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Original

Oxidative stress induces phosphorylation of the ABC transporter, ATP-binding protein, in *Porphyromonas gingivalis*

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Abstract: The Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is a major causative agent of periodontal disease. Although *P. gingivalis* is an anaerobic bacterium, it exhibits aerotolerance and can survive in periodontal pockets, indicating that it must possess a mechanism for protection against oxidative stress, although the precise details are still unclear. Recently, phosphorylation signaling has been implicated in the regulation of bacterial virulence. In the present study, to examine the effect of oxidative stress on phosphorylation of proteins in *P. gingivalis*, we analyzed oxidative stress-induced alterations of phosphorylated proteins using two-dimensional electrophoresis with phosphoprotein staining coupled with MALDI-TOF mass spectrometry analysis. Among the phosphorylated proteins analyzed, we identified an increase in phosphorylation of the ABC transporter, ATP-binding protein (PG0258). Since the ABC transporter family is known to be involved in lipopolysaccharide (LPS) biosynthesis, we examined the level of LPS using an endotoxin assay and found that LPS production was increased in *P. gingivalis*. Our present findings suggest that the early response of *P. gingivalis* to oxidative stress could trigger the development and progression of periodontal disease through enhancement of LPS production by phosphorylation of the ABC transporter, ATP-binding protein. (J Oral Sci 52, 561-566, 2010)

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Introduction

The Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is a major causative agent in the initiation and progression of severe periodontal disease, in which the supporting connective tissue and bone surrounding the teeth are destroyed, ultimately resulting in tooth loss (1). *P. gingivalis* expresses a number of potential virulence factors, including cysteine proteases, gingipains, fimbriae and lipopolysaccharide (LPS), all of which may contribute to the pathogenesis of periodontitis. Bacterial colonizers within periodontal pockets encounter the cells and extracellular effector molecules of the host immune system, and their ability to withstand and even modulate the host's immune responses is a key determinant of their long-term survival and pathogenic potential. Indeed, both innate and acquired defense mechanisms are operational within the periodontal pocket (2). For instance, although by definition *P. gingivalis* cannot grow under aerobic conditions, it exhibits a high degree of aerotolerance, enabling it to survive within periodontal pockets despite frequent exposure to aerobic conditions (3). To accomplish aerotolerance, *P. gingivalis* must express proteins that counter oxidative stress. Superoxide dismutase (SOD) and alkyl hydroperoxide reductase C subunit (AhpC) are known to be involved in *P. gingivalis* aerotolerance, and

both SOD and AhpC have also been identified in the anaerobic bacterium *Bacteroides fragilis* (4,5). However, the system responsible for countering oxidative stress in *P. gingivalis* has not yet been characterized.

In this connection, for a complete understanding of how these antioxidant mediators function within *P. gingivalis*, precise details of their post-translational modification are crucial. Protein phosphorylation is one of the most frequently occurring forms of post-translational modification, and plays a central role in a variety of fundamental cellular functions in eukaryotic cells (6). Although protein phosphorylation in bacteria has been investigated less intensively, initial studies have revealed that bacteria use histidine/aspartate phosphorylation, which represents a paradigm for bacterial signal transduction (7). Interestingly, bacterial pathogens have developed a variety of strategies for interacting with host cells, enabling the bacteria to survive and propagate. In the process of pathogenesis, phosphorylation occurs during cell-cell interaction and adhesion, translocation of bacterial effectors into host cells, and changes in host cellular structure and function induced by infection, suggesting that signaling phosphorylation may be involved in the regulation of bacterial virulence (8). The genome of *P. gingivalis* W83 has been completely sequenced (2), and is now available on the genome project database on the web site of The Institute for Genomic Research Comprehensive Microbial Resource (TIGR-CMR). We have comprehensively examined the whole-cell proteomics of *P. gingivalis* W83 using analytical techniques such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS), together with reference to the genome project database, and this has now facilitated a comprehensive understanding of the molecular biology of *P. gingivalis* (9).

Although the regulatory networks involving protein kinases and phosphatases in bacterial signaling cascades represent an emerging theme, little is known about the different protein partners, kinases and their substrates, in *P. gingivalis*. We have previously reported our use of 2-DE and peptide mass fingerprinting for proteomic analysis of the effect of oxidative stress on protein expression in *P. gingivalis* W83 (10,11). In the present study, we examined the effect of oxidative stress on protein phosphorylation in *P. gingivalis*.

Materials and Methods

Bacterial culture conditions

P. gingivalis W83 was obtained from the Forsyth Institute (Boston, MA, USA) and grown anaerobically (80% N_2, 10% H_2, 10% CO_2) for 48 h in Todd-Hewitt broth supplemented with hemin (0.01 μg/ml) and vitamin K1 (0.01%). To examine the effect of oxidative stress, 40-ml samples of the cell culture grown to stationary phase were incubated under aerobic conditions at 37°C with shaking for 10, 20 or 30 min.

Sample preparation and 2-DE

For 2-DE, a 40-ml culture sample was first treated with 10 ml of 50% trichloroacetic acid and then pelleted by brief centrifugation, after which the cell pellet was washed with 20 ml of 50% acetone and divided into 20 1-ml aliquots that were washed 5 times with acetone and air-dried. 2-DE was then carried out according to the instructions of the instrument's manufacturer (GE Healthcare UK Ltd., Buckinghamshire, UK) (12). Briefly, whole-cell *P. gingivalis* preparations (1 mg) were solubilized in 1 ml of solubilization solution (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 1 mM EDTA, and 0.005% bromophenol blue) and applied to immobilized pH gradient (IPG) strips (length, 11 cm; linear pH range, 4-7; GE Healthcare UK Ltd.) using the in-gel sample rehydration technique. The first-dimensional electrophoresis step was carried out using Multiphore II (GE Healthcare UK Ltd.), as indicated in Table 1. After equilibrating the IPG strips in equilibration solution (1.5 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.005% bromophenol blue) containing 1% DTT and 2.5% iodoacetamide, the second-dimensional electrophoresis step, involving 12.5% SDS-PAGE (13), was carried out.

2-DE gel staining

Following 2-DE, the gels were stained using Pro-Q® Diamond (Molecular Probes, Inc., Eugene, OR, USA) as follows. The gels were immersed in Fix Solution (50% methanol, 10% trichloroacid) and incubated overnight at room temperature with gentle agitation. After a second fixation (1 h), the gels were washed 4 times with distilled water for 15 min each time with gentle agitation, and then incubated for 4 h in the dark with Pro-Q® Diamond phosphoprotein gel stain under gentle agitation. After the staining, the gels were destained 4 times with Destain Solution (20% acetonitrile, 50 mM sodium acetate) for 1 h each at room temperature to remove background staining, and then protected from light (14). The stained gels were then scanned at 340 nm using an Alpha Imager™ 3400 (Alpha Innotech Corp., San Leandro, CA, USA), after which total protein was stained using colloidal Coomassie Brilliant Blue (CBB, PhastGel Blue R-350, Amersham Biosciences). Briefly, the gels were washed with 7% acetic acid for 30 min and then stained with 0.02% CBB for 24 h. After staining, the gels were again scanned using an Alpha Imager™ 3400.

Image analysis

The protein expression profile was analyzed using the software package Phoretix™ 2D Evolution (Nonlinear Dynamics, Newcastle upon Tyne, UK), which has the ability to create a "virtual profile" of averaged gels and enables both automated and manual spot detection (15). In order to obtain accurate and reliable results, it is essential to minimize errors as much as possible. To accomplish this, the 2-DE was repeated four times using identical samples, after which three gels with fine resolution that contained well over 300 protein spots when stained with CBB were selected and used for calculation of the averaged gels. Two parameters were applied to create the averaged gels: 'Maximum number of gels from which a spot may be absent' and 'Include absent spots in calculations'. Setting the first parameter to 0 means that a spot must be present in all of the sub-gels to be represented in the averaged gel. The second parameter means that when computing the volume of an averaged spot, absent spots are regarded as having zero volume. This approach enables each gel to be analyzed separately, and the differences between gels to be quantified. Normalization of spot volumes in a gel was performed using the 'total spot volume normalization' option. With this method, the volume of each spot is divided by the total volume of all of the spots in the gel.

In-gel tryptic digestion and MALDI-TOF MS analysis

Protein spots showing different expression profiles in the presence or absence of aeration were semi-automatically excised, trypsinized, concentrated, desalted, and eluted using a high-throughput gel-excise processor (Xcise, Shimadzu Biotech, Kyoto, Japan). Concentration and desalination of peptides were accomplished using a ZipTip -C18 pipette tip (Millipore Corporation, Bedford, MA, USA). Peptides were eluted directly onto the MALDI target using 1 ml of a saturated solution of ?-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% (v/v) trifluoroacetic acid.

Peptide mass analyses were then carried out using an AXIMA-CFR (Shimadzu Biotech) MALDI-TOF mass spectrometer in positive reflection mode at an accelerating voltage of 20 kV. Histograms against time were constructed using the Phoretix 2D Evolution package.

Protein identification by Mascot software

Peptide mass fingerprint analysis was carried out to identify proteins. Preliminary amino acid sequences for *P. gingivalis* W83 were obtained from TIGR (www.tigr.org) and input to a computer to create an internal database. Proteins were then identified using the database search engine MASCOT (Matrix Science, Boston, MA, USA) with the following search parameters: trypsin was used as the enzyme, one missed cleavage was allowed, and carbamidomethyl and oxidized methionine were set as fixed and variable modifications, respectively. Proteins were identified using the probability-based molecular weight search score. Protein scores exceeding 46 were regarded as significant ($P < 0.05$) from the *P. gingivalis* TIGR database.

Endotoxin assay

LAL solution (Limulus II single test, Wako, Osaka, Japan) and samples were mixed in a test tube. The sample suspension in the tube was incubated statically at 37°C for 60 min, and then the change in turbidity at 430 nm during coagulation of the sample in the tube was measured.

Results

P. gingivalis proteins were extracted and separated on an immobilized pH4-7 gradient strip, after which they were separated by 12.5% SDS-PAGE, then stained with CBB and Pro-Q® diamond (Fig. 1). As shown in Fig. 1, phosphorylation of a protein spot indicated by the white

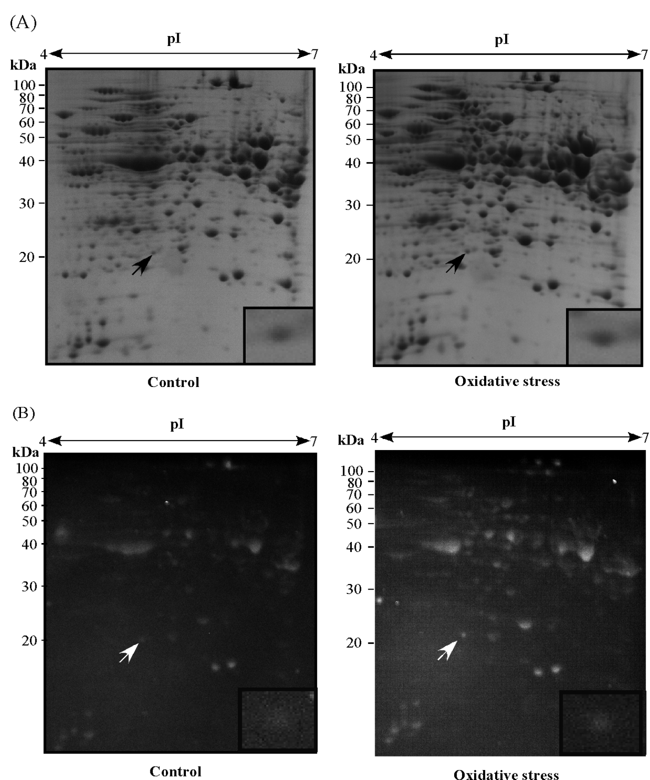

Fig. 1 Representative 2-DE gel showing a *P. gingivalis* cell preparation stained with CBB (A) and Pro-Q® diamond (B). Experiments were carried out in triplicate, after which the gels were stained with Pro-Q® diamond and CBB, and averaged. The averaged gels were examined using image matching software to detect the spots.

arrow increased with aeration time.

Next, we analyzed the time-dependent alteration of protein expression and phosphorylation under aerobic conditions, and histograms showing the protein expression profiles in oxidatively stressed *P. gingivalis* were constructed using Phoretix 2D Evolution. Phosphorylation of the protein (white arrow in Fig. 1) was significantly increased by oxidative stress at 10 min, although the level of protein expression was unchanged as shown in Fig. 2.

Finally, the protein spot was cut out from the gel and in-gel digested with trypsin, then the molecular masses of the digested peptides were analyzed using MALDI-TOF-MASS. These peptide mass values are shown in Fig. 3 A. Using MASCOT analysis software with a *P. gingivalis* database containing amino acid sequences obtained from TIGR-CMR, the probability-based Mowse score was 91.5, as shown in Fig. 3B. The MASCOT search instructions indicated that a score of more than 46 was significant for specific identification of proteins in the case of the TIGR-*P. gingivalis* database. The protein spot described above was successfully identified as the ABC transporter, ATP-binding protein (PG0258), with a high probability score. Matched peptides are shown in Fig. 3C.

Since the ABC transporter is known to be involved in LPS synthesis, we measured endotoxin activity in order to examine whether oxygen stress stimulates LPS production. As shown in Fig. 4, oxygen stress enhanced endotoxin activity at 60 and 90 min.

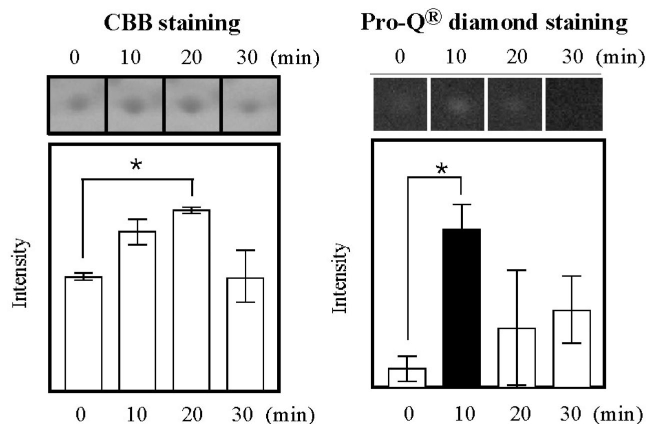

Fig. 2 Expanded 2-DE patterns showing oxidative stress-induced changes in protein phosphorylation and expression in *P. gingivalis*. Histograms showing the protein expression profiles in *P. gingivalis* under oxidative stress were constructed using the Phoretix 2D Evolution software package. Phosphorylation of the ABC transporter (PG0258) was increased more than 3-fold by oxidative stress within 10 min, although the level of protein expression was unchanged.

Discussion

For many years after the discovery that protein phosphorylation was catalyzed by protein kinases, the prevailing view was that these enzymes were present only in eukaryotes (16). However, since the identification of bacterial homologues (17), genomics studies have now demonstrated that protein kinases and phosphatases are also widespread in prokaryotes (18). Recently, protein phosphorylation has been shown to play an essential role in a variety of fundamental cellular functions in bacteria (19).

In this study, alteration of protein phosphorylation in *P.*

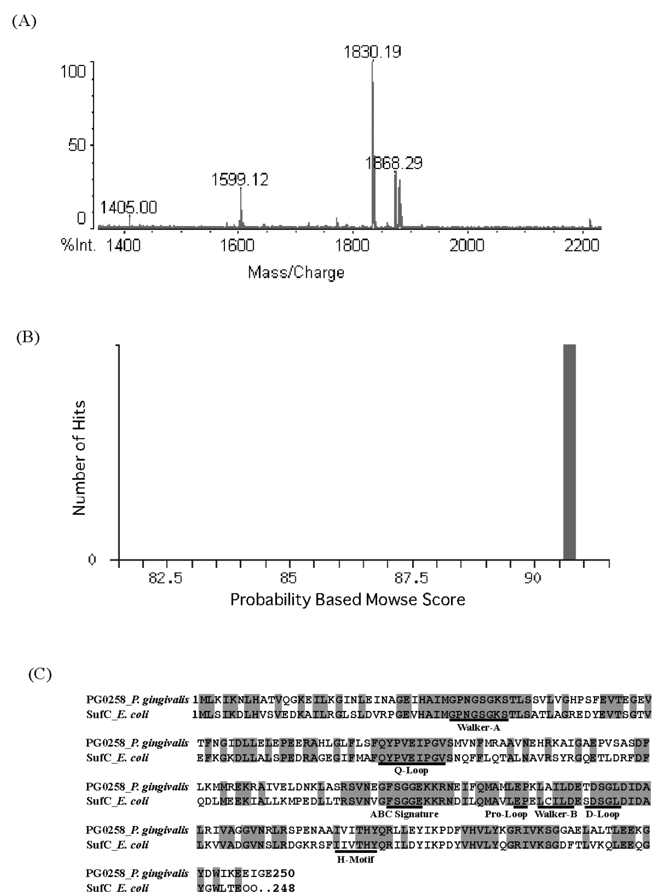

Fig. 3 Identification of the phosphorylated protein. The protein spot was cut out from the gel and in-gel digested with trypsin, then the molecular masses of the digested peptides were analyzed using MALDI-TOF-MASS. (A) Trypsin-digested peptide mass values. (B) Probability based the Mowse score, which was 91.5. The MASCOT search instructions indicated that a score of 46 was significant for specific protein identification. The protein spot of interest was successfully identified as the ABC transporter (PG0258). (C) ATP-binding protein with a high probability score. Underlining indicates peptides matched with the ABC transporter (PG0258).

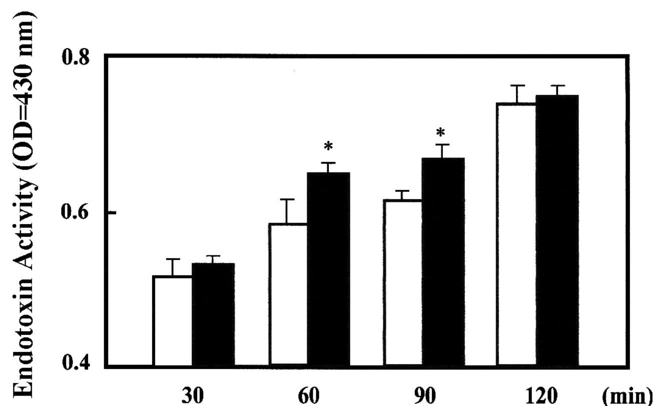

Fig. 4 Endotoxin activity from *P. gingivalis* with or without oxidative stress was extracted using the hot phenol method. The activity of the extracted endotoxin was measured by *Limulus* endotoxin assay. $*P < 0.05$, $n = 3$.

gingivalis was found for several protein spots (data not shown) using proteomic technology after subjecting the cells to oxidative stress. Because we found it difficult to adequately explain all the results of the proteome analysis, we randomly chose a single protein (PG0258). The phosphorylation of PG0258 was markedly increased by oxidative stress within 10 min. PG0258, an ABC transporter, and the SufC homolog, which shares 59% sequence identity, have strictly conserved Walker-A (P-loop) and Walker-B motifs (Fig. 3) and ATPase activity (20,21).

The iron-sulfur cluster assembly SUF (mobilization of sulfur) system is one of three iron-sulfur assembly systems present in a wide range of bacteria. The SUF system is composed of a six-gene cluster with protein complexes responsible for cysteine desulfuration, cytosolic ATPase activity, and a protein scaffold for assembly of the iron and sulfur cluster and its transfer to protein targets (22). The phosphorylation may also play a structural role, affecting the functionality of the ABC transporter, and it has been reported that binding of substrates to periplasmic substrate-binding proteins of ABC transporters in Gram-negative bacteria initiates a cascade of conformational changes across the membrane, resulting in hydrolysis of ATP in the cytosol, and thus promoting the transport process. In *Phormidium laminosum*, it is noteworthy that the phosphorylation/dephosphorylation events of nitrate transport involve a multi-subunit ABC transporter (ATP-binding cassette) (23).

Among the various virulence factors involved in bacterial pathogenesis, special attention has recently been paid to the cell wall components, exopolysaccharides (24). A major breakthrough was the demonstration of a biological link between the activity of certain protein kinases and phosphatases, and the production and/or transport of surface polysaccharides. In addition, genetic studies have revealed a key pathogenetic role of some serine/threonine kinases. From a general standpoint, the demonstration of a direct relationship between protein phosphorylation and bacterial virulence represents a novel concept of great importance when attempting to understand the molecular and cellular mechanisms that underlie pathogenesis.

Since ABC transporters are able to export a wide diversity of substrates, including sugars and drugs, across biological membranes, the present results offer new perspectives for clarifying the molecular mechanisms involved in the physiology and pathogenicity of *P. gingivalis*. It is known that the ABC transporter exports the nascent polymer across the inner membrane prior to completion of the LPS molecule, and that determination of LPS chain length is dependent on the ABC transporter pathway, which is widespread among Gram-negative bacteria (25).

Within periodontal pockets, *P. gingivalis* would be frequently subjected to oxidative stress; according to our findings, oxidative stress enhances PG0258 phosphorylation within as little as 10 min. These findings suggest that protein phosphorylation, which appears to be a component of the early response to oxidative stress, may be one of the key determinants of virulence in *P. gingivalis* and, perhaps, the development and progression of periodontal disease. Taken together with existing knowledge, our present findings allow us to hypothesize that oxygen stress enhances phosphorylation of the ABC transporter homolog and stimulates the production of LPS, thus contributing to the virulence of *P. gingivalis*.

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