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(54) Title: IMMUNOLOGY TREATMENT FOR BIOFILMS

(57) Abstract: The invention provides a composition for use in raising an immune response to P. gingivalis in a subject, the composition comprising an amount effective to raise an immune response of at least one polypeptide having an amino acid sequence substantially identical to at least 50 amino acids, or an antigenic or immunogenic portion, of one of the polypeptides corresponding to accession numbers selected from the group consisting of AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117 and AAQ67118. The invention also provides a method of preventing or treating a subject for P. gingivalis infection comprising administering to the subject a composition of the invention

IMMUNOLOGY TREATMENT FOR BIOFILMS

Field of the invention

The present invention relates to compositions and methods for preventing or altering bacterial biofilm formation and/or development such as those containing *Porphyromonas gingivalis*. In particular the present invention relates to the use and inhibition of polypeptides which are regulated during growth as a biofilm or under haem-limitation, to modulate biofilm formation and/or development. The present invention relates to the identification of polypeptides which may be used as the basis for an antibacterial vaccine or an immunotherapeutic / immunoprophylactic.

Background of the invention

Many bacterial treatments are directed to bacteria in a planktonic state. However, bacterial pathologies include bacteria in a biofilm state. For example, *Porphyromonas gingivalis* is considered to be the major causative agent of chronic periodontal disease. Tissue damage associated with the disease is caused by a dysregulated host immune response to *P. gingivalis* growing as a part of a polymicrobial bacterial biofilm on the surface of the tooth. Bacterial biofilms are ubiquitous in nature and are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces (1). These sessile bacterial cells adhering to and growing on a surface as a mature biofilm are able to survive in hostile environments which can include the presence of antimicrobial agents, shear forces and nutrient deprivation.

The Centers for Disease Control and Prevention estimate that 65% of human bacterial infections involve biofilms. Biofilms often complicate treatment of chronic infections by protecting bacteria from the immune system, decreasing antibiotic efficacy and dispersing planktonic cells to distant sites that can aid reinfection (2,3). Dental plaque is a classic example of a bacterial biofilm where a high diversity of species form a heterogeneous polymicrobial biofilm growing on the surface of the tooth. The surface of the tooth is a unique microbial habitat as it is the only hard, permanent, non-shedding surface in the human body. This allows the accretion of a substantial bacterial biofilm over a lengthy time period as opposed to mucosal surfaces where epithelial cell shedding limits

development of the biofilm. Therefore, the changes to the *P. gingivalis* proteome that occur between the planktonic and biofilm states are important to our understanding of the progression of chronic periodontal disease.

P. gingivalis has been classified into two broad strain groups with strains including W50 and W83 being described as invasive in animal models whilst strains including 381 and ATCC 33277 are described as non-invasive (4,5). Griffen *et al.* (6) found that W83/W50-like strains were more associated with human periodontal disease than other *P. gingivalis* strains, including 381-like strains, whilst Cutler *et al.* (7) demonstrated that invasive strains of *P. gingivalis* were more resistant to phagocytosis than non-invasive strains. Comparison of the sequenced *P. gingivalis* W83 strain to the type strain ATCC 33277 indicated that 7% of genes were absent or highly divergent in strain 33277 indicating that there are considerable differences between the strains (8). Interestingly *P. gingivalis* strain W50 forms biofilms only poorly under most circumstances compared to strain 33277 which readily forms biofilms (9). As a consequence of this relatively few studies have been conducted on biofilm formation by *P. gingivalis* W50.

Quantitative proteomic studies have been employed to determine proteome changes of human bacterial pathogens such as *Pseudomonas aeruginosa, Escherichia coli and Streptococcus mutans* from the planktonic to biofilm state using 2D gel electrophoresis approaches, where protein ratios are calculated on the basis of gel staining intensity (10-12). An alternative is to use stable isotope labelling techniques such as ICAT, iTRAQ or heavy water ($H_2{}^{18}O$) with MS quantification (13). The basis for $H_2{}^{18}O$ labelling is that during protein hydrolysis endopeptidases such as trypsin have been demonstrated to incorporate two ^{18}O atoms into the C-termini of the resulting peptides (14,15). In addition to use in the determination of relative protein abundances (16-19), ^{18}O labelling in proteomics has also been used for the identification of the protein C-terminus, identification of N-linked glycosylation after enzymatic removal of the glycan, simplification of MS/MS data interpretation and more recently for validation of phosphorylation sites (20-23). The $^{16}O/^{18}O$ proteolytic labelling method for measuring relative protein abundance involves digesting one sample in $H_2{}^{16}O$ and the other sample in $H_2{}^{18}O$. The digests are then combined prior to analysis by LC MS/MS. Peptides eluting from the LC column can be quantified by measuring the relative signal

intensities of the peptide ion pairs in the MS mode. The incorporation of two ^{18}O atoms into the C-terminus of digested peptides by trypsin results in a mass shift of +4 m/z allowing the identification of the isotope pairs.

Due to the complexity of the proteome, prefractionation steps are advantageous for increasing the number of peptide and protein identifications. Most prefractionation steps involve a 2D LC approach at the peptide level after in-solution digestion (24,25). However due to potential sample loss during the initial dehydration steps of the protein solution, SDS PAGE prefractionation at the protein level followed by $^{16}O/^{18}O$ labelling during in gel digestion has also been carried out successfully, (26-29). The $^{16}O/^{18}O$ proteolytic labelling is a highly specific and versatile methodology but few validation studies on a large scale have been performed (30). An excellent validation study was carried out by Qian *et al* (18) who labelled two similar aliquots of serum proteins in a 1:1 ratio and obtained an average ratio of 1.02 ± 0.23 from 891 peptides. A more recent study by Lane *et al* (26) further demonstrated the feasibility of the $^{16}O/^{18}O$ method using a reverse labelling strategy to determine the relative abundance of 17 cytochrome P450 proteins between control and cytochrome P450 inducers treated mice that are grafted with human tumours.

Summary of the invention

This invention is illustrated by reference to a sample system whereby *P. gingivalis* W50 is grown in continuous culture and a mature biofilm developed on the vertical surfaces in the chemostat vessel over an extended period of time. The final biofilm is similar to that which would be seen under conditions of disease progression, thus allowing a direct comparison between biofilm and planktonic cells. $^{16}O/^{18}O$ proteolytic labelling using a reverse labelling strategy was carried out after SDS-PAGE prefractionation of the *P. gingivalis* cell envelope fraction followed by coupling to off-line LC MALDI TOF-MS/MS for identification and quantification. Of the 116 proteins identified, 81 were consistently found in two independent continuous culture studies. 47 proteins with a variety of functions were found to consistently increase or decrease in abundance in the biofilm cells providing potential targets for biofilm control strategies. Of these 47 proteins

the present inventors have selected 24 proteins which they believe are particular useful as targets in treatment and/or prevention of *P. gingivalis* infection.

Accordingly, the present invention is directed in a first aspect towards a polypeptide which modulates biofilm formation. In one form, the microorganisms in the biofilm are bacteria. In one form, the bacteriais from the genus *Porphyromonas.* In one embodiment, the bacteria is *P. gingivalis* and the polypeptide has an amino acid sequence selected from the group consisting of the sequences corresponding to the accession numbers listed in Table 4. The invention extends to sequences at least 80% identical thereto, preferably 85% , 90%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The invention also includes a polypeptide corresponding to accession number AAQ65742 (version 0.1) and a polypeptide at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical hereto.

Preferably, the polypeptide is at least 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of any one of the sequences corresponding to the accession numbers listed in Table 4.

One aspect of the invention is a composition for use in raising an immune response directed against *P. gingivalis* in a subject, the composition comprising an effective amount of at least one polypeptide of the first aspect of the invention or an antigenic or immunogenic portion thereof. The composition may optionally include an adjuvant and a pharmaceutically acceptable carrier. Thus, the composition may contain an antigenic portion of such a polypeptide instead of the full length polypeptide. Typically, the portion will be substantially identical to at least 10, more usually 20 or 50 amino acids of a polypeptide corresponding to the sequences listed in Table 4 and generate an immunological response. In a preferred form, the composition is a vaccine.

The invention also provides a composition that raises an immune response to *P. gingivalis* in a subject, the composition comprising an amount effective to raise an immune response of at least one antigenic or immunogenic portion of a polypeptide corresponding to accession numbers selected from the group consisting of AAQ65462,

AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117 and AAQ67118.

In another embodiment, there is provided a composition for use in raising an immune response directed against *P. gingivalis* in a subject, the composition comprising an effective amount of at least one polypeptide corresponding to an accession number selected from the group consisting of AAQ65462, AAQ66991, AAQ65561 and AAQ66831.

In another embodiment, there is provided a composition for use in raising an immune response directed against *P. gingivalis* in a subject, the composition comprising an effective amount of a polypeptide corresponding to accession number AAQ65742.

In another embodiment, there is provided a composition for use in raising an immune response to *P. gingivalis* in a subject, the composition comprising amount effective to raise an immune response of at least one polypeptide having an amino acid sequence substantially identical to at least 50 amino acids of a polypeptide expressed by *P. gingivalis* and that is predicted by the CELLO program to be extracellular.

In another embodiment, there is provided a composition for use in raising an immune response to *P. gingivalis* in a subject, the composition comprising an amount effective to raise an immune response of at least one polypeptide having an amino acid sequence selected substantially identical to at least 50 amino acids of a polypeptide that causes an immune response in a mouse or a rabbit.

In one embodiment, there is provided an isolated antigenic polypeptide comprising an amino acid sequence comprising at least 50, 60, 70, 80, 90 or 100 amino acids substantially identical to a contiguous amino acid sequence of one of the sequences corresponding to the accession numbers listed in Table 4. The polypeptide may be purified or recombinant.

In another embodiment there is a composition for the treatment of periodontal disease comprising as an active ingredient an effective amount of at least one polypeptide of the first aspect of the invention.

In another embodiment there is a composition for the treatment of *P. gingivalis* infection comprising as an active ingredient an effective amount of at least one polypeptide of the first aspect of the invention.

Another aspect of the invention is a method of preventing or treating a subject for periodontal disease comprising administering to the subject a composition according to the present invention as described above.

Another aspect of the invention is a method of preventing or treating a subject for *P. gingivalis* infection comprising administering to the subject a composition according to the present invention as described above.

In another aspect of the invention there is a use of a polypeptide of the invention in the manufacture of a medicament for the treatment of *P. gingivalis* infection.

In another aspect of the invention there is a use of a polypeptide of the invention in the manufacture of a medicament for the treatment of periodontal disease.

The invention also extends to an antibody raised against a polypeptide of the first aspect of the present invention. Preferably, the antibody is specifically directed against one of the polypeptides corresponding to the accession numbers listed in Table 4.The antibody may be raised using the composition for raising an immune response described above.

In one embodiment, there is provided an antibody raised against a polypeptide wherein the polypeptide corresponds to an accession number selected from the group consisting of AAQ65462, AAQ66991, AAQ65561 and AAQ66831.

In one embodiment, there is provided an antibody raised against a polypeptide wherein the polypeptide corresponds to accession number AAQ65742.

Another aspect of the invention is a composition useful in the prevention or treatment of periodontal disease, the composition comprising an antagonist or combination of antagonists of a *P. gingivalis* polypeptide of the first aspect of the present invention and a pharmaceutically acceptable carrier, wherein the antagonist(s) inhibits *P. gingivalis* infection. The antagonist(s) may be an antibody. The invention also includes use of an antagonist or combination of antagonists in the manufacture of a medicament useful for preventing or treating periodontal disease.

In a further aspect of the present invention there is provided an interfering RNA molecule, the molecule comprising a double stranded region of at least 19 base pairs in each strand wherein one of the strands of the double stranded region is substantially complementary to a region of a polynucleotide encoding a polypeptide which modulates biofilm formation as described above. In one embodiment, one of the strands is complementary to a region of polynucleotide encoding a polypeptide transcript of the sequences listed in Table 4.

Brief description of the drawings

Figure 1: $^{16}O/^{18}O$ quantification of specific BSA ratios. Quantification of known amounts of BSA was carried out in the same manner as for the biofilm and planktonic samples reported in the experimental procedures to validate the methodology. Briefly pre-determined amounts of BSA were loaded in adjacent lanes of a NuPAGE gel followed by excision of bands of equal size, normal or reverse proteolytic labelling, nanoHPLC and MALDI TOF-MS/MS. (A) MS spectra of BSA tryptic peptide, RHPEYAVSVLLR at known ^{16}O:^{18}O labelling ratios 1:1 (i), 2:1 (ii), 1:5 (iii) and 10:1 (iv) showing the characteristic doublet isotopic envelope for ^{16}O and ^{18}O labelled peptide (S0, S2 and S4 are the measured intensities of the isotopic peaks) (B) SDS PAGE gel of known BSA ratios used for the quantification procedure.

Figure 2: Typical forward and reverse MS and MS/MS spectra from *P. gingivalis* sample. (i,ii) Zoomed portion of mass spectra showing the [M+H]+ parent precursor ion of the normal and reverse labelled peptide GNLQALVGR belonging to PG2082 and

showing the typical 4Da mass difference in a 1:1 ratio (iii,iv) mass spectrum showing the [M+H]+ parent precursor ion of the normal and reverse labelled peptide YNANNVDLNR belonging to PG0232 and showing the typical 4Da mass difference in a 2:1 ratio (v, vi) MS/MS spectrum of heavy labelled (+2 18O) YNANNVDLNR and unlabelled YNANNVDLNR peptide characterized by the 4 Da shift of all Y ions.

Figure 3: Correlation of normal/reverse labelled technical replicates. Log10 transformed scatter plot comparison of peptide abundance ratio of the normal (Bio18, Plank16) and reverse (Plank18, Bio16) labelling for both biological replicates. The abundance ratios of the reverse labelled peptides have been inversed for a direct comparison. (A) Biological replicate 1 (B) Biological replicate 2

Figure 4: Distribution and correlation of protein abundances of biological replicates. (A) Normalized average fold change for the 81 quantifiable proteins identified in both biological replicates displayed a Gaussian-like distribution. The abundance ratio of each protein was further normalized to zero (R – 1) and ratios smaller than 1 were inverted and calculated as (1 – (1/R)) (18). All 81 quantifiable proteins from each biological replicate were sorted by increasing ratios (Biofilm/Planktonic) and divided equally into six groups with equal number of proteins (A-F). Groups C and D represents proteins not significantly regulated (< 3 SD from 1.0). (B) Distribution of proteins based on rankings. Insert: ranking table for the determination of similarity between both biological replicates. Proteins were ranked in descending order with 1 having the highest similarity when both biological replicates fell within the same group and 6 having the least similarity.

Figure 5: Breakdown of the 116 proteins identified in this study based on identification in one or both biological replicates and number of unique peptides identified. The proteins identified from both biological replicates (81) are presented in table 2. Legend shows number of unique peptides identified per protein.

Detailed description of the embodiments

The invention provides method for treating a subject including prophylactic treatment for periodontal disease. Periodontal diseases range from simple gum inflammation to serious disease that results in major damage to the soft tissue and bone that support the teeth. Periodontal disease includes gingivitis and periodontitis. An accumulation of oral bacteria at the gingival margin causes inflammation of the gums that is called 'gingivitis.' In gingivitis, the gums become red, swollen and can bleed easily. When gingivitis is not treated, it can advance to 'periodontitis' (which means 'inflammation around the tooth.'). In periodontitis, gums pull away from the teeth and form 'pockets' that are infected. Periodontitis has a specific bacterial aetiology with *P. gingivalis* regarded as the major aetiological agent The body's immune system fights the bacteria as the plaque spreads and grows below the gum line. If not treated, the bones, gums, and connective tissue that support the teeth are destroyed. The teeth may eventually become loose and have to be removed.

Using proteomic a strategy the present inventors identified and quantified the changes in abundance of 116 *P. gingivalis* cell envelope proteins between the biofilm and planktonic states, with the majority of proteins identified by multiple peptide hits. . The present inventors demonstrated enhanced expression of a large group of cell-surface located C-Terminal Domain family proteins including RgpA, HagA, CPG70 and PG99. Other proteins that exhibited significant changes in abundance included transport related proteins (HmuY and IhtB), metabolic enzymes (FrdA and FrdB), immunogenic proteins and numerous proteins with as yet unknown functions.

As will be well understood by those skilled in the art alterations may be made to the amino acid sequences of the polypeptides that have been identified as having a change in abundance between biofilm and planktonic states. These alterations may be deletions, insertions, or substitutions of amino acid residues. The altered polypeptides can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by site-directed mutagenesis on the encoding DNA). It is intended that such altered polypeptides which have at least 85%, preferably at least 90%, 95%, 96%, 97%, 98% or 99% identity with the sequences set out in the Sequence

Listing are within the scope of the present invention. Antibodies raised against these altered polypeptides will also bind to the polypeptides having one of the sequences to which the accession numbers listed in Table 4 relate.

Whilst the concept of conservative substitution is well understood by the person skilled in the art, for the sake of clarity conservative substitutions are those set out below.

Gly, Ala, Val, Ile, Leu, Met;

Asp, Glu, Ser;

Asn, Gln;

Ser, Thr;

Lys, Arg, His;

Phe, Tyr, Trp, His; and

Pro, Nα-alkalamino acids.

The practice of the invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, and immunology well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T. A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D. M. Glover and B. D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F. M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present). The disclosure of these texts are incorporated herein by reference.

An 'isolated polypeptide' as used herein refers to a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it naturally occurs or the polypeptide or peptide may be synthetically synthesised. Preferably, the polypeptide is

also separated from substances, for example, antibodies or gel matrix, for example, polyacrylamide, which are used to purify it. Preferably, the polypeptide constitutes at least 10%, 20%, 50%, 70%, and 80% of dry weight of the purified preparation. Preferably, the preparation contains a sufficient amount of polypeptide to allow for protein sequencing (ie at least 1,10, or 100 mg).

The isolated polypeptides described herein may be purified by standard techniques, such as column chromatography (using various matrices which interact with the protein products, such as ion exchange matrices, hydrophobic matrices and the like), affinity chromatography utilizing antibodies specific for the protein or other ligands which bind to the protein.

The terms 'peptides, proteins, and polypeptides' are used interchangeably herein. The polypeptides of the present invention can include recombinant polypeptides including fusion polypeptides. Methods for the production of a fusion polypeptide are known to those skilled in the art.

An 'antigenic polypeptide' used herein is a moiety, such as a polypeptide, analog or fragment thereof, that is capable of binding to a specific antibody with sufficiently high affinity to form a detectable antigen-antibody complex. Preferably, the antigenic polypeptide comprises an immunogenic component that is capable of eliciting a humoral and/or cellular immune response in a host animal.

In comparing polypeptide sequences, 'substantially identical' means 95% or more identical over its length or identical over any 10 contiguous amino acids.

A 'contiguous amino acid sequence 'as used herein refers to a continuous stretch of amino acids.

A 'recombinant polypeptide' is a polypeptide produced by a process that involves the use of recombinant DNA technology.

A reference to 'preventing' periodontal disease means inhibiting development of the disease condition, but not necessarily permanent and complete prevention of the disease.

In determining whether or not two amino acid sequences fall within a specified percentage limit, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignments of sequences. In such comparisons or alignments, differences will arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity or similarity between two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. For example, amino acid sequence identities or similarities may be calculated using the GAP programme and/or aligned using the PILEUP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux et al et al., 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar residues and to minimise the number and length of sequence gaps in the alignment. Alternatively or in addition, wherein more than two amino acid sequences are being compared, the Clustal W programme of Thompson et al, (1994) is used.

The present invention also provides a vaccine composition for use in raising an immune response directed against *P. gingivalis* in a subject, the composition comprising an immunogenically effective amount of at least one polypeptide of the first aspect of the invention and a pharmaceutically acceptable carrier.

The vaccine composition of the present invention preferably comprises an antigenic polypeptide that comprises at least one antigen that can be used to confer a protective response against *P. gingivalis*. The subject treated by the method of the invention may be selected from, but is not limited to, the group consisting of humans, sheep, cattle, horses, bovine, pigs, poultry, dogs and cats. Preferably, the subject is a human. An immune response directed against *P. gingivalis* is achieved in a subject, when there is

development in the host of a cellular and/or antibody-mediated response against the specific antigenic polypeptides, whether or not that response is fully protective.

The vaccine composition is preferably administered to a subject to induce immunity to *P. gingivalis* and thereby prevent, inhibit or reduce the severity of periodontal disease. The vaccine composition may also be administered to a subject to treat periodontal disease wherein the periodontal disease is caused, at least in part, by *P. gingivalis*. The term 'effective amount' as used herein means a dose sufficient to elicit an immune response against *P. gingivalis*. This will vary depending on the subject and the level of *P. gingivalis* infection and ultimately will be decided by the attending scientist, physician or veterinarian.

The composition of the present invention comprises a suitable pharmaceutically-acceptable carrier, such as a diluent and/ or adjuvant suitable for administration to a human or animal subject. Compositions to raise immune responses preferably comprise a suitable adjuvant for delivery orally by nasal spray, or by injection to produce a specific immune response against *P. gingivalis*. A composition of the present invention can also be based upon a recombinant nucleic acid sequence encoding an antigenic polypeptide of the present invention, wherein the nucleic acid sequence is incorporated into an appropriate vector and expressed in a suitable transformed host (eg. *E. coli, Bacillus subtilis, Saccharomyces cerevisiae*, COS cells, CHO cells and HeLa cells) containing the vector. The composition can be produced using recombinant DNA methods as illustrated herein, or can be synthesized chemically from the amino acid sequence described in the present invention. Additionally, according to the present invention, the antigenic polypeptides may be used to generate *P. gingivalis* antisera useful for passive immunization against periodontal disease and infections caused by *P. gingivalis*.

Various adjuvants known to those skilled in the art are commonly used in conjunction with vaccine formulations and formulations for raising an immune response. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete

Freunds adjuvant (IFA), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminium monostrearate), oil emulsions, Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels such as aluminium salts. Other examples include oil in water emulsions such as SAF-1, SAF-0, MF59, Seppic ISA720, and other particulate adjuvants such as ISCOMs and ISCOM matrix. An extensive but exhaustive list of other examples of adjuvants are listed in Cox and Coulter 1992 [In: Wong WK (ed.) *Animals parasite control utilising technology*. Bocca Raton; CRC press et al., 1992; 49-112]. In addition to the adjuvant the vaccine may include conventional pharmaceutically acceptable carriers, excipients, fillers, buffers or diluents as appropriate. One or more doses of a composition containing adjuvant may be administered prophylactically to prevent periodontal disease or therapeutically to treat already present periodontal disease.

In another preferred composition the preparation is combined with a mucosal adjuvant and administered via the oral or nasal route. Examples of mucosal adjuvants are cholera toxin and heat labile *E. coli* toxin, the non-toxic B sub-units of these toxins, genetic mutants of these toxins which have reduced toxicity. Other methods which may be utilised to deliver the antigenic polypeptides orally or nasally include incorporation of the polypeptides into particles of biodegradable polymers (such as acrylates or polyesters) by micro-encapsulation to aid uptake of the microspheres from the gastrointestinal tract or nasal cavity and to protect degradation of the proteins. Liposomes, ISCOMs, hydrogels are examples of other potential methods which may be further enhanced by the incorporation of targeting molecules such as LTB, CTB or lectins (mannan, chitin, and chitosan) for delivery of the antigenic polypeptides to the mucosal immune system. In addition to the composition and the mucosal adjuvant or delivery system the composition may include conventional pharmaceutically acceptable carriers, excipients, fillers, coatings, dispersion media, antibacterial and antifungal agents, buffers or diluents as appropriate.

Another mode of this embodiment provides for either a live recombinant viral vaccine, recombinant bacterial vaccine, recombinant attenuated bacterial vaccine, or an inactivated recombinant viral vaccine which is used to protect against infections caused by *P. gingivalis*. Vaccinia virus is the best known example, in the art, of an infectious

virus that is engineered to express vaccine antigens derived from other organisms. The recombinant live vaccinia virus, which is attenuated or otherwise treated so that it does not caused disease by itself, is used to immunise the host. Subsequent replication of the recombinant virus within the host provides a continual stimulation of the immune system with the vaccine antigens such as the antigenic polypeptides, thereby providing long lasting immunity. In this context and below, 'vaccine' is not limited to compositions that raise a protective response but includes compositions raising any immune response.

Other live vaccine vectors include: adenovirus, cytomegalovirus, and preferably the poxviruses such as vaccinia (Paoletti and Panicali, U.S. Patent No. 4,603,112) and attenuated *salmonella* strains (Stocker *et al.*, U.S. Patent Nos. 5,210.035; 4,837,151; and 4,735,801; and Curtis *et al.* et al., 1988, Vaccine 6: 155-160). Live vaccines are particularly advantageous because they continually stimulate the immune system which can confer substantially long-lasting immunity. When the immune response is protective against subsequent *P. gingivalis* infection, the live vaccine itself may be used in a protective vaccine against *P. gingivalis.* In particular, the live vaccine can be based on a bacterium that is a commensal inhabitant of the oral cavity. This bacterium can be transformed with a vector carrying a recombinant inactivated polypeptide and then used to colonise the oral cavity, in particular the oral mucosa. Once colonised the oral mucosa, the expression of the recombinant protein will stimulate the mucosal associated lymphoid tissue to produce neutralising antibodies. For example, using molecular biological techniques the genes encoding the polypeptides may be inserted into the vaccinia virus genomic DNA at a site which allows for expression of epitopes but does not negatively affect the growth or replication of the vaccinia virus vector. The resultant recombinant virus can be used as the immunogen in a vaccine formulation. The same methods can be used to construct an inactivated recombinant viral vaccine formulation except the recombinant virus is inactivated, such as by chemical means known in the art, prior to use as an immunogen and without substantially affecting the immunogenicity of the expressed immunogen.

As an alternative to active immunisation, immunisation may be passive, i.e. immunisation comprising administration of purified immunoglobulin containing an antibody against a polypeptide of the present invention.

The antigenic polypeptides used in the methods and compositions of the present invention may be combined with suitable excipients, such as emulsifiers, surfactants, stabilisers, dyes, penetration enhancers, anti-oxidants, water, salt solutions, alcohols, polyethylene glycols, gelatine, lactose, magnesium sterate and silicic acid. The antigenic polypeptides are preferably formulated as a sterile aqueous solution. The vaccine compositions of the present invention may be used to complement existing treatments for periodontal disease.

The invention also provides a method of preventing or treating a subject for periodontal disease comprising administering to the subject a vaccine composition according to the present invention. Also provided is an antibody raised against a polypeptide of the first aspect of the present invention. Preferably, the antibody is specifically directed against the polypeptides of the present invention.

In the present specification the term 'antibody' is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), chimeric antibodies, diabodies, triabodies and antibody fragments. The antibodies of the present invention are preferably able to specifically bind to an antigenic polypeptide as hereinbefore described without cross-reacting with antigens of other polypeptides.

The term 'binds specifically to' as used herein, is intended to refer to the binding of an antigen by an immunoglobulin variable region of an antibody with a dissociation constant (Kd) of 1μM or lower as measured by surface plasmon resonance analysis using, for example a BIAcore™ surface plasmon resonance system and BIAcore™ kinetic evaluation software (eg. version 2.1). The affinity or dissociation constant (Kd) for a specific binding interaction is preferably about 500 nM to about 50 pM, more preferably about 500 nM or lower, more preferably about 300 nM or lower and preferably at least about 300 nM to about 50 pM, about 200 nM to about 50 pM, and

more preferably at least about 100 nM to about 50 pM, about 75 nM to about 50 pM, about 10 nM to about 50 pM.

It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full length antibody. Examples of binding fragments of an antibody include (I) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody; (v) a dAb fragment which consists of a VH domain, or a VL domain ; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv). Other forms of single chain antibodies, such as diabodies or triabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites.

Various procedures known in the art may also be used for the production of the monoclonal and polyclonal antibodies as well as various recombinant and synthetic antibodies which can bind to the antigenic polypeptides of the present invention. In addition, those skilled in the art would be familiar with various adjuvants that can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freud's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*. Antibodies and antibody fragments may be produced in large amounts by standard techniques (eg in either tissue culture or serum free using a fermenter) and purified

using affinity columns such as protein A (eg for murine Mabs), Protein G (eg for rat Mabs) or MEP HYPERCEL (eg for IgM and IgG Mabs).

Recombinant human or humanized versions of monoclonal antibodies are a preferred embodiment for human therapeutic applications. Humanized antibodies may be prepared according to procedures in the literature (e.g. Jones et al. 1986, Nature 321: 522-25; Reichman et al. 1988 Nature 332: 323-27; et al. 1988, Science 1534-36). The recently described 'gene conversion metagenesis' strategy for the production of humanized monoclonal antibody may also be employed in the production of humanized antibodies (Carter et al. 1992 Proc. Natl. Acad. Sci. U.S.A. 89: 4285-89). Alternatively, techniques for generating the recombinant phase library of random combinations of heavy and light regions may be used to prepare recombinant antibodies (e.g. Huse et al. 1989 Science 246: 1275-81).

As used herein, the term 'antagonist' refers to a nucleic acid, peptide, antibody, ligands or other chemical entity which inhibits the biological activity of the polypeptide of interest. A person skilled in the art would be familiar with techniques of testing and selecting suitable antagonists of a specific protein, such techniques would including binding assays.

The antibodies and antagonists of the present invention have a number of applications, for example, they can be used as antimicrobial preservatives, in oral care products (toothpastes and mouth rinses) for the control of dental plaque and suppression of pathogens associated with dental caries and periodontal diseases. The antibodies and antagonists of the present invention may also be used in pharmaceutical preparations (eg, topical and systemic anti-infective medicines).

The present invention also provides interfering RNA molecules which are targeted against the mRNA molecules encoding the polypeptides of the first aspect of the present invention. Accordingly, in a seventh aspect of the present invention there is provided an interfering RNA molecule, the molecule comprising a double stranded region of at least 19 base pairs in each strand wherein one of the strands of the double

stranded region is complementary to a region of an mRNA molecule encoding a polypeptide of the first aspect of the present invention.

So called RNA interference or RNAi is known and further information regarding RNAi is provided in Hannon (2002) *Nature* 418: 244-251, and McManus & Sharp (2002) *Nature Reviews: Genetics* 3(10): 737-747, the disclosures of which are incorporated herein by reference.

The present invention also contemplates chemical modification(s) of siRNAs that enhance siRNA stability and support their use *in vivo* (see for example, Shen *et al.* (2006) *Gene Therapy* 13: 225-234). These modifications might include inverted abasic moieties at the 5' and 3' end of the sense strand oligonucleotide, and a single phosphorthioate linkage between the last two nucleotides at the 3' end of the antisense strand.

It is preferred that the double stranded region of the interfering RNA comprises at least 20, preferably at least 25, and most preferably at least 30 base pairs in each strand of the double stranded region. The present invention also provides a method of treating a subject for periodontal disease comprising administering to the subject at least one of the interfering RNA molecules of the invention.

The compositions of this invention can also be incorporated in lozenges, or in chewing gum or other products, e.g. by stirring into a warm gum base or coating the outer surface of a gum base, illustrative of which are jelutong, rubber latex, vinylite resins, etc., desirably with conventional plasticizers or softeners, sugar or other sweeteners or such as glucose, sorbitol and the like.

In a further aspect, the present invention provides a kit of parts including (a) a composition of polypeptide inhibitory agent and (b) a pharmaceutically acceptable carrier. Desirably, the kit further includes instructions for their use for inhibiting biofilm formation in a patent in need of such treatment.

Compositions intended for oral use may be prepared according to any method known in

the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Throughout this specification the word 'comprise', or variations such as 'comprises' or 'comprising', will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive. The invention specifically includes all combinations of features described in this specification.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following Examples.

Growth and harvesting of P. gingivalis for biofilm v planktonic studies

Porphyromonas gingivalis W50 (ATCC 53978) was grown in continuous culture using a model C-30 BioFlo chemostat (New Brunswick Scientific) with a working volume of 400 mL. Both the culture vessel and medium reservoir were continuously gassed with 10% CO_2 and 90% N_2. The growth temperature was 37°C and the brain heart infusion growth medium (Oxoid) was maintained at pH 7.5. Throughout the entire growth, redox potential maintained at -300 mV. The dilution rate was 0.1 h^{-1}, giving a mean generation time (MGT) of 6.9 h. Sterile cysteine-HCl (0.5 g/L) and haemin (5 mg/L) were added. The culture reached steady state approximately 10 days after inoculation and was maintained for a further 30 days until a thick layer of biofilm had developed on the vertical surfaces of the vessel.

All bacterial cell manipulations were carried out on ice or at 4°C. During harvesting, the planktonic cells were decanted into a clean container and the biofilm washed twice gently with PGA buffer (10.0 mM NaH_2PO_4 , 10.0 mM KCl, 2.0 mM, citric acid, 1.25 mM $MgCl_2$, 20.0 mM $CaCl_2$, 25.0 mM $ZnCl_2$, 50.0 mM $MnCl_2$, 5.0 mM $CuCl_2$, 10.0 mM $CoCl_2$, 5.0 mMH_3BO_3 , 0.1 mMNa_2MoO_4 , 10 mM cysteine-HCl with the pH adjusted to 7.5 with 5 M NaOH at 37°C) followed by harvesting of the biofilm into a 50 mL centrifuge tube.

Planktonic and biofilm cells were then washed 3 times (7000 g) with PGA buffer and both samples resuspended to a final volume of 30 mL with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, pH 8.0, proteinase inhibitor inhibitor (Sigma)) and

lysed by 3 passages through a French Press Pressure Cell (SLM, AMINCO) at 138 MPa. The lysed cells were centrifuged at 2000 g for 30 min to remove any unbroken cells. The supernatant was further centrifuged at 100000 g for 1 h to separate the lysed cells into their soluble and insoluble (cell envelope) fractions. The cell envelope fraction was further washed 3 times with wash buffer at 100000 g, for 20 min each to remove any soluble contaminations. All samples were then frozen and stored at –80°C.

Growth and harvesting of P. gingivalis for haem-limitation and excess studies

P. gingivalis W50 was grown in continuous culture using a Bioflo 110 fermenter/bioreactor (New Brunswick Scientific) with a 400 mL working volume. The growth medium was 37 g/L brain heart infusion medium (Oxoid) supplemented with 5 mg/mL filter sterilized cysteine hydrochloride, 5.0 μg/mL haemin (haem-excess) or 0.1 μg/mL haemin (haem-limited). Growth was initiated by inoculating the culture vessel with a 24 h batch culture (100 mL) of *P. gingivalis* grown in the same medium (haem-excess). After 24 h of batch culture growth, the medium reservoir pump was turned on and the medium flow adjusted to give a dilution rate of 0.1 h^{-1} (mean generation time (MGT) of 6.9 h). The temperature of the vessel was maintained at 37°C and the pH at 7.4 ± 0.1. The culture was continuously gassed with 5% CO_2 in 95% N_2. Cells were harvested during steady state growth, washed three times with wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$) at 5000 g for 30 min and disrupted with 3 passes through a French Pressure Cell (SLM, AMINCO) at 138 MPa. The lysed cells were then centrifuged at 2000 g for 30 min to remove unbroken cells followed by ultracentrifugation at 100000 g, producing a soluble (supernatant) and membrane fraction. All fractions were carried out on ice.

Preparation and analysis of ^{18}O proteolytic labelled biofilm and planktonic cell envelope fraction

The cell envelope fraction was first resuspended in 1 mL of ice cold wash buffer containing 2% SDS, then sonication and vortexing were carried out to aid resuspension of the pellet. The final step in resuspension involved use of a 29-guage-insulin needle to help break up particulates. The mixture was then centrifuged at 40000 g to remove

insoluble particles and the protein concentration of the supernatant was determined using the BCA reagent (Pierce) according to the manufacturer's instructions.

The resuspended samples were subjected to precipitation using 5 volumes of ice cold acetone overnight at -20°C which further helped to inactivate any proteolytic activity. After acetone precipitation, both samples were resuspended to a final concentration of 3 mg/mL with 25 mM Tris pH 8.0 and 1% SDS assisted by intermittent sonication, vortexing and the use of a 29-guage-insulin needle. A second BCA protein assay was then carried out to standardize the final protein amount.

Gel electrophoresis on a NuPAGE gel was carried out as per manufacturer's protocol using MOPs running buffer (NuPAGE, Invitrogen) except the samples were boiled at 99°C for 5 min prior to loading onto a 10-well 10% NuPAGE gel with MOPs as the running buffer. The biofilm and planktonic samples (30 µg each) were loaded in adjacent lanes on the gel. SDS-PAGE was then carried out at 126 V (constant) at 4°C until the dye front was approximately 1 cm from the bottom of the gel. For the biological replicate, the gel used was a 4-12% NUPAGE gradient gel using MOPs as the running buffer to give a similar but not exact pattern of separation so as to overcome the potential variation of a protein band being separated into two fractions. Staining was carried out overnight in Coomassie brilliant blue G-250 (31) followed by overnight destaining in ultrapure H_2O.

The two gel lanes were divided into 10 gel bands of equal sizes using a custom made stencil and each section cut into approximately 1 mm^3 cubes. Destaining was carried out 3 times in a solution of 50 mM NH_4HCO_3/ACN (1:1). After destaining, the gel cubes were dehydrated with 100% ACN, followed by rehydration/reduction with a solution of 10 mM dithiothreitol in ABC buffer (50 mM NH_4HCO_3) at 56°C for 30 min. The excess solution was removed before adding 55 mM iodoacetamide in ABC buffer for 60 min at room temperature in the dark. After the alkylation reaction, the gel cubes were washed 3 times in ABC buffer, followed by dehydration twice in 100% ACN for 10 min. The gel cubes were further dried under centrifugation using a speedvac for 90 min. Digestion was carried out in 60 µL solution per gel section containing 2 µg of sequence grade modified trypsin (Promega) and ½ strength ABC buffer made up in either $H_2{}^{16}O$ or

$H_2{}^{18}O$ ($H_2{}^{18}O$, >97 % purity, Marshall Isotopes) for 20 h at 37°C. After digestion, the peptides were twice extracted from the gel using a solution of 50% ACN/0.1% TFA in their respective water ($H_2{}^{16}O/H_2{}^{18}O$) and 0.1% TFA with the aid of sonication for 5 min each. The pooled extract was boiled at 99°C for 5 min to inactivate the trypsin followed by freeze drying for 48 h.

The freeze-dried peptides were resuspended in a solution of 5% ACN/0.1% TFA in their respective water ($H_2{}^{16}O/H_2{}^{18}O$) just before analysis using nanoHPLC and MALDI TOF-MS/MS analysis. The peptide solution (20 µL) was then loaded onto an Ultimate Nano LC system (LC Packings) using a FAMOS autosampler (LC Packings) in advanced µL pickup mode. The samples were first loaded onto a trapping column (300 µm internal diameter x 5 mm) at 200 µL/min for 5 min. Separation was achieved using a reverse phase column (LC Packings, C18 PepMap100, 75 µm i.d. x 15 cm, 3 µm, 100Å) with a flow rate of 300 nL/min, and eluted in 0.1% formic acid with an ACN gradient of 0-5 min (0%), 5-10 min (0-16%), 10-90 min (16-80%), 90-100 min (80-0%).

Eluents were spotted straight onto pre-spotted anchorchip plates (Bruker Daltonics) using the Proteineer Fc robot (Bruker Daltonics) at 30 s intervals. Prior to spotting, each spot position was pre-spotted with 0.2 µL of ultrapure H_2O to reduce the concentration of the acetonitrile during the crystallization process with the matrix. The plate was washed with 10 mM ammonium phosphate and 0.1% TFA and air-dried before automated analysis using a MALDI-TOF/TOF (Ultraflex with LIFT II upgrade, Bruker Daltonics). MS analysis of the digest was initially carried out in reflectron mode measuring from 800 to 3500 Da using an accelerating voltage of 25 kV. All MS spectra were produced from 8 sets of 30 laser shots, with each set needing to have a signal to noise, S/N >6, Resolution >3000 to be included. Calibration of the instrument was performed externally with $[M+H]^+$ ions of the prespotted internal standards (Angiotensin II, Angiotensin I, Neurotensin, Renin_substrate and ACTH_Clip) for each group of four samples. LIFT mode for MALDI-TOF/TOF was carried out in a fully automated mode using the Flexcontrol and WarpLC software (Bruker Daltonics). In the TOF1 stage, all ions were accelerated to 8 kV and subsequently lifted to 19 kV in the LIFT cell and all MS/MS spectra were produced from accumulating 550 consecutive laser shots.

Selection of parent precursors was carried out using the WarpLC software (ver 1.0) with the LC MALDI SILE (Stable Isotope Labelling Experiment) work flow. Only the most abundant peak of each heavy or light pair separated by 4 Da was selected, providing its S/N was >50. Compounds separated by less than six LC MALDI fractions were considered the same and therefore selected only once.

Peak lists were generated using Flexanalysis 2.4 Build 11 (Bruker Daltonics) with the Apex peak finder algorithm with S/N > 6. The MS scan was smoothed once with the Savitzky Golay algorithm using a width of 0.2 m/z and baseline subtraction was achieved using the Median algorithm with flatness of 0.8.

Protein identification was achieved using the MASCOT search engine (MASCOT version 2.1.02, Matrix Science) on MS/MS data queried against the *P. gingivalis* database obtained from The Institute for Genomic Research (TIGR) website (www.tigr.org). MASCOT search parameters were: charge state 1+, trypsin as protease, one missed cleavage allowed and a tolerance of 250 ppm for MS and 0.8 m/z for MS/MS peaks. Fixed modification was set for carbamidomethyl of cysteine and variable modification was C-terminal ^{18}O labelled lysine and arginine residues.

A reverse database strategy as described previously (32) was employed to determine the minimum peptide MASCOT score required to omit false positives for single peptide identification. Briefly, the database consists of both the sequence of every predicted *P. gingivalis* protein in its normal orientation and the same proteins with their sequence reversed (3880 sequences). The whole MS/MS dataset was then searched against the combined database to determine the lowest Mascot score to give 0% false positives. A false positive was defined as a positive match to the reversed sequence (bold red and above peptide threshold score). A false positive rate for single hit peptides was determined to be 0.5% with Mascot peptide ion scores of >threshold and <25. When the Mascot peptide ion score was >30, there was no match to the reverse database. In order to increase the confidence of identification for single hits peptide, we used a minimum Mascot peptide ion score of >50 which gives a two order of magnitude lower probability of incorrect identification than if a score of 30 was used, according to the Mascot scoring algorithm.

The matched peptides were evaluated using the following criteria, i) at least 2 unique peptides with a probability based score corresponding to a *p*-value <0.05 were regarded as positively identified (required bold red matches) where the score is –log X 10log(P) and P is the probability that the observed match is a random event (33), ii) where only one unique peptide was used in the identification of a specific protein (identification of either heavy or light labelled peptide is considered as one) the MASCOT peptide ion score must be above 50 or that peptide is identified in more than one of the four independent experiments (2 biological replicates and 2 technical replicates).

Due to the mixed incorporation of one or two ^{18}O atoms into the peptides, the contribution of the natural abundance of the ^{18}O isotope and the $H_2{}^{18}O$ purity (a=0.97), the ratios of the peptides R were mathematically corrected using equation:

$$R = (I_1 + I_2) / I_0 \qquad (1)$$

I_0, I_1 and I_2 were calculated according to the following equations (27),

$$I_1 = \frac{aS_2 - [aJ_2 - 2(1-a)J_4]S_0 - 2(1-a)S_4}{a^2 - (2 - a - a^2)J_2 + 2(1-a)^2 J_4} \qquad (2)$$

$$I_0 = S_0 - (1-a) I_1 \qquad (3)$$

$$I_2 = \frac{1}{a^2}(S_4 - J_4 I_0 - J_2 I_1) \qquad (4)$$

Where S_0, S_2 and S_4 are the measured intensities of the monoisotopic peak for peptide without ^{18}O label, the peak with 2 Da higher than the monoisotopic peak, and the peak with 4Da higher than the monoisotopic peak respectively (Fig 1A). J_0, J_2 and J_4 are the corresponding theoretical relative intensities of the isotopic envelope of the peptide calculated from MS-Isotope (http://prospector.ucsf.edu). However when the intensity of

the second isotopic peaks (S_1 and S_5) was more intense than the first isotopic peaks (S_0 and S_4), the ratio was simply calculated as S_1 divided by S_5. This was true especially for large peptides above 2000 m/z where the contribution of the fifth isotopic peak of the ^{16}O labelled peptide to the S_4 peak becomes significant. Calculation of mixed $^{16}O^{18}O$ incorporation was determined by the difference in the experimental S_2 and theoretical S_2 (J_2) as a percentage of experimental S_4.

Protein abundance ratios were determined by averaging all identified peptides of the same protein, even when the same protein was identified in more than one gel section. The data from each 'normal' replicate was combined with the inversed ratios from its respective 'reverse' replicate providing an average ratio and standard error for each protein in each biological replicate. Normalization of both the biological replicates was then carried out similarly to that previously reported (34,35). Briefly the averaged ratio for each biological replicate was multiplied by a factor so that the geometric mean of the ratios was equal to one.

Preparation and analysis of ICAT labelled haem-limited and excess cells

Protein labelling and separation were based on the geLC-MS/MS approach (Li et al., 2003) using the cleavable ICAT reagent (Applied Biosystems). Another proteomic approach has been taken in PCT/AU2007/000890 which is herein incorporated by reference. Protein was first precipitated using TCA (16%) and solubilised with 6 M urea, 5 mM EDTA, 0.05% SDS and 50 mM Tris-HCl pH 8.3. Protein concentration was determined using the BCA protein reagent and adjusted to 1 mg/ml. 100 µg of protein from each growth condition was individually reduced using 2 μL of 50 mM Tris(2-carboxy-ethyl)phosphine hydrochloride for 1 h at 37°C. Reduced protein from the haem-limitation growth condition was then alkylated with the $ICAT_{heavy}$ reagent and protein from haem-excess growth condition with the $ICAT_{light}$ reagent. The two samples were then combined and subjected to SDS-PAGE on a precast Novex 10% NUPAGE gel (Invitrogen). The gel was stained for 5 min using SimplyBlue™ SafeStain (Invitrogen) followed by destaining with water. The gel lane was then excised into 20 sections from the top of the gel to the dye front.

The excised sections were further diced into 1 mm^3 cubes and in-gel digested overnight and extracted twice according to the above procedure. The pooled supernatant was dried under reduced vacuum to about 50 μL followed by mixing with 500 μL of affinity load buffer before loading onto the affinity column as per manufacturer's instruction (Applied Biosystems). Eluted peptides were dried and the biotin tag cleaved with neat TFA at 37°C for 2 h followed by drying under reduced vacuum. The dried samples were suspended in 35 μL of 5% acetonitrile in 0.1% TFA.

MS was carried out using an Esquire HCT ion trap mass spectrometer (Bruker Daltonics) coupled to an UltiMate Nano LC system (LC Packings – Dionex). Separation was achieved using a LC Packings reversed phase column (C18 PepMap100, 75 µm i.d. x 15 cm, 3 µm, 100Å), and eluted in 0.1% formic acid with the following acetonitrile gradient: 0-5 min (0%), 5-10 min (0-10%), 10-100 min (10-50%), 100-120 min (50-80%), 120-130 min (80-100%).

The LC output was directly interfaced to the nanospray ion source. MS acquisitions were performed under an ion charge control of 100000 in the m/z range of 300-1500 with maximum accumulation time of 100 ms. When using GPF three additional m/z ranges (300-800, 700-1200 and 1100-1500) were used to select for precursor ions and each m/z range was carried out in duplicate to increase the number of peptides identified. MS/MS acquisition was obtained over a mass range from 100-3000 m/z and was performed on up to 10 precursors for initial complete proteome analysis and 3 for ICAT analysis for the most intense multiply charged ions with an active exclusion time of 2 min.

Peak lists were generated using DataAnalysis 3.2 (Bruker Daltonics) using the Apex peak finder algorithm with a compound detection threshold of 10000 and signal to noise threshold of 5. A global charge limitation of +2 and +3 were set for exported data. Protein identification was achieved using the MASCOT search engine (MASCOT 2.1.02, Matrix Science) on MS/MS data queried against the *P. gingivalis* database obtained from The Institute for Genomic Research (TIGR) website (www.tigr.org). The matched peptides were further evaluated using the following criteria, i) peptides with a probability based Mowse score corresponding to a *p*-value of at most 0.05 were

regarded as positively identified, where the score is –log X 10(log(P)) and P is the probability that the observed match is a random event ii) where only one peptide was used in the identification of a specific protein and the MASCOT score was below 30, manual verification of the spectra was performed. To increase confidence in the identification of ICAT-labelled proteins especially for those with single peptide hits, additional filters were applied as follows: i) the heavy and light peptides of an ICAT pair must have exhibited closely eluting peaks as determined from their extracted ion chromatograms ii) for proteins with a single unique peptide, this peptide must have been identified more than once (e.g in different SDS-PAGE fractions or in both the light and heavy ICAT forms iii) if a single peptide did not meet the criteria of (ii), the MASCOT score must have been ≥ 25, the expectation value ≤ 0.01 and the MS/MS spectrum must have exhibited a contiguous series of 'b' or 'y'-type ions with the intense ions being accounted. Determinations of false positives were as described above.

The ratio of isotopically heavy ^{13}C to light ^{12}C ICAT labelled peptides was determined using a script from DataAnalysis (Bruker Daltonics) and verified manually based on measurement of the monoisotopic peak intensity (signal intensity and peak area) in a single MS spectrum. The minimum ion count of parent ions used for quantification was 2000 although >96% of both heavy and light precursor ions were >10000. In the case of poorly resolved spectra, the ratio was determined from the area of the reconstructed extracted ion chromatograms (EIC) of the parent ions. Averages were calculated for multiple peptides derived from a single parent protein and outliers were removed using the Grubb's test with $\alpha = 0.05$.

The cellular localization of P. gingivalis proteins was predicted using CELLO (http://cello.life.nctu.edu.tw (36)). Extracellular, outer membrane, inner membrane and periplasmic predictions were considered to be from the envelope fraction.

The concentrations of short-chain fatty acids (SCFA) in cell-free culture supernatants (uninoculated, haem-excess and haem-limited) were determined by capillary gas chromatography based on the derivatization method of Richardson *et al.* (37).

The correlation coefficient (r) between both biological replicates was evaluated using the Pearson correlation coefficient function from Microsoft Excel. The coefficient of variance (CV) was calculated by the standard deviation of the peptide abundance ratios divided by the mean, expressed as a percentage.

Extraction of nucleic acids for transcriptomic analysis

RNA was extracted from 5 mL samples of *P. gingivalis* cells harvested directly from the chemostat. To each sample 0.2 volumes of RNA Stabilisation Reagent (5% v/v phenol in absolute ethanol) were added. Cells were pelleted by centrifugation (9000 *g*, 5 min, 25°C), immediately frozen in liquid nitrogen and stored at -70°C for later processing. Frozen cells were suspended in 1 mL of TRIzol reagent (Invitrogen) per 1 x 10^{10} cells and then disrupted using Lysing Matrix B glass beads (MP Biomedicals) and the Precellys 24 homogeniser (Bertin Technologies, France). The glass beads were removed by centrifugation and the RNA fraction purified according to the TRIzol manufacturer's (Invitrogen) protocol, except that ethanol (at a final concentration of 35%) rather than isopropanol was added at the RNA precipitation stage and samples were then transferred to the spin-columns from the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare). RNA was purified according to the manufacturer's instructions from the binding step onwards, including on-column DNAse treatment to remove any residual DNA. RNA integrity was determined using the Experion automated electrophoresis station (Bio-Rad).

Genomic DNA was extracted from *P. gingivalis* cells growing in continuous culture using the DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer's instructions.

Microarray design, hybridization and analysis

Microarray slides were printed by the Australian Genome Research Facility and consisted of 1977 custom designed 60-mer oligonucleotide probes for the predicted protein coding regions of the *P. gingivalis* W83 genome including additional protein coding regions predicted by the Los Alamos National Laboratory Oralgen project. Microarray Sample Pool (MSP) control probes were included to aid intensity-dependent

normalisation. The full complement of probes was printed 3 times per microarray slide onto Corning UltraGAPS coated slides.

Slides were hybridised using either haeme-excess or heme-limited samples labelled with Cy3, combined with a universal genomic DNA reference labelled with Cy5 (GE Lifesciences). cDNA was synthesized from 10 µg of total RNA using the SuperScript plus indirect cDNA labelling system (Invitrogen), with 5 µg of random hexamers (Invitrogen) for priming of the cDNA synthesis reaction. cDNA was labelled with Cy3 using the Amersham CyDye post-labelling reactive dye pack (GE Lifesciences) and purified using the purification module of the Invitrogen labelling system. Cy5-dUTP labelled genomic cDNA was synthesized in a similar manner from 400 ng of DNA, using the BioPrime Plus Array CGH Indirect Genomic Labelling System (Invitrogen).

Prior to hybridisation, microarray slides were immersed for 1 h in blocking solution (35% formamide, 1% BSA, 0.1% SDS, 5X SSPE [1X SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA]) at 42°C. After blocking slides were briefly washed in H_2O followed by 99% ethanol and then dried by centrifugation. Labelled cDNAs were resuspended in 55 µL of hybridization buffer (35% formamide, 5X SSPE, 0.1% SDS, 0.1 mg mL^{-1} Salmon Sperm DNA) denatured at 95°C for 5 min then applied to slides and covered with LifterSlips (Erie Scientific). Hybridisation was performed at 42°C for 16 h. Following hybridisation slides were successively washed in 0.1% SDS plus 2X SSC [1X SSC is 150 mM NaCl 15 mM sodium citrate] (5 min at 42 °C, all further washes performed at room temperature), 0.1% SDS plus 0.1X SSC (10 min), 0.1X SSC (4 washes, 1 min each), and then quickly immersing in 0.01X SSC, then 99% ethanol and using centrifugation to dry the slides.

Slides were scanned using a GenePix 4000B microarray scanner and images analysed using GenePix Pro 6.0 software (Molecular Devices). Three slides were used for each treatment (haeme-limitation or haeme-excess) representing three biological replicates.

Image analysis was performed using the GenePix Pro 6.0 software (Molecular Devices), and "morph" background values were used as the background estimates in further analysis. To identify differentially expressed genes the LIMMA software package was

used with a cut off of P <0.005. Within array normalisation was performed by fitting a global loess curve through the MSP control spots and applying the curve to all other spots. The Benjamini Hochberg method was used to control the false discovery rate to correct for multiple testing.

Gene predictions were based on the *P. gingvalis* W83 genome annotation from the The Institute for Genomic Research (TIGR, www.tigr.org). Operon prediction was carried out from the Microbesonline website (http://microbesonline.org)

Response of P. gingivalis to haeme-limitation as determined using DNA microarray analysis

A DNA microarray analysis of the effect of haeme-limited growth on *P. gingivalis* global gene expression was carried out under identical growth conditions employed for the proteomic analysis. Analysis of data from three biological replicates identified a total of 160 genes that showed statistically significant differential regulation between haeme-excess and haeme-limitation, with the majority of these genes showing increased levels of expression under conditions of heme-limitation and only 8 genes being down-regulated. Many of the up-regulated genes were predicted to be in operons and the majority of these showed similar changes in transcript levels (**Table 3 and 5**). There was broad agreement between the transcriptomic and proteomic data with a significant correlation between the two data sets where differential regulation upon haeme-limitation was observed [Spearman's correlation 0.6364, $p < 0.05$]. However for some of the proteins showing differences in abundance from the proteomic analysis, the transcriptomic analysis of the corresponding genes did not detect any statistically significant differences in the abundance of the mRNA. The microarray analyses tended to identify only those genes encoding proteins that had large changes in abundance as determined by the proteomic analysis (**Tables 3 and 5**). Where protein and transcript from the same gene were found to be significantly regulated by haeme-limitation the majority showed the same direction of regulation. The exceptions were two gene products, PG0026 a CTD family putative cell surface proteinase and PG2132 a fimbrillin (FimA). These proteins decreased in abundance in the proteomic analysis under haeme-limitation but were predicted to be up-regulated by the transcriptomic analysis. Both these proteins are cell surface located and it is quite possible that they are either

released from the cell surface or post-translationally modified which could preclude them from being identified as up-regulated in the proteomic analysis.

In addition to the gene products discussed in more detail below transcription of several genes of interest were significantly up-regulated including the genes of a putative operon of two genes, PG1874 and PG1875, one of which encodes Haemolysin A; eight concatenated genes PG1634-PG1641 of which PG1638 encodes a putative thioredoxin and PG1043 that encodes FeoB2, a manganese transporter. PG1858 which encodes a flavodoxin was the most highly up-regulated gene at 15.29-fold. Of the 152 significantly up-regulated genes ~55 have no predicted function.

Continuous culture and biofilm formation

P. gingivalis W50 was cultured in continuous culture over a 40 day period during which the cell density of the culture remained constant after the first 10 days with an OD_{650} of 2.69 ± 0.21 and 2.80 ± 0.52 for biological replicates 1 and 2 respectively. This equates to a cell density of ~3 mg cellular dry weight/mL. Over this time period a biofilm of *P. gingivalis* cells developed on the vertical glass wall of the fermenter vessel. This biofilm was ~2 mm thick at the time of harvest.

Validation of $^{16}O/^{18}O$ quantification method using BSA

To determine the accuracy and reproducibility of the $^{16}O/^{18}O$ quantification method, known amounts of BSA were loaded onto adjacent gel lanes to give ratios of 1:1, 1:2, 1:5 and 10:1 (Fig 1B). The bands were subjected to in-gel tryptic digestion in the presence of either $H_2{}^{16}O$ or $H_2{}^{18}O$, mixed and then analyzed by LC MALDI-MS/MS. A typical set of spectra for a single BSA tryptic peptide across the four ratios shows the preferential incorporation of two ^{18}O atoms, which is seen most clearly by the predominance of the +4 Da peak in the 10:1 BSA ratio, and by the almost symmetrical doublet in the 1:1 spectrum, simplifying both quantification and identification (Fig 1A). The average incorporation of a single ^{18}O atom was estimated to be <7% based on the 1:1 labelling (Supplementary Table). The calculated average ratios for all identified BSA peptides were 0.98 ± 0.12, 2.22 ± 0.26, 4.90 ± 0.75 and 10.74 ± 2.04 for ratios of 1:1 (triplicate), 2:1 (and 1:2), 1:5 and 10:1, respectively indicating a good dynamic range, high accuracy of ± 2-11% and a low CV ranging from 11.75% to 18.95% (Table 1). The

reproducible accuracy of the 1:1 mixture (performed in triplicate) implies that labelling bias was very low. This was further confirmed by comparing normal and reverse labelled BSA at a 2:1 ratio, using only peptides that were identified in both experiments. The normal ratio was determined to be 2.11 ± 0.33 while the reverse was 2.30 ± 0.20 (Table 1).

Experimental design for quantitative analysis of biofilm and planktonic samples

The design of this study involved the use of two biological replicates, that is two independent continuous cultures, each one split into a biofilm sample obtained from the walls of the vessel, and a planktonic sample obtained from the fluid contents of the vessel. Two technical replicates for each biological replicate were performed, and although we had established that there was no significant labelling bias with BSA, we chose to utilize the reverse labelling strategy as there is a lack of $^{16}O/^{18}O$ labelling validation studies that have been conducted on complex biological samples (30). Therefore in total there were four experiments, each consisting of 10 LC-MALDI MS/MS runs stemming from 2×10 gel segments.

Figure 2 shows typical MS and MS/MS spectra of two normal and reverse labelled peptides from the biofilm/planktonic samples illustrating the typical reverse labelling pattern. As with the BSA data, it could be seen that there was a high level of double ^{18}O incorporation with the average mixed incorporation calculated to be <15% for all peptides, confirming that the $^{16}O/^{18}O$ proteolytic labelling method was also effective with complex samples (data not shown). The predominance of doubly labelled peptides was further confirmed by the relatively few Mascot hits to the +2 Da species. MS/MS spectra of the heavy labelled peptides further revealed the expected +4 Da shifts in the Y ions (Fig 2).

The cell envelope proteome of planktonic and mature biofilm P. gingivalis cells

We have identified and determined the relative abundance of 116 proteins from 1582 peptides based on the selection criteria described in the experimental procedures section. Of the proteins identified, 73.3% were identified by more than 2 unique peptides, 12.9% were from 1 unique peptide but identified in both biological replicates and 13.8% were identified only by 1 unique peptide with Mascot peptide ion score of

>50 (Fig 5). CELLO (36) predicted 77.6% of these proteins to be from the cell envelope thereby showing the effectiveness of this cell envelope enrichment method. Bioinformatics classification by TIGR (www.tigr.org) and ORALGEN oral pathogen sequence databases (www.oralgen.lanl.gov) predicted a large percentage of the identified proteins to be involved in transport, have proteolytic activities, or cell metabolism functions. Interestingly 55% of all identified proteins were of unknown functions.

To compare technical replicates of the biological data, the Log_{10} transformed protein abundance ratios of each pair of normal and reverse labelled experiments were plotted against each other (Fig 3). Linear regression of these plots indicated that each pair is highly correlated with R^2 values of 0.92 and 0.82 for biological replicate 1 and 2, respectively. The slope of each linear fit was also similar to the expected value of 1.0 at 0.97 and 0.93 for biological replicate 1 and 2, respectively indicating no labelling bias between the technical replicates (Fig 3). The protein abundance ratios from the technical replicates were averaged to give a single ratio for each biological replicate.

Before comparing the average data for the two biological replicates, the protein abundance ratios of each biological replicate were normalized to give an average mean ratio of 1.0. A plot of the normalized protein abundance ratios from both the biological replicates exhibits a Gaussian-like distribution closely centered at zero (Fig 4A) similar to that described by others (40,41). There was a significant positive correlation between the two biological replicates (Pearson's correlation coefficient $r = 0.701$, $p < 0.0001$) indicating that the growth of the biofilm/planktonic cultures and all downstream processing of the samples could be reproduced to a satisfactory level. To determine which proteins were consistently regulated in the two biological replicates, a simple ranking chart was constructed where proteins were divided into 6 groups (A-F) according to their abundance ratio and then ranked 1-6 according to group-based correlation, with those ranked 1 having the highest similarity when a protein from both biological replicates fell within the same group (Fig 4B). Using the ranking chart, we were able to determine that 34 out of 81 (42%) of the proteins identified from both replicates were ranked number one, considerably higher than the value expected for a random correlation which would be 17% (or 1/6). The majority of the remaining proteins

were ranked number two, and therefore in total, 70 proteins (86.4%) were considered to be similarly regulated between the two experiments (ranked 1 or 2; Table 2).

Based on the measured standard deviation (±0.26) of the 2:1 BSA labelling experiment (Table 1), protein abundance changes were deemed to be biologically significant when they differed from 1.0 by >3 standard deviations (either >1.78 or <0.56) (18,42). Using this criteria, the abundance of 47 out of the 81 proteins identified in both replicates were significantly changed (based on the average ratios), and of these, 42 were ranked either 1 or 2 (Table 2). Of the 42 proteins ranked 1 and 2, 24 had significantly increased in abundance and 18 had decreased in abundance.

Enzymes of metabolic pathways showing co-ordinated regulation

Twenty proteins involved in the glutamate/aspartate catabolism were identified in the haem-limited vs haem-excess study using ICAT labelling strategies (**Table 3**). Of those, enzymes catalyzing six of the eight steps directly involved in the catabolism of glutamate to butyrate were identified and found to have increased 1.8 to 4 fold under haem-limitation (**Table 3**). Although the other two catalytic enzymes (PG0690, 4-hydroxybutyrate CoA-transferase and PG1066, butyrate-acetoacetate CoA-transferase) were not detected using ICAT, they were found to be present in a separate qualitative study at comparable high ion intensities to those proteins reported in **Table 3** (not shown) and belong to operons shown to be upregulated. On the other hand, the effect of haem-limitation on the abundances of the enzymes of the aspartate catabolic pathway was mixed, with the enzymes catalyzing the breakdown of aspartate to oxaloacetate in the oxidative degradation pathway being unchanged and the enzymes involved in the conversion of pyruvate to acetate showing an increase of 2 to 4.4 fold.

The abundance of two iron containing fumarate reductase enzymes, FrdA (PG1615) and FrdB (PG1614) that together catalyse the conversion of fumarate to succinate via the reductive pathway from aspartate, was significantly reduced in cells cultured in haem-limitation (**Table 3**). These two proteins, that are encoded in an operon (Baughn et al., 2003), show similar changes in abundance in response to haem-limitation (FrdA L/E=0.35; FrdB L/E=0.25).

Analysis of organic acid end products

The amounts of acetate, butyrate and propionate in the spent culture medium of *P. gingivalis* grown under haem limitation were 13.09 ± 1.82, 7.77 ± 0.40 and 0.71 ± 0.05 mmole/g cellular dry weight, respectively. Levels of acetate, butyrate and propionate in the spent culture medium of *P. gingivalis* grown in haem excess were 6.00 ± 0.36, 6.51 ± 0.04 and 0.66 ± 0.07 mmole/g cellular dry weight, respectively.

The above results illustrate the changes in protein abundance that occur when planktonic *P. gingivalis* cells adhere to a solid surface and grow as part of a mature monospecies biofilm. It is the first comparative study of bacterial biofilm versus planktonic growth to utilize either the geLC MS approach of Gygi's group (46) or the $^{16}O/^{18}O$ proteolytic labelling method to determine changes in protein abundances as all other such studies published to date have utilized 2D gel electrophoresis based methods (10-12). A two technical replicate and two biological replicate $^{16}O/^{18}O$ reverse labelling approach was successfully employed to quantitate and validate the changes in protein abundance.

Continuous culture of P. gingivalis

In this study *P. gingivalis* W50 was cultured in continuous culture as opposed to the more traditional methodology of batch culture. Batch culture introduces a large range and degree of variation into bacterial analyses due to interbatch variables such as: size and viability of the inoculum, exact growth stage of the bacterium when harvested, levels of available nutrients in the medium and redox potential of the medium, amongst other factors. In continuous culture the bacterium is grown for many generations under strictly controlled conditions that include growth rate, cell density, nutrient concentrations, temperature, pH and redox potential. (44,47,48). A previous study has demonstrated a high level of reproducibility of *Saccharomyces cerevisiae* transcriptomic analyses continuously cultured in chemostats in different laboratories (49). Furthermore in our study the growth of both biofilm and planktonic cells was carried out in a single fermentation vessel, reducing variability as compared to separate cultivations. The consistent changes in *P. gingivalis* cell envelope protein abundances between biological replicates of 86.4% of the identified proteins (ranked 1 and 2) seen in this study

illustrate the applicability of the continuous culture system and the $^{16}O/^{18}O$ proteolytic labelling strategy to the analysis of the effect of biofilm growth on the *P. gingivalis* proteome.

Efficiency of ^{18}O labelling

The basic proteomic method employed in this study was the geLC MS method (46,50) due to the high resolution and solubility of membrane proteins that the SDS-PAGE method affords. This method was combined with a single ^{18}O labelling reaction during the in-gel digestion procedure similar to that described by others (26-29). Efficient labelling should result in the incorporation of two ^{18}O atoms into the C-terminus of each peptide and should be resistant to back-exchange with ^{16}O. This was found to be the case in our study with BSA where the level of single ^{18}O atom incorporation was estimated to be <7% and the mean ratios obtained for various BSA experiments were found not to significantly favour ^{16}O (Table 1) suggesting that back exchange with normal water was not a problem. Similar results were also obtained for the biological samples. A crucial step for efficient ^{18}O labelling was the need for the complete removal of the natural $H_2{}^{16}O$ followed by resolubilization of the protein in $H_2{}^{18}O$ before tryptic digestion employing a 'single-digestion' method. Although a number of studies have used a 'double digestion' method (51,52), the single digestion method has the advantage of giving a higher efficiency of ^{18}O labelling as in the double digestion method some tryptic peptides were unable to exchange either of their C-terminal ^{16}O atoms for an ^{18}O atom after the initial digestion (53). We further utilized an in-gel digestion method where the protein is retained in the gel matrix during the initial dehydration step using organic solvents as in any standard in-gel digestion protocol. Complete removal of any trace natural $H_2{}^{16}O$ was achieved through lyophilization by centrifugation under vacuum while the protein was still within the gel matrix to prevent further adsorptive losses during the initial lyophylization step. Rehydration and in-gel digestion was carried out in $H_2{}^{18}O$ containing a large excess of trypsin which was also reconstituted in $H_2{}^{18}O$. During the digestion procedure, tryptic peptides liberated from the gel after the incorporation of the first ^{18}O atom can undergo the second carbonyl oxygen exchange process mediated by the excess trypsin. This should promote the replacement of the second carbonyl oxygen since peptides liberated would have higher solubility than proteins thereby resulting in a higher level of doubly ^{18}O labelled tryptic

peptides (Figs 1 and 2; (54)). In order to prevent back exchange with normal water, trypsin was deactivated by boiling which has been previously shown to be effective (51,54). In addition, the dried, deactivated mix was only resuspended and mixed immediately prior to injection onto a nanoLC to minimize spontaneous exchange, although this spontaneous exchange has been shown to be low (15,40).

Reverse labelling

In the case of stable isotope labelling and quantification using MS, errors are potentially introduced during the labelling and ionization process. These errors include the potential different affinity of the label and the possible suppression effect of the heavy or light labelled peptides during the MALDI process (13,55). Traditional technical replicates which involve repeating the same labelling could result in an uncorrected bias towards a particular label or increased random error of specific peptides due to contaminating peaks. Our normal and reverse labelled technical replicates demonstrated a high degree of correlation with scatter plot gradients of 0.97 (R^2 = 0.92) and 0.93 (R^2 = 0.82) for biological replicates 1 and 2, respectively (Fig 3) which is close to the expected ratio of 1.0 for no labelling bias. These gradients also indicate that the method was reproducible with respect to protein estimation, gel loading, gel excision and in-gel digestion. The lack of bias suggests normalization routines like dye swap or LOWESS data normalization routinely used in microarray experiments (35) might be unnecessary. However samples that are considerably more complex than the bacterial cell envelopes used in this study may still require reverse labelling validation as when one considers the influence of minor contaminating peptides on the calculation of the $^{18}O/^{16}O$ ratios and the need to verify peptides with extreme changes. The reverse-label design in addition to providing an estimate and means for correcting systematic errors had the further benefit of allowing both the heavy and light labelled peptides to be readily identified since the MS/MS acquisition method selected only the most intense peptide in each heavy/light pair to fragment. In this way the possibility of incorrect assignment is reduced. To our knowledge, this is the first report of reverse $^{16}O/^{18}O$ labelling in a complex biological sample other than the recent quantitation of seventeen cytochrome P450 proteins (26,30).

Biofilm vs planktonic culture

We have demonstrated a strong positive correlation between the biological replicates ($r = 0.701$, $p < 0.0001$) indicating that there was reproducibility in biofilm formation and development. This was also seen by the finding that 70 out of 81 quantifiable proteins were observed to exhibit similar ratios in both biological replicates (Table 2, ranked 1 or 2). More than three quarters of the *P. gingivalis* proteins identified in this study were identified by >2 unique peptides, further increasing the confidence of identification and quantification of this labelling procedure. Of the 81 proteins consistently identified from both biological replicates, 47 significantly changed in abundance from the planktonic to biofilm state. The change in abundance of a percentage of the detected proteome, especially in the cell envelope, is consistent with other studies on biofilm forming bacteria such as *Pseudomonas aeruginosa,* where over 50% of the detected proteome was shown to exhibit significant changes in abundance between planktonic and mature biofilm growth phases. (12). We further observed a wide range of responses in the cell envelope proteome of *P. gingivalis* to growth as a biofilm. A number of proteins previously demonstrated to be altered in abundance in response to biofilm culture were also found to have changed in abundance in our study. Remarkably some proteins were observed to have changed in abundance by up to fivefold (Table 2) suggesting some major shifts in the proteome in response to biofilm culture.

C-Terminal Domain family

P. gingivalis has recently been shown to possess a novel family of up to 34 cell surface-located outer membrane proteins that have no significant sequence similarities apart from a conserved C-Terminal Domain (CTD) of approximately 80 residues (31,56). The *P. gingivalis* CTD family of proteins includes the gingipains (RgpA [PG2024], RgpB [PG0506], Kgp [PG1844]); Lys- and Arg-specific proteinases and adhesins, that are secreted and processed to form non-covalent complexes on the cell surface and are considered to be the major virulence factors of this bacterium (57-61). Gingipains have been linked directly to disease pathogenesis due to their ability to degrade host structural and defense proteins and the inability of mutants lacking functional Kgp or RgpB to cause alveolar bone loss in murine periodontal models (62). Although these CTD family proteins have a variety of functions the known and putative functions of the CTD family proteins are strongly focused towards adhesive and proteolytic activities and also include the CPG70 carboxypeptidase (63), PrtT thiol proteinase, HagA

haemagglutinin, *S. gordonii* binding protein (PG0350, (64)) a putative haemagglutinin, putative thiol reductase, putative fibronectin binding protein, putative Lys-specific proteinase (PG0553) and a putative von Willebrand factor domain protein amongst others. The majority of these proteins are likely to play important roles in the virulence of the bacterium as they are involved in extracellular proteolytic activity, aggregation, haem/iron capture and storage, biofilm formation and maintenance, virulence and resistance to oxidative stress. The CTD has been proposed to play roles in the secretion of the proteins across the outer membrane and their attachment to the surface of the cell, probably via glycosylation (56,65,66). In this work we were able to quantify nine CTD family proteins consistently regulated in both replicates (Table 2) and all except PG2216 and PG1844 (Kgp) had increased in abundance during the biofilm state. The significant increase in the abundance of many of this group of proteins therefore suggests they play important functional roles during the biofilm state.

The major cell surface proteases of *P. gingivalis* RgpA, Kgp are known to be actively involved in peptide and haem acquisition, especially from haemoglobin and the release of haem at the cell surface (67,68). During the biofilm state, there was an average 2.7 fold increase in the abundance of RgpA. HagA which contains the adhesin domains that are also found in RgpA and Kgp that are responsible for haemagglutination and hemoglobin binding of *P. gingivalis* (69) was also higher in abundance in the biofilm state.

Kgp in contrast was observed to be significantly lower in abundance in biofilm cells of *P. gingivalis*. This could be due to a decrease in Kgp abundance or may be due to the release of Kgp from the *P. gingivalis* cell surface during biofilm culture. Kgp is essential for *P. gingivalis* to hydrolyze haemoglobin at surface-exposed Lys residues which leads to the release and uptake of peptides and haem (67,70). The adhesion domains of Kgp are involved in haemoglobin binding and Genco *et al* (70) have proposed that Kgp acts as a haemophore, that like siderophores, is released from the cell surface to scavenge haem from the environment. Kgp with bound haem is then proposed to bind to HmuR, an outer membrane TonB-linked receptor, reported to be required for both haemoglobin and haem utilization and deliver haem to the cell (71). Interestingly, HmuY a protein that is encoded in an operon with HmuR, was also more abundant in biofilm cultured cells.

The hmu locus contains 6 genes (*hmuYRSTUV*) and has been suggested to belong to the multigenic cluster encoding proteins involved in the haem-acquisition pathways similar to the Iht and Htr systems (72). HmuY was shown to be required for both haemoglobin and haem utilization and is regulated by iron availability (72,73). Although HmuR was not identified in our study, the operonic nature of *hmuR* and *hmuY* and other evidence suggests that their expression is similarly regulated and they act in concert for haem utilization (71,74). The decrease in abundance of Kgp and the increase in abundance of HmuY is therefore consistent with its proposed role as a hemophore and haem limitation in biofilm growth (see below).

CPG70 (PG0232) a CTD family protease that has been demonstrated to be involved in gingipain processing was also consistently higher in abundance in biofilm culture possibly indicating a role in the remodeling of cell surface proteins during biofilm growth (63,75). A CTD family putative thioredoxin (PG0616) was also significantly higher in abundance in the biofilm state. PG0616 has been characterized as HBP35, a haem binding protein having coaggregation properties (76). Of particular note was the increased abundance of the immunoreactive 46 kDa antigen, PG99 by an average factor of 5.0 in biofilm cells (Table 2). This was the highest observed increase in protein abundance in this study, and since PG99 is both immunogenic and a CTD family member and therefore most probably located on the cell surface, this protein represents a good potential target for biofilm disruptive agents.

Transport proteins

Two putative TonB dependent receptor family proteins (PG1414 and PG2008) and a putative haem receptor protein (PG1626) also show significant increase in abundance. The exact functions of these proteins are unknown, however a COG search on the NCBI COG database resulted in hits to the P functional class of outer membrane receptor proteins involving mostly Fe transport (77). Interestingly we also observed an increased abundance of the intracellular iron storage protein ferritin (PG1286). The consistent increases in abundance of these iron/haem transporting and storage proteins could be an indication of haem/iron limitation, especially within the deeper layers of the biofilm since ferritin is important for *P. gingivalis* to survive under iron-depleted conditions (78).

It is likely that both haemoglobin and haem would not diffuse far into the biofilm due to the high proteolytic activity, high haemoglobin and haem binding and storage capacities of *P. gingivalis*. It is also possible that ferrous iron transport via FeoB1 plays a more important role in the iron metabolism of this species in the deeper layers of the biofilm which may also explain the increase in ferritin as there would be little chance of cell surface storage of iron as haem (45,79). *P. gingivalis* grown under conditions of haem limitation exhibits an increase in intracellular iron, indicating that PPIX is the growth limiting factor and that ferrous iron is accumulated via the FeoB1 transporter (45).

IhtB (PG0669) and a putative TonB dependent receptor (PG0707) both showed a decrease in abundance in the biofilm state (Table 2). IhtB is a haem binding lipoprotein that also has been proposed to function as a peripheral outer membrane chelatase that removes iron from haem prior to uptake by *P. gingivalis* (80). A similar decrease in abundance of two proteins potentially involved in haem/Fe uptake during the biofilm state that coincided with an increase in abundance of many others indicates a shift in either the types of receptors being used for uptake or more likely a change in the substrate being used. Taken together from the above observations, it appears that *P. gingivalis* growing in a biofilm is likely to be haem starved. The higher abundance of some transport and binding proteins therefore suggests them being more crucial during the biofilm state and thus possible antimicrobial drug targets.

There is a higher abundance of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) during the biofilm state compared to the planktonic which is consistent with previous results obtained for *Listeria monocytogenes* and *Pseudomonas aeruginosa* (12,106). Although GAPDH is classified as a tetrameric NAD-binding enzyme involved in glycolysis and gluconeogenesis, there have been numerous reports of this protein being multifunctional and when expressed at the cell surface of Gram-positive bacteria, it appeared to be involved in binding of plasmin, plasminogen and transferrin (107,108). Interestingly coaggregation between *Streptococcus oralis* and *P. gingivalis* 33277 has been shown to be mediated by the interaction of *P. gingivalis* fimbriae and *S. oralis* GAPDH (109). The exact role, if any, of GAPDH in substrate binding in *P. gingivalis* however remains to be answered.

Biofilm formation

There was a significantly higher abundance of the universal stress protein (UspA) in the planktonic cells as compared to the biofilm cells. The production of Usp in various bacteria was found to be stimulated by a large variety of conditions, such as entry into stationary phase, starvation of certain nutrients, oxidants and other stimulants (110,111). The increased abundance in planktonic phase cells is consistent with the fact that *P. gingivalis* has evolved to grow as part of a biofilm and that planktonic phases are likely to be more stressful. Expression of UspA in *P. gingivalis* is thought to be related to biofilm formation as inactivation of *uspA* resulted in the attenuation of early biofilm formation by planktonic cells (112). In this study the biofilm has been established and reached maturation, it therefore appears to have lesser need for UspA as compared to free floating planktonic cells.

A homologue of the internalin family protein InlJ (PG0350) was observed to be higher in abundance during the biofilm state. PG0350 has been shown to be important for biofilm formation in *P. gingivalis* 33277 as gene inactivation resulted in reduced biofilm formation (39). Higher levels of PG0350 in the biofilm could suggest that this protein might be required not just for initial biofilm formation but acts an adhesin that binds *P. gingivalis* to each other or extracellular substrates within the biofilm.

Proteins with unknown functions

The largest group of proteins identified in this study was 41 proteins with unknown functions including four proteins that were identified for the first time in this study (Table 2). Of the 41 proteins identified, 37 were predicted to be from the cell envelope and within this group 17 proteins show significant changes between the biofilm and planktonic cells. The majority of these proteins have homology to GenBank proteins with defined names but not well-defined functions. Of particular interest are several proteins that were consistently found to substantially increase in abundance in the biofilm state, namely PG0181, PG0613, PG1304, PG2167 and PG2168.

The above results represent a large scale validation of the $^{16}O/^{18}O$ proteolytic labelling method as applied to a complex mixture, and are the first to use this approach for the comparison of bacterial biofilm and planktonic growth states. A substantial number of proteins with a variety of functions were found to consistently increase or decrease in abundance in the biofilm cells, indicating how the cells adapt to biofilm conditions and also providing potential targets for biofilm control strategies.

Table 1: Quantification of predetermined BSA ratios using $^{16}O/^{18}O$ proteolytic labelling

Expected ratio 1:1a)	Triplicate analysis			Mean ratio (±SD)
CCTESLVNR	0.83	0.84	0.88	0.85 ± 0.03
DLGEEHFK	0.95	1.06	0.85	0.95 ± 0.10
EACFKVEGPK	1.09	1.12	1.09	1.10 ± 0.02
ECCDKPLLEK	1.01	0.96	0.87	0.94 ± 0.07
EYEATLEECCAK	1.05	1.01	1.05	1.04 ± 0.02
LVTDLTKVHK	0.86	0.91	1.02	0.93 ± 0.08
RHPEYAVSVLLR	1.07	0.96	0.94	0.99 ± 0.07
YICDNQDTISSK	1.00	1.15	1.03	1.06 ± 0.08
Average	0.98 ± 0.10	1.00 ± 0.10	0.97 ± 0.09	0.98 ± 0.08
Average of all peptides ID**				0.98 ± 0.12
CV of all peptides ID				13.1%

	Expected ratio 2:1 b) (18O/16O)	Expected ratio 1:2 b) (18O/16O)	
QTALVELLK	1.92	0.44 (2.27)	2.10
LVNELTEFAK	2.45	0.46 (2.17)	2.31
RHPEYAVSVLLR	1.82	0.42 (2.36)	2.09
LGEYGFQNALIVR	2.21	0.43 (2.31)	2.26
MPCTEDYLSLILNR	2.59	0.40 (2.50)	2.55
KVPQVSTPTLVEVSR	2.35	0.39 (2.57)	2.46
LFTFHADICTLPDTEK	1.72	0.44 (2.27)	2.00
RPCFSALTPDETYVPK	1.82	0.52 (1.92)	1.87
Average	2.11 ± 0.33	2.30 ± 0.20	2.24 ± 0.24
Average of all peptides ID***			2.22 ± 0.26
CV of all peptides ID			11.75%

Expected ratio 1:5		Expected ratio 10:1	
	(18O/16O)		(18O/16O)
AEFVEVTK	0.232 (4.32)	AEFVEVTK	12.38
CCTESLVNR	0.184 (5.42)	QTALVELLK	10.40
SHCIAEVEK	0.176 (5.67)	LVNELTEFAK	14.17
ECCDKPLLEK	0.169 (5.91)	FKDLGEEHFK	9.41
HPEYAVSVLLR	0.218 (4.58)	HPEYAVSVLLR	11.76
YICDNQDTISSK	0.187 (5.36)	YICDNQDTISSK	10.16
LKECCDKPLLEK	0.252 (3.97)	RHPEYAVSVLLR	10.14
SLHTLFGDELCK	0.183 (5.45)	SLHTLFGDELCK	7.58
RHPEYAVSVLLR	0.201 (4.97)	EYEATLEECCAK	14.07
VPQVSTPTLVEVSR	0.206 (4.86)	ETYGDMADCCEK	12.67
ECCHGDLLECADDR	0.298 (3.35)	LGEYGFQNALIVR	9.36
LFTFHADICTLPDTEK	0.210 (4.76)	VPQVSTPTLVEVSR	8.34
		KVPQVSTPTLVEVSR	8.86
		LFTFHADICTLPDTEK	11.08
		RPCFSALTPDETYVPK	10.26
Average	0.210 ± 0.04 (4.90 ± 0.75)		10.74 ± 2.04
CV of all peptides ID	15.26%		18.95%

a) For expected ratio of 1:1, only peptides that were identified in all three separate experiments are included in this table

b) For expected ratio of 2:1 and 1:2, only peptides that were identified in both experiments are included in this table

** n=55 *** n=24

Table 2: List of the 81 proteins identified from both biological replicates of the *P. gingivalis* cell envelope fraction. An abundance ratio of >1 indicates a higher abundance of the protein in the biofilm with respect to the planktonic state. If the ratio differs from one with more than 3-fold SD (0.26) from the predetermined BSA ratios (>1.78 or <0.56), the proteins were considered to have significantly changed. Based on their mean ratios, proteins highlighted in grey represent significant changes.

		Biological replicate 1					Biological replicate 2					
Tgr #	Protein	Loca	# peptide for quant	Mascot scoreb	Norm Ratio (B/P)c	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group) e
CTD family proteins												
	Proteinases											
PG0232	Zinc carboxypeptidase, CPG70	OM	13	71/20	1.67	0.08	26	74/21	2.53	0.08	15	3 (DF)
PG0553	Extracellular protease, lysyl endopeptidase precursor (API)	OM/EX	1	65/23	3.33		2	62/19	3.45		2	1 (FF)
PG1424	Peptidylarginine deiminase	PP	9	86/22	0.49	0.05	15	89/20	3.16	0.33	10	4 (BF)
PG1837	Hemagglutinin protein HagA	OM	3 (15)*	59/20	2.73	0.44	3 (15)*	42/21	4.21	0.36	3	1 (FF)
PG1844	Lysine-specific cysteine proteinase	OM	3 (13)*	78/20	0.43	0.04	9 (24)*	77/18	0.40	0.04	10	1 (BB)
PG2024	Arginine-specific protease ArgI polyprotein (RgpA)	OM	3 (12)*	76/22	1.63	0.10	3 (22)*	72/20	3.78	0.61	4	3 (DE)
	Haem binding											
PG0616	Thioredoxin, putative	PP/CY	14	96/20	2.23	0.20	14	96/20	3.69	0.20	6	2 (EF)

		Biological replicate 1					Biological replicate 2					
Tigr #	Protein	Loca	# peptide for quant	Mascot scoreb	Norm Ratio (B/P)c	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group) e
	Biofilm related											
PG0350	Internalin-related protein	OM/EX	2	42/21	1.99		2	53/21	4.27		3	2 (EF)
	Protein with unknown functions											
PG1798	Immunoreactive 46 kDa antigen PG99	PP/EX	1	36/20	5.94		1	30/21	4.07		1	1 (FF)
PG2216	Unnamed protein (conserved)	OM/EX	2	36/22	0.72	NA	4	51/21	0.68	0.06	3	1 (CC)
Transport												
PG0669	Haem-binding protein IhtB	OM	16	120/21	0.25	0.03	14	69/20	0.16	0.02	6	1 (AA)
PG0707	TonB-dependent receptor, P92	OM	5	110/21	0.14	0.02	30	117/20	0.31	0.04	17	1 (AA)
PG0782	MotA/TolQ/ExbB proton channel family protein	IM	7	80/19	0.86	0.14	4	78/19	0.92	0.12	7	1 (CC)
PG1006	Putative TonB dependent receptor	OM/EX	6	59/21	1.42	0.12	9	90/20	1.50	0.11	7	1 (DD)
PG1414	TonB linked outer membrane receptor, PG47	OM	11	88/21	3.81	0.43	3	49/19	3.69	NA!	7	1 (FF)
PG1551	HmuY protein	UN	4	75/20	2.57	0.71	4	74/20	2.78	0.21	1	1 (DD)
PG1626	Possible outer membrane-associated protein P58 (putative haem receptor protein)	OM	41	113/21	2.37	0.22	37	122/19	3.68	0.26	17	2 (EF)
PG2008	TonB-dependent receptor, P90	OM	24	68/21	2.08	0.17	26	112/20	3.12	0.21	20	2 (EF)
PG0185	RagA protein	OM	58	89/21	0.42	0.02	109	126/20	0.79	0.04	24	2 (BC)
PG0186	Lipoprotein RagB	OM	51	142/22	0.34	0.04	56	142/19	0.26	0.02	24	1 (AA)

		Biological replicate 1					Biological replicate 2					
Tigr #	Protein	Loca	# peptide for quant	Mascot score[b]	Norm Ratio (B/P)[c]	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group)[e]
PG1010	ABC transporter, ATP-binding protein	IM	1	69/21	1.41		2	60/19	1.26		2	1 (DD)
PG1762	Protein-export membrane protein SecD/protein-export membrane protein SecF	IM	14	91/22	0.76	0.07	4	99/20	0.48	0.09	10	2 (CB)
PG2082	POT family protein	IM	13	75/23	0.80	0.06	6	48/22	0.52	0.10	4	1 (CC)
Iron/haem storage and oxidative stress response												
PG0090	Dps family protein	CY	3	72/19	1.37	0.15	1	40/21	1.27		2	1 (DD)
PG1286	Ferritin	CY	5	73/22	1.50	0.04	7	124/19	2.42	0.14	3	2 (DE)
Biofilm and invasion related												
PG0159	Endopeptidase PepO	PP/CY	8	122/20	0.35	0.08	3	60/19	0.18	NA!	3	2 (BA)
PG0245	Universal stress protein family	CY	1	67/22	0.41		2	64/20	0.25		1	2 (BA)
PG2132	Fimbrilin	EX	1	31/22	1.14		2	66/20	0.85		3	2 (DC)
Energy Metabolism												
PG0249	Oxaloacetate decarboxylase, putative	CY	1	44/22	3.50		1	37/21	2.00		1	2 (FE)
PG0306	Electron transport complex, RnfABCDGE type, G subunit	PP	2	108/22	1.08		1	61/19	0.72		1	2 (DC)
PG1084	Thioredoxin family protein	CY	2	87/22	1.07		1	132/20	0.14		1	4 (DA)
PG1612	Methylmalonyl-CoA	CY	1	65/21	0.73		2	55/21	0.42		1	2 (CB)

		Biological replicate 1					Biological replicate 2					
Tigr#a	Protein	Locb	# peptide for quant	Mascot scorebf	Norm Ratio (B/P)c	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group) e
	decarboxylase, alpha subunit (mmdA)											
PG1614	Fumarate reductase, iron-sulfur protein (frdB)	UN	2	60/20	0.15		2	53/21	0.19		2	1 (AA)
PG1615	Fumarate reductase, flavoprotein subunit (frdA)	UN	3	58/19	0.06	NA!	1	43/20	NA**		3	NA
PG1704	Thiol:disulfide interchange protein dsbD, putative	IM	3	64/23	2.14		3	68/20	0.46	0.08	1	4 (EB)
PG2181	NADH:ubiquinone oxidoreductase, Na translocating, B subunit (nqrB)	IM	1	91/21	1.32		1	69/20	0.38		1	3 (DB)
PG2124	Glyceraldehyde 3-phosphate dehydrogenase	CY	3	105/22	3.39	0.62	2	94/19	2.45		3	2 (FE)
PG2157	Glutamine cyclotransferase-related protein	PP/EX	2	70/21	0.11		3	53/20	0.45	NA!	3	2 (AB)
Ribosomal proteins												
PG0167	Ribosomal protein L25	CY	4	57/20	0.27	0.05	2	50/21	0.30		3	1 (AA)
PG0375	Ribosomal protein L13 (rplM)	CY	2	69/21	0.28		1	116/20	0.18		2	1 (AA)
PG0392	Ribosomal protein L10 (rplJ)	CY	2	97/21	0.33		5	68/22	0.37	0.02	3	2 (AB)
PG0393	Ribosomal protein L7/L12	CY	6	81/20	0.68	0.03	4	79/21	0.56	0.03	4	2 (CB)

		Biological replicate 1					Biological replicate 2					
Tigr #	Protein	Loca	# peptide for quant	Mascot scoreb	Norm Ratio (B/P)c	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group) e
Proteins with unknown functions												
	Integral outer membrane proteins											
PG0027	Probable integral outer membrane protein P40	OM	11	143/21	0.74	0.06	23	111/20	0.81	0.02	11	1 (CC)
PG0613	Possible outer membrane associated protein P23	EX	3	55/19	4.41	0.56	1	43/20	3.24		2	1 (FF)
PG0694	Outer membrane protein 40	OM	37	116/20	3.62	0.37	77	86/20	3.39	0.24	13	1 (FF)
PG0695	Outer membrane protein 41	OM	100	110/21	2.31	0.21	53	124/19	3.18	0.20	19	2 (EF)
PG1652	Probable integral outer membrane protein P64	OM	2	53/20	1.04		14	67/20	1.70	0.27	5	2 (CD)
PG1786	Probable integral outer membrane protein P30	EX/OM	5	56/21	0.99	0.17	5	56/26	1.33	0.08	3	2 (CD)
PG1823	Probable integral outer membrane protein P20	PP/OM	26	117/21	0.39	0.03	18	91/20	0.98	0.10	7	2 (BC)
PG2106	Probable integral outer membrane protein P22	IM/OM	15	73/20	0.31	0.04	13	103/17	0.45	0.04	7	2 (AB)
	Lipoproteins											
PG0188	Lipoprotein, putative	PP/EX	7	72/23	0.92	0.09	4	40/20	1.18	0.11	5	2 (CD)
PG0241	Lipoprotein, putative	OM/EX	4	93/20	0.45	0.08	2	44/20	0.16		3	2 (BA)
PG0906	Lipoprotein, putative	PP/CY	1	42/22	0.48		5	97/20	0.40	0.05	3	1 (BB)
PG2173	Outer membrane lipoprotein Omp28	PP/OM	8	109/22	0.77	0.1	12	89/18	0.71	0.04	7	1 (CC)

		Biological replicate 1					Biological replicate 2						
Tigr#	Protein	Local	# peptide for quant	Mascot score[b]	Norm Ratio (B/P)c	SEd	# peptide for quant	Mascot score	Norm ratio (B/P)	SE	Total unique peptides	Rank (Group)e
	Others proteins											
PG0179	Unnamed protein (conserved)	PP/EX	1	64/22	1.23		1	61/19	3.55		1	3 (DF)
PG0181	Immunoreactive 32 kDa antigen PG49	PP/OM	4	46/20	2.08	0.84	1	31/21	3.14		3	2 (EF)
PG0217f	Unnamed protein	OM	4	74/21	1.58	0.34	7	79/18	1.78	0.07	5	2 (DE)
PG0218	Unnamed protein	OM	4	61/20	2.10	0.55	11	55/20	1.59	0.17	4	2 (ED)
PG0287f	Unnamed protein	IM/OM	3	77/21	1.32	0.06	7	53/20	1.90	0.21	5	2 (DE)
PG0409	Unnamed protein (conserved)	PP	4	82/22	2.20	0.38	2	69/19	2.28		2	1 (EE)
PG0423	Unnamed protein (conserved)	CY	2	90/23	3.62		1	72/21	0.68		1	4 (FB)
PG0452	Unnamed protein (conserved)	OM	1	53/20	0.39		3	81/20	0.40	0.11	2	1 (BB)
PG0448	Unnamed protein (conserved)	OM	11	74/22	1.05	0.11	10	58/20	1.44	0.08	7	1 (DD)
PG0449	TPR domain protein	PP	2	68/23	1.28		1	64/18	1.19		1	1 (DD)
PG0602	Unnamed protein (conserved)	OM	3	63/21	0.65	0.07	1	37/21	NA**		2	NA
PG0914	Unnamed protein	OM	1	66/21	2.23		1	27/22	2.91		2	2 (EF)
PG1028	TPR domain protein	PP	2	78/21	0.37		5	70/19	0.48	0.08	4	1 (BB)
PG1035	Unnamed protein (conserved)	OM	1	42/21	0.31		5	62/21	1.16	0.16	3	4 (AD)
PG1304	Unnamed protein (conserved)	OM	3	52/20	2.80	1.00	1	34/20	2.93		1	2 (EF)
PG1356	Unnamed protein (conserved)	CY	2	60/21	0.31		1	43/21	0.12		2	1 (AA)

		Biological replicate 1					Biological replicate 2					
Tigr #	Protein	Loca[a]	# peptide for quant	Mascot score[b]	Norm Ratio (B/P)[c]	SEd[d]	# peptide for quant	Mascot score	Norm ratio (B/P)	SE[e]	Total unique peptides	Rank (Group)[e]
PG1382	Unnamed protein (conserved)	OM	3	63/21	1.89	0.29	1	51/21	4.43		2	2 (EF)
PG1493	Unnamed protein (conserved)	OM	6	67/22	1.85	0.15	8	79/17	2.20	0.17	13	1 (EE)
PG1621	Unnamed protein (conserved)	OM	2	51/21	1.10		4	60/21	0.42	0.05	2	2 (CB)
PG1684f	Unnamed protein	EX/IM	1	58/22	1.25		1	73/20	0.79		1	2 (DC)
PG1715	Unnamed protein (conserved)	OM	4	51/20	1.10	0.04	13	71/20	0.97	0.14	3	2 (DC)
PG1881	Unnamed protein (conserved)	OM	2	49/22	0.61		4	48/19	0.82	0.16	3	1 (CC)
PG1889f	Unnamed protein	CY	12	99/20	0.16	0.02	8	85/20	0.25	0.03	6	1 (AA)
PG2049	Unnamed protein	IM/CY	3	48/21	0.63	0.06	1	27/21	0.82		1	1 (CC)
PG2167	Immunoreactive 53 kDa antigen PG123	OM	7	64/20	2.84	0.73	4	45/20	2.26	0.06	3	1 (EE)
PG2168	Unnamed protein (conserved)	UN	3	47/21	4.90	1.97	4	74/21	2.04	0.14	4	2 (FE)
PG2174	Unnamed protein	OM	5	58/21	0.78	0.06	15	102/21	0.37	0.04	7	2 (CB)

a Locations as determined by the CELLO program; EX: Extracellular, OM: Outer membrane, IM: Inner membrane, PP: Periplasm, CY: Cytoplasm; UN: unknown
b Maximum Mascot peptide ion score / identity threshold
c Normalized ratio; B = Biofilm, P= Planktonic, Normalization process as described in experimental procedures
d SE = Standard error of the mean
e Ranking and grouping as described in Fig 4B
f Proteins identified only in this study
! SE measurements not carried out due to unresolved/overlapping of one of the 3 peptides
* Due to presence of identical peptides between these proteins, ratios derived were from peptides that were unique to these proteins only. Values in parenthesis are total number of peptides matched.
** Due to unresolved/overlapping peaks

Table 3. Proteomic and transcriptomic analyses of genes products involved in glutamate/aspartate catabolism in *P. gingivalis* during growth in heme-limitation compared to heme-excess. Shading indicates proteins that are predicted to be encoded in operons.

No	Tigr Acc#	Protein and peptide sequence identified	Score[1]	N[2]	n- ICAT[3]	Proteomics Fold change[4]	SD (±)	Transcriptomics Fold change	Transcriptomics P-value
1	PG0329	*Formiminotransferase-cyclodeaminase-related protein*		2	1	2.9	-	NS[5]	
		. IMEC*VPNFSEGR	30/14						
2	PG0548	*Pyruvate ferredoxin/flavodoxin oxidoreductase family protein*		1	1	2.0	-	NS	
		. IAGELLPC*VFHVSAR	33/15						
3	PG0687	*Succinate-semialdehyde dehydrogenase*		27	6	4.0	1.6	1.77	0.066
		. AFDNGIIC*SGEQSIIYNEADK	37/18						
		. C*SAHAVR	22/16						
		. EYQATHNQEAVDNIC*R	18/13						
		. GVGAEDVIC*K	43/13						
		. NHGAYFC*DEAEGDR	53/14						
		. TC*NAIIIAPHPR	66/13						
4	PG0689	*NAD-dependent 4-hydroxybutyrate dehydrogenase*		12	3	2.8	0.8	1.93	8.892E-05
		. ELIIVPTTC*GTGSEVTNISIAEIK	35/19						
		. ILNC*QPEYVYPK	41/18						
		. LDELLGC*LLTK	35/14						
5	PG0690[6]	*4-hydroxybutyrate-CoA transferase*						3.31	0.03
6	PG0691[6]	*NifU-like protein*						2.60	0.0002
7	PG0692	*4-hydroxybutyryl-CoA dehydratase*		5	2	2.9	0.7	1.65	0.054
		AGNYMIDLLLANVC*K	42/15						
		TASC*FQR	20/15						
8	PG1067	*Hypothetical protein*		2	1	2.4		NS	
		TDISESAADVLDEPIVVC*R	64/14						
9	PG1068	*Conserved hypothetical protein*		3	2	1.7	0.1	NS	
		MIITAAIC*GAEVLK	40/12						
		AVC*PDVIIQFSTGGAVGMTNDER	38/15						
10	PG1075[6]	*Butyrate-acetoacetate CoA transferase*						1.4	0.05
11	PG1076	*Acyl-CoA dehydrogenase, short-chain specific*		3	2	1.8	0.2	NS	
		LYC*AETAMDMTTK	26/14						
		SIAQFQNTQFQLADLQC*R	23/19						
12	PG1078	*Electron transfer flavoprotein, alpha subunit*		5	2	2.0	0.4	NS	
		VTAILC*GYK	22/15						
		TGLTADC*TSLEIGDER	55/15						
13	PG1079[6]	*Enoyl-CoA*						1.3	0.04
14	PG1081	*Acetate kinase*		9	3	3.5	0.9	NS	
		VLVLNC*GSSSVK	42/14						
		AC*EILGLDYDK	29/15						
		VEEC*IPLAPLHNPANEK	42/14						
15	PG1082	*Phosphotransacetylase*		5	2	4.4	1.6	NS	
		AAELVENPLYLGC*LIVK	52/15						

No	Tigr Acc#	Protein and peptide sequence identified	Score[1]	N[2]	n- ICAT[3]	Proteomics Fold change[4]	SD (±)	Transcriptomics Fold change	Transcriptomics P-value
		GC*SVEDIYR	45/15						
16	PG1232	*Glutamate dehydrogenase, NAD-specific*		10	2	2.3	1.2	NS	
		. C*MLDLR	28/14						
		. LRPESTGFGAVYFVQNMC*K	54/15						
17	PG1271	*Ornithine aminotransferase*		3	2	0.09	-	NS	
		. AVIIVC*DGNFHGR	42/19						
		. YFDFLSAYSAVNQGHC*HPK	32/19						
18	PG1417	*Fumarate hydratase class I, anaerobic*		3	2	1.0	0.2	NS	
		. GQLPFC*QDTGTAIILGK	57/15						
		. HGASC*PVGMGVSC*SADR	18/16						
19	PG1612	*Methylmalonyl-CoA decarboxylase, alpha subunit*		2	2	2.4	-	NS	
		. FNGQSVGIVANQPQVMAGC*LDSNASR	28/14						
		. C*TNFGIDK	21/15						
20	PG1614	*Fumarate reductase, iron-sulfur protein (FrdB)*		6	2	0.25	0.1	NS	
		MDELGFGNG*TNTR	45/15						
		APVVFDHDC*R	36/13						
21	PG1615	*Fumarate reductase, flavoprotein subunit (FrdA)*		1	1	0.35	-	NS	
		LAEVSNAIIDQC*VAQGVPFAR	29/14						
22	PG1741	*Aspartate ammonia-lyase*		4	2	1.0	0.2	NS	
		. C*GLHEFNLPAMQPGSSIMPGK	24/14						
		. VNPVIPEVMNQIC*YK	20/15						
23	PG1810	*2-oxoglutarate oxidoreductase, beta subunit*		3	1	2.5	0.5	NS	
		. IADMLALLDGTC*LVTR	54/16						
24	PG1949	*Malate dehydrogenase*		3	1	1.0	0.2	NS	
		. LTPNLC*LYDPFAVGLEGVAEEIR	35/15						

1 Highest scoring peptide score/threshold score (P=0.05)
2 Total number of independent peptide identification events for each protein
3 Number of unique ICAT-labelled peptides identified for each protein
4 Average ratios of all quantified peptides for each protein in fold change (Heme-limitation/excess)
5 NS no statistically significant change detected
6 Only identified in the microarray analysis
C* Denotes ICAT-modified cysteine

Table 4. The 24 *P. gingivalis* polypeptides selected as targets for inhibition of biofilm formation.

Tigr #	Protein	Predicted location	Ratio (B/P) Biol. Rep 1	Ratio (B/P) Biol. Rep 2	Rank (Group)	Accession Number	Accession Number Version
CTD family proteins							
PG0232	Zinc carboxypeptidase, CPG70	OM	1.67	2.53	3 (DF)	AAQ65462	AAQ65462.1
PG0553	Extracellular protease, lysyl endopeptidase precursor (API)	OM/EX	3.33	3.45	1 (FF)	AAQ65742	AAQ65742.1
PG2024	Arginine-specific protease Argl polyprotein (RgpA)	OM	1.63	3.78	3 (DE)	AAQ66991	AAQ66991.1
	Haem binding						
PG0616	Thioredoxin, putative	PP/CY	2.23	3.69	2 (EF)	AAQ65800	AAQ65800.1
	Biofilm related						
PG0350	Internalin-related protein	OM/EX	1.99	4.27	2 (EF)	AAQ65561	AAQ65561.1
	Adhesins						
PG1837	Hemagglutinin protein HagA	OM	2.73	4.21	1 (FF)	AAQ66831	AAQ66831.1
	Protein with unknown functions						
PG1798	Immunoreactive 46 kDa antigen PG99	PP/EX	5.94	4.07	1 (FF)	AAQ66797	AAQ66797.1
Transport							
PG1414	TonB linked outer membrane receptor, PG47	OM	3.81	3.69	1 (FF)	AAQ66469	AAQ66469.1
PG1551	HmuY protein	OM	2.57	2.78	1 (DD)	AAQ66587	AAQ66587.1
PG1626	Possible outer membrane-associated protein P58 (putative haem receptor protein)	OM	2.37	3.68	2 (EF)	AAQ66654	AAQ66654.1
PG2008	TonB-dependent receptor, P90	OM	2.08	3.12	2 (EF)	AAQ66977	AAQ66977.1
Proteins with unknown functions							
	Integral outer membrane proteins						
PG0613	Possible outer membrane associated protein P23	EX	4.41	3.24	1 (FF)	AAQ65797	AAQ65797.1
PG0694	Outer membrane protein 40	OM	3.62	3.39	1 (FF)	AAQ65867	AAQ65867.1
PG0695	Outer membrane protein 41	OM	2.31	3.18	2 (EF)	AAQ65868	AAQ65868.1
	Others proteins						
PG0181	Immunoreactive 32 kDa antigen PG49	PP/OM	2.08	3.14	2 (EF)	AAQ65416	AAQ65416.1
PG0218	Unnamed protein	OM	2.10	1.59	2 (ED)	AAQ65449	AAQ65449.1

Tigr #	Protein	Predicted location	Ratio (B/P) Biol Rep 1	Ratio (B/P) Biol Rep 2	Rank (Group)	Accession Number*	Accession Number Version
PG0914	Unnamed protein	OM	2.23	2.91	2 (EF)	AAQ66051	AAQ66051.1
PG1304	Unnamed protein (conserved)	OM	2.80	2.93	2 (EF)	AAQ66377	AAQ66377.1
PG1382	Unnamed protein (conserved)	OM	1.89	4.43	2 (EF)	AAQ66444	AAQ66444.1
PG1493	Unnamed protein (conserved)	OM	1.85	2.20	1 (EE)	AAQ66538	AAQ66538.1
PG2167	Immunoreactive 53 kDa antigen PG123	OM	2.84	2.26	1 (EE)	AAQ67117	AAQ67117.1
PG2168	Unnamed protein (conserved)	UN	4.90	2.04	2 (FE)	AAQ67118	AAQ67118.1
	Energy Metabolism						
PG1614	Fumarate reductase, iron-sulfur protein (frdB)	UN	0.15	0.19	1(AA)	AAQ66642	AAQ66642.1
PG1615	Fumarate reductase, flavoprotein subunit (frdA)	UN	0.06		NA	AAQ66643	AAQ66643.1

* These accession numbers provide a sequence for the *P. gingivalis* proteins referred to in the specification. Sequences corresponding to the accession numbers are incorporated by reference.

Table 5: Proteomic and transcriptomic analyses of *P. gingivalis* grown in heme-limitation compared to heme-excess. Shading indicates proteins that are predicted to be encoded in operons.

No	Tigr Acc#	Protein and peptide sequence identified	Score[1]	N[2]	n- ICAT[3]	Proteomics Fold change[4]	SD (±)	Transcriptomics Fold change	Transcriptomics P-value
Iron transport and related proteins									
13	PG0644[6]	*HtrB (Tla) TonB-linked receptor*						2.05	1.54E-04
18	PG1552	*HmuR*		1	1	4.0		3.13	0.003
		MNSDELFEEITYPGYTIC*R	25/15						
21	PG1019	*Hypothetical protein*		2	1	25.0		2.57	0.003
		TYMIDTNDSENDC*IAR	70/14						
22	PG1020[6]	Conserved hypothetical protein; possible outer membrane receptor protein						3.36	3.0E-04
Others									
33	PG1874[6]	*Conserved hypothetical protein*						1.52	0.05
34	PG1875[6]	*Hemolysin A*						1.40	0.05

1 Highest scoring peptide score/threshold score (P=0.05)
2 Total number of independent peptide identification events for each protein
3 Number of unique ICAT-labelled peptides identified for each protein
4 Average ratios of all quantified peptides for each protein in fold change (Heme-limitation/excess)
5 NS no statistically significant change detected
6 Only identified in microarray analysis
C* Denotes ICAT-modified cysteine

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CLAIMS

1. A composition for use in raising an immune response to *P. gingivalis* in a subject, the composition comprising an amount effective to raise an immune response of at least one antigenic or immunogenic portion of a polypeptide corresponding to accession numbers selected from the group consisting of AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117 and AAQ67118.

2. A composition of claim 1 wherein the portion has an amino acid sequence that is substantially identical to at least 50 amino acids of one of the polypeptides.

3. A composition of claim 1 or 2 wherein the polypeptide corresponds to an accession number selected from the group consisting of AAQ65462, AAQ66991, AAQ65561 and AAQ66831.

4. A composition of claim 1 or 2 wherein the polypeptide corresponds to accession number AAQ65742.

5. A composition for use in raising an immune response to *P. gingivalis* in a subject, the composition comprising amount effective to raise an immune response of at least one polypeptide having an amino acid sequence substantially identical to at least 50 amino acids of a polypeptide expressed by *P. gingivalis* and that is predicted by the CELLO program to be extracellular.

6. A composition for use in raising an immune response to *P. gingivalis* in a subject, the composition comprising an amount effective to raise an immune response of at least one polypeptide having an amino acid sequence selected substantially identical to at least 50 amino acids of a polypeptide that causes an immune response in a mouse or a rabbit.

7. A method of preventing, inhibiting or treating a subject for periodontal disease comprising administering to the subject an effective amount of composition according to any one of claims 1 to 6.

8. A method of preventing or treating a subject for *P.gingivalis* infection comprising administering to the subject a composition according to any one of claims 1 to 7.

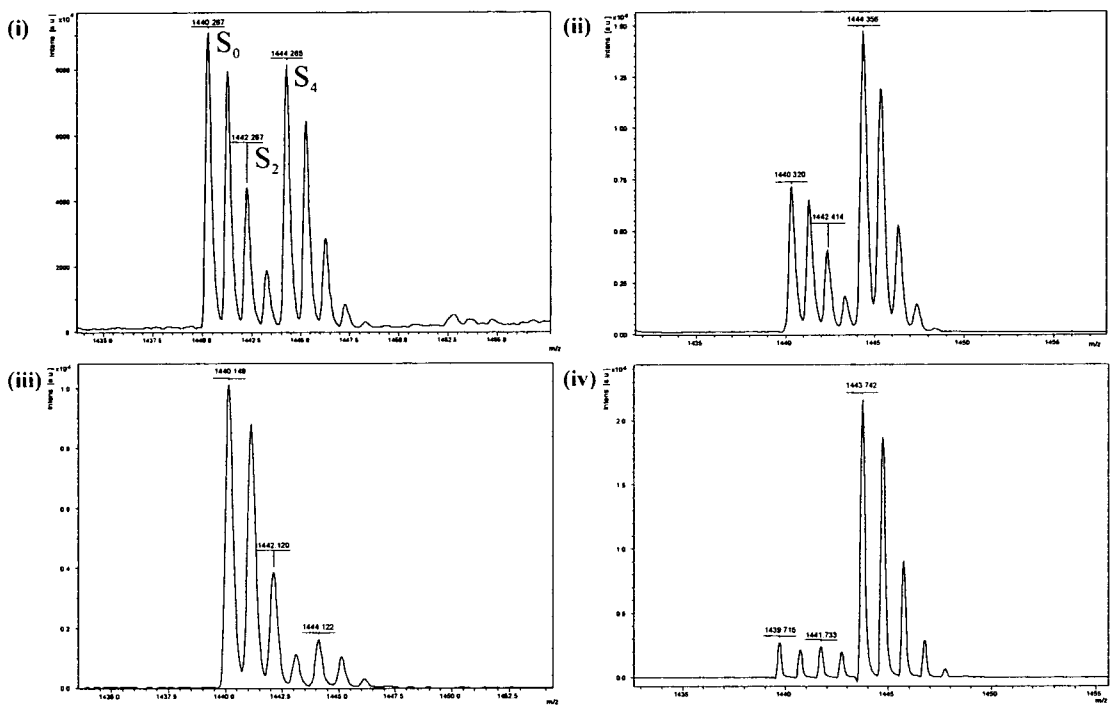
9. An antibody raised against an antigenic region of a polypeptide having an amino acid sequence, the sequence being substantially identical to at least 50 amino acids of one of the polypeptides corresponding to accession numbers AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117 and AAQ67118.

10. An antibody of claim 9 wherein the polypeptide corresponds to an accession number selected from the group consisting of AAQ65462, AAQ66991, AAQ65561 and AAQ66831.

11. An antibody of claim 9 wherein the polypeptide corresponds to an accession number selected from the group consisting of AAQ65742.

Figure 1

A


(i)
1440.287
S0
1444.285
S4
1442.287
S2
(ii)
1444.356
1440.320
1442.414
(iii)
1440.148
1442.120
1444.122
(iv)
1443.742
1439.715
1441.733

B

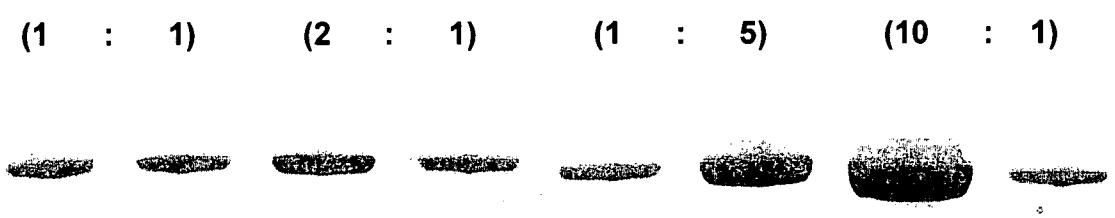

(1 : 1)
(2 : 1)
(1 : 5)
(10 : 1)

Figure 2

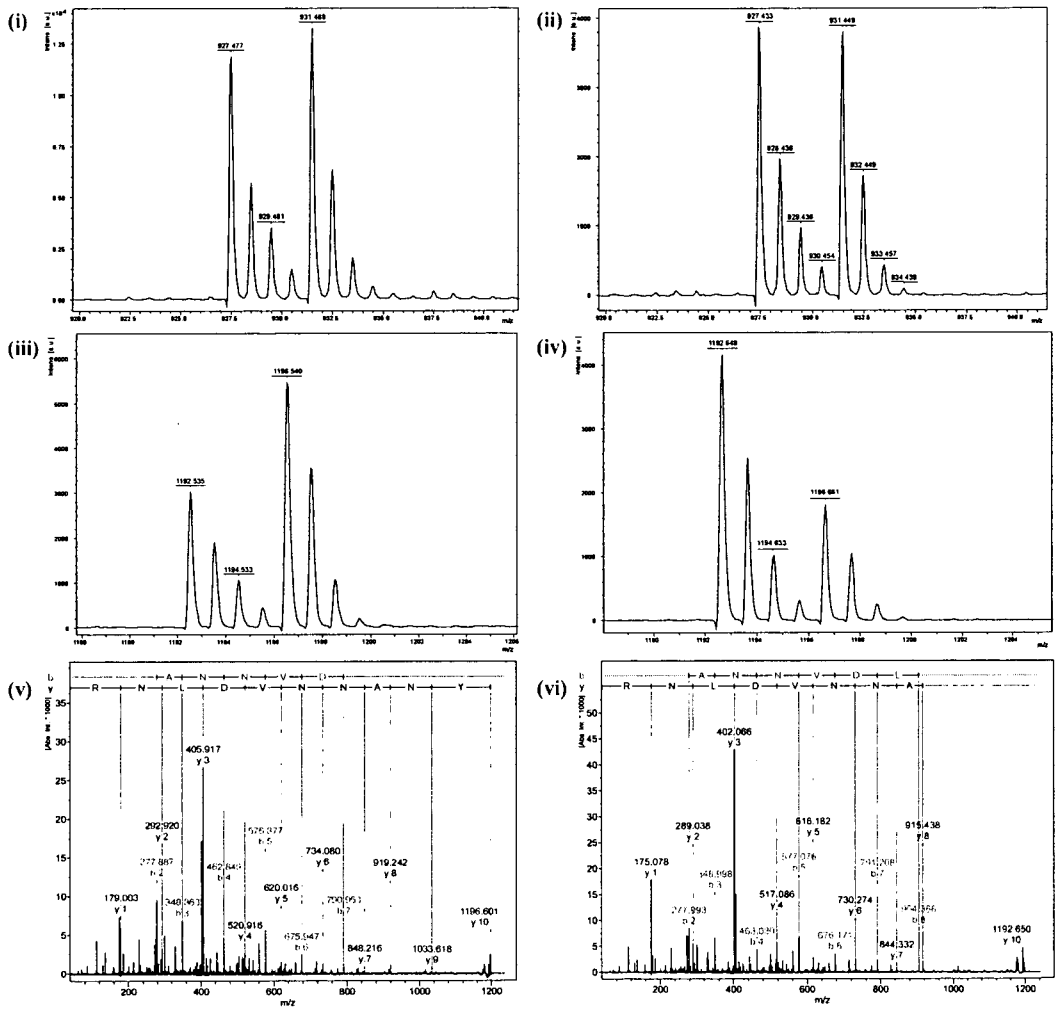
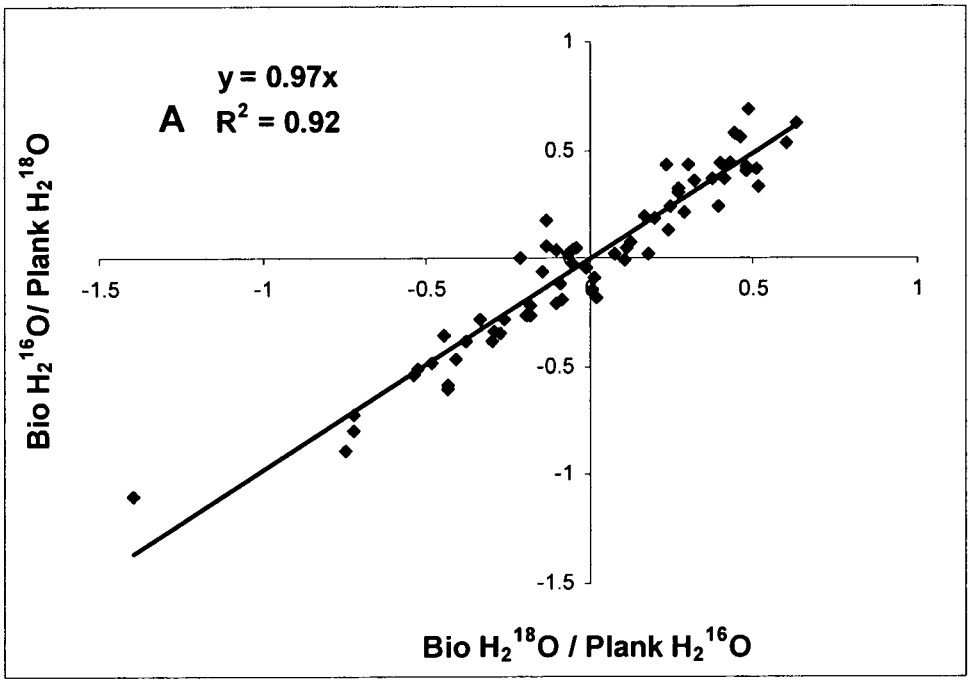

(i)
927.477
931.469
929.481
(ii)
927.433
931.449
929.438
932.449
930.454
933.457
934.438
(iii)
1196.540
1192.535
1194.533
(iv)
1192.648
1194.653
1196.661
(v)
R N L D V N N A N Y
405.917 y 3
292.920 y 2
734.080 y 6
919.242 y 8
620.016 y 5
179.003 y 1
520.918 y 4
848.216 y 7
1033.618 y 9
1196.601 y 10
m/z
(vi)
A N N V D L
R N L D V N N A
402.086 y 3
289.038 y 2
616.182 y 5
915.438 y 8
175.078 y 1
517.086 y 4
730.274 y 6
1192.650 y 10
m/z

Figure 3


A
y = 0.97x
R2 = 0.92
Bio H2 16O/ Plank H2 18O
Bio H2 18O / Plank H2 16O
1
0.5
-0.5
-1
-1.5
-1.5
-1
-0.5
0.5
1

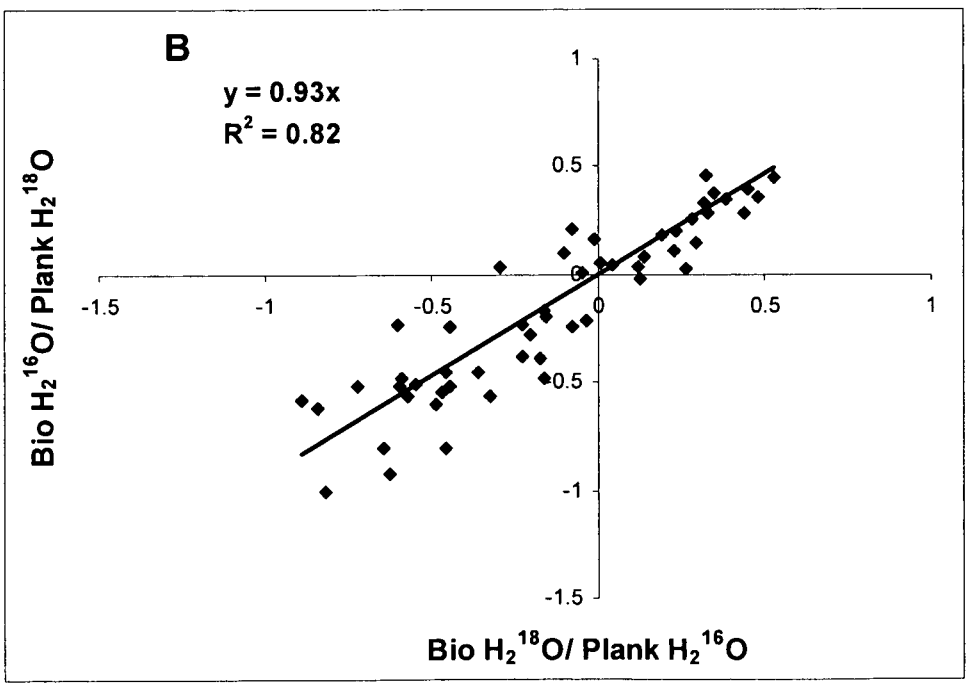

B
y = 0.93x
R2 = 0.82
Bio H2 16O/ Plank H2 18O
Bio H2 18O/ Plank H2 16O
1
0.5
0
-0.5
-1
-1.5
-1.5
-1
-0.5
0
0.5
1

Figure 4

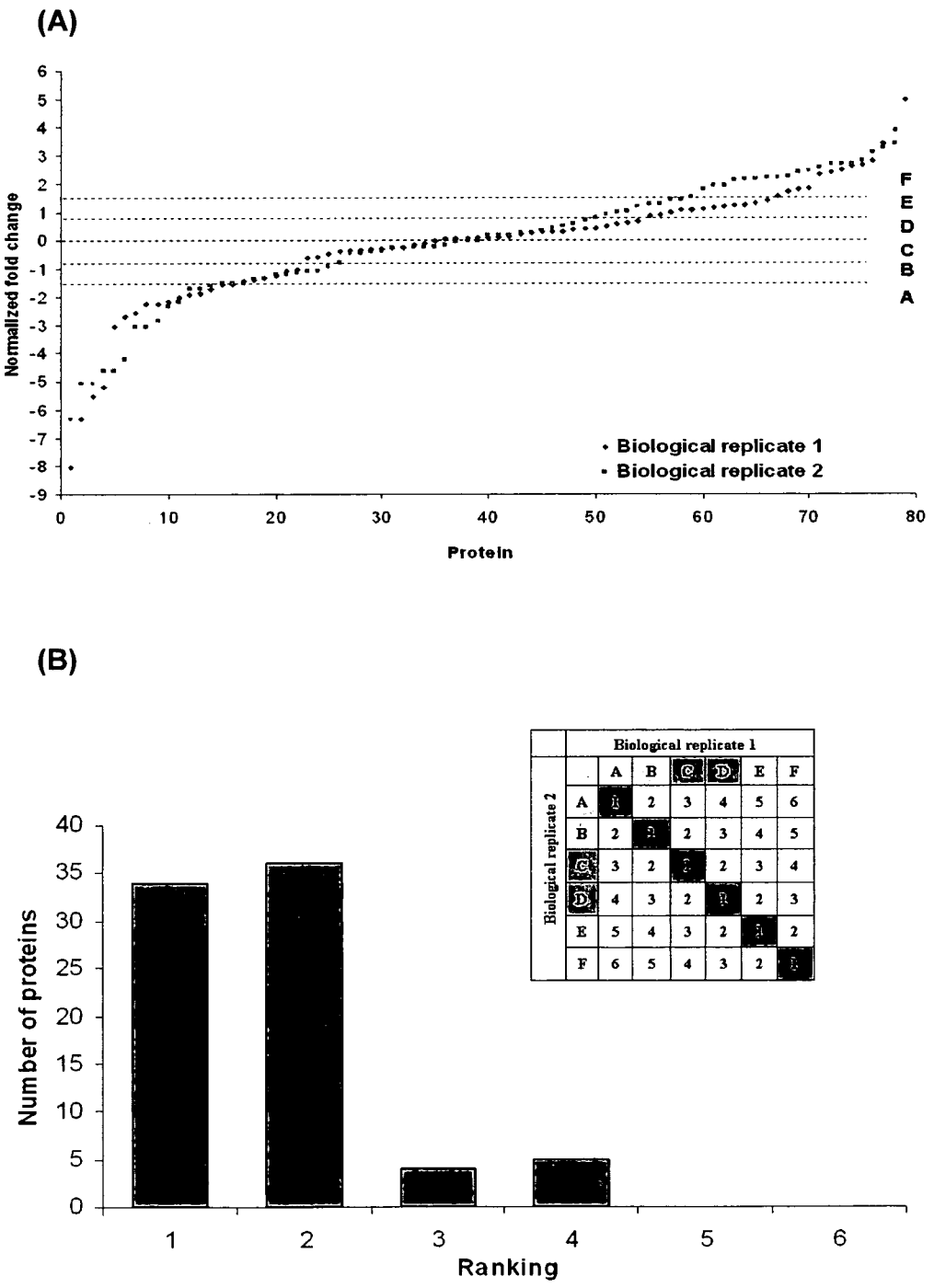
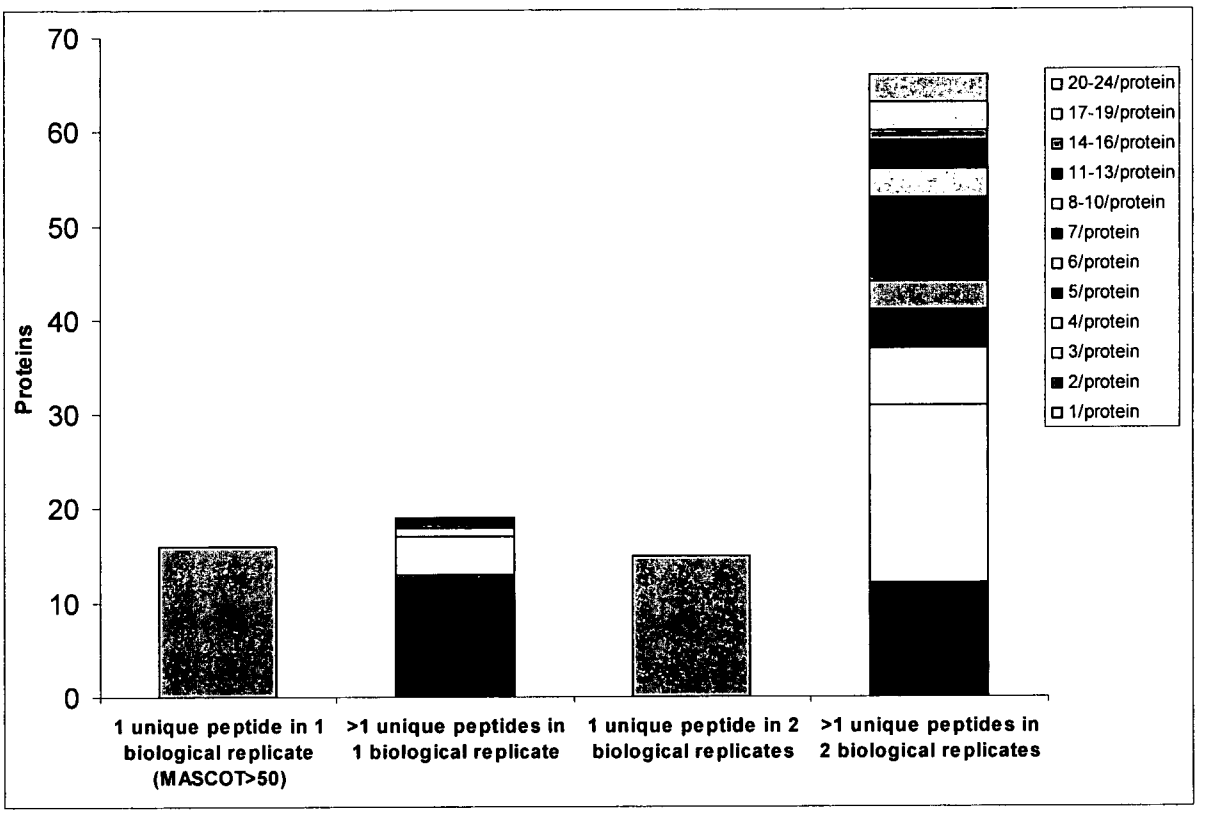

(A)
Normalized fold change
Protein
Biological replicate 1
Biological replicate 2
F
E
D
C
B
A
(B)
Biological replicate 1
Biological replicate 2
Number of proteins
Ranking

Figure 5


70
60
50
40
30
20
10
0
Proteins
1 unique peptide in 1 biological replicate (MASCOT>50)
>1 unique peptides in 1 biological replicate
1 unique peptide in 2 biological replicates
>1 unique peptides in 2 biological replicates
20-24/protein
17-19/protein
14-16/protein
11-13/protein
8-10/protein
7/protein
6/protein
5/protein
4/protein
3/protein
2/protein
1/protein

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001018

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 15/31 (2006.01) *A61K 38/16* (2006.01) *A61K 39/02* (2006.01) *C07K 14/195* (2006.01)
A61K 39/40 (2006.01) *A61P 43/00* (2006.01) *A61K 38/00* (2006.01) *A61K 39/00* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GENOMEQUEST, MEDLINE, CAPLUS, WPIDS: (AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117, AAQ67118, Porphyromonas gingivalis, immune, extracellular, periodontal, antibody)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1999029870 A1 (CSL LIMITED [AU/AU]) 17 June 1999 (See abstract, page 1 lines 5-10, page 7 lines 34-36, page 8 lines 5-11, page 14, lines 4-10 and claims 1-10 and 16. See also SEQ ID NO: 415, 426, 437, 438, 441, 455, 457, 463, 518 & 528)	1-11
X	WO 2005019249 A2 (UNIVERSITY OF FLORIDA [US/US]) 03 March 2005 (See abstract and pages 5, 6 and 28-30. See also SEQ ID NO 186, 285 & 286)	1-3 & 6-10
X	US 7204991 B2 (BARR et al) 17 April 2007 (See abstract and column 3, lines 33-55, Example 2 and claims. See also SEQ ID 3&4)	1-2 & 6-9

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2008	12 SEP 2008
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **RAMILA DEWALAGAMA** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6222 3659

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU2008/001018

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nelson K.E et al, 'Complete genome sequence of the oral pathogenic bacterium Porphyromonas gingivalis Strain W83' Journal of Bacteriology. 2003, 185(18):5591-5601. GenPet Accession No: AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117, AAQ67118 (02 September 2003) (See whole document)	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001018

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

[See Supplemental Box 1]

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001018

Supplemental Box 1
(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. In coming to this conclusion the International Searching Authority has considered PCT Rule 13.2, which states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features", where the "special technical features" make a contribution over the prior art. The international searching authority has identified 21 separate inventions.

The claims are directed to a composition for use in raising an immune response to *P.gingivalis* in a subject, the composition comprising an amount effective to raise an immune response to one of 21 polypeptides listed in claim 1. The claims are also directed to an antibody raised against an antigenic region of a polypeptide having an amino acid sequence, the sequence being substantially identical to one of 21 polypeptides listed in claim 9. Each of the 21 polypeptides represents a special technical feature.

The only common feature linking the polypeptides identified in claims 1 and 9 are that they are derived from *P. gingivalis*. Polypeptides with accession numbers AAQ65462, AAQ65742, AAQ66991, AAQ65561, AAQ66831, AAQ66797, AAQ66469, AAQ66587, AAQ66654, AAQ66977, AAQ65797, AAQ65867, AAQ65868, AAQ65416, AAQ65449, AAQ66051, AAQ66377, AAQ66444, AAQ66538, AAQ67117, AAQ67118 derived from *P. gingivalis* are already known from the following citations and therefore is not considered to be a special technical feature.

- WO1999/29870 A1 (CSL, LIMITED AU/AU) 17 June 1999
- WO2005/019249 A2 (UNIVERSITY OF FLORIDA US/US) 3 March 2005
- US7204991 B2 (CSL Limited) 17 April 2007
- Nelson, K.E et al, 2003. Journal of Bacteriology. 185(18):5591-5601.

Please note that WO1999/29870, WO2005/019249 and US7204991 further discloses compositions for use in raising an immune response directed against *P. gingivalis* in a subject, the compositions comprising an effective amount of a polypeptide (which correspond to the polypeptides claimed in the instant claims). Each of these citations also discloses antibodies which are raised against these polypeptides.

Therefore, the invention is not linked so as to form a single inventive concept. Hence, it is considered that each claimed polypeptide in the present application represents a separate invention. Therefore, a lack of unity exists and the claims appear to represent 21 separate inventions.

Furthermore, in relation to the claimed polypeptides, it is appropriate to apply the Markush approach. In using the Markush approach to analyse the unity of invention, it is noted that although the 21 polypeptides share a common source (*P. gingivalis*), the polypeptides do not share any significant structural element or a single recognised class embracing all the polypeptides claimed. Therefore, according to Markush practice, it is appropriate to classify each polypeptide individually.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/001018

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9929870	AU	16487/99	CA	2313823	EP	1037999
		EP	1681350	EP	1908772	NZ	504811
		US	6444799	US	2002172976	US	2007189981
		ZA	9811333				
WO	2005019249	AU	2004266213	CA	2535799	EP	1660524
		US	2006078950				
US	7204991	AU	52042/01	CA	2407603	EP	1276762
		NZ	521797	US	2003215402	WO	0183530

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX