Gene Expression Profiling during Growth *in vitro* Using a Custom-made *Porphyromonas gingivalis* Gene Array

Koichi Hiratsuka^{1,2}, and Yoshimitsu Abiko^{1,2}

¹Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, and ²Research Institute of Oral Science, Matsudo, Chiba 271-8587, Japan

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Abstract

A custom-made microarray for *Porphyromonas gingivalis* was constructed to investigate the gene expression profiling during its *in vitro* growth. There was a strong positive correlation between the results of both microarray and *quantitative real-time PCR* analyses ($r = 0.910$, $p < 0.01$). Many transcripts changed dramatically during the late-log to the early stationary phase. In a stationary phase, most of the transcripts decreased. However, transcripts related to the transposon function, oxidative stress, and energy metabolism were increased or sustained at high levels. More notably, the transcripts related to protein folding and protein stabilization, such as *dnak*, *groEL*, *groES*, and *hspG*, increased significantly after 10h in the stationary phase, suggesting that these chaperone proteins may play an important role in bacterial survival. All together, our custom array is useful for transcriptional research for *P. gingivalis*. It provides that the gene expressions are growth phase-specific and are changed dramatically in response to environmental change during bacterial culture.

Introduction

Porphyromonas gingivalis, a Gram-negative bacterial pathogen, is closely associated with adult periodontal disease. This bacterium is known to possess many virulence factors, including fimbriae, proteases, hemagglutinins, and capsular polysaccharides(1). Microorganisms are often able to adapt to large changes in their environment. This is particularly true for organisms living in the mouth, where they are exposed to dramatic changes in oxygen tension, osmolarity, temperature, pH, and bacterial cell density(2). Moreover, several reports have demonstrated that the expression of putative virulence factors in *P. gingivalis* is regulated by environmental conditions, such as hemin, pH, and temperature(3-5). Thus, adaptation to the environmental changes in the mouth must involve direct changes in the synthesis of virulence factors.

Cultivated bacteria are usually used in an *in vitro* study. However, it seems that researchers may not consider the

bacterial growth phase carefully, even if they use the bacteria during exponential growth. In comparison with the eukaryote, it is well known that bacterial transcripts are breakable and the cell division is performed in a short time, indicating that the bacterial gene expressions are changeable during its growth. Therefore, it is important and interesting to know the profiling of interested gene expressions during growth.

Recent technological advances have made it possible to study global gene expressions in both prokaryotes and eukaryotes by using DNA microarray. For example, the global pattern of growth phase-dependent gene expression is analyzed in *Helicobacter pylori* during growth *in vitro*, finding that the late-log phase may be the most virulent and pathogenic phase of growth(6). The gene expression patterns of biosynthetic, regulatory, and ribosomal proteins in *Streptomyces coelicolor* are analyzed from alterations in gene expression during the growth(7). Also, the investigation under iron starvation conditions in *H. pylori* demonstrates that gene expressions between exponential and stationary phases are different responses for growth-

Correspondence to :

Koichi Hiratsuka

E-mail : hiratsuka.koichi@nihon-u.ac.jp

inhibiting stress (8).

P. gingivalis is one of the most well-studied oral microorganisms. Also, the genome sequence of *P. gingivalis* strain W83 (9), ATCC33277 (10), and TDC60 (11) were completed. However, it is difficult for us to expect that commerce-based microarrays for oral microorganisms are released. In this report, we prepared a custom-made microarray for virulence-associated genes in *P. gingivalis* and examined their gene expression profiles during *in vitro* growth.

Materials and Methods

Microarray procedure

DNA probes for spotting on an array were amplified by PCR from 92 virulence-related genes of *P. gingivalis*. The average size of these amplicons was 615 bp. In addition, three kinds of human gene encodings β -actin, GAPDH and lactoferrin receptor were amplified as the external controls.

Purified PCR products were diluted to a final concentration of 50 ng μL^{-1} in Microarray Crosslinking Regent D (Amersham Bioscience Corp., Piscataway, NJ, USA), transferred to a 384-well microtiter plate, and then printed onto silane- and metal-coated glass slides (Microarray Slide Type 7; Amersham Bioscience) using a GEN III Microarray Spotter (Molecular Dynamics Inc., Sunnyvale, CA, USA) at 55% relative humidity. After drying for 1 h in 55% to 60% relative humidity, the arrays were fixed by crosslinking at 100 mJ $(\text{cm}^2)^{-1}$ in a UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA) and then stored to dry in a desiccator at room temperature. Six replicates of each PCR product were then printed onto glass slides, yielding 570 spots per array.

Preparation of total RNA and fluorescently labeled cDNA

P. gingivalis W83 was grown in brain heart infusion (BBL Microbiology Systems, Cockeysville, MD, USA) containing 0.25% yeast extract supplemented with hemin ($5 \mu\text{g ml}^{-1}$) and menadion ($1 \mu\text{g ml}^{-1}$) in an anaerobic chamber containing 80% N₂, 10% H₂, and 10% CO₂. Before initiating bacterial growth, the fresh growth medium was incubated in an anaerobic chamber for 3 days to remove residual oxygen as much as possible.

P. gingivalis was grown in the early-log phase (EL; OD₅₅₀ = 0.40–0.50), mid-log phase (ML; OD₅₅₀ = 1.00–1.10), late-log phase (LL; OD₅₅₀ = 1.55–1.65), early stationary phase (ST-E; OD₅₅₀ = 1.80–1.82), or 10 h after the stationary phase (ST-10; OD₅₅₀ = 1.80–1.82) (Fig. 1A). Total RNA was isolated using a FastPrep FP120 Instrument (Qbiogene,

CA, USA) with Trizol Regent (Invitrogen, CA, USA) as previously described (12). RNA concentrations and degradations were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

A CyScribe First-Strand cDNA Labeling Kit (Amersham Bioscience) was used for a direct labeling procedure. The Cy5-labeled ML sample (control), Cy3-labeled EL, LL, ST-E, or ST-10 samples (subject), and identical amounts of Cy3- and Cy5-pre labeled human genes (external controls) were mixed together, precipitated with ethanol, and resuspended in 55 μL of DW (DNase/RNase-free, Invitrogen, Carlsbad, CA, USA).

Hybridization and scanning

The 55 μL of labeled cDNA mixture was denatured at 95 °C for 3 min, cooled on ice, and mixed with an equal volume of Hybridization Buffer (Amersham Bioscience) and 110 mL of formamide (hybridization cocktail, total volume = 220 mL). The array was placed in the slide chamber on an Automated Slide Processor (Amersham Bioscience), hybridized with 200 μL of the hybridization cocktail at 42 °C for 12 h, washed with washing buffer I (1X SSC with 0.2% SDS) and buffer II (0.1X SSC with 0.2% SDS) twice for 10 min at 45 °C, and also washed with 0.1X SSC once for 4 min at 45 °C.

Microarrays were scanned at 10 mm with a confocal fluorescence laser scanner (GenePix 4000; Amersham Bioscience). The emitted fluorescent signal was detected with a photomultiplier tube at 532 nm (Cy3) or 635 nm (Cy5). The laser power and the photomultiplier tube voltage were set to 100% and 500, respectively. The scanned images were processed as TIFF images, and the pixel intensities of each hybridization spot were analyzed quantitatively using GenePix 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.

Microarray data mining and analysis

The contamination of dusts in an array increases remarkably in the signal intensities of the gene spot and the background. Therefore, we did not use a mean but a median for the determination of signal intensities of the DNA spot and the background. The signal intensity of an individual DNA spot was determined as follows: [a median of signal intensities in a DNA spot area] – [a median of signal

intensities in the background (blank) area]. The same DNA probe was spotted in four different places on one microarray. An average of the signal intensities of an individual DNA spot was calculated as an experimental value for every microarray plate. In addition, the presence or absence of an individual gene expression was determined for every microarray plate. The average intensity of the DNA spots that were lower than that of the background + 3SDs was judged as an absence of individual gene expression.

Experiments were performed with quadruplicates using independently obtained samples. Therefore, an average intensity of microarray plates ($n = 4$) was used as the final signal intensity of each DNA spot. Also, the genes that were judged as present for their expressions more than three times in a quadruplicate were targeted for subsequent analysis.

Intensities of the external controls were equalized and used for normalization. Induction ratios were obtained by dividing background-corrected signal intensities of experimental samples (EL, LL, ST-E or ST-10) by background-corrected intensities of control samples (ML).

Global gene expression was compared among growth phases using Welch ANOVA, and then subjected to hierarchical cluster analysis using the average linkage clustering method (13). To compare between growth stages, *t*-test was used. Values of $p < 0.05$ were considered significant.

Quantitative real-time PCR (QRT-PCR)

Quantitative detection was performed with a DNA Engine Opticon™ Continuous Fluorescence Detection System Version 1.0 (MJ Research, Alameda, CA, USA) using a QuantiTect SYBR Green PCR Kit (Qiagen). The primer sequences are listed in Table 1.

The qRT-PCR experiments were performed with quadruplicates using samples obtained independently and used in microarray experiments. The standard curve for each gene was created by analyzing serial 10-fold dilutions of the *P. gingivalis* chromosome. The copy number of each transcript was determined using each standard curve. The fold change of each gene expression was calculated from the mRNA copy number in EL, LL, ST-E, or ST-10 samples divided by that in the ML sample. The resulting average fold changes from four replicates were compared with those obtained from the microarray to determine their correlation.

Table 1 Primer sequences used for QRT-PCR

Gene Name	Tiger ID	Primer	Primer Sequence (5'-3')
<i>16S rRNA</i>	PG0726	F	AAGCGGAGGAACATGTGGTT
		R	GGCACTTAAGCCGACACCTC
<i>kbp</i>	PG1844	F	AATACGCCGGTTCTGTATGCT
		R	TGTCACCAACCAAGCCAAG
<i>rgpA</i>	PG2024	F	AGCACGCTTACCCTTACAGTAGTT
		R	TCAAGAACAATCTCGGCCTGACCG
<i>dnaK</i>	PG1208	F	ATCGTCTGATTGAGGCATGTGTGGC
		R	TTCTTGCCGGTAGCTTTGTCATGAG
<i>sod</i>	PG1545	F	AATTCCACCACGGTAAGCACC
		R	GAGCCGAATTGTTTGTTCGAT
<i>prrC</i>	PG1542	F	CGGGCAAGTGCTATCTAAGC
		R	CGTAGCTCCTGAGCCGTAAC
<i>tpx</i>	PG1729	F	TCAATACTAATGGACAGCTCCCGG
		R	GTTTTCTGCTCACTACAACGACGGC
<i>oxyR</i>	PG0270	F	GAGCTAAGCTTTTCGACAGG
		R	AGCAAGTCGTCTTCAAGACC
<i>groEL</i>	PG0520	F	TCGCGTAGAAGATGCCTTGA
		R	CGTCCTTGCCCTTCTTTACC
<i>groES</i>	PG0521	F	TGAACATCAAACCATTTGGCAGACCG
		R	TCTATTTCAGTGCCGGCATATTTGC
<i>dnaK</i>	PG1208	F	ATCGTCTGATTGAGGCATGTGTGGC
		R	TTCTTGCCGGTAGCTTTGTCATGAG
<i>htpG</i>	PG0045	F	GCAGCCAAATCTTCTTTTCACCG
		R	CTTTTCTCTCATCGAGCAGCTTGGC

Results

Global gene Expression profiling during bacterial growth

The reliability of the microarray data was shown in Fig. 1B. The cDNA from the ML sample was labeled with Cy3 or Cy5, separately. Both different fluorescent-labeled cDNAs were mixed and then cohybridized on an array. The result revealed a linear correlation with no more than a two-fold change in the relative expression level.

Based on our statistical analysis of 95 genes including three external controls, we subjected 92 to further analysis. On the whole, transcription levels were increased during both EL and LL, as compared to ML, and were then reduced after LL. During ST-E, almost all of the transcripts reduced significantly, and the transcription levels were the lowest during ST-10. Conversely, some of transcripts increased after the ST. The standard deviation was at a maximum during ST-E, and it was approximately four times that of EL and LL (Fig. 1C and 1D).

The expression ratios ($n = 32$) determined by microarray analysis were confirmed by the QRT-PCR assay (Fig. 2). The gene expression ratio of *16S rRNA*, *kbp*, *rgpA*, *dnaK*, *sod*, *prrC*, *tpx*, and *oxyR* was calculated from their expressions in EL, LL, ST-E, and ST-10 samples divided by

Fig. 1 Bacterial growth and the gene expression profiling. (A) a growth curve of *P. gingivalis* W83 *in vitro*. Arrows indicate the growth stages studied. EL: early-log phase, ML: mid-log phase, LL: late-log phase, ST-E: early stationary phase, ST-10: 10 h after the stationary phase. (B) a scatter plot analysis of the cohybridization using Cy3 and Cy5 fluorescently labeled cDNA from ML sample. Dashed lines show two-fold. (C) a profiling of all mRNA expressions (n = 92) during growth. They expressed as fold change in comparison with the ML. (D) a profiling of the average of all mRNA expressions during growth. Data are presented as means $\pm$ SD.

that in the ML sample. There was a strong positive correlation between the results of both analyses ($r = 0.910$, $p < 0.01$).

Hierarchical cluster analysis

Hierarchical cluster analysis of the 92 genes was performed. The kinetics of the changes in their expression during growth were divided into five clusters and evaluated

(Fig. 3, Table 1). The genes belonging to clusters 1 and 2 accounted for 62% of the target genes in this study. In both clusters, gene transcription was sharply lower in ST-E, but then declined more gradually during the period from ST-E to ST-10 in cluster 2 than in cluster 1. Cluster 1 contained many genes encoding components of peptidoglycan (*murC* and *murG*) (14, 15), cell division-associated proteins (*ftsA* and *ftsZ*) (16), a fimbriin and the surrounding ORFs (one

Fig. 2 Correlation of DNA microarray and real-time PCR assays. The expression ratios ($n = 32$) determined by microarray analysis were confirmed by QRT-PCR assay. n : sample number, r : correlation coefficient, Y : regression equation.

upstream and four downstream ORFs). Cluster 2 contained genes encoding proteins involved in protein synthesis (*ef-g* and *ef-tu*) (17), RNA synthesis and processing (*rbfA*, *rpoB*, and *rpoC*) (18) and cell growth (*ftsQ*, *gyrB*, *surE*, and *uip*) (19-21). Most hemin-associated proteins and proteases tested belonged to cluster 1 or 2.

The kinetics of the changes in the transcription of the genes in clusters 3 and 4 differed considerably from the others. Cluster 3 contained five genes that showed increased transcription at ST-10 after reaching a minimum during ST-E. Four of those five genes, *dnaK*, *groEL*, *groES* and *htpG*, encode proteins involving protein folding and stabilization (22). Cluster 4 contained 14 genes that showed increased transcription after ML, which remained high even at ST-10. This cluster contained genes involved in the transposon function (*ISPG1*, *KIS-2*, and *ISPG5*) (23), oxidative stress (*dps*, *oxyR*, and *rbr*) (24-26) and energy metabolism (*mcmA* and *mcmB*) (27). Cluster 5 contained 20 genes that showed comparatively small fluctuations in transcription during growth.

Characterization of each growth stage

Gene transcriptions occurring in pairs of sequential growth stages were compared. During EL, 16 transcriptions, including *ahpF*, *ahpC*, *dps*, *ftn*, *cydA*, *sod*, *tpx*, *groEL* and *rbr*, were significantly higher than during ML. During LL, while 30 transcriptions of the 31 genes were higher than during ML. Among them were cysteine proteases (*HGP15*, *rgpA*, and *rgpB*) (28), energy metabolism-associated genes

(*hbp35*, *gdh*, *cydA*, and *rbr*) (29) and stress-responsive genes (*dps*, *ahpFC*, *ftn*, *tpx*, and *sod*) (30, 31), as well as *recA*-like protein and transposase (*ISPG1* and *ISPG5*). During ST-E, 72 transcriptions were at a lower level than during LL; five gene expressions (*ef-g*, *folK*, *hmuY*, *PG35*, and *PG50*) (17, 32) showed changes of 50-fold or greater; 14 showed 10- to 50-fold; 33 showed 2- to 10-fold. Conversely, 10 transcriptions were increased; among these, six (*fimS/fimR*, *ISPG1*, *PG27*, *PG47*, *HGP15*, and *rbr*) (33) increased more than two-fold. A comparison of ST-E and ST-10 showed that the reductions in gene expression persisted; and indeed, 80% of the genes showed the minimum transcription levels at ST-10. By contrast, transcription of 10 genes (*dnaK*, *ef-g*, *ef-tu*, *groEL*, *groES*, *htpG*, *hup*, *mcmB*, *oxyR*, and *porT*) (17, 22, 25, 27, 30, 31) was higher at ST-10 than in ST-E (Fig. 4).

Discussion

We have developed a custom DNA microarray for *P. gingivalis*. A total of four microarray plates (four identical DNA spots/ a microarray) were used for gene expression analyses in each growth stage compared with ML. The reliability of the microarray data revealed a linear correlation with no more than a two-fold change in the relative expression level. In addition, QRT-PCR assays confirmed the validity of the expression ratios by microarray analysis. These results indicate the custom-made microarray is useful for a research of global gene expression in *P. gingivalis*.

The reliability of a relative comparison depends largely on the normalization of unwanted variations between samples. In eukaryotes, the average transcriptional levels of all genes on the chromosome are frequently used for normalization. Alternatively, constantly expressed genes, often selected from among housekeeping genes such as β -actin and GAPDH, are used as internal controls for normalization of the results. In prokaryotes, on the other hand, 16S rRNA has been frequently used for normalization. However, the expression is not stable during the growth because it depends on protein expressions. More notably, the expressions decreased remarkably after ST, as shown in this study. In addition, the rRNA copy number, as determined by QRT-PCR, was approximately 1,000 to 50,000-fold higher than our interested inducible genes (data not shown). All together, pre-labeled external controls (β -actin, GAPDH, and lactoferrin receptor genes in humans) were used for normalization among samples.

Little is known about whether the difference of the

Fig. 3 Hierarchical cluster analysis and the kinetics of the changes in gene expression during the growth of *P. gingivalis*. Shown are the distributions of individual gene expression levels; individual growth phases (EL, ML, LL, ST-E, and ST-10; columns), the 92 genes (rows), and the kinetics of changes in gene expression in each cluster (graphs). The scale bar indicates the color-coding of the RNA levels relative to the level in EL.

Table 2 Gene list spotted on a *P. gingivalis* array

Gene ID	TIGR ID	Description	Main role ^b
Cluster 1^a			
<i>hbp35</i>	PG0616	thioredoxin, hemin-binding protein	Energy metabolism
<i>PG0668</i>	PG0668	hypothetical tonB-linked outer membrane receptor	Transport and binding proteins
<i>PG2133</i>	PG2133	lipoprotein	Cell envelope
<i>PG2136</i>	PG2136	hypothetical protein	
<i>PG2134</i>	PG2134	lipoprotein	Cell envelope
<i>PG2135</i>	PG2135	lipoprotein	Cell envelope
<i>fimA</i>	PG2132	fimbriin	Cell envelope
<i>ftsZ</i>	PG0584	cell division	Cellular processes
<i>pfk</i>	PG0163	phosphofructokinase	Energy metabolism
<i>recA</i>	PG0881	rec A-like protein	DNA metabolism
<i>fur</i>	PG0465	ferric uptake transcriptional regulator	Regulatory functions
<i>kgp</i>	PG1844	lysine-specific cysteine proteinase	Protein fate
<i>gdh</i>	PG1232	NAD-dependent glutamate dehydrogenase	Energy metabolism
<i>ftn</i>	PG1286	ferritin	Transport and binding proteins
<i>ftsA</i>	PG0583	cell division	Cellular processes
<i>murG</i>	PG0580	UDP-N-acetylglucosamine	Cell envelope
<i>pepO</i>	PG0159	endopeptidase	Protein fate
<i>ragA</i>	PG0185	tonB-dependent outer membrane receptor	Unknown function
<i>murC</i>	PG0581	UDP-N-acetylmuramate	Cell envelope
<i>fetB</i>	PG0669	heme-binding protein	Transport and binding proteins
<i>luxS</i>	PG0498	autoinducer-2 production protein	Cellular processes
<i>PG2131</i>	PG2131	60kDa protein	Unknown function
<i>PG2226</i>	PG2226	hypothetical protein	
Cluster 2			
<i>mfa1</i>	PG0176	cell surface protein (fimbriin-related)	Cell envelope
<i>PG2223</i>	PG2223	glycosyl transferase, group 2 family protein	Cell envelope
<i>prtQ</i>	PG0753	protease	Protein fate
<i>PG2224</i>	PG2224	putative epithelial cell attachment protein	Cellular processes
<i>ftsQ</i>	PG0582	cell division protein	Cellular processes
<i>hemK</i>	PG0156	modification methylase	Unknown function
<i>rgpB</i>	PG0506	Arg-gingipain-B (RGP-B)	Protein fate
<i>surE</i>	PG2163	survival protein	Cellular processes
<i>sod</i>	PG1545	superoxide dismutase	Cellular processes
<i>hemH</i>	PG0127	ferrochelatase	Biosynthesis of cofactors, prosthetic groups, and carriers
<i>PG0188</i>	PG0188	lipoprotein	Cell envelope
<i>dpp IV</i>	PG0724	dipeptidyl peptidase IV; serine protease	Protein fate
<i>porR</i>	PG1138	pigmentation and extracellular proteinase regulator	Regulatory functions
<i>upp</i>	PG0752	Uracil phosphoribosyltransferase	Purines, pyrimidines, nucleosides, and nucleotides
<i>porS</i>	PG1137	porS protein	Unknown function
<i>PG0209</i>	PG0209	formate/nitrite transporter	Transport and binding proteins
<i>folK</i>	PG1541	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	Biosynthesis of cofactors, prosthetic groups, and carriers
<i>arcA</i>	PG1424	peptidylarginine deiminase	Cellular processes
<i>PG0922</i>	PG0922	membrane protein	Cell envelope
<i>prtC</i>	PG1542	collagenase	Protein fate
<i>PG0924</i>	PG0924	5-nucleotidase	Cell envelope
<i>gyrB</i>	PG1702	DNA gyrase subunit B (topoisomerase)	DNA metabolism
<i>hmuR</i>	PG1552	tonB-dependent receptor HmuR	Transport and binding proteins
<i>hmuY</i>	PG1551	tonB-dependent receptor HmuY	Transport and binding proteins
<i>rbfA</i>	PG0923	Ribosomal binding factor A	Transcription
<i>PG2008</i>	PG2008	hypothetical tonB-linked outer membrane receptor	
<i>PG0707</i>	PG0707	hypothetical tonB-linked outer membrane receptor	Transport and binding proteins
<i>rpoC</i>	PG0395	DNA-dependent RNA polymerase	Transcription
<i>rpoB</i>	PG0394	DNA-dependent RNA polymerase	Transcription
<i>fusA</i>	PG1940	translation elongation factor G (EF-G)	Protein synthesis
<i>rpsA</i>	PG1297	ribosomal protein S1	Protein synthesis
<i>tufA</i>	PG0387	translation elongation factor Tu (EF-Tu)	Protein synthesis

(continued)

Gene ID	TIGR ID	Description	Main role ^b
Cluster 3			
<i>dnaK</i>	PG1208	DNA replication	Protein fate
<i>groEL</i>	PG0520	heat shock protein 60 (GroEL)	Protein fate
<i>groES</i>	PG0521	heat shock protein 10 (GroES)	Protein fate
<i>htpG</i>	PG0045	high temperature protein ; (Hsp90 homologue)	Protein fate
<i>porT</i>	PG0751	porT protein	Unknown function
Cluster 4			
<i>dps</i>	PG0090	DNA-binding stress protein	Cellular processes
<i>mutA</i>	PG1656	methylmalonyl-CoA mutase	Energy metabolism
<i>htrA</i>	PG0648	htrA protein (ABC solute binding protein)	Transport and binding proteins
<i>ISPG4</i>	PG0050	transposase	Mobile and extrachromosomal element functions
<i>tlyC</i>	PG0272	hemolysin-related protein with CBS domains	Unknown function
<i>oxyR</i>	PG0270	redox-sensitive transcriptional activator	Regulatory functions
<i>PG1432</i>	PG1432	sensor histidine kinase	Signal transduction
<i>ISPg1</i>	PG0184	transposase	Mobile and extrachromosomal element functions
<i>mutB</i>	PG1657	methylmalonyl-CoA mutase	Energy metabolism
<i>rbr</i>	PG0195	rubrerythrin	Energy metabolism
<i>PG1317</i>	PG1317	hypothetical outer membrane protein	
<i>ISPg5 (orf2)</i>	PG0008	transposase orf2	Other categories
<i>PG1414</i>	PG1414	hypothetical tonB-linked outer membrane receptor	
Cluster 5			
<i>ISPg5 (orf1)</i>	PG0009	transposase orf1	Other categories
<i>nosR</i>	PG1623	membrane-bound regulatory protein	Regulatory functions
<i>PG1759</i>	PG1759	adhesin protein	Cell envelope
<i>atoS</i>	PG0017	sensor histidine kinase	Signal transduction
<i>phyA</i>	PG1875	hemolysin A	Cellular processes
<i>bcp</i>	PG0880	bacterioferritin comigratory protein	Unknown function
<i>PG0938</i>	PG0938	calcium ion-transporting ATPase	Transport and binding proteins
<i>comM</i>	PG1768	competence protein ComM	Transport and binding proteins
<i>hemN</i>	PG1505	oxygen-independent coproporphyrinogen III oxidase	Hypothetical proteins
<i>rgpA</i>	PG2024	Arg-gingipain-A (RGP-A)	Cellular processes
<i>ahpC</i>	PG0618	alkyl hydroperoxide reductase subunit C	Cellular processes
<i>tpx</i>	PG1729	thiol peroxidase	Cellular processes
<i>tla</i>	PG1844	TonB-linked adhesin.	
<i>cydB</i>	PG0899	cytochrome d oxidase subunit II	Energy metabolism
<i>feoB</i>	PG1043	ferrous iron transport protein B	Transport and binding proteins
<i>cydA</i>	PG0900	cytochrome d oxidase subunit I	Energy metabolism

^a The numbers indicate the result by a hierarchical cluster analysis divided into five clusters.^b The classifications are described according to that in TIGR database.

bacterial growth stage used in our study will influence our target gene expressions. For study, most researchers use bacterial cells grown in the logarithm phase, especially from the mid-log to late-log phase in order to keep the cell mass. Therefore, we examined a relative rate of the gene expressions in other growth phases normalized with gene expressions in ML.

In this study, dramatic changes of transcripts in EL and LL were shown rather than in ML. In addition, most transcripts declined after LL. Interestingly, transcripts in cluster 4 continued increasing even in the stationary phase. Among these, transposon is known to be one of the mobile

DNA elements that mediate genome rearrangement by inserting themselves within and between genomes. It is noteworthy that their transcription increased significantly during the period from LL to ST, as we have found that spontaneous mutation often occurs in long-term cultures.

Correspondingly, the gene expressions in cluster 3 were also characteristic. They were minimized in ST-E and subsequently increased in an ST-10, but nevertheless the expression levels of 80% genes showed the minimum. Four of five genes in cluster 3 were stress proteins (*dnaK*, *groEL*, *groES*, and *htpG*). This is indicative of a cellular response to environmental stresses such as oxidative stress, osmotic

Fig. 4 The end-point RCR (30 cycles) analysis of the gene expression profiles about protein folding and stabilization at individual growth phases in *P. gingivalis*. EL: early-log phase, ML: mid-log phase, LL: late-log phase, ST-E: early stationary phase, and ST-10: 10 h after stationary phase.

stress and superfluous accumulation of the metabolic products, that could affect protein folding and stability.

P. gingivalis exhibits a complex response to oxidative stress, which accounts for its aerotolerance (24-26). The expression of genes such as *ahpCF*, *dps*, *ftn*, *cydA*, *sod*, *tpx*, and *groEL* increased during EL, suggesting these genes may have responded to residual oxygen in the culture medium, present despite incubation in an anaerobic chamber three days before use.

Thompson et al. (6) used a DNA microarray to analyze the global pattern of the growth phase-dependent gene expression in a *Helicobacter pylori* culture. Cluster analysis of the expression profiles revealed a major switch in gene expression during the LL to ST phase transition, which was termed "the Log-Stat switch." Statistical analysis of the genes that were significantly induced or repressed during the Log-Stat switch showed that many of these genes were related to virulence. Similarly, our data showed a significant increase or decrease in the expression of virulence-related genes during the transition from LL to ST-E, suggesting that it may correspond to the most important growth phase for the virulence of *P. gingivalis*.

In conclusion, virulence-relative gene expressions in *P. gingivalis* are suggested to be regulated during growth in a

way that enables the metabolism to be adapted to the environmental conditions, and the expressions of numerous genes are growth phase-specific. For *in vitro* study, especially, bacteria samples during LL to ST-E provide us with more important information as opposed to ML by showing dramatic transcriptional changes at those times. Our findings also give us a need to pay attention to the sampling time of bacterial cells and support that microarray techniques constitute a powerful new tool with which to study how gene expression changes during bacterial growth, enabling characterization of microbial communities and their biological functions.

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