Oral Microbiology Immunology 2004: 19: 88–94
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

The effect of oxygen on the growth and physiology of *Porphyromonas gingivalis*

P. I. Diaz*, A. H. Rogers
Microbiology Laboratory, Dental School, The University of Adelaide, Australia

Diaz PI, Rogers AH. The effect of oxygen on the growth and physiology of Porphyromonas gingivalis.
Oral Microbiol Immunol 2004: 19: 88–94. © Blackwell Munksgaard, 2004.

Oxygen constitutes a constant challenge for the survival of strict anaerobes in the oral environment. The aim of this study was to investigate the effect of oxygen on the physiology and growth of *Porphyromonas gingivalis* in a continuous culture system when grown under conditions of hemin limitation and excess. Results showed that, when grown in the presence of hemin at 0.5 mg/l, *P. gingivalis* could tolerate low levels of oxygen, being able to reach steady-state when 6% oxygen was present in the incoming gas mixture. When the hemin concentration was increased to 5 mg/l, the culture tolerated 10% oxygen. Anaerobically-grown cells were coccoid in shape, whereas those grown in the presence of oxygen were bacillary. Acetate was the predominant end-product in cultures grown in the presence of oxygen or in cultures hemin-limited. Despite some changes in the activity of Arg- and Lys-gingipain, most of the proteolytic activity was retained in the presence of oxygen. Activity of each of the three anti-oxidant enzymes tested (NADH oxidase, NADH peroxidase and SOD) was detected under all conditions and usually increased under oxygenated environments. Higher activities were also seen in the hemin-limited cultures. These results show some of the changes that occur in the physiology of *P. gingivalis* as a result of oxidative stress and confirm that hemin has a protective effect on the growth of the microorganism in the presence of oxygen.

Key words: anti-oxidant enzymes; continuous culture; haemin; oxidative stress; *Porphyromonas gingivalis*; proteolytic activity

Anthony H. Rogers, Microbiology Laboratory, Dental School, The University of Adelaide. North Terrace, Adelaide, South Australia 5005, Australia
E-mail: tony.rogers@adelaide.edu.au

Current address: *National Institutes of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892-4350, USA.
Accepted for publication October 2, 2003

Porphyromonas gingivalis is a gram-negative anaerobic cocco-bacillus strongly implicated in the etiology of adult periodontitis. Some strains of *P. gingivalis* produce a capsular polysaccharide material, which increased the virulence of the microorganism when tested in a mouse model (15). Many other virulence factors, including outer membrane vesicles, adhesins, lipopolysaccharides, hemolysins and proteinases are also produced by *P. gingivalis* and are thought to contribute to its pathogenicity (10).

P. gingivalis is dependent on nitrogenous substrates for energy (28). When the microorganism was grown in Tryptone medium the main fermentation end-products were ammonia, butyrate, acetate and propionate, together with small amounts of isobutyrate and isovalerate (33). Due to their low molecular weights, these end-products can penetrate the periodontal tissues and potentially disturb host cell activity and defense systems (14). For example, butyrate is a potent inhibitor of the proliferation of gingival fibroblasts, potentially reducing the ''wound healing'' or reparative ability of these cells (2).

Another important feature of the growth of *P. gingivalis* is its preference for protoporphyrin IX (heme or hemin) as an iron source (22). Heme can be obtained *in vivo* by *P. gingivalis via* the proteolysis of hemoglobin and the heme-carrying plasma proteins haptoglobin and hemopexin (3) and is transported into the cell in an energy-dependent process regulated by the levels of available hemin (6). When the heme molecule, Fe (II)PPIX, is released from hemoglobin, it is converted into dimers in a reaction involving oxygen and is accumulated on the cell surface in the m-oxo dimeric form, $[Fe\ (III)PPIX]_2O$, a mechanism thought to provide anti-oxidant protection to the cells (31). It has also been suggested that aggregated dimers could provide anti-oxidant protection, serving as a physical barrier to environmental oxygen, as well as acting as a buffer system to hydrogen peroxide, due to a catalase-like activity inherent to the layer (30).

Another important characteristic of *P. gingivalis* is that it produces very active proteinases, whose main role is to provide (oligo)peptides for growth, although they might also contribute to invasion and destruction of the periodontal tissues and to modulation and evasion of the immune system (16). The main *P. gingivalis* proteinases belong to the cysteine proteinase

family, with specificity for arginine and lysine residues, and are commonly designated Arg-gingipain (Rgp) and Lys-gingipain (Kgp). Several functions have been assigned to these proteinases that appear to contribute to the virulence of the microorganism. For example, spontaneous *P. gingivalis* mutants with reduced Rgp and Kgp activities and wild-type cells treated with a proteinase inhibitor have been reported to have a decreased virulence in animal models (13). Moreover, a functional *kgp* gene appears to be necessary for the binding of *P. gingivalis* to hemoglobin and for the accumulation of hemin on the surface of the microorganism, as well as being necessary, in part, for hemagglutination activity (25).

It is relevant to study the effect of oxygen on the physiology of oral microorganisms as oxygen represents a constant challenge for their survival in the oral environment. In order to colonize oral surfaces, bacteria first have to survive in oral fluids, where they are exposed to oxygen and high oxidation-reduction potentials. It has been suggested that oral anaerobes encounter residual amounts of oxygen in the early stages of dental plaque development and even in established periodontal pockets (19). Indeed, the E_h values of a healthy gingival sulcus are around +75 mV (12), while periodontal pockets have been reported to possess residual oxygen at one tenth the level in air-saturated water (which is 0.021 μmol/ml) (23) and E_h values ranging from +14 mV to –157 mV (12). The growth of *P. gingivalis* under oxygenated environments might alter the physiology of the microorganism and affect the expression of different proteins, including virulence factors. Although some information exists on the oxidative stress mechanisms associated with *P. gingivalis*, many questions remain. The few studies on the anti-oxidant enzymes of *P. gingivalis* (1) have been conducted in batch culture, a situation in which close control over individual environmental factors is not accurate and is not likely to simulate *in vivo* conditions. On the other hand, continuous culture in a chemostat is considered to be the best *in vitro* model for the study of oral microorganisms, as it most closely resembles natural conditions, in which bacteria grow at sub-maximal rates due to the limited availability of one or more nutrients (34). The generation times of microorganisms in dental plaque vary between 8 and 12 h (7), a situation that can be reproduced in the chemostat, allowing close control over relevant environmental factors such as pH, temperature and redox-potential (E_h).

Accordingly, the aim of the present study was to investigate the effect of oxygen on the physiology and growth of *P. gingivalis* in a continuous culture system. An analysis of its cell morphology, fermentation end-products, anti-oxidant enzyme levels and proteinase activities was carried out to determine possible physiological changes occurring in the microorganism in an oxygenated environment. These studies were also conducted with cultures of the microorganism grown under conditions of hemin excess and hemin limitation in order to confirm whether hemin increases the tolerance of the microorganism to oxygen.

Materials and methods

Microorganism and maintenance of the strain

P. gingivalis W50 (ATCC 53978) was maintained short-term on anaerobic blood agar plates, incubated at 37°C in an atmosphere of 5% H_2, 5% CO_2 and 90% N_2.

Continuous culture growth conditions

P. gingivalis was grown in continuous culture in BM medium (29) supplemented with 5 mg/l hemin (hemin excess) and 0.5 g/l of cysteine. Growth in a 365 ml working-volume chemostat was initiated by inoculating the culture vessel with a 24 h batch culture of the microorganism grown in the same medium. After 16–24 h of batch culture growth, the medium reservoir pump was turned on and the medium flow adjusted to give a dilution rate of 0.069/h ($t_d = 10$ h), which was kept constant in all the experiments. The temperature was maintained at 36°C and the pH controlled at 7.4 by the automatic addition of 2N KOH. The culture was sparged with the appropriate gas mixture at a flow rate of 300 cm^3/min. After about 7 generations, under all conditions, and based upon lack of change in the optical density (OD_{560nm}), the culture was considered to have achieved steady-state. At this stage and for 3 consecutive days, an appropriate volume of culture was removed from the chemostat and analyzed for its optical density, cell dry weight, and viability (4). The redox potential (E_h) of the culture at each gassing stage was monitored with a redox electrode (model Pt 4805-DPAS-SC-K85/120, Mettler Toledo, Switzerland). Initially, *P. gingivalis* was grown with an anaerobic gaseous atmosphere of N_2/CO_2 (90 : 5) and then the incoming gas mixture was adjusted to contain increasing levels of oxygen, as follows: $N_2/CO_2/O_2$ (92 : 5 : 3), $N_2/CO_2/O_2$ (89 : 5 : 6), $N_2/CO_2/O_2$ (85 : 5 : 10) and air/CO_2 (95 : 5).

Effect of hemin on the continuous culture growth and oxygen tolerance

To determine the putative protective effect upon oxidative stress of hemin, *P. gingivalis* was cultured under hemin limitation and hemin excess conditions. The tolerance to oxygen of cells grown under both conditions was then compared. A concentration of hemin, growth restrictive but producing similar biomass and cell numbers to hemin-excess conditions, using the anaerobic gas phase, was determined as follows. Growth was initiated by inoculating the chemostat with 30 ml of a batch culture of *P. gingivalis* W50 grown anaerobically overnight with hemin in excess (5 mg/l). The gas mixture used for all experiments was N_2/CO_2 (90 : 5). Repeat experiments were carried out utilizing the following concentrations of hemin in the growth medium: 5, 1, 0.7, 0.5, 0.2 and 0.05 mg/l. After 12–15 generations, cultures were shown to have reached steady-state and growth parameters (viable counts and optical density) were determined.

The optimum hemin-limited concentration which produced cell numbers similar to those obtained under hemin excess was then used to grow *P. gingivalis* under the various oxygenated conditions described previously.

After hemin had been added to the uninoculated growth medium, an absorbance spectrum was obtained in order to identify the state of the hemin species in the solution. This spectrum revealed that the hemin in the growth medium occurred as both Fe (III) monomers (A_{365nm}) and dimers (A_{385nm}), but dimers constituted the majority of the heme species.

Analysis of morphologic changes

Continuous cultures of *P. gingivalis* were sampled at steady-state and prepared immediately for scanning electron microscopy (SEM) analysis as described previously (4). Samples were coated with platinum and analyzed using a Philips XL30 Field Emission Scanning Electron Microscope.

To determine the effect of oxygen on capsule production by *P. gingivalis*, samples from steady-state cultures, grown under anaerobic and oxygenated conditions, were stained with 10% nigrosin and counterstained with Maneval's stain (containing

Table 1. The effect of oxygen on the growth of *P. gingivalis* under hemin limitation and excess

Gas phase	E_h (mV)[a]		Viable counts (mean ± SD of Log_{10} CFU/ml, $n = 3$)		Dry weight (mean ± SD, mg/ml, $n = 3$)		Optical density (560 nm) (mean ± SD, $n = 3$)	
	Excess[b]	Lim[c]	Excess	Lim	Excess	Lim	Excess	Lim
N_2/CO_2 (95 : 5)	−507	−487	9.84 ± 0.12	9.79 ± 0.10	1.40 ± 0.11	1.23 ± 0.12	1.43 ± 0.18	1.34 ± 0.09
$N_2/CO_2/O_2$ (92 : 5 : 3)	−437	−420	9.41* ± 0.10	9.36* ± 0.20	0.95** ± 0.10	0.79** ± 0.01	0.93** ± 0.06	0.77** ± 0.04
$N_2/CO_2/O_2$ (89 : 5 : 6)	−423	−385	9.25** ± 0.22	9.15** ± 0.10	0.83** ± 0.07	0.75** ± 0.08	0.91** ± 0.06	0.79** ± 0.01
$N_2/CO_2/O_2$ (85 : 5 : 10)	−398	NS[d]	9.05** ± 0.18	NS[d]	0.85** ± 0.11	NS[d]	0.98** ± 0.05	NS[d]

[a]E_h (redox potential) is an average of five separate readings, differing from each other less than 5%, taken at steady-state.
[b]Excess – hemin excess (5 mg/l).
[c]Lim – hemin limitation (0.5 mg/l).
[d]NS = the culture did not survive.
*$P < 0.05$ for results compared to anaerobic growth under the same hemin concentration.
**$P < 0.001$ for results compared to anaerobic growth under the same hemin concentration.

fuchsin and methanol). Samples were examined under light microscopy.

Effect of oxygen on the formation of acidic metabolic end-products

The effects of oxygen on the formation of acidic metabolic end-products by *P. gingivalis* were compared for hemin-limited and -excess cultures grown anaerobically and stressed with oxygen. Cell-free culture filtrates were prepared under each gaseous condition at steady-state and stored at –20°C until analyzed. A high pressure liquid chromatography system (HPLC) was used for acidic end-product analysis, following the methods of Guerrant et al. (8).

Effect of oxygen on the activity of Arg- and Lys-proteinases

When steady-state was achieved at each growth condition (Tables 1), 20 ml of cell culture was removed from the chemostat and centrifuged (8000 × *g*, 4°C for 30 min). Cell pellets were resuspended in 5 ml of 0.1 M Tris–HCl, 10 mM L-cysteine and 10 mM $CaCl_2$ (pH 8.0) buffer. Protein content in both whole cell suspensions and supernatants was assayed using a BCA Protein Assay Kit (Pierce, Rockford, IL). Enzyme activity was measured in 1 ml (total volume) of the same buffer containing 0.5 mM DL-BApNA (N-α-benzoyl-DL-arginine-p-nitroanilide, Sigma, St. Louis, MO) or 0.25 mM AcLyspNA (N-α-acetyl-lysine-p-nitroanilide, Bachem, Bubendorf, Switzerland) by measuring the free p-nitroanilide released from the substrates when 10 μg of protein sample was added to the reaction mixture. The change in absorbance of this solution at 405 nm was recorded spectrophotometrically, at 0.25-min intervals, for 5 min. One unit was defined as the amount of p-nitroanilide (in nmol) released by 1 mg of protein sample per minute.

Effect of oxygen on the activity of anti-oxidant enzymes

The activity of the enzymes NADH oxidase, NADH peroxidase and SOD, involved in the detoxification of oxygen or its radicals, was assayed in cells grown in continuous culture, in anaerobic and aerated environments. The preparation of cell extracts and enzyme assays were carried out as described previously (4). NADH oxidase and NADH peroxidase activities were assayed at 25°C following the methods of Higuchi (9). NADH oxidase was assayed by monitoring, spectrophotometrically, the oxidation of β-NADH at A_{340nm}. NADH peroxidase was assayed under anaerobic conditions, achieved in a Thunberg-type cuvette, using the same reaction mixture as for NADH oxidase, but with the addition of 0.3 mM H_2O_2. One unit of activity for both enzymes was defined as the amount of extract that catalyzed the oxidation of 1 nmol NADH/min. SOD activity was measured at 550 nm by competitively inhibiting the reduction of cytochrome c at 25°C, following the methods of McCord & Fridovich (21). One unit of SOD was defined as the amount of extract that decreased the rate of reduction of cytochrome c by 50%.

Statistical analysis

Data were expressed as means ± standard deviations. Differences between means were analyzed for statistical significance using Student's *t*-test.

Results

Growth of *P. gingivalis* under oxygenated atmospheres in hemin-limited and -excess conditions

As seen in Fig. 1, hemin limitation was almost relieved when the concentration

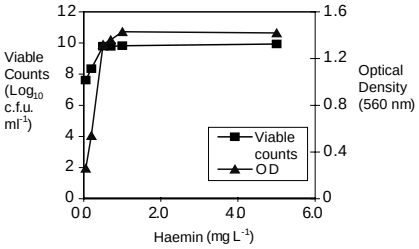

Fig. 1. The effect of hemin concentration on the steady-state growth of *P. gingivalis* W50 grown under anaerobic conditions (N_2/CO_2, 95 : 5).

reached 0.5 mg/l, a result similar to that obtained by McKee et al. (22). This concentration was therefore used for all ''hemin-limited'' experiments.

Table 1 shows the effect of oxygen on the growth parameters of *P. gingivalis* W50 when grown under hemin limitation and excess. Under hemin excess the organism was able to survive all the different gaseous atmospheres tested, with the exception of air:CO_2 (95 : 5), under which the culture washed out. Under the conditions at which the culture survived, a decrease in cell viability was always observed when the gas phase was changed to contain oxygen. As expected, the culture E_h increased as the oxygen concentration in the gas phase was increased. Nevertheless, under the gas phase containing 10% oxygen, the cells were still able to create a reduced environment ($E_h = –398$ mV). It is worth noting that the E_h of the uninoculated medium was also measured under the different gaseous atmospheres tested. The E_h of the medium under the anaerobic gas phase was about –350 mV, but when oxygen was introduced, only positive E_h values were recorded. Although no attempt was made to grow *P. gingivalis de novo* under those positive E_h values, since growth was always initiated anaerobically,

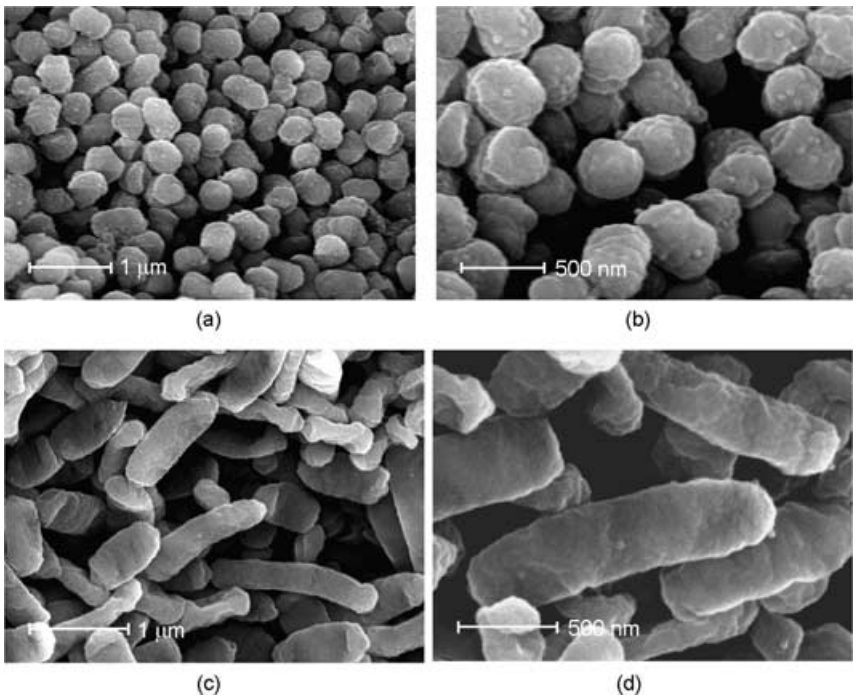

Fig. 2. SEM of *P. gingivalis* W50 grown under anaerobic conditions ($NO_2/CO_2/O_2$, 5 : 10) (c and d).

they do give an indication of the ability of the microorganism to reduce the environment.

Table 1 also shows that there were no significant differences in the culture growth parameters between hemin excess and the chosen hemin-limited condition under anaerobic growth. However, hemin-limited cultures appeared to be more affected by oxygen than those grown under hemin excess, as shown by the decreased viability and the observation that steady-state was not achieved at the most highly oxygenated condition tested ($N_2/CO_2/O_2$; 85 : 5 : 10), when growth occurred under hemin limitation. Interestingly, this culture did not obey wash-out kinetics but disappeared slowly until the optical density was undetectable. This phenomenon could be explained by the appearance of thin biofilms that formed over some of the chemostat inserts as the culture declined. Moreover, analysis of the cultures' E_h under all gas phases (Table 1) indicated that cultures grown under hemin excess were able to achieve a more reduced environment than those grown in hemin-limited conditions.

Morphologic changes in *P. gingivalis* grown under anaerobic and oxygenated environments

Figure 2 shows the change in the cell shape of *P. gingivalis* as a consequence of exposure to oxygen. Anaerobically grown cells are coccoid, whereas cells stressed with oxygen clearly grow in a bacillary form, increasing by at least threefold in length. This change was consistent through all the continuous culture experiments and was observed under both hemin limitation and hemin excess.

Utilizing the staining procedure described above, it was possible to visualize *P. gingivalis* capsule but no changes in the capsule were observed when cells grown under oxygen were compared with those grown anaerobically.

The effect of oxygen on the formation of acidic metabolic end-products

Tables 2 and 3 show the effect of oxygen on the formation of acidic end-products by *P. gingivalis* grown under hemin excess (5 mg/l) and limitation (0.5 mg/l). The main fermentation end-product under hemin excess and anaerobic growth was butyrate, followed by acetate (Table 2). However, when oxygen was introduced into the system, acetate increased, becoming the main product, whereas butyrate decreased. Changes, some of them statistically significant, also occurred in other fermentation end-products; for example, succinate and isobutyrate decreased under oxygen stress, while propionate increased. Changes in isovalerate were not consistent. In contrast, under hemin-limited conditions, the main end-product was acetate,

Table 2. The effect of oxygen on the fermentation end-products of *P. gingivalis* grown under hemin-excess conditions (5 mg/l)

Gas phase	Dry weight[b]	Succinate[a]	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate
N_2/CO_2 (95 : 5)	1.40	0.61 ± 0.2	7.25 ± 0.2	2.79 ± 0.2	2.16 ± 0.4	8.99 ± 0.3	1.94 ± 0.9
$N_2/CO_2/O_2$ (92 : 5 : 3)	0.95	0.72 ± 0.1	$9.15 \pm 0.1^*$	2.08 ± 0.4	$0.19 \pm 0.3^*$	$4.82 \pm 0.2^*$	1.39 ± 0.8
$N_2/CO_2/O_2$ (89 : 5 : 6)	0.83	0.23 ± 0.3	$13.27 \pm 0.5^*$	3.36 ± 0.8	1.04 ± 0.8	$5.20 \pm 0.8^*$	2.40 ± 0.3
$N_2/CO_2/O_2$ (85 : 5 : 10)	0.85	0.22 ± 0.2	$13.00 \pm 0.1^*$	3.28 ± 0.7	1.28 ± 0.2	$5.58 \pm 0.4^*$	2.40 ± 0.5

[a]End-products in mmol/g cells dry weight.
[b]Dry weights in mg/ml.
$^*P < 0.001$ for results compared to anaerobic gas phase; $n = 4$.

Table 3. The effect of oxygen on the fermentation end-products of *P. gingivalis* grown under hemin-limited conditions (0.5 mg/l)

Gas phase	Dry weight[b]	Succinate[a]	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate
N_2/CO_2 (95 : 5)	1.23	0.15 ± 0.1	7.55 ± 0.2	1.63 ± 0.6	0	5.70 ± 0.2	1.12 ± 0.4
$N_2/CO_2/O_2$ (92 : 5 : 3)	0.79	0.13 ± 0.2	$12.09 \pm 0.8^*$	1.85 ± 0.2	0	5.75 ± 0.1	1.43 ± 0.2
$N_2/CO_2/O_2$ (89 : 5 : 6)	0.75	0.43 ± 0.3	$13.44 \pm 0.6^*$	$3.47 \pm 0.3^*$	0	5.70 ± 0.3	2.20 ± 0.2

[a]End-products in mmol/g cells dry weight.
[b]Dry weights in mg/ml.
$^*P < 0.001$ for results compared to anaerobic gas phase; $n = 4$.

Table 4. The effect of oxygen on proteinase activity of *P. gingivalis* when grown under hemin-excess conditions

	Lys-proteinase activity[a]		Arg-proteinase activity[a]	
Gas phase	CP	SN	CP	SN
N_2/CO_2 (95 : 5)	190 ± 13	132 ± 20	606 ± 16	259 ± 11
$N_2/CO_2/O_2$ (92 : 5 : 3)	176 ± 8	85 ± 34	891* ± 52	98* ± 29
$N_2/CO_2/O_2$ (89 : 5 : 6)	138* ± 9	69* ± 7	750* ± 32	101* ± 13
$N_2/CO_2/O_2$ (85 : 5 : 10)	136* ± 3	64* ± 8	785* ± 26	83* ± 44

[a]Specific activity associated with the resuspended cell pellets (CP) and supernatants (SN) is presented as mean ± SD (nmol·mg/protein/min), $n = 4$.
*$P < 0.05$ for results compared to anaerobic growth.

which increased under oxygen stress, as it did in the hemin-excess, oxygen-stressed, culture (Table 3). Butyrate, however, remained unchanged through all gas phases, whereas succinate, propionate and isovalerate slightly increased when oxygen was introduced into the system. A difference observed in all gas phases, when hemin-limited growth was compared to hemin-excess growth, was the complete disappearance of isobutyrate under hemin limitation.

The effect of oxygen on the activity of Arg- and Lys-proteinases (Rgp and Kgp)

Tables 4 and 5 record the effect of oxygen on the activity of Rgp and Kgp when the microorganism was cultured under hemin-excess and -limited conditions, respectively. There was an increase in Rgp activity present in the whole-cell fraction and a decrease in the activity found in the supernatants, irrespective of the hemin concentration. However, the total activity (cell pellet plus supernatant), did not appear to increase, except for the culture grown under 3% oxygen. It appears that under oxygen stress, an increased proportion of enzyme activity remains cell-bound. On the other hand, Kgp activity appears to decrease in both whole cells and supernatants as oxygen is introduced into the system. This observation was again consistent for both hemin concentrations, although under hemin limitation the decrease in Lys-gingipain was not statistically significant at the levels tested.

The effect of oxygen on the activity of the anti-oxidant enzymes of *P. gingivalis* grown under hemin-excess and hemin-limited conditions

The activity of anti-oxidant enzymes detected in *P. gingivalis* is presented in Tables 6 and 7. The activity of each of the three enzymes tested (NADH oxidase, NADH peroxidase and SOD) was detected under all conditions and the activities generally increased under oxygenated environments compared with the activities obtained for anaerobically-grown cells.

Discussion

When the ability of *P. gingivalis* to survive in the presence of oxygen was tested in continuous culture, a decrease in cell viability was observed as the oxygen concentration was increased. This result might indicate that oxygen stress produces an increased demand for amino acid-derived ATP for the maintenance of internal homeostasis. When the cultures reached steady-state under the different oxygenated atmospheres, the E_h decreased to around –400 mV, an indirect indication that the oxygen present had been consumed. The only condition under which the cells could not survive (under hemin excess) was when air:CO_2 (95 : 5) was applied to the system. During wash-out, the E_h of this culture increased gradually, reaching positive values, indicating that the culture's ability to reduce the environment had been exceeded by the incoming oxygen in the gas phase.

When the effect of hemin, in excess or limitation, was tested on the ability of *P. gingivalis* to withstand varying amounts of oxygen in the environment, it was observed that hemin-excess cultures were better able to tolerate oxygen than hemin-limited cultures. Hemin-limited cell pellets were normally white, whereas pellets from the hemin-excess cultures were brown – indicative of the amount of hemin bound to the cell surface under the two conditions. This protective effect of hemin could be attributed to the buffer capacity of the m-oxo layer of dimers formed over the surface of the cells (30). The absorbance spectrum

Table 5. The effect of oxygen on proteinase activity of *P. gingivalis* when grown under hemin-limited conditions

	Lys-proteinase activity[a]		Arg-proteinase activity[a]	
Gas phase	CP	SN	CP	SN
N_2/CO_2 (95 : 5)	103 ± 17	20 ± 5	406 ± 15	175 ± 21
$N_2/CO_2/O_2$ (92 : 5 : 3)	82 ± 7	16 ± 8	534* ± 32	76* ± 17
$N_2/CO_2/O_2$ (89 : 5 : 6)	79 ± 6	15 ± 3	502* ± 41	64* ± 18

[a]Specific activity associated with the resuspended cell pellets (CP) and supernatants (SN) is presented as mean ± SD (nmol·mg/protein/min), $n = 4$.
*$P < 0.05$ for results compared to anaerobic growth.

Table 6. The effect of oxygen on the activity of the anti-oxidant enzymes of *P. gingivalis* under hemin-excess conditions

Gas condition	E_h (mV)	NADH oxidase	NADH peroxidase	SOD[b]
N_2/CO_2 (95 : 5)	−507	5.49 ± 0.51[a]	8.41 ± 0.61	8.94 ± 0.05
$N_2/CO_2/O_2$ (92 : 5 : 3)	−437	7.08 ± 1.11	8.33 ± 0.51	10.44 ± 0.51*
$N_2/CO_2/O_2$ (89 : 5 : 6)	−423	10.3 ± 1.23**	12.53 ± 0.81**	10.71 ± 0.33*
$N_2/CO_2/O_2$ (85 : 5 : 10)	−398	9.78 ± 1.95**	12.29 ± 0.70**	10.83 ± 0.29**

[a]Mean ± SD of activity (units/mg protein), $n = 4$.
[b]Superoxide dismutase.
*$P < 0.05$ for results of anaerobic versus 3%, 6% or 10% O_2.
**$P < 0.001$ for results of anaerobic versus 3%, 6% or 10% O_2.

Table 7. The effect of oxygen on the activity of the anti-oxidant enzymes of *P. gingivalis* under hemin-limited conditions

Gas phase	E_h (mV)	NADH oxidase	NADH peroxidase	SOD[b]
N_2/CO_2 (95 : 5)	−487	8.15 ± 0.72[a]	9.04 ± 0.81	8.55 ± 0.62
$N_2/CO_2/O_2$ (92 : 5 : 3)	−420	12.77 ± 1.43*	11.12 ± 1.06**	8.45 ± 0.52
$N_2/CO_2/O_2$ (89 : 5: 6)	−385	19.83 ± 1.18**	18.11 ± 1.82**	10.67 ± 0.57*

[a]Mean ± SD of activity (units/mg protein), $n = 4$.
[b]Superoxide dismutase.
*$P < 0.05$ for results of anaerobic versus 3% O_2 or 6% O_2.
**$P < 0.001$ for results of anaerobic versus 3% O_2 or 6% O_2.

indicated that hemin in the growth medium (pH 7.4) was already in the dimeric form, in accordance with the fact that no reducing agents, other than cysteine, were added. Therefore, the protection offered by the ''dimer layer'', in the present study, is more likely to be due to the exclusion of oxygen from the surface of the cell, rather than to the reaction of monomers with oxygen. This contrasts with the *in vivo* situation, in which an additional mechanism would be operating; that is, the reaction of heme, (Fe (II)PPIX), after being sequestered from hemoglobin, with oxygen to form dimers that bind to the surface of the cell. Moreover, in the system utilized here, the catalase-like activity present in the dimers (31) could also have provided some protection against any hydrogen peroxide formed outside the cell membrane, as a result of the reaction of oxygen with some components of the complex medium or with the cell surfaces.

Recently, the role of a gene involved in DNA repair after oxidative stress in many microorganisms has been studied in *P. gingivalis*. *RecA* is a gene involved in *P. gingivalis* virulence in the murine mouse model (17) and although no direct evidence exists of the mechanism by which *recA* modulates virulence in this microorganism, it has been proposed that, as the gene is involved in DNA repair (5), its role might be to allow the cells to survive DNA damage caused by oxidative stress during infection. Interestingly, it has been shown that the expression of *recA* is increased under hemin-limiting growth conditions (18), a finding that could be linked to the increased susceptibility of hemin-limited cells to oxidative stress because of the absence of the μ-oxo dimer layer.

SEM images showed that when the cells grew in an oxygenated atmosphere they became elongated, growing as a bacillus or a short rod. Interestingly, a similar elongation occurs in *Fusobacterium nucleatum* in the presence of oxygen, when grown in a continuous culture system (4). Further studies are necessary to clarify this morphologic phenomenon in which there is a decrease in the surface area to which the cell mass is exposed to oxygenated liquid.

The production of a capsular material by *P. gingivalis*, when grown anaerobically and under oxygenated conditions, was also investigated. Although it has been reported that oxidative stress increases the capsular material in other microorganisms, such as *Actinobacillus actinomycetemcomitans* (27) and *Pseudomonas aeruginosa* (26), this was not observed in *P. gingivalis*.

The presence of oxygen was also seen to have an effect on the formation of acidic end-products. An increase in acetate and a decrease in butyrate were the main changes noted when cells growing under hemin excess were exposed to oxygen. In other microorganisms, acetate has also been seen to increase under oxygenated environments, coincident with an increase in the enzyme NADH oxidase (32). A similar shift could be occurring in *P. gingivalis*. According to Takahashi et al. (33), more reduced electron carriers are necessary for the formation of butyrate from aspartate than for the formation of acetate from the same amino acid. Therefore, in the presence of oxygen, the cells could be diverting their metabolism to the production of acetate, instead of butyrate, thus allowing the NADH oxidase to utilize the ''extra'' NADH for oxygen detoxification. However, the decrease in butyrate could also be a consequence of the inactivation by oxygen of some of the enzymes belonging to the pathway responsible for its production. For example, 4-hydroxybutyryl-CoA dehydratase, an enzyme involved in the metabolism of glutamate to butyrate, has been shown to lose its activity under aerobic conditions (24). Interestingly, the levels of butyrate in the present experiments were low under all conditions – anaerobic and oxygenated – in hemin-limited cells. As this reduction is certainly not due to the presence of oxygen in the environment, it might also indicate that some of the enzymes in the butyrate pathway require iron for activity or as part of their structure.

Results also showed that NADH oxidase activity increased with oxygen in both hemin-limited and hemin-excess cultures. However, the magnitude of the increase, after a determined oxygen concentration was applied to the culture, was higher under hemin limitation. For instance, the increase in activity under hemin limitation from anaerobic to 6% oxygen was 1.4-fold, while under hemin excess the activity of the enzyme increased only 0.8-fold. Similarly, NADH peroxidase activity doubled from anaerobic growth to 6% oxygen under hemin limitation, while the increase under hemin excess was only 0.4-fold. If the increase in the activity of these enzymes reflects the environmental pressure exerted on the cells, these results seem to confirm that the cultures grown under hemin limitation were more stressed by oxygen than those grown under hemin excess. The heme layer formed over the surface of hemin-excess cells might isolate the cells from exposure to oxygen as well as functioning as a peroxidase-like system (31). In contrast, hemin-limited cells probably increased the expression of anti-oxidant enzymes to compensate for the lack of the heme layer. On the other hand, SOD activity increased only slightly under the oxygenated atmospheres tested. In this respect, these results differ from those obtained previously by Amano et al. (1) who reported that, in batch grown cells, the SOD activity of *P. gingivalis* 381 was induced more by aeration than was its NADH oxidase activity.

A comparison of the Rgp activity from hemin-limited and -excess cultures further confirmed that the cells grown with 0.5 mg/l of hemin were hemin-limited, as the Rgp activity was lower than at 5 mg/l. This result is in accord with previous reports on the activity of this enzyme under conditions of hemin excess and hemin limitation (20). In essence, the enzyme assays showed that the cell-associated Rgp activity was increased by oxygen stress, while the corresponding supernatant activity decreased. Kgp activity decreased in both fractions under oxygenated environments. The reasons for these changes remain unclear. Perhaps, oxygen could affect the processing of Rgp in such a way that it impedes enzyme liberation into the extracellular environment. Maintaining the activity levels of Rgp under oxidative stress could also be significant *in vivo* as this enzyme has been shown to be responsible for interfering with the activity of polymorphonuclear leukocytes (11), which generate active oxygen species. On the other hand, the decrease in the total Kgp activity could represent a real decrease in its transcription or translation or, alternatively, oxygen could have affected the catalytic properties of the enzyme, perhaps due to protein oxidation. Nevertheless, it seems that in the presence of moderate amounts of oxygen, *P. gingivalis* is capable of retaining most of its proteolytic activity, an indication that the cells might still be able to display virulence in moderately oxygenated environments.

Although *P. gingivalis*, when compared to other anaerobes, possesses a low tolerance to oxygen (4), the microorganism could be classified as an aerotolerant anaerobe. That is, while it is incapable of colony formation on plates incubated aerobically, it can tolerate, for a limited time, the presence of atmospheric oxygen and it will grow in the presence of oxygen concentrations lower than that in air. A certain degree of aerotolerance might be a requirement for all strict anaerobic species present in the oral environment in order to persist as part of the indigenous flora and eventually

proliferate in an adequate anaerobic niche. This study also showed that the presence of a hemin-rich environment increases the tolerance to oxygen of *P. gingivalis*. Thus, the proliferation of the microorganism in a hemin-rich environment, such as the periodontal sulcus, is favored from both a nutritional and an oxygen-tolerance perspective. The growth of *P. gingivalis* under oxygenated environments is also accompanied by changes in fermentation end-products, cell morphology, anti-oxidant enzymes and proteinase activity. Although the levels of proteinase activities were maintained at a high level under oxygenated conditions, the decrease in the production of butyrate, the most harmful end-product for gingival tissues (2), indicates that the microorganism might not reach its maximum pathogenic potential until establishing an anaerobic environment. The results obtained in this study assist in our understanding of how this and other obligatory anaerobic microorganisms adapt to the unfavorably oxygenated conditions of the mouth at the different stages of colonization and disease progress. Understanding such mechanisms might increase our knowledge of the ecology of dental plaque.

Acknowledgments

Technical support from The Centre for Electron Microscopy of South Australia (CEMSA) is gratefully acknowledged. P. I. Diaz was supported by an IPRS scholarship from The University of Adelaide. We thank Dr. John Smalley, University of Liverpool, UK, for helpful comments.

References.

1. Amano A, Tamagawa H, Takagaki M, Murakami Y, Shizukuishi S, Tsunemitsu A. Relationship between enzyme activities involved in oxygen metabolism and oxygen tolerance in black-pigmented *Bacteroides*. J Dent Res 1988: **67**: 1196–1199.
2. Bartold PM, Gully NJ, Zilm PS, Rogers AH. Identification of components in *Fusobacterium nucleatum* chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation. J Periodont Res 1991: **26**: 314–322.
3. Carlsson JW, Höfling JF, Sundqvist GK. Degradation of albumin, haemopexin, haptoglobin and transferrin by black-pigmented *Bacteroides* species. J Med Microbiol 1984: **18**: 39–46.
4. Diaz PI, Zilm PS, Rogers AH. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. Microbiol 2002: **148**: 467–472.
5. Fletcher HM, Morgan RM, Macrina FL. Nucleotide sequence of the *Porphyromonas gingivalis* W83 *recA* homolog and construction of a *recA*-deficient mutant. Infect Immun 1997: **65**: 4592–4597.
6. Genco CA, Dixon DW. Emerging strategies in microbial haem capture. Mol Microbiol 2001: **39**: 1–11.
7. Gibbons RJ. Bacteriology of dental caries. J Dent Res 1964: **43**: 1021–1028.
8. Guerrant GO, Lambert MA, Moss CW. Analysis of short-chain fatty acids from anaerobic bacteria by high-performance liquid chromatography. J Clin Microbiol 1982: **16**: 355–360.
9. Higuchi M. Reduced nicotinamide adenine dinucleotide oxidase involvement in defense against oxygen toxicity of *Streptococcus mutans*. Oral Microbiol Immunol 1992: **7**: 309–314.
10. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000 1999: **20**: 168–238.
11. Kadowaki T, Yoneda M, Okamoto K, Maeda K, Yamamoto K. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. J Biol Chem 1994: **269**: 21371–21378.
12. Kenny EB, Ash AA, Jr. Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. J Periodontol 1969: **40**: 630–633.
13. Kesavalu L, Holt SC, Ebersole JL. Trypsin-like protease activity of *Porphyromonas gingivalis* as a potential virulence factor in a murine lesion model. Microb Pathogen 1996: **20**: 1–10.
14. Kurita-Ochiai T, Ochiai K, Fukushima K. Butyric-acid induces apoptosis in murine thymocytes and splenic T- and B-cells occurs in the absence of p53. J Dent Res 2000: **79**: 1948–1954.
15. Laine ML, van Winkelhoff AJ. Virulence of six capsular serotypes of *Porphyromonas gingivalis* in a mouse model. Oral Microbiol Immunol 1998: **13**: 322–325.
16. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 1998: **62**: 1244–1263.
17. Liu Y, Fletcher HM. Environmental regulation of *recA* gene expression in *Porphyromonas gingivalis*. Oral Microbiol Immunol 2001: **16**: 136–143.
18. Liu Y, Fletcher HM. The *recA* gene in *Porphyromonas gingivalis* is expressed during infection of the murine host. Oral Microbiol Immunol 2001: **16**: 218–223.
19. Marquis RE. Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. J Indust Microbiol 1995: **15**: 198–207.
20. Marsh PD, Mc Dermid AS, McKee AS, Baskerville A. The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. Microbiology 1994: **140**: 861–865.
21. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969: **244**: 6049–6055.
22. McKee AS, McDermid AS, Baskerville A, Dowsett AB, Ellwood DC, Marsh PD. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. Infect Immun 1986: **52**: 349–355.
23. Mettraux GR, Gusberti FA, Graf H. Oxygen tension (pO_2) in untreated human periodontal pockets. J Periodontol 1984: **55**: 516–521.
24. Müh U, Buckel W, Bill E. Mössbauer study of 4-hydroxybutyryl-CoA dehydratase. Probing the role of an iron-sulfur cluster in an overall non-redox reaction. Eur J Biochem 1997: **248**: 380–384.
25. Okamoto K, Nakayama K, Kadowaki T, Abe N, Ratnayake DB, Yamamoto K. Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. J Biol Chem 1998: **273**: 21225–21231.
26. Sabra W, Kim E-J, Zeng A-P. Physiological responses of *Pseudomonas aeruginosa* PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. Microbiology 2002: **148**: 3195–3202.
27. Scannapieco FA, Millar SJ, Reynolds HS, Zambon JJ, Levine MJ. Effect of anaerobiosis on the surface ultrastructure and surface proteins of *Actinobacillus actinomycetemcomitans* (*Haemophilus actinomycetemcomitans*). Infect Immun 1987: **55**: 2320–2323.
28. Shah HN, Gharbia SE. Ecological events in subgingival dental plaque with reference to *Bacteroides* and *Fusobacterium* species. Infection 1989: **17**: 264–268.
29. Shah HN, Williams RA, Bowden GH, Hardie JM. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. J Appl Bacteriol 1976: **41**: 473–95.
30. Smalley JW, Silver J, Marsh PJ, Birss AJ. The periodontopathogen *Porphyromonas gingivalis* binds iron protoporphyrin IX in the mu-oxo dimeric form: an oxidative buffer and possible pathogenic mechanism. Biochem J 1998: **331**: 681–685.
31. Smalley JW, Birss AJ, Silver J. The periodontal pathogen *Porphyromonas gingivalis* harnesses the chemistry of the micro-oxo bishaem of iron protoporphyrin IX to protect against hydrogen peroxide. FEMS Microbiol Lett 2000: **183**: 159–164.
32. Stanton TB, Jensen NS. Purification and characterization of NADH oxidase from *Brachyspira* (*Serpulina*) *hyodysenteriae*. J Bacteriol 1993: **175**: 2980–2987.
33. Takahashi N, Sato T, Yamada T. Metablolic pathways for cytotoxic end product formation from glutamate- and aspartate-containing peptides by *Porphyromonas gingivalis*. J Bacteriol 2000: **182**: 4704–4710.
34. Van der Hoeven JS, de Jong MH. Continuous culture studies and their relation to the *in vivo* behaviour of oral bacteria. In: Dean ACR, Ellwood DC, Evans CGT, eds. Continuous culture. Biotechnology, medicine and the enviroment. Chichester: Ellis Horwood, 1984: 89–109.