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Adaptation of *Porphyromonas gingivalis* to microaerophilic conditions involves increased consumption of formate and reduced utilization of lactate

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Porphyromonas gingivalis, previously classified as a strict anaerobe, can grow in the presence of low concentrations of oxygen. Microarray analysis revealed alteration in gene expression in the presence of 6 % oxygen. During the exponential growth phase, 96 genes were upregulated and 79 genes were downregulated 1.4-fold. Genes encoding proteins that play a role in oxidative stress protection were upregulated, including alkyl hydroperoxide reductase (*ahpCF*), superoxide dismutase (*sod*) and thiol peroxidase (*tpx*). Significant changes in gene expression of proteins that mediate oxidative metabolism, such as cytochrome *d* ubiquinol oxidase-encoding genes, *cydA* and *cydB*, were detected. The expression of genes encoding formate uptake transporter (PG0209) and formate tetrahydrofolate ligase (*fhs*) was drastically elevated, which indicates that formate metabolism plays a major role under aerobic conditions. The concomitant reduction of expression of a gene encoding the lactate transporter PG1340 suggests decreased utilization of this nutrient. The concentrations of both formate and lactate were assessed in culture supernatants and cells, and they were in agreement with the results obtained at the transcriptional level. Also, genes encoding gingipain protease secretion/maturation regulator (*porR*) and protease transporter (*porT*) had reduced expression in the presence of oxygen, which also correlated with reduced protease activities under aerobic conditions. In addition, metal transport was affected, and while iron-uptake genes such as the genes encoding the haemin uptake locus (*hmu*) were downregulated, expression of manganese transporter genes, such as *feoB2*, was elevated in the presence of oxygen. Finally, genes encoding putative regulatory proteins such as extracellular function (ECF) sigma factors as well as small proteins had elevated expression levels in the presence of oxygen. As *P. gingivalis* is distantly related to the well-studied model organism *Escherichia coli*, results from our work may provide further understanding of oxygen metabolism and protection in other related bacteria belonging to the phylum *Bacteroidetes*.

Abbreviations: ECF, extracellular function; formyl-THF, 10-formyl-tetrahydrofolate; GNAT, Gcn5-related *N*-acetyltransferase; JCVI, J. Craig Venter Institute; RNAP, RNA polymerase; TIGR, The Institute for Genomic Research.

The microarray data discussed in this paper have been deposited in the Gene Expression Omnibus (GEO) repository available at the National Center for Biotechnology Information (NCBI) under the accession number GSE17960.

Four supplementary figures, showing aerobic growth of *P. gingivalis* W83, a genomic view of the regulated genes, selected pathways affected by the presence of oxygen and the lactate utilization locus, and two supplementary tables, listing genes upregulated and downregulated in bacteria grown in the presence of oxygen, are available with the online version of this paper.

INTRODUCTION

Porphyromonas gingivalis is a Gram-negative bacterium that plays a major role in the development of periodontal disease (Ezzo & Cutler, 2003; Slots, 1986). This disease is a chronic inflammatory condition of the supporting tissues of the teeth, and affects 10–15 % of adults (Fox, 1992). Studies have also indicated that chronic periodontitis may be associated with increased risk of cardiovascular diseases (Beck *et al.*, 1998; Miyakawa *et al.*, 2004), diabetes (Pucher & Stewart, 2004), rheumatoid arthritis (Mercado *et al.*, 2003), and spontaneous preterm birth and preterm low birth weight (Lin *et al.*, 2007). Finally, recent studies have shown increased numbers of several oral bacteria, including *Porphyromonas* spp. and *Prevotella* spp., in oral squamous cell carcinoma, raising the possibility that the presence of certain bacteria is a risk factor for the development of oral cancer (Nagy *et al.*, 1998). In order to prevent onset and progression of these diseases, the elimination of pathogenic micro-organisms is important. Due to increasing antibiotic resistance, the identification of new targets for the development of novel therapies is needed. So far, virulence factors such as adhesins and proteases have been the major targets of intensive investigation. However, since metabolism is crucial for both microbe–microbe interactions and host–microbe communication, knowledge of *P. gingivalis* metabolic processes will help not only to produce a better understanding of the above interactions but also to identify novel targets for subsequent drug development.

P. gingivalis has been classified as a strictly anaerobic bacterium (reviewed by Lamont & Jenkinson, 1998). Inspection of its genome, however, reveals the presence of genes encoding components of aerobic respiration, including cytochrome *bd* oxidase (encoded by *cydA* and *cydB*) (Nelson *et al.*, 2003). Cytochrome *bd* is a haem protein and has a high affinity for oxygen (Cotter *et al.*, 1997; D'mello *et al.*, 1996; Govantes *et al.*, 2000) (reviewed by Gennis & Stewart, 1996). In addition, a related bacterium, *Bacteroides fragilis*, has recently been demonstrated to grow aerobically and consume oxygen (Baughn & Malamy, 2004). CydA and CydB have been shown to be required for oxygen consumption by *B. fragilis* (Baughn & Malamy, 2004). These results are in agreement with findings from the Rogers laboratory and our recent studies that demonstrate that *P. gingivalis* can grow in the presence of 6 % oxygen (Diaz & Rogers, 2004; He *et al.*, 2006). Although the above data show that *P. gingivalis* can grow aerobically, gene expression under such conditions remains unknown.

Knowledge of the transcriptome of aerobically grown bacteria is of major significance. Although *P. gingivalis* grows optimally without oxygen and is found mainly in anaerobic periodontal pockets, to reach the periodontal pocket it is transferred through different sites in the oral cavity (saliva, tongue, buccal mucosa), where it is exposed to oxygen (Dahlen *et al.*, 1992; Lamont & Jenkinson, 2000; van Steenbergen *et al.*, 1993). Thus, it must cope with the oxidative stress resulting from oxygen exposure, and it probably employs oxygen metabolism pathways similar to those of *B. fragilis* (Baughn & Malamy, 2004). Although research focusing on the oxidative stress defence mechanisms of *P. gingivalis* is under way in several laboratories, so far the oxidative metabolism of anaerobic bacteria remains largely unknown (Diaz *et al.*, 2006; Meuric *et al.*, 2008). As it is the cellular physiology that determines the ability both to grow and survive, as well as to express virulence properties, knowledge regarding the basic cell physiology under various environmental conditions is necessary for a comprehensive understanding of the pathogenicity of *P. gingivalis*. We hypothesize that interference with the mechanisms required for growth and survival in the presence of oxygen will inhibit bacterial growth and reduce survival of the bacterium before it reaches its favoured niche, the periodontal pocket, where it grows very rapidly.

The availability of genomic sequences enables the application of genome-based approaches to search for targets required for growth in the presence of oxygen. Both the annotated genome and genomic microarrays are available to those who study *P. gingivalis*. We hypothesized that genes whose products are required for growth and survival with oxygen will be overexpressed in cultures grown in the presence of oxygen. On the other hand, genes whose products are detrimental for growth with oxygen will be downregulated. To simultaneously interrogate the transcription of all genes present on the *P. gingivalis* W83 genome, microarray analysis was performed. The results were analysed using a variety of tools available for interpreting high-throughput data (Hendrickson *et al.*, 2008). Finally, bioinformatics searches were done to identify targets with unique functions such as the novel lactate utilization operon.

METHODS

Bacterial strains and growth conditions. *P. gingivalis* strain W83, a strain that is virulent in a murine abscess model and for which the sequence is available, was used in this study (Lewis & Macrina, 1998; Nelson *et al.*, 2003). The bacterium was grown in brain heart infusion (BHI) broth supplemented with haemin (5 μg ml^{-1}) and menadione (1 μg ml^{-1}). Anaerobic cultures were prepared in an anaerobic atmosphere consisting of 80 % N_2, 10 % CO_2 and 10 % H_2. Aerobic cultures were grown in the presence of 6 % oxygen (microaerophilic atmosphere consisted of 6 % O_2, 80 % N_2, 7 % CO_2 and 7 % H_2). These conditions were generated using Anoxomat Mark II anaerobic system (Mart Microbiology). Three independent cultures for both conditions were prepared on different days to ensure the biological significance of the results.

RNA preparation. RNA was isolated from bacteria grown to various growth phases [early exponential phase OD_{660} = 0.5, late exponential phase OD_{660} =0.9 and stationary phase (overnight cultures)] using an RNeasy mini kit (Qiagen).

Microarray analysis. Differentially cyanine-labelled cDNAs were prepared by reverse-transcribing 10 μg total RNA with ArrayScript reverse transcriptase (Applied Biosystems/Ambion), and dye was

incorporated into the reverse-transcribed cDNAs (Cy3 and Cy5, GE Healthcare). Genomic microarrays were obtained from the J. Craig Venter Institute (JCVI; formerly the Institute for Genomic Research, TIGR). These arrays contained 70-mer oligonucleotide probes for all predicted ORFs present on the *P. gingivalis* W83 genome. Probes were spotted in quadruplicate on the arrays. Microarray hybridization and washing were done as described previously, with minor modifications (Diaz *et al.*, 2006). DTT (0.1 mM) was added to post-hybridization washes and the slides were protected from the effects of ozone by the application of DyeSaver (Genishphere). Hybridized cDNA was detected with an Axon 4200A microarray scanner. At least two biological replicates using RNA derived from cultures prepared on different days and two technical duplicates were used for each time point. Images were inspected for quality, and spot intensities were determined using GenePix v6.0 software. Data were analysed for significant differences using the Significance Analysis for Oral Pathogen Microarray Data (SAOPMD) tools available at the Bioinformatics Resource for Oral Pathogens (BROP) at The Forsyth Institute (www.brop.org). This software is based on Linear models for Microarray Data (LIMMA) for statistical significance. All repeats within (four repeats per gene per array) and between arrays were combined to generate the microarray results.

Validation of gene expression data. The differential expression of selected genes was validated by real-time quantitative RT-PCR using the SYBR green-based detection system on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). Primers were designed using Primer3 software, available at http://frodo.wi.mit.edu. Experimental samples were tested in triplicate under the conditions recommended by the manufacturer using 20 ng total RNA (except for 16S rRNA, for which 0.2 ng total RNA was used). Reverse transcriptase was omitted in samples serving as negative controls. cDNA was synthesized with the AffinityScript Multi Temperature cDNA Synthesis kit (Stratagene). Quantitative PCR was done using the standard curve mode protocol (a calibration curve was constructed using serial fivefold dilutions of 100 ng total RNA) and a thermal profile consisting of one cycle of 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. RNA quantity was normalized using a probe specific for 16S rRNA.

Pathway analysis. Microarray data were examined for significance by importing the data into the BioCyc Omics Viewer available at http://biocyc.org/. The data were interpreted using the *P. gingivalis* W83 database. Genes were considered as differentially regulated based on a 0.3 cutoff (absolute value, logarithmic scale). A genomic view and a cellular metabolic view were generated.

Formate and lactate assays. Formate concentrations were determined using an enzymic method. Briefly, cells and supernatants from *P. gingivalis* cultures grown in BHI media for 16 h anaerobically and in the presence of 6 % oxygen were harvested by centrifugation. Reactions were prepared by mixing 100 µl diluted supernatant samples (1 : 10 dilution), 250 µl PBS, 100 µl NAD (0.02 M) and 50 µl formate dehydrogenase (FDH; 8 U ml^{-1}). The mixtures were incubated at 37 °C for 2 h. FDH activity was monitored spectrophotometrically by measuring the absorbance at 340 nm using a Fluorostar Galaxy spectrophotometer (BMG Labtechnologies). To determine the formate concentrations in cells, the bacteria were lysed using Lysing Matrix B Tubes (MP Biomedicals), and 100 µl cell lysate was used for the assays. A calibration curve was generated by using serial dilutions of sodium formate in assays performed in parallel with the experimental samples.

A lactate assay was done using a commercially available kit from Eton Bioscience. This assay is based on reduction of tetrazolium salt in an NADH-dependent reaction to give formazan, which exhibits maximum absorbance at 490 nm. Briefly, 20 µl diluted samples were mixed with 20 µl lactate assay solution (containing lactate dehydrogenase and tetrazolium salt) in a 96-well transparent, flat-bottommed microplate. The plate was covered and incubated at 37 °C for 1 h. The reaction was stopped by adding 50 µl of 0.5 M acetic acid per well, followed by brief gentle agitation. Then the absorbance was measured at 490 nm using a microplate reader. A standard curve was prepared in parallel using twofold dilutions of a lactate standard (2.5 mM).

Protease activity. Protease activities were determined as described previously (Lewis & Macrina, 1998). Briefly, bacterial cultures were grown aerobically and in the presence of 6 % oxygen until they reached the mid-exponential phase of growth (OD_{660}=0.4). The cells were separated from the culture supernatant by centrifugation. Equal numbers of the cells (2.5×10^3) and 20 µl of supernatants from each culture condition were used to perform protease assays. The assays were done using chromogenic substrates [N^{α}-benzoyl-L-arginine *p*-nitroanilide (Bz-Arg-pNA) and N^{α}-benzyloxycarbonyl-L-lysine *p*-nitroanilide (Z-LysX-pNA) from Novabiochem] in the presence of Tris/HCl buffer, pH 8.5. Protease activity was assessed by determination of a yellow colour measured spectrophotometrically at 405 nm.

RESULTS AND DISCUSSION

Growth in microaerophilic conditions

P. gingivalis is able to grow in the presence of 6 % oxygen (Fig. 1). An approximately 30 % reduction in growth rate was observed in the mid-exponential growth phase for bacteria cultured in the presence of oxygen when compared with those grown anaerobically. The magnitude of the reduction decreased to 15 % at late exponential phase. Inspection of the growth curves of the bacterium grown in anaerobic and microaerophilic conditions (Fig. 1) thus revealed no drastic differences in growth dynamics of *P. gingivalis*, indicating that this bacterium can grow nearly equally under both conditions. This is noteworthy, given

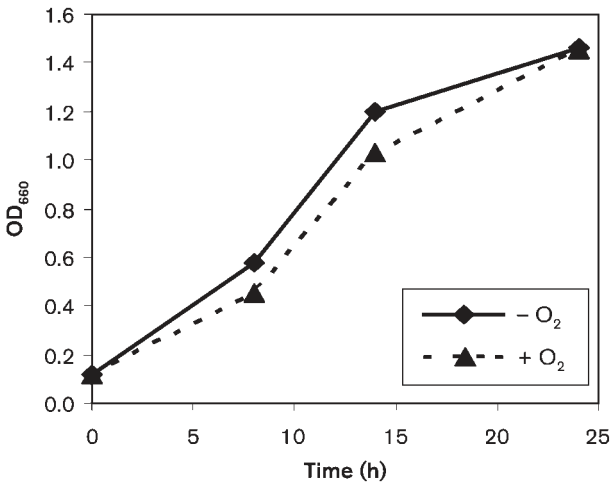

Fig. 1. Aerobic growth of *P. gingivalis* W83. Growth of *P. gingivalis* W83 in anaerobic and microaerophilic (6% oxygen) conditions, as determined by OD_{660}.

that this organism is classified as a strictly anaerobic bacterium and that exclusively anaerobic conditions are recommended to grow the organism (Duerden, 1980; Loesche *et al.*, 1992). Our laboratory and previous studies have demonstrated that *P. gingivalis* can withstand prolonged exposure to oxygen; thus, clinical samples predicted to contain this bacterium can be stored for a short time in the presence of low concentrations of oxygen. However, to preserve survivability of other organisms that may be present in the samples and which may require strictly anaerobic conditions, storage of clinical samples in the absence of oxygen may be prudent until we learn more about the metabolic capacities of the various micro-organisms present in the oral cavity.

These results are also significant in terms of the oxidative stress potential of this bacterium. So far, the knowledge of aerobic metabolism in anaerobic bacteria is very limited. We suggest that *P. gingivalis* must possess mechanisms allowing it to withstand low oxygen concentrations for prolonged periods of time. Although genome sequence analysis did reveal the presence of genes encoding proteins that may play a role in oxygen consumption and aerobic metabolism, such as cytochrome *d* ubiquinol oxidase, CydAB, other genes involved in the process still need to be defined.

Genomic view of oxygen-regulated genes

Although there may be multiple levels of regulation of genes within an operon, it is generally accepted that the genes are regulated in the same fashion. In the present study, most of the regulated genes were found to be clustered together in the genome (Supplementary Fig. S2). Such coordinate regulation of multiple genes having operonic organization (e.g. the *feoB2* and *hmu* loci reported previously) is an excellent way to validate the accuracy of our microarray results (Dashper *et al.*, 2005; Lewis *et al.*, 2006).

Gene regulation in aerobic and anaerobic conditions

Analysis of our microarray results revealed that in the mid-exponential growth phase (culture density reaching OD_{660}=0.5), 96 genes were 1.4-fold upregulated when the bacteria were grown aerobically (in the presence of 6 % oxygen) (Table 1, listing upregulated genes except ones annotated as hypothetical ones, and Supplementary Table S1, listing all upregulated genes). The transcriptional profile of the cultures in the late exponential growth phase (OD_{660} ~0.9) also resembled that of the early phase (with culture density OD_{660} ~0.5) (results not shown). To gain insight into the metabolic context of the transcriptome, the microarray data were examined using the metabolic pathway analysis program available at http://biocyc.org/. The results are shown in Fig. 2, and detailed pathways are also included in Supplementary Fig. S3. The overview in Fig. 2 shows 340 genes, including the upregulated genes in red and the downregulated genes in yellow. The regulation values were based on the fold change, for which the threshold was set at 0.3 (based on a logarithmic scale) for the analysis. Several anabolic pathways, such as those for formyl tetrahydrofolate biosynthesis, arginine and serine biosynthesis, panthotenate biosynthesis, and *de novo* synthesis of purines and pyrimidines, were upregulated. However, the lysine degradation, aspartate oxidation, pentose phosphate and fatty acid oxidation pathways, and formaldehyde assimilation, were downregulated (Fig. 2 and Supplementary Fig. S3). As expected, oxidative stress response mechanisms, including removal of peroxides and superoxides, were upregulated. In addition, metal transport was differentially regulated, with upregulation of manganese transport and downregulation of iron transport. Finally, it is noteworthy that differential regulation of nutrient transporters was observed, with upregulation of the formate/nitrate transporter PG0209 and downregulation of lactate permease PG1340 (Fig. 2, Tables 1 and 2).

The exact pathways in which the various players may be involved need further investigation, as the *P. gingivalis* database has been computationally derived and needs extensive curation to be accurate. So far, the most comprehensively curated (and thus the most accurate) database is that for *Escherichia coli* K-12.

Oxygen detoxification genes are induced in aerobic conditions

Our results demonstrate upregulation of the alkylhydroperoxide reductase (*ahp*) locus consisting of *ahpC* and *ahpF* in cells grown in aerobic conditions (Table 1, Fig. 2). PG0617 was also upregulated, although less than 1.4-fold, thus suggesting that this gene is part of the same operon (results not shown). The upregulation of these genes upon exposure to oxygen has been demonstrated before (Diaz *et al.*, 2004, 2006). Also, as alkyl hydroperoxidases are indispensable for defence against intracellularly generated peroxides, our data confirm that *P. gingivalis* is indeed capable of oxidative metabolism, resulting in the generation of intracellular peroxides as by-products (see below). Upregulation of expression of a gene encoding superoxide dismutase, *sod* (PG1545) (Table 1, Fig. 2), was also detected, which is consistent with its role in protecting bacteria from atmospheric oxygen (Nakayama, 1994). These data are again in agreement with earlier results that demonstrate upregulation of *sod* expression upon exposure to oxygen (Ohara *et al.*, 2006). In addition, this study showed that PG1190, which encodes glycerate dehydrogenase (HprA), was significantly upregulated (Table 1, Fig. 2). This enzyme catalyses the reduction of hydroxypyruvate to D-glycerate (using NADH as an electron donor) and is predicted (BioCyc database) to have a role in photorespiration (although such a process has yet to be demonstrated). These results indicate that glycerate metabolism is elevated in aerobically grown bacteria. On

Table 1. Genes upregulated under microaerophilic conditions in *P. gingivalis* W83 during the mid-exponential phase of growth

Gene ID*	M†	Fld‡	*P* value	Repeat§	Common name
PG0619	4.301474	19.718449	0.000000	23	Alkyl hydroperoxide reductase, F subunit
PG1827	4.011012	16.122599	0.000000	23	RNA polymerase sigma-70 factor, ECF subfamily
PG0900	2.752273	6.737781	0.000000	24	Cytochrome *d* ubiquinol oxidase, subunit I
PG0618	2.550736	5.859333	0.000000	24	Alkyl hydroperoxide reductase, C subunit
PG2213	2.266013	4.809922	0.000017	24	Nitrite reductase-related protein
PG1190	1.899944	3.731987	0.000000	24	Glycerate dehydrogenase
PG1868	1.827187	3.548446	0.000000	24	Membrane protein, putative
PG1729	1.826209	3.546040	0.000000	24	Thiol peroxidase
PG0275	1.773292	3.418332	0.000000	24	Thioredoxin family protein
PG1842	1.746192	3.354718	0.000000	24	Acetyltransferase, GNAT family
PG0433	1.672085	3.186748	0.000001	23	Tetrapyrrole methylase family protein
PG0209	1.618581	3.070728	0.000000	24	Formate–nitrite transporter
PG1642	1.568171	2.965285	0.000000	24	Cation-transporting ATPase, E1-E2 family, authentic frameshift
PG0899	1.531452	2.890766	0.000012	24	Cytochrome *d* ubiquinol oxidase, subunit II
PG1321	1.523558	2.874992	0.000000	23	Formate–tetrahydrofolate ligase
PG1545	1.376314	2.596043	0.000000	24	Superoxide dismutase, Fe–Mn
PG1286	1.286740	2.439761	0.000001	24	Ferritin
PG0432	1.273873	2.418099	0.000002	24	NOL1-NOP2-sun family protein
PG1043	1.212650	2.317630	0.000000	24	Ferrous iron transport protein B
PG0047	1.206248	2.307367	0.000000	24	Cell division protein FtsH, putative
PG2205	1.200092	2.297543	0.000000	24	2-Dehydropantoate 2-reductase
PG0889	1.141490	2.206088	0.000000	24	Peptidase, M24 family
PG1124	1.102806	2.147720	0.000000	24	ATP : cob(I)alamin adenosyltransferase, putative
PG0459	1.083449	2.119096	0.003701	20	ISPg5, transposase Orf1
PG1134	1.080530	2.114813	0.000000	24	Thioredoxin reductase
PG1641	1.057968	2.081997	0.000000	24	Phosphotyrosine protein phosphatase
PG2008	1.025836	2.036139	0.000000	24	TonB-dependent receptor, putative
PG0890	0.984835	1.979087	0.000000	24	Alkaline phosphatase, putative
PG1044	0.978094	1.969862	0.000000	23	Iron-dependent repressor, putative
PG0034	0.956851	1.941068	0.000000	24	Thioredoxin
PG1088	0.845295	1.796632	0.000000	24	Acetyltransferase, GNAT family
PG1213	0.820160	1.765602	0.176489	18	RNase H
PG0010	0.770883	1.706314	0.000045	24	ATP-dependent Clp protease, ATP-binding subunit ClpC
PG1269	0.762336	1.696235	0.026306	22	Delta-1-pyrroline-5-carboxylate dehydrogenase
PG1636	0.734038	1.663288	0.000001	24	FtsK-SpoIIIE family protein
PG1640	0.733573	1.662752	0.003768	24	DNA damage-inducible protein F
PG1541	0.730168	1.658832	0.000000	24	2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase
PG2009	0.701519	1.626216	0.148599	20	DNA repair protein RecO, putative
PG1042	0.689485	1.612708	0.000000	24	Glycogen synthase, putative
PG0299	0.687504	1.610495	0.005657	19	ISPg3, transposase, truncation
PG0090	0.670898	1.592063	0.004132	24	Dps family protein
PG1542	0.666572	1.587297	0.000000	24	Collagenase
PG0258	0.644826	1.563551	0.000002	24	ABC transporter, ATP-binding protein
PG0095	0.641523	1.559976	0.236406	22	DNA mismatch repair protein MutS
PG0720	0.637839	1.555997	0.000000	24	DNA-binding response regulator
PG0993	0.619548	1.536394	0.035660	12	Transposase, ISPg2-related, truncation
PG1501	0.608565	1.524742	0.000000	24	Transcriptional regulator, TetR family
PG1505	0.608501	1.524674	0.104468	11	Radical *S*-adenosylmethionine (SAM) domain protein
PG0017	0.603115	1.518993	0.000137	20	Sensor histidine kinase
PG0620	0.584758	1.499787	0.001129	24	ATP-dependent protease La
PG0273	0.572313	1.486905	0.000033	24	4-Phosphopantetheinyl transferase family protein
PG1540	0.570984	1.485537	0.000001	24	SAM : tRNA ribosyltransferase-isomerase
PG1089	0.568501	1.482982	0.000005	24	DNA-binding response regulator RprY
PG0740	0.565106	1.479496	0.070047	14	NLP-P60 family protein
PG1036	0.562349	1.476671	0.001795	24	Excinuclease ABC, A subunit
PG1638	0.561521	1.475824	0.001220	23	Thioredoxin family protein

Table 1. cont.

Gene ID*	M†	Fld‡	*P* value	Repeat§	Common name
PG0245	0.543118	1.457119	0.000908	24	Universal stress protein family
PG0046	0.537684	1.451641	0.000002	24	Phosphatidate cytidylyltransferase
PG0063	0.536831	1.450782	0.000021	22	Outer membrane efflux protein
PG1435	0.533697	1.447634	0.013341	8	Integrase
PG0924	0.528935	1.442864	0.165218	24	5-Nucleotidase, lipoprotein e(P4) family
PG1758	0.522016	1.435960	0.074702	24	Ribosomal protein S15
PG1467	0.507143	1.421233	0.348358	16	Methyltransferase, UbiE-COQ5 family
PG1383	0.497821	1.412079	0.131557	23	Amino acid exporter, putative

*Gene ID according to JCVI (formerly TIGR).
†M=log(spot intensity under aerobic conditions/spot intensity under anaerobic conditions).
‡Fld=fold change (ratio of transcript abundance in aerobic conditions to that in anaerobic conditions).
§Repeat: number of spots used for the analysis.

the other hand, hydroxypyruvate has high antioxidant capacity and can react with peroxide (Perera *et al.*, 1997). Thus, HprA may simply reduce oxidized pyruvate, which then serves as a peroxide sink.

Thioredoxin-encoding genes (PG0034, PG0275, PG1134 and PG1638) were significantly upregulated in mid-exponential cells (Table 1, Fig. 2). The thioredoxin system (thioredoxin, thioredoxin reductase and NADPH) is a disulfide-reducing system present in a variety of bacteria and has been demonstrated to protect cells against oxidative stress. Our results are also consistent with other reports that demonstrate induction of these genes upon exposure to oxygen in *P. gingivalis* (Diaz *et al.*, 2006; Meuric *et al.*, 2008) as well as in the related bacterium *B. fragilis* (Rocha *et al.*, 2007).

The thiol peroxidase gene, PG1729, was also upregulated. Thiol peroxidase (Tpx) has peroxidase activity and is an antioxidant enzyme present in a variety of bacteria (Wan *et al.*, 1997; Zhou *et al.*, 1997).

PG0090, encoding Dps, a ferritin-like iron-binding protein, was upregulated in the presence of oxygen. This protein has been shown to be upregulated under aerobic conditions as well as to have a role in protection against peroxide stress (Ueshima *et al.*, 2003). Another iron binding protein-encoding gene, PG1286 (ferritin), had elevated expression (Table 1). Ferritin has been shown to be required for *P. gingivalis* survival under iron-depleted conditions; however, it is not required for protection of the bacterium against oxidative stress either generated by peroxides or resulting from exposure to atmospheric oxygen (Ratnayake *et al.*, 2000).

Finally, a group of genes encoding chaperonins, such as the ATP-dependent Clp protease (PG0010) and the ATP-dependent protease encoded by PG0620, were induced by oxygen (Table 1). Clp proteases as well as chaperonins have been shown to play a role in the survival of *P. gingivalis* in the presence of environmental stress (Capestany *et al.*, 2008).

Central metabolism transcripts adapt to the presence of oxygen

Aerobic metabolism in *P. gingivalis* is largely unknown. The upregulation of genes mediating aerobic metabolism, including cytochrome *d* ubiquinol oxidase, subunit I CydA (PG0899), cytochrome *d* ubiquinol oxidase and subunit II CydB (PG0900) was observed in this study (Table 1). This locus also contained an ORF encoding a protein of unknown function (PG0901) that was upregulated in the presence of oxygen (Supplementary Table S1). This indicates that it was a member of the same transcriptional unit as the *cydAB* operon. This locus was drastically upregulated in cells at mid-exponential phase, which would be in agreement with the data showing that the locus is indispensable for oxygen utilization, as shown for another member of the phylum *Bacteroidetes*, *B. fragilis* (Baughn & Malamy, 2004).

It is noteworthy that the gene encoding the formate–tetrahydrofolate ligase (Fhs) PG1321 was upregulated in the presence of oxygen (Table 1, Fig. 2 and Supplementary Fig. S3). Fhs catalyses the formation of 10-formyl-tetrahydrofolate (formyl-THF), which is used directly in purine biosynthesis and formylation of Met-tRNA (initiator of protein synthesis in prokaryotes and mitochondria). Formyl-THF is also used indirectly in the biosynthesis of methionine, serine, glycine and thymine, and thus is central to *de novo* purine and amino acid biosynthesis (Fig. 3). Formyl-THF is synthesized by activation of formate, and Fhs is speculated to scavenge formate. Fhs has also been found in anaerobic bacteria such as *Clostridium* spp. (Whitehead *et al.*, 1988). Some bacteria (e.g. *E. coli*) can form formyl-THF independently of formate by the serine hydroxymethyltransferase degradation of serine to glycine and 5,10-methylene-THF, which can then be converted to 5,10-formyl-THF by dehydrogenase and cyclooxygenase (Dev & Harvey, 1982). Since not all organisms have Fhs, this suggests that some organisms are not able to incorporate formate into the

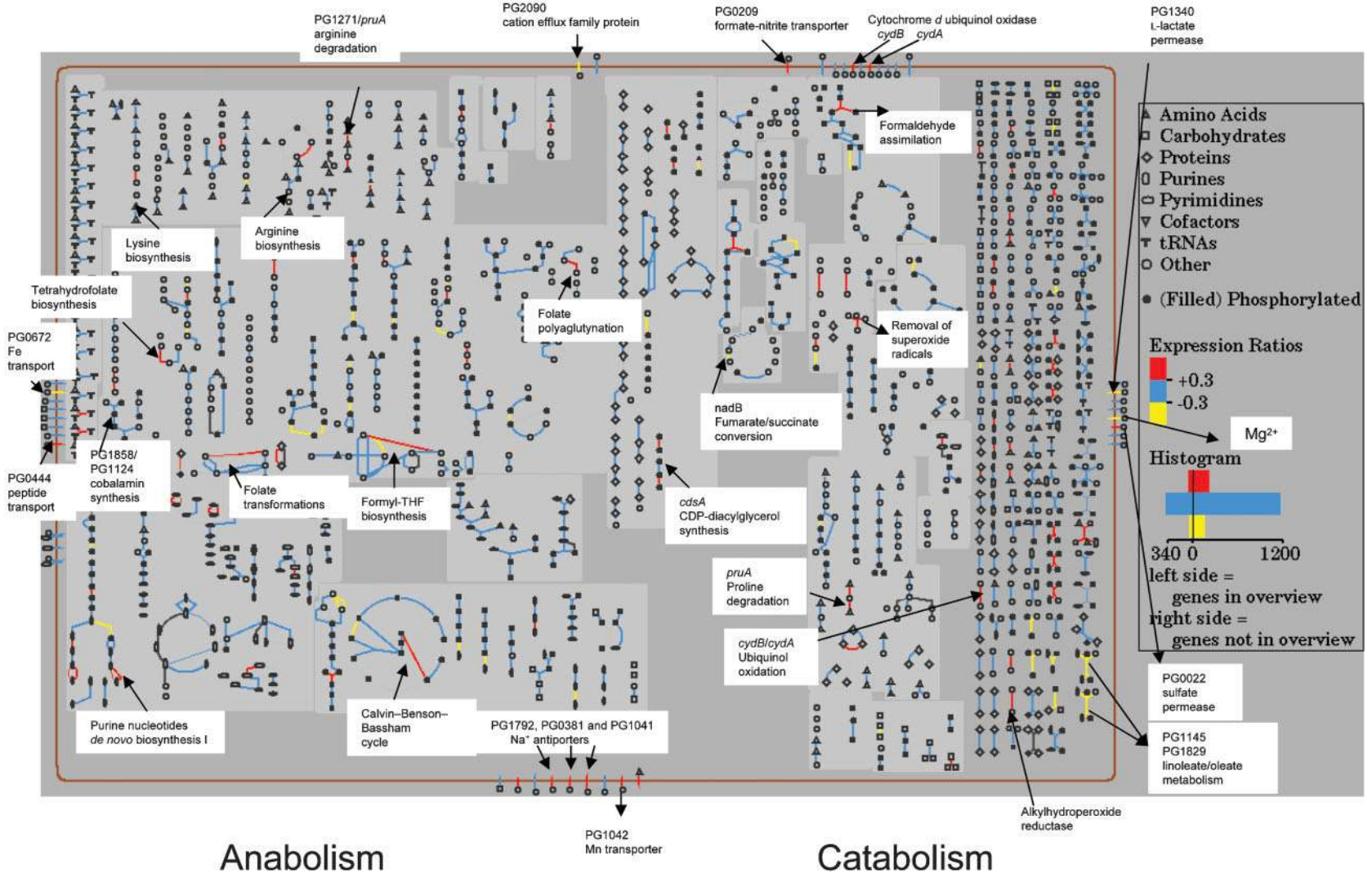

Fig. 2. View of the metabolic pathways of oxygen-regulated genes in *P. gingivalis*. *P. gingivalis* W83 was grown anaerobically and in the presence of 6 % oxygen. Transcriptional data derived from cultures harvested at the mid-exponential phase of growth (OD=0.5) were used. The colour designation is as follows: red, upregulation; yellow, downregulation; blue, no significant change in expression under aerobic (6 % oxygen) conditions when compared with anaerobically grown bacteria. Genes regulated at >0.3 on a logarithmic scale are shown as regulated.

carbon pool (Whitehead *et al.*, 1988). It is noteworthy that in eukaryotes a trifunctional enzyme composed of three proteins (synthetase, cyclohydrolyse and dehydrogenase) exists (Caperelli *et al.*, 1980). The eukaryotic synthetase domain has approximately 50 % homology with bacterial Fhs (Whitehead & Rabinowitz, 1988). Formate is abundant in the oral cavity, as it is a metabolic product of several saccharolytic bacteria, such as the oral streptococci *Actinomyces* sp. and *Lactobacillus* sp. (Iwami *et al.*, 1992; Takahashi & Yamada, 1999). Streptococci are dominant in supragingival plaque, and as the first colonizers of a tooth they establish conditions for binding and accumulation of subsequent bacteria. Thus, Gram-negative anaerobic bacteria such as *P. gingivalis* bind to streptococci and can then use the formate produced by streptococci. Consistent with the predicted elevated use of formate by the Fhs enzyme of *P. gingivalis* in aerobic conditions, the gene encoding the formate/nitrate transporter activity, PG0209, was upregulated in the presence of oxygen (Table 1, Fig. 2). The role of the transporter was further determined by assessing the levels of formate consumption of aerobically and anaerobically grown bacteria. As shown in Fig. 4(a), *P. gingivalis* grown in the presence of 6 % oxygen had 80 % reduced levels of formate in culture supernatants, which was consistent with elevated nutrient consumption by bacterial cells. This would be mediated through upregulation of the formate transporter, which transports formate into the cells. Similar results were obtained when the formate concentration in bacterial cells was assessed; 18 % reduced levels of formate were detected in aerobically grown cells when compared with anaerobically cultured bacteria, thus indicating that the formate is used once transported into bacterial cell (Fig. 4a).

As described above, the formate–nitrite transporter (PG0209) was upregulated in the presence of oxygen (Table 1, Fig. 2), thus demonstrating that formate and nitrite may be substrates that are metabolized preferentially under aerobic conditions. Besides the role of formate in the generation of formate–THF as described above, it may also play a role in metabolism. It could serve as an electron donor for *P. gingivalis* that is capable of fumarate respiration. The system appears to be used widely by anaerobic bacteria such as *Wolinella recta* (Ohta *et al.*, 1991). However, a gene encoding formate dehydrogenase activity was not identified in this study. It is possible that such an enzyme differs from the well-characterized one in other bacteria, and thus one of the hypothetical proteins of unknown function may encode a novel formate dehydrogenase in *P. gingivalis*. It is possible that the PG0209 protein also transports nitrite. Nitrite is abundant in the oral environment as it is generated by the reduction of nitrate by oral bacteria (Doel *et al.*, 2005); however, its role in *P. gingivalis* metabolism has yet to be established. It is possible that it plays a role in respiratory metabolism. However, as the expression of PG1821, which encodes the cytochrome *c* nitrite reductase small subunit NrfH, and the expression of PG1820, which encodes the cytochrome *c* nitrite reductase catalytic subunit NrfA, were unaffected in cells at the mid-exponential growth phase, this nitrite metabolism may not be regulated by the availability of oxygen.

Interestingly, PG1340, which encodes L-lactate permease, was downregulated under aerobic conditions (Table 2, Fig. 2). This indicates that lactic acid transport was also reduced, consistent with the reduction of fermentative metabolism in the presence of oxygen. Our lactate uptake assays showed that *P. gingivalis* grown under anaerobic conditions indeed consumes more lactate from the culture medium (Fig. 4b) and at the same time accumulates more lactate in cells (Fig. 4b). Such data suggest that the bacterium utilizes lactate. It is probable that lactate is used as a substrate for respiratory metabolism. This is based on reports demonstrating that other members of the phylum *Bacteroidetes*, such as *Bacteroides* spp. strain JW20 isolated from termite hindguts, have the ability to ferment lactate to propionate and acetate (Schultz & Breznak, 1979). To test the feasibility of this hypothesis, the *P. gingivalis* W83 genome was searched for the presence of genes encoding proteins involved in lactate utilization. Comparative analysis of lactate metabolism systems in other bacteria led to the identification of the novel three-gene locus PG1171–73, which encodes the putative L-lactate dehydrogenase system composed of an Fe–S oxidoreductase subunit (YkgE), an iron–sulfur cluster binding subunit (YkgF), and a hypothetical protein (YkgE family protein). In other bacteria, such as *Campylobacter jejuni*, *Pasteurella multocida*, *Corynebacterium diphtheriae*, *Bacillus clausii*, *Vibrio vulnificus* and *Helicobacter pylori*, this gene cluster is preceded by an ORF encoding L-lactate permease (Supplementary Fig. S4), while in *P. gingivalis* the gene encoding L-lactate permease is located elsewhere in the genome. It is noteworthy that the novel lactate dehydrogenase region is also similar to a region in other members of the order *Bacteroidales*, i.e. *Prevotella intermedia*, *Bacteroides thetaiotaomicron* and *B. fragilis* (Supplementary Fig. S4). Although the ability of lactate to enhance the growth of *Bacteroides* isolated from termite hindguts has been demonstrated, the role of lactate in promoting growth of other members of the phylum *Bacteroidetes* has yet to be determined (Schultz & Breznak, 1979). Also, although cross-feeding of lactate among oral microorganisms has been shown (e.g. *Veillonella atypica*–*Streptococcus gordonii*), this is the first indication that such nutritional interdependence may also include *P. gingivalis* (Egland *et al.*, 2004). Our data indicate that lactate plays a role in energy metabolism in *P. gingivalis*, and the newly identified lactate utilization locus will serve as a platform for future experimental approaches leading to detailed analysis of the lactate utilization mechanisms in these species. As lactate may be abundant due to the presence of lactate producers such as oral streptococci and *Lactobacillus* spp., mechanisms allowing utilization of this substrate seem beneficial for survival and growth of *P. gingivalis* in the oral cavity. Many members of the order

Table 2. Genes downregulated under microaerophilic conditions in *P. gingivalis* W83 during the mid-exponential phase of growth

Gene ID*	M†	Fld‡	*P* value	Repeat§	Common name
PG0656	−1.167622	0.445154	0.018084	24	Ribosomal protein L34
PG2046	−1.066924	0.477336	0.072498	20	tRNA(Ile)-lysidine synthetase
PG0214	−0.955201	0.515770	0.000000	24	RNA polymerase sigma-70 factor, ECF subfamily
PG1518	−0.846663	0.556069	0.002512	13	ISPg8, transposase, degenerate
PG0538	−0.719822	0.607172	0.000071	22	Outer membrane efflux protein
PG1340	−0.675188	0.626251	0.011193	23	L-Lactate permease
PG1378	−0.670723	0.628192	0.011882	16	A–G-specific adenine glycosylase
PG2040	−0.659376	0.633152	0.017171	24	DNA-binding protein, histone-like family
PG1138	−0.637244	0.642940	0.070058	24	Pigmentation and extracellular proteinase regulator
PG2061	−0.629681	0.646319	0.013140	24	Dihydrofolate reductase
PG2105	−0.600443	0.659551	0.157164	24	Lipoprotein, putative
PG0539	−0.596809	0.661215	0.000705	22	Efflux transporter, membrane fusion protein (MFP) component, RND family
PG2152	−0.592482	0.663201	0.147504	24	DNA-binding protein, histone-like family
PG2038	−0.578090	0.669850	0.004687	22	*N*-Acetylmuramoyl-L-alanine amidase, putative
PG1176	−0.559966	0.678318	0.324451	19	ABC transporter, ATP-binding protein, putative
PG1551	−0.548684	0.683643	0.000332	24	HmuY protein
PG1497	−0.546073	0.684882	0.002012	22	DNA-binding protein, histone-like family
PG1663	−0.543447	0.686129	0.000121	24	ABC transporter, ATP-binding protein
PG1828	−0.524139	0.695374	0.000010	24	Lipoprotein, putative
PG0058	−0.524078	0.695403	0.025044	24	Nicotinate (nicotinamide) nucleotide adenylyltransferase
PG0738	−0.521129	0.696826	0.089663	24	Cytidine–deoxycytidylate deaminase family protein
PG0928	−0.519755	0.697490	0.005323	24	Response regulator
PG0199	−0.513812	0.700370	0.000018	23	TatD family protein
PG1480	−0.496718	0.708717	0.001912	19	Conjugative transposon protein TraI
PG1829	−0.496710	0.708721	0.000006	24	Long-chain fatty acid CoA ligase, putative
PG0264	−0.488815	0.712610	0.198323	24	Glycosyltransferase, group 2 family protein
PG2222	−0.477651	0.718146	0.072609	22	Acyltransferase, HtrB-MsbB family
PG1127	−0.475972	0.718982	0.000163	24	Transcriptional regulator, AsnC family
PG2032	−0.474327	0.719803	0.024341	24	Primosomal protein N
PG0751	−0.459947	0.727013	0.000102	24	PorT protein
PG0075	−0.455514	0.729250	0.096559	24	Phosphoribosylformylglycinamidine cyclo-ligase, putative
PG1690	−0.447940	0.733089	0.048362	24	Sua5-YciO-YrdC-YwlC family protein
PG0985	−0.443237	0.735483	0.000002	22	RNA polymerase sigma-70 factor, ECF subfamily
PG2091	−0.441659	0.736288	0.037867	24	Dihydroneopterin aldolase
PG0675	−0.440779	0.736737	0.136953	24	Indolepyruvate ferredoxin oxidoreductase, alpha subunit
PG1224	−0.440290	0.736986	0.094382	24	ABC transporter, periplasmic substrate-binding protein, putative, degenerate
PG0254	−0.438424	0.737941	0.018955	24	N utilization substance protein A, putative
PG2215	−0.434300	0.740053	0.000674	24	Mannose-1-phosphate guanylyltransferase
PG0704	−0.429853	0.742338	0.000406	24	Phosphoglycerate mutase family protein
PG0022	−0.426994	0.743810	0.014995	24	Sulfate permease family protein
PG1714	−0.409966	0.752641	0.082550	24	Pyridoxamine-phosphate oxidase
PG0369	−0.409651	0.752805	0.110729	24	Phosphopantetheine adenylyltransferase
PG1606	−0.403236	0.756161	0.052477	24	Ion transporter
PG1345	−0.402356	0.756621	0.002762	22	Glycosyltransferase, group 1 family protein
PG1237	−0.400268	0.757718	0.145483	24	Transcriptional regulator, LuxR family
PG1576	−0.395648	0.760148	0.049670	24	L-Aspartate oxidase
PG0350	−0.395366	0.760297	0.000050	24	Internalin-related protein
PG1380	−0.390353	0.762943	0.103174	24	ABC transporter, ATP-binding protein
PG2090	−0.388911	0.763706	0.026713	24	Cation efflux family protein

*Gene ID according to JCVI (formerly TIGR).

†M=log(spot intensity under aerobic conditions/spot intensity under anaerobic conditions).

‡Fld=fold change (ratio of transcript abundance in aerobic conditions to that in anaerobic conditions).

§Repeat: number of spots used for the analysis.

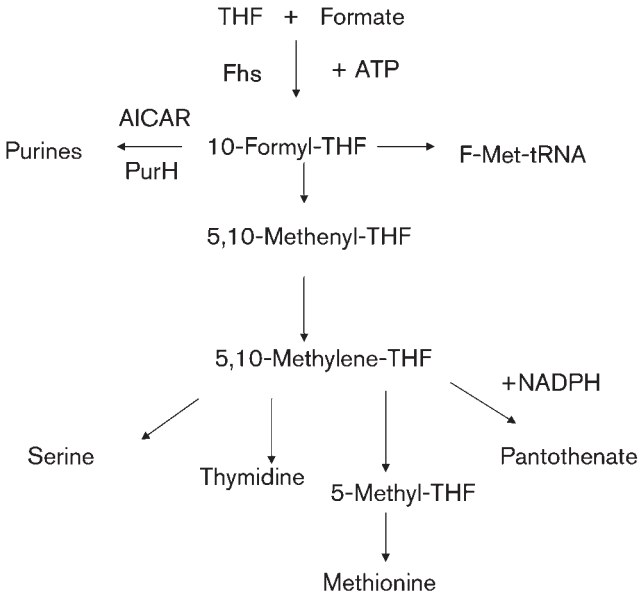

Fig. 3. Role of Fhs in purine and amino acid biosynthesis in *P. gingivalis*.

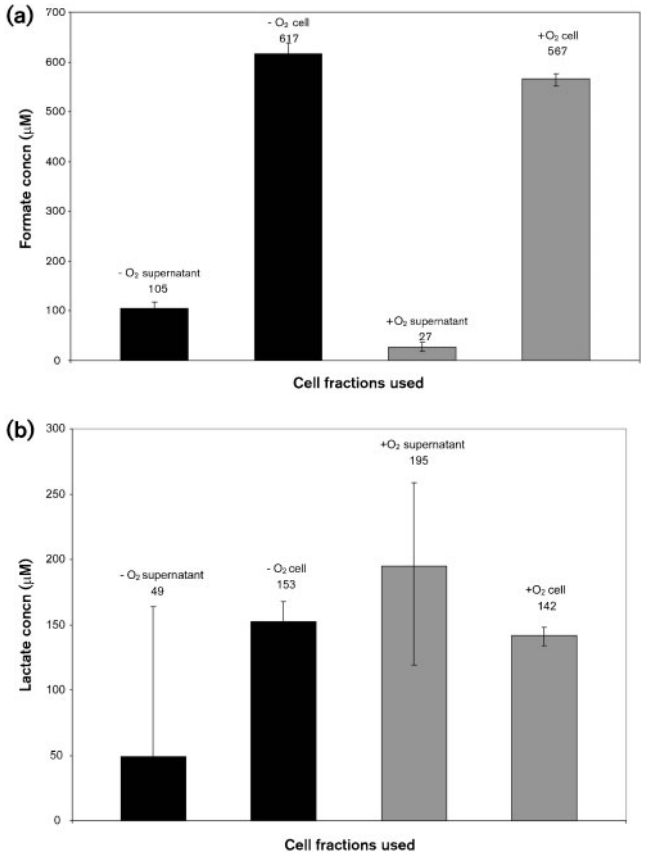

Fig. 4. Effect of oxygen on metabolite utilization by *P. gingivalis* W83. Formate (a) and lactate (b) levels in culture supernatants (designated as 'supernatant') and in bacterial cells (designated as 'cell') were determined in *P. gingivalis* W83 cultures grown anaerobically ($-O_2$) and in the presence of 6 % oxygen ($+O_2$). The numbers above the bars indicate the mean value derived from an experiment performed in triplicate.

Bacteroidales can also utilize carbohydrates (Koropatkin *et al.*, 2008; Scholle *et al.*, 1990). Although *P. gingivalis* has two pathways for lactate fermentation from glucose and fructose (Fig. 2), these pathways may be incomplete in *P. gingivalis* W83 due to a mutation in the glucose kinase gene *glk*, but may function in other *P. gingivalis* strains (Naito *et al.*, 2008).

Several genes encoding other factors in the metabolism of *P. gingivalis* were also regulated. Thus, genes encoding anabolic proteins such as PG1271, involved in arginine biosynthesis (acetylornithine aminotransferase); PG2205, encoding 2-dehydropantoate 2-reductase; PanE (Table 1), which mediates pantothenate synthesis (Supplementary Fig. S3a); PG0653, encoding a protein that belongs to the SerB family and has a role in the serine biosynthesis pathway; PG0030, encoding cytidine deaminase, involved in salvage pathways of pyrimidine deoxyribonucleotides (Supplementary Fig. S3b); PG1123, encoding PurB; and PG1129, encoding Nrd, were upregulated (the last two mediate the *de novo* biosynthesis of purine nucleotides; Supplementary Fig. S3b). As expected, PG1541, encoding FolK, a member of the tetrahydrofolate biosynthesis pathway, was also upregulated (Table 1, Supplementary Fig. S3c, d). Finally, PG1042, which encodes a putative glycogen synthase, was upregulated (Table 1). This indicates that energy storage is an important strategy under aerobic conditions.

The expression of PG1614, which encodes fumarate reductase, the major enzyme that plays a role in anaerobic metabolism, was unaffected. This indicates that under microaerophilic conditions both aerobic and anaerobic modes of metabolism may function. However, PG0690, which encodes 4-hydroxybutyrate coenzyme A transferase, was slightly downregulated (ratio 0.8, results not shown). This transferase mediates the catabolism of glutamate to butyrate (Takahashi *et al.*, 2000). Other downregulated genes included those mediating the biosynthesis of NAD from aspartate: PG0058, encoding nicotinate (nicotinamide) nucleotide adenylyltransferase (NadD), and PG1576, encoding L-aspartate oxidase (NadB) (Table 2, Fig. 2 and Supplementary Fig. S3f). PG1829, encoding a putative long-chain fatty acid CoA ligase, was also downregulated (Table 2), indicating that fatty acid β-oxidation is also reduced in aerobically grown bacteria (Supplementary Fig. S3f). Finally, PG1067, encoding a hypothetical protein possibly involved in lysine fermentation to acetate and butyrate, was downregulated (Supplementary Table S2, Supplementary Fig. S3f).

PG2061 encoding FolA, a dihydrofolate reductase that converts dihydrofolate into tetrahydrofolate, had reduced expression (Table 1, Supplementary Fig. S3c, d). The

mechanism of FolA regulation in prokaryotes has been shown to be mediated by the TyrR regulator (Yang *et al.*, 2007), while in eukaryotes it has been shown to involve growth-dependent regulation (Slansky & Farnham, 1996). Such downregulation would thus be consistent with the slightly reduced growth rate of the bacterium under aerobic conditions. It is also possible that there are significant variations in this enzyme among anaerobic bacteria and as such regulation of its expression may differ as well (Roth *et al.*, 1989). Finally, it may not be necessary to reduce dihydrofolate to tetrahydrofolate, since tetrahydrofolate synthesis is upregulated (Table 1). Such a scenario would be plausible, considering that reduction may be more difficult to achieve for anaerobes under aerobic conditions.

Acylation mechanisms

Several members of the Gcn5-related *N*-acetyltransferase (GNAT) family were upregulated. GNATs are abundant in nature and catalyse the transfer of an acetyl group to a nitrogen atom on an acceptor molecule (Vetting *et al.*, 2005). Although a variety of GNAT substrates are known (e.g. aminoglycosides, glucosamine 1-phosphate), most still need to be functionally characterized. The most drastically upregulated in the presence of oxygen was the two-component locus composed of PG1841, which encodes a hypothetical protein, and PG1842, which encodes *N*-acetyltransferase (GNAT family) (Table 1). As this acetyltransferase is located within the *hagA* locus (PG1840), it is possible that it is involved in acylation of the HagA adhesin (Han *et al.*, 1996; Lewis & Macrina, 1999). Another upregulated GNAT locus was PG1088 (Table 1). Expression of other GNATs, encoded by PG1254, PG1358 and PG1751, was not significantly altered. On the other hand, expression of PG2145, which encodes polysaccharide deacetylase (xylanase/chitin deacetylase) (PgdA), was slightly downregulated (ratio 0.77, results not shown). These data indicate that acylation plays an important role in *P. gingivalis* grown in the presence of oxygen. Detailed functional analysis of the GNAT family members and substrates is thus warranted to gain a more comprehensive understanding of the adaptation of *P. gingivalis* to oxygen.

Metal transport

Changes in the expression of metal transporters were observed in this study. Although metals are essential nutrients, they can also play an important role in defence against oxidative stress. In addition, they may be toxic. In particular, iron transport, which is required for reactions leading to the generation of hydroxyl radicals from peroxide, must be tightly regulated (Touati, 2000). On the other hand, the structurally similar metal manganese is indispensable for growth of *P. gingivalis* in the presence of oxygen (He *et al.*, 2006). As a consequence of being a cofactor of antioxidant enzymes, such as superoxidase dismutase (SOD), this metal has antioxidant properties (Archibald & Fridovich, 1982; Hiraoka *et al.*, 2000; Nakayama, 1990, 1994; Yim *et al.*, 1990). In agreement with the functional properties of these metals, the down-regulation of iron transporters such as the major haemin uptake system *hmu* (PG1551) was observed (Dashper *et al.*, 2005; Lewis *et al.*, 2006) (Table 2). On the other hand, iron-storage proteins such as the chaperone like protein-encoding gene *dps* (PG0090) and the ferritin-encoding gene PG1286 were upregulated in mid-exponential phase cells (Table 1).

Consistent with the antioxidative role of manganese, upregulation of expression of the genes encoding manganese transporters, such as *feoB2* (PG1043), was observed (Table 1) (Dashper *et al.*, 2005; He *et al.*, 2006). It is noteworthy that the expression of five ORFs forming the locus PG1042–PG1044 was upregulated, thus indicating operonic organization of the locus (Table 1, Supplementary Fig. S2).

Regulators

Several genes encoding regulatory proteins were regulated upon transition into anaerobic conditions. A sigma factor encoded by PG1827 [extracellular function (ECF) sigma 70, SigH] was drastically upregulated (Table 1). Sigma factors are components that mediate genome-wide regulatory processes. They bind reversibly to DNA-dependent RNA polymerase (RNAP) to form holoenzyme. This in turn enables binding of holoenzyme to specific promoters and leads to mRNA synthesis from these promoters. Although holoenzyme can drive the synthesis of mRNA, this process can also be regulated by several transcriptional regulators, activators or repressors. The DNA-dependent RNAP of *P. gingivalis* has been reported to differ from that of *E. coli*. The differences were at the protein level as well as in the ability of the polymerase to recognize *E. coli* promoters (purified *P. gingivalis* polymerase binds to *P. gingivalis* promoters but not to *E. coli* promoters). Thus, ECF sigma factors of *P. gingivalis* may differ from the well-characterized factors in *E. coli*, both at the protein level as well as in regard to their target recognition (Klimpel & Clark, 1990).

So far, promoters of the *Bacteroides* group have not been well defined, and this study indicates that there are significant differences in both the consensus sequences as well as in the position of the promoters (promoters of several *P. gingivalis* genes are located far upstream of the translational start site, e.g. the promoter of the *hmu*, *fimA* and *feoB* loci) (Lewis *et al.*, 2006). Therefore, further investigation of the promoter regions as well as of the transcription process in *Bacteroides* is warranted. So far, sigma factors have not been characterized in *Bacteroides*. Thus, our studies, which demonstrate regulation of selected factors in *P. gingivalis*, will serve as a platform for future experimental investigations that will shed light on the transcription process in this group of bacteria. This is in agreement with our observation that the sigma factors identified as regulated in our study share significant

similarities to putative sigma factors in other bacteria belonging to the phylum *Bacteroidetes*. The RNAP sigma factor encoded by PG1827 belongs to the SigH ECF subfamily. The ECF sigma factor SigH has been demonstrated to play a role in the regulation of expression of oxidative stress mechanisms in other bacteria, including *Mycobacterium tuberculosis* and *Salmonella enterica* (Bang *et al.*, 2005; Manganelli *et al.*, 2002). Thus, the upregulation of the *sigH* gene in *P. gingivalis* grown under aerobic conditions indicates that it also may play a role in the regulation of oxidative stress protection mechanisms in this bacterium.

Another sigma factor encoded by PG0214 (RNAP sigma-70, ECF subfamily) was significantly downregulated (Table 2). Also, the sigma factor encoded by PG0985, defined as a possible RNAP sigma-24 factor, was downregulated (Table 2). This protein has similarity to putative ECF sigma factors from a variety of bacteria belonging to the phylum *Bacteroidetes*. For example, *B. fragilis* and *B. thetaiotaomicron* were found to have a protein that is 26 % identical and 48 % similar over the entire 167 amino acid region to the sigma factor encoded by PG0985.

Among other regulators, PG1089, which encodes the DNA-binding response regulator RprY, was upregulated (Table 1). Such regulation was consistent with the reported role of this protein in oxidative stress protection in *P. gingivalis* (Duran-Pinedo *et al.*, 2007). Also, while PG1501, which encodes a TetR-like transcriptional regulator, was upregulated, PG1237, encoding a LuxR-like regulator, had reduced expression (Tables 1 and 2). Interestingly, expression of the oxidative stress regulator OxyR (encoded by PG0270) was not significantly affected.

Virulence factors

In order to establish itself in the oral cavity, *P. gingivalis* binds other organisms as well as host components. *P. gingivalis* W83 does not produce the major fimbrillin composed of FimA, due to a mutation in the *fimA* gene (Fujiwara *et al.*, 1993; Nelson *et al.*, 2003), although expression of PG2132, which encodes the fimbrillin, was not altered (results not shown). However, it does possess the *mfa* locus (PG0178), encoding a 67 kDa fimbrillin forming short fimbria. This locus has been shown to play a role in adherence to other organisms such as *Streptococcus gordonii*; however, it also inhibits invasion of host cells (Capestany *et al.*, 2008; Park *et al.*, 2005). It is noteworthy that this locus was upregulated under aerobic conditions (1.4-fold, results not shown), which indicates that it plays a role during the early colonization stages when the organism may be present in the supragingival environment. Thus, our findings are significant, as they indicate upregulation of adhesins that in turn facilitate initial adhesion and allow colonization of the oral cavity. On the other hand, PG1138, encoding the major pigmentation and extracellular proteinase activity regulator PorR, was downregulated (Table 2) (Shoji *et al.*, 2002). This regulator has been demonstrated to play a role in modulating the activity of the major group of *P. gingivalis* proteases, including Rgp and Kgp (Curtis *et al.*, 2001). Furthermore, the protease transporter-encoding gene, *porT* (PG0751) (Nguyen *et al.*, 2009; Sato *et al.*, 2005), was also downregulated (Table 2). Indeed, analysis of the protease activities of Rgp and Kgp demonstrated that both were reduced in aerobically grown bacteria, which was in agreement with the transcriptional data (Fig. 5a, b). The Arg-X protease activity was reduced by 35 and 30 % in culture supernatants and cells, respectively, in aerobically grown bacteria when compared with anaerobically grown cells (Fig. 5a). Similarly, although the extracellular Lys-X specific activity was moderately regulated (by 18 %), the cell-associated activity was reduced by 20 % when the bacteria were grown in the presence of oxygen (Fig. 5b). On the other hand, PG1542, encoding collagenase, and PG0889, encoding peptidase, were upregulated (Table 1).

Transcription of two glycosyltransferase-encoding genes, PG0264 and PG1345, was downregulated (Table 2), indicating that post-transcriptional modifications of the bacterial proteins may also be reduced when *P. gingivalis* is grown in the presence of oxygen.

Genes PG0890 and PG1641 encode two phosphatases that were upregulated. Alkaline phosphatase PepP (encoded by PG0890) may play a role in various processes, including folate biosynthesis (e.g. phosphorylation of formate for formyl-THF synthesis), gamma-hexachlorocyclohexane degradation, and glycerolipid metabolism. *pepP* is part of a two-gene locus with PG0889, encoding alkaline peptidase (probable XAA_PO aminopeptidase), present upstream. The second phosphatase, phosphotyrosine protein phosphatase (encoded by PG1641), has recently been shown to play a role in the regulation of a variety of processes in *P. gingivalis* (Maeda *et al.*, 2008).

Finally, a gene, PG0350, encoding LnlJ, which interferes with heterotypic bacterial biofilm formation, was downregulated (Table 2) (Capestany *et al.*, 2006), suggesting elevated ability of the bacterium to bind streptococcal species when exposed to oxygen.

In summary, the upregulation of genes encoding adhesins and phosphatases, and the downregulation of protease-encoding genes and proteins interfering with adhesion in the presence of oxygen, were observed.

Small and hypothetical proteins

It is noteworthy that in the presence of oxygen drastic upregulation was detected of the expression of genes encoding small proteins. Such regulation indicates that these proteins play a role in bacterial growth in the presence of oxygen. The most drastically upregulated locus was that composed of PG2212 and PG2213 (Table 1). PG2212 is a 282 bp gene that encodes a 94 amino acid hypothetical protein lacking structural properties or similarities to other proteins. It is located downstream of PG2213, which encodes a small 60 amino acid protein predicted to be a putative nitrite reductase. The latter has

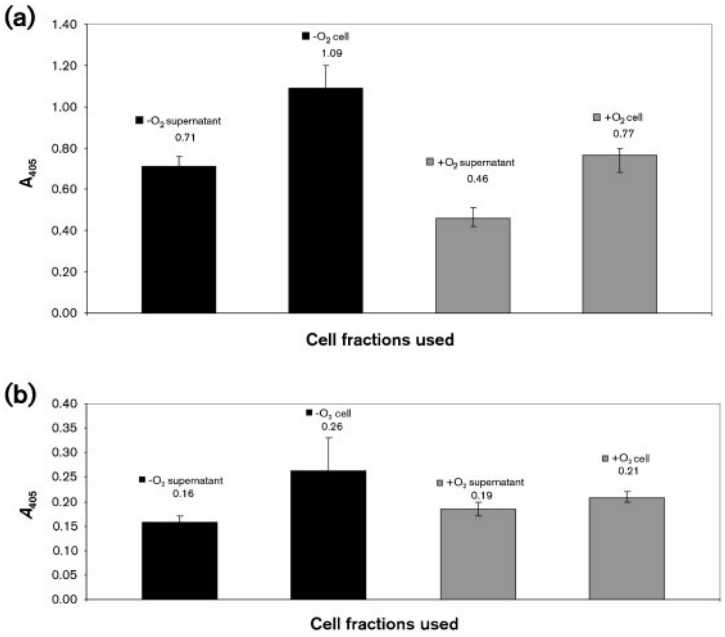

Fig. 5. Effect of oxygen on protease activity in *P. gingivalis* W83. Arg-X protease activity (a) and Lys-X protease activity (b) in culture supernatants (designated as 'supernatant') and in bacterial cells (designated as 'cell') were determined in *P. gingivalis* W83 cultures grown anaerobically ($-O_2$) and in the presence of 6 % oxygen ($+O_2$). A_{405} was used to measure protease activity (see Methods). The numbers above the bars indicate the mean value derived from an experiment performed in triplicate.

numerous hits to a small segment of the large subunit of nitrite reductase NirB (Wang & Gunsalus, 2000). Reductases are iron–sulfur proteins that have a role in oxidative stress protection. The function of this protein in *P. gingivalis* is not yet known; however, it may have redox or regulatory functions (or both). This is a very small protein compared with other reductase proteins, and is therefore quite unusual.

Large numbers of the regulated genes encoded hypothetical proteins (approximately 40 % of the total regulated genes) (Supplementary Tables S1 and S2). These data verify the presence of transcripts for those genes and suggest that the gene products play a role in the adaptation of *P. gingivalis* to oxygen.

Verification of microarray results by quantitative RT-PCR

The microarray results for several genes were verified by quantitative RT-PCR. As shown in Table 3, *ahpC*, *dps*, *reg*

Table 3. qRT-PCR analysis of expression of selected genes in *P. gingivalis* W83 in the mid-exponential phase of growth

Gene name/ORF*	Expression ratio†‡	Primers
hmuY/PG1551	0.45	5′-CCGACTATAAGAACGATTTGAACTG-3′
		5′-CTTACCACTTTCGCCACAATTG-3′
dps/PG0090	11.00	5′-CAGGGAAAATCGACGAGGTA-3′
		5′-GGAGAGGAAACCGATCATCA-3′
sod/PG1545	2.90	5′-CTTTGGTTCGGGCTGGGTAT-3′
		5′-ATTGGGTTCCTTCTCGATGGA-3′
ahpC/PG0618	32.00	5′-CTCACTCGCGATCTGGGTAT-3′
		5′-TGTGCAGCCTTGATCTTACG-3′
reg/PG1044	2.20	5′-TGCTGCACAAACAAGGCTAC-3′
		5′-TATCCGCTCCTGCTCTTCAT-3′
feoB2/PG1043	1.90	5′-ACCTCCGAGGATGAGGAGAT-3′
		5′-TCGTCCATTAGATTGGCACA-3′
feoB1/PG1294	0.80	5′-GCACCCTCGGTGTCATCTAT-3′
		5′-AACTGCCCACTTCCAATGTC-3′
16S	1.00	5′-AGGCGGAATTCGTGGTGTAG-3′
		5′-TTTGATACCCACGCCTTCGT-3′

*Designation based on the JCVI (TIGR) nomenclature.

†The expression ratio was calculated by dividing the quantity of transcript under aerobic conditions by that under anaerobic conditions.

‡The experiment was performed in triplicate; mean values were used to obtain the ratio.

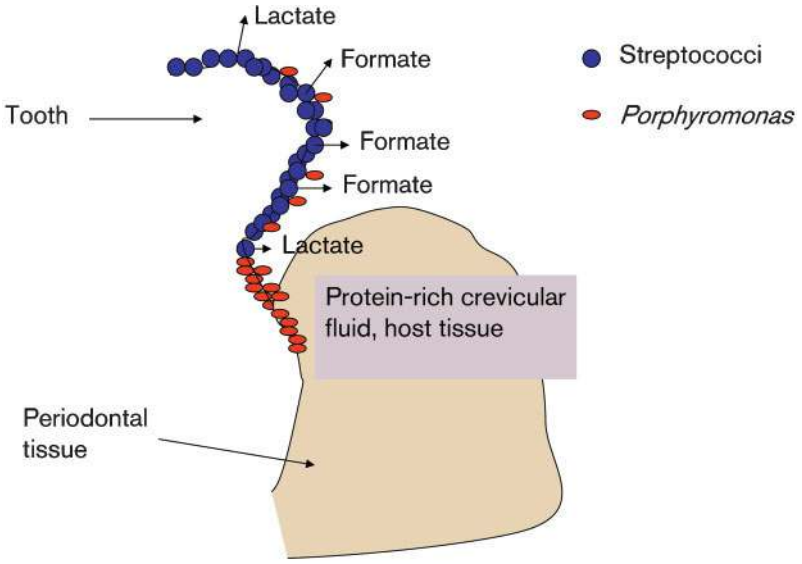

Fig. 6. Nutrient availability in supragingival and subgingival environments. Early colonizers such as streptococci produce formate and lactate, which is then available for late colonizers such as *P. gingivalis* that bind to supragingival surfaces. Once in subgingival pockets, proteins derived from crevicular fluid or host tissues are available to satisfy the nutritional needs of the bacterium.

and *feoB2* were upregulated, and *hmuY* and *feoB1* were downregulated. The similar profiles of expression of all genes examined to those observed in our microarray analysis experiments confirmed the changes in transcript abundance and validated our microarray data.

Conclusions

This work shows that *P. gingivalis* grows similarly in 6 % oxygen and under anaerobic conditions. This serendipitous finding allowed the comparison of the transcriptional profile of anaerobically grown bacteria with that of aerobically grown ones. As expected, the transcription of genes encoding oxidative stress protection and aerobic respiration mechanisms was upregulated in microaerophilic conditions. Some of this regulation was growth phase-dependent. However, for the first time, to our knowledge, the roles of formate and lactate metabolism were revealed. Such findings are novel and add significantly to known information regarding *P. gingivalis* metabolism, which has mainly focused on the utilization of amino acids. The fact that the organism can also utilize formate and lactate is of utmost importance, as these are products of *Streptococcus* metabolism. As streptococci are the early colonizers and set the stage for colonization by later inhabitants such as *Porphyromonas* species, nutritional interdependence would be expected to play a significant role. Also, at the time of initial colonization by *P. gingivalis*, when the organism first colonizes the supragingival plaque, proteins are less available and the organism may need to rely on other nutrients, for both anabolic and ATP-generating purposes, such as formate or lactate (Fig. 6). As it transits into the anaerobic periodontal pockets with protein-rich crevicular fluid, especially abundant in periodontally compromised patients, peptides and amino acids would be available to satisfy the nutritional needs of *P. gingivalis*. In agreement with the elevated availability of proteins in anaerobic subgingival pockets, we also observed elevated protease activity in bacteria grown in the absence of oxygen. Thus, an insight into forms of metabolism other than protein-based ones may provide targets for interventional strategies that are preventive in nature. It is noteworthy that in addition to genes encoding the expected proteins, the possible roles of multiple gene products yet to be implicated in metabolism or oxidative stress protection, such as novel small proteins and differentially regulated hypothetical proteins, have been elucidated. Finally, information regarding possible regulatory mechanisms governing the adaptation of the organism to aerobic conditions has been outlined. Although many questions still remain, altogether this work provides new insights into the complex metabolic capabilities of *P. gingivalis* that are dependent on the presence of oxygen, and postulates new hypotheses that are expected to stimulate future investigations.

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