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Gene expression profiling and characterization under hemin limitation in *Porphyromonas gingivalis*

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Abstract: Hemin is an important nutrient for Porphyromonas gingivalis growth and pathogenicity. We examined the gene expression profile of P. gingivalis, including genes involved in its pathogenicity, at various growth stages under hemin-standard and limited conditions by using a custom-made microarray. The transcription of many genes decreased after late-log and mid-log phases under hemin-standard and limited conditions, respectively. We focused on two groups of genes while comparing gene expression profiles under hemin-standard and limited conditions by gene tree analysis. Genes belonging to group A maintained high transcriptional levels, whereas genes in group B were expressed at low levels under standard hemin conditions. However, group B genes increased remarkably under hemin-limited conditions. Groups A and B contained genes involved in regulatory functions and protein fate, respectively. Genes related to energy metabolism, transport, and protein binding were present in both groups. Our results suggest that P. gingivalis experienced severe stress under hemin-limited conditions, and growth phase-dependent changes in transcription levels were observed for many genes. Moreover, increased expression of genes involved in energy metabolism suggests that hemin is related not only to pathogenicity, but also energy metabolism. (J. Oral Sci. 47, 191-197, 2005)

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Introduction

Microorganisms are often able to adapt to changes in their environment. Adaptation to these changes involves a directed change in the synthesis of virulence factors (1). This is particularly true for organisms living in the mouth, where they are exposed to dramatic changes in oxygen tension, redox potential, osmolarity, temperature, pH, and the bacterial cell density (2). Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, is implicated as one of the major pathogens of adult periodontal disease (3). This species possesses many virulence factors, including fimbriae, proteases, hemagglutinins, and capsular polysaccharides, which allow this organism to cause disease (2). In general, microorganisms must scavenge essential nutrients, including iron, which play a crucial role in the establishment and progression of infections (4). Iron is a constituent of several important metabolic enzymes and is essential for the growth of almost all microorganisms. P. gingivalis has several mechanisms to utilize iron from some iron-containing molecules such as hemoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin, and cytochrome C (5-7).

Numerous investigations have examined the effects of iron depletion on bacteria, identifying an accompanying decrease in virulence expression (8-11). Significant decreases in gingipain enzyme activity were found in the wild-type strains grown under iron-depleted conditions (8). Promoter activity of *fim*A gene, which encodes fimbrillin, decreased by approximately 50% in response to hemin limitation (9). Transcription of hemagglutinin-associated genes such as *hagB* and *hagC* decreased under heminlimited or non-hemin conditions (10). Trypsin-like enzyme activity in culture supernatant was also lower at each growth phase under hemin-limited than standard hemin conditions (11). Kesavalu et al. (8) demonstrated that P. gingivalis cells grown under iron-depleted conditions for multiple passages showed significantly decreased lesion size in mice, in contrast to cells grown under iron-normal and iron-elevated conditions. Conversely, other reports have shown increases in virulence under hemin limitation (11-14). The binding (12) and transferring (13) of hemoglobin increased considerably when the organisms were grown under hemin-limited conditions, and collagenolytic activity was generally higher in heminlimited cultures (11). Moreover, cells and vesicles from hemin-limited cultures for hemagglutination of sheep erythrocytes exhibited higher titers than their hemin-excess counterparts (14). These reports showed a discrepancy in the virulence of P. gingivalis under hemin limitation. There were few reports that showed the obvious growth phase on sample collection. In addition, little is known about changes in global gene expression of virulence-related proteins during each growth phase under hemin limitation. Therefore, the variability in pathogenicity between reports in response to hemin is likely a result of differences in gene expression during bacterial growth phases at the time of sample collection.

Recent technology advances have made it possible to study global gene expression in both prokaryotes and eukaryotes by using DNA microarrays (15-18). The complete genome sequence of *P. gingivalis* strain W83 has been previously determined (19) and is available for designing *P. gingivalis* microarrays. In the present study, we prepared a custom-made DNA array of pathogenicityassociated genes and investigated the possible role of hemin in the virulence of *P. gingivalis* by comparing the transcriptional profile under hemin-limited and standard hemin conditions *in vitro*.

Materials and Methods

Microarray procedure

Ninety-three pathogenicity-related genes that had been previously described in detail were selected and amplified from *P. gingivalis* genomic DNA. The average size of these amplicons was 615 bp. Human genes encoding β actin, GAPDH, and lactoferrin receptor were also amplified for printing on the array. All DNA probes were printed as a block of three on both the right and left sides of a glass slide, yielding six replicates of each PCR product and 570 spots per array. Purified PCR products were diluted to a final concentration of 50 ng/µl in Microarray Crosslinking Reagent D (Amersham Bioscience, Piscataway, NJ, USA) and then transferred to a 384 - well microtiter plate for printing. The purified products were printed onto silane- and metal-coated glass slides (Microarray Slide Type 7; Amersham Bioscience) by using a GEN III Microarray Spotter (Molecular Dynamics, Sunnyvale, CA, USA) under 55% relative humidity. After drying under 55 - 60% relative humidity for 1 h, the arrays were fixed by UV cross-linking at 100 mJ/cm² in a UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA), and then stored dry in a desiccator at room temperature.

Media and growth conditions

P. gingivalis strain W83 was subcultured by serial passage in hemin-free brain heart infusion (BBL Microbiology Systems, Cockeysville, MD, USA) containing 0.25% yeast extract supplemented with menadion (1 μ g/ml). The bacteria were then transferred and cultured in fresh medium with hemin (5 μ g/ml or 0.001 μ g/ml) to early-log phase (EL), mid-log phase (ML), late-log phase (LL), or stationary phase (ST) (Fig. 1). Before inoculating the bacteria, fresh growth medium was incubated in an anaerobic chamber for several days to remove residual oxygen.

Preparation of total RNA and fluorescent-labeled cDNA

After harvesting the cells by centrifugation, cells were re-suspended in 300 µl of DNase, RNase free water (DW; Invitrogen, Carlsbad, CA, USA). For isolation of total



Fig. 1 Growth under different hemin concentrations in *P. gingivalis*. *P. gingivalis* strain W83 was subcultured by serial passage in hemin-free broth. The bacteria was transferred and cultured in fresh medium with hemin (5 μ g/ml or 0.001 μ g/ml) to early-log phase (EL), mid-log phase (ML), late-log phase (LL), or stationary phase (ST).

RNA, the cell suspensions were transferred into FastPrep tubes containing Lysing Matrix B (Qbiogene, Carlsbad, CA, USA) and 900 μ l of Trizol Reagent (Invitrogen), and disrupted by using a FastPrep FP120 Instrument (Qbiogene) at a speed rating of 6.5 for 40 s. After centrifugation in 200 μ l of chloroform, the supernatants were treated with chloroform, precipitated with isopropanol, resuspended in DW, and stored at -70°C. The concentration of total RNA was calculated based on absorbance measured at 260 nm. In addition, the degradation of total RNA was examined by using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples used in this study exhibited A_{260}/A_{280} and 23S rRNA/16S rRNA ratios of at least 1.8 and 1.6, respectively.

The CyScribe First-Strand cDNA Labeling Kit (Amersham Bioscience) was used for labeling. Briefly, 25 µg of total RNA was used for synthesis of fluorescently labeled cDNA by reverse transcription with Cy5 - dUTP (Amersham Bioscience), SuperscriptII (Invitrogen), and random nonamer primers for 1.5 h at 42°C. The cDNA was treated with NaOH, neutralized with HEPES, and then purified to remove unincorporated dye. The Cy5-labeled samples and identical amounts of Cy5-prelabeled human genes were mixed together, precipitated with ethanol, and then resuspended in 55 µl of DW.

Hybridization and scanning

Hybridization and washing steps were carried out using an Automated Slide Processor (ASP; Amersham Bioscience) by following the manufacturer's instructions. The resuspended samples were denatured at 95°C. for 3 min, cooled on ice, and mixed with an equal volume of Hybridization Buffer (Amersham Bioscience) and 110 µl of formamide (total volume; 220 µl). The arrays were incubated at 55°C for 2h in pretreatment solution (2×SSPE, 0.2% SDS), washed with MilliQ water twice at room temperature, dried, and placed in the ASP. The hybridization cocktail (200 µl) was injected into the slide chamber and hybridization was carried out at 42°C for 12h. The arrays were washed at 45°C with washing buffer I ($1 \times SSC$, 0.2% SDS) twice for 5 min, with washing buffer II $(0.1 \times SSC,$ 0.2% SDS) twice for 5 min, and with $0.1 \times SSC$ for 30 sec prior to being air-dried in the dark. Three microarray replicates were analyzed for each growth stage.

Microarrays were scanned at 10 µm with a confocal fluorescence laser scanner (GenePix 4000; Amersham Bioscience). The emitted fluorescent signal was detected by a photomultiplier tube (PMT) at 532 nm (Cy3) or 635 nm (Cy5). For array experiments, the laser power was 100% and the PMT voltage was 500. The scanned images were processed as TIFF images and the pixel intensities of each hybridization spot were analyzed quantitatively by using GenePixTM Pro 3.0 software (Axon Instruments, Redwood City, CA, USA). A grid of individual circles defining the location of each DNA spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. Median signal intensity was determined for each spot. The local background signal was subtracted automatically from the hybridization signal of each separate spot.

Microarray data mining and analysis

The average of the signal intensities from blank portions on the array was taken as background. At each growth stage, genes with median signal intensities lower than the median background intensity + 3SDs were treated as an absence of expression. Intensities of the pre-labeled human controls were equalized and used for normalization among different arrays. Global gene expression was compared among growth phases using the Kruskal-Wallis test (*P*-value cutoff, 0.05; multiple testing correction; Benjamini and Hochberg False Discovery Rate), and then subjected to gene tree analysis based on standard correlation.

Results and Discussion

P. gingivalis W83 cells were grown under two different hemin concentrations -5 μ g/ml, the standard concentration observed *in vitro*, and 0.001 μ g/ml, the lowest hemin concentration to sustain growth of *P. gingivalis*. As shown in Fig. 1, a delay in growth and a decrease in population were observed under hemin restrictions. On the basis of the growth curve, we designated the EL, ML, and LL phases of growth, and defined the ST, in which the number of viable cells remained approximately constant.

In the present study, we compared global gene expression under different hemin conditions during growth phase by using custom-made DNA microarrays based on PCR products from P. gingivalis. RNA samples were isolated at time points covering the entire growth phase. Although investigators often use an averaged transcriptional level of all mRNAs at each point to normalized control (i.e. global normalization), we used pre-labeled human external controls mixed in hybridization solution for the normalization. The global normalization is, in general, used effectively when large numbers of probes are spotted on an array and the probes do not belong to a restricted category. In a previous study, quantitative real-time PCR analysis demonstrated that the levels of 16S ribosomal RNA were not stable during growth phase and the amount of rRNA was significantly higher than that of other mRNAs from inducible genes (data not shown).

In the present study, 90 of the 93 initially monitored genes

were analyzed further. The gene expression profiles and average expression levels during growth phase are shown in Fig. 2. The mRNA expression of each gene did not change significantly from EL to LL under standard hemin conditions (Fig. 2A, 2C). As a whole, mRNA expression of each gene varied under hemin limited conditions (Fig. 2B) more than under standard hemin conditions (Fig. 2A). Under standard hemin conditions (Fig. 2C), the average and standard deviation of the transcripts did not vary over time from EL to LL, suggesting that many genes might be constitutively expressed. However, a significant reduction in many transcripts was observed during ST. Likewise, a significant reduction was observed in many genes after exposing the ML cells to hemin-limited conditions (Fig. 2D). Many bacteria have evolved the ability to condition culture medium by secreting extracellular signaling molecules, termed autoinducers, in association with growth phase to control expression of specific genes, a process termed quorum sensing (20-22). luxS-dependent quorum sensing in P. gingivalis has recently been described, and expression of *luxS* was found to be higher in LL than in EL, suggesting that the quorum sensing may play an important role for the virulence of P. gingivalis. Our data showed that a reduction in gene expression under heminlimited conditions was not observed during the shift from LL to ST, but it was observed during the transition from ML to LL. This suggests that the quorum sensing system



Fig. 2 Expression profiles of 90 *P. gingivalis* W83 genes. A and B represent profiles of individual genes, whereas C and D depict average profiles under standard hemin concentration (5 μ g/ml; A, C) or hemin-limited conditions (0.001 μ g/ml; B, D). Levels of mRNA expression during growth were expressed as the foldchange relative to the level seen during EL. Data are presented as means ± SD.

might not work well under hemin limitation.

Figure 3 exhibits the results of gene tree analysis identifying related gene expression patterns during growth. Under hemin limitation, the expression of most genes increased at EL and ML, and then decreased significantly after LL. These data suggest that *P. gingivalis* may have a regulatory switch that initiates pathogenesis during the middle of the exponential-phase growth under hemin limitation.



Fig. 3 Gene tree analysis based on standard correlation of the changes in gene expression under various hemin concentrations during growth of *P. gingivalis*. Distributions of individual gene expression levels are shown. Individual growth phases (E, early-log phase; M, midlog phase; L, late-log phase; or S, stationary phase), hemin concentrations (Cont.; standard hemin conditions; or Limit.; Hemin-limited conditions) (columns), and levels of the 90 genes (rows) are shown. The scale bar indicates the color coding of the RNA levels.

Two gene groups showed significant differences in transcription profiles under hemin-standard and heminlimited conditions (Fig. 3A, 3B). Group A contained 14 genes that showed comparatively small transcriptional fluctuations under standard hemin conditions, but which exhibited increases in transcription from EL to ML under hemin limitation (Fig. 3A). Group A contained many genes related to energy metabolism (mutA and mutB), regulatory functions (nosR and fur), and transport and binding proteins (htrA, comM, and PG0938, PG1759) (Table 1). Group B contained 11 genes that were downregulated under standard hemin conditions, but were upregulated throughout bacterial growth under hemin limitation (Fig. 3B, Table 2). Group B contained genes involved in protein fate (groES and dnaK), transport and binding proteins (PG0668 and fetB), and energy metabolism (gdh and rbr).

The *mutA* and *mutB* genes in group A encode subunits of methylmalonyl-CoA mutase, which catalyzes the interconversion of methylmalonyl-CoA and succinyl-CoA. This enzyme is known to play a role in the process that provides the amino acid metabolism intermediate to the TCA cycle (23). The mut genes exhibited a typical expression pattern in group A (Fig. 3A). P. gingivalis is asaccharolytic and cannot utilize carbohydrates as carbon/energy sources (24). Our data suggest that mut genes expression stability may play an important role in basic energy metabolism in P. gingivalis and that expression is up-regulated under hemin limitation. On the other hand, gdh gene expression increases at ML and LL, and these increases are particularly enhanced under hemin-limited conditions (Fig. 3B). The gdh gene encodes NADdependent glutamate dehydrogenase, which catalyzes the interconversion of glutamic acid and 2-oxoglutaric acids. This enzyme is known to participate in energy metabolism, amino acid metabolism, and nucleic acid metabolism (23).

P. gingivalis binds hemin on its surface and transports the entire molecule into the cell by an energy-dependent mechanism (25). Our data suggest that *P. gingivalis* that

Table 1

reside in low-hemin environments may compensate by upregulating *mut* gene and *gdh* gene products to sustain energy production. Interestingly, genes encoding competence protein (*comM*) and transcriptase (*ISPG4* and *ISPG5*) in group A, and transcriptase (*ISPG1*) in group B were up-regulated under hemin limitation. Our data suggest that these genes might play a role in adaptation to environmental change through increased expression.

Iron acquisition is essential for P. gingivalis survival in a healthy periodontal environment. As shown in Fig. 3B, expression of fetB, PG0668, and PG1414 genes, which encode transport and binding proteins, increased under hemin-limited conditions. FetB exhibits sequence similarity to the periplasmic binding proteins necessary for transporting siderophores through the periplasmic space of gram-negative bacteria (26). PG0668 and PG1414 are tonB-linked outer membrane receptors that transport hemin into the periplasmic space, from where it is further transported into the cell by a multicomponent periplasmic binding protein-dependent, ATP binding cassette transport system (27). The *rbr* gene (group B) encodes rubrerythrin, which contains a mixed Zn, Fe binuclear site (28). Rbr protein has multiple roles, not only in protein quality control, but also as an indirect regulator of transcription (29). Our data suggest that P. gingivalis might adapt to environmental change by increasing the expression of these genes and that these genes products might participate in the hemin uptake pathway under hemin limitation.

The *groES*, *dnaK* and *dps* genes in group B encode known stress response proteins. Most studies of Dps have focused on its role in oxidative damage protection, particularly against peroxides (30,31). Dps protects the cell not only from oxidative stress but also from multiple stresses such as UV and gamma irradiation, iron and copper toxicity, thermal stress, and acid and base shock (32). In addition, the Dps protein is induced by nutritional stress in *Bacteroides fragilis* (33). Our data show that the bacterial population continued to increase after ML until ST under hemin limitation, although the expression many

Tab	le	2
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Gene ID	TIGR ID	Description	Main role*
hemN	oxygen-independent coproporphyrinogen III oxidase	PG1505	Hypothetical proteins
phyA	hemolysin A	PG1875	Cellular processes
nosR	membrane-bound regulatory protein	PG1623	Regulatory functions
fur	ferric uptake transcriptional regulator	PG0465	Regulatory functions
ISPg5 (orf2)	transposase orf2	PG0008	Other categories
mutA	methylmalonyl-CoA mutase alpha-subunit	PG1656	Energy metabolism
mutB	methylmalonyl-CoA mutase beta-subunit	PG1657	Energy metabolism
ISPG4	transposase	PG0050	Mobile and extrachromosomal element functions
htrA	htrA protein (ABC solute binding protein)	PG0648	Transport and binding proteins
atoS	sensor histidine kinase	PG0017	Signal transduction
comM	competence protein ComM	PG1768	Transport and binding proteins
PG0938	calcium ion-transporting ATPase	PG0938	Transport and binding proteins
PG1759	adhesin protein	PG1759	Cell envelope
htpG	high temperature protein ; (Hsp90 homologue)	PG0045	Protein fate

"The classifications are described according to that in TIGR database.

Gene ID	TIGR ID	Description	Main role*
groES	heat shock protein 10 (GroES)	PG0521	Protein fate
dps	DNA-binding stress protein	PG0090	Cellular processes
PG1414	hypothetical tonB-linked outer membrane receptor	PG1414	
PG0668	hypothetical tonB-linked outer membrane receptor	PG0668	Transport and binding proteins
gdh	NAD-dependent glutamate dehydrogenase	PG1232	Energy metabolism
fetB	heme-binding protein	PG0669	Transport and binding proteins
PG1432	sensor histidine kinase	PG1432	Signal transduction
ISPg1	transposase	PG0184	Mobile and extrachromosomal element functions
PG1317	hypothetical outer membrane protein	PG1317	
dnaK	DNA replication	PG1208	Protein fate
rbr	rubrerythrin	PG0195	Energy metabolism

The classifications are described according to that in TIGR datab

arrayed genes exhibited remarkable decreases after ML. The stress-related proteins, which exhibited increased mRNA expression throughout bacterial growth, might play a role in protecting the proteins associated with growth and cell division by refolding misfolded proteins. The biological activities of proteins related to bacterial growth or cell division might be maintained until the late phase of growth.

In conclusion, we studied transcription profiles of *P. gingivalis* virulence genes during growth under heminlimited conditions. Our analysis suggested that *P. gingivalis* may control the expression of many genes required for metabolism, stress response, and hemin acquisition in order to adapt to environmental changes. Our analysis also revealed that the expression of many genes changes drastically during bacterial growth. Microarray technology is very useful for analysis of gene expression changes under various conditions throughout *P. gingivalis* growth, and is an extremely powerful tool for characterizing global gene expression changes.

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