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Original

Monitoring of *dnaK* gene expression in *Porphyromonas gingivalis* by oxygen stress using DNA microarray

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Abstract: ***Porphyromonas gingivalis*, a Gram-negative anaerobe associated with adult periodontitis, expresses numerous potential virulence factors. *dnaK*, a member of the heat shock protein family, functions as a molecular chaperone and plays a role in microbial pathogenicity. However, little is known regarding its gene expression caused by oxygen stress in *P. gingivalis*. In the present study, a custom-made DNA microarray was designed and used to monitor *dnaK* gene expression in *P. gingivalis* caused by oxygen stress. The results demonstrated that *dnaK* mRNA was up-regulated in a short time, and the DNA microarray results were confirmed by real-time polymerase chain reaction analysis. These findings suggest that oxygen stress stimulates gene expression of *dnaK* and may have a relationship to the aerotolerance activity of this organism as well as its expression of pathogenesis. (J. Oral Sci. 46, 93-100, 2004)**

Key words: *Porphyromonas gingivalis*; oxygen stress; *dnaK*; gene expression; DNA microarray.

Introduction

Microorganisms adapt to various environmental conditions by switching on and off their expression of virulence genes. This biological process allows for optimal growth and survival within different environmental niches, and the adaptation strategy includes sensing and responding to changes in oxygen tension (1). Especially for anaerobes, it is critical that virulence genes are expressed in an oxygen rich condition during the infection process. Atmospheric oxygen is metabolically converted to reactive oxygen species in bacterial cells. While reactive oxygen species are also generated by polymorphonuclear leukocytes and attack invading bacterial cells it is widely recognized that two cellular systems function to protect organisms from oxidative stresses (2, 3).

Porphyromonas gingivalis, a Gram-negative anaerobe, is most strongly associated with adult periodontitis and expresses numerous potential virulence factors, such as fimbriae, hemagglutinins, and lipopolysaccharides (4). This microorganism, an obligate anaerobe, exhibits a relatively high degree of aerotolerance (5) and possesses superoxide dismutase (SOD) in response to oxidative stress (6), which is an important factor in its relative aerotolerance.

P. gingivalis also responds to environmental stressors by up-regulating a set of genes encoding heat shock protein (HSP) (7), which is found in some of the most common immunodominant antigens expressed by bacteria (8). *Escherichia coli* DnaK functions as a molecular chaperone to mediate protein folding, protein translocation, and repair of unfolded proteins damaged by environmental stress (9). However, little is known regarding *dnaK* expression caused by oxygen stress in *P. gingivalis*.

The recent advent of DNA microarray technology allows a thorough analysis of gene expression patterns in different environmental conditions (10). In this approach, individual DNA probes are arrayed on a small glass surface, with labeled first strand cDNA from specific tissue or cell sources hybridized onto the array. The amount of

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fluorescence at each DNA probe spot correlates with the abundance of specific mRNA transcripts in the cell. We have constructed a custom-made DNA microarray that contained virulent genes including *dnaK* in the Genebank and TIGR databases (11).

In the present study, we monitored gene expression change of *dnaK* gene expression using the DNA microarray with oxygen stress in *P. gingivalis*. Further, the DNA microarray result was confirmed using a real-time polymerase chain reaction (PCR) analysis.

Materials and Methods

Bacterial culture

P. gingivalis W83 strains were grown in brain heart infusion (BBL Microbiology Systems, Cockeysville, MD, USA) 0.25% yeast extract supplemented with hemin (5 μg/ml) and menadion (1 μg/ml). All cultures were incubated at 37°C in an anaerobic chamber containing 80% N_2, 10% H_2, and 10% CO_2.

For the oxygen stress treatment, 20 ml of cell culture at late-log phase was transferred to sterile flasks and vigorously shaken at 37°C in air by using a thermostatic shaking incubator (TAL-RS310, Thomas, Tokyo, Japan). Cells were harvested by centrifugation at 8,000 × *g* for 30 min at 4°C and resuspended in DNAase and RNAase free water for total RNA extraction.

Purification of RNA

Cells were transferred into FastPrep tubes containing Lysing Matrix B (Qbiogene Inc., CA, USA) with 900 μl of Trizol Regent (Invitrogen Corp., CA, USA). After disruption of the cells using a cell homogenizer (FP120; Qbiogene) at a speed rating of 6.5 for 40 sec, the cells were incubated on ice for 2 min. The RNA fraction was then extracted with chloroform and isopropanol, and stored with 80% ethanol. Each sample was resuspended with DW (Invitrogen), and then its quality and concentration was examined using an Aligent 2100 bioanalyzer with a RNA Nano LabChip Kit (Aligent Technology, CA, USA).

Preparation of fluorescent-labeled cDNA.

For a direct fluorescent labeling cDNA preparation, a CyScribe First-Strand cDNA Labeling Kit (Amersham Bioscience, NJ, USA) was used. Briefly, 25 μg of total RNA and 2 μg of random nonamers were denatured by heating at 70°C for 5 min and kept at 25°C for 10 min. Each reaction was then reverse-transcribed with superscript II with either Cy3-dUTP or Cy5-dUTP for 1.5 h at 42°C. Template RNA was destroyed using 2 μl of 2.5 M NaOH at 37°C for 15 min and neutralized with 10 μl of 2 M HEPES buffer, and then purified using a QIA quick® PCR Purification Kit (Qiagen, CA, USA).

Preparation of DNA microarrays

Chromosomal DNA was prepared from *P. gingivalis* strains by the method of Smith *et al*. (12). To construct the microarrays, 123 genes, including *dnaK*, were selected from the GeneBank and TIGR databases, and the specific region from the genomic DNA was designed and amplified by PCR. Information for the DNA nucleotide sequence of *dnaK* was obtained from *P. gingivalis* 381. PCR was carried out using Taq DNA polymerase (Perkin Elmer Cetus, CT, USA) in a DNA thermal cycler (Gene amplification PCR system, Perkin Elmer Cetus) and all PCR products were verified by agarose gel electrophoresis to ensure that only a single product of the expected length had been amplified with each primer pair. Purified PCR products were diluted to a final concentration of 50 ng/μl using Microarray Crosslinking Reagent D (Amersham Bioscience Corp.) and then transferred to a 384-well microplate for spotting. DNA samples were spotted on a silane-coated Microarray Slide Type 7 (Amersham Bioscience) using a GEN III Microarray Spotter (Amersham Bioscience), and fixed by UV crosslinking at 100 mJ/cm^2 in a UV Stratalinker 1800 (Stratagene, CA, USA). Each glass slide contained 6 replicates of the gene probe.

Microarray analysis

Hybridization and washing steps were carried out using an automated hybridization and washing machine (Automated Slide Processor, Amersham Bioscience) as per the manufacturer's recommendations. Briefly, the DNA spotted glass slides were pre-treated with a pretreatment solution containing 2 × SSPE and 0.2% SDS at 55°C for 2 h. Fifty-five microliters of dye-labeled sample was mixed with 200 μl of the hybridization cocktail (Amersham Bioscience) and denatured at 95°C for 3 min. Fifty-five microliters of the mixture was mixed with 110 μl of formamide and the solution was then injected into the slide chamber of the hybridization and washing machine. This was followed by hybridization carried out at 42°C for 12 h. Following hybridization, the slides were washed twice with washing buffer I, (1 × SSC, 0.2% SDS), at 65°C for 5 min, and were washed twice by washing solution II, (0.1 × SSC, 0.2% SDS), at room temperature for 5 min, and were washed once with 0.1 × SSC at room temperature for 30 sec, prior to being air dried in the dark.

The microarrays were scanned with a scanning laser confocal fluorescence microscope (GenePix, Amersham Bioscience). The emitted fluorescent signal was detected by a photo-multiplier tube at 570 nm for Cy3 or at 670

nm for Cy5. The scanned image displays were saved as 16-bit TIFF files and analyzed by quantifying the pixel density of each hybridization spot using the software (ImaGene version 3.0, Biodiscovery, CA, USA). The local background signal was subtracted automatically from the hybridization signal of each separate spot. Fluorescence intensity values for the 3 pre-labeled human genes used as positive controls were averaged and then used for the

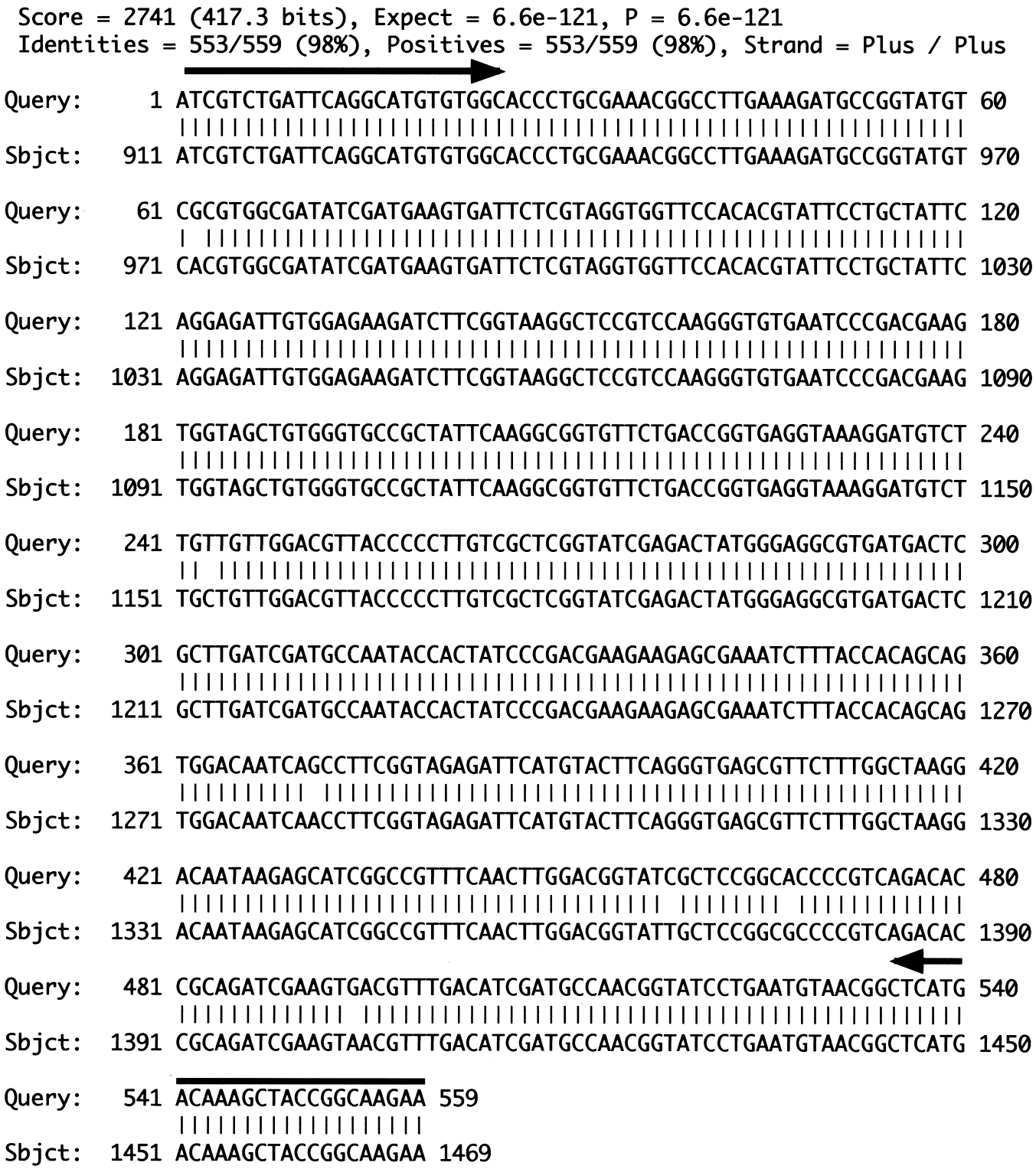

Fig. 1 Nucleotide homology search of *dnaK* PCR products spotted on microarray with *dnaK* gene database of *P. gingivalis* W83. Query, PCR products from the *P. gingivalis* 381 chromosomal DNA template; Sbjct, *dnaK* sequence of *P. gingivalis* W83 in the DNA database. Arrow lines indicated PCR primer regions.

normalization of each chip. Statistical analysis was performed using GeneSpring software (Silicon Genetics, CA, USA).

Real-time PCR analysis

The design of the oligonucleotide primers for the *dnaK* gene was evaluated using Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primer sequences and other information for *dnaK* are listed in Fig. 1.

Two-step quantitative detection of the *dnaK* gene of *P. gingivalis* was performed as follows. Total RNAs were reverse-transcribed using Superscript II and Random Primer (Gibco BRL, MD, USA), and PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen). The amplification reactions were performed in 20 µl of final volume containing 10 µl of 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.25 µM *dnaK*-specific DNA primers, and 5 µl of 500-fold diluted cDNA solution. To reduce the variability between replicates, PCR premixes, which contained all reagents except for cDNA, were prepared and aliquoted into 0.2 ml thin-wall strip tubes (MJ Research Inc., MA, USA). The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 min and 40 cycles at 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. All experiments were performed in quadruplicate for each data point. All PCR reactions were performed using an OPTICON™ DNA Engine (MJ Research).

Results

Identification of microarray

We used *P. gingivalis* W83 strain to monitor *dnaK* gene expression caused by oxygen stress and the *dnaK* probe was spotted onto the microarray using *P. gingivalis* 381 chromosomes as the template DNA. In order to confirm the quality and usefulness of the *dnaK* probe, DNA sequences of *dnaK* PCR products from *P. gingivalis* 381 chromosomal DNA templates were examined and a DNA sequence homology search with *dnaK* in W83 was also carried out. As shown in Fig. 1, PCR products on the microarray from *P. gingivalis* 381 had a 98% homology with the *dnaK* gene from the W83 strain. This result showed that our *dnaK* probe was useful for analyzing the *dnaK* mRNA level in the W83 cells.

dnaK transcript profiling using microarray

Gene transcription profiles for *P. gingivalis* W83 treated with oxygen for 15, 45, and180 min, were compared with the control, which was cultured in an anaerobic condition. Each time point examination was carried out using 2 sets of DNA microarray data generated from 5 independent

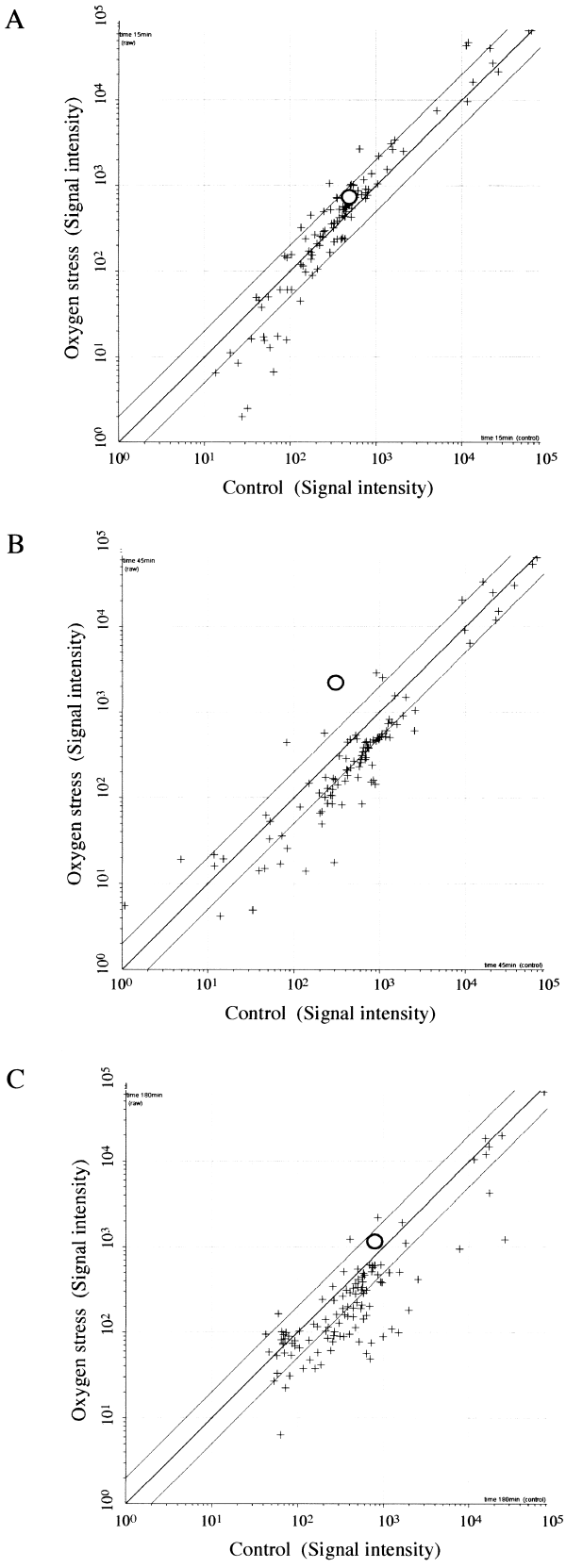

Fig. 2 DNA microarray analysis of mRNA level changes in *P. gingivalis* W83 by oxygen stress. A, 15 min; B, 45 min; C, 180 min after oxygen stress. Circle, dnaK; cross dots, other 122 genes from *P. gingivalis* W83 on microarray.

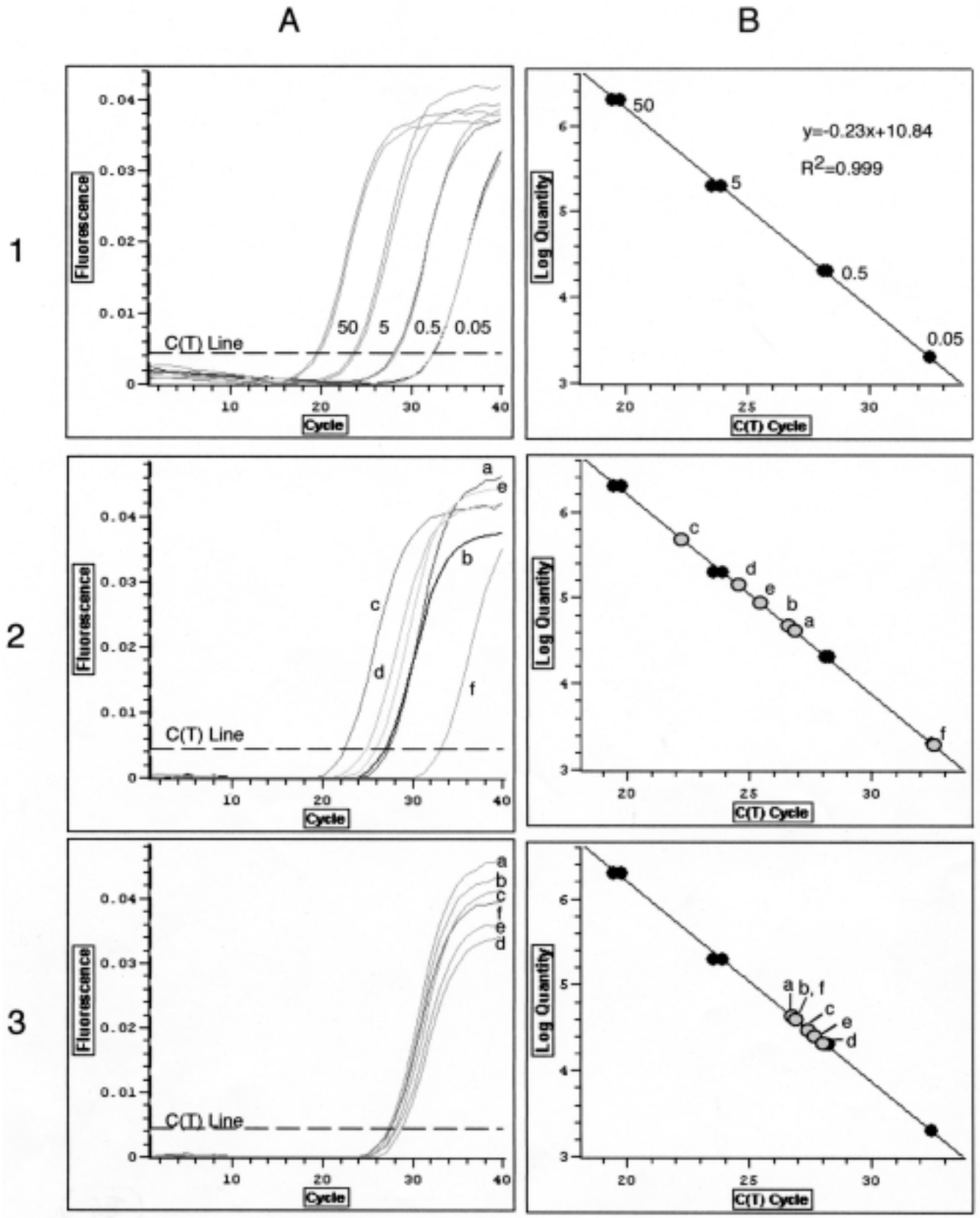

Fig. 3 Real time PCR quantitation of *dnaK* gene Expression. A, Fluorescent detection curves of *dnaK* gene amplification. B, Standard curve of *dnaK* mRNA for quantitation. 1, Standard samples; 10-fold serial dilutions from 0.05 ng to 50 ng of chromosomal DNA from *P. gingivalis*. 2, RNA samples from aerobic cell cultures under oxygen stress. 3, RNA samples from anaerobic cell cultures used as controls. Incubation time; a, 0 min; b, 15 min; c, 45 min; d, 90 min; e, 180 min; f, 360 min.

microarray slides. Scatter plots of a total of 123 genes from each time point of the samples are shown in Fig. 2. The most striking difference caused by oxygen stress in *P. gingivalis* W83 cultures was the up-regulation of *dnaK* gene expression (open circle in Fig. 2) after 45 min of oxygen treatment.

Evaluation of *dnaK* gene expression by real time-PCR

Microarray analysis showed that *dnaK* expression was increased by oxygen stress, therefore, we also examined *dnaK* mRNA levels from *P. gingivalis* W83 cultures after oxygen stress treatments of 45, 90, 180 and 360 min using real-time quantitative RT-PCR analysis to assess the reliability of the microarray results. Figure 3 shows PCR exponential curves (Fig. 3A) and the copy number standards of the *dnaK* templates (Fig. 3B) at the selected time points of oxygen treatment. The mRNA copy number from each sample was determined by copy number standards. A reliable quantification with high linearity for *dnaK* mRNA with and without oxygen treatments at each time point was obtained, and the normalized *dnaK* mRNA copy number is shown in Fig. 4. Quantitative real-time PCR assay results of *dnaK* expression were very similar to the microarray results. The ratio and copy number of *dnaK* mRNA were increased at 45 min, decreased at 90 min, and nearly totally diminished at 360 min.

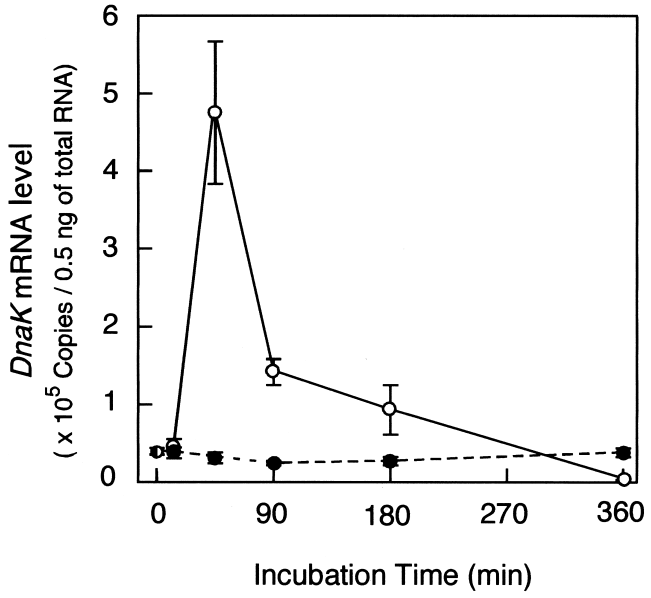

Fig. 4 Estimation of *dnaK* mRNA copy number using real time PCR. Experiments were performed in quadruplicate for each data point. ○, oxygen stress; ●, control (without oxygen stress). Vertical bars are mean ± SE.

Discussion

In the present study, we demonstrated that *dnaK* gene expression was increased by oxygen stress using a custom-made DNA microarray. Oxygen stress significantly increased the *dnaK* mRNA level after 45 min. The DNA microarray method is becoming widely used for large-scale gene expression profiling, and methods for assessing statistical significance have also begun to be developed. One problem underlying the microarray technology is that it can produce false positive results, and the results must be confirmed using additional methods, such as the RT-PCR analysis. However, some problems occur when using an endpoint PCR method that uses gel electrophoresis to measure mRNA levels, as endpoint PCR analysis can only provide semi-quantitative results, and the differences between varying concentrations of PCR reaction components are difficult to discern due to reagents that may be limiting as the reaction progresses. In contrast, real-time PCR analysis, in which the accumulation of PCR products is measured and reported during each amplification cycle, makes it possible to quantitatively analyze data.

In this study, in order to validate our microarray expression data, we prepared mRNA samples from varying time cultures following oxygen stress treatment and reexamined *dnaK* mRNA levels using real-time quantitative RT-PCR. The results showed that oxygen stress increased *dnaK* mRNA levels in *P. gingivalis* cells at 45 min, which were decreased at 90 min, and almost completely negligible after 360 min. These results were consistent for fold change and direction of change in almost all instances between the microarray data and real-time quantitative RT-PCR results, and suggest that the custom-made microarray is useful and accurate for analysis of *dnaK* gene expression.

HSPs are classified into groups or families according to their molecular mass in kilodaltons, and encoded by genes that increase their expression during stress conditions such as heat shock, heavy metals, alcohol, inflammation, and oxidative stress. Under these conditions, HSP increases cell survival rate by protecting and disaggregating stress-labile proteins (13). In non-stress conditions, they have multiple housekeeping functions, such as folding and translocating proteins, activating transcription factors, and protein degradation (14,15). HSP may also play a role in microbial pathogenicity, and antigenic components observed in a variety of bacterial infections and those involved in the pathogenesis of diseases have been identified as members of HSP families (16). The dominant antigens are found in microorganisms, and are capable of inducing strong humoral and cellular responses (17).

Oxidative conditions for anerobic bacteria are stressful and lead to toxicity; however, the molecular events

contributing to lethality are poorly defined. During macrophage killing of bacteria, the organisms are first engulfed by endocytosis into phagosomes, which then fuse with lysosomes to form phagolysosomes (18). In the present study, we found that oxygen stress increased *dnaK* gene expression. Although *dnaK* plays an important role to help this microorganism survive under oxygen stress, oxygen stress may increase *dnaK* expression over a period of time and then abrogate it almost completely over a longer period in *P. gingivalis*. Thus, encountering a long period of exposure to a stressful oxygen condition is likely to cause severe damage because a very low expression of *dnaK* leads to a lethal situation for this microorganism.

The *dnaK* family is comprised of highly conserved molecular chaperones that regulate a wide variety of cellular processes during normal and stress conditions (19). The chaperone activity of *dnaK* involves an ATP-controlled cycle of polypeptide binding and release, during which a newly synthesized or translocated polypeptide is promoted to correct folding by preventing aggregation and misfolding (20). *P. gingivalis dnaK* has been molecularly cloned, and it was shown that the gene encoded 641 amino acids with a molecular mass of 69, 136 (21). GroEL-like proteins isolated from *Actinobacillus actinomycetemcomitans, P. gingivalis, and Bacteroides forsythus* show a high degree of homology between their N-terminal amino acid sequences. These findings suggest that dnaK- and GroEL-like proteins from periodontal pathogens are well conserved, and that GroEL-like proteins resemble each other closely (22).

The role of the immune response to HSP in infectious and autoimmune diseases is still controversial, though there is evidence that microbial HSPs are immunodominant antigens of many microorganisms. Lopatin et al. (23) studied the relationship between immunity to HSPs and periodontal disease status, and found that patients with higher levels of the anti-*dnaK* antibody tended to have significantly healthier periodontal tissues, reflecting the presence of protective anti-HSP antibodies. On the other hand, HSPs have been increasingly recognized as important molecules in infectious and autoimmune diseases, as they are strongly immunogenic, with immune responses to microbial HSP speculated to initiate chronic inflammatory diseases that have autoimmune responses to human HSP. It has also been postulated that periodontopathic bacteria stimulate host cells in the periodontium to up-regulate the expression of HSP, which in turn may stimulate the production of proinflammatory cytokines involved in tissue destruction with periodontal disease (24). Kirby et al. (25) reported that a molecular chaperone, GroEL, from *A. actinomycetemcomitans* has the capacity to stimulate potent bone-resorbing activity. Further, Shelburne et al. (26) compared *dnaK* gene transcription results to clinical data from periodontal sites, and found correlations between disease status and elevated expression of *dnaK*.

Further studies are needed to elucidate the role of DnaK caused by oxygen stress in the progression of periodontitis. Since DnaK is an important virulent factor, it is not only essential to determine the expression change of *dnaK* due to oxygen stress, but also to be able to monitor different conditions and challenges that may arise. Such experiments using a custom microarray are in progress in our laboratory.

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References

1. Forng RY, Champagne C, Simpson W, Genco CA (2000) Environmental cues and gene expression in *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Oral Dis 6, 351-365
2. Farr SB, Kogoma T (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev 55, 561-585
3. Miller RA, Britigan BE (1997) Role of oxidants in microbial pathophysiology. Clin Microbiol Rev 10, 1-18
4. Holt SC, Kesavalu L, Walker S, Genco CA (1999) Virulence factors of *Porphromonas gingivalis*. Periodontol 2000 20,168-238
5. Nakayama K (1994) Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. J Bacteriol.176, 1939-1943
6. Lynch MC, Kuramitsu KH (1999) Role of superoxide dismutase activity in the physiology of *Porphyromonas gingivalis*. Infect Immun 67, 3367-3375
7. Maeda H, Miyamoto M, Hongyo H, Nagai A, Kurihara H, Muryama Y (1994) Heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*: molecular cloning and sequence analysis of its gene and purification of the recombinant protein. FEMS Microbiol Lett 124, 121-122.
8. Zugel U, Kaufmann H (1999) Immune response against heat shock proteins in infectious diseases. Immunobiology 201, 22-35

9. Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92, 351-366
10. Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM (1999) Expression profiling using cDNA microarrays. Nat Genet. 21, 10-14
11. Hiratsuka K, Kiyama-Kishikawa M, Shibata Y, Hamajima S, Abiko Y (2003) Microanalysis of virulence gene expression in *Porphyromonas gingivalis* during culture pahse. J Dent Res 82 , B-281 (abstract)
12. Smith GL, Sansone C, Socransky SS (1989) Comparison of two methods for the small- scale extraction of DNA from subgingival microorganisms. Oral Microbiol Immunol 4, 135-140
13. SkowyraD, Georgopoulos C, Zylicz M (1990) The *Escherichia coli dnaK* gene product, the hsp70 homologue, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. Cell 62, 939-944
14. Helmbrecht,K., Zeise,E. and Rensing,L. (2000) Chaperone in cell cycle regulation and mitogenic signal transduction: a review. Cell Prolif 33, 341-365
15. Herendeen SL, Van Bogelen RA, Neidhardt FC (1979) Levels of the major proteins of *Escherichia coli* during growth at different temperatures. J Bacteriol 139, 185-194
16. Gaston JSH, Life PF, Bailey LC, Bacon PA (1989) *In vitro* responses to a 65-kilodalton mycobacterial protein by synovial T cells from inflammatory arthritis patients. J Immunol 143, 1289-1295
17. Lathigra RB, Butcher PD, Garbe TR, Young DB (1991) Heat-shock proteins as virulence factors of pathogens. Curr Top Microbiol Immunol 167, 125-143
18. Hassett D J, Cohen MS (1989) Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. FASEB J 3, 2574-2582
19. Boorstein WR, Ziegelhoffer T, Craig EA (1994) Molecular evolution of the Hsp70 multigene family. J Mol Evol 38, 1-17
20. Liberek K, Marszalek J, Ang D, Georgopoulos C, Zylicz M (1991) *Escherichia coli* DnaJ and GrpE Heat Shock Proteins Jointly Stimulate ATPase Activity of DnaK, Proc Natl Acad Sci USA, l 88, 2874-2878
21. Yoshida A, Nakano Y, Yamashita Y, Oho T, Shibata Y, Ohishi M, Koga T (1999) A novel *dnaK* operon from *Porphyromonas gingivalis*. FEBS Lett 446, 287-291
22. Hinode D, Nakamura R, Grenier D, Mayrand D (1998) Cross-reactivity of specific antibodies directed to heat shock proteins from periodontopathogenic bacteria and of human origin. Oral Microbiol Immunol 13, 55-58
23. Lopatin DE, Shelburne CE, Van Poperin N, Kowalski CJ, Bagramian RA(1999) Humoral immunity to stress proteins and periodontal disease. J Periodontol 70, 1185-1193
24. Yamazaki K, Ohsawa Y, Tabeta K, Ito H, Ueki K, Oda T, Yoshie H, Seymour GJ (2002) Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. Infect Immun 70, 2492-2501
25. Kirby AC, Meghji S, Nair SP, White P, Reddi K, Nishihara T, Nakashima K, Willis AC, Sim R, Wilson M, Henderson B (1995) The potent bone-resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. J Clin Invest 96, 1185-1194
26. Shelburne CE, Gleason RM, Germaine GR, Wolff LF, Mullally BH, Coulter WA, Lopatin DE (2002) Quantitative reverse transcription polymerase chain reaction analysis of *Porphyromonas gingivalis* gene expression in vivo. J Microbiol Methods 49, 147-156