Interaction of a putative transcriptional regulatory protein and the thermo-inducible cts-52 mutant repressor in the Bacillus subtilis phage φ105 genome

Annie Y. Chan¹ and Boon. L. Lim¹*

¹ Department of Zoology, The University of Hong Kong, Pokfulam Road, Hong Kong, China

*Author of correspondence:

Department of Zoology, The University of Hong Kong, Pokfulam Road, Hong Kong, China.

Tel: +852 2299-0826, Fax: +852 2559-9114

Email: bllim@hkucc.hku.hk

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Abbreviations: a.a., amino acids; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase; K_D, equilibrium dissociation constant; mtcφ105, cts-52 mutant repressor; ORF, open-reading frame; O_R, operator; SPR, Surface plasmon resonance; wtcφ105, wild-type repressor
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Abstract

A 144 amino acid cts-52 mutant repressor (mtcφ105) located in the EcoRI-F immunity region (immF) of Bacillus subtilis phage φ105 is involved in the control mechanism of a thermo-inducible expression system. Adjacent to the repressor gene, an open-reading frame, designated ORF4, encodes a polypeptide of 90 amino acid residues, which shares a 37% homology with the amino acid sequence of the repressor. Based on protein sequence alignment, a DNA-binding α-helix-β-turn-α-helix (HTH) motif was identified in the N-terminal region (residues 18–37) of the repressor as well as in the polypeptide of ORF4 (residues 22–41). In vivo expression of the mutant repressor and ORF4 were confirmed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis. To study their DNA binding properties, the wild-type repressor (wtcφ105) and the mutant repressor mtcφ105, which has a Thr17 to Ile substitution, were overexpressed in Escherichia coli and purified for affinity assays. Their affinities towards six operator sites at various temperatures were elucidated by surface plasmon resonance (SPR). Our data showed that a temperature shift does not influence the wtcφ105-operators’ binding affinity, while the binding of mtcφ105 to the operators was temperature sensitive. This explains how thermo-induction triggers the release of the mutant repressor and renders heterologous gene expression. Interestingly, mtcφ105 and ORF4 demonstrated
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a large affinity discrepancy towards individual operators at different temperatures.
mRNA levels monitored by real-time RT-PCR indicated a suppression of mtcφ105 expression, but a stimulation of ORF4 transcription after thermo-induction. Our data suggested that ORF4 might be a counter protein to the phage repressor in the modulation of the two divergent-oriented promoters P_M and P_R within the immF region.
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Introduction

Immediately after temperate bacteriophage infection, lysogeny is tightly regulated, by repressing the lytic promoter. Switching from lysogeny to lytic development, or prophage induction, occurs after the host SOS system is activated in response to DNA damage, for example, after ultraviolet (UV) radiation\textsuperscript{1,2}. In the \textit{Bacillus subtilis} phage φ105, a thermo-inducible prophage mutant that carries a cts mutation was discovered two decades ago\textsuperscript{3}. Dhaese and colleagues\textsuperscript{4} demonstrated that an open-reading frame (ORF), designated cφ105, was responsible for the maintenance of lysogeny (Figure 1). It suppressed phage DNA replication and adjacent bacterial genes transcription before thermo-induction. Using chloramphenicol acetyl transferase (CAT) activity assays, transcriptional control in the immunity region was found to rely on two divergently orientated promoters, P\textsubscript{M} (Left) and P\textsubscript{R} (Right)\textsuperscript{5,6}. Similarly to coliphage λcI, wtcφ105 is both a negative and positive regulator of transcription. It stimulates P\textsubscript{M}, the promoter for its own gene, but represses P\textsubscript{R}, which probably signals the onset of a lytic pathway\textsuperscript{6}. The repressor was shown specifically to recognise six operator sites that are identified within the 3.2 kb immunity region fragment\textsuperscript{6-11}. Similarly to the recently reported operator sites identified in two mycobacteriophages, L5\textsuperscript{12} and Bxb1\textsuperscript{12,13}, the 14 bp-core operator sequence 5’-GACGGAATAAAG-3’ of wtcφ105 is lacking in two-fold rotational symmetry\textsuperscript{10,11}. 

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A thermo-inducible expression system based on phage φ105 has been established in *Bacillus subtilis*\textsuperscript{14,15}. Unlike other *Bacillus* expression systems that employed plasmid-based, constitutive promoters, this prophage expression system is capable of driving recombinant protein expression in a thermo-inducible manner. The complete suppression of heterologous protein expression before thermo-induction is desirable for expressing toxic products. The genetic switch results from a mutation (*cts*-52) on the wtcφ105 that renders the expression system thermo-inducible\textsuperscript{16–18}. As shown in previous studies\textsuperscript{19,20}, induction of heterologous gene expression in the φ105MU331 system was achieved by a temperature shift from 37°C to 50°C. The aim of our study was to examine whether transcriptional control is closely regulated by altered affinities between the mutant repressor and the six operators at different temperatures. To address the effect of the *cts*-52 mutation\textsuperscript{18}, site-directed polymerase chain reaction (PCR) mutagenesis was performed to generate a wild-type copy of wtcφ105\textsuperscript{8,21,22}.

A model for the role of cooperativity in the regulation of genetic switches is most characterised in the λ phage system. Pairs of cI dimers interact cooperatively to occupy adjacent operator sites at O\textsubscript{R} and at O\textsubscript{L}. These cI tetramers repress the lytic promoter P\textsubscript{R} and activate its own transcription from P\textsubscript{RM}. Transcription of cI is in turn negatively regulated by the Cro protein, which primarily represses P\textsubscript{RM}\textsuperscript{23–25}. A more complicated immunity mechanism has recently been characterised in *Salmonella*.
phage P22; this involves three immunity systems\textsuperscript{26--29}, showing that some phages utilise more than one immunity repressor to safeguard the control of immunity. In the \(\phi\)105 genome, an ORF adjacent to the repressor gene, denoted \textit{ORF4}, which is situated downstream from \(P_R\)\textsuperscript{10,11}, has never been studied (Figure 1). Sequence analysis showed that ORF4 is potentially another transcriptional regulatory protein. ORF4 shares a 37\% sequence identity with \textit{mtc}\(\phi\)105 and both proteins carry a typical DNA-binding \(\alpha\)helix-\(\beta\)turn-\(\alpha\)helix (HTH) motif at their N-termini. To examine whether ORF4 is also a transcriptional regulator that acts upon \(P_M\) and \(P_R\), it was overexpressed and its binding affinities towards the operators were compared with that of the repressor. The mRNA levels of \textit{mtc}\(\phi\)105 and \textit{ORF4} were also monitored by real-time RT-PCR so as to determine the influence of temperature shift on \textit{mtc}\(\phi\)105 and \textit{ORF4} transcription. Based on these findings, a model of the impact of thermo-induction on the transcriptional regulation within the \textit{immF} region is proposed.
Results

Sequence analysis of mtcφ105 and ORF4

Based on the results of nucleotide sequencing, the *cts*-52 mutation designated by previous studies\(^\text{16,17}\) was found to be a T to C transition that is equivalent to the *cts*-23 mutation\(^\text{3,4,18}\). This point mutation not only leads to an amino acid (a.a.) substitution from Thr17 to Ile (Figure 2A), but also renders the repressor thermo-inducible. Analysis of the φ105 genome sequence found that ORF4 coding sequence is located adjacent to the gene of the cφ105 repressor and shares a 37% a.a. sequence identity. A DNA-binding HTH motif was identified in the N-terminal regions (residues 18–37) of the 144 a.a *cts*-52 mutant repressor as well as of the 90 a.a. polypeptide of ORF4 (residues 22–41). Such similarities indicated that ORF4 is potentially another DNA-binding protein within the *immF* region and it is likely to interact with the same six operator sites as the repressor.

After the a.a. sequence of ORF4 was deduced by DNA sequencing, the sequence was blasted in GenBank and showed a high homology to the consensus sequence of a large DNA-binding protein family, termed HTH3. *λcI*, *λCro* and P22 are the prominent representatives in this family. The alignment of the protein sequences of the DNA-binding proteins (Figure 2B) is based, in part, on sequence homology and
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also on structural homology among them. The region of maximal sequence homology is found within a 20-residue sequence that aligns with the \( \alpha2 \) and \( \alpha3 \) helices of \( \lambda cI \), \( \lambda Cro \) and P22\(^{27,28,30}\).

The φ105 genome was searched for sequences that are closely related to the 14 bp-core region. A consensus sequence template was determined using the core sequences in \( OR1, OR2, OR3, OR4, OR5 \) and \( OR6 \). At least 28 regions were found to align with the consensus sequence \( GWCGKRAATWCMAK \) (W=A or T, K=G or T, R=A or G, M=A or C), including the \( OR1–OR6 \) (data not shown). In the thermo-inducible expression system\(^{14,15} \), the gene of interest is cloned downstream of a strong holin gene promoter\(^{14} \). However, no such consensus sequence was identified at the –35 or –10 RNA recognition regions of the holin gene promoter. A direct control of heterologous gene expression in the phage expression system is therefore unlikely.

**Identification of a novel protein designated ORF4**

To determine whether the \( ORF4 \) is a functional gene or a pseudogene, a RT-PCR was performed. Total RNA was extracted from wild-type \( B. subtilis \) strain 168 and the lysogen \( IA304(\phi105MU331) \). Contamination of genomic DNA was prevented by DNaseI treatment. As shown in Figure 3, PCR products of both ORF4 and the
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repressor were amplified from strain IA304(φ105MU331), but not from B. subtilis strain 168. The identity of the PCR product was verified by Southern hybridisation (data not shown). Samples that had not been reverse transcribed showed no detectable amplification (lane 3 and 7), indicating the absence of contaminating DNA.

Western blot analysis further verified the in vivo expression of mtcφ105 repressor and ORF4 coding sequence in IA304(φ105MU331) (Figure 4). Data showed that the sizes of mtcφ105 and ORF4 proteins were approximately 16 kDa and 13 kDa, respectively.

mtcφ105 and ORF4 transcription levels in the course of thermo-induction

To analyze the effect of thermo-induction on mtcφ105 and ORF4 transcription, total RNA was extracted at four time points: before induction, 10 minutes, 30 minutes and 60 minutes after induction. The expression levels of each target sequence were compared using the $2^{\Delta\Delta C_T}$ method. PCR efficiency of the target coding sequences (mtcφ105 and ORF4) and internal control (16SrRNA) was shown to be similar using real-time RT-PCR and TaqMan detection (Figure 5). Thus, relative quantification by the $2^{\Delta\Delta C_T}$ method was validated in these experiments. According to the data collected, the transcript level of mtcφ105 decreased moderately after thermo-induction and then gradually recovered (Figure 6). Following a 60 minute incubation, the expression
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level increased by three-fold. In the case of ORF4, expression was dramatically increased by 65-fold immediately after thermo-induction, followed by a moderate drop to approximately 30-fold. These observations suggested that the two ORFs were functional and expressed in IA304(φ105MU331), but that the amounts of transcript were not equivalent at various time points. In general, this indicated the PM and PR activities are influenced by a temperature shift.

Kinetic analysis of protein and operator sites interaction

To assess the interaction of the two proteins with PM and PR, real-time protein-DNA interaction is recorded continuously in terms of resonance units over a function of time, leading to a sensorgram which allows us to study the association and dissociation of protein-DNA complexes within the same experiment\textsuperscript{32}. To address the specificity of protein–operator interaction, two control experiments, using double-stranded oligonucleotides without a consensus sequence and a sensor flow cell containing no DNA as negative controls were performed. The control experiments showed no non-specific binding between the reference cell and the non-specific oligonucleotides (data not shown). Prior to the calculation of the equilibrium dissociation constant, all resonance units in the kinetic measurements were subtracted with the reference data.
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To evaluate the accuracy of the surface plasmon resonance (SPR) technique, equilibrium dissociation constants of repressor–operator site interactions obtained from SPR were compared with the results generated from *in vitro* repressor-binding competition experiments. Both experiments provided similar results. At room temperature (22.5°C), mtcφ105 showed the strongest binding to OR3 (K_D ~ 10^{-11} M), followed by OR1, OR2, then OR6 (Table 1). In contrast, ORF4 showed a very weak interaction (K_D ~ 10^{-7} M) towards OR3 that overlapped with its own gene. Both mtcφ105 and ORF4 showed low intrinsic affinity towards OR4 and OR5 at 22.5°C. This could be explained by the fast association and dissociation rates of the complexes. Surprisingly, although OR1, OR2 and OR3 bear an identical consensus sequence, they differ in protein-binding affinity. Seemingly, the flanking regions of these operators to some extent lead to discrepancies in DNA conformation, thereby affecting the binding affinities towards mtcφ105 and ORF4. In short, we conclude that both mtcφ105 and ORF4 are DNA-binding proteins that bind to multiple but non-identical 14-bp operator sites within the *immF* region. Specific binding to all operator sites was obtained, both with mtcφ105 and ORF4.

In order to examine the effect of temperature on these DNA–protein interactions, SPR analyses were performed at 37°C, with or without prior incubation of the proteins at 50°C (Table 1). Obviously, a temperature shift leads to an alternation in binding
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behaviour between the proteins and operators. At 37°C, both proteins showed an increase in affinity towards OR4 and OR5. The affinities of mtcφ105 towards OR2, OR3, OR4 and OR6 were strong and similar (KD ~ 10⁻¹⁰M). The interactions between mtcφ105 and OR1 or OR5 were ten-fold (KD ~ 10⁻⁹M) weaker. In the case of ORF4, a weak association with OR3 (KD ~ 10⁻⁸M) was recorded, while it was found to have comparable affinities (KD ~ 10⁻¹⁰M) towards the other five operator sites.

To determine whether thermo-induction alters these affinities, protein samples were incubated at 50°C for 10 minutes and then reincubated at 37°C before analysis. SPR analysis was then carried out at 37°C. Our data indicated that there was more than a ten-fold decrease in affinity in the interaction of mtcφ105 towards OR3, OR4, OR5 and OR6 when compared with that obtained without heat treatment. Under the same conditions, the binding profile of ORF4 was also altered. ORF4 showed a very strong affinity towards OR1 (KD ~ 10⁻¹³M) after heat incubation, i.e. a 10,000-fold increase. Additionally, the binding between ORF4 and OR3 seemed to be stronger than that measured before induction (KD ~ 10⁻⁹M).

Mutational analysis of thermo-inducible repressor

A wild-type repressor was prepared by site-directed PCR mutagenesis. Interactions between the wild-type repressor and the six operator sites were assayed at 37°C,
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before and after heat treatment (Table 2). At 37°C, the binding behaviour of the
wild-type repressor was similar to that of the mutant repressor. However, unlike the
mutant repressor, the binding affinities of the wild-type repressor towards the six
operators were not affected by heating. Even after heat treatment, no significant
alternation in binding was observed.
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Discussion

Most of the studies on bacteriophage φ105 focused on the regulatory role of cφ105 in maintaining lysogeny but ignored the existence of ORF4 in the close proximity. Our data suggested that ORF4 is a functional polypeptide and its expression is regulated by the repressor.

The repressor and ORF4 isolated from IA304(φ105MU331) are likely to belong to the large family of HTH DNA-binding proteins, designated as HTH3. Both proteins showed homology to the α3 helix of λcI that presents DNA recognition specificity. Western blot analysis indicated that proteins of the two ORFs were expressed in vivo and were able specifically to bind to the six individual operators. It is believed that ORF4 is potentially another DNA-binding protein within the immF region of the φ105 genome.

To determine whether both proteins bind to various operators at different affinities and to examine whether their affinities are affected by a temperature shift, protein-DNA interaction was measured in vitro using SPR technology. The SPR technique has an advantage over traditional electrophoretic mobility shift assays, since it can also measure the association and dissociation rates of the protein–DNA interaction. Therefore, this technique prevents underestimation of an affinity constant.
as a result of a high dissociation rate of the complexes, and vice versa.

In the $\phi 105MU331$ expression system, cells bearing a heterologous gene construct were grown at 37°C and then thermo-induced at 50°C. Subsequently, heterologous protein was detected after 30 minutes’ incubation at 37°C. To imitate this physical change, purified proteins were incubated at the desired temperature prior to SPR. The activities of the $P_M$ and $P_R$ promoter were also monitored by their gene products, $mtc\phi 105$ and $ORF4$ mRNAs, using real-time RT-PCR analysis. Based on these findings, a model of $P_M$ and $P_R$ modulation within the immF region is proposed and illustrated in Figure 7. Before thermo-induction, mtc$\phi 105$ could bind to all six operators with a similar level of affinities. It is likely that its binding to $O_R1$, $O_R2$ and $O_R4$ could stimulate $P_M$, while its interaction with $O_R5$ and $O_R6$ could suppress $P_R$, as proven by the CAT activity assay. Moreover, binding of mtc$\phi 105$ to $O_R3$ indefinitely hinders $ORF4$ expression (Figure 7A). The above assumptions are confirmed by the transcript levels of mtc$\phi 105$ (1.05 x $10^5$ copies/0.1 µg total RNA) and ORF4 (2.29 x $10^2$ copies/0.1 µg total RNA) at the lysogeny state (data not shown).

After thermo-induction, the affinities of mtc$\phi 105$ towards $O_R4$–$6$ were moderately reduced (30–60 fold). Release of mtc$\phi 105$ from $O_R3$, $O_R5$ and $O_R6$ derepressed $P_R$ activity and permitted $ORF4$ transcription. Thus, a dramatic increase in $ORF4$ transcript level was observed (>60 fold). Thermo-induction also induced a drastic
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increase (>1000 fold) in the affinity of ORF4 towards O\(_R\)1 (Figure 7B). A moderate fall in mtc\(\phi\)105 transcript level was observed after thermo-induction (Figure 6), which could be either attributed to the strong binding of ORF4 to O\(_R\)1, or the loss of binding of mtc\(\phi\)105 to O\(_R\)4, or both.

Our data further confirmed that wtc\(\phi\)105 is insensitive to thermo-induction, which is also supported by Rutberg L and colleagues\(^5\). Hence, the cts-52 mutation on mtc\(\phi\)105 is the sole critical protein responsible for the modulation of the genetic switch in the \(\phi\)105\textit{MU331} expression system.

In the \(\phi\)105\textit{MU331} expression system, the expression of the heterologous gene was controlled by the promoter of the holin gene, which is located 10.6 kb from the \textit{immF} region. Although thermo-induction was found to turn on the promoter activity of the holin gene\(^19\), it was deemed unlikely that mtc\(\phi\)105 nor ORF4 had any direct interaction with this promoter, as no consensus operator sequence was identified at its upstream region. It is supposed that the binding of mtc\(\phi\)105 to certain operator sequences in the \(\phi\)105 genome will block either the expression of ORF4 or other trans factor necessary for triggering heterologous gene expression. To further elucidate the sequence of events, a microarray displaying all 51 ORFs (GenBank accession number: AB016282) in the bacteriophage genome in both the forward and reverse directions should provide more information.
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In summary, we conclude that ORF4 is a putative DNA-binding protein. It is a novel transcriptional regulator discovered in bacteriophage φ105. Both mtcφ105 and ORF4 respond to thermo-induction, which in turn directs the expression of the heterologous gene in the φ105MU331 system. It is likely that mtcφ105 and ORF4 in bacteriophage φ105 resemble the genetic switch of the lambda phage cI and Cro system. These two proteins may be tightly counterbalanced to respond to environmental stress.
Materials and Methods

Bacterial strains

Details of the bacterial strains and phage employed in this study are listed in Table 3. *E. coli* strain JM109 (Stratagene) was used for plasmid construction and *E. coli* strain BL21(DE3) (Stratagene) was used as an expression host for the pGEX vectors (Amersham Biosciences).

Wild-type *B. subtilis* strain 168 was used in this study as a control. The detection of *mtc*φ105 and ORF4 was performed in *B. subtilis* strain IA304 carrying a φ105MU331 prophage designated IA304(φ105MU331). This φ105 derivative prophage carries an *ind* mutation, which restores almost normal competence, and a *cts*-52 mutation, which confers thermo-induction.

One-step RT-PCR and Southern Blot analysis

To detect the presence of *mtc*φ105 and ORF4 mRNA transcripts in *B. subtilis* IA304 (φ105MU331), one-step RT-PCR and Southern hybridisation were performed. Single colonies of *B. subtilis* strain 168 and IA304 (φ105MU331) were cultivated for 12 hours at 37°C and 280 rpm in 5 ml brain heart infusion (BHY) medium (3.7%(w/v) (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 5 µg/ml erythromycin). One millilitre of the overnight culture was sub-cultured the next day in 15 ml BHY medium without
antibiotic until the OD$_{600}$ reached 3.0. The culture was then heat induced in a 50°C water bath for 4 minutes, with vigorous shaking, followed by re-incubation at 37°C. Bacterial cells were collected at four time points: before induction, and 10 minutes, 30 minutes and 60 minutes after induction.

Total RNA was extracted by the RNease Mini Kit (QIAGen) and treated with DNase I (DNA-free kit, Ambion) to remove contaminating DNA. After removal of DNase I, the integrity of the RNA samples was monitored by gel electrophoresis and OD$_{260}$ measurement. Fifty nanograms of total RNA were added as the PCR template in individual PCR reactions using the OneStep RT-PCR System (QIAGen). Additionally, two negative controls were prepared, either in the absence of RNA templates or by omitting the reverse transcription step. Two pairs of gene-specific primers, ORF1BamS/ORF1EcoA and ORF4BamS/ORF4EcoA (Table 4), were employed for mtc$\phi105$ and ORF4 amplification, respectively. Each primer was added at a final concentration of 0.6 $\mu$M. The RNA samples were reverse-transcribed at 50°C for 30 minutes, followed by incubation at 95°C for 15 minutes before amplification. Forty amplification cycles were then carried out by denaturation of cDNA at 94°C for 30 seconds, annealing at 50°C for 40 seconds and polymerization at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes. The same volume of PCR products was loaded onto 1% (w/v) formaldehyde gel and visualised under UV
transillumination. To avoid cross-contamination, filtered tips and separate areas were used for RNA extraction and RT-PCR.

The identity of the PCR product was verified by non-radioactive Southern hybridization by applying gene-specific DNA probes labelled with digoxigenin (Roche). An \textit{mtc}\textit{φ}105-specific probe was synthesized by PCR using the primer pair ORF1PS/ORF1PA, whereas the \textit{ORF4}-specific probe was synthesized using the primer pair ORF4PS/ORF4PA. All working procedures were followed as recommended by the manufacturer. Chemiluminescent detection was conducted using CSPD (Roche) as substrate.

**Real-time RT-PCR**

We assessed the relative quantity of \textit{mtc}\textit{φ}105 and \textit{ORF4} mRNA transcripts at various time points using real-time RT-PCR (Bio-Rad iCycler iQ Detection System) and the TagMan MGB probes (Applied Biosystems) (Table 4). One hundred nanograms of total RNA was added to 25 µl of TaqMan EZ RT-PCR Core Reagent (Applied Biosystems) in each reaction. No Template Controls (NTC) were prepared in parallel reactions to test for contamination. All the reaction mixtures were transferred to a 96-well PCR plate (Bio-Rad) and subjected to initial AmpErase UNG (uracil N-glycosylase) treatment at 50°C for 2 minutes, followed by reverse transcription at
60°C for 30 min. Deactivation of UNG was carried out at 95°C for 5 minutes and a PCR amplification protocol of 40 cycles of denaturation at 94°C for 20 seconds and annealing at 60°C for 1 minute was performed.

We adopted a relative quantification approach in which the expression levels of the target genes were compared to the data obtained at a time point before thermo-induction. The mean fold change in expression of the target gene at each time point was calculated using the $2^{\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T, \text{Target}} - C_{T, \text{16SrRNA}})_{\text{Time x}} - (C_{T, \text{Target}} - C_{T, \text{16SrRNA}})_{\text{Time 0}}$. The amount of mtcφ105 and ORF4 mRNA transcripts was normalised to the expression of B. subtilis 168 16SrRNA in each sample. To validate the $\Delta\Delta C_T$ calculation, the amplification efficiency of the target and internal control were assessed by preparing a serial dilution of total RNA. For each dilution, samples were amplified using primers and fluorogenic probes for mtcφ105, ORF4 and 16SrRNA. The average $C_T$ was determined and a plot of $C_T$ versus the log total RNA dilution was analysed. When the slope is close to zero, the PCR efficiency of the target and internal control is similar.

**DNA manipulation and plasmid construction**

In order to overexpress mtcφ105 and ORF4 proteins, plamids pCφ105 and pORF4 were constructed. Gene-specific primer pairs composed of specific restriction sites at the 5’ends, ORF1BamS/ORF1EcoA and ORF4BamS/ORF4EcoA, (Table 4), were
used for mtcφ105 and ORF4 amplification, respectively. Genomic DNA from B. subtilis IA304 (φ105MU331), employed as a template, was firstly denatured at 94°C for 3 minutes, followed by 30 amplification cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds and polymerization at 75°C for 1 minute. A final extension was carried out at 75°C for 5 minutes. All amplification steps were performed using Pfu polymerase (Invitrogen) to enhance fidelity. After restriction enzyme digestion, the PCR products were ligated to the BamHI and EcoRI sites of the expression vector pGEX-2T (Amersham Biosciences), at a position downstream of the glutathione-S-transferase (GST)-tag. The recombinant plasmids designated pCφ105 and pORF4 were transformed into JM109 by electroporation. Positive clones were selected on Luria-Bertani (LB) agar plates supplemented with 100 µg/ml ampicillin and PCR screened. All plasmids were sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Subsequently, the clones were transformed into the expression E. coli host BL21(DE3).

**Site-directed PCR mutagenesis**

Site-directed PCR mutagenesis was performed in a three-step PCR reaction. Template DNA used for PCR mutagenesis was pCφ105, and the primers are listed in Table 4. The first PCR was carried out with primer ORF1BamHS and primer wtA. The second PCR was performed using primer wtS and primer ORF1EcoA. The DNA fragments
bearing the wild-type cφ105 sequences were annealed in a 1:1 ratio by heating at 95°C for 10 minutes and then allowed to cool down at room temperature for 1 hour. A final PCR was performed using primer pairs ORF1BamS/ORF1EcoA. Finally, the PCR product was digested and ligated to pGEX-2T (Amersham Biosciences) as described above.

**Protein overexpression and purification in E. coli**

LB broth supplemented with 2% (w/v) glucose and 100 µg/ml ampicillin was used to grow BL21(DE3) strain. Induction was achieved by the addition of 0.1 mM IPTG (Sigma) when the cells reached the log phase. The cells were then incubated at 25°C for 3 hours. Expression of both fusion proteins, GST-mtcφ105 and GST-ORF4, was confirmed by SDS-PAGE and Western blot analysis using anti-GST antibodies (Amersham Biosciences).

The fusion proteins were purified using Glutathione Sepharose4B matrix (Amersham Biosciences). After sample loading and column washing, thrombin was loaded for the cleavage of the GST-tag. Target proteins were then eluted. The protein identities were verified by using the Protein Sequencer G1000A System (Hewlett Packard).

**SDS-PAGE and Western blot analysis of mtcφ105 and ORF4** expression in *B.*
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Twenty $\mu$g of protein extracts from various time points were examined on 15% SDS-PAGE, followed by Western Blot analysis. Purified mtc$\phi 105$ and ORF4 polypeptides were applied as positive controls. Protein concentration was determined by a Bio-Rad dye-binding assay, using IgG as the standard.

**Kinetic analysis of protein-DNA interaction**

To study the interaction of mtc$\phi 105$ and ORF4 with the six operator sequences, BIAcore 2000 (BIAcore AB) was used. By monitoring the adsorption of biomolecules on a sensor chip, real-time protein–DNA interaction was measured. This optical technique measures changes in refractive index in the vicinity of the surface. Such changes are directly proportional to the change in adsorbed mass and are expressed in resonance units (RU); 1000 RU corresponds to a surface concentration of ~1 ng/mm².

Prior to DNA immobilisation on the sensor chip matrix, biotinylated oligonucleotides were annealed to their complementary oligonucleotides (Table 4), by being heated at 95°C for 10 minutes and allowed to cool at room temperature for 1 hour. The double-stranded oligonucleotides individually possessed of the six operator-consensus sequences were denoted, in order, as $O_{R1}$, $O_{R2}$, $O_{R3}$, $O_{R4}$, $O_{R5}$ and $O_{R6}$. Addition of a spacer at the N or C terminus was based on the sequence of the parent DNA
sequences to avoid steric hindrance during protein binding. A pair of duplex oligonucleotides without the consensus sequence was designed to test for non-specific binding.

Biotinylated double-stranded oligonucleotides were immobilised on a streptavidin-coated SA chip (Amersham Biosciences). The procedure was followed according to the method described by Blaesing and colleagues. About 100 RU DNA was immobilised on the sensor chip matrix. This relatively low concentration of DNA avoids mass transport limited conditions that may interfere with kinetic measurements.

In each sensor chip, a blank surface without DNA was included as a reference. After oligonucleotide immobilisation, the sensor chip surface was subsequently saturated with biotin.

In order to examine the binding between mtcφ105 and ORF4 with the six operator sequences, purified proteins were diluted to a concentration range from 0.8 nM to 40 nM, so as to perform a precise kinetic measurement. Analyses were performed at a flow rate of 100 µl/min in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v)). To simulate thermo-induction, protein samples were incubated at 50°C for 10 minutes and then at 37°C for 10 minutes before analysis. At every measurement, 250 µl protein was injected. Refractive deviations of the different sensor chip surfaces were normalised at the beginning of
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Each measurement. For regeneration, the sensor chip surface was briefly pulsed by 10 mM glycine-HCl, pH 3.0, to remove bound protein. Data analysis was performed with BIAevaluation 2.1 software (BIAcore). All data were handled after subtraction of the reference data obtained from a sensor flow cell containing no DNA (data not shown). These data were successfully fitted to a 1:1 Langmuir binding model.

Statistics

The results of the quantitative PCR data were analysed by One Way ANOVA, using SPSS version 11.0, and ranked by Turkey test into different groups. The relationship between the level of expression corresponding to each studied coding sequence and the time point after thermo-induction was evaluated by simple linear regression. The significance of the model was assessed by analysis of variance of the regression. Statistical significance was set at a p-value of less than 0.05.

Acknowledgements

We would like to thank Dr. Thomas Leung for providing the Bacillus strains and Dr. Van-Kaer for his advice on protein expression. We are also grateful to Miss Elisa Lau for her assistance in the operation of the BIAcore biosensor.
References


Interaction of cφ105 and ORF4 with operators


Interaction of cφ105 and ORF4 with operators


Interaction of cφ105 and ORF4 with operators


Interaction of cφ105 and ORF4 with operators

operator DNA fragments with the lambda-cro repressor coupled with changes in their conformation. *EMBO J.* 6:1129-1135.


Figure legends

Figure 1. Orientation of the six operator sites and location of the two ORFs ($c\phi105$ and $ORF4$) within the imm$F$ region$^6$. Generally, OR1, OR2 and OR4 sites are located closer to P$_M$. OR1 and OR2 are positioned upstream from the RNA polymerase recognition site (-35), while OR4 overlaps with this sequence. OR5 and OR6 are positioned near to P$_R$ and the latter overlaps with the RNA polymerase recognition site (-10). OR3 is located within the coding sequence $ORF4$.

Figure 2. Protein alignment. (A) Comparison of mtC$\phi105$ and ORF4 polypeptides. Identical residues are shaded by grey boxes. The Thr17$\rightarrow$Ile mutation that renders mtC$\phi105$ thermo-inducible is squared. (B) Alignment of the $\alpha$helix-$\beta$turn-$\alpha$helix motif. Conserved residues are underlined and the asterisk indicates the position of “turn”. The ninth glycine residue is an amino acid favourable for forming a short loop between the two helices.

Figure 3. RT-PCR analysis. mtC$\phi105$ (lane 4) and $ORF4$ transcripts (lane 8) were detected in $B. subtilis$ IA304($\phi105MU331$) but not in $B. subtilis$ strain 168 (lanes 2 and 6). Two negative controls, without the addition of RNA templates (lanes 1 and 5)
Interaction of cφ105 and ORF4 with operators

or by omitting the reverse transcription step (lanes 3 and 7), are shown.

Figure 4. Western blot analysis of the mtcφ105 and ORF4 proteins after thermo-induction. The protein bands of 16 kDa and 13 kDa were mtCφ105 and ORF4, respectively.

Figure 5. Validation of the $2^{\Delta\Delta C_T}$ method in relative quantification of mtcφ105 and ORF4 transcript levels. The data were fitted using least-squares linear regression analysis (n=4). When the slope is close to zero, the PCR efficiency of the target and internal control is similar.

Figure 6. Fold-changes in the mRNA levels of mtcφ105 and ORF4. The data were normalised to the expression of 16SrRNA at various time points. Horizontal bars represent the geometric means for four replicates at each time point (n=4).

Figure 7. A model on the modulation of P_M and P_R by mtcφ105 and ORF4 at the immF region of the Bacillus phage φ105 genome. Interaction of mtcφ105 and ORF4 with individual operators are illustrated (A) before and (B) after thermo-induction. (*) represents a competition; □ represents promoter activation; △, represents promoter repression.)
Interaction of cφ105 and ORF4 with operators

Table 1. Equilibrium dissociation constants of mtcφ105 and ORF4 towards the six operator sites.

Table 2. Differences in the equilibrium dissociation constants between the mutant repressor and wild-type repressor.

Table 3. Bacterial strains and bacteriophage employed in this study.

Table 4. Oligonucleotides for PCR, RT-PCR, DIG-probe, TaqMan® probe synthesis and surface plasmon resonance.
Interaction of cφ105 and ORF4 with operators

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>mtcφ105 K_{D}(nM)</th>
<th>ORF4 K_{D}(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_b1</td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>O_b2</td>
<td>0.89</td>
<td>0.17</td>
</tr>
<tr>
<td>O_b3</td>
<td>0.03</td>
<td>719</td>
</tr>
<tr>
<td>O_b4</td>
<td>N.D b</td>
<td>N.D b</td>
</tr>
<tr>
<td>O_b5</td>
<td>N.D b</td>
<td>N.D b</td>
</tr>
<tr>
<td>O_b6</td>
<td>3.29</td>
<td>3.43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>22.5°C a</th>
<th>37°C a</th>
<th>50°C → 37°C a</th>
<th>22.5°C a</th>
<th>37°C a</th>
<th>50°C → 37°C a</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_b1</td>
<td>0.60</td>
<td>1.01</td>
<td>0.30</td>
<td>0.09</td>
<td>0.25</td>
<td>0.00018</td>
</tr>
<tr>
<td>O_b2</td>
<td>0.89</td>
<td>0.26</td>
<td>0.24</td>
<td>0.17</td>
<td>0.11</td>
<td>0.87</td>
</tr>
<tr>
<td>O_b3</td>
<td>0.03</td>
<td>0.36</td>
<td>21.9</td>
<td>719</td>
<td>42.4</td>
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<tr>
<td>O_b4</td>
<td>N.D b</td>
<td>0.64</td>
<td>24.2</td>
<td>N.D b</td>
<td>0.44</td>
<td>0.92</td>
</tr>
<tr>
<td>O_b5</td>
<td>N.D b</td>
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<td>38.3</td>
<td>N.D b</td>
<td>0.98</td>
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<tr>
<td>O_b6</td>
<td>3.29</td>
<td>0.52</td>
<td>26.1</td>
<td>3.43</td>
<td>0.18</td>
<td>0.29</td>
</tr>
</tbody>
</table>

a Real-time interaction was monitored at 22.5°C, 37°C or after incubation at 50°C prior to analysis at 37°C. Results were obtained from two independent measurements, with protein concentrations ranging from 0.8 nM to 40 nM. These data successfully fitted into the 1:1 Langmuir binding model.

b Not determined because of fast association and dissociation rate.
Interaction of cφ105 and ORF4 with operators

Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild-type cφ105 $K_D$ (nM)</th>
<th>Mutant cφ105 $K_D$ (nM)</th>
<th>Wild-type cφ105 $K_D$ (nM)</th>
<th>Mutant cφ105 $K_D$ (nM)</th>
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</thead>
<tbody>
<tr>
<td>O1</td>
<td>1.18</td>
<td>1.01</td>
<td>1.04</td>
<td>0.30</td>
</tr>
<tr>
<td>O2</td>
<td>0.28</td>
<td>0.26</td>
<td>0.13</td>
<td>0.24</td>
</tr>
<tr>
<td>O3</td>
<td>0.52</td>
<td>0.36</td>
<td>0.28</td>
<td>21.9</td>
</tr>
<tr>
<td>O4</td>
<td>0.59</td>
<td>0.64</td>
<td>0.28</td>
<td>24.2</td>
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<tr>
<td>O5</td>
<td>1.73</td>
<td>1.23</td>
<td>1.72</td>
<td>38.3</td>
</tr>
<tr>
<td>O6</td>
<td>0.26</td>
<td>0.52</td>
<td>0.30</td>
<td>26.1</td>
</tr>
</tbody>
</table>

* Real-time interaction was monitored at 37°C or after incubation at 50°C prior to analysis at 37°C.

Results were obtained from two independent measurements, with protein concentrations ranging from 0.8 nM to 40 nM. These data successfully fitted into the 1:1 Langmuir binding model.
### Table 3

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>ATCC 27370&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IA304</td>
<td>trpC2 metB52 sin-l SPβS</td>
<td>Leung <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17(rK- mK+) supE44 relA1 D(lac-proAB) F’ traD36 proAB lacPZDM15</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>E. coli B F- dcm ompT hsdS(rK- mK-) gal l (DE3)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Bacteriophage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ105MU331</td>
<td>ind-125 cts-52Ω[lacZ'[ClaI]-ermC-cat'[NcoI]]331A(DI:1t)</td>
<td>Leung <em>et al.</em>, 1995</td>
</tr>
</tbody>
</table>

<sup>a</sup> ATCC= American Type Culture Collection
### Interaction of φ105 and ORF4 with operators

**Table 4**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Orientation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene-specific primers for PCR and RT-PCR</td>
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<td></td>
</tr>
<tr>
<td>ORF1BamS</td>
<td>AAGGATCCATGACTGAGGGCAAGAA</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>ORF1EcoA</td>
<td>AGAATTCCTATCCTGTATCTTTTCT</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>ORF4BamS</td>
<td>AAGGATCCATGCTGGATGGGAAAAAGCT</td>
<td>Sense</td>
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<tr>
<td>ORF4EcoA</td>
<td>AGAATTTCTCTCCTAGCGCTCCTTCT+CA</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>16SF</td>
<td>TCCGCAATGGACGAAAGTCT</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>16SA</td>
<td>ACATCGCAGAAACCTTCATCA</td>
<td>Antisense</td>
<td></td>
</tr>
</tbody>
</table>

| Primers for PCR DIG-DNA probe preparation |
| ORF1PS     | AACTGGCTGAAAGCCAAATCT | Sense |
| ORF1PA     | AGTCTTTTCTTTTCTTCAT | Antisense |
| ORF4PS     | CACTTGAAACAGACGAAATGG | Sense |
| ORF4PA     | TCCTCAACTACTTTGATTTCC | Antisense |

| Primers for site-directed PCR mutagenesis |
| wtS        | GGAACGTAATTTACCCAGAATGAA | Sense |
| wtA        | GTTGCAACTCTGGTTAATTACCTTCC | Antisense |

| Sequences of TaqMan MGB probe applied |
| FAM'ORF1   | FAM-CCCTGTTGGGCT | Sense |
| FAM'ORF4   | FAM-GTATCCGAACGGCA | Sense |
| VIC'16SrRNA| VIC-ACGGAGCAAGCCGGTGTA | Sense |

| Oligonucleotides for surface plasmon resonance |
| B'Or1S     | 5' (B)-aaaaaaGACGGAAATAACAGAtatttt | Sense |
| B'Or2S     | 5' (B)-taaattGACGGAAATAACAGAaatt | Sense |
| B'Or3S     | 5' (B)-aaaaatGACGGAAATAACAGTagttg | Sense |
| B'Or4S     | 5' (B)-ccgaatGTCGGAAATAACAGTactaa | Sense |
| B'Or5S     | 5' (B)-aaaaatGACGAAATCCAGAaatt | Sense |
| B'Or6S     | 5' (B)-caattgtcagctaaagatgaagca | Sense |
| B'Non-boxS | 5' (B)-caattgtcagctaaagatgaagca | Sense |

1. All Taqman MGB probes are 3’ labelled with non-fluorescent minor groove binder as the quencher.
2. Complementary oligonucleotides without labelling were prepared for double-strand annealing.

---

39
A

mtcφ105

1 -----MTVGQRIKAIRKERKLIQVQLAEKANLSRSYLADIERDRYPSLSTLEAVAGALGI
ORF4

1 MLDGKKLALIKORRKSKEEKALTERKLQTEMAKALGMSRTYLDIENGRLPSTKLSRINILNL

mtcφ105

57 QVSAIVGEETLIKEEQAEYNSKEEKDIAKRMEEIRKDLEKSDKLSFSGEPMSQEAVEFLM
ORF4

61 DLNLKLMTEIQVVEE--G------------------------------HYDRAAGTCRRCA-------

mtcφ105

117 EAMEHIVRQTQRINKKYTPKKYRNDDQE
ORF4

-------------------------------------

B

mtcφ105

1QVLOAEKANLSRSYLADIER20
ORF4

1QTEMALKGMSRTYLDIEN20
P22

1QAAALGKMVGSNVAISQWER20
λCro

1QTKTDGPLVQSAINKAIH20
λCI

1QESVADKMQGQSGVGAFLN20

--αhelix--βturn-----αhelix--
\[ y = 0.0155x + 14.91 \]
\[ R^2 = 0.0065 \]

\[ y = -0.012x + 19.67 \]
\[ R^2 = 0.3034 \]
Fold changes in the expression of mtc phi 105

Fold changes in the expression of ORF4
A  Before thermo-induction

B  After thermo-induction