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Controlled cellular redox, repressive hemin utilization and adaptive stress responses are crucial to metronidazole tolerance of *Porphyromonas gingivalis* persisters

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Clinical Relevance

Scientific rationale for the study: Microbial persisters are critical for recalcitrance and relapse of infectious and inflammatory diseases. It remains unknown whether and how *P. gingivalis* forms persisters in response to periodontal treatment. The understanding of its persister formation in oral/periodontal niches may help develop new strategies for effective prevention and management of periodontitis.

Principal findings: The ability of *P. gingivalis* to form metronidazole-tolerant persisters is mainly determined by regulation of cellular redox state, and hemin could be an important mediator of *P. gingivalis* persistence.

Practical implications: *P. gingivalis* persisters as specific phenotypes may represent an undefined mechanism underlying persistent periodontal infection and inflammation.

Conflict of interest and source of funding

The authors declared that they have no conflict of interest.

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Abstract

Background: Antimicrobial-tolerant microbial persisters critically account for various infections and inflammation. This study identified the characteristics of *Porphyromonas gingivalis* persisters, and explored their underlying survival mechanisms through proteomic profiling.

Methods: *P. gingivalis* cultured with different concentrations of hemin was treated with 100 μg/ml of metronidazole (MTZ). The viability of *P. gingivalis* persisters was determined by colony-forming unit assay and LIVE/DEAD staining. The proteomic signature of *P. gingivalis* persister fractions was examined using LC-MS/MS and bioinformatic analysis.

Results: A small fraction of *P. gingivalis* persisters survived from lethal MTZ treatment without heritability. At late exponential phase, the frequency of these persisters significantly increased when incubated with 1 μg/ml of hemin compared to 10 μg/ml. Higher levels of *P. gingivalis* persisters formed at stationary phase than the late exponential phase. High-throughput proteomic analysis showed that the persisters markedly downregulated multiple proteins involved in electron transfer and heme/iron utilization essential for redox regulation and MTZ activation. Moreover, the persisters

enabled to shut down major cellular activities (e.g. translation) and overexpress stress proteins.

Conclusion: The presence and survival of metronidazole-tolerant *P. gingivalis* persisters may be dominated by regulation of cellular redox state and enhanced via repression of heme/iron utilization, dormancy and stress responses.

INTRODUCTION

Microbial persisters represent a small subpopulation of phenotypic variants highly tolerant to antimicrobials in a community of genetically susceptible microorganisms, and they are distinct from drug-resistant mutants with acquired genetic resistance (Lewis, 2010). In the presence of lethal antimicrobials, the bulk of a microbial population is killed but the remaining drug-tolerant persisters survive. They resume growth once antimicrobial treatment ceases, and form a new population as sensitive to antimicrobials as the original one. Persisters have been identified in various microbes, e.g., *Escherichia coli*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*; and they are deemed as the main culprit for the recalcitrance of long-lasting infections and chronic inflammation (Helaine & Kugelberg, 2014). The formation and survival of persisters are mainly attributed to their dormancy and multiple cellular pathways (Maisonneuve & Gerdes, 2014). However, microbial persistence involves complex survival mechanisms that need to be further investigated (Helaine & Kugelberg, 2014).

Severe periodontitis is the 6th most prevalent disease in humans with a global prevalence of 11.2%; and yet it is a leading cause of multiple tooth loss and edentulism in adults with huge healthcare burden worldwide (Jin et al., 2016; Kassebaum et al., 2014;

Listl et al., 2015; Tonetti et al., 2017). *Porphyromonas gingivalis* as a keystone periodontopathogen critically contributes to the breakdown of host-microbe symbiosis and resultant immuno-inflammatory destruction of tooth-supporting tissues (Hajishengallis et al., 2012). Its presence in oral niches is closely linked to unresolved periodontal lesions and progressive bone loss (Chaves et al., 2000; Mombelli et al., 2000). The patients with persistence of *P. gingivalis* and other pathogens respond unfavorably to comprehensive periodontal therapy (Colombo et al., 2012). Adjunctive use of systemic antibiotics like metronidazole may reduce the levels of periodontal pathogens, and offer additional benefits to treatment outcomes (Keestra et al., 2015; Soares et al., 2014). Although *P. gingivalis* develops various strategies to evade and undermine host defense, it remains unknown whether it harbors persisters and the underlying survival mechanisms.

At diseased sites, the influx of inflammatory exudates and vascular ulceration provide high amount of hemoproteins. The virulence of *P. gingivalis* and its interactions with host cells are markedly affected by various microenvironmental factors such as hemin and temperature (Al-Qutub et al., 2006; Curtis et al., 2011; Herath et al., 2013). Notably, mass spectrometry-based high-throughput proteomics provides a powerful platform for protein profiling of targeting biological systems (Zhang et al., 2013), and it has been increasingly applied in biomedical studies on microbial persisters (Conlon et al., 2013; Li et al., 2015). This study investigated the characteristics of metronidazole-tolerant *P. gingivalis* persisters, and explored their survival mechanisms via the high-throughput shotgun proteomic approach.

MATERIALS AND METHODS

Bacterium and culture conditions

P. gingivalis (ATCC 33277) was inoculated on horse blood agar (HBA) plates and incubated in an anaerobic chamber (5% CO_2, 10% H_2 and 85% of N_2) at 37°C. Planktonic *P. gingivalis* cultures were prepared in trypticase soy broth (BD, Sparks, MD) supplemented with yeast extract (5 mg/ml; BD), vitamin K (1 µg/ml; Sigma-Aldrich, St. Louis, MO) and filter-sterilized hemin (0, 1 and 10 µg/ml; Sigma-Aldrich). Bacterial growth was monitored by measuring the optical density (OD660) in SpectraMax® M2 Multimode Microplate Reader (Molecular Devices Ltd., Sunnyvale, CA).

Antimicrobial susceptibility

Stock solutions of metronidazole (MTZ) (Sigma-Aldrich) were prepared in sterile water. The minimum inhibitory concentration (MIC) of MTZ against *P. gingivalis* was determined by broth microdilution method following the standard (M11-A8) of Clinical and Laboratory Standards Institute (2012). Serial 2-fold dilutions of MTZ (100 µl) were made in culture media with hemin (0, 1 and 10 µg/ml) in 96-well plates (Thermo Fisher Scientific, Waltham, MA). Bacterial suspension (100 µl) was added with approximately 1×10^6 colony-forming units (CFU)/ml. The plates were incubated anaerobically at 37°C for 48 h. All experiments were repeated three times.

P. gingivalis persister viability

P. gingivalis was grown in broth containing 10 µg/ml of hemin (hemin-repletion), 1 µg/ml of hemin (hemin-limitation) or no added hemin (hemin-deficiency) for 48 h. The cultures

were diluted in fresh media to OD660 of 0.1 and incubated to late exponential phase (40 h) or stationary phase (88 h) followed by MTZ treatment (100 μg/ml) in fresh media. At 0, 6 and 24 h, an aliquot of the cultures was washed twice with PBS, serially diluted and plated on HBA for counting CFU using Autoplate® Spiral Plating System (Advanced Instruments, Norwood, MA). The colonies were analyzed 4 to 7 days after incubation. The survival rate of persisters was calculated on the viable cells of total cells in an untreated culture.

Heritability of *P. gingivalis* persistence

P. gingivalis colonies recovered from 6-h MTZ (100 μg/ml) treatment of a late exponential-phase culture under hemin-limitation condition were inoculated into fresh broth with 1 μg/ml hemin. After 48-h incubation, the bacterial suspension was diluted to OD660 of 0.1 and grown to late exponential phase (40 h) for quantitation of persisters. The MIC of MTZ against *P. gingivalis* recovered from the persisters was determined as aforementioned.

LIVE/DEAD staining and confocal laser scanning microscopy (CLSM)

The viability of MTZ-treated, late exponential-phase *P. gingivalis* was further evaluated by LIVE/DEAD staining and CLSM. After 6 and 24 h MTZ (100 μg/ml) treatments, the cells were washed twice with PBS and stained with LIVE/DEAD BacLight Viability kit (Molecular Probes, Eugene, OR) in dark for 30 min. The untreated samples served as the controls. The stained samples were placed onto a glass slide under a coverslip, and assessed by a CLSM system (FLUOVIEW FV 1000; Olympus, Tokyo, Japan).

Protein extraction and digestion

The late exponential-phase *P. gingivalis* cultured under conditions of hemin-limitation and hemin-repletion was treated with MTZ (100 μg/ml) for 6 h. The MTZ-exposed cells and the untreated controls from three biological replicates were washed twice with PBS and lysed by B-PER® Bacterial Protein Extraction Reagent with Enzymes (Pierce Biotechnology, Rockford, IL) as per manufacturer's instructions. After centrifugation (15,000 g) for 5 min, the protein concentration in supernatants was determined by Bradford assay (Bio-Rad, Hercules, CA). Proteins were precipitated in acetone at -20°C for 30 min and re-suspended in 0.1 M Tris-HCl containing 8 M urea for in-solution trypsin digestion (Li et al., 2015). Each sample (100 μg) was reduced with 20 mM dithiothreitol for 20 min, and alkylated with 25 mM of iodoacetamide for 30 min at room temperature. Subsequently, proteins were digested with sequencing-grade trypsin (Promega, Madison, WI) in an enzyme-to-protein ratio of 1:100 (w/w) at 37°C for 16 h. The peptide mixtures were acidified by formic acid to stop trypsin digestion. After centrifugation, the supernatants were desalted on StageTips (Rappsilber et al., 2007), dried down in SpeedVac (Thermo Fisher Scientific) and re-suspended with 0.1% formic acid.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The peptide samples were analyzed by LC-MS/MS using a nanoflow high-performance liquid chromatography (HPLC) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Briefly, the samples were loaded with auto-sampler into a self-packed PicoTip column with a 360 μm outer diameter, 75 μm inner diameter and 15

μm tip (New Objective, Woburn, MA) packed with 8-10 cm of ODS-A C_{18} 5 μm phase (YMC, Allentown, PA). The peptides were rinsed with 0.1% formic acid for 5 min, and separated with a linear gradient from 2-35% of 100% acetonitrile and 0.1% formic acid over 150 min at a flow rate of 200 nl/min. Full-mass spectra were acquired in a data-dependent manner cycling via a full-scan (300-1800 *m/z*) followed by collision-induced dissociation scans on the 20 most abundant ions. The dynamic exclusion time was set to 60 s.

Proteomic data processing

The proteomic raw data available via ProteomeXchange with identifier-PXD008976 were analyzed with the MaxQuant software (Version 1.5.0.25; Max-Planck Institute of Biochemistry, Martinsried, Germany) (Cox & Mann, 2008). MS/MS spectra were searched using the Andromeda search engine against *P. gingivalis* (ATCC 33277) database (UniProt release 2014-10). Enzyme specificity was set to trypsin, allowing a maximum of two missed cleavages. Initial and main searches were included with the peptide mass tolerance set at 20 ppm and 4.5 ppm, respectively. Carbamidomethylation was selected as the fixed modification, and methionine oxidation and N-terminal acetylation were variable modifications. The minimum peptide length was 7 amino acids. The false discovery rate (FDR) was set to 0.01 both at peptide and protein levels. Label-free quantitation was performed in MaxQuant to determine the normalized protein intensity. When a set of identified peptides was shared by multiple proteins, they were pooled as one protein group. The proteins with valid intensity values in all samples were subjected to hierarchical clustering and statistical analysis. Relative protein expression

was determined by *t*-test with a cut-off permutation-based FDR value of 0.05, using the Perseus software (Version 1.5.0.9; Max-Planck Institute of Biochemistry) (Cox & Mann, 2012). The proteins with significantly differential expression should have at least 2-fold difference in abundance.

Statistical analysis

Quantitative data were shown as mean±SD. Statistical significance was analyzed by one-way ANOVA/Tukey's test as appropriate. A *p*-value <0.05 indicated significant difference.

RESULTS

Characterization of *P. gingivalis* persisters and effects of hemin on their persistence

P. gingivalis cultured with different levels of hemin supplementation displayed similar growth profiles, and no significant difference was found in the CFU both at 40 h and 88 h. The MIC (0.125 μg/ml) of MTZ against *P. gingivalis* was not affected by hemin availability. At late exponential phase, the 6-h MTZ (100 μg/ml) treatment killed the majority of *P. gingivalis* cells, but failed to eradicate them. Some of the surviving cells died at a lower rate during further treatment for 24 h (Fig. 1A). The biphasic pattern of killing reflected the presence of drug-tolerant persisters. After 6-h treatment, *P. gingivalis* cells grown under the hemin-deficiency and hemin-limitation conditions gave rise to about 10- and 14-folds higher frequencies of persister formation, with reference to those cultured under hemin-repletion condition (Fig. 1B). Significant difference existed between hemin-limitation and hemin-repletion cultures ($p<0.05$). After 24-h treatment,

the proportions of MTZ-tolerant persisters decreased to a similar level under various hemin conditions (Fig. 1B). The tolerance of *P. gingivalis* persisters was further examined by treating stationary-phase cultures with MTZ for 6 h. As compared to late exponential-phase *P. gingivalis* cells, the levels of MTZ-tolerant persisters in stationary-phase cultures increased around 44-, 10- and 430-folds under the conditions of hemin-deficiency, hemin-limitation and hemin-repletion, respectively (Fig. 1C). No significant difference occurred among them. The MIC of MTZ against the cells recovered from the persisters remained unchanged, and they produced a similar level of persisters as the original cultures when re-treated with MTZ (100 μg/ml) (Fig. 1D). CLSM confirmed the presence of *P. gingivalis* persisters in their late exponential phase exposed to MTZ for 6 and 24 h (Fig. 2).

Protein expression profiles of *P. gingivalis* persisters under hemin-limitation condition

A total of 327 proteins/protein groups were identified under the hemin-limitation condition. Of them, 310 were present in the persister fractions of *P. gingivalis*, 280 in the untreated controls and 263 in common. The statistical analysis was limited to 183 proteins with consistent representation in all samples (Fig. 3A). Interestingly, MTZ treatment resulted in 18 upregulated proteins and 13 downregulated ones in the persister fractions with reference to the controls (>2-fold difference; permutation-based FDR, 0.05) (Fig. 3B and Table S1). Herein, gene names were used to simply refer to the corresponding proteins.

Notably, the expression of multiple proteins responsible for redox regulation and electron transfer was significantly altered. The persisters showed increased intensity of probable thiol-disulfide oxidoreductase (*PGN_1181*) and decreased expression of *PGN_1659*, *PGN_1753*, *PGN_0660*, *PGN_0893* and *PGN_1396*. Additionally, the expression of methionine gamma-lyase (*PGN_1618*) was greatly inhibited (Fig. 4A).

A variety of proteins related to heme/iron uptake were differentially regulated. The persisters displayed highly overexpressed putative MotA/TolQ/ExbB proton channel protein (*PGN_0806*) and TonB-linked receptor Tlr (*PGN_0683*) in association with energy-dependent hemin transport across the outer membrane of Gram-negative bacteria. There was overexpression of putative v-type ATPase subunit E (*PGN_1764*) involved in proton pump and creation of membrane potential. However, hemagglutinin protein HagA required for hemagglutination and hemin binding was significantly downregulated. Furthermore, extensively reduced expression was detected in outer membrane proteins 40 and 41 (*PGN_0728* and *PGN_0729*) presumably accounting for hemin binding (Fig. 4B).

In addition, the *P. gingivalis* persisters greatly increased the expression of peptidyl-prolyl isomerases (PPIases; *PGN_1510* and *PGN_0742*) and TPR domain protein (*PGN_0876*) implicated in protein folding. Tyrosine recombinase XerC essential to chromosome stability at cell division was enriched. Several ribosomal proteins involved in translational regulation were identified, including upregulated *rplJ* and *rplY*, and downregulated *rpsA* and *rpsM*. The level of elongation factor G (EF-G) was markedly lowered, and *fabH* associated with fatty acid biosynthesis was greatly downregulated.

Protein expression profiles of *P. gingivalis* persisters under hemin-repletion condition

Overall, 326 proteins/protein groups were mapped under the hemin-repletion condition (301 in *P. gingivalis* persister fractions and 312 from the controls). Of them, 287 proteins existed in both conditions, and 204 proteins in all replicates were eligible for statistical analysis (Fig. 3C). There were 23 upregulated and 27 downregulated ones in the persister fractions with reference to the controls (>2-fold difference; permutation-based FDR, 0.05) (Fig. 3D and Table S2). Among them, 20 proteins were differentially up- or down-regulated in a similar fashion under both hemin-limitation and hemin-repletion conditions (Table 1).

Moreover, a series of additional proteins associated with cellular redox regulation and heme/iron utilization differentially expressed under hemin-repletion condition. Regarding the proteins involved in electron transfer and redox control, the expression of *PGN_1173* increased, whereas that of thioredoxin (*PGN_0033*), rubrerythrin (*PGN_0302*), *PGN_1529* and *PGN_1530* decreased (Fig. 4A). Regarding heme/iron utilization, the persisters showed considerable increase in the expression of hemagglutinin-related protein (*PGN_1519*), and decreased production of arginine-specific cysteine proteinase RgpA and 35 kDa hemin binding protein (HBP35). The abundance of V-type ATPase subunit B (*PGN_1761*) was elevated. Interestingly, ferritin as a key iron-storage protein was significantly upregulated (Fig. 4B). Furthermore, there was increased expression of 10 kDa chaperonin (*groS*), acyl carrier protein and elongation factor Ts. The abundance of putative cell division trigger factor (*PGN_0791*) and proline--tRNA ligase (*proS*) was reduced.

DISCUSSION

In the present study, *P. gingivalis* incubated with varying concentrations of hemin exhibited similar profiles of planktonic growth in consistence with the previous observations (Al-Qutub et al., 2006; Xie & Zheng, 2012). The same MIC endpoints of MTZ against *P. gingivalis* cultured with different levels of hemin suggest that its susceptibility to MTZ may be independent of hemin availability. Notably, despite that *P. gingivalis* was susceptible to MTZ, a small subpopulation of its persisters survived this lethal treatment, i.e. the MTZ-tolerant *P. gingivalis* persisters. These surviving cells are not resistant mutants, and the regrown cells indeed form a new subset of persisters. The level of persisters is higher during stationary phase compared to exponential state in a bacterial population (Keren et al., 2004; Willenborg et al., 2014). In line with these reports, the present study demonstrates the increased survival of MTZ-tolerant *P. gingivalis* persisters in stationary-phase cultures with reference to those at late exponential phase, possibly due to starvation-related stringent response, accumulation of quorum sensing molecules and actions of certain endogenous proteins (Leszczynska et al., 2013; Vega et al., 2012). It is noted that approximately 13 μg/ml of MTZ could be detected in gingival crevice fluid from periodontitis patients after administration with 500 mg MTZ 2-3 times daily for at least 2 days (Pahkla et al., 2005). The ability of *P. gingivalis* persisters to withstand lethal concentration of MTZ far exceeding clinically relevant doses may critically account for the persistence of periodontal infection and inflammation.

P. gingivalis existing at different microenvironmental hemin levels displays distinct phenotypes, which facilitates its survival and contributes to a dysregulated host response (Darveau, 2010). Interestingly, the 6-h MTZ treatment of late exponential-phase *P. gingivalis* generated a relatively higher level of persisters under both hemin-limitation and hemin-deficiency conditions than hemin-repletion status. Hemin level may serve as an important mediator of MTZ tolerance in *P. gingivalis* persisters. Nevertheless, the hemin-mediated discrepancy in *P. gingivalis* persistence was offset with prolonged treatment and it was not found in stationary-phase cultures, suggesting that hemin may mainly regulate MTZ tolerance of the growing *P. gingivalis* cells at the early treatment period. Further investigation is required to clarify this point.

MTZ enters bacterial cells as a prodrug, and it is activated by intracellular reduction through the transfer of an electron to the nitro group of MTZ subsequently converted to a short-lived nitroso free radical (Lofmark et al., 2010). Activation of MTZ is attributed to various electron transfer proteins, e.g., ferredoxin, flavodoxin, thioredoxin reductase, pyruvate-ferredoxin oxidoreductase (PFOR) and hydrogenases (Chong et al., 2014; Kaakoush et al., 2009; Leitsch et al., 2011). As persisters constitute only a tiny portion of the whole microbial population and yet represent a transient physiological state, it is extremely challenging to completely isolate them from non-viable cells following antimicrobial treatment (Helaine & Kugelberg, 2014). Therefore, in the present study we collected *P. gingivalis* persister-containing, MTZ-treated cells for further proteomic analysis as previous studies on other microorganisms (Li et al., 2015; Van Acker et al., 2013). The MTZ-tolerant *P. gingivalis* persister fractions exhibited distinct proteomic profiles from the controls. The expression of 20 proteins was similarly up- or down-

regulated under both hemin-limitation and hemin-repletion conditions. *PGN_1753* as a subunit of 2-oxoglutarate oxidoreductase catalyzes the production of ferredoxin (Dorner & Boll, 2002). *PGN_0660* is a subunit of alkyl hydroperoxide reductase and serves as a reducing agent in *E. coli* (Seaver & Imlay, 2001). *PGN_0893* functions in the generation of fumarate as an anaerobic electron acceptor (Maga et al., 2013). *PGN_1618* uses methionine to generate 2-oxobutyrate and acts as a substrate for PFOR or pyruvate dehydrogenase complex (Sato & Nozaki, 2009). *PGN_1396*, a class III ribonucleotide reductase, exhibits reductive activity and requires thioredoxin reductase or glutathione reductase for regeneration (Gon et al., 2006). Indeed, the decreased expression of these proteins could inhibit the electron transfer essential to the activation of MTZ for their survival. Additionally, the persisters showed elevated abundance of *PGN_1181* for regulation of cellular thiol-disulfide redox environment. Besides the commonly regulated proteins under both conditions, *PGN_1659* was downregulated in the persisters under hemin-limitation condition, whereas those under hemin-repletion condition displayed overexpression of *PGN_1173* and lower level of thioredoxin, rubrerythrin, *PGN_1529* and *PGN_1530*. These proteins are closely linked with redox regulation and electron transfer. Taken together, these findings suggest that cellular redox control may critically account for the tolerance of *P. gingivalis* persisters to MTZ.

Hemin is used essentially by *P. gingivalis* and provides its protection against oxidative stress. Impaired hemin biosynthesis or uptake impedes electron transport and inhibits MTZ activation (Chong et al., 2014; Lewis et al., 2006). It is of note that the expression of various heme/iron utilization-associated proteins was significantly altered in MTZ-tolerant *P. gingivalis* persisters. Under both hemin-limitation and hemin-repletion

conditions, the persisters downregulated the expression of hemin binding proteins (HagA, *PGN_0728* and *PGN_0729*), indicating the inhibition of hemin uptake. However, the increased expression of *PGN_0806* and *PGN_1764* involved in hemin transport suggests the need of certain amount of hemin. Under hemin-repletion condition, more heme/iron utilization-related proteins were involved, including downregulated RgpA and HBP35 as well as upregulated *PGN_1519*, *PGN_1761* and ferritin. Both RgpA and HBP35 play an important role in hemin binding (Olczak et al., 2005), and Ferritin acts in deposition of excess iron and detoxification. These findings suggest that *P. gingivalis* persisters weaken the uptake and utilization of heme/iron in response to MTZ, especially at high hemin levels. The decreased heme/iron utilization may therefore promote the survival of MTZ-tolerant *P. gingivalis* persisters. This could to some extent explain the higher persister frequency under conditions of hemin-limitation and hemin-deficiency than that of hemin-repletion.

Furthermore, the translation process may be inhibited in *P. gingivalis* persisters. Under hemin-limitation condition, the intensity of translation-related proteins (EF-G, *rpsA* and *rpsM*) decreased, while a translational repressor protein rplJ was upregulated. *rpsA* is required for translation of most natural mRNAs in *E. coli* (Skorski et al., 2006). Meanwhile, there was increased expression of *rplY* for accurate translation under stress conditions. Under hemin-repletion condition, EF-G and *rpsM* were downregulated together with *proS*. Additionally, the aforementioned *PGN_1396* catalyzes rate-limiting step in DNA biosynthesis, and its lower intensity under hemin-limitation condition may result from the action of MTZ as a DNA biosynthesis inhibitor or an activator in response to MTZ treatment in the persisters (Logan et al., 2003). Under hemin-repletion condition,

cell division may be suppressed due to downregulation of *PGN_0791*. As dormancy reflects persister tolerance, the shutdown of translation, DNA replication and cell division collectively enhance the survival of *P. gingivalis* persisters. Moreover, several stress proteins were identified with higher expression, e.g., XerC under hemin-limitation condition and *groS* under hemin-repletion condition. The increased expression of PPIases and TPR domain protein under both conditions facilitates proper protein folding and degradation of misfolded proteins. Further investigation may confirm this assumption.

Within the limitations of the experiments concerned, this study provides the first evidence on the proteomic profiles of MTZ-tolerant *P. gingivalis* persisters. The regulation of cellular redox state critically accounts for their formation and survival. Hemin may be an important mediator of their tolerance to MTZ. Repression of heme/iron utilization, dormancy and adaptive stress response may collectively contribute to the survival of *P. gingivalis* persisters. Due to current lack of reliable techniques for complete isolation of microbial persisters, further functional studies are required for better understanding their survival mechanisms. The current findings suggest that MTZ-tolerant *P. gingivalis* persisters may represent an underlying cause of persistent periodontal infection and inflammation. As such, targeting *P. gingivalis* persisters at oral/periodontal niches could be a potential strategy for effective management of periodontal diseases.

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FIGURE LEGENDS

***Fig*. 1.** The characteristics of MTZ-tolerant *P. gingivalis* persisters. (**A)** Time-dependent killing of late exponentially growing *P. gingivalis* by MTZ (100 μg/ml) at different levels of hemin. (**B)** Effect of hemin on the survival of late exponentially growing *P. gingivalis* exposed to MTZ (100 μg/ml) for 6 and 24 h. Percentage survival refers to the ratio of persisters to the total number of cells in an untreated culture. **p*<0.05. **(C)** Effect of hemin on the survival of stationary-phase *P. gingivalis* exposed to MTZ (100 μg/ml) for 6 h. (**D)** Non-inheritable persistence. *P. gingivalis* was cultured to late exponential phase (Growth) and treated with 100 μg/ml of MTZ (Treatment) for 6 h under hemin-limitation condition. Colonies recovered from the surviving persisters were inoculated into fresh media (Regrowth) and subjected to MTZ treatment again (Retreatment). Data are outputs from at least three (A-C) or two (D) independent experiments.

***Fig*. 2.** LIVE/DEAD staining of late exponentially growing *P. gingivalis* at different levels of hemin exposed to MTZ (100 μg/ml) for 6 h **(A)** and 24 h **(B)**. Live cells stain green and dead cells are red/yellow-colored. One representative field from two experiments is shown. Scale bar = 20 μm.

***Fig*. 3.** The protein expression profiles of MTZ-tolerant *P. gingivalis* persister fractions. **(A & C)** Heat maps of the proteins identified in the persister fractions and untreated controls of *P. gingivalis* under hemin-limitation (HL) **(A)** and hemin-repletion (HR) **(C)** conditions. The samples of persisters and controls are clustered separately, and they display distinct protein expression profiles. Both persister and control samples are highly

reproducible, with an average Pearson correlation coefficient of 0.966 and 0.950 respectively under hemin-limitation condition as well as 0.971 and 0.963 respectively under hemin-repletion condition. **(B & D)** Relative quantitation of proteins in *P. gingivalis* persister fractions as compared to untreated controls under hemin-limitation **(B)** and hemin-repletion **(D)** conditions. Differentially expressed proteins obtained by statistical analysis at a cut-off FDR value of 0.05 are plotted, and those with >2-fold difference in abundance are considered to be statistically significant.

***Fig*. 4.** The differentially expressed proteins involved in cellular redox regulation **(A)** and heme/iron utilization **(B)** in MTZ-tolerant *P. gingivalis* persister fractions as compared to untreated controls, under both hemin-limitation and hemin-repletion conditions. The yellow and orange colors represent Log2 [Persisters/Controls] ratio. The gray color denotes that data are unavailable. HL, hemin-limitation. HR, hemin-repletion.

TABLE 1 The differentially expressed proteins in MTZ-tolerant *P. gingivalis* persister fractions under both hemin-limitation and hemin-repletion conditions

UniProt accession no.	Description of proteins	Genes	Log_2 fold change (Persisters/Controls)		*p*-values	
			Hemin-limitation	Hemin-repletion	Hemin-limitation	Hemin-repletion
B2RKY4	Putative peptidyl-prolyl cis-trans isomerase	*PGN_1510*	6.00	3.33	3.37E-04	1.03E-04
B2RHL9	Putative uncharacterized protein	*PGN_0345*	4.08	2.34	4.37E-05	1.29E-03
B2RIY0	Putative MotA/TolQ/ExbB proton channel protein	*PGN_0806*	3.40	2.24	6.67E-04	4.49E-03
B2RKY7	Putative uncharacterized protein	*PGN_1513*	2.75	2.39	1.04E-03	2.71E-02
B2RJ50	TPR domain protein	*PGN_0876*	2.62	1.32	3.81E-05	7.22E-03
B2RJL1	Putative uncharacterized protein	*PGN_1037*	2.16	1.51	6.79E-03	1.69E-02
B2RJV3	Putative uncharacterized protein	*PGN_1129*	1.54	1.51	1.17E-02	2.02E-04
B2RMC1	Immunoreactive 23 kDa antigen	*PGN_1998*	1.52	1.42	1.27E-02	2.53E-04
B2RK05	Probable thiol:disulfide oxidoreductase	*PGN_1181*	1.27	1.12	1.69E-02	3.51E-03
B2RLN8	Putative v-type ATPase subunit E	*PGN_1764*	1.25	1.28	2.07E-02	1.90E-02
B2RLM7	Putative 2-oxoglutarate oxidoreductase alpha subunit	*PGN_1753*	-1.12	-1.77	5.57E-04	3.91E-03
B2RLK7	Hemagglutinin protein HagA	*hagA*	-1.53	-2.22	5.07E-03	1.30E-04
B2RII4	Putative alkyl hydroperoxide	*PGN_0660*	-1.55	-2.09	1.96E-02	9.61E-03

	reductase C subunit					
B2RJ67	Fumarate hydratase class I anaerobic	*PGN_0893*	-1.67	-3.05	5.79E-03	5.68E-05
B2RL92	Methionine gamma-lyase	*PGN_1618*	-2.15	-1.43	2.76E-03	4.52E-04
B2RKM0	Anaerobic ribonucleoside-triphosphate reductase	*PGN_1396*	-2.46	-2.32	2.58E-03	2.91E-04
B2RLZ4	Elongation factor G (EF-G)	*fusA*	-2.70	-2.88	6.54E-03	9.26E-03
B2RIQ3	Outer membrane protein 41	*PGN_0729*	-2.85	-2.85	8.81E-04	8.56E-05
B2RLW8	30S ribosomal protein S13	*rpsM*	-3.10	-2.09	9.87E-04	5.13E-03
B2RIQ2	Outer membrane protein 40	*PGN_0728*	-3.66	-3.13	3.03E-05	1.58E-05