter Steeg & Hoeven 1990	Ny375, (University of Nijmegen)	*Prevotella intermedia* Ny365 *Bifidobacterium adolescentis* Ny369 *Eubacterium alactolyticum* PS406 *E. brachy* Ny402 *E. lentum* PS538 *E. nodatum* Ny393 *E. saburream* Ny396 *Fusobacterium nucleatum* Ny373 *Lactobacillus catenaforme* Ny374 *Peptostreptococcus anaerobius* Ny407 *P. micros* Ny370 *Propionibacterium acnes* Ny371 *Streptococcus mitis* PS301 *Veillonella parvula* Ny368 or culture filtrates (medium: ECBA or PY + 10% inactivated human serum).	Growth by counting and O.D. measurement. pH and redox-potential also measured.	*P. intermedia*, *E. nodatum*, *V. parvula*, *F. nucleatum* or their culture filtrates enhanced growth of *T. denticola* in PY medium with serum. Continuous co-culture of *T. denticola*, *F. nucleatum* and *P. intermedia* enhanced growth of the spirochete.
Grenier 1992a	ATCC 35405	*Porphyromonas gingivalis* ATCC 33277, FDC 381, HW11D-5 *P. asaccharolytica* ATCC 25260, BM4 *P. endodontalis* ATCC 35406 *Prevotella intermedia* 5W2 *P. melaninogenica* ATCC 25845 *P. loescheii* ATCC 15930 *P. denticola* ATCC 33185 *Bacteroides levii* ATCC 29147.	Visual coaggregation assay.	Only *P. gingivalis* form large coaggregate with *T. denticola*. Heating both bacteria was required to abolish coaggregation, bimodal coaggregation between the 2 bacteria suggested.
Grenier 1992b	ATCC 35405	*Porphyromonas gingivalis* ATCC 33277.	Co-culture growth in BHI-K. Or growth in culture filtrate of interacting bacteria.	Mutual symbiotic enhancement of growth of *P. gingivalis* and *T. denticola*. *P. gingivalis* provided isobutyric acid for *T. denticola* growth.

Table 4.5 cont'd. Studies on *Treponema denticola* interaction with periodontopathogens and other members of subgingival plaque flora.

Author/Year	*Treponema denticola* strain used	Interacting bacterial species	Assay	Results Observed
Nilius *et al.* 1993	ATCC 33520, ATCC 33521, ATCC 35405; D65BR1, N39, IPP, (VPI & SU)	*Porphyromonas gingivalis* ATCC 33277. Culture filtrates. (medium: Wilkins-Chalgren broth plus hemin and menadione) and culture filtrate extracts.	Growth by O.D. measurement.	*P. gingivalis* heat liable proteinaceous factor(s) > 50 kDa enhanced growth of *T. denticola*. Fatty acid from *P. gingivalis* culture filtrate had no effect.
Onagawa *et al.* 1994	ATCC 33520, ATCC 33521, ATCC 35404, ATCC 35405; MI-2, MAK-1, HAR-2, TAK-3, MIY-4, NOM-5, (Tokyo Dental College)	*Porphyromonas gingivalis* FDC 381, ATCC 33277; 16-1, Tokyo Dental College.	Visual coaggregation assay and phase-contrast microscopy.	Except strain MIY-4, all *T. denticola* coaggregated with *P. gingivalis*. Cells and extracted hemagglutinin, heated *P. gingivalis* FDC 381 lost coaggregation ability with ATCC 33520. No significant effect on coaggregation by serum, saliva, sugars, amino acids or EDTA.
Kolenbrander *et al.* 1995	ATCC 33520, ATCC 33521, ATCC 35404, ATCC 35405; N16B1, N17A1, (VPI & SU); GM-1, MS25, (UTHSC-SA)	*Fusobacterium nucleatum* subsp. *nucleatum* PK 1594; *F. nucleatum* subsp. *fusiforme* NCTC 11326; *F. nucleatum* subsp. *polymorphum* PK 1909, ATCC 10953; *F. nucleatum* subsp. *vincentii* ATCC 49256; *F. periodontium* ATCC 33693; *F. simiae* ATCC 33568; *F. ulcerans* ATCC 49185 (skin); *F. mortiferum* ATCC 25557 (max. abscess).	Visual coaggregation assay + reversal of coaggregation by sugars or L-arginine. Inhibition of coaggregation by heating, protease treatment.	*T. denticola* coaggregate with oral *Fusobacterium* strains. Coaggregation could be reversed by d-galactosamine or lactose but not by L-arginine.
Grenier 1996a	ATCC 35405	Subgingival bacterial isolates from periodontal healthy individuals.	Plaque bacteria on *T. denticola* spread on NOS plate – identification of isolates inhibiting growth of *T. denticola*.	*Staphylococcus aureus* (produced bacteriocin like substance) and *Streptococcus mutans* (produced lactic acid) isolates found to be inhibitory to *T. denticola*. Isolates of *S. aureus* and *S. mutans* were also inhibitory to *Porphyromonas gingivalis* and *Prevotella intermedia*.

Table 4.5 cont'd. Studies on *Treponema denticola* interaction with periodontopathogens and other members of subgingival plaque flora.

Author/Year	*Treponema denticola* strain used	Interacting bacterial species	Assay	Results Observed
Yao *et al.* 1996	GM-1, (UTHSC-SA)	*Bacteroides forsythus* 9610 *Porphyromonas gingivalis* ATCC 33277 *Streptococcus crista* CC5A.	^{3}H-TdR labeled *P. gingivalis* or *S. crista*; ^{35}S-methionine labeled *T. denticola*. Quantified nitrocellulose blot assay in 0.1% Tween 20-KCl, 2h, 20°C.	*T. denticola* adhere to all bacteria tested, demonstrating saturation kinetics. Treponeme bound best to *B. forsythus* and *P. gingivalis*. *T. denticola-B. forsythus* binding pair was only affected by heating either of the binding pair. *T. denticola-P. gingivalis* binding pair was affected by rhamnose, fucose, arginine, diluted serum or saliva and heating both binding pair. *P. gingivalis* fimbriae were not involved. *T. denticola-S. crista* binding pair was affected by D-GluNAc, diluted saliva, and heating of *S. criata*.

Table 4.6 Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Nitzan *et al.* 1978	Ichelson 2, Ichelson 3, N-39, TD2, Ambigua, MRB, FM, TRRD, IPP, ST-10, T-32A, Miller, (VPI & SU)	Fibrinolytic activities by i) fibrin-agar plate assay, ii) 2 fibrin plate assays.	Column semi-purified culture supernatant.	All strains but ST-10 possessed fibrinolytic activity. *T. denticola* fibrinolysin of approx. 1,000 kDa identified. Heating to 75°C, 10 min or pH 5.5-9.5 did not affect fibrinolytic activities.
Fitzgerald & Gannon 1983	Unknown, (University of Minnesota)	Viscometry techniques to measure hyaluronan degradation.	Cell bound hyaluronidase activity.	*T. denticola* did not degrade hyaluronan.
Mäkinen *et al.* 1986	ATCC 35405; Al_2, Spira, Corha, H_{22}, Sim, Myr and H_1, (University of Michigan)	Hydrolysis of chromogenic collagenase substrates: PZ-PLGPA, azocoll azocasein; 2NAs: PNA, OH-P-NA, Pyr-NA, and BANA.	FPLC purified sonic extract of *T. denticola*. PNA enzyme > 100 kDa, BA*p*NA enzyme=45 kDa.	ATCC 35405 produced proline iminopeptidases. All strains had strong BANA digesting ability, however, could not digest azocoll, elastin-orcein, and serum albumin. *p*CMBA inhibit PNA enzyme activities. Purified PNA enzyme lost most activities with storage. BANA/BA*p*NA activities last at least 2 month with storage.
Ohta *et al.* 1986	ASLM	Hydrolysis of i) chromogenic substrates: BA*p*NA, SAAPNA, BAEE, N-α-*p*-tosyl-L-arginine methyl ester, N-α-*p*-tosyl-L-lysine methyl ester; ii) azocasein; iii) azocoll. Other assays included BSA zymography; gelatin degradation by plate diffusion assay; TCA soluble substances release post-digestion of: heat-denatured casein, and urea-denatured hemoglobin.	FPLC purified *T. denticola* 69 kDa (SDS-PAGE) or 50 kDa (gel filtration) BANA/BA*p*NA degrading enzymes.	69 kDa BA*p*NA peptidase could not hydrolyze, Ala-, Arg-, Gly-, Ho-Pro-, Ile-, Leu-, Lys-, Met-, Phe-, Pro-, Ser, Tyr-, Try-, and Val-2Nas, nor SAAPNA, casein, hemoglobin, azocasein, azocoll, BSA, and gelatin. The 69 kDa BA*p*NA enzyme was unaffected by metal chelaters, or sulfhydryl agents while on the other hand affected by Mn^{2+}, Hg^{2+}, Co^{2+} and Zn^{2+}, DFP, and TLCK.

Table 4.6 cont'd Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Uitto *et al.* 1986	Clinical isolates, (University of McGill)	Hydrolysis of: radiolabeled soluble tendon type I collagen or insoluble lens capsule basement membrane (Type IV) collagen, 25°C, 16 h. SDS-PAGE analysis of degradation production.	Detergent soluble, sonicated, filtered, *T. denticola* extract.	*T. denticola* extract degraded type IV but not type I collagen. However, *T. denticola* extracts were able to activate latent HGF collagenase to degrade host collagen. Gelatinase and trypsin-like activity present in extract. Low elastase-like activity detected.
Mäkinen *et al.* 1987	ATCC 35405	Hydrolytic activities of PNA and 24 other 2NAs.	FPLC purified sonic extract of *T. denticola.*	BANA peptidase, and 3 PNA peptidases identified. The dominant PNA peptidase was a sulfhydryl peptidase which activities were unaffected by metal chelators.
Mikx & de Jong 1987	$L_{12}D$, (University of Pennsylvania)	Hydrolysis of FITC labeled human keratin preparation by *T. denticola* whole cell.		Keratin degradation ability demonstrated by *T. denticola.* Keratin degraded into small peptides.
Sela *et al.* 1988	ATCC 35405	Normal human PMN. Assay of LPS induced superoxide ($O_2^{\cdot}$) or H_2O_2 production, 37°C, 10 min.	Phenol extracted LPS.	PMN remained viable throughout the study period. *T. denticola* phenol extract inhibited PMN $O_2^{\cdot}$ production.
Suideo *et al.* 1988	OT2, OT3, ST1, ST2, (Sunstar Inc.)	Peptidase activities: amino (9), dipeptides (7), and oligopeptides (6), BANA, BLNA, BFNA	*T. denticola* whole cell.	*T. denticola* strongly hydrolyzed BANA, Cbz-GGANA, BVGANA.
Suzuki & Watanabe 1988	Unnamed, (University of Shizuko)	Hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside.	Column purified sonic *T. denticola* extract with β-D-galactosidase activities	Suggested *T. denticola* β-D-galactosidase might be constitutively expressed. Enzyme optimal pH at 4.5-5.5. Fe (II) ion stimulated enzyme activity while thioglycolate and cysteine were inhibitory.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Uitto *et al.* 1988a	ATCC 33520, ATCC 35405	Degradation of i) ^{3}H-type IV collagen from mouse EHS tumor organ culture, or ii) acid solublized bovine lens capsule extract by spirochete extract at 28°C or 35°C respectively (analyzed by SDS-PAGE). Degradation of ^{14}C-type I collagen (by SDS-PAGE), ^{14}C-gelatin (by scintillation counting, ^{125}I-fibronection (by SDS-PAGE), elastin (Chromogenic substrate used), casein (agarose gel diffusion assay), BA*p*NA, SAAPNA, and SAAANA by *T. denticola* extract were also studied.	Sonicated, filtered, *T. denticola* extract.	*T. denticola* extract degraded all substrates tested except collagen type I.
Uitto *et al.* 1988b	ATCC 33520, ATCC 35405	1) hydrolysis of transferrin, α_1-antitrypsin, fibrinogen, IgG, BSA by substrate gel zymography, 2) hydrolysis of BSA, α_1-antitrypsin, transferrin, fibrinogen, IgG, IgA, gelatin and soluble type I collagen by enzymatic digestion followed by SDS-PAGE. 3) Spectrophotometrical analysis regarding digestion of synthetic chromogenic substrates: 2NAs, azocoll, azocasein, and rhemazo billiant blue elastin. Effects of various compounds on the CTLP activities were also evaluated.	Gel purified 95 kDa CTLP from ATCC 35405.	*T. denticola* CTLP hydrolyzed all proteins tested. Rhemazo brilliant blue elastin was not digested and not was BA*p*NA, TGPLNA, ATNA, GNA, PNA and MNA. The enzyme specifically digested SAAPNA. Heating at 50°C, 1 h. destroyed the activity. Reducing agents and EDTA, EGTA increased the enzyme activities. PMSF, TPCK, sulfhydryl reagents and human serum reduced CTLP enzyme activity.
Miyazawa 1989	ATCC 35405	Hydrolysis of BANA, BAGFPMNA, Cbz-GAMNA and Cbz-PAGPMNA.	Whole *T. denticola*.	All substrates hydrolyzed by *T. denticola*.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Siboo *et al.* 1989	ATCC 33520, ATCC 35404, ATCC 35405	PLC activity assay using chromogenic substrate: NPPC. PLC activity in spent media, cytosol fractions and membrane fractions were tested. Inhibition by EDTA was also recorded.	PMSF and TPCK treated protein from i) spent medium (filtered); ii) sonicated extract: supernatant = cytosol fraction (filtered); resuspended pellet = membrane fraction.	*T. denticola* cytosol and spent media but not membrane fraction, contained PLC activity. PLC activity inhibited by EDTA.
Grenier *et al.* 1990	ATCC 35405	Reconstituted basement membrane (Matrigel), type IV collagen, laminin and fibronectin degradation.	Gel purified *T. denticola* 95 kDa CTLP.	95 kDa CTLP was membrane bound. CTLP completely degraded fibronectin, laminin, partially degraded type IV collagen and Matrigel.
Kurimoto *et al.* 1990	Unknown	Effect of *T. denticola* LPS material on human PMN lysosomal enzyme release. *Limulus* amoebocyte lysate clotting activity and systemic effect of *T. denticola* LPS like material to rabbit were also studied.	Crude *T. denticola* LPS extract by phenol/H_2O or phenol/chloroform/ petroleum-ether.	*Limulus* amoebocyte lysate clotting activity of *T. denticola* LPS extract was relatively weak. *T. denticola* LPS extract effect on PMN lysosomal enzyme release was inconclusive. *T. denticola* LPS extract was pyrogenic to rabbits.
Mäkinen *et al.* 1990a	ATCC 35405; Myr, Sim, Corha, H, Spira, AI_2, H_{22}, unnamed *T. denticola* isolates: n=22, (University of Michigan)	Hydrolysis of chromogenic collagen substrates: PZ-PLGPA, azocoll, azocasein, elastin-orcein; 2NAs: BANA, BA*p*NA; and FALGPA.	FPLC partially purified sonic extract of *T. denticola*.	Semi-purified *T. denticola* enzyme could not digest azocasein, serum albumin, nor 2NAs tested. The PZ-PLGPA enzyme was inhibited by EDTA, *p*CMBA and PA. SH group did not affect the enzyme activities.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Mäkinen *et al.* 1990b	ASLM, (University of Michigan)	Detection of BA*p*NA/BANA digestion activities. Study of inactivation of enzyme activities by carboxyl-group reagent.	50-65 kDa benzoylarginine peptidase (Same as OPase in ATCC 35405?).	The benzoylarginine peptidase was irreversibly inactivated by EEDQ, WRK and EDC, which substantiated the possibility of a reactive carbonyl group being in the enzyme. This was a character shared by serine proteases including trypsin.
Norton Hughes & Yotis 1990	ATCC 33520	Analysis of acid phosphatase activity using pNPP as substrate, various pH, 60 min, 37°C. Inactivation of acid phosphatase by diazo-NDS also tested.	Acid phosphatase from i) French-pressed cell extract; ii) membrane, iii) cytoplasmic, and iv) osmotic shock fluid.	Optimal pH for *T. denticola* acid phosphatase were at 4.8 & 6.2. F^- ion affect the acid phosphatase activities. Acid phosphatase might be periplasmic located.
Yoshida *et al.* 1990	ATCC 35405	Hydrolysis of PZ-PLGPA, azocoll, azocasein, azoalbumin, BANA. Effect of pH, temperature, reducing agent, cations, EDTA, protease inhibitors.	Detergent extract of *T. denticola* protein.	*T. denticola* detergent extract could digest PZ-PLGPA, could not hydrolyze azocasein. DTT, glutathione, H_2O_2 and EDTA inhibited the PZ-PLGPA-digesting activity. TPLK, TLCK, and anti-trypsin did not affect the activity.
Chan *et al.* 1991	ATCC 35404	Analysis of PLC activity by hydrolysis of chromogenic substrate: NPPC microassay.	Affinity purified from culture supernatant a 60 kDa PLC using L-α-phosphatidylcholine (α-lecithin) column or HIAC.	The *T. denticola* PLC was of pI 5.5.
Mikx 1991	ATCC 33520; Ny375, Ny576, B11, B12, (University of Nijmegen)	API ZYM assay for peptidase, glycosidase and esterase activities of whole *T. denticola*.		Glycosidase activities: α-L-fucosidase; peptidase activities: +; esterase activities: +.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Miyamoto *et al.* 1991	Johnson, (Nihon Dental University)	Periodontitis patient serum antibodies against recombinant *T. denticola* 53 kDa lipoprotein detected by western immunoblotting: AP, n=6; healthy, n=2.	Recombinant *T. denticola* 53 kDa lipoprotein.	All AP patient samples were highly reactive to sonic extract of *T. denticola*. 1/6 patient sera reacted with the 53 kDa lipoprotein band. None of the healthy subject sera reacted with that protein.
Suzuki & Watanabe 1991	ATCC 35405	PLC activity assay using chromogenic substrate: NPPC. PLC activity in spent medium, outer sheath preparation, and outer sheath sonic extract. Inhibition by cation, EDTA, protease inhibitors also recorded.	*T. denticola* sonic extract.	*T. denticola* PLC inhibited by Cu^{2+}, EDTA and TPCK. DTT and Mn^{2+} enhanced the PLC activities.
Weinberg & Holt 1991	ATCC 35404; GM-1, MS25, SR-4, SR-5, C-1, C-2, C-3, (UTHSC-SA)	Effect of anti-*T. denticola* GM-1 64 kDa IgG or Fab fragments pretreatment of GM-1 and ATCC 35405 in their ability to adhere to HGF. ^{125}I-labeled spirochete assay.	HPLC semi-purified *T. denticola* GM-1 64 kDa major OM protein	Native major OM protein of GM-1 presented itself in oligomeric forms between 116-162 kDa. 64 kDa protein appeared to be glycoprotein. Antibodies against major OM protein of GM-1 did not cross-react with ATCC 35404 or SR-4 major OM protein. Anti-64 kDa Fab fragments inhibited GM-1 attachment to HGF by 78%. Amino acid analysis revealed a fragment of the protein 100% homologous with an integrin alpha subunit of a human leukocyte adhesion glycoprotein p150,95.
Haapasalo *et al.* 1992	ATCC 35405	Binding of laminin, fibronectin, biotinylated fibrinogen and gelatin onto blot of *T. denticola* proteins transferred from a SDS-PAGE gel. Binding detected by specific antibodies or streptavidin-alkaline phosphatase (modified western blot assay)	Semi-purified *T. denticola* 53 kDa and 72 kDa proteins.	Fibronectin, laminin, and fibrinogen attached to the 53 kDa surface protein and the 72 kDa protein of *T. denticola*. Pre-treating the blot with fibrinogen inhibited subsequent binding of fibronectin and laminin.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Mäkinen *et al.* 1992	ATCC 35405	Hydrolysis of azocoll, azocasein, FALGPA, 2NAs, BANA, BA*p*NA, and PZ-PLGPA.	FPLC purification of soluble sonic extract of *T. denticola*, a 62 kDa protein (FALGPA-peptidase).	62-kDa endopeptidase was a metallopeptidase with activities not dependent on-SH or serine residue. The enzyme could digest FALGPA, bradykinin and bradykinin-related peptides. The enzyme could not hydrolyze collagen types I and IV or other collagenase substrates or other proteins.
Mikx *et al.* 1992	ATCC 33520	API (extended API and API ZYM) 2-NAs (total 68) assay for *N*-acetyl arylamidase activities: amino, dipeptides, and oligopeptides. Arginine aminopeptidase or trypsin-like activity: BAMC; phenylalanine aminopeptidase or chymotrypsin-like activity: Cbz-FNA, PHAMC, SPLP; proline aminopeptidase or iminopeptidase activity: Cbz-PNA, PRAMC.	Whole *T. denticola* in continuous culture.	ATCC 33520 demonstrated to have AFPANA and FPANA (elastase like), BAMC, SPLP, and PRAMC degrading activities. BAMC degradation might be mediated by a thiol protease. The CTLP activities of ATCC 33520 whole cell, however was not similar to that of ATCC 35405, i.e. could not be inhibited by PMSF and marginally affected by $HgCl_2$. PRAMC peptidase activity blocked by $HgCl_2$.
Sorsa *et al.* 1992	Unknown, wrong ATCC no. quoted	Detection of the activation of latent human fibroblast (HGF) and human PMN interstitial collagenases (proMMP-1 and proMMP-8 respectively), 1 h incubation at 37°C using soluble type I collagen.	Preparative gel purified 95 kDa CTLP.	CTLP had no collagenolytic activity. CTLP, however, activated latent collagenases (both HGF and PMN) CTLP had no effect on TIMP-1-collagenase complex.
Uitto *et al.* 1992	Unknown strain	Induction of degradation by PLE cells of radio-labeled soluble type I and IV collagens, 28°C; reaction product analyzed by SDS-PAGE.	*T. denticola* whole cell, extract, and gel purified CTLP.	*T. denticola* whole cell and CTLP could degrade native collagen IV, denatured collagen I and synthetic collagen substrates. Triple helical collagen I could not be degraded. *T. denticola* or CTLP could induce PLE cells to secrete collagenase. *T. denticola* extract had no effect on TIMP-mediated inhibition of gingival fibroblast collagenase nor on fibroblast collagenase itself.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Egli *et al.* 1993	ATCC 35404, ATCC 35405	Black lipid bilayer model membrane assay of purified *T. denticola* protein. Morphology of *T. denticola* OM was also studied by EM.	*T. denticola* 53 kDa surface protein	*T. denticola* 53 kDa OM protein could reconstitute channels (single channel conductance of 1.8 nS in 0.1M KCl) in black lipid bilayer model membrane. Regular hexagonal array was observable under EM from *T. denticola* OM preparations.
Grenier & Uitto 1993	ATCC 35405	Toxicity of *T. denticola* peptidoglycan and LPS-like material towards PLE cells: LDH assay and MTT. Morphological effects on PLE cells were also studied by EM.	*T. denticola* peptidoglycan and LPS-like materials.	LPS-like material exerted negligible toxic effect on PLE. *T. denticola* peptidoglycan was highly toxic to PLE.
Syed *et al.* 1993	ATCC 35405; strains number 1-7, (University of Michigan)	Hydrolysis of chromogenic substrate such as 2NA: PNA, Pyr-NA, BANA, BA*p*NA, γGNA, collagenases, azocasein, PZ-PLGPA, FALGPA. Determination of SOD, catalase and peroxidase activities	Cell suspensions, cell sonic extract supernatant. Enzymes were prepared from column purified sonic extract supernatant.	*T. denticola* could grow in the medium used (under screw cap) in aerobic or anaerobic environment. Morphology or motility of cells were not affected. Most protease activities of *T. denticola* remained unchanged. Anaerobically grown cells had higher BANA activities.
Chu *et al.* 1994	ATCC 33520, ATCC 35404; GM-1, MS-25, (UTHSC-SA)	Spirochete passaged > 3x in iron depleted medium. Iron-binding protein detection by lithium dodecyl sulfate-PAGE followed by TMBZ staining. Hemin binding also measured.	*T. denticola* OM preparation by freeze-thawing or action of EDTA-Mg^{2+} then subjected to detergent extraction followed by gel purification of 44 kDa hemin-binding protein.	In hemin limitation, 4 peptides: 73 (membrane), 44, 43 (OS) and 16 (soluble cell fraction) kDa were over expression as the expense of a 54 kDa peptide. Only the 44 kDa protein bound to TMBZ, the 43 kDa protein bound weakly.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Chu & Holt 1994 and Chu *et al.* 1997	ATCC 35404	Determination of hemolysis and hemoxidation activity of sheep RBCs. α C-N and β C-S lyase activities on cystathionine or S-aminoethyl-L-cysteine with homocysteine, cysteamine formation detected by GC-MS.	46 kDa cysteine desulfhydrase-hemolysin or cystalysin. Preparative gel purified native and recombinant protein.	Hemolysis and hemoxidation activities of the cysteine or sulfhydryl requiring hemolysis were sensitive of proteinase K, pronase, DTT, and heating at 80°C. PMSF, TLCK, benzamidine had no effect on the activities. βME stimulate such activities. Cystalysin was not affected by TLCK, pronase or proteinase K. Suggested cystalysin might play important role of H_2S production *in vivo*.
Kokeguchi *et al.* 1994	ATCC 35405	Periodontitis patient (AP and RPP) sera antibodies against *T. denticola* 53 kDa protein detected by Western blotting assay: AP, n=8; RPP, n=10; healthy, n=4.	Column purified Zwittergent 3-14 extracted *T. denticola* 53 kDa surface protein.	All periodontitis patient sera samples were highly reactive to sonic extract of *T. denticola* ATCC 35405 by ELISA. 3/8 AP, and 6/10 RPP patient sera reacted strongly with 53 kDa protein. None of the healthy subject sera reacted with the 53 kDa band.
Mäkinen *et al.* 1994	ATCC 35405	Hydrolysis of Cbz-GPNA, and HBPs: bradykinin, substance P, neurotensin, angiotensins, oxytocin, vasopressin, and human endothelin fragment 22-38. EEDQ inhibition was also tested.	Triton X-100 *T. denticola* extract, FPLC purified 76 kDa POPase, EC 3.4.21.26.	The 76 kDa POPase hydrolyzed tetra-peptide ($P_3P_2P_1P'_1$) up to 3 kDa peptides. An imino acid residue in position P_1 was absolutely necessary. Cbz-GPNA hydrolysis inhibited by: insulin B-chain, human endothelin-1, neuropeptide Y, substance P, T-kinin neurotensin, and bradykinin. POPase was a serine endopeptidase. Activities did not affect by metal ions. POPase suggested to be outer membrane or periplasmic bound. *In vivo* substrates of POPase suggested to be natural, proline containing HBPs.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Rosen *et al.* 1994	ATCC 33520, ATCC 35404; GM-1, (UTHSC-SA)	Substrate gel zymography. Proteins used: Collagen type IV, fibrinogen, albumin.	Filtered (0.2 μm) culture supernatant. Triton X-114 crude *T. denticola* cells extract. *T. denticola* crude OM by freeze-thawing.	Proteolytic enzymes (fibrinolytic) of size ranging from 91-228 kDa. Protease enriched by detergent extraction and might associate with outer membrane. pH optimal at 7-8. Protease activity could be blocked by sulfhydryl group reagents such as *p*CMBA, and $HgCl_2$. Activity also blocked by PMSF and $ZnCl_2$. The protease might be a member of *T. denticola OM* oligomer.
Mäkinen *et al.* 1995a	ATCC 35405	Detection of BA*p*NA degradation. Effect on BA*p*NA digestion by different treatment.	Triton X-100 extracted, FPLC purified, 78 kDa OPase.	OPase hydrolyzed X-Arg-*p*-nitroaniline but not protein. Enzyme activities were not cation, or-SH dependent. 78 kDa OPase was sensitive to chlorhexidine acetate. OPase belonged to serine peptidases . OPase: 50-65 kDa benzoylarginine peptidase equivalent in strain ASLM. BA*p*NA hydrolysis followed normal Michallis-Menten kinetics.
Mäkinen *et al.* 1995b	ATCC 35405	Hydrolysis of HBPs: substance P, bradykinin, Angiotensins; synthetic substrates: NSP, SAAPNA.	Combined action of purified *T. denticola* CTLP, POPase, and FALGPA-peptidase on HBPs.	Inactivated substance P could be hydrolyzed, by CTLP (with high affinity), POPase and FALGPA-peptidase; Bradykinin-by FALGPA-peptidase and POPase; Angiotensin I to Angiotensin II by CTLP then POPase 95 kDa CTLP activity was based on active seryl group, active imidazole group and active carboxyl group. CTLP not affected by metal ion. CTLP hydrolyzed SAAPNA, calf thymus histone, human plasma fibrinogen, caseins, and gelatin. Therapeutic level of CHX activated the hydrolysis of SAAPNA by *T. denticola* whole cell or CTLP. However, the converse was observed with NSP hydrolysis.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Rosen *et al.* 1995	ATCC 33520, ATCC 35404; GM-1, (UTHSC-SA)	EM visualization of *T. denticola* vesicles. Substrate gel zymography. Protein used: fibrinogen. SDS-PAGE assay of breakdown products from, fibrinogen, fibronectin or collagen IV. Chromogenic substrate hydrolysis: SAAPNA, BA*p*NA, γGNA, PNA, *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide, FALGPA. Effect of protease inhibitors: DFP, PMSF, TPCK, E-64, Cbz-Fak, Cbz-FFK, Cbz-FCK, PA, phenylboromic acid, chymostatin, and pepstatin were tested.	Filtered (0.2 μm) culture supernatant, concentrated to retrieve precipitated extracellular vesicles. Crude *T. denticola* OM prepared by freeze-thawing. Gel purified 91 kDa protease.	*T. denticola* produced 50 – 100 nm vesicles *in vitro* with protein composition similar to outer sheath of the spirochete. 4 proteolytic bands visualized in zymogram ranged 91-228 kDa. A 91 kDa protease was purified. Upon heating, it dissociated into 72, 38 and 35 kDa peptides. The 91 kDa protease was inhibited by all serine protease inhibitors tested except TLCK. Other inhibitors for which were sulfhydryl group reagents: *p*CMBA, $HgCl_2$ and $ZnCl_2$.
Sorsa *et al.* 1995	Unknown, wrong ATCC no. quoted	Detection of the activation of latent dental plaque collagenase (extract) using conventional and affinity chromatography and soluble type I collagen.	Preparative gel purified 95 kDa CTLP.	CTLP activated latent dental plaque collagenase.
Uitto *et al.* 1995	ATCC 35405	Immuno-detection of HGF and PLE cells related fibronectin after CTLP treatment. PLE cells multilayer permeability assay.	Gel purified *T. denticola* 95 kDa CTLP.	Peri-PLE/HGF cells fibronectin was degraded by CTLP. CTLP treated PLE cells multilayer showed increased permeability.
Ding *et al.* 1996	ATCC 35405	Healthy human PMN incubated with *T. denticola* membrane components, 37°C, 5-120 min. measurement of MMP-8 release versus soluble type I collagen; MMP-9 release versus gelatin zymography; elastase versus SAAPVNA; cathepsin G versus SAAPNA; NGAL versus western blotting.	Column purified 53 kDa outer membrane protein containing some CTLP. Peptidoglycan and LPS extracts also tested.	Partially-purified 53 kDa protein induced MMP-8, elastase and cathepsin G release from PMN. All membrane components triggered release of MMP-9 and NGAL from PMN. PMN remained viable throughout study period.
Fenno *et al.* 1996	ATCC 35405	Attachment assay of recombinant Msp onto immobilized laminin, fibronectin and BSA using modified ELISA assay.	Msp.	Recombinant Msp adhered to immobilized laminin and fibronectin. Attachment of *T. denticola* to immobilized laminin and fibronectin was increased by pre-treatment of substrate with rMsp.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Grenier 1996b	ATCC 35405; e', ((McGill University)	Degradation of host protease inhibitors: α-1-antitrypsin, antichymotrypsin, α_2-macroglobulin, antithrombin III, antiplasmin, cystatin C. Anaerobic activation of plasminogen, 2h. 37°C.	Gel purified *T. denticola* 95kDa CTLP.	*T. denticola* CTLP degraded completely α-1-antitrypsin, antichymotrypsin, antithrombin III, antiplasmin but only partially degraded α_2-macroglobulin and cystatin. Whole cells of both stains degraded completely antithrombin III, partially degraded the rest of host protease inhibitor tested except α-1-antitrypsin which could not be digested. *T. denticola* CTLP had no plasminogen activation activity.
Ishihara *et al.* 1996	ATCC 35405	Hydrolysis of i) natural substrates: human plasma fibronectin, human plasma α-1-antitrypsin, human IgG, human placenta collagen type IV; ii) chromogenic substrates: SAAPNA, SAAPLNA + 11 other 2NAs. Effect of protease inhibitors also tested.	Column purified *T. denticola* CTLP, doublet of 98 and 100 kDa (dentilisin).	CTLP doublet became 3 peptides (38, 43 and 72 kDa) after subjected to boiling. Purified CTLP hydrolyzed all natural substrate tested to different degree. SAAPNA activity of CTLP inhibited by PMSF, DFF, PMBS; partially affected by EDTA, $ZnCl_2$ and soybean trypsin inhibitor. 72 kDa-CTLP fragment could be located on *T. denticola* surface.
Leung *et al.* 1996	ATCC 35405	Microscopic and modified ELISA assays for *T. denticola* attachment to PLE cells monolayer. Effects of i) serum proteins: BSA, fibrinogen and fibronectin; ii) specific antibodies: anti-*T. denticola*-IgG, anti-95 kDa CTLP IgG; iii) chemical or physical pre-treatment: PMSF, *p*CMBA, cysteine, mixed glycosidase, proteinase K, heating and low pH.	*T. denticola* whole cell and purified 95 kDa CTLP.	*T. denticola* attached to PLE cells in a selective fashion. FBS and fibrinogen inhibited *T. denticola* binding to PLE cells while BSA and fibronectin did not. Proteinase K, 60°C, pH 3.2 pre-treatment of *T. denticola* inhibited the attachment of the spirochetes to PLE cells so as PMSF, *p*CMBA, and specific antibodies against CTLP. The CTLP might be involved in binding of *T. denticola* to epithelial cells.
Mäkinen *et al.* 1996	ATCC 35405	PNA digestion.	FPLC purified 30.4 kDa PIPases (EC 3.4.11.5). Likely to be tetrameric *in vivo*.	30.4 kDa PIPase (Pro-Y) could not degrade Pro-Asp nor tripeptides except Pro-Gly-Gly. Replacing 'Pro'-Y with other amino acid resulted in no hydrolysis. Diethylpyrocarbonate inactivated the proposed outer membrane bound enzyme.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Mathers *et al.* 1996	ATCC 35405	Patch clamp assay. Binding of Msp complex to HeLa cell (membrane) proteins detected by immunoprecipitation.	Semi-purified detergent extract of *T. denticola*-Msp complex.	Msp complex bound to a 65 kDa cell surface protein and a 96 kDa cytoplasmic protein of HeLa cell. The Msp complex depolarized and increased the conductance of the HeLa cell membrane. Short-lived channel with a slope conductance of 0.4 nS in physiological normal saline was detected.
Schade *et al.* 1996	ATCC 35405; D39DPI, (VPI & SU)	Protease assays using fluorescence polarization, substrate: BODIPY-α-casein.	*T. denticola* whole cell.	*T. denticola* degraded BODIPY-Z-casein.
Scott *et al.* 1996	ATCC 35405	Hydrolysis of hyaluronan, chondroitin-4-sulphate and chondroitin-6-sulphate, 3 h. at 37°C. Inhibition by Cd, Hg, Cu and Mn ions, EDTA, SDS, *p*CMBA, PMSF, TPCK, soybean trypsin inhibitor, DTT, glutathione and L-cysteine were also tested. The effect of gold sodium thiomalate, rabbit anti-*A. mellifera*-venom antibodies were also tested.	*T. denticola* culture supernatant hyaluronidase or chondroitinase activities and affinity column purified 59 kDa HGase (EC 3.2.1.35).	*T. denticola* produce extracellular HGase. No effect of the HGase activities by the inhibitor tested except gold sodium thiomalate and anti-bee-venom antibodies. Anti-bee-venom (including anti-bee-Hase) cross react with *T. denticola* HGase and were used to demonstrate the outer membrane location of *T. denticola* HGase.
Mäkinen & Mäkinen 1997	ATCC 35405	Hydrolysis of GNA.	Triton X-100 *T. denticola* extract, FPLC purified 26 kDa GGT, EC 2.3.2.2., a metabolite transporting agent.	Native GGT existed as a 213 kDa oligomer and may aggregate with carbohydrate. The GGT activities increased with presence of glutathione or thiol group. GGT activities not affected by metal ions. Proposed GGT to be on outer membrane of *T. denticola*.

Table 4.6 cont'd. Studies investigating virulence or pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Assay	Virulence factor(s) Studied	Remarks
Sela *et al.* 1997	ATCC 35404; GM-1, (UTHSC-SA)	Healthy human PMN. PMN LDCL assayed after challenge by dLPP. Lysozyme activities, βG, LDH were also measured.	Triton X-114 extraction of *T. denticola*; detergent phase retrieved; protein ppt. by cold acetone at –20°C; delipidated ⇒ dLPP.	dLPP appeared to be similar to crude *T. denticola* detergent extract. 53 kDa protein and its oligomeric native forms were lipoprotein. dLPP induced LDCL. dLPP induced lysozyme release from PMN, but not βG. A 14 kDa *T. denticola* compound had no effect on PMN.
Fenno *et al.* 1998a	ATCC 35405	Adherence and cytotoxic effects of CTLP, Msp and rMsp on PLE cells. Hemolytic activity of Msp and rMsp. Pore forming activity of rMsp in model membrane (black lipid bilayer assay), and epitheloid (HeLa) cell membrane (patch clamp).	Gel purified Msp, rMsp, and CTLP.	Msp, rMsp and CTLP bound to FPLE cells. Specific antibodies blocked adhesion of Msp while adhesion of CTLP was partially blocked by combined serine protease inhibitors and specific antibodies. Msp and CTLP were cytotoxic to PLE cells. The cytotoxic activities could be inhibited by the same treatments that inhibited adherence. Msp, rMsp hemolysed human erythrocytes. CTLP did not lyse erythrocytes. Anti-rMsp IgG reduced Msp and rMsp hemolytic activities. Mean single channel conductance of rMsp was 10.3 nS at 10 mV, 1 M KCl. rMsp could generate large conductance channel on HeLa cell.

Table 4.7 Studies genetically identifying and characterizing virulence/pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Virulence factor identify	Gene(s) Reported	Remarks
Que & Kuramitsu 1990	ATCC 35405	Protein with chymotrypsin-like activities.	*prtA* – unknown gene size or sequence.	Recombinant pXQ 27.2 subclone (containing *prtA* gene) product revealed one SAAPNA hydrolyzing (67 kDa) and one PZ-PLGPA degrading enzyme (36 kDa). Anti-native CTLP recognized the 67 kDa protein only. The column purified recombinant 67 kDa *prtA* gene product degraded type IV collagen, laminin, and fibronectin but not collagen type I.
MacDougall *et al.* 1991	ATCC 33520	Proteases: including a 95 kDa protein with chymotrypsin-like activities.	Nil.	Recombinant *Hind* III fragment of λTdT20 subclone in pJDC9 *E. coli* expressed "Trypsin like" activity. *E. coli* infected by λtDC12 expressed a protein with protease activity (degrading albumin) of approx. 95 kDa.
Miyamoto *et al.* 1991	Johnson, (Nihon Dental University)	Immunogenic lipo-protein of *T. denticola*.	*TdpA*-1416 bp, 472 amino acids, MW 53 kDa. GenBank: D00598.	The *tdpA* gene is conserved in *T. denticola* strains ATCC 33520, ATCC 35404, ATCC 35405, OKA-3, S-2 and treponeme strains E-21, E-30, and Y-181.
Ishihara *et al.* 1992	ATCC 33520	Aspartate carbamyl-transferase.	ATCase gene-1425 bp, 475 amino acids, MW 54.9 kDa. GenBank: D10052.	Recombinant TD12 subclone (containing ATCase gene) product revealed the presence of an ATCase protein. Potential Shine-Dalgarno sequence 7-11 bp up stream. Potential –10 and –35 promoter sequences identified. *T. denticola* ATCase gene product showed 33.8% homology with that of *E. coli*.
Arakawa & Kuramitsu 1994	ATCC 35405	Protein with chymotrypsin-like activities.	*prtB*-822 bp, 274 amino acids, MW 30.4 kDa GenBank: L25603.	Recombinant PrtB protease could be inhibited by PMSF, DFP, TPCK but not TLCK. Zn, EDTA also inhibited the protease. Recombinant PrtB protein digested BSA, human serum album, casein and hemolyzed sheep erythrocytes. It could not degrade collagen types I, IV, fibronectin, fibrinogen, gelatin, IgG and transferrin.
Ishihara & Kuramitsu 1995	ATCC 35405	Neutral phosphatase.	*phoN*-1026 bp, 342 amino acid, MW 37.9 kDa GenBank: L25421.	PhoN phosphatase was membrane associated. Potential Shine-Dalgarno sequence 8-12 bp up-stream.

Table 4.7 cont'd. Studies genetically identifying and characterizing virulence/pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Virulence factor identify	Gene(s) Reported	Remarks
Chu *et al.* 1995, 1997	ATCC 35404	Hemolysin or cystalysin or cysteine desulfhydrase.	*hly*-1197 bp, 399 amino acid, MW 46.2 kDa.	Hly in transformants (*E. coli*) produced hemolysis and hemoxidation. *hly* deduced amino acid sequence is homologous to pyridoxal-phosphate-dependent aminotransferase. Postulated that the cystalysin may produce H_2S *in vivo*.
Heinzerling *et al.* 1995, 1997	ATCC 35405	Endoflagellar gene operon.	*flgB* operon: $flgB_T$, $flgC_T$, $fliE_T$, $fliF_T$, $fliG_T$, $fliH_T$, $fliI_T$, $fliJ_T$, $flgB_T$ – 420 bp, 140 amino acids, MW 15.9 kDa. $flgC_T$-453 bp, 151 amino acids, MW 16.5 kDa. $fliE_T$- 366 bp, 122 amino acid, MW 13.3 kDa. $fliF_T$- MW 65.1 kDa. $fliG_T$-MW 39 kDa, GenBank U15609 $fliH_T$-996 bp, 332 amino acid, MW 75.0 kDa. $fliI_T$-1413 bp, 471 amino acid, MW 51.3 kDa. $fliJ_T$-459 bp, 153 amino acid.	Operonic arrangement of seven genes: 4 flagellar basal body genes-homologous to *Salmonella typhimurium*, *flgB*, *flgC*, *fliE*, *fliF*; a flagellar switch component-homologous to *S. typhimurium fliG*; 2 putative flagellar export proteins genes-homologous *to S. typhimurium fliH*, *fliI* and One gene resembling *S. typhimurium fliJ* was also identified (*fliJ* function unknown). Evidence for cotranscription was identified. Conservation of flagella genes between *T. denticola* and other gram-negative bacteria was observed. A $gufA_T$ gene (protein trafficking and degradation) located 5' to the first putative flagellar gene was also identified. Expression of $fliG_T$ was capable of affecting flagellar function in other organisms.
Fenno *et al.* 1996, 1997, 1998a	ATCC 35405; ATCC 33520, ATCC 35404, OTK, (University of Minnesota)	Msp.	*msp*-1629 bp, 543 amino acids, MW 58.2 kDa. GenBank: U29399, U44255, U44256.	The Msp peptide had a signal sequence with a potential cleavage site for signal peptidase I. Potential –35 and –10 promoter regions and ribosome binding site were identified. Transcription termination sequence of *msp* was also reported. *msp* locus was conserved in all strains tested. High sequence homology in the flanking regions and signal peptide coding regions of the *msp* genes from various *T. denticola* strains tested. However, significant differences between regions encoding mature peptide could be identified in strains ATCC 33520 and OTK. The Msp peptide was reported to be homologous to deduced products of a number of open reading frames in the *T. pallidum* genome.

Table 4.7 cont'd. Studies genetically identifying and characterizing virulence/pathogenic factors of *Treponema denticola*.

Author/Year	*Treponema denticola* strain used	Virulence factor identify	Gene(s) Reported	Remarks
Ishihara *et al.* 1996	ATCC 35405	Dentilisin, 72 kDa fragment of 95 kDa CTLP.	*prtP*-2166 bp, 722 amino acids, MW 77.5 kDa; 43 kDa gene-1065 bp, 355 amino acids; MW 38.9 kDa.	The *prtP* gene product appeared to be composed of a signal peptide region (26 amino acids) followed by a prosequence and the mature protein domain. The deduced amino acid sequence suggested that the 72-kDa protein is a CTLP. Nature of the 43 kDa protein was not reported.43 kDa gene located upstream of *prtP* gene. Potential Shine-Dalgarno sequence 11-15 bp (*prtP*) or 14-19 bp (43 kDa gene) up-stream. Potential –10 and –35 promoter sequences identified. *prtP* gene product shared some homologies with subtilisin of *Bacillus subtilis*.
Kataoka *et al.* 1997	ATCC 35405	MCP	*dmcA*, MW57 kDa. GenBank: U33210	The *dmcA* gene located upstream of *prtB*. The rMCP cross-reacted with anti-*E. coli* MCP serum, showing marked homology from corresponding protein of *E. coli*. A *dmcA* mutant (HL0501) was constructed which found to be defective in ability to migrate towards nutrients. Protein methylation in *dmcA* mutant was also altered.
Fenno *et al.* 1998b	ATCC 35405	Msp, CTLP	Allelic replacement mutation of *T. denticola* at: i) *msp* gene by pkx 241-HE, or pkx241-PE; ii) CTLP locus by pCTLP-KE.	Isogenic mutants in the genetic loci encoding Msp and CTLP were constructed. All mutant strains lacked the Msp hexagonal array OM structure. CTLP mutant (strain CKE) produced no CTLP and had aberrant Msp expression. m*sp* mutant at the centrally located *Hin*dIII site (strain MHE) lacked Msp and produced increased levels of CTLP, while *msp* mutant at the *Pst*I site near the 3' end (strain MPE) produced small amounts of a truncated Msp, but produced no CTLP. Structural interactions (in transport and assembly) between Msp and CTLP was proposed.

Table 4.8 A summary of the interactions that occurred between *T. denticola* and PLE cells with an attempt to relate and hypothesize the *T. denticola* induced cytopathic or cytotoxic effects.

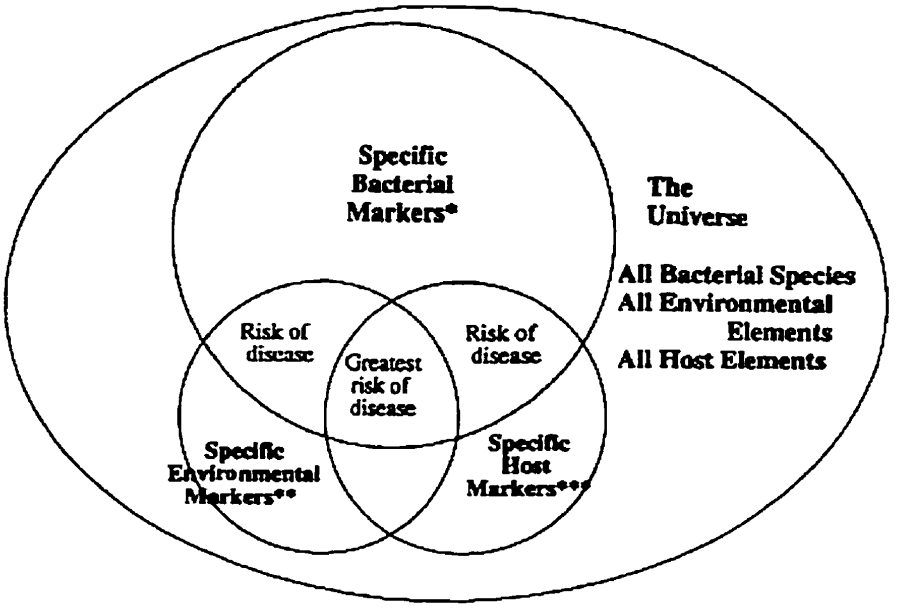
Step	Stage of *T. denticola* infection	PLE cell cellular response	Consequence	Suggested pathogenic mechanisms
(1)	Initial exposure of *T. denticola* to JE or PLE cells monolayer.	"selective binding" of *T. denticola* to PLE cells.	Attachment of *T. denticola* onto PLE cells that possess "special receptor(s): e.g. fibronectin on PLE cells, molecules that resemble the HeLa cell 65 kDa membrane protein.	*T. denticola* motility (Heinzerling *et al.* 1995, 1997); chemotactic ability (Kataoka *et al.* 1997); molecules that could mediate attachment such as CTLP, Msp or other proteinaous factors (Leung *et al.* 1996, Fenno *et al.* 1998); *T. denticola* CTLP might uncover cryptic binding sites for attachment mediated by CTLP or Msp etc.
(2)	Induction of cytopathic effect on PLE cells and preparation for more attachment to PLE cells.	i) In affected PLE cells; reduction in quantity of cellular cytoskeleton and altered location of actin filaments; reduction in cell size and start of the process of losing confluency or integrity of PLE cell monolayer.	Some PLE cells loose volume control ability, contraction and blebbing of affected cells is evident. Affected cells also depolarized.	*T. denticola* 59 kDa HGase (Scott *et al.* 1996) could degrade the hyaluronan covering the PLE cell monolayer. Upset of PLE cell volume and electrolyte regulation as per effect of *T. denticola* Msp forming pores on PLE cell membrane (Mathers *et al.* 1996). Direct action of CTLP on PLE cell attachment apparatus or molecules (Uitto *et al.* 1995), plus activation of latent MMP (Sorsa *et al.* 1992, 1995, Ding *et al.* 1996), resulting in autodegradation of attachment molecules in substratum (Ellen *et al.* 1994b). *T. denticola* 60 kDa PLC (Chan *et al.* 1991) might also affect stability of PLE cell membranes, producing harmful effects. Further increase in *T. denticola* attachemnt as in step (1).
		ii) Unaffected PLE cells migrate and expand to keep confluency of monolayer culture (may resemble wound healing of junctional/pocket epithelium in chronic periodontitis).	The subpopulation of spreading or migrating cells increases, these might possess more receptors for *T. denticola* binding.	
(3)	Induction of cytotoxic effects and PLE cells killing.	i) Gradual reduction in cellular viability; leaching out of LDH to culture medium; cytoplasmic changes including vacuolation, blebbing, lost of microvilli, cytoplasmic condensation and condensation of nuclear chromatin.	Apoptotic change of PLE cells, cytotoxicity or loss of PLE cell viability.	Direct cytotoxic action by *T. denticola* CTLP; cytotoxic effect of Msp or Msp complex by creating pores on PLE cells (Mathers *et al.* 1996, Fenno *et al.* 1998). Other *T. denticola* cytotoxic factors e.g. metabolic products: H_2S, short chain fatty acids; peptidoglycan from disintegrated *T. denticola*, PLC etc. may be in action too (Chan *et al.* 1991, Grenier & Uitto 1993, Chu *et al.* 1995, 1997).
		ii) Detachment of PLE cells.	Detached PLE cells with cellular structures broken down and loss of cytoplasmic integrity.	As above.

Table 4.9 A summary of postulated interactions that might occur between *T. denticola* and human periodontal tissue *in vivo*.

Step	Stage of *T. denticola* infection	Host response	Postulated consequence	Suggested pathogenic mechanisms
(1)	Initial contact or colonization of subgingival area: adhesion to established subgingival biofilm or host tissue.	Initial host inflammatory response; production of inflammatory mediators, and HBPs (Fig. 1.6 A & B). Hydrolysis of epithelium hyaluronan, hemagglutination, lysis of blood clot	Host inflammatory response stimulated and modulated.	Motility (Heinzerling *et al.* 1995, 1997); chemotactic ability (Kataoka *et al.* 1997); metabolic products: e.g. short chain fatty acids, H_2S; LPS; CTLP, POPase FAGPA-peptidase etc. (Grenier *et al.* 1990, Chu *et al.* 1995, 1997, Mäkinen & Mäkinen 1996,) modulated local level of HBPs. Attachment mediated by Msp, CTLP and maybe other *T. denticola* proteinaous factors (Leung *et al.* 1996, Fenno *et al.* 1998). Hydrolysis of hyaluronan by *T. denticola* 59 kDa HGase (Scott *et al.* 1996). *T. denticola* 60 kDa PLC (Chan *et al.* 1991), 46 kDa cystalysin (Chu *et al.* 1995, 1997) may also be involved Ability to carry along coaggregated periodontopathogens to the vicinity of periodontal pockets – such periodontopathogens may produce pathogenic effects in human (Grenier.1992a, Onagawa *et al.* 1994, Kolenbrander *et al.* 1995, Yao *et al.* 1996)
(2)	Innate host defense activation			
	- complement activation	Complement activation via classic and alternate pathways.	Localized inflammatory response.	*T. denticola* activating both classical and alternative complement pathways (Schenkein & Berry 1991).
	- protease inhibitors	Transferrin, α_1-antitrypsin, antichymotrypsin, α_2-macroglobulin etc. (Table 1.8).	Inactivate/abort host attempt in defense and control of local tissue destruction.	CTLP (Uitto *et al.* 1988b, Grenier *et al.* 1990, Grenier 1996b).
	- PMN	PMN $O_2^{\cdot}$ production inhibited. Limited or slow degranulation induced. Small amount of lysozyme release. Induced MMP-8, elastese and cathepsin G release.	Render host PMN defense ineffective. Induction of host related connective tissue breakdown.	Protease resistance treponeme OM structure. *T. denticola* LPS and partially purified 53 kDa protein may be involved (Sela *et al.* 1988, Ding *et al.* 1996). 53 kDa dLPP induced PMN LDLL and lysozyme release (Sela *et al.* 1997.

Table 4.9 cont'd. A summary of postulated interactions that might occur between *T. denticola* and human periodontal tissue *in vivo*.

Step	Stage of *T. denticola* infection	Host response	Postulated consequence	Suggested pathogenic mechanisms
(3)	Adaptive host defense activation - humoral	Increased anti-*T. denticola* Ig levels in AP subjects, however in severe periodontitis subjects, such response is minimal.	Severely impaired humoral immune response in subjects may relate to extensive tissue destruction.	Not yet identified. *T. denticola* Msp or 53 kDa protein is one of the immunogenic proteins (Haapasalo *et al.* 1992); *T. denticola* LPS is also immunogenic in diseased subjects (Yotis *et al.* 1995). *T. denticola* CTLP may degrade Ig in the vicinity of the pocket (Uitto *et al.* 1988b).
	- cellular	Inhibited human lymphocyte blastogenic response to mitogens. Polyclonal B cells activation (mouse model).	Upset of the regular adaptive cellular immune response in host.	Factors related to such action have not yet been identified. Such factor from *T. denticola* is heat liable.
(4)	Localized damage/destruction of host cells and living tissues - pocket epithelial cells - extracellular matrix molecules - hemolysis	Development of chronic inflammatory state: by attempted wound healing from host to repair epithelial/connective tissue breakdown.	Enabling a favorable habitat for *T. denticola* survival; *T. denticola* invasion is rare.	CTLP, POPase, PSPase etc. that modulate host inflammatory response (Mäkinen *et al.* 1994). CTLP, Msp, 46 kDa hemolysin etc. induced direct cellular/tissue damage and/or activation of latent plaque/tissue/PMN collagenase, MMP etc. (Sorsa *et al.* 1992, 1995, Chu *et al.* 1995, 1997, Ding *et al.* 1996, Fenno *et al.* 1998). Periodontopathogens co-adhering to *T. denticola* might directly or indirectly create tissue destruction.
(5)	Invasion and/or induction of alveolar bone resorption - indirect degradation of periodontal fibres	Degradation of periodontal fibres, activation of local osteoclastic activities probably due to transient alteration of host defense that favors tissue destruction.	Loss of periodontal attachment and bone support; occasional occurrence.	As above. *T. denticola* LPS maybe responsible for triggering bone resorption (Gopalsami *et al.* 1993, Table 4.3).



***Bacteria**
Periodontopathogens

****Environmental markers**
Smoking
Poor oral hygiene
Initial probing depth and attachment loss
Plaque retaining factors
Educational attainment
Professional dental visits
Subgingival temperature
Other resident bacteria

*****Host markers**
Age
Race
Number of teeth
Diabetes
Gingival crevicular fluid substances
Prostaglandin E
Beta glucuronidase
Lactate dehydrogenase
Neutral protease
PMN function

Figure 4.1 Bacterial risk assessment model for progressing periodontitis. (From Wolff et al. 1994.)

CHAPTER V Conclusions

As outlined in the review article by Fenno and McBride (1998), the prevalence of and the role as specific potential pathogens of spirochetes in certain severe periodontal conditions and their intimate contact with epithelial tissue had been studied with great interest. However, progress in characterization of spirochetes lagged in comparison to other potential periodontal pathogens, largely due to technical difficulties in isolating and culturing spirochetes from dense, microbially complex subgingival plaque. Oral spirochetes were subsequently ignored in many studies of the microbial etiology of periodontal diseases, especially in those using viable counts of potential pathogens as indicators of association with disease (Fenno & McBride 1998). *T. denticola* is by far the best characterized of the seven currently cultivable oral spirochete species. It exhibits numerous behaviors and products consistent with an important role in periodontal disease etiology. In addition to aiding in defining the etiology of periodontal disease, characterization of *T. denticola* disease-causing behaviors may enhance understanding of similar behaviors in other pathogenic spirochetes. The objective of the present study was to investigate biological activities of *T. denticola* relative to its potential virulence. The present investigation concentrated on certain highly expressed outer membrane components that mediate its adherence, proteolytic activity, and cytotoxic effects.

An *in vitro* junctional epithelial cell model was used to study the above mentioned actions and the potential virulence factors of the *T. denticola* outer membrane (OM). Study of the *T. denticola* whole cell, partially purified, or purified OM elements interaction with host eukaryotic cells revealed the following:

1. *T. denticola* could adhere to PLE cells and other eukaryotic cells in culture with varying

affinities. The spirochete – eukaryotic cell binding appeared to be the result of a complicated spirochete – host cell interaction process.

2. *T. denticola* induced cytopathic and cytotoxic changes in eukaryotic cells in culture. Cytopathic changes induced membrane disruption, vacuolation, loss of cell contacts, loss of cell size control and cytoskeletal rearrangement. Cytotoxic effects included detachment, cell lysis and apoptotic changes of PLE cells.
3. Major *T. denticola* OM protein elements, namely, chymotrypsin-like protease (CTLP) and major surface protein (Msp) were isolated for various experiments. CTLP had been well characterized previously. Msp was found to be a major oligomeric protein consisting of 53 kDa protein subunits on the OM of *T. denticola* where CTLP also co-localized. Msp appeared to constitute one of the major elements of the hexagonal structure of the spirochete that also possessed porin function on an artificial lipid bilayer.
4. *T. denticola* CTLP and Msp were involved in the attachment to and cytotoxicity towards PLE cells.
5. Purified CTLP, semi-purified Msp (or Msp complex), and purified Msp could attach to PLE (or FPLE) cells and were also found to be cytotoxic to PLE cells.
6. Msp complex bound to a putative epithelial cell (HeLa cell) surface receptor and was shown to form large conductance ion channels (0.4 nS) on the HeLa cell cytoplasmic membrane.

T. denticola in vitro infection was carried out using porcine PLE cells as a junctional epithelial cell model. In conclusion, *T. denticola* ATCC 35405 can adhere to and is cytotoxic towards epithelial cells and the adhesion and cytotoxicity are mediated by OM components including Msp and CTLP. Experiments suggested that the strong proteolytic activities of CTLP

and the pore-forming activity of Msp were responsible for the cytopathic and cytotoxic effects of the putative periodontopathogen *T. denticola*.

Bibliography

Addison, W.C. 1979. The distribution of nuclei in human odontoclasts in whole cell preparations. Arch. Oral Biol. **23**:1167-1171.

Adonogianaki, E., Moughal, N.A., Mooney, J., Stirrups, D., Kinane, D.F. 1994. Acute-phase proteins in gingival crevicular fluid during experimentally induced gingivitis. J. Periodont. Res. **29**:196-202.

Akiyama, H. 1977. Endotoxin-like substance extracted from pure cultivated oral spirochetes Shigaku **65**:688-706.

American Academy of Periodontology 1989. World Workshop in Clinical Periodontics. American Academy of Periodontology: Chicago

American Academy of Periodontology 1996. Annals of Periodontology. American Academy of Periodontology:Chicago.

American Association for Periodontology 1996. Consensus Report. Periodontal diseases: Pathogenesis and microbial factors. Ann. Periodont. **1**:926-932.

Anderson, G.S., Stern I.B. 1966. The proliferation and migration of the attachment epithelium on the cemental surface of the rat incisor. Periodontics **4**:115-123.

Arakawa, S., Kuramitsu, H.K. 1994. Cloning and sequence analysis of a chymotrypsinlike protease from *Treponema denticola*. Infect. Immun. **62**:3424-3433.

Armitage, G.C., Dickinsen, W.R., Jenderseek, R.S., Levine, S.M., Chambers, D.W. 1982. Relationship between the percentage of subgingival spirochetes and the severity of periodontal disease. J. Periodontol. **53**:550-556.

Ashimoto, A., Chen, C., Bakker, I., Slots, J. 1996. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol. Immunol. **11**:266-273.

Attström, R., van der Velden, U. (eds)1994. Consensus report of session I. *In* Lang, N.P., Karring, T. (eds), Proceedings of the first European Workshop on Periodontology. pp. 120-126. Quintessence: London.

Baehni, P.C., Song, M., McCulloch, C.A.G. , Ellen, R.P. 1992. *Treponema denticola* induces actin rearrangement and detachment of human gingival fibroblasts. Infect. Immun. **60**:3360-3368.

Bartold, P.M. 1995. Turnover in periodontal connective tissues: dynamic homeostasis of cells, collagen and ground substances. Oral Diseases **1**:238-253.

Beachey, E.H. 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325-345.

Beauséjour, A., Deslauriers, N., Grenier, D. 1997. Activation of the interleukin-1β precursor by *Treponema denticola*: a potential role in chronic inflammatory periodontal diseases. Infect. Immun. 65:3199-3202.

Benz, R. 1988. Structure and function of porins from Gram-negative bacteria. Annu. Rev. Microbiol. **42**:359-393.

Benz, R., Bauer, K. 1988. Permeation of hydrophilic molecules through the outer membrane of Gram-negative bacteria. Eur. J. Biochem **176**:1-19.

Benz, R., Janko, K., Läuger, P. 1979. Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta **551**:238-247.

Benz, R., Schmid, A., Hancock, R.E.W. 1985. Ion selectivity of gram negative bacterial porins. J. Bacteriol. **162**:722-727.

Berg, H. C. 1976. How spirochetes may swim. J. Theor. Biol. **56**:269-273.

Berkovitz, B.K.B., Moxham, B.J., Newman, H.N. (eds) 1995. The Periodontal Ligament in Health and Disease. 2nd Ed. Mosby-Wolfe: London.

Berkovitz, B.K.B., Shore, R.C. 1995. Cells of the periodontal ligament. *In* Berkovitz, B.K.B., Moxham, B.J., Newman, H.N. (eds), The Periodontal Ligament in Health and Disease. 2nd Ed. Pp. 9 – 33. Mosby-Wolfe: London.

Berrier, C., Coulombe, A., Houssin, C., Ghazi, A. 1992. Fast and slow kinetics of porin channels from *Escherichia coli* reconstituted into giant liposomes and studied by patch clamp. FEBS Lett. **306**:251-256.

Berrier, C., Coulombe, A., Houssin, C., Ghazi, A. 1993. Voltage-dependent cationic channel of *Escherichia coli*. J. Membr. Biol. **133**:119-127.

Bevilocqua, M.P., Pober, J.S., Wheller, M.E. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J. Clin. Invest. **76**:2003-2011.

Bickel, M. 1993. The role of interleukin-8 in inflammation and mechanisms of regulation. J. Periodontol. **64**(5 suppl):456-460.

Birkedal-Hansen, H. 1993. Role of cytokines and inflammatory mediators in tissue destruction. J. Periodont. Res. **28**:500-510.

Boehringer, H., Berthold, P.H., Taichman, N.S. 1986. Studies on the interaction of human neutrophils with plaque spirochetes. J. Periodontal Res. **21**:195-209.

Boehringer, H., Taichman, N.S., Shenker, B.J. 1984. Suppression of fibroblast proliferation by oral spirochetes. Infect. Immun. **45**:155-159.

Brumpt, E. 1925. Les spirochetoses, pp. 535-578. *In* Roger, G.H., Widal, F. Tessier, P.J. (eds), Noveau traite de medecine. Masson et Cie: Paris.

Brunette, D.M., Heersche, J.N.M., Purdon, A.D., Sodek, J., Moe, H.K., Assuras, J.N. 1979. *In vitro* cultural parameters and protein and prostaglandin secretion of epithelial cells derived from porcine rests of Malassez. Arch. Oral Biol. **24**:199-203.

Brunette, D.M., Melcher, A. H., Moe, H. K. 1976. Culture and the origin of epithelium-like and fibroblast-like cells from porcine periodontal ligament explants and cell suspensions. Arch. Oral. Biol. **21**:393-400.

Buechner, M., Delcour, A.H., Martinac, B., Adler, J., Kung C. 1990. Ion channel activities in the *Escherichia coli* outer membrane. Biochim. Biophys. Acta **1024**:111-121.

Byers, M.R., Mecific, K.B., Kimberly, C.L. 1987. Numerous nerves with calcitonin gene-related peptide-like immunoreactivity innervated junctional epithelium of rats. Brain Res. **419**:311-314.

Canale-Parola, E. 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. **41**:181-204.

Canale-Parola, E. 1978. Motility and chemotaxis of spirochetes. Ann. Rev. Microbiol. **32**:69-99.

Canale-Parola, E. 1984. The Spirochetes, *In* Krieg, N.R., Holt, J.G. (eds), Bergey's Manual of Systematic Bacteriology. Vol 1. pp. 38-70. Williams and Wilkins: Baltimore.

Carlsson, J. 1988. What is a periodontal pathogen? *In* Guggenheim, B. (ed.), Periodontology Today, pp. 104-111. S. Karger: Basel.

Carranza Jr., N., Riviere, G.R., Smith, K.S., Adams, D.F., Maier, T. 1997. Differential attachment of oral treponemes to monolayers of epithelial cells. J. Periodontol. **68**:1010-1018.

Chan E.C.S., Qui, Y.S., Siboo, R., Noble, P. 1995. Evidence for two distinct locomotory phenotypes of *Treponema denticola* ATCC 35405. Oral Microbiol. Immunol. **10**:122-124.

Chan, E.C.S., Siboo, I.R., Siboo, R. 1991. Purification of phospholipase C by hydrophobic interaction affinity chromatography. J. Chromatography **568**:85-92.

Chan, E.C.S., Siboo, R., Keng, T., Psarra, N., Hurley, R., Cheng, S.L., Iugovaz, I. 1993. *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolated from periodontal pockets. Int. J. Syst. Bacteriol. **43**:196-203.

Charon, N.W., Goldstein, S.F., Block, S.M., Curci, K., Ruby, J.D., Kreiling, J.A.,

Limberger, R.J. 1992a. Morphology and dynamics of protruding spirochete. J. Bacteriol. **174**:832-840.

Charon, N.W., Greenberg, E.P., Koopman, M.B.H., Limberger, R.J. 1992b. Spirochete chemotaxis, motility, and the structure of the spirochetal periplasmic flagella. Res. Microbiol. **143**:597-603.

Cheng, S.-L., Chan, E.C.S. 1983. The routine isolation, grown, and maintenance of the intermediate-size anaerobic oral spirochetes from periodontal pockets. J. Periodontal Res. **18**:362-368.

Choi, B.K., Paster, B.J., Dewhirst, F.E., Göbel, U.B. 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect. Immun. **62**:1889-1895.

Chu, L., Burgum, A., Kolodrubetzi D., Holt, S.C. 1995. The 46-kilodalton-hemolysin gene from *Treponema denticola* encodes a novel hemolysin homologous to aminotransferases. Infect. Immun. **63**:4448-4455.

Chu, L., Ebersole, J.L., Kurzban, G.P., Holt, S.C. 1997. Cystalysin, a 46-kilodalton cysteine desulfhydrase from *Treponema denticola*, with hemolytic and hemoxidative activities. Infect. Immun. **65**:3231-3238.

Chu, L., Holt, S.C. 1994. Purification and characterization of a 45 kDa hemolysin from *Treponema denticola* ATCC 35404. Microb. Pathog. **16**:197-212.

Chu, L., M. Song, Holt, S.C. 1994. Effect of iron regulation on expression and hemin-binding function of outer-sheath proteins from *Treponema denticola.* Microb. Pathog. **16**:321-335.

Cimasoni, G. 1983. The Crevicular Fluid Updated. S. Karger: Basel.

Cimasoni, G., McBride, B.C. 1987. Adherence of *Treponema denticola* to modified hydroxyapatite. J. Dent. Res. **66**:1727-1729.

Cockayne, A., Sanger, R., Ivic, A., Strugnell, R.A., MacDougall, J.H., Russel, R.R.B., Penn, C.W. 1989. Antigenic and structural analysis of *Treponema denticola.* J. Gen. Microbiol. **135**:3209-3218.

Cover, T.L., Puryear, W., Perez-Perez, G.I., Blaser, M.J. 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59**:1264-1270.

Cunningham, T.M., Walker, E.M., Miller, J.N., Lovett, M.A. 1988. Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. J. Bacteriol. **170**:5789-5796.

Dahle, U.R., Olsen I., Tronstad, L., Caugant, D.A. 1995. Population genetic analysis of oral treponemes by multilocus enzyme electrophoresis. Oral Microbiol. Immunol. **10**:265-270.

Dahle, U.R. Tronstad, L., Olsen, I. 1993. Spirochaetes in oral infections. Endod. Dent. Traumatol. **9**:87-94.

Dahle, U.R. Tronstad, L., Olsen, I. 1996. 3-hydroxy fatty acids in a lipopolysaccharide-like material from *Treponema denticola* strain FM. Endod. Dent. Traumatol. **12**:202-205.

Dahlquist, F.W. 1978. The meaning of Scatchard and Hill plots. Methods Enzymol. **48**:270-299.

Darveau, R.P., Tanner, A., Page, R.C. 1997. The microbial challenge in periodontitis. Periodontol. 2000 **14**:12-32.

Dawson, J.R., Ellen, R.P. 1990. Tip-oriented adherence of *Treponema denticola* to fibronectin. Infect. Immun. **58**:3924-3928.

Dawson, J.R. Ellen, R.P. 1994. Clustering of fibronectin adhesions toward *Treponema denticola* tips upon contact with immobilized fibronectin. Infect. Immun. **62**:2214-2221.

de Filippo, A.B., Ellen, R.P., McCulloch, C.A.G. 1995. Induction of cytoskeletal rearrangements and loss of volume regulation in epithelial cells by *Treponema denticola*. Archs. Oral Biol. **40**:199-207.

Delcour, A.H., Adler, J., Kung, C. 1991. A single-amino acid substitution alters conductance and gating of OmpC porin of *Escherichia coli*. J. Membr. Biol. **119**:267-275.

Delcour, A.H., Adler, J., Kung, C., Martinac, B. 1992. Membrane-derived oligosaccharides (MDOs) promote closing of an *E. coli* porin channel. FEBS Lett. **304**:216-220.

DiPasquale, A., Bell, P.B. 1974. The upper cell surface: its inability to support active cell movement in culture. J. Cell Biol. **62**:198-214.

DiRienzo, J.M., Cornell, S., Boehringer, H. 1991. Use of randomly cloned DNA fragments for the identification of oral spirochetes. Oral Microbiol. Immunol. **6**:88-96.

Ding, Y.-L., Uitto, V.-J., Haapasalo, M., Lounatmaae, K., Konttinen, Y.T., Salo, T., Grenier, D., Sorsa, T. 1996. Membrane components of *Treponema denticola* trigger proteinase release from human polymorphonuclear leukocytes. J. Dent. Res. **75**:1986-1993.

Dolby, A.E. 1986. The host defense system of the mouth. *In* Ivonyi, L. (ed), Immunological Aspects of Oral Diseases. pp. 1-11. MTP Press: Lancaster.

Ebersole, J.L., Kesavalu, L., Schmeider, S.L., Machen, R.L., Holt, S.C. 1995. Comparative virulence of periodontopathogens in a mouse abscess model. Oral Diseases **1**:115-128.

Egli, C., Leung, W. K., Müller, K.-H., Hancock, R.E.W., McBride, B.C. 1993. Pore-forming properties of the major 53-kilodalton surface antigen from the outer sheath of *Treponema*

denticola. Infect. Immun. **61**:1694-1699.

Ellen, R.P., Dawson, J.R., Yang, P.F. 1994a. *Treponema denticola* as a model for polar adhesion and cytopathogenicity of spirochetes. Trend. Microbiol. **2**:114-119.

Ellen, R.P., Song, M., McCulloch, C.A.G. 1994b. Degradation of endogenous plasma membrane fibronectin concomitant with *Treponema denticola* 35405 adhesion to gingival fibroblasts. Infect. Immun. **62**:3033-3037.

Embery, G., Waddington, R.J., Hall, R.C. 1995. The ground substance of the periodontal ligament. *In* Berkovitz, B.K.B., Moxham, B.J., Newman, H.N. (eds), The Periodontal Ligament in Health and Disease. 2nd Ed. Pp. 83-106. Mosby-Wolfe: London.

Engler, W.O., Ramfjord, S.P., Hiniker, J.J. 1965. Development of epithelial attachment and gingival sulcus in rhesus monkeys. J. Periodontol. **36**:44-57.

Eriksen, S., Olsnes, S., Sandvig, K., Sand, O. 1994. Diphtheria toxin at low pH depolarizes the membrane, increases the membrane conductance and induces a new type of ion channel in Vero cells. EMBO J. **13**:4433-4439.

Fenno, J.C., Hannam, P.M., Leung, W.K., Tamura, M., McBride, B.C. 1998a. Cytopathic effects of the major surface protein (Msp) and the chymotrypsin-like protease (CTLP) of *Treponema denticola*. . Infect. Immun. **66**:1869-1877.

Fenno, J.C., McBride, B.C. 1998. Virulence factors of oral treponemes. Anaerobe **4**:1-17.

Fenno, J.C., Müller, K.-H., McBride, B.C. 1996. Sequence analysis, expression, and binding activity of recombinant major outer sheath protein (Msp) of *Treponema denticola*. J. Bacteriol. **178**:2489-2497.

Fenno, J.C., Wong, G.W.K., Hannam, P.M., McBride, B.C. 1998b. Mutagenesis of outer membrane virulence determinants of the oral spirochete *Treponema denticola*. FEMS Microbiol. Lett. In press.

Fenno, J.C., Wong, G.W.K., Hannam, P.M., Müller, K.-H., Leung, W.K., McBride, B.C. 1997. Conservation of *msp*, the gene encoding the major outer membrane protein of oral *Treponema* spp. J. Bacteriol. **179**:1082-1089.

Finlay, B.B. 1990. Cell adhesion and invasion mechanisms in microbial pathogenesis. Curr. Opin. Cell Biol. **2**:815-820.

Firestone, G.L., Winguth, S.D. 1990. Immunoprecipitation of proteins. Methods Enzymol. **182**:688-700.

Fitzgerald, T.J., Gannon, E.M. 1983. Further evidence for hyaluronidase activity of *Treponema pallidum*. Can. J. Microbiol. **29**:1507-1513.

Galdiero, F., Cippollaro de L'Ero, G., Benedetto, N., Galdiero, M., Tufano, M.A. 1993. Release of cytokines induced by *Salmonella typhimurium* porins. Infect. Immun. **61**:155-161.

Garrison, S.W., Nichols, F.C. 1989. LPS elicited secretory response in monocytes: altered release of PGE_2 but not IL-1 beta in patients with adult periodontitis. J. Periodont. Res. **24**:88-95.

Gazi, M.I., Cox, S.W., Clark, D.T., Eley, B.M. 1996. A comparison of cysteine and serine proteinases in human gingival crevicular fluid with tissue, saliva and bacterial enzymes by analytical isoelectric focusing. Archs. Oral Biol. **41**:393-400.

Genco. R.J. 1990. Pathogenesis and host responses in periodontal disease. *In* Genco, R.J., Goldman, H.M., Cohen, D.W. (eds.), Contemporary Periodontics. Chapter 14, pp. 184-193. C.V. Mosby: St. Louis.

Genco, R.J., Loos, B.G. 1991. The use of genomic DNA fingerprinting in studies of the epidemiology of bacteria in periodontitis. J. Clin. Periodontol. **18**:396-405.

Ghosh, C.R., Stevens, K.M. 1967. Complement-fixing and hemagglutinating antibodies to *Treponema microdentium*. Proc. Soc. Exp. Biol. Med. **24**:559-562.

Gibbson, R.J. 1989. Bacterial adhesion to oral tissue: a model for infectious diseases. J. Dent. Res. **68**:750-760.

Gibbons, R.J. Moreno, E.C., Spinell, D.M. 1976. Model delineating the effects of a salivary pellicle on the adsorption of *Streptococcus miteor* onto hydroxyapatite. Infect. Immun. **14**:1109-1112.

Gopalsami, C., Yotis., W., Corrigan, K., Schade, S., Keene, J., Simonson, L. 1993. Effect of outer membrane of *Treponema denticola* on bone resorption. Oral Microbiol. Immunol. **8**:121-124.

Grenier, D. 1991. Characteristics of hemolytic and hemagglutinating activities of *Treponema denticola*. Oral Microbiol. Immunol. **6**:246-249.

Grenier, D. 1992a. Demonstration of a bimodal coaggregation reaction between *Porphyromonas gingivalis* and *Treponema denticola*. Oral Microbiol. Immunol. **7**:280-284.

Grenier, D. 1992b. Nutritional interactions between two suspected periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*. Infect. Immun. **60**:5298-5301.

Grenier, D. 1996a. Antagonistic effect of oral bacteria towards *Treponema denticola*. J. Clin. Microbiol. **34**:1249-1252.

Grenier, D. 1996b. Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*. Microbiol. **142**:955-961.

Grenier, D., Uitto, V.-J. 1993. Cytotoxic effect of peptidoglycan from *Treponema denticola*. Microb. Pathog. **15**:389-397.

Grenier, D., Uitto, V.-J., McBride, B.C. 1990. Cellular location of a *Treponema denticola* chymotrypsinlike protease and importance of the protease in migration through the basement membrane. Infect. Immun. **58**:347-351.

Gunsolley, J.C., Tew, J.G., Connor, T., Burmeister, J.A., Schenkein, H.A. 1991. Relationship between race and antibody reaction with periodontitis-associated bacteria. J. Periodont. Res. **26**:59-63.

Haapasalo, M., Hannam, P.M., McBride, B.C., Uitto, V.-J. 1996. Hyaluronan, a possible ligand mediating *Treponema denticola* binding to periodontal tissue. Oral Microbiol. Immunol. **11**:156-160.

Haapasalo, M., Müller, K.-H., Uitto, V.-J., Leung, W.K., McBride, B.C. 1992. Characterization, cloning, and binding properties of the major 53-kilodalton *Treponema denticola* surface antigen. Infect. Immun. **60**:2058-2065.

Haapasalo, M., Singh, U., McBride, B.C., Uitto, V.-J. 1991. Sulfhydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. Infect. Immun. **59**:4230-4237.

Haffajee, A.D., Cugini, M.A., Dibart, S., Smith, C., Kent, Jr., R.L., Socransky, S.S. 1997. The effect of SRP on the clinical and microbiological parameters of periodontal diseases. J. Clin. Periodontol. **24**:324-334.

Hager, D.A., Burgess, R.R. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. **109**:76-86.

Haines, K.A., Reibman, J., Tang, X.Y., Blake, M., Weissmann, G. 1991. Effects of protein I of *Neisseria gonorrhoeae* on neutrophil activation: generation of diacylglycerol from phosphatidylcholine via a specific phospholipase C is associated with exocytosis. J. Cell Biol. **114**:433-442.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflüg. Arch. Eur. J. Physiol. **391**:85-100.

Hancock, R.E.W. 1987. Role of porins in outer membrane permeability. J. Bacteriol. **169**:929-933.

Hancock, R.E.W., Benz, R. 1986. Demonstration and chemical modification of a specific phosphate-binding site in the phosphate-starvation-inducible outer membrane porin protein P of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **860**:699-707.

Hanks, C.T., Syed, S.A., Craig, R.G., Hartrick, J.M., van Dyke, T.E. 1991. Modeling bacterial damage to pulpal cells *in vitro*. J. Endodontics **17**:21-25.

Harper, D.S., Lamster, I.B., Celenti, R. 1989. Relationship of subgingival plaque flora to lysosomal and cytoplasmic enzyme activity in gingival crevicular fluid. J. Clin. Periodontol. **16:**164-169.

Hart, T.C., Kornman, K.S. 1997. Genetic factors in the pathogenesis of periodontitis. Periodontol. 2000 **14**:202-215.

Heinzerling, H.F., Olivares, M., Burne, R.A. 1997. Genetic and transcriptional analysis of *flgB* flagellar operon constituents in the oral spirochete *Treponema denticola* and their heterologous expression in enteric bacteria. Infect. Immun. **65**:2041-2051.

Heinzerling, H.F., Penders, J.E.C., Burne, R.A. 1995. Identification of a *fliG* homologue in *Treponema denticola*. Gene **161**:69-73.

Henderson, B., Poole, S., Wilson, M. 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60**:316-341.

Heussen, C., Dowdle, E.B. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gel containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102**:196-202.

Höök, M., McGavin, M.J., Switalski, L.M., Raja, R., Raucir, G., Lindgren, P.E. 1990. Interaction of bacteria with extracellular matrix proteins. Cell. Differ. Dev. **32**:433-438.

Hudson, M.J. 1991. The spirochaetes. *In* Duerden, B.I., Drasar, B.S. (eds), Anaerobes in Human Disease. Chapter 8, pp. 108-132. Edward Arnold: London.

Hurlen, B., Olsen, I., Lingaas, E., Midtvedt, T. 1984. Neutrophil phagocytosis of *Treponema denticola* as indicated by extracellular release of lactoferrin. Acta Path. Microbiol. Scand. Sect. B. **92**:171-173.

Ingman, T., Sorsa, T., Michaelis J., Konttinen, Y.T. 1994. Immuno-histochemical study of neutrophil- and fibroblast-type collagenases and stromelysin-1 in adult periodontitis. Scand. J. Dent. Res. **102**:342-349.

Irwin, C.R., Schor, S.L., Ferguson, M.W.J. 1994. Effects of cytokines on gingival fibroblasts *in vitro* are modulated by the extracellular matrix. J. Periodont. Res. **29**:309-317.

Ishihara, K. 1988. Immunomodulating activity of oral *Treponema* strains on proliferation of mouse lymphocytes. Shikwa Gakuho **88**:1833-1845.

Ishihara, K., Ishihara, M., Takozol, I., Okuda, K. 1992. Cloning and expression of the

aspartate carbamoyltransferase gene from *Treponema denticola*. Appl. Environ. Microbiol. **58**:3399-3403.

Ishihara, K., Miura, T., Kuramitsu, H.K., Okuda, K. 1996. Characterization of the *Treponema denticola prtP* gene encoding a prolyl-phenylalanine-specific protease (dentilisin). Infect. Immun. **64**:5178-5186.

Ishihara, K., Takazoe, I., Okuda, K. 1992. Immunomodulating activity of oral *Treponema* strains on proliferation of mouse lymphocytes. Bull. Tokyo Dent. Coll. **33**:45-50.

Ishikawa, I., Nakashima, K., Koseki, T., Nagasawa, T., Watanabe, H., Arakawa, S., Nitta, H., Nishihara, T. 1997. Induction of the immune response to periodontopathic bacteria and its role in the pathogenesis of periodontitis. Periodontol. 2000 **14**:79-111.

Jacob, E., Meiller, T.F., Nauman, R.K. 1982. Detection of elevated serum antibodies to *Treponema denticola* in humans with advanced periodontitis by an enzyme-linked immunosorbent assay. J. Periodontal Res. **17**:145-153.

Jin, L.J., Söder, P-Ö, Leung, W.K., Corbet, E.F., Samaranayake, L.P., Davies, W.I.R. 1998. Granulocyte elastase activity and PGE_2 levels in gingival crevicular fluid in relation to the presence of subgingival periodontopathogens in a group of Chinese subjects with untreated adult periodontitis. Eur. J. Oral Sci. submitted.

Joe, A., Yamamoto, A., McBride, B.C. 1993. Characterization of recombinant and native forms of a cell surface antigen of *Porphyromonas (Bacteroides) gingivalis*. Infect. Immun. **61**:3294-3303.

Johnson, R.C. 1976. Comparative spirochete physiology and cellular composition. *In* Johnson, R.C. (ed.), The Biology of the Parasitic Spirochetes pp. 39-48. Academic Press: New York.

Johnson, R.C. 1977. The Spirochetes. Ann. Rev. Microbiol **31**:89-106.

Johnson, T.C., Reinhardt, R.A., Payne, J.B., Dyer, J.K., Patil, K.D. 1997. Experimental gingivitis in periodontitis-susceptible subjects. J. Clin. Periodontol. **24**:618-625.

Kataoka, M., Li, H., Arakawa, S., Kuramitsu, H. 1997. Characterization of a methyl-accepting chemotaxis protein gene, *dmcA*, from the oral spirochete *Treponema denticola*. Infect. Immun. **65**:4011-4016.

Katsumoto, T., Naguro, T., Iino, A., Takagi, A. 1981. The effect of tannic acid on the preservation of tissue culture cells for scanning electron microscopy. J. Elect. Micro. **30**:177-182.

Kelleher, P.J., Juliano, R.L. 1984. Detection of proteases in polyacrylamide gels containing covalently bound substrates. Anal. Biochem. **136**:470-475.

Kerr, J.F.R., Gobé, G.C., Winterford, C.M., Harmon, B.V. 1995. Anatomical methods in

cell death. Methods Cell Biol. **46**:1-27.

Kesavalu, L., Walker, S.G., Holt, S.C., Crawley, R.R., Ebersole, J.L. 1997. Virulence characteristics of oral treponemes in a murine model. Infect. Immun. **65**:5096-5102.

Kessel, M., Brennan, M.J., Trus, B.L., Bischer, M.E., Steven, A.C. 1988. Naturally crystalline porin in the outer membrane of *Bordetella pertussis*. J. Mol. Biol. **203**:275-278.

Keulers, R.A.C. 1995. Attachment of *Treponema denticola*, in particular strain ATCC 33520, to epithelial cells and erythrocytes. An *in vitro* study. Ph.D. thesis, University of Nijmegen: the Netherlands.

Keulers, R.A.C., Maltha, J.C., Mikx, F.H.M., Wolters-Lutgerhorst, J.M.L. 1993a. Attachment of *Treponema denticola* strains to monolayers of epithelial cells of different origin. Oral Microbiol. Immunol. **8**:84-88.

Keulers, R.A.C., Maltha, J.C., Mikx, F.H.M., Wolters-Lutgerhorst, J.M.L. 1993b. Attachment of *Treponema denticola* strains ATCC 33520, ATCC 35405, B11 and Ny541 to a morphologically distinct population of rat palatal epithelial cells. J. Periodont. Res. **28**:274-280.

Keulers, R.A.C., Maltha, J.C., Mikx, F.H.M., Wolters-Lutgerhorst, J.M.L. 1993c. Involvement of treponemal surface-located protein and carbohydrate moieties in the attachment of *Treponema denticola* ATCC 33520 to cultured rat palatal epithelial cells. Oral Microbiol. Immunol. **8**:236-241.

Kigure, T., Saito, A., Seida, K., Yamada, S., Ishihara, K., Okuda, K. 1995. Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. J. Periodont. Res. **30**:332-341.

Kinane, D., Mooney, J., MacFarlane, T., McDonald, M. 1993. Local and systemic antibody response to putative periodontopathogens in patients with chronic periodontitis: correlation with clinical indices. Oral Microbiol. Immunol. **8**:65-68.

Klinge, B., Kuvatanasuhati, J., Attström, R., Kalfas, S., Edwardsson, S. 1992. The effect of topical metronidazole therapy on experimentally-induced periodontitis in the beagle dog. J. Clin. Periodontol. **19**:702-707.

Klitorinos, A., Noble, P., Siboo, R., Chan, E.C.S. 1993. Viscosity-dependent locomotion of oral spirochetes. Oral Microbiol. Immunol. **8**:242-244.

Ko, K.S.-C., Glogaurer, M., McCulloch, C.A.G., Ellen, R.P. 1998. *Treponema denticola* outer membrane inhibits calcium flux in gingival fibroblasts. Infect. Immun. **66**:703-709.

Kokeguchi, S., Miyamoto, M., Kato, K., Tanimoto, I., Karihara, H., Murayama, Y. 1994. Isolation and characterization of a 53 kDa major cell envelope protein antigen from *Treponema denticola* ATCC 35405. J. Periodont. Res. **29**:70-78.

Kolenbrander, P.E., London, J. 1993. Adhere today, here tomorrow: oral bacterial adherence. J. Bacteriol. **175**:3247-3252.

Kolenbrander, P.E., Parrish, K.D., Andersen, R.N., Greenberg, E.P. 1995. Intergeneric coaggregation of oral *Treponema* spp. With *Fusobacterium* spp. and intrageneric coaggregation among *Fusobacterium* spp. Infect. Immun. **63**:4584-4588.

Koreniewski, C., Callewaert, D.M. 1983. An enzyme-release assay for natural cytotoxicity. J. Immunol. Meth. **64**:313-320.

Kornman, K.S., Page, R.C., Tonetti, M.S. 1997. The host response to the microbial challenge in periodontitis: assembling the players. Periodontol. 2000 **14**:33-53.

Koseki, T., Benno, Y., Zhang-Koseki, Y.J., Umeda, M., Ishikawa, I. 1996. Detection frequencies and the colony-forming unit recovery of oral treponemes by different cultivation methods. Oral Microbiol. Immunol. **11**:203-208.

Kropinski, A.M., Parr, T.R., Angus, B.L., Hancock, R.E.W., Ghiorse, W.C., Greenberg, E.P. 1987. Isolation of the outer membrane and characterization of the major outer membrane protein from *Spirochaeta aurantia*. J. Bacteriol. **169**:172-179.

Kueng, W., Silber, E., Eppenberger, U. 1989. Quantification of cells cultured on 96-well plates. Anal. Biochem. **182**:16-19.

Kurimoto, T., Suzuki, M., Watanabe, T. 1990. Chemical composition and biological activities of lipopolysaccharides extracted from *Treponema denticola* and *Treponema vincentii*. Shigaku **78**:208-232.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**:680-685.

Lai, C.-H., Listgarten, M.A., Evian, C.I., Dougherty, P. 1986. Serum IgA and IgG antibodies to *Treponema vincentii* and *Treponema denticola* in adult periodontitis, juvenile periodontitis and periodontally healthy subjects. J. Clin. Periodontol. **13**:752-757.

Lakey, J.H., Pattus, F. 1989. The voltage-dependent activity of *Escherichia coli* porins in different planar bilayer reconstitutions. Eur. J. Biochem. **186**:303-308.

Leschine, S.B., Canale-Parola, E. 1980. Rifampin as a selective agent for isolation of oral spirochetes. J. Clin. Microbiol. **12**:792-795.

Leung, W.K., Haapasalo, M., Uitto, V.-J., Hannam, P.M., McBride, B.C. 1996. The surface proteinase of *Treponema denticola* may mediate attachment of the bacteria to epithelial cells. Anaerobe **2**:39-46.

Li, H., Ruby, J., Charon, N., Kuramitsu, H. 1996. Gene inactivation in the oral spirochete

Treponema denticola: Construction of an *flgE* mutant. J. Bacteriol. **178**:3664-3667.

Li, H., Kuramitsu, H.K. 1996. Development of a gene transfer system in *Treponema denticola* by electroporation. Oral Microbiol Immunol **11**:161-165.

Lindhe, J., Socransky, S.S. 1979. Chemotaxis and vascular permeability produced by human periodontopathic bacteria. J. Periodontal Res. **14**:138-146.

Lisanti, M.P., Sargiacomo, M., Graeve, L., Saltiel, A.R., Rodriguez-Boulan, E. 1988. Polarized apical distribution of glycosylphosphatidylinositol-anchored proteins in a renal epithelial cell line. Proc. Natl. Acad. Sci. USA **85**:9557-9561.

Listgarten, M.A. 1976. Structure of the microbial flora associated with periodontal health and disease in man. J. Periodontol. **47**:1-18.

Listgarten, M.A. 1982. Colonization of subgingival areas by motile rods and spirochetes: clinical implications. *In* Genco, R.J., Mergenhagen, S.E. (eds), Host-Parasite Interactions in Periodontal Diseases pp. 112-120. American Society for Microbiology: Washington, D.C.

Listgarten, M.A., Helldén, L. 1978. Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans. J. Clin. Periodontol. **5**:115-132.

Listgarten, M.A., Lindhe, J., Helldén L. 1978. Effect of tetracycline and/or scaling on human periodontal disease. Clinical, microbiological, and histological observations. J. Clin. Periodont. **5**:246-271.

Listgarten, M.A., Mayo, H.E., Tremblay, R. 1975. Development of dental plaque on epoxy resin crowns in man. A light and electron microscopic study. J. Periodontol. **46**:10-26.

Löe, H., Theilade, E., Jensen, S.B. 1965. Experiment gingivitis in man. J. Periodontol. **36**:177-187.

Loesche, W.J. 1993. Bacterial mediators in periodontal disease. Clin. Infectious. Dis. **16**:S203-S210.

Loesche, W.J., Lopatin, D.E., Stoll, J., van Poperin N., Hujoel, P.P. 1992. Comparison of various detection methods for periodontopathic bacteria: Can culture be considered the primary reference standard? J. Clin. Microbiol. **30**:418-426.

MacDougall, J.H., Beighton, D., Russell, R.R.B. 1991. Cloning and expression of protease genes from *Treponema denticola* in *Escherichia coli*. Oral Microbiol. Immunol. **6**:270-274.

MacNeil, D., Krystal, G., Fairhurst, M., Waterfield, J.D. 1986. Correction of defective 1L 3 responses of T-lymphocytes from autoimmune mice. J. Immunol. **137**:3464-3468.

Makela, M., Salo, T., Uitto, V.-J., Larjava, H. 1994. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. J. Dent. Res.

73:1397-1406.

Mäkinen, K.K., Chen, C.-Y., Mäkinen, P.-L. 1996. Proline iminopeptidase from the outer cell envelope of the human oral spirochete *Treponema denticola* ATCC 35405. Infect. Immun. **64**:702-708.

Mäkinen, K.K., Chen, C.-Y., Mäkinen, P.-L., Ohta, K., Loesche, W.J. 1990b. The benzoylarginine peptidase from *Treponema denticola* (strain ASLM), a human oral spirochete: evidence for active-site carboxyl groups. Molecular Microbiol. **4**:1413-1417.

Mäkinen, K.K., Mäkinen, P.-L. 1996. The peptidolytic capacity of the spirochete system. Med. Microbiol. Immunol. **185**:1-10.

Mäkinen, K.K., Mäkinen, P.-L., Loesche, W.J., Syed, S.A. 1995a. Purification and general properties of an oligopeptidase from *Treponema denticola* ATCC 35405 – a human oral spirochete. Arch. Biochem. Biophys. **316**:689-698.

Mäkinen, K.K., Mäkinen, P.-L., Syed, S.A. 1992. Purification and substrate specificity of an endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35405, active on furylacryloyl-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267**:14285-14293.

Mäkinen, K.K., Syed, S.A., Mäkinen, P.-L., Loesche, W.J. 1986. Benzoylarginine peptidase and iminopeptidase profiles of *Treponema denticola* strains isolated from the human periodontal pocket. Curr. Microbiol. **14**:85-89.

Mäkinen, K.K., Syed, S.A., Mäkinen, P.-L., Loesche, W.J. 1987. Dominance of iminopeptidase activity in the human oral bacterium *Treponema denticola* ATCC 35405. Curr. Microbiol. **14**:341-346.

Mäkinen, K.K., Syed, S.A., Salvader, S.L., Mäkinen, P.-L. 1990a. Hydrolysis of the Leu-Gly bond of phenylazobenzyl-oxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (a substrate of microbial collagenases) by treponemes isolated from the subgingival plaque of periodontitis patients. Curr. Microbiol. **20**:69-74.

Mäkinen, P.-L., Mäkinen, K.K. 1997. γ-glutamyltransferase from the outer cell envelope of *Treponema denticola* ATCC 35405. Infect. Immun. **65**:685-691.

Mäkinen, P.-L., Mäkinen, K.K., Syed, S.A. 1994. An endo-acting proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. Infect. Immun. **62**:4938-4947.

Mäkinen, P.-L., Mäkinen, K.K., Syed, S.A. 1995b. Role of the chymotrypsin-like membrane-associated proteinase from *Treponema denticola* ATCC 35405 in inactivation of bioactive peptides. Infect. Immun. **63**:3567-3575.

Maltha, J.C., Mikx, F.H.M., Kuijpers, F.J. 1985. Necrotizing ulcerative gingivitis in beagle dogs. III. Distribution of spirochetes in interdental gingival tissues. J. Periodont. Res. **20**:522-

531.

Mangan, D.F., Laughon, B.E., Bower, B., Lopatin, D.E. 1982. *In vitro* lymphocyte blastogenic responses and titers of humoral antibodies from periodontitis patients to oral spirochete isolates. Infect. Immun. **37**:445-451.

Manthey, C., Vogel, S.N. 1994. Interactions of lipopolysaccharide with macrophages. *In* Zwilling, B.S., Eisenstein, T.K. (eds), Macrophage-pathogen interactions pp. 63-81. Marcel Dekker: New York.

Marsh, P., Martin, M. 1992. Oral Microbiology. 3rd Ed. Chapman & Hall: London.

Martin, A., Clynes, M. 1993. Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. Cytotechnol. **11**:49-58.

Masuda, K., Kawata, T. 1982. Isolation, properties, and reassembly of outer sheath carrying a polygonal array from an oral treponeme. J. Bacteriol. **150**:1405-1413.

Mathers, D.A., Leung, W.K., Fenno, J.C., Yan, H., McBride, B.C. 1996. Major surface protein complex of *Treponema denticola* depolarizes and induces ion channels in HeLa cell membranes. Infect. Immun. **64**:2904-2910.

McCulloh, C.A. 1994. Host enzymes in gingival crevicular fluid as diagnostic indicators of periodontitis. J. Clin. Periodontol. **21**:497-506.

McCulloch, C.A.G., Barghana, U., Melcher, A.H. 1989. Cell death and the regulation of populations of cells in the periodontal ligament. Cell Tissue Res. **255**:129-138.

Meghi, S., Henderson, B., Nair, S.P., Tufano, M.A. 1997. Bacterial porins stimulate bone resorption. Infect. Immun. **65**:1313-1316.

Meikle, M.C., Hembry, R.M., Holley, J., Horton, C., McFarlane, C.G., Reynolds, J.J. 1994. Immunolocalization of matrix metalloproteinases, and TIMP-1 (tissue inhibitor of metalloproteinases) in human gingival tissues from periodontitis patients. J. Periodont. Res. **29**:118-126.

Mendieta, C., Reeve, C., Romero, J. 1985. Biosynthesis of prostaglandins in gingiva of patients with chronic periodontitis. J. Periodontol. **56**:44-47.

Merrilees, M.J., Sodek, J., Aubin, J.E. 1983. Effect of cells of epithelial rests of Malassez and endothelial cells on synthesis of glycosaminoglycans by periodontal ligament fibroblasts *in vitro*. Dev. Biol. **97**:146-153.

Mikx, F.H.M. 1991. Comparison of peptidase, glycosidase and esterase activities of oral and non-oral *Treponema* species. J. Gen. Microbiol. **137**:63-68.

Mikx, F.H.M., de Jong, M.H. 1987. Keratinolytic activity of cutaneous and oral bacteria.

Infect. Immun. **55**:621-625.

Mikx, F.H.M., Keulers, R.A.C. 1992. Hemagglutination activity of *Treponema denticola* grown in serum-free medium in continuous culture. Infect. Immun. **60**:1761-1766.

Mikx, F.H.M., Maltha, J.C., van Campen, G.J. 1990. Spirochetes in early lesions of necrotizing ulcerative gingivitis experimentally induced in beagles. Oral Microbiol. Immunol. **5**:86-89.

Mikx, F.H.M., van Campen, G.J. 1982. Microscopical evaluation of the microflora in relation to necrotizing ulcerative gingivitis in the beagle dog. J. Periodontol. Res. **17**:576-584.

Miyamoto, M., Noji, S., Kokeguchi, S., Kato, K., Karihara, H., Murayama, Y., Taniguchi, S. 1991. Molecular cloning and sequence analysis of antigen gene *tdpA* of *Treponema denticola*. Infect. Immun. **59**:1941-1947.

Miyazawa, Y. 1989. Correlation between subgingival microflora and peptidase activity in periodontal pockets measured with synthetic substrates. Nippon Shishubyo Gakkai Kaishi **31**:43-54.

Moore, W.E.C., Moore, L.V.H. 1994. The bacteria of periodontal diseases. Periodontol. 2000 **5**:66-77.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., Hash, D.E., Burmeister, J.A., Ranney, R.R. 1982. Bacteriology of severe periodontitis in young adult humans. Infect. Immun. **38**:1137-1148.

Morgan, H., Lonsdale, J.T., Adler, G. 1990. Polarity-dependent voltage-gated porin channels from *Escherichia coli* in lipid bilayer membranes. Biochim. Biophys. Acta **1021**:175-181.

Morinushi, T., Lopatin, D.E., Syed, S.A., Bacon, G., Kowalski, C.J., Loesche, W.J. 1989. Humoral immune response to selected subgingival plaque microorganisms in insulin-dependent diabetic children. J. Periodontol. **64**:199-204.

Morris, E.J., McBride, B.C. 1984. Adherence of *Streptococcus sanguis* to saliva-coated hydroxyapatite: evidence for two binding sites. Infect. Immun. **43**:656-663.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. **65**:55-63.

Moss-Salentijn, L., Hendricks-Klyvert, M. 1990. Dental and Oral Tissues. An Introduction. 3rd Ed. Lea & Febiger: Philadelphia.

Mousques, T., Listgarten, M.A., Phillips, R.W. 1980. Effect of scaling and root planing on the composition of the human subgingival microbial flora. J. Periodontal Res. **15**:144-151.

Nagata, E., Kondo, T., Ayasaka, N., Nakata, M., Tanako, T. 1992. Immunohistochemical

study of nerve fibers with substance P-or calcitonin gene-related peptide-like immunoreactivity in the junctional epithelium of developing rats. Arch. Oral Biol. **37**:655-662.

Nakamura, Y., Umemoto, T., Nakatani, Y., Namikawa, I, Wadood A. 1993. Common and specific antigens of several treponemes detected by polyclonal antisera against major cellular proteins. Oral Microbiol. Immunol. **8**:288-294.

Nakashima, K., Demeurisse, C., Cimasoni, G. 1994. The recovery efficiency of various materials for sampling enzymes and polymorphonuclear leukocytes from gingival crevices. J. Clin. Periodontol. **21**:479-483.

Nakatani, Y., Umemoto, T., Nakamura, Y., Noda, M., Ito, U., Namikawa, I. 1992. Adherence of an oral spirochete *Treponema denticola* to glass surface *in vitro*. Jpn. J. Oral Biol. **34**:374-383.

Newman, G.R. 1989. LR white embedding medium for colloidal gold methods. *In* Hayat, M.A. (ed), Colloidal gold: principles, methods, and applications, vol. 2, pp. 47-73. Academic Press: New York.

Newman, H.N. 1990. Plaque and chronic inflammatory periodontal disease. A question of ecology. J. Clin. Periodontol. **17**:533-541.

Newman, M.G., Socransky, S.S. 1977. Predominant cultivable microbiota in periodontosis. J. Periodont. Res. **12**:120-128.

Nilius, A.M., Spencer, S.C., Simonson, L.G. 1993. Stimulation of *in vitro* growth of *Treponema denticola* by extracellular growth factors produced by *Porphyromonas gingivalis*. J. Dent. Res. **72**:1027-1031.

Nisengard, R.J., Newman, M.G., Zambon, J.J. 1988. Periodontal disease. *In* Newman, M.G., Nisengard, R.J., (eds), Oral Microbiology and Immunology. Section IV. Oral health and disease. Chapter 28, pp. 411-437, W.B. Saunders: Philadelphia.

Nitzan, D., Sperry, J.F., Wilkins, T.D. 1978. Fibrinolytic activity of oral anaerobic bacteria. Arch. Oral Biol. **23**:465-470.

Norton Hughes, C.A., Yotis, W.W. 1990. A study of the acid phosphatase of *Treponema denticola*. Zbl. Bakt. **274**:195-202.

Novak, M.J., Cohen, H.J. 1991. Depolarization of polymorphonuclear leukocytes by *Porphyromonas* (*Bacteroides*) *gingivalis* 381 in the absence of respiratory burst activation. Infect. Immun. **59**:3134-3142.

Offenbacher, S. 1996. Periodontal diseases: pathogenesis. Ann. Periodontol. **1**:821-878.

Ohta, K., Mäkinen, K.K., Loesche, W.J. 1986. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect.

Immun. **53**:213-220.

Olsen, I. 1984. Attachment of *Treponema denticola* to cultured human epithelial cells. Scand. J. Dent. Res. **92**:55-63.

Olsen, I., Lingaas, E., Hurlen, B., Midtvedt, T. 1984. Scanning and transmission electron microscopy of the phagocytosis of *Treponema denticola* and *Escherichia coli* by human neutrophils *in vitro*. Scand. J. Dent. Res. **92**:282-293.

Onagawa, M., Ishihara, K., Okuda, K. 1994. Coaggregation between *Porphyromonas gingivalis* and *Treponema denticola*. Bull. Tokyo Dent. Coll. **35**:171-181.

Osborn, L. 1990. Leukocyte adhesion to endothelium in inflammation. Cell **62**:3-6.

Page, R. C. 1991. The role of inflammatory mediators in the pathogenesis of periodontal disease. J. Periodont. Res. **26**:230-242.

Page, R.C., Kornman, K.S. (eds) 1997. The pathogenesis of periodontitis. Periodontol. 2000 vol. 14.

Page, R.C., Schroeder, H.E. 1990. Structure and pathogenesis. *In* Schluger, S., Yuodelis, R., Page, R.C., Johnson, R.H. (eds.) Periodontal Diseases. Chapter 7, pp. 183-220. Lea & Febiger: Philadelphia.

Pan, Y.-M. 1993. *In vitro* model of periodontal epithelium. Ph.D. thesis, University of British Columbia: Vancouver.

Pan, Y.-M. 1994. Personal communication.

Pan, Y.-M., Firth, J.D., Salonen, J.I., Uitto, V.-J. 1995. Multilayer culture of periodontal ligament epithelial cells: a model for junctional epithelium. J. Periodont. Res. **30**:97-107.

Parratt, L.G. 1961. Probability and Experimental Errors in Science. John Wiley and Sons: New York.

Paster, B.J., Dewhirst, F.E., Weisburg, W.G., Tordoff, L.A., Fraser, G.J., Hespell, R.B., Stanton, T.B., Zablen, L., Mandelco, L., Woese, C.R. 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. **173**:6101-6109.

Patti, J.M., Höök, M. 1994. Microbial adhesins recognizing extracellular matrix molecules. Curr. Opin. Cell Biol. **6**:752-758.

Payne, N.R., Horowitz, M.A. 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med. **166**:1377-1389.

Pederson, E.D., Miller, J.W., Matheson, S., Simonson, L.G., Chadwick, D.E., Covill, P.J., Turner, D.W., Lamberts, B.L., Morton, H.E. 1994. Trypsin-like activity levels of *Treponema*

denticola and *Porphyromonas gingivalis* in adults with periodontitis. J.Clin. Periodontol. **21**:519-525.

Pietrantonio, F., Noble, P.B., Amsel, R., Chan, E.C.S. 1988. Locomotory characteristics of *Treponema denticola*. Can. J. Microbiol. **34**:748-752.

Putnins, E.E. 1995. Growth factor induction of epithelial cell proliferation and matrix metalloproteinase secretion. Ph.D. thesis, University of British Columbia: Vancouver.

Qiu, Y.-S., Klitorinos, A., Rahal, M.D., Siboo, R., Chan, E.C.S. 1994. Enumeration of viable oral spirochetes from periodontal pockets. Oral Microbiol. Immunol. **9**:301-304.

Que, X.-C., Kuramitsu, H.K. 1990. Isolation and characterization of the *Treponema denticola prtA* gene coding for chymotrypsinlike protease activity and detection of a closely linked gene encoding PZ-PLGPA-hydrolyzing activity. Infect. Immun. **58**:4099-4105.

Rachel, R., Engel, A.M., Huber, R., Stetter, K.O., Baumeister, W. 1990. A porin-type protein is the main constituent of the cell envelope of the ancestral eubacterium *Thermatoga maritima*. FEBS Lett. **262**:64-68.

Reijntjens, F.M.J., Mikx, F.H.M., Wolters-Lutgerhorst, J.M.L., Maltha, J.C. 1986. Adherence of oral treponemes and their effect on morphological damage and detachment of epithelial cells *in vitro*. Infect. Immun. **51**:642-647.

Renart, J., Sandoval, I.V. 1984. Western blots. Methods Enzmol. **104**:455-460.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in the electron microscope. J Cell Biol. **17**:208.

Riviere, G.R., Smith, K.S., Carranza Jr., N., Tzagaroulaki, E., Kay, S.L., Dock, M. 1995. Subgingival distribution of *Treponema denticola*, *Treponema socranskii*, and pathogen-related oral spirochetes: Prevalence and relationship to periodontal status of sampled sites. J. Periodontol. **66**:829-837.

Riviere, G.R., Smith, K.S., Carranza, Jr., N., Tzagaroulaki, E., Kay, S.L., Dock, M., Zhu, X., DeRouen, T.A. 1996. Association between *Porphyromonas gingivalis* and oral treponemes in subgingival plaque. Oral Microbiol. Immunol. **11**:150-155.

Roberts, C.J., Birkenmeier, T., McQuillan, J., Akiyama, S., Yamada, S., Chen, W.-T., Yamada, K., McDonald, J. 1988. Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J. Biol. Chem. **263**:4586-4592.

Romanos, G.E., Schröter-Kermani, G., Hinz, N., Wachtel, H.C., Bernimoulin, J.P. 1991. Immunohistochemical localization of collagenous components in healthy periodontal tissues of the rat and marmoset (*Callithrix jacchus*). II. Distribution of collagen types IV, V and VI. J. Periodont. Res. **26**:323-332.

Romanos, G.E., Schröter-Kermani, G., Hinz, N., Wachtel, H.C., Bernimoulin, J.P. 1992. Immunohistochemical localization of collagenous components in healthy periodontal tissues of the rat and marmoset (*Callithrix jacchus*). I. Distribution of collagen types I and III. J. Periodont. Res. **27**:101-110.

Rosen, G., Naor, R., Kutner, S., Sela, M. 1994. Characterization of fibrinolytic activities of *Treponema denticola*. Infect. Immun. **62**:1749-1754.

Rosen, G., Naor, R., Rahamin, E., Yishai, R., Sela, M.N. 1995. Proteases of *Treponema denticola* outer sheath and extracellular vesicles. Infect. Immun. **63**:3973-3979.

Ruby, J.D. 1995. *Treponema denticola* periplasmic flagella and motility. Ph.D. thesis, West Virginia University: Morgantown.

Ruby, J.D., Li, H., Kuramitsu, H., Norris, S.J., Goldstein, S.F., Buttle, K.F., Charon, N.W. 1997. Relationship of *Treponema denticola* periplasmic flagella to irregular cell morphology. J. Bacteriol. **179**:1628-1635.

Salvi, S.E., Lawrence, H.P., Offenbacher, S., Beck, J.D. 1997. Influence of risk factors on the pathogenesis of periodontitis. Periodontol. 2000 **14**:173-201.

Salyers, A.A., Whitt, D.D. 1994. Bacterial Pathogenesis. A Molecular Approach. Chapter 4. Virulence factors that damage the host pp. 47-62. ASM Press: Washington, D.C.

Sasaki, N., Nakagawa, T., Seida, K., Ishihara, K., Okuda, K. 1989. Clinical, microbiological and immunological studies of post-juvenile periodontitis. Bull. Tokyo Dent. Coll. **30**:205-211.

Sauve, R., Simmoneau, C., Monette, R., Roy, G. 1986. Single-channel analysis of the potassium permeability in HeLa cancer cells: evidence for a calcium-activated potassium channel of small unitary conductance. J. Membr. Biol. **92**:269-282.

Schade, S.Z., Jolley, M.E., Sarauer, B.J., Simonson, L.G. 1996. BODIPY-α-casein, a pH-independent protein substrate for protease assays using fluorescence polarization. Anal. Biochem. **243**:1-7.

Schenkein, H.A., Berry, C.R. 1991. Activation of complement by *Treponema denticola*. J. Dent. Res. **70**:107-110.

Schreoder H.E., Münzel-Pedrazzoli S. 1970. Morphometric analysis comparing junctional and oral epithelium of normal human gingiva. Helv. Odontol. Acta **14**:53-66.

Schuster, G.S. (ed.) 1990. Oral Microbiology and Infectious Disease. 3rd ed. B.C. Decker: Philadelphia.

Scott, D., Klitorinos, A., Chan, E.C.S., Siboo, R. 1997. Visualization of an extracellular mucoid layer of *Treponema denticola* ATCC 35405 and surface sugar lectin analysis of some

Treponema species. Oral Microbiol. Immunol. **12**:121-125.

Scott, D., Siboo, I.R., Chan, E.C.S., Siboo, R. 1996. An extracellular enzyme with hyaluronidase and chondroitinase activities from some oral anaerobic spirochetes. Microbiol. 142:2567-2576.

Scragg, M., Ferreira, L. 1991. Evaluation of differential staining procedures for quantification of fibroblast cultured in 96-well plates. Anal. Biochem. **198**:80-85.

Sedgley, C.M., Samaranayake, L.P., Darvell, B.W. 1996. The adherence of oral isolates of *Enterobacteriaceace* to HeLa cells: an *in vitro* method using image analysis. A.P.M.I.S. **104**:583-590.

Sela, M.N., Weinberg, A., Borinsky, R., Holt, S.C., Dishon, T. 1988. Inhibition of superoxide production in human polymorphonuclear leukocytes by oral treponemal factors. Infect. Immun. **56**:589-594.

Shenker, B.J., Listgarten, M.A., Taichman, N.S. 1984. Suppression of human lymphocyte responses by oral spirochetes: A monocyte-dependent phenomenon. J. Immunol. **132**:2039-2045.

Siboo, R., Al-Joburi, W., Gornitsky, M., Chan, E.C.S. 1989. Synthesis and secretion of phospholipase C by oral spirochetes. J. Clin. Microbiol. **27**:568-570.

Simonson, L.G., Goodman, C.H., Bial, J.J., Morton, H.E. 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56**:726-728.

Simonson, L.G., McMahon, K.T., Childers, D.W., Morton, H.E. 1992. Bacterial synergy of *Treponema denticola* and *Porphyromonas gingivalis* in a multinational population. Oral Microbiol. Immunol. 7:111-112.

Skogaard, M. 1965. Turnover of the gingival epithelium in marmosets. Acta Odontol. Scand. **23**:623-643.

Slots, J., Emrich, L.J., Genco, R.J., Rosling, B.G. 1985. Relationship between some subgingival bacteria and periodontal pocket depth and gain or loss of periodontal attachment after treatment of adult periodontitis. J. Clin. Periodontol. **12**:540-552.

Smibert, R.M. 1984. Genus III. *Treponema* Schandinn 1905, pp. 49-57. *In* Krieg, N.R., Holt, J.G. (eds.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins: Baltimore.

Snyderman, R., McCarty, G.A. 1982. Analogous mechanisms of tissue destruction in rheumatoid arthritis and periodontal disease. *In* Genco, R.J., Mergenhagen, S.E. (eds.), Host Parasite Interactions of Periodontal Diseases pp. 354-362. American Society for Microbiology: Washington, D.C.

Socransky, S.S. 1979. Criteria for the infectious agents in dental caries and periodontal disease.

J. Clin. Periodontol. **6**(7 Extra issue):16-21.

Socransky, S.S., Haffajee, A.D. 1991. Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. J. Periodont. Res. **26**:195-212.

Socransky, S.S., Manganiello, J.E. 1973. The oral microbiology of man from birth through senility. J. Periodontol. **42**:485-496.

Söder, P.-Ö, Jin, L.J., Söder, B. 1993. DNA probe detection of periodontopathogens in advanced periodontitis. Scand. J. Dent. Res. **101**:363-370.

Sorsa, T., Ding, Y.-L., Ingman, T., Salo, T., Westerlund, U., Haapasalo, M., Tschesche, H., Konttinen, Y.T. 1995. Cellular source, activation and inhibition of dental plaque collagenase. J. Clin. Periodontol. **22**:709-717.

Sorsa, T., Ingman, T., Suomalainen, K., Haapasalo, M., Konttinen, Y.T., Lindy, O., Saari, H., Uitto, V.-J. 1992. Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. Infect. Immun. **60**:4491-4495.

Squier, C.A., Johnson, N.W., Hackemann, M. 1975. Structure and function of normal human oral mucosa. *In* Dolby, A.E. (ed.). Oral Mucosa in Health and Disease. Chapter 1, pp. 1-112. Blackwell scientific: Oxford.

Stadnyl, A.W. 1994. Cytokine production by epithelial cells. FA-SEB J. **8:**1041-1047.

Staggs, T.M., Greer, M.K., Baseman, J.B., Holt, S.C., Tryon, V.V. 1994. Identification of lactoferrin-binding proteins from *Treponema pallidum* subspecies *pallidum* and *Treponema denticola*. Molecular Microbiol. **12**:613-619.

Stanislawski, L., Simpson, W.A., Hasty, D., Sharon, N., Beachey, E.H., Ofek, I. 1985. Role of fibronectin in attachment of *Streptococcus pyogenes* and *Escherichia coli* to human cell lines and isolated oral epithelial cells. Infect. Immun. **48**:257-259.

Stashenko, P., Fujiyoshi, P., Obernesser, M.S., Prostak, L., Haffajee, A.D., Socransky, S.S. 1991a. Levels of interleukin-1β in tissue from sites of active periodontal disease. J. Clin. Periodontol. **18**:548-554.

Stashenko, P., Jandinski, J.J., Fujiyoski, P., Rynar, J., Socransky, S.S. 1991b. Tissue levels of bone resorptive cytokines in periodontal disease. J. Periodontol. 62**:504-509.**

Steinberg, A.I. 1970. Evidence for the presence of circulating antibodies to an oral spirochete in the sera of clinic patients. J. Periodontol. **41**:213-214.

Steinberg, A.I., Gershoff, S.N. 1968. Quantitative differences in spirochetal antibody observed in periodontal disease. J. Periodontol. **39**:286-289.

Steinberg, A.I., Socransky, S.S., Gershoff, S.N., Weinstock, A. 1966. Use of tanned-cell hemagglutination to demonstrate circulating antibody against an oral spirochete. J. Bacteriol. **91**:2114.

Steven, A.C., ter Heggler, B., Muller, R., Kistler, K., Rosenbusch, J.P. 1977. Ultrastructure of a periodic protein layer in the outer membrane of *Escherichia coli*. J. Cell Biol. **72**:292-301.

Su, H., Zhang, Y.-X., Barrera, O., Watkins, N.G., Caldwell, H.D. 1988. Differential effect of trypsin on infectivity of *Chlamydia trachomatis*. Loss of infectivity requires cleavage of major outer membrane protein variable domains I and IV. Infect. Immun. **56**:2094-2100.

Suido, H., Eguchi, T., Tanaka, T., Nakamura, M. 1988. Identification of periodontopathic bacteria based upon their peptidase activities. Adv. Dent. Res. **2**:304-309.

Suzuki, J.B., Park, S.K., Falkler, Jr. W.A. 1984. Immunologic profile of juvenile periodontitis. I. Lymphocyte blastogenesis and the autologous mixed lymphocyte response. J. Periodontol. **55**:453-460.

Suzuki, M., Watanabe, T. 1988. Study on β-D-galactosidase produced in *Treponema denticola*. Shigaku **75**:1229-1237.

Suzuki, M., Watanabe, T. 1991. Preliminary study on phospholipase-C in *Treponema denticola* ATCC 35405 and *Treponema vincentii* ATCC 35580. Shigaku **78**:1038-1046.

Syed, S.A., Mäkinen, K.K., Mäkinen, P.-L., Chen, C.-Y., Muhammad Z. 1993. Proteolytic and oxidoreductase activity of *Treponema denticola* ATCC 35405 grown in an aerobic and anaerobic gaseous environment. Res. Microbiol. **144**:317-326.

Taichman, N.S., Boehringer, C.H., Lai, C.-H., Shenker, B.J., Tsai, C.-C., Berthold, P.H., Listgarten, M.A., Shapiro, I.S. 1982. Pathobiology of oral spirochetes in periodontal disease. J. Periodontal Res. **17**:449-451.

Taichman, N.S., Klass, J.E., Shenker, B.J., Macarak, E.J., Boehringer, H., Tsai, C.-C. 1984. Suspected periodontopathic organisms alter *in vitro* proliferation of endothelial cells. J. Periodontal Res. **19**:583-586.

Takahashi, A., Yamaguchi, H., Miyamoto, H. 1993. Change in K^+ current of HeLa cells with progression of the cell cycle studied by patch clamp technique. Am. J. Physiol. **265**:C328-C336.

Takahashi, T., Kato, T., Okuda K. 1996. B-cell activation of outer membrane preparations from *Porphyromonas* gingivalis and *Treponema denticola*. Bull. Tokyo Dent. Coll. **37**:9-15.

Tall, B.D., Nauman, R.K. 1994. Outer membrane antigens of *Treponema* species. J. Med. Microbiol. **40**:62-69.

ter Steeg, P.F., van der Hoeven, J.S. 1990. Growth stimulation of *Treponema denticola* by periodontal microorganisms. Antonie van Leeuwenhoek **57**:63-70.

ter Steeg, P.F., van der Hoeven, J.S., de Jong, M.H., van Muster, P.J.J., Jansen, M.J.H. 1988. Modeling the gingival pocket by enrichment of subgingival microflora in human serum in chemostats. Microb. Ecol. Health Dis. **1**:73-84.

Tew, J.G., Smibert, R.M., Scott, E.A., Burmeister, J.A., Ranney, R.R. 1985. Serum antibodies in young adult humans reactive with periodontitis associated treponemes. J. Periodontal Res. **20**:580-590.

Theilade, J., Attström, R. 1985. Distribution and ultrastructure of subgingival plaque in beagle dogs with gingival inflammation. J. Periodont. Res. **20**:131-145.

Thomas, D.D. 1996. Aspects of adherence of oral spirochetes. Crit. Rev. Oral. Biol. Med. **7**:4-11.

Thomas, D.D., Baseman, J.B., Alderete, J.F. 1985. Fibronectin mediates *Treponema pallidum* cytadherence through recognition of fibronectin cell-binding domain. J. Exp. Med. **161**:514-525.

To, L., Sasaki, S., Socransky, S.S. 1978. "Piggyback" hypothesis of subgingival colonization of non-motile microorganisms. I. Migration through liquids. J. Dent. Res. **57**(Special Issue):316 (abstr. 968).

Tonetti, M.S. 1997. Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. J. Periodont. Res. **32**:104-108.

Tonetti, M.S., Gerber, L., Lang, N.P. 1994. Vascular adhesion molecules and initial development of inflammation in clinically healthy human keratinized mucosa around teeth and osseointegrated implants. J. Periodont. Res. **29**:386-392.

Tufano, M.A., Sommese, L., Galdiero, F. 1986. Some biological activities of *Eikenella corrodens* major outer membrane proteins. Eur. J. Epidemiol. **2**:305-311.

Uitto, V.-J., Chan, E.C.S., Quee, T.C. 1986. Initial characterization of neutral proteinase from oral spirochetes. J. Periodontol. Res. **21**:95-100.

Uitto, V.-J., Haapasalo, M., Laakso, T., Salo, T. 1988a. Degradation of basement membrane collagen by proteases from some anaerobic oral micro-organisms. Oral Microbiol. Immunol. **3**:97-102.

Uitto, V.-J., Grenier, D., Chan, E.C.S., McBride, B.C. 1988b. Isolation of a chymotrypsinlike enzyme from *Treponema denticola*. Infect. Immun. **56**:2717-2722.

Uitto, V.-J., Grenier, D., Pan, Y.-M., McBride, B.C., Cawston, T. 1992. The collagenolytic activity of *Treponema denticola*. Matrix **Suppl. 1**:141-142.

Uitto, V.-J., Larjava, H. 1991. Extracellular matrix molecules and their receptors. An overview with special emphasis on periodontal tissues. Crit. Rev. Oral Biol. Med. **2**:323-354.

Uitto, V.-J., Larjava, H., Peltonen, J., Brunette, D.M. 1992 Expression of fibronectin and integrins in cultured periodontal ligament epithelial cells. J. Dent. Res. **71**:1203-1211.

Uitto, V.-J., Pan, Y.-M., Leung, W.K., Larjava, H., Ellen, R.P., Finlay, B.B., McBride, B.C. 1995. Cytopathic effects of *Treponema denticola* chymotrypsin-like proteinase on migrating and stratified epithelial cells. Infect. Immun. **63**:3401-3410.

Umemoto, T., Namikawa, I., Suido, H., Asai, S. 1989. A major antigen on the outer envelope of a human oral spirochete, *Treponema denticola*. Infect. Immun. **57**:2470-2474.

Umemoto, T., Zambon, J.J., Genco, R.J., Namikawa, I. 1988. Major antigens of human oral spirochetes associated with periodontal disease. Adv. Dent. Res. **2**:292-296.

van Rensburg, B.G.J. 1995. Oral Biology. Quintessence: Chicago.

Vasiliev, J.M., Gelfand, I.M., Domnina L.V., Zacharova, O.S., Ljubimov, A.V. 1975. Contact inhibition of phagocytosis in epithelial sheets: alternations of cell surface properties induced by cell-cell contacts. Proc. Natl. Acad. Sci. USA **72**:719-722.

Wang, B., Holt, S.C. 1993. Interaction of *Treponema denticola* with Hep-2 cells. J. Dent. Res. **72** (Suppl):324 (abstr. 1770).

Weber, F. H., Canale-Parola, E. 1984. Pectinolytic enzymes of oral spirochetes from humans. Appl. Environ. Microbiol. **48**:61-67.

Weinberg, A., Holt, S.C. 1990. Interaction of *Treponema denticola* TD-4, GM-1 and MS25 with human gingival fibroblasts. Infect. Immun. **58**:1720-1729.

Weinberg, A., Holt, S.C. 1991. Chemical and biological activities of a 64-kilodalton outer sheath protein from *Treponema denticola* strains. J. Bacteriol. **173**:6935-6947.

Williams, D.M., Hughes, F.T., Odell, E.W., Forthing, F.M. 1992. Pathology of Periodontal Disease. Oxford University Press: Oxford.

Wolf, V., Lange, R., Wecke, J. 1993. Development of quasi-multicellular bodies of *Treponema denticola*. Arch. Microbiol. **160**:206-213.

Wolff, L., Dahlén, G., Aeppli, D. 1994. Bacteria as risk makers for periodontitis. J. Periodontol. **65**:498-510.

Xu, G.Z., Shi, B., MacGroarty, E.J., Tien, H.T. 1986. Channel-closing activity of porins from *Escherichia coli* in bilayer lipid membranes. Biochim. Biophys. Acta **862**:57-64.

Yang, P.F., Song, M., Grove, D.A., Ellen, R.P. 1998. Filamentous actin disruption and diminished inositol phosphate response in gingival fibroblasts caused by *Treponema denticola*. Infect. Immun. **66**:696-702.

Yao, E.S., Lamont, R.J., Leu, S.P., Weinberg, A. 1996. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol. Immunol. **11**:35-41.

Yoshida, M., Suzuki, M., Watanake, T. 1990. Study of collagenase isolated from the outer envelopes of *Treponema denticola* ATCC 35405. Shigaku **77**:1687-1706.

Yotis, W.W., Macaluso, F., Gopalsami, C. 1995. Immunochemical features of macromolecule of *Treponema denticola*. J. Basic Microbiol. **35**:255-268.

Zar, J. H. 1996. Biostatistical Analysis. Prentice-Hall: New Jersey.