Effect of alkaline growth pH on the expression of cell envelope proteins in *Fusobacterium nucleatum*

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Fusobacterium nucleatum is a Gram-negative anaerobic organism that plays a central role in the development of periodontal diseases. The progression of periodontitis is associated with a rise in pH of the gingival sulcus which promotes the growth and expression of virulence factors by periodontopathogenic bacteria. We have previously reported that the expression of specific cytoplasmic proteins is altered by a shift in growth pH. In the present study we have compared cell envelope protein expression of *F. nucleatum* during chemostat growth at pH 7.2 and 7.8. From a total of 176 proteins resolved from the cell envelope, 15 were found to have altered expression in response to an increase in growth pH and were identified by MS. Upregulated proteins included an outer membrane porin which has been identified as playing a role in virulence, a periplasmic chaperone which assists in the folding of outer membrane proteins, and a transporter thought to be involved with iron uptake. Proteins downregulated at pH 7.8 were consistent with our previous findings that the bacterium reduces its catabolism of energy-yielding substrates in favour of energy-storage pathways. Among the downregulated proteins, two transporters which are involved in the uptake of C4 dicarboxylates and phosphate were identified. A putative protease and an enzyme associated with the metabolism of glutamate were also identified. A high proportion of the cell envelope proteins suggested by these data to play a role in the organism's response to alkaline growth pH may have arisen by lateral gene transfer. This would support the hypothesis that genes that provide an ability to adapt to the changing conditions of the oral environment may be readily shared between oral bacteria.

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INTRODUCTION

Periodontal diseases are widespread and do not have a single causative microbial agent. Periodontitis is, however,

associated with the proliferation of the anaerobic pathogenic species that belong to the so-called 'red complex' (Socransky *et al.*, 1998). These organisms normally inhabit healthy subgingival sites within the oral cavity, but the allogenic factors that cause an increase in their proportions during the transition from health to disease are poorly understood. It is believed that, in the early stages, a combination of host factors and the presence of *Fusobacterium nucleatum* and *Prevotella intermedia* leads to the alkalization of the gingival sulcus which is conducive to the proliferation of more acid-sensitive (red complex) bacteria (Socransky & Haffajee, 2005; Takahashi, 2003). Resolving the magnitude of the pH increase in the gingival crevice has proved inconclusive, as Vroom *et al.* (1999), using pH-sensitive dyes, showed that plaque organisms in a biofilm create a 'mosaic of micro-

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Abbreviations: ACN, acetonitrile; CE, cell envelope; 2DE, 2D electrophoresis; ESI, electron spray ionization; FA, formic acid; HGR, habitat gene reservoir; HGT, horizontal gene transfer; IPG, immobilized pH gradient; TBP, tributyl phosphine.

A supplementary figure, showing estimated ribosome-binding strengths for gene FN0689 and its two flanking genes, and two supplementary tables, showing up- or downregulated proteins from the cell envelope of *Fusobacterium nucleatum* following chemostat growth at pH 7.8 relative to pH 7.2, and potential ecoparalogues in the *F. nucleatum* ATCC 25586 genome, are available with the online version of this paper.

environments' which have differing pH values. Gradients in the biofilm are not uniform and adjacent zones, with significantly different pHs, are detected. Bickel & Cimasoni (1985) showed that the pH in periodontal pockets may be as high as 9.0, but the interaction of reducing compounds with the metallic antimony pH electrodes used may have led to artefactual results (Eggert *et al.*, 1991). Using glass micro-electrodes, Eggert and co-workers reported that 7% of subjects with periodontal disease had sites measuring pH 7.6 or higher (Eggert *et al.*, 1991), and concluded that there was no correlation between increased pH and periodontal pockets compared with healthy sites. However, metabolism of proteins derived from gingival crevicular fluid by plaque inhabitants would be expected to promote the alkalization of the environment, a notion supported by the findings of Marsh *et al.* (1993) that the growth pH optimum of *Porphyromonas gingivalis* is pH 7.5 and that alkaline pH is also associated with an increased proteolytic activity of the organism.

As with many other oral bacteria of importance, the genome of *F. nucleatum* has been sequenced (Kapatral *et al.*, 2002, 2003; Karpathy *et al.*, 2007), and this has facilitated the investigation of the proteome in response to environmental changes. We have previously investigated the response of *F. nucleatum* to changes in external pH (Zilm *et al.*, 2007) and reported the regulation of cytoplasmic proteins associated with nutrient metabolism and energy storage. We have also demonstrated that, at a growth pH above 8.2, *F. nucleatum* co-adheres and forms homogeneous biofilms (Zilm & Rogers, 2007). These investigations suggest that the organism has a coordinated response to an alkaline environment.

An organism's regulation of cell envelope (CE) proteins in response to environmental stresses has been termed the extracytoplasmic stress response (ESR) (Rowley *et al.*, 2006). The ESR to environmental alkalization has, to our knowledge, not been investigated in *F. nucleatum* and could be considered the first step in understanding the signal transduction pathways which lead to changes in gene expression.

The sequencing of the genomes of oral bacteria has also led to the investigation of niche-specific genes which are the result of lateral transfer between species. These genes have been described as the habitat gene reservoir (HGR), and are retained if they increase the recipient's adaptation to the environment (Legault *et al.*, 2006). In contrast to other oral organisms, between 20 and 45% of *F. nucleatum* genes are reported to have been acquired by horizontal gene transfer (HGT), maybe as a result of its ability to aggregate with many plaque inhabitants (Mira *et al.*, 2004). The transcription and subsequent translation of genes into functional proteins, however, depends on the taxonomic 'distance' between donor and recipient and on the 'new' genomic context of the recipient, which may have a different set of promoters, ribosome-binding sites and regulatory regions (Mira, 2008; Sorek *et al.*, 2007). In terms

of functionality, it is therefore important to demonstrate protein expression of laterally transferred genes associated with the HGR.

Another challenge associated with the study of gene expression in response to a single change in environmental conditions has been to eliminate the influence of other confounding factors. In some of the early and most insightful studies on bacterial ecology in dental plaque, continuous culture was used in an attempt to simulate *in vivo* growth conditions (Ellwood & Hunter, 1976; Marsh *et al.*, 1993, 1994; McDermid *et al.*, 1988; McKee *et al.*, 1986; Rogers *et al.*, 1986, 1991, 1992; Tempest, 1969). The post-genomic era has seen the resurgence of this method for the culture of bacteria and its advantages for functional genomic studies have been reviewed (Hoskisson & Hobbs, 2005).

In the present study, we aim to provide an insight into how *F. nucleatum* copes with the alkalization of the gingival sulcus by examining the change in expression of CE proteins. We have also undertaken a genomic comparison of the genes found to be regulated by external pH to establish whether they also constitute part of the HGR shared by other plaque inhabitants.

METHODS

Materials and reagents. Amino acids, endonucleases and Bacterial Protease Inhibitor Cocktail were purchased from Sigma Aldrich, and DeStreak reagent from GE Healthcare. All SDS-PAGE and 2D electrophoresis (2DE) chemicals, solubilization buffer, immobilized pH gradient (IPG) strips, IEF equipment, densitometer and image analysis software (PD-Quest 7.2) used for proteomic analysis were purchased from Bio-Rad Laboratories. Brain heart infusion broth and anaerobic blood agar plates were purchased from Oxoid.

Micro-organism. *F. nucleatum* ATCC 10953, subspecies *polymorphum* (Dzink *et al.*, 1990), was maintained on anaerobic blood agar plates, and the preparation of starter cultures for chemostat inoculation has been described previously (Zilm *et al.*, 2007).

Growth media and culture conditions. The organism was grown under nitrogen limitation in a chemically defined growth medium (CDM) based on that of van der Hoeven *et al.* (1985). The concentrations of the energy-yielding amino acids glutamic acid (40 mM), lysine and histidine (10 mM each), and glucose (20 mM) were raised to increase growth yields. Thioglycollic acid (0.5 g l⁻¹) was added to maintain a low redox potential, as was Tween 80 (0.2 g l⁻¹) to aid cell dispersion.

F. nucleatum was grown anaerobically using a model C-30 BioFlo Chemostat (New Brunswick Scientific) with a culture volume of 365 ml, as described previously (Rogers *et al.*, 1991). The flow rate of the medium was set at 27.5 ml h⁻¹ ($D=0.075$ h⁻¹), giving an estimated generation time of 9.2 h, which is typical of natural ecosystems such as dental plaque (Hamilton *et al.*, 1979; Socransky *et al.*, 1977). Anaerobic growth conditions and sampling of the culture, under steady-state growth conditions, have been described previously (Zilm *et al.*, 2007). Growth pH was maintained at either 7.2 or 7.8 by the automatic addition of 2 M KOH.

Cell harvesting. After equilibration, cell culture samples were harvested over five consecutive days and pooled, as described previously (Zilm *et al.*, 2007).

CE sample preparation for proteomic analysis. Cell lysis in the presence of endonucleases (DNase and RNase) and protease inhibitor cocktail was performed as described previously (Zilm *et al.*, 2007). Disrupted cell suspensions were centrifuged (20 000 g, 30 min at 4 °C) to collect the CE. The CE fraction was then washed twice in sodium phosphate buffer (0.05 M, pH 7.5) and resuspended in an aliquot (1 ml) of either solubilization buffer [5 M electrophoresis grade urea, 2 M thiourea, 40 mM Tris, 2 % CHAPS, 2 % sulfobetaine 3-10 (SB 3-10), 2 mM tributyl phosphine (TBP), 0.2 % (w/v) ampholytes] or SDS gel buffer (Laemmli, 1970). Samples were left for 1 h at room temperature before carefully (to avoid foaming) aspirating the solution with a fine-gauge needle. Samples were then clarified by centrifugation (20 000 g, 60 min at 15 °C) to remove insoluble material and stored at -80 °C.

2DE. Protein quantification was performed using an RC DC protein assay kit (Bio-Rad Laboratories) in accordance with the manufacturer's instructions. IEF was performed on 11 cm pre-cast IPG strips with pH ranges of 4–7 and 7–10 using a Protean IEF cell. Briefly, 0.35 mg cell protein was cup-loaded (anode end) onto an 11 cm IPG strip which had been passively rehydrated for 20 h in 0.2 ml solubilization buffer containing DeStreak reagent (15 mg ml⁻¹) instead of TBP. IEF was run using a customized method aimed at optimizing protein separation. Briefly, the voltage was gradually increased using linear ramping to 4000 V over 7.5 h as follows: 150 V for 1 h, 300 V for 3 h, 600 V for 1.5 h, 1200 V for 1.5 h, followed by rapid ramping to 4000 and 8000 V over 1.5 and 1 h, respectively. Focusing occurred for 8000 V h with a 50 µA per strip current limit and the temperature was maintained at 20 °C. After 8000 V h had been achieved, the strips were maintained at 500 V until the IEF strips were removed and placed at -20 °C. Between three and six replicate IPG strips per sample were run concurrently. After IEF, proteins were separated in the second dimension, as previously described (Zilm *et al.*, 2007).

1D electrophoresis (SDS-PAGE). CE proteins solubilized in SDS buffer were boiled for 90 s and allowed to cool before being separated by SDS-PAGE using discontinuous gels (12 % T 3.3 % C resolving gel and 6 % stacking gel) (Laemmli, 1970).

Coomassie blue staining, and image acquisition and analysis. Gels were stained with Coomassie blue R-250, destained, and then scanned and analysed as described previously (Zilm *et al.*, 2007). Replicate groups, representing each growth pH in the matchset, contained a minimum of three gels. Analysis sets containing proteins that showed significant ($P < 0.01$ and $0.01 < P < 0.05$) quantitative changes were identified and the 'spots' excised from the gels for MS analysis and protein identification. Differences in the mean protein quantity between replicate groups were analysed for statistical significance using Student's *t* test.

MS and protein identification. Gel spots containing proteins were excised from the gel and digested with trypsin. Protein spots were excised from each gel and placed into 1.5 ml capped tubes (Eppendorf). The spots were destained and digested with 100 ng (10 ng µl⁻¹ in 5 mM ammonium bicarbonate) modified porcine trypsin (sequencing grade, Promega) per sample. Tryptic peptides were extracted from the gel pieces with 50 % acetonitrile (ACN), 0.3 % formic acid (FA) in water. The volumes of the final samples were reduced from ~120 to ~1 µl by vacuum centrifugation. The peptides were then diluted to ~5 µl with FA30 (seven parts 0.1 % FA, three parts ACN).

MALDI MS (MS and MS/MS). One microlitre of each digested protein sample was applied to a 600 µm AnchorChip (Bruker Daltonic) following the method of Zhang *et al.* (2005). MALDI-TOF mass spectra were acquired at random locations over the matrix

surface spot using a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonic) in reflectron mode and controlled by flexControl software (version 3, Bruker Daltonic). External calibration was performed using peptide standards (Bruker Daltonic). Between three and six of the most highly abundant sample ions (not trypsin or keratin) were selected for MALDI-TOF/TOF analysis, which was performed in LIFT mode using the same spot on the target.

Mass spectra data acquired by MS and MS/MS were analysed using flexAnalysis (version 3, Bruker Daltonic), and then exported to BioTools (version 3.1, Bruker Daltonic), and the MS and corresponding MS/MS data were combined and used to interrogate an in-house Mascot database search engine (version 2.2, Matrix Science; <http://www.matrixscience.com>) using the following parameters:

Taxonomy: Eubacteria

Database: NCBI non-redundant 20080214

Enzyme: trypsin

Fixed modifications: carbamidomethyl

Variable modifications: oxidation of Met

Mass tol MS: 50 p.p.m.

MS/MS tol: 0.5 Da

Missed cleavages: 1

Protein identification was based upon the MOWSE and probability scores generated by the software. Based on the combined MS/MS data, samples that returned a positive 'hit' were submitted independently to Mascot. Predicted molecular masses and isoelectric points of identified proteins were calculated using Compute pI/Mw from the Expert Protein Analysis System (http://au.expasy.org/tools/pi_tool.html).

Liquid chromatography-electron spray ionization (LC-ESI) MS.

Samples which did not give sufficient spectra using MALDI MS for accurate protein identification were further analysed using LC-ESI ion trap MS. Samples (2.5 µl) were diluted to 6 µl with 3 % ACN and 0.1 % FA, and 5 µl of each sample was introduced into an Agilent Protein ID Chip column assembly (40 nl trap column with 0.075 × 43 mm C-18 analytical column) housed in an Agilent HPLC-Chip Cube Interface which was connected to an HCTultra 3D ion-trap mass spectrometer (Bruker Daltonic). After the column had been equilibrated with 4 % ACN/0.1 % FA at a flow rate of 0.5 µl min⁻¹, the samples were eluted over 32 min with an ACN gradient (4–31 %).

Ionizable species ($300 < m/z < 1200$) were trapped, and one or two of the most intense ions eluted were fragmented by collision-induced dissociation. Peak detection of MS and MS/MS spectra was achieved using DataAnalysis software (version 3.4, Bruker Daltonic) and the data were imported into BioTools. MS/MS data were searched as described above, but with an MS mass tolerance and MS/MS tol. of 0.3 and 0.4 Da, respectively, and a peptide charge of 1+, 2+ and 3+.

GRAVY, PSORTb and BOMP analysis. Prediction of translated protein hydrophobicity was derived using the Grand Average of Hydrophobicity (GRAVY) (<http://www.expasy.org/tools/protparam.html>).

Prediction of bacterial protein subcellular localization (Gardy *et al.*, 2005) was derived from <http://www.psorth.org/psorth/index.html> using the Gram-negative option. Protein localization was based on scores out of 10.

Predicted β -barrel integral outer membrane proteins (BOMPs) (Berven *et al.*, 2004) were identified (<http://www.bioinfo.no/tools/bomp>) with an E-value of less than $1e^{-10}$.

Comparative genomic analysis. The presence of paralogous genes was determined by amino acid sequence similarity among all proteins in *F. nucleatum*, considering that two ORFs were part of a gene family if (i) their BLAST E-value was lower than 10^{-5} , (ii) the amino acid identity was higher than 30 %, and (iii) the sequence alignment was at least 60 % of the total protein length, following the method of Pushker *et al.* (2004).

Potential HGT events were detected by a combination of three methods based on sequence similarity, phylogenetic trees and DNA compositional features (Mira, 2008).

Sequence similarity. Protein sequences of *F. nucleatum* subsp. *nucleatum* ATCC 25586 were retrieved from <ftp://ftp.ncbi.nih.gov/genomes/Bacteria> and a BLASTP (Altschul *et al.*, 1997) search of each protein was performed against the non-redundant database. Top hits for each protein were recorded after filtering those with E-values $>10^{-5}$ and sequence identity and length lower than 30 and 50 %, respectively. All recorded hits were then assigned to one of eight categories as belonging to the Bacteroides, Firmicutes, α -, β -, γ -Proteobacteria, δ -, ϵ -Proteobacteria, Spirochaetes, other Bacteria, Archaea or Eukaryotes/no hit. Hits to Firmicutes (the group to which *Fusobacterium* appears to be most closely related) were refined by further BLASTP analysis between *F. nucleatum* and all sequenced bacteria available from this group. If the gene had a homologue in only one genus from all the available low-GC Gram-positive species, it was considered an HGT event from/to this group. If it was present in more than one genus it was considered vertically inherited and consistent with the ribosomal phylogeny.

Phylogenetic trees. For each *F. nucleatum* gene, the protein sequences of up to 50 best BLAST hits (E-value $<10^{-5}$) were retrieved. All sequences were then automatically aligned with CLUSTAL W (Thompson *et al.*, 1994) with default parameters. A neighbour-joining tree with 1000 bootstrap replicates was generated from the resulting alignment. Phylogenetic incongruence was detected in well-resolved trees (trees should have more than four hits), and bootstrap

values at the nodes chosen for a decision on taxonomic assignment should be >500 following the protocol of Mira *et al.* (2004).

DNA compositional features. Significant deviations in codon usage, amino acid composition and G + C content were considered evidence of potential HGT (Garcia-Vallve *et al.*, 2000). Significant differences in oligonucleotide use between a given region and the rest of the genome were detected using the OligoWeb resource (<http://insilico.ehu.es/oligoweb>) and also considered indicative of potential HGT.

RESULTS

Growth of *F. nucleatum* in a chemostat

We have previously reported changes in cellular yields and nutrient utilization by *F. nucleatum* when grown at pH 7.2 and 7.8 in a chemostat (Zilm *et al.*, 2007). Briefly, optimal cellular yields ($700 \text{ mg protein ml}^{-1}$) were obtained at pH 7.4, while growth at pH 7.2 and 7.8 resulted in decreased cellular yields of 10 and 13 %, respectively. Growth above pH 7.2 was also characterized by the consumption of all exogenous nutrients from which *F. nucleatum* derives energy (Rogers *et al.*, 1991).

2DE of CE proteins expressed by *F. nucleatum*

Representative 2DE and SDS-PAGE gels are shown in Fig. 1. The separation of proteins by SDS-PAGE (Fig. 1a) proved inadequate in resolving all proteins, and better resolution was achieved with 2DE (Fig. 1b, c).

Based on staining with Coomassie blue R250, a total of 176 protein 'spots' were used for 2DE analysis, and included

Fig. 1. Separation of envelope proteins in *F. nucleatum* grown by continuous culture at pH 7.2 in a chemostat and separated by (a) SDS-PAGE and (b, c) 2D-PAGE and stained with Coomassie blue R250. The horizontal axes represent the pH range used for IEF, pH 4–7 (b) and pH 7–10 (c), and the vertical axes represent molecular mass (kDa). 2D-PAGE gels are representative gels from each replicate group.

proteins within the isoelectric point (pI) range of 4–10 and a molecular mass of 10–80 kDa. *In silico* representation of the *F. nucleatum* proteome indicates that the majority of cellular proteins are within this range (Zilm *et al.*, 2007). From a total of ~2361 proteins (subsp. *polymorphum*), 272 proteins have been annotated as transporters and ~20 % of all genes (subsp. *nucleatum*) are translated into proteins associated with the CE (<http://www.oralgen.lanl.gov/>).

The degree of solubility of the CE fraction was also investigated. Of the total protein associated with the CE, 11.5 mg was initially solubilized in 2DE buffer as described above. Protein assay of the insoluble proteins, collected by centrifugation, revealed that ~78 % of the protein was solubilized (data not shown).

Proteins regulated by alkaline pH

Gels were arranged into two matchsets (pI 4–7 and 7–10) containing three to five replicate gels from each growth pH. Gels were normalized using the total density of all detected spots in the matchset gels. The correlation coefficient between all member gels ranged from 0.69 to 0.86. Proteins were considered to be regulated by pH if, after normalization, the average mean spot density between replicate groups of gels representing growth at pH 7.2 was significantly ($P < 0.01$ or $0.01 > P < 0.05$) different from those representing growth at pH 7.8 (Supplementary Table S1). Quantification was based on a synthetically derived, Gaussian image of the matchset member gels, and 16 proteins were identified as showing significant regulation (Table 1) in response to growth pH. Fig. 2 shows the relative position of each regulated protein, and spot quantities (shown in the margins) provide a qualitative assessment of the level of up- or downregulation and the standard error between each group.

Identification of regulated proteins

Regulated protein spots were excised from the 2DE gels and, following in-gel digestion, were initially identified by MALDI-TOF MS (Table 1). If MOWSE (molecular mass search) scores were below or close to the cut-off threshold, individual peptide ions were analysed using ion-trap MS. Protein identifications were also supported by comparing observed and predicted pIs and molecular masses. All proteins showed relatively small variations in molecular masses and pIs (Supplementary Table S1). Due to the sensitivity of the MS analysis, some spots did not give a single positive identification (Table 1), and two protein spots (spot pairs, SSP3202/3201) were identified as the same protein having two locations on the gel (Fig. 2). The relative proportion of proteins within spots containing more than one protein was determined by calculation of the Exponentially Modified Protein Abundance Index (emPAI) score (Supplementary Table S1).

Two programs (PSORTb and BOMP) were used to predict the location of proteins in the CE. Spot 3802 was the only

regulated protein that contained a single β -barrel structure (BOMP analysis), representative of proteins found in the outer membrane (data not shown). PSORTb analysis of regulated proteins predicted that six proteins (including 3802) were associated with the CE (Table 1). Of these, two proteins (5204 and 3301) were predicted to be located in the bacterial periplasm and one (3104) was predicted to be associated with the cytoplasmic membrane. The remaining four proteins (0202, 0203, 1102, 3002) were predicted to be associated with the CE, but PSORTb was unable to further resolve their location. The relative hydrophobicity of regulated proteins was determined by their GRAVY scores (Table 1). Two proteins (3101 and 3104) with positive GRAVY values were considered hydrophobic, while the remaining proteins had negative GRAVY values and were thus considered hydrophilic.

In response to an increase in growth pH to 7.8, five proteins (0202, 0203, 3101, 3202/3201 and 3802) were upregulated. Of these, spots 0202, 0203, 3101 and 3802 were upregulated more than threefold, and the remaining proteins showed a minimum twofold and maximum 10-fold downregulation in response to growth at pH 7.8 (Table 1).

Analysis of the *F. nucleatum* (subsp. *nucleatum*) annotated proteome shows ~20 % of proteins as 'hypothetical', with unknown functions (<http://www.oralgen.lanl.gov/>).

Table 1 shows that seven of the proteins (42 %) were identified as being 'hypothetical', but are now shown to be true proteins and associated with the organism's coordinated response to increasing external pH.

Comparative genomic analysis

The proteins shown to respond to alkaline growth pH were investigated further to determine whether the genome contained paralogous sequences (i.e. genes showing significant sequence similarity that could be involved in related functions) and whether they had been acquired by HGT (Table 2). Many trees were difficult to resolve because of a low phylogenetic signal or because the protein gave an insufficient number of hits in public databases. Among those that could be resolved, only FN1439 and FN1549 showed a phylogeny congruent with vertical inheritance, and several indicated clear transfers from phylogenetically unrelated species (Table 2). These genes also showed signs of HGT in their sequence composition, in agreement with previous work that indicates that CE genes are over-represented among potential HGT events in *F. nucleatum* (Mira *et al.*, 2004). Genes FN1252 and FN1856 appeared to be transferred to/from other oral species, despite the low number of oral bacteria currently sequenced.

The paralogues analysis showed that four of the 15 proteins under study had related ORFs in the genome. Some of them are proteins of unknown function, and their sequence similarity to the ones identified in the present work would therefore suggest that they are also located in the CE. It has

Table 1. Up- or downregulated proteins isolated from the CE of *F. nucleatum* following growth in a chemostat at pH 7.8 relative to pH 7.2

SSP*	Mean spot quantity ratio [†] pH 7.8/7.2	P [‡]	GRAVY score [§]	PSORTb location	NCBI¶ accession no.	Gene ID#	Protein identification**
0202	3.2	9.1×10^{-6}	-0.77	Non-cytoplasmic	148322602	FNP_0033	Hypothetical protein
0203	44.7	1.4×10^{-18}	-0.29	Non-cytoplasmic	148323088	FNP_0530	34 kDa membrane antigen precursor
1001	0.5	9.0×10^{-3}	-0.37	Unknown	148323877	FNP_1344	Hypothetical protein
1102	0.5	4.8×10^{-3}	-0.82	Non-cytoplasmic	148322846	FNP_0281	Hypothetical membrane-spanning protein
					148322503	FNP_2364	
2101	0.5	1.8×10^{-3}	-0.67	Cytoplasm	148322305	FNP_2161	Hypothetical protein
2505††	0.2	3.1×10^{-3}	-0.20	CE	148323554	FNP_1008	Hypothetical protein
3502	0.54	9.2×10^{-3}	-0.30	Periplasm	148323082	FNP_0524	Dicarboxylate:proton TRAP-T family tripartite ATP-independent transporter binding protein
3002††	0.3	3.4×10^{-4}	-0.86	Non-cytoplasmic	148322844	FNP_0279	Hypothetical protein
3101††	6.7	3.1×10^{-3}	0.014	Cytoplasm	148323151	FNP_0594	Hypothetical protein
3104††	0.5	3.1×10^{-3}	0.19	Cytoplasmic membrane	148323515	FNP_0969	Butyrate-acetoacetate CoA transferase subunit B
3202	1.9	8.1×10^{-4}	-0.68	Cytoplasm	148322857	FNP_0293	Peptidyl prolyl <i>cis-trans</i> isomerase
3201	2.23	1.5×10^{-3}	-0.68	Cytoplasm	148322857	FNP_0293	Peptidyl prolyl <i>cis-trans</i> isomerase
3301††	0.1	7.3×10^{-3}	-0.3	Periplasm	148323082	FNP_0524	Dicarboxylate:proton (H ⁺) TRAP-T family tripartite, ATP-independent transporter binding protein, C4 dicarboxylate binding protein
4302††	0.4	2.8×10^{-3}	-0.07	Cytoplasm	148322296	FNP_2151	Stomatin-like protein
5204	0.2	4.0×10^{-2}	-0.01	Periplasm	148323210	FNP_0657	Phosphonate ABC superfamily ATP binding cassette transporter binding protein
3802	3.2	4.1×10^{-2}	-0.44	Outer membrane	148322338	FNP_2196	OmpIP family outer membrane porin

*Standard SPot numbers assigned by PDQuest analysis software; refer to Fig. 2.

†Ratio of protein quantity (after normalization) for each protein identified from *F. nucleatum* grown at pH 7.8 compared with growth at pH 7.2 (mean of 4–5 replicates).

‡Significance level using Student's *t* test.

§Grand Average of Hydrophobicity score of the identified protein (<http://au.expasy.org/tools/protparam.html>).

||Predicted subcellular location of identified bacterial proteins assigned using PSORTb v2.0 (<http://www.psort.org/>). For CE, expected location includes periplasm, outer membrane and cytoplasmic membrane.

¶National Center for Biotechnology Information.

#Gene identification code from genome-sequencing project (<http://www.oralgen.lanl.gov/>).

**Protein name or description of function from annotations in the NCBI database.

††Proteins identified using MALDI-TOF/TOF. All others were identified using ESI ion trap MS.

recently been shown that paralogous proteins are in most cases very divergent, with only a few cases above 50 % amino acid identity (Sanchez-Perez *et al.*, 2008). These instances of highly similar paralogues have been related to pairs of proteins performing the same function under different environmental conditions. The presence of these so-called 'ecoparalogues' is more frequent in species undergoing severe environmental fluctuations and is more apparent among CE proteins (which are extracellularly exposed) (Sanchez-Perez *et al.*, 2008). It is therefore interesting that two potential pairs of ecoparalogues were found among the proteins under study (Table 2). Given that fluctuations in external pH could influence the functionality of CE proteins, a list of highly similar

paralogues in the *F. nucleatum* genome was identified. This shows that there are a significant number of potential ecoparalogues, that many of them display important differences in pI, and that they may be localized in the CE (Supplementary Table S2).

DISCUSSION

The recent genomic sequencing of *F. nucleatum* subsp. *polymorphum* (FNP) has confirmed the phenotypic heterogeneity seen amongst *F. nucleatum* subspecies and casts serious doubt on the validity of differentiating disease-related subspecies based on the electrophoretic migration

Fig. 2. Gaussian image of proteins expressed by *F. nucleatum* grown in a chemostat at pH 7.2 and 7.8 and separated by 2D-PAGE using a pI range of 4–7 (a) and 7–10 (b). Numbered spots represent proteins which were significantly regulated by culture pH. The level of expression of numbered spots is represented by quantity graphs shown in the left and right margins. The right-hand columns of each pair represent growth at pH 7.8 and the left-hand columns represent growth at pH 7.2. Results represent the mean of 3–5 replicates.

of two intracellular enzymes (glutamine dehydrogenase and 2-oxoglutarate reductase) (Gharbia & Shah, 1992; Karpathy *et al.*, 2007; Morris *et al.*, 1996). *F. nucleatum* subsp. *polymorphum* represents a separate phylogenetic branch which includes several human pathogens (Conrads, 2002; Gmur *et al.*, 2006; Goldstein *et al.*, 1995). At a species level, its metabolic versatility enables it to create and survive conditions conducive to the establishment of pathogenic obligate anaerobes (Diaz *et al.*, 2002). Increasing alkalinity of the gingival sulcus has been identified as one of the disease-associated conditions potentially initiated by *F. nucleatum* (Takahashi, 2003, 2005). In this study we have examined changes in the CE proteome of *F. nucleatum* subsp. *polymorphum* caused by a change in growth pH from 7.2 to 7.8.

One of the problems when dealing with the separation of CE proteins using conventional proteomic technologies is the relatively poor solubility of hydrophobic membrane proteins, the abundance of which is usually far less than that of hydrophilic cytoplasmic proteins found in whole-cell lysates. In dealing with these problems, we have partially purified the CE of *F. nucleatum* by centrifugation,

and the solubility of proteins has been optimized by using a solution containing multiple chaotropes and strong detergents. Unlike our previous study (Zilm *et al.*, 2007), in which we looked at the protein expression of cytoplasmic proteins within the pI range 4–7, we have currently achieved a greater proteome coverage by separating CE proteins within the pI range 4–10 (Zilm *et al.*, 2007).

Parenthetically, it is worth noting that two hydrophobic, pH-regulated proteins (positive GRAVY score, Table 1) were separated using our 2DE protocol. 1D SDS-PAGE has the advantage that most hydrophobic membrane proteins, with the exception of proteins containing many transmembrane (TMR) regions, solubilize in strong ionic detergents such as SDS (Cordwell, 2006). The disadvantage of SDS-PAGE is that the separation of complex mixtures is difficult when determining the level of expression of individual proteins (Fig. 1).

Nevertheless, the separation of complex mixtures of proteins by 2DE does not completely eliminate the potential for multiple proteins to be separated in the same

Table 2. Phylogenetic characteristics of genes regulated by alkalinity

SSP	NCBI accession no.	Gene ID	Phylogenetic tree*	DNA composition†	Paralogues (%)‡	<i>F. nucleatum</i> subsp. <i>polymorphum</i> homologue (%)‡	Comments
202	19704771	FN1439	Consistent with 16S–23S rDNA phylogeny	Normal	FN0228 (32)	148322602	Clear native gene
203	19704587	FN1252	Clear HGT from Proteobacteria	GC content different, excess of proline		148323088 (96)	HGR gene (transferred to many oral species)
1001	19704024	FN0689	Uncertain	GC3 different	FN0688	148323877 (90)	Paralogous genes are contiguous and may recombine
	66475006				FN0690 (48–51)		
3101	19704527	FN1192	No tree§	GC1 different		148323151 (94)	Could be not annotated in other genomes due to very short length. No BLAST hits to viral databases
2505	19705204	FN1899	No tree§	GC content different	FN1590 (90)	148323554 (93)	No BLAST hits to viral databases
3502							
2101	34762380	FNV2041	Present in Fn, Fv, Fp. Low similarity to Proteobacteria and one clostridium	Unusual codon usage and amino acid frequencies		148322305 (84)	Potential transfer
3002	19703594	FNO249	No tree§	Normal		148322844 (90)	
3104	19705161	FN1856	Base of Firmicutes. Top hit to <i>P. gingivalis</i>	GC1 different	FN0273 (53)	148323515 (<i>atoA</i> , 95)	Likely a native gene transferred towards <i>Porphyromonas</i>
3202	57117487	FN0263	Tree uncertain, but Fn within proteobacterial cluster	GC1 and GC2 different		148322857 (87)	Potential HGT
3201							Universally distributed protein
3301	19704593	FN1258	Likely HGT from Proteobacteria	Normal		148323082 (97)	
4302	19704881	FN1549	Consistent with 16S–23S rDNA phylogeny	Normal		148322296 (95)	Clear native gene
5204	19704470	FN1135	Ambiguous. At the base of Firmicutes and Proteobacteria	Normal		148323210 (96)	
3802	19705216	FN1911	No tree§	Normal		148322338 (95)	Many repeats belonging to different antigen families
1102	19703596	FN0251	Ambiguous. More similar to Proteobacteria but gene also present in Firmicutes	GC content different; excess of methionine and glutamic acid		148322846 (93)	

*Phylogenetic assignment based on a maximum-likelihood tree performed with the 50 BLAST top hits of each gene. Fn, Fv and Fp represent *Fusobacterium nucleatum*, *F. nucleatum* subsp. *vincentii* and *F. polymorphum*, respectively.

†Significantly different GC content, codon use or amino acid composition is indicated, following Garcia-Vallve *et al.* (2000).

‡Figures in parentheses represent the percentage amino acid similarity. Genes in bold type indicate potential ecoparalogues (proteins performing the same function optimally under different environmental conditions, such as differences in pH). Blank cells indicate that the gene is a singleton.

§No tree could be drawn because no hits were retrieved in the database outside the Fusobacteria.

'spot' (Campostrini *et al.*, 2005). In the present study, if more than one protein was identified in each spot, the relative abundance of each protein was determined by calculating the emPAI score (Exponentially Modified Protein Abundance Index) from the MS/MS data, and the assumption was made that the most abundant protein would account for the observed regulation (Ishihama *et al.*, 2005). Supplementary Table S1 shows the calculated emPAI scores for each identified protein. In some cases (spots 3502, 3201, 3301, 5204), identified proteins also matched homologues in *F. nucleatum* subsp. *vincentii* (FNV) and *nucleatum* (FNN) (Supplementary Table S1). Proteins identified as being regulated by increasing pH are shown in Table 1. With the exception of spot 0203, all regulated proteins matched best to the subspecies *polymorphum* used in this study. Spot 0203 was unique in that the peptides of the identified protein matched better to the homologue from subspecies *nucleatum*. The reason for this is unclear but may be a result of the heterogeneous nature of the *F. nucleatum* genome.

Proteins downregulated by increasing pH

A rise in pH from 7.2 to 7.8 was associated with the downregulation of two transport proteins. One of these, C4 dicarboxylate binding protein (3301), is an extracytoplasmic solute receptor (ECSR)-dependent uptake system which, unlike many prokaryote transport proteins, uses a proton motive force instead of ATP to drive solute uptake. Described as a tripartite ATP-independent periplasmic (TRAP-T) transporter for C4 dicarboxylates (e.g. malate, succinate and fumarate), the transporter has been well characterized in *Rhodobacter capsulatus* (Forward *et al.*, 1997), and consists of an ECSR and two integral membrane proteins. Homologues of *R. capsulatus* proteins have been found in many Gram-negative bacteria, and SSP 3301 represents the periplasmic component of the TRAP-T uptake system found in *F. nucleatum*. The 10-fold downregulation of this protein appears to be correlated with the rise in alkalinity of the environment, and the reduced uptake of C4 dicarboxylates may be related to the general decrease in ATP synthesis and fall in cell yield associated with growth at pH 7.8 (Zilm *et al.*, 2007). Hypothetically, the downregulation may also be associated with a reduced proton gradient, which may help the organism to maintain a neutral cytoplasmic pH during an increase in external pH.

The multiple-component uptake system for the transport of phosphonate (SSP 5204) was also downregulated at pH 7.8. Unlike TRAP-T transporters, this uptake system consists of a periplasmic binding protein, and the translocation of phosphonate across the inner membrane is coupled with ATP hydrolysis. Phosphonate uptake represents one of three ways in which prokaryotes acquire phosphorus, and the downregulation of the phosphonate-binding protein (PBP) is consistent with the decreased ATP synthesis associated with growth at high pH (Rogers *et al.*,

1991). The reduced uptake and dissociation of phosphonates may also contribute to the maintenance of a neutral periplasmic pH, despite an elevated external pH.

The reduced ability to acquire substrates required for ATP synthesis and growth at pH 7.8 was also reflected in the 2.5-fold downregulation of the stomatin-like protein (SSP 4302) (Table 1). Annotated as an integral membrane protease (<http://www.oralgen.lanl.gov/>), this protein represents one of the little-known proteins associated with the transport and degradation of proteins in *F. nucleatum* (Kapatral *et al.*, 2002). Its function and potential substrates are unknown.

A rise in pH also produced twofold downregulation of butyrate-acetoacetate CoA transferase (SSP 3104, Table 1), a metabolic enzyme associated with energy production from amino acids such as glutamate (Barker, 1981). The short-chain fatty acid thioesters butanoyl-CoA and acetoacetyl-CoA are the preferred substrates, and the products of the reaction (butyric acid and acetoacetate) cause acidification of the environment. The downregulation of this enzyme is consistent with the overall decrease in metabolic activity previously reported in *F. nucleatum* when grown at pH 7.8 (Zilm *et al.*, 2007). The decreased production of butyric acid would promote the alkalization of the environment caused by the fermentation of amino acids derived from proteins. Importantly, butyric acid production is considered one of several virulence factors in fusobacteria, since butyric acid may slow wound healing by causing mitotic arrest in human gingival fibroblasts (Bartold *et al.*, 1991).

Five proteins (SSP 1001, 1102, 2101, 2505, 3002) whose expression was downregulated in response to growth pH were represented in the *F. nucleatum* subsp. *polymorphum* genome but were identified as having no known function. With the exception of spot 2505, all of the hypothetical proteins had an observed molecular mass of 20 kDa or less, and their expression may play an important role in the organism's response to pH. The potential function and identification of these proteins will form the basis for future investigations. An interesting case is that of SSP 1001, because the gene could be an adhesin and is flanked by two paralogous ORFs of high similarity (Table 1), both of which lack ribosome-binding sites (Supplementary Fig. S1) and may therefore not be translated. This could be indicative of intragenomic recombination between the gene encoding SSP 1001 and its two flanking ORFs, as has been shown in cases of antigens undergoing combinatorial gene conversion among contiguous ORFs (Santoyo & Romero, 2005).

Proteins upregulated by increasing pH

Two hypothetical proteins (SSP 0202 and 3101) were upregulated in response to growth at pH 7.8. PSORTb analysis predicted that one protein (3101), which had a positive GRAVY score, was associated with the cytoplasm.

The cellular location of spot 0202 could not be predicted using the PSORTb algorithm, although the detection of a signal peptide suggests that the protein is transported across the cytoplasmic membrane.

The outer membrane protein (OMP) SSP 3802 (Table 1) was upregulated about threefold and belongs to the Omp IP family of porins. It has been identified as one of the 132 proteins that play a role in bacterial virulence (Karpathy *et al.*, 2007) and is a putative surface antigen containing variable number repeat (VNR) motifs (<http://www.oralgene.lanl.gov/>). Five of the 14 predicted outer membrane proteins identified in *F. nucleatum* subsp. *nucleatum* (Kapatral *et al.*, 2002) have molecular masses above 188 kDa and so homologues in subsp. *polymorphum* would not be resolved by the 2DE gels used in this study.

The approximately twofold upregulation of the predicted peptidyl prolyl *cis-trans* isomerase (PPI) (SSP 3202 and 3201, Table 1) may affect the expression of outer membrane proteins. Although PSORTb analysis predicted that the location of the protein was cytoplasmic, PPIs are a family of soluble proteins found in the periplasm of Gram-negative bacteria that act as chaperones for the folding of proteins such as outer membrane proteins and pilus components (Matsuzaki *et al.*, 2000).

In response to growth at pH 7.8, spot 0203 (Table 1) displayed the greatest upregulation (>44 fold) and is identified as a pathogen-specific membrane antigen (<http://www.oralgene.lanl.gov/>). As with many proteins associated with the *F. nucleatum* proteome, it is uncharacterized but is thought to be a high-affinity Fe^{2+} transport protein found in the CE. Iron is an essential element required for the growth of periodontal pathogens such as *P. gingivalis*. The onset of periodontal disease is associated with destruction of host tissues, and the subsequent bleeding releases iron in the form of haem and haem proteins (Ruby & Goldner, 2007). Perhaps the increased ability to sequester iron from the environment is in response to competition for iron caused by the proliferation of pathogenic species. We have also previously reported (Zilm *et al.*, 2007) the upregulation (71-fold) of cytoplasmic flavodoxin, which is usually upregulated in response to iron limitation. It appears, therefore, that proteins associated with iron metabolism and uptake form part of the organism's co-ordinated response to elevated pH.

Proteins acquired by HGT

The phylogenetic analysis shows that only two of the up- or downregulated proteins present a tree pattern that is congruent with the ribosomal phylogeny (Table 2), whereas using the same protocol over 50% of all CE proteins were found to be native. Some proteins are short and do not show significant sequence similarity hits, suggesting that they could be of phage origin (transferred by phage transduction), currently underrepresented in public databases. Between 30 and 50% of genes in Table 2

show some evidence for HGT, especially from Proteobacteria, and have significant hits to phylogenetically unrelated oral species such as *P. gingivalis* and *Treponema denticola*. Thus, we suggest that these HGR genes could be good targets for a vaccine, as their cognate proteins are extracellularly exposed, highly expressed and present in several pathogenic species of the subgingival dental plaque.

Conclusion

We have examined the proteomic changes associated with the CE when *F. nucleatum* is grown at physiological (pH 7.2) and elevated pH (7.8), thought to be consistent with the pH of the diseased gingival sulcus. The results presented allow gene expression to be compared with the vast amount of published physiological data and follow on from our previous examination of cytoplasmic proteins regulated by growth pH (Zilm *et al.*, 2007). Growth at pH 7.8 produced changes in CE proteins which were associated with decreased uptake of exogenous C4 dicarboxylates and phosphorus. Reduced metabolic activity was also seen in the downregulation of a membrane protease and an enzyme associated with amino acid fermentation. This overall decrease in cell metabolic activity was also consistent with the expression of cytoplasmic proteins during growth at pH 7.8 (Zilm *et al.*, 2007). Interestingly, the increased expression of an outer membrane porin at pH 7.8 was also associated with the upregulation of a periplasmic chaperone responsible for the correct folding of proteins as they transit the periplasm destined for the outer membrane. It should be emphasized that, although pH and other physicochemical properties may be controlled intracellularly, their effect will be important for the proteins exposed to the environment. Thus, it is interesting that many proteins predicted to be located in or on the outer membrane have paralogous genes of high sequence similarity (Supplementary Table S2) that may be performing the same function under different environmental conditions (for instance under pH fluctuations). Two of these proteins appear to be downregulated at pH 7.8 (Table 2), and further studies could indicate whether this is an extended phenomenon and whether their paralogous counterparts are overexpressed under the same circumstances.

Unfortunately, the annotation of sequenced genomes is not keeping pace with the rate at which prokaryote genomes are being sequenced. Indeed, this study has identified a number of hypothetical proteins which are represented in the *F. nucleatum* genome but currently have no known function. Thus, the potential role of these proteins in response to elevated growth pH remains to be resolved.

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