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(54) Title: PORPHYROMONAS GINGIVALIS POLYPEPTIDES USEFUL IN THE PREVENTION OF PERIODONTAL DISEASE

(57) Abstract: The invention is directed to vaccine compositions and methods based on *P. gingivalis* proteins identified to be regulated by haem availability that can be used in the prevention and treatment of periodontal disease. In particular, two specific internalin-like *P. gingivalis* proteins, namely PG0350 and PG1374 involved in the internalization of *P. gingivalis* by host cells, the hypothetical protein, PG1019 purported to be a cell surface lipoprotein and the alkyl hydroperoxide reductase protein, PG0618 have been identified as useful targets for the prevention and treatment of periodontal disease.

**PORPHYROMONAS GINGIVALIS POLYPEPTIDES USEFUL IN THE  
PREVENTION OF PERIODONTAL DISEASE**

**FIELD OF THE INVENTION**

The present invention relates to compositions and methods of isolating *Porphyromonas gingivalis* (*P. gingivalis*) proteins useful in the prevention of and treatment of periodontal disease. More particularly, the invention is directed to vaccine compositions and methods based on *P. gingivalis* proteins identified to be regulated by haem availability that can be used in the prevention and treatment of periodontal disease.

**BACKGROUND OF THE INVENTION**

10 Periodontal disease is a chronic bacterial infection that affects the gums and bone supporting the teeth. Periodontal disease begins when the bacteria in plaque (the sticky biofilm that constantly forms on teeth) causes the gums to become inflamed. Periodontal disease can affect the gingival tissue (gums); periodontal membrane (connective tissue embedded in the cementum and alveolar bone); cementum (mineralized connective tissue covering the roots of 15 the teeth); and the alveolar bone (bone socket). Depending on the progression of the disease, there may occur a destruction of periodontal membranes, alveolar bone loss, and apical migration of the connective tissue attachment. Advanced periodontal disease may result in the formation of periodontal pockets harbouring bacterial plaque, and progressive loosening and eventual loss of teeth. Periodontal disease includes gingivitis that can advance to 20 periodontitis. Chronic periodontitis is an inflammatory disease of the supporting tissues of teeth that is associated with specific bacteria in subgingival dental plaque. The disease has been estimated to affect around 35% of dentate adults and is a major cause of tooth loss in the Western world<sup>(1)</sup>. *P. gingivalis*, a member of the normal oral microflora of subgingival dental 25 plaque, has been implicated as one of the major opportunistic pathogens in the progression of this disease<sup>(2)</sup>.

*P. gingivalis* is a black-pigmented, asaccharolytic, Gram-negative anaerobic, cocco-bacillus, that relies on the fermentation of amino acids for energy production<sup>(3)</sup>. Like most bacteria, *P. gingivalis* has an essential growth requirement for iron that it preferentially acquires in the

form of haem, a molecule comprised of a protoporphyrin IX ring (PPIX) with a co-ordinated central ferrous atom<sup>(4)</sup>. This utilization of haem as an iron source may reflect the inability of *P. gingivalis* to synthesize PPIX de novo<sup>(5)</sup>. Haem is preferentially obtained from

5 haemoglobin, and is acquired through the activity of the cell-surface Arg- and Lys-specific proteinase/adhesin complex<sup>(4,6,7)</sup>, possibly in conjunction with a TonB-linked outer membrane receptor, HmuR<sup>(8)</sup>. Unlike aerobic or facultative bacteria that obtain iron using siderophores *P. gingivalis* does not produce siderophores and lacks the ferric reductase activity usually associated with siderophore-mediated iron acquisition<sup>(9,10)</sup>. *P. gingivalis* stores haem on its surface in the form of  $\mu$ -oxo bis-haem, which has inherent catalase activity that helps to

10 protect the cell from oxidative attack<sup>(11)</sup>. For *P. gingivalis* to be able to compete with the large numbers and diversity of bacteria within the micronutrient-limiting environment of the oral cavity<sup>(12)</sup> it not only has to establish itself but also has to evade or overcome numerous host defences.

15 The initiation and progression of periodontal disease is associated with bleeding at the site of disease, thereby providing an elevated level of haemoglobin. Therefore in order to help understand the mechanism by which *P. gingivalis* establishes and proliferates in subgingival plaque and initiates disease it is important to determine the changes in relative protein abundances of *P. gingivalis* during the transition from micronutrient poor (haem-limitation) to rich (haem-excess) conditions.

20 Although many proteins have been associated with growth under haem-limitation<sup>(9,13)</sup>, no extensive work on the *P. gingivalis* proteome or the changes to the proteome during haem-limitation has been reported.

25 In developing compositions which would be useful in the prevention and treatment of periodontal disease it is desirable to identify agents that interfere and prevent the initial stages of the disease process.

The present inventors have now developed methods for identifying specific *P. gingivalis* proteins regulated by haem availability that can be used as suitable targets for the prevention and treatment of periodontal disease.

**SUMMARY OF THE INVENTION**

The present inventors have successfully developed methods of identifying *P. gingivalis* proteins regulated by haem availability that are responsible for *P. gingivalis* metabolism, virulence and invasion of host cells. In particular, two specific internalin-like *P. gingivalis* proteins, namely PG0350, PG1374 involved in the internalization of *P. gingivalis* by host cells, a hypothetical protein, PG1019 purported to be a cell surface lipoprotein and an alkyl hydroperoxide reductase protein, PG0618 have been identified as useful targets for the prevention and treatment of periodontal disease.

5 A first aspect of the invention is an isolated antigenic *P. gingivalis* polypeptide, the polypeptide being selected from the group consisting of:

- (i) the PG0350 protein having the amino acid sequence of SEQ ID NO:1;
- (ii) the PG1374 protein having the amino acid sequence of SEQ ID NO:2;
- (iii) the PG1019 protein having the amino acid sequence of SEQ ID NO:3;
- (iv) the PG0618 protein having the amino acid sequence of SEQ ID NO:4;
- 10 (v) an amino acid sequence at least 85% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4; or
- (vi) an amino acid sequence comprising at least 10 amino acids identical to a contiguous amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.

20 A second aspect of the invention is a vaccine 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 and a pharmaceutically acceptable carrier.

A third aspect of the invention is a method of preventing or treating a subject for periodontal disease comprising administering to the subject a vaccine composition according to the present invention.

5 A fourth aspect of the invention is an antibody raised against a polypeptide of the first aspect of the present invention. Preferably, the antibody binds specifically to the polypeptides of the present invention.

A fifth aspect of the invention is a composition useful in the prevention or treatment of periodontal disease, the composition comprising an antibody of the fourth aspect of the present invention and a pharmaceutically acceptable carrier.

10 In a sixth aspect of the present invention there is provided a method of identifying a *P. gingivalis* polypeptide involved in the progression of periodontal disease, wherein the method comprises the steps of:

a) determining the relative amount of a polypeptide or peptide thereof produced by *P. gingivalis* grown under haem limited conditions; and

15 b) determining the relative amount of the polypeptide or peptide thereof produced by *P. gingivalis* grown under higher haem conditions than step a);

wherein an increase in the amount of the polypeptide or peptide fragment thereof detected in step a) compared to step b) indicates that the polypeptide is involved in the progression of periodontal disease.

20 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 SEQ ID NO: 5 or SEQ ID NO: 6 or SEQ ID NO: 7 or SEQ ID NO: 8.

25 In an eighth aspect of the present invention there is provided for the use of at least one polypeptide of the first aspect of the present invention in the manufacture of a medicament for the treatment of periodontal disease in a subject.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows a diagrammatic representation of the combined strategy used to identify proteins from *P. gingivalis* grown under haem-limitation. The lysed cells were prefractionated into soluble and insoluble fractions using ultra-centrifugation, followed by 5 analysis of these two fractions. The separation and analysis procedure consists of two main methods; LCMS with gas phase fractionation and geLCMS.

Figure 2 shows a graph indicating the codon adaptation index (CAI) distribution of the identified *P. gingivalis* proteome and the predicted *P. gingivalis* genome calculated using INCA<sup>(14)</sup>. Ribosomal and tRNA synthases were defined as highly expressed genes. Genes 10 with less than 100 codons were excluded, bringing the total calculated genes to 1685.

Figure 3 shows the analysis and identification of PG0390 based on detection of a single peptide (A) Total ion chromatogram. (B) Mass spectrum at 54.8min, insert showing an enlarged ICAT peptide ion pair at 703.9 and 708.4 m/z of ratio 1:2 (L/H). (C) Product ion spectrum for precursor 703.9 m/z. This peptide ion was identified as having the sequence 15 LVDLNC\*FDIK (MASCOT score = 40; C\* denotes ICAT modified cysteine).

Figure 4 shows the distribution of protein abundance based on ratio of haem-limitation over haem-excess (every one unit on the Log2 scale indicates a two fold change).

Figure 5 shows binding to KB cells by *P. gingivalis* W50 (♦) and ECR312 (■). The assay was carried out with two biological replication (n=6). Insert shows the gating of live KB cells 20 based on forward and side scattering properties (top left), five peaks representing FITC fluorescence of bound *P. gingivalis* W50 to KB cells at *P. gingivalis*:KB cell ratios of 250 to 1250 (top right) and ECR312 (bottom right).

Figure 6 shows the amino acid sequence of PG0350 protein (referred to as SEQ ID NO:1 as also indicated in the sequence listing).

25 Figure 7 shows the amino acid sequence of PG1374 protein (referred to as SEQ ID NO:2 as also indicated in the sequence listing).

**Figure 8** shows the amino acid sequence of PG1019 protein (referred to as SEQ ID NO:3 as also indicated in the sequence listing).

**Figure 9** shows the amino acid sequence of PG0618 protein (referred to as SEQ ID NO:4 as also indicated in the sequence listing).

## 5 DETAILED DESCRIPTION OF THE INVENTION

The present invention advantageously provides the identification of *P. gingivalis* proteins regulated by haem availability as useful targets for the prevention and treatment of periodontal disease. Preferably, the invention provides the identification of *P. gingivalis* proteins upregulated by haem-limitation as useful targets for the prevention and treatment of periodontal disease.

In particular, the invention provides an isolated antigenic *P. gingivalis* polypeptide, the polypeptide being selected from the group consisting of:

- (i) the PG0350 protein having the amino acid sequence of SEQ ID NO:1;
- (ii) the PG1374 protein having the amino acid sequence of SEQ ID NO:2;
- (iii) the PG1019 protein having the amino acid sequence of SEQ ID NO:3;
- (iv) the PG0618 protein having the amino acid sequence of SEQ ID NO:4;
- (v) an amino acid sequence at least 85% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4; or
- (vi) an amino acid sequence comprising at least 10 amino acids identical to a contiguous amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.

Preferably, the isolated antigenic polypeptide is 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.

Preferably, the isolated antigenic polypeptide comprises an amino acid sequence comprising at least 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids identical to a contiguous amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.

5 In a preferred embodiment, the antigenic polypeptides comprise amino acid sequences that compose the hydrophilic, surface-exposed regions of the PG1374 or PG0350 or PG1019 or PG0618 protein.

10 The terms "peptides, proteins, and polypeptides" are used interchangeably herein. The polypeptides of the present invention can include recombinant polypeptides such as fusion polypeptides. Methods for the production of a fusion polypeptide are well-known to those skilled in the art.

15 As will be well understood by those skilled in the art alterations may be made to the amino acid sequences set out in the Sequence Listings. 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 set out in the Sequence Listings.

20 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;

25 Ser, Thr;

Lys, Arg, His;

Phc, Tyr, Trp, His; and

Pro, N $\alpha$ -alkalainino acids.

The practice of the invention will employ, unless otherwise indicated, conventional techniques of chcmistry, 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, Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984)<sup>(15)</sup>, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989)<sup>(16)</sup>, Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991)<sup>(17)</sup>, Glover & 5 Haines (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996)<sup>(18)</sup>, and Ausubel et al., (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present)<sup>(19)</sup>. 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 20% of dry weight of the purified preparation. Preferably, the preparation contains a sufficient amount of polypeptide to allow for protein sequencing (i.e. 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 25 utilizing antibodies specific for the protein or other ligands which bind to the protein.

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.

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

- 5 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
- 10 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
- 15 Research Park, Madison, Wisconsin, United States of America<sup>(20)</sup>. The GAP programme utilizes the algorithm of Needleman and Wunsch<sup>(21)</sup> 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<sup>(22)</sup> is used.
- 20 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 effective amount of at least one polypeptide of the first aspect of the invention and a pharmaceutically acceptable carrier.

- 25 The vaccine composition of the present invention preferably comprises an antigenic polypeptide that comprises at least one antigen that can be used to confer immunity 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 the subject's immune system produces antibodies against the specific antigenic polypeptides.

The vaccine composition is preferably administered to a subject to induce immunity to *P. gingivalis* and thereby prevent periodontal disease. The term "effective amount" as used

5 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 vaccine composition of the present invention comprises a suitable pharmaceutically-acceptable carrier, such as diluent and/ or adjuvant suitable for administration to a human or 10 animal subject. The vaccine preferably comprises a suitable adjuvant for delivery orally by nasal spray, or by injection to produce a specific immune response against *P. gingivalis*. A vaccine 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 (e.g.

15 *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, COS cells, CHO cells and HeLa cells) containing the vector. The vaccine 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 20 against periodontal disease and infections caused by *P. gingivalis*.

Various adjuvants known those skilled in the art are commonly used in conjunction with vaccine formulations. 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

25 include incomplete Freunds adjuvant (IFA), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminium monostearate), 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 30 but exhaustive list of other examples of adjuvants are listed in Cox and Coulter 1992<sup>(23)</sup>. 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 the vaccine containing adjuvant may be administered prophylactically to prevent periodontal disease or therapeutically to treat already present periodontal disease.

5 In another preferred vaccine 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  
10 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  
15 antigenic polypeptides to the mucosal immune system. In addition to the vaccine and the mucosal adjuvant or delivery system the vaccine 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,  
20 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 cause disease by itself, is  
25 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.

Other live vaccine vectors include: adenovirus, cytomegalovirus, and preferably the poxviruses such as vaccinia<sup>(24)</sup> and attenuated salmonella strains<sup>(25-28)</sup>. Live vaccines are  
30 particularly advantageous because they continually stimulate the immune system which can

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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 5 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 10 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 15 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 20 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 stearate 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.

25 A third aspect of the invention is a method of preventing or treating a subject for periodontal disease comprising administering to the subject a vaccine composition according to the present invention.

In the present method a subject is treated 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. Bacteria, mainly Gram-negative species including *P. gingivalis* cause 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 5 "periodontitis" (which means "inflammation around the tooth."). In periodontitis, gums pull away from the teeth and form "pockets" that are infected. 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.

10 A fourth aspect of the invention 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 15 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 20 antigen by an immunoglobulin variable region of an antibody with a dissociation constant (Kd) of 1 $\mu$ M or lower as measured by surface plasmon resonance analysis using, for example a BIAcore<sup>TM</sup> surface plasmon resonance system and BIAcore<sup>TM</sup> kinetic evaluation software (e.g. 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 25 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)).

10 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.

15 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 aluminum hydroxide, surface active substances such as lysolecithin, pluronics 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)

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

25 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<sup>(29, 30)</sup>. The recently described "gene conversion metagenesis" strategy for the production of humanized monoclonal antibody may also be

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employed in the production of humanized antibodies<sup>(31)</sup>. Alternatively, techniques for generating the recombinant phage library of random combinations of heavy and light regions may be used to prepare recombinant antibodies<sup>(32)</sup>.

5 The present invention also provides a composition useful in the prevention or treatment of periodontal disease, the composition comprising an antagonist of a *P. gingivalis* polypeptide of the first aspect of the present invention and a pharmaceutically acceptable carrier, wherein the antagonist inhibits *P. gingivalis* infection.

10 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. Possible antagonists of PG0350, PG1374, PG1019 and PG0618 are preferably antibodies, either monoclonal or polyclonal, which will inhibit the binding of these proteins to host cells or other substrates or they may be proteins or peptides that interfere with the binding of these 15 proteins.

20 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).

In a sixth aspect of the present invention there is provided a method of identifying a *P. gingivalis* polypeptide involved in the progression of periodontal disease, wherein the method comprises the steps of:

25 a) determining the relative amount of a polypeptide or peptide thereof produced by *P. gingivalis* grown under haem limited conditions; and

b) determining the relative amount of the polypeptide or peptide thereof produced by *P. gingivalis* grown under higher haem conditions than step a);

wherein an increase in the amount of the polypeptide or peptide fragment thereof detected in step a) compared to step b) indicates that the polypeptide is involved in the progression of periodontal disease.

In order to grow *P. gingivalis* under haem limited conditions, it is preferred that the concentration of haemin is about 0.1  $\mu$ g/ml to about 0.5  $\mu$ g/ml. Haem limiting conditions are achieved when the cell density of the *P. gingivalis* cells is significantly lower than that observed under the growth conditions of step (b) of the method of the invention. The higher haem conditions of step (b) is preferably achieved using a concentration of haemin of above 5  $\mu$ g/ml.

10 A comparison of the relative amounts of a polypeptide in the haem limited and higher haem conditions can be preferably determined by using a differential proteomic approach. Preferably, the amount of a polypeptide is determined by qualitative proteomic analysis commonly used in the art, such as but not limited to, a combined strategy of in-solution and in-gel digestion and LC-MS/MS, analysis using stable isotope labelling strategies (ICAT) in 15 combination with MS.

The isolated antigenic *P. gingivalis* polypeptides identified according to the method defined in the sixth aspect of the invention can be used as targets for treating and preventing periodontal disease. In particular, the isolated polypeptides can be used to develop vaccine compositions against *P. gingivalis*, for instance *P. gingivalis* infection, such as periodontal disease.

20 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 25 of SEQ ID NO: 5 or SEQ ID NO: 6 or SEQ ID NO: 7 or SEQ ID NO: 8.

So called RNA interference or RNAi is well 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*

5 *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 10 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.

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 example.

15 **EXAMPLE 1**

**1. Materials and Methods**

**1.1 Bacterial strain and chemicals**

*P. gingivalis* W50 (ATCC 53978) was obtained from the culture collection of the Centre for

Oral Health Science, The University of Melbourne. Chemicals used were ultra high purity

20 except for MS work where LC MS grade reagents were used (Sigma, Riedel-de Haen).

**1.2 Growth and harvesting of *P. gingivalis***

*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/mL brain heart infusion medium (Oxoid) supplemented with 5 mg/mL filter sterilized

25 cysteine hydrochloride, 5.0  $\mu$ g/mL haemin (haem-excess) or 0.1  $\mu$ g/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<sup>-1</sup> (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<sub>2</sub> in 95% N<sub>2</sub>. 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<sub>2</sub>) 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.

### 1.3 Preparation of samples for non-quantitative proteome analysis

Non-quantitative proteome analysis was carried out using two methods, in-solution digestion with trypsin followed by LCMS with gas phase fractionation (GPF) and in-gel digestion followed by LCMS (geLC-MS) as part of a combined strategy (Figure 1). For the in-solution digestion method, protein was boiled at 95°C for 3 min, precipitated with TCA (16%) and resuspended in solubilization buffer (8 M Urea, 50 mM Tris-HCl pH 8.3, 5 mM EDTA, 0.05% SDS). Protein concentration was determined with a BCA protein reagent (Pierce) and adjusted to 2 µg/µL. Reduction was carried out with 1 mM DTT for 30 min and alkylation using 10 mM iodoacetamide for 60 min. The solution was diluted with water to a final concentration of 1 M urea before digestion. Digestion was carried out using sequencing-grade modified trypsin (Promega) at a ratio of 1:100 w/w trypsin to protein at 37°C for 16 h. The digestion was terminated by formic acid addition to a final concentration of 1% v/v. Peptides were then desalted using Sep-Pak C18 cartridges (Waters), dried using a vacuum centrifuge (Thermosavant) and resuspended in 5% acetonitrile in 0.1% TFA. An amount of peptide equivalent to 2 µg was injected for each LC-MS/MS analysis.

For the geLC-MS method, 25 µg of protein was separated on a precast Novex 12% Tris-HCl glycine gel (Invitrogen) and stained overnight with Commassie Brilliant Blue G-250 (Sigma). The gel was divided into thirty individual sections which were then excised and cut into approximately 1 mm<sup>3</sup> cubes. Destaining was carried out three times with a solution of 50% ethanol and 25 mM ammonium bicarbonate (ABC) buffer followed by dehydration with

100% ethanol. Reduction was carried out by incubating the dehydrated gel cubes with 10 mM DTT in 25 mM ABC for 60 min at 56°C. The reduction solution was then replaced with 55 mM of iodoacetamide in 25 mM ABC and incubated for 45 min. The gel cubes were washed twice in 50 mM ABC and dehydrated with 100% ethanol. Thirty  $\mu$ L of modified sequencing-grade trypsin at a concentration of 5  $\mu$ g/mL in 25 mM ABC buffer and 1 mM CaCl<sub>2</sub> was added and incubated at 4°C for 30 min. Excess trypsin solution was removed and 15  $\mu$ L of 25 mM ABC buffer was added. Digestion was carried out overnight at 37°C and terminated by TFA addition to a final concentration of 0.1% v/v. The supernatant was then transferred to an eppendorf tube. To the gel pieces 50  $\mu$ L of 50% ethanol in 0.1% TFA was added and sonicated for 15 min. The process was repeated and all supernatants derived from one gel section were pooled and dried to about 10  $\mu$ L using a vacuum centrifuge.

#### 1.4 Preparation of samples for quantitative ICAT analysis

Protein labelling and separation were based on the gelC-MS/MS approach<sup>(33)</sup> using the cleavable ICAT reagent (Applied Biosystems). 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  $\mu$ g of protein from each growth condition was individually reduced using 2  $\mu$ L 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<sub>heavy</sub> reagent and protein from haem-excess growth condition with the ICAT<sub>light</sub> 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<sup>TM</sup> 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<sup>3</sup> 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  $\mu$ L followed by mixing with 500  $\mu$ L 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  $\mu$ L of 5% acetonitrile in 0.1% TFA.

### 1.5 Liquid chromatography and mass spectrometry

MS was carried out using an Esquire HCT ion trap mass spectrometer (Bruker Daltonics)

5 coupled to an UltiMate Nano LC system (LC Packings – Dionex). Separation was achieved using a LC Packings reversed phase column (C18 PepMap100, 75  $\mu$ m i.d. x 15 cm, 3  $\mu$ m, 100 $\text{\AA}$ ), 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

10 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 15 initial complete proteome analysis and 3 for ICAT analysis for the most intense multiply charged ions with an active exclusion time of 2 min.

### 1.6 Protein identification for non-quantitative proteome analysis

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

20 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](http://www.tigr.org)). The matched peptides were further evaluated using the following criteria, i) peptides with a probability based Mowse 25 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.

### 1.7 Protein identification for ICAT

To increase confidence in the identification of ICAT-labeled 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

5 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  $\geq 25$ , the expectation value  $\leq 0.01$  and the MS/MS spectrum must have exhibited a contiguous series of 'b' or 'y'-type ions with the intense ions being accounted.

### 10 1.8 Estimation of false positive

To independently estimate the level of false positive assignments, a reverse database of *P. gingivalis* was created by reversing the order of the amino acid sequences for each protein such that the database is identical in size to the normal database in terms of the protein

number, size and distribution of amino acids<sup>(34)</sup>. The false positive rate was thus estimated as

15  $N_R / N_F$  where  $N_R$  = number of peptides identified with the reverse database (MASCOT score of peptide above threshold for the reverse database) and  $N_F$  = number of peptides identified with the normal database (MASCOT score of peptide above threshold for normal database). False positives were determined from the comprehensive proteome analysis ( $N_F = 18375$  peptides) and quantitative ICAT analysis ( $N_F = 530$  peptides).

### 20 1.9 Quantification of relative abundance

The ratio of isotopically heavy <sup>13</sup>C to light <sup>12</sup>C ICAT labeled 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

25 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  $\alpha = 0.05$ .

### 1.10 Genome analysis

The cellular localisation of *P. gingivalis* proteins was predicted using CELLO (<http://cello.life.nctu.edu.tw><sup>(35)</sup>) and transmembrane helices using TMHMM 2.0 ([www.cbs.dtu.dk/services/TMHMM-2.0](http://www.cbs.dtu.dk/services/TMHMM-2.0)) based on the sequence obtained from TIGR. To estimate the relative expression level of the proteins identified as compared to the theoretical proteome CAI values were calculated based on the coding sequence of *P. gingivalis* from genebank (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Porphyromonas\_gingivalis\_W83/) using the program INteractive Codon Analysis 1.12a (<http://www.bioinfo-hr.org/inca>,<sup>(14)</sup> with ribosomal proteins and tRNA synthases being defined as highly expressed genes. Operon prediction was carried out from the Microbesonline website (<http://microbesonline.org><sup>(36)</sup>).

### 1.11 Construction of ECR312 mutant

*P. gingivalis* W50 Open Reading Frame PG1374 potentially encodes an immunoreactive 47 kDa antigen (PG97) based on the *P. gingivalis* W83 genome ([www.tigr.org](http://www.tigr.org)). To construct *P. gingivalis* PG1374 mutant, a 672 bp upstream fragment of the PG1374 gene with flanking ApaI and AatII restriction sites (underlined) was generated from the W50 genomic DNA by PCR with primers ECR312ApaI-For (5' - AGAGGGCCCTAGCAATCATTGATTGCT - 3') and ECR312AatII-Rev (5' - TGCGACGTCGTGTTACCAATAGAGGATT - 3'). This fragment was cloned into AatII and BamHI sites on pAL30, pGem<sup>®</sup>T-easy (Promega) containing a subcloned *ermF* cassette<sup>(37)</sup> to create pAL36. Similarly, a 565 bp fragment downstream of PG1374 with flanking PstI and NdeI restriction sites was amplified with ECR312PstI-For2 (5' - TGACTGCAGGCTTCGACCTGGATCTT - 3') and ECR312NdeI-Rev2 (5' - TCGCATATGAAGAAATAAGTGCCGTCGG - 3') primers, and cloned into PstI and NdeI restriction sites in pAL36. The resulting plasmid having *ermF* cassette flanked with upstream and downstream fragments of the PG1374 open reading frame (designated as pAL36.1) was linearized with *Sca*I and transformed into *P. gingivalis* W50 as previously described<sup>(38)</sup>. Transformed cells were selected after 7 days of incubation at 37°C under anaerobic conditions on HBA plate containing 10 µg mL<sup>-1</sup> erythromycin. Confirmation of DNA integration was performed by PCR analysis and the resulting mutant was designated as ECR312.

### 1.12 Antibiotic protection invasion assay

To compare the invasion efficiencies of W50 and ECR312, an antibiotic protection assay was carried out as described previously in a 24-well cell culture plate<sup>(39)</sup>. Briefly, a fixed number of *P. gingivalis* cells were allowed to invade a KB monolayer (~10<sup>5</sup> cells in each well). Non invaded or adhered cells were killed by further incubation for 1 h with gentamicin (300 µg/mL) and metronidazole (200 µg/mL). Colony forming units of invaded bacteria were then enumerated on horse blood agar plates.

### 1.13 Cell binding assays

The cell binding assay was carried out as described previously<sup>(40)</sup>. Briefly, *P. gingivalis* was first grown to mid log phase to a cell density of ~ 2.9 x 10<sup>9</sup> cells/mL. The cells were then washed followed by labelling with 500 µg of fluorescein isothiocyanate (FITC) (Invitrogen) resuspended in 500 µL DMSO followed by incubation at 37°C for 45 min with shaking. After incubation, the cells were further washed, resuspended in incomplete Earl's minimum essential medium (JRH Biosciences) and the *P. gingivalis* cells adjusted based on cell counts using a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA). The green emission of FITC was measured with a 525-nm filter (FL1). The multiparametric data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA). All measurements were done in duplicate, and for quantitation of FITC fluorescence, mean fluorescence intensity (MFI) values were used.

Binding of the wild type *P. gingivalis* and ECR312 was carried out in parallel by inoculating 200 µL of cell suspension onto the KB cells at 5% CO<sub>2</sub> atmosphere at 37°C for 40 min. Following incubation the supernatant containing the KB cells and bacteria were transferred to a 1.5 mL tube. The remaining bound cells were then detached off the well with 200 µL of Trypsin-EDTA mixture (JRH Bioscience) for 5 min at 37°C and pooled with the corresponding collected supernatants. 500 µL of complete EMEM was then added to inactivate the trypsin followed by three washes and final suspension in 1 mL PBS. The bound cells were counted on the flow cytometer as described earlier.

## 2. Results and discussion

### 2.1 Growth of *P. gingivalis* in continuous culture

When grown in continuous culture in a rich medium containing excess haem *P. gingivalis* W50 achieved a steady state cell density approximately 48 h after inoculation of  $2.03 \pm 0.04$  mg cellular dry weight/mL. When the concentration of haemin in the growth medium was decreased from 5.0 µg/ml to 0.1 µg/ml, a significantly lower steady state cell density of  $0.99 \pm 0.20$  mg cellular dry weight/mL was achieved demonstrating that haem availability was limiting growth. The effect of haem-limitation on cell density was alleviated when haem was added back into the culture.

The growth of *P. gingivalis* during haem-limitation was in agreement with previous studies using chemostats with a *P. gingivalis* mean generation time of 6.9 h<sup>(41, 42)</sup>. Due to the inability of *P. gingivalis* to synthesize PPIX, this essential nutrient is thought to be acquired through proteolysis of haemoglobin and other haem containing plasma proteins<sup>(9)</sup> and a deficiency in haem was thus reflected in the significantly lower cell density.

### 2.2 Proteome analysis of *P. gingivalis* grown under haem-limitation

The proteome of *P. gingivalis* grown under haem-limitation was extensively analysed by two different approaches. Using in-solution digestion followed by LCMS with gas phase fractionation, 344 proteins were identified. In the gelLCMS approach, 385 proteins were identified while 247 proteins were found by both approaches. With the combined strategy a total of 478 proteins were identified (see Table 1) with an estimated false positive rate of 0.4% calculated from searches against the *P. gingivalis* reverse database. 77.0% of all proteins were identified by  $\geq 2$  unique peptides or by  $\geq 2$  identical peptides from independent LCMS runs (from different m/z ranges or SDS PAGE bands). The 478 identified proteins represent ~ 25% of all the 1988 assigned protein-encoding genes identified by whole-genome analysis<sup>(43)</sup>. Although a quarter of the total predicted proteome was identified this figure is higher if the actual number of genes expressed during any one growth condition is taken into account. In *Pseudomonas aeruginosa* and *Bacillus subtilis* the percentage of total ORFs transcribed during a single growth condition was estimated to be 60% and 40%, respectively

(<sup>44, 45</sup>). By examining the duty cycle limitation of their mass spectrometers Zhang and co-workers (<sup>46</sup>) estimated that around 60% of *P. gingivalis* predicted ORFs were expressed under their growth condition. Therefore based on these figures between 41-62% of *P. gingivalis* proteins expressed under the present growth conditions have been identified. The functional classification of the 478 identified proteins is shown in Table 1.

**Table 1.** Coverage of the theoretical proteome of *P. gingivalis* using the combined strategy shown in Figure 1.

Functional class of proteins <sup>a</sup>	Proteins ID/ Total Protein <sup>b</sup>	% of class ID
Energy metabolism	78/140	55.7
Protein synthesis	68/117	58.1
Fatty acid and phospholipid metabolism	9/16	56.3
Protein fate	37/75	49.3
Purines, pyrimidines, nucleosides, and nucleotides	22/44	50.0
Central intermediary metabolism	11/23	47.8
Cellular processes	12/46	26.1
Cell envelope	32/140	22.8
Amino acid biosynthesis	2/19	10.5
Signal transduction	2/12	16.6
Unknown function	47/201	23.4
Transcription	7/31	22.6

Transport and binding proteins	20/119	16.8
DNA metabolism	14/81	17.3
Biosynthesis of cofactors, prosthetic groups, and carriers	14/88	15.9
Hypothetical proteins (includes conserved)	96/695	13.8
Regulatory functions	5/47	10.6
Other categories	2/134	1.5

<sup>a</sup> Functional classification data obtained from TIGR ([www.tigr.org](http://www.tigr.org))

<sup>b</sup> Some proteins have been assigned to more than one functional class

To date, there is only one reported attempt to identify *P. gingivalis* proteins globally using a multidimensional proteomics approach<sup>(46)</sup>. The present inventors have identified 478 *P. gingivalis* W50 proteins that were expressed during continuous culture under haem-limitation compared to the study of Zhang and co-workers<sup>(46)</sup> where 1014 *P. gingivalis* ATCC 33277 proteins were identified when this strain was cultured in keratinocyte growth medium and the same medium exposed to secreted epithelial cell components. As the strain used, the growth conditions and the processing of MS data were different in the two studies, it is difficult to make direct comparisons between the two datasets. Nevertheless, 75 proteins were uniquely identified in the present methods.

Using CELLO to predict the subcellular protein localisation, most of the proteins identified in this study were predicted to be from the cytoplasm (347 out of a predicted 1350 proteins), followed by the periplasmic space (48/113), outer membrane (47/154), inner membrane (24/256) and extracellular (12/35). As expected, a low percentage of predicted inner membrane proteins were identified. To further increase the confidence of predicting inner membrane proteins the Transmembrane Hidden Markov Model (TMHMM) was used. Using the TMHMM approach 20 out of the 242 proteins predicted to have >2 transmembrane domains (TMD) were identified and 5 out of 44 proteins predicted to have >10 TMDs were

identified. Notably, all 12 of the membrane proteins with >10 transmembrane domains detected were identified from the in-solution digestion method.

### 2.3 Significance of identified proteins

Highly expressed genes in many bacteria often have a strong composition bias in terms of codon usage. The Codon Adaptation Index (CAI) can be used to predict the expression level of a gene based on its codon sequence, with a higher CAI value indicating increased expression<sup>(47)</sup>. Of 1685 genes in the *P. gingivalis* genome that have >100 codons, almost 92% have CAI values between 0.62 and 0.80 (Figure 2). Although this range is narrow compared with eukaryotic organisms<sup>(48)</sup> it is similar to the thermophile *Thermoanaerobacter tengcongensis* where 89% of the predicted genes have CAI values between 0.35 and 0.50<sup>(49)</sup>. The CAI values of the genes encoding the 478 identified proteins in this study have a similar distribution to the theoretical proteome although there was a bias towards the detection of higher abundance proteins. Despite this bias, a number of proteins encoded by genes of very low CAI were identified. This result clearly exemplifies the problem of the large dynamic range of protein abundance in cells, showing it is currently not possible to detect all proteins at once.

The functional classes of proteins with the highest percentage of identified proteins are those involved in energy metabolism (Table 1), typically those involved in fermentation (95%, CAI 0.70-0.80), glycolysis (82%, CAI 0.71-0.83) and metabolism of amino acids and amines (81%, CAI 0.71-0.84). This was largely expected as essential proteins involved in basic metabolic functions such as energy metabolism have been shown to be very abundant in the bacterial cell. Most importantly almost 90% of these proteins are predicted to be in the cytoplasm, which also made detection easier compared with membrane proteins. The functional classes of proteins represented least are those involved in transpositioning, hypothetical proteins and regulatory functions.

Although the complete genome of *P. gingivalis* has been sequenced, many critical questions regarding cellular functions remain unanswered. Proteomic studies that identify the translated gene products therefore help provide additional insights into the functional genome. For example *P. gingivalis* is known to be asaccharolytic<sup>(3)</sup> although the genome

contains putative ORFs for all enzymes of the glycolytic pathway<sup>(43)</sup>. The poor utilization of this pathway has been attributed to the glucose kinase gene being interrupted by an insertion element<sup>(43)</sup>. In keeping with this finding glucose kinase, or another glycolysis-specific enzyme, phosphofructokinase was not identified. In contrast, all enzymes involved in gluconeogenesis were found, suggesting glucose necessary for processes such as polysaccharide biosynthesis may be derived via this pathway.

#### 2.4 Response of *P. gingivalis* to haem-limitation as determined using ICAT

To carry out the quantitative ICAT analysis of the *P. gingivalis* response to haem-limitation, the gelLCMS approach was chosen, as the in-solution ICAT method was unsatisfactory due to the presence of strong interfering triply charged ions. The presence of these triply charged ions resulted in very low number of protein identifications. The ICAT labelled soluble and insoluble protein fractions were therefore independently separated by SDS-PAGE and each gel lane divided into 20 sections for in-gel tryptic digestion followed by affinity purification and LCMS. In total 142 proteins were identified. No matches to the reverse database were obtained indicating a low level of false positive identification. Considering proteins detected in both fractions, 53 proteins (34.0%) were identified based on the presence of two or more unique peptides with a probability based Mowse score corresponding to a *p*-value of at most 0.05, 60 proteins (38.5%) were identified based on the presence of one unique peptide identified from two or more different fractions or both ICAT labelling states (of those 58 have MASCOT score of  $\geq 25$ ) and 43 of the proteins (27.5%) were identified on the basis of a single unique peptide having a MASCOT score  $\geq 25$ , expectation value of  $\leq 0.01$ , a contiguous series of 'b' or 'y'-type ions and the intensities of ions being accounted for when interpreted manually. An example of a protein identification based on the analysis of a single unique peptide is shown in Figure 3.

Of the proteins identified, 103 were found in the soluble fraction, 53 in the insoluble fraction and 14 proteins in both fractions. In response to the change in environmental conditions from haem-excess to haem-limitation 70 of the identified proteins exhibited at least a two-fold change in abundance (Figure 4). Of these, the abundance of 53 proteins increased more than 2 fold and the abundance of 17 proteins decreased more than 2 fold during haem-limitation.

In order to assess the reproducibility of the present data and to increase protein identification for the insoluble fractions, cells were harvested from the chemostat on different days during haem-limitation and excess growth. A different extraction method followed by ICAT labelling was then repeated with modified protocols (not shown). The data obtained were reproducible with proteins showing similar abundance ratios for selected proteins shown in Table 2 (e.g., PG0695 L/H = 1.1, relabelling = 1.3; PG0350 L/H = 3.2, relabelling = 2.6; PG0159 L/H = 2.0, relabelling = 2.2; PG0232 L/H = 0.4, relabelling = 0.4). To further verify the data, the relative abundance of those identified proteins that are predicted to be encoded by genes forming an operon were compared<sup>(50)</sup>. Five groups of proteins were found to be encoded by predicted operons or by genes grouped at specific loci (Table 2 - shaded). In each case the abundance of the encoded proteins appeared to be similar. One of the predicted operons encodes the outer membrane proteins, Omp40 (PG0694) and Omp41 (PG0695) whose abundance were unchanged at a ratio of 1.1 (haem-limitation/haem-excess, L/E). These proteins have high sequence similarity to the OmpA-like porins of Gram-negative bacteria<sup>(51)</sup> and are thought to provide a physical linkage between the outer membrane and the peptidoglycan layer. These structural proteins would not be expected to vary in abundance with a change in environmental haem levels. The remaining four predicted operons were found to be associated with glutamate or aspartate catabolism.

Table 2. Expression data of selected proteins in *P. gingivalis* during growth in haem-limitation.

No	Tigr Acc#	Protein and peptide sequence identified	Score <sup>1</sup>	N <sup>2</sup>	n- ICAT <sup>3</sup>	Fold change <sup>4</sup>	SD (±)
<b>Proteinases</b>							
1	PG2024/ PG0506	<i>Arginine-specific protease</i> ( <i>RgpA<sub>Ca</sub>/RgpB</i> ) .GQDEMNEILC*EK .C*YDPGVTPK	11 51/14 24/14	2	0.39	0.1	
2	PG2024/ PG0506	<i>Arginine-specific protease</i> ( <i>RgpA/RgpB</i> adhesins)	19	3	0.95	0.2	

30

.DAGMSAQSHYC\*VEV 34/15

K

.EGLTAT\*TFEEDGVAAG 44/13

NHEYC\*VEVK

.C\*VNVTVNSTQFNPVK 59/15

3 PG0232 *Zinc carboxypeptidase* 4 1 0.40 0.1  
 .C\*QILIENHDKR 21/18  
 .YPSLC\*TTSVIGK 56/19

4 PG0026 *Hypothetical protein* 5 2 0.46 0.1  
 (Homology to Arg  
 proteases)  
 .C\*VVNSPGGQTASMAK 30/14  
 .FSNLPVLGGESC\*R 58/14

**Invasion related proteins**

5 PG0350 *Internalin related protein* 11 4 3.2 0.6  
 .FVPYNNDDEGGEENVC 35/13  
 \*TTEHVEMAK  
 .HIMELSEADVEC\*TIK 46/14  
 .ILHC\*NNNQLTALNLSA 23/15  
 NTK  
 .LDLPANADIETLNC\*SK 52/13

6 PG1374 *Immunoreactive 47KDa* 5 2 6.5 0.7  
 protein  
 .GLSVLVC\*HISNQIAGEE 27/15  
 MTK

7 PG0159 *Endopeptidase PepO* 6 1 2.0 0.3  
 .METELAQIC\*YSK 55/13

8 PG2132 *Fimbrillin FimA* 2 1 0.50 -  
 .YDASNELRPTILC\*IYG 45/16  
 K

**Iron transport and related proteins**

9	PG1552	<i>HmuR</i>		1	1	4.0	-
		.MNSDELFEETYPGYTI	25/15				
		C*R					
10	PG1019	<i>Hypothetical protein</i>		2	1	25.0	-
		.TYMIDTNDSENDC*IAR	70/14				
11	PG1286	<i>Ferritin</i>		2	1	1.2	-
		.FGSVLEVFQQVYEH*EC	73/13				
		K					
12	PG0090	<i>Dps family protein</i>		3	1	1.1	0.1
		.EEHELVC*AASTLK	36/13				
13	PG0618	<i>Alkyl hydroperoxide reductase subunit C</i>		1	1	41.6	-
		.AAQYVAAHDGQVC*P	36/15				
		AK					

**Others**

14	PG0694	<i>Omp40</i>		5	1	1.1	0.1
		.RPVSC*PECPEPTQPTVT	26/16				
		R					
15	PG0695	<i>Omp41</i>		12	1	1.1	0.1
		.RPVSC*PECPEVTPVTK	39/15				

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

5 4 Average ratios of all quantified peptides for each protein in fold change (Haem-limitation/excess)

\* Denotes ICAT modified cysteine

## 2.5 Host cell invasion related proteins

Three proteins possibly involved in invasion of host cells, internalin related protein (PG0350), immunoreactive 47 kDa protein (PG1374) and endopeptidase PepO (PG0159) were higher in abundance during haem-limitation (Table 2). During an antibiotic protection invasion assay

5 *P. gingivalis* lacking a functional PG1374 had approximately 50% lower invasion capability into epithelial cells as compared to the wild type (W50,  $32625 \pm 2582$  cfu/mL, ECR312  $16250 \pm 1089$  cfu/mL ;  $p<0.01$ , Student's T-test). In a separate binding assay, there is no significance difference in the adherence of the PG1374 mutant as compared to the wild type W50 (Figure 5).

10 PG1374 and PG0350 belong to a new class of cysteine containing protein with leucine rich repeat domains similar to the *L. monocytogenes* internalin protein InlJ<sup>(52)</sup>. In *L. monocytogenes*, there are at least fifteen members of the internalin family and all have been found to share certain structural features consisting of a signal peptide, N-terminal leucine rich repeat domain followed by a conserved inter-repeat region. Many of these proteins are 15 involved in the cellular invasion process<sup>(53)</sup>. It has not been demonstrated why multiple internalins exist, but they are proposed to confer tropism toward different cell types<sup>(54)</sup>.

20 The higher abundance of PG1374 and PG0350 during haem limitation (6.5 and 3.2 fold increase respectively) in the current work suggests the expression of these two proteins is stimulated during low haem growth conditions. From the sequence information and predicted structure, more than half of the internalin LRR residues face outwards and are variable, suggesting them to be for protein-protein interaction surfaces specific to the different internalin classes<sup>(55)</sup>. PG1374 and PG0350 both possesses a signal peptide and are part of the novel class 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<sup>(56)</sup>. In addition PG1374 is strongly immunogenic when probed with sera from human periodontitis patients<sup>(57)</sup> which further suggests it to be involved in cell surface protein interactions.

25 The process of internalization of *P. gingivalis* into gingival epithelial cells is thought to involve a coordinated process of attachment and invasion mediated by fimbriae and a variety

of cell surface proteinases<sup>(58-60)</sup>. A *P. gingivalis* 33277 mutant lacking a functional putative internalin (PG0350) was shown to exhibit similar invasive characteristics but reduced biofilm formation capability compared to the wild type bacteria<sup>(46, 61)</sup>. The similar invasion was attributed to the presence of fimbriae that also play a role in epithelial cell invasion by strain 5 33277 although there is also a possibility that the similar invasiveness of this mutant was due to the presence of a second putative internalin protein (PG1374) encoded in the *P. gingivalis* genome. A double knockout of these two putative internalin proteins would potentially shed light on their possible cooperative invasive roles.

The 50% reduction in epithelial cell invasion by ECR 312 and no difference in cell binding 10 clearly demonstrate that the observed reduced invasion into epithelial cells by *P. gingivalis* deficient in PG1374 is not due to lesser adherence but a real defect in the invasion process (Figure 5). Bacterial invasion has been shown to be a highly complex process involving numerous proteins and receptors<sup>(62)</sup>. The involvement of multiple factors involved in *P. gingivalis* invasion has been demonstrated by Lamont's group at the University of Florida and 15 includes haloacid dehalogenases, endopeptidases, a cation-transporting ATPase and an ATP-binding cassette transporter<sup>(60, 63)</sup>. The discovery of PG1374 as an epithelial cell invasion related protein therefore adds to the list of proteins involved in this complex host cell invasion process by bacterial pathogens.

For many bacterial pathogens, it has been well established that iron availability influences 20 virulence and the invasion process, but little is known about the influence of haem on the expression of *P. gingivalis* invasion genes. We were unable to perform binding and invasion assay on haem-limited cells because of the high mortality rate of the *P. gingivalis* from the oxidative stress likely due to lack of the protective layer of the  $\mu$ -oxo bis-haem form of iron PPIX<sup>(64-66)</sup>. However in this study, we have shown that the levels of the putative invasion 25 related proteins PcpO, PG0350 and PG1374 increased under haem-limitation and PG1374 is involved in the cell invasion.

These experiments described above are the first quantitative proteomic analysis of the 30 response of *P. gingivalis* to a change in environmental conditions and demonstrates the utility of the stable isotope labelling approach combined with complete proteome analyses. *P. gingivalis* responds to limitation of the essential micronutrient haem by increasing the

abundance of a number of proteins linked to the oxidative stress response, virulence and invasion of host cells.

## 2.6 Cell surface located protein PG1019

A *P. gingivalis* hypothetical protein, PG1019 was observed to be 25 times more abundant when the bacterium was grown under haem-limitation. Bioinformatic analyses suggest that PG1019 is a lipoprotein that is encoded by a gene located immediately upstream of a gene encoding a putative outer membrane receptor protein (PG1020) in a predicted operon.

Multiple alignment (not shown) of PG1020 with known *P. gingivalis* TonB-linked outer membrane receptors shows the presence of a putative TonB box (residues 118-126), that is one of the characteristics of TonB-linked receptors<sup>(67)</sup> and a conserved region (residues 236-272) which Simpson and co-workers<sup>(68)</sup> refer to as the TonB box IV region. TonB-linked outer membrane receptors have been implicated with many iron, iron complex and other micronutrient uptake systems. The high abundance of PG1019 under haem limited growth conditions would be consistent with this protein being an accessory lipoprotein to a TonB-linked system involved in the transport of iron/iron complexes into the cell or the sensing of environmental iron or iron complexes, although this remains to be demonstrated. In addition to the proteomic data a transcriptomic analysis using custom made *P. gingivalis* DNA microarrays of *P. gingivalis* W50 compared to a mutant lacking a function *feoB1* gene (*P. gingivalis* FB1) was performed. The wild type W50 and FB1 mutant were both grown in continuous culture in haem excess conditions. *P. gingivalis* FB1 has approximately half the cellular iron content of the wild type W50<sup>(69)</sup>. Genes that show an increase in transcription are therefore likely to be upregulated in response to the decrease in intracellular or environmental iron content. Both PG1019 and PG1020 were significantly upregulated to similar levels in the FB1 mutant compared to the wild type. PG1019 showed a Log2 increase of 2.46 and PG1020 showed a Log2 increase of 2.33 at a significance level of P<0.05 in biological replicates, this is further evidence that these genes are located in an operon. Further a separate transcriptomic DNA microarray analysis of *P. gingivalis* W50 indicated that there was little or no expression of the PG1019 gene during haem-excess growth.

## 2.7 Alkyl hydroperoxide reductase protein, AhpC (PG0618)

The most substantial change in *P. gingivalis* protein abundance during the transition from haem-excess to haem-limitation was observed with an alkyl hydroperoxide reductase protein, AhpC (PG0618, Table 2) which is a peroxide-scavenging enzyme that has been shown to play an important role in peroxide resistance in *P. gingivalis*<sup>(65)</sup>. In *P. gingivalis*, formation

5 of a layer of the  $\mu$ -oxo bis-haem form of iron PPIX with oxygen on the cell surface is thought to act as an oxidative buffer due to its inherent catalase-like activity<sup>(11)</sup>. This layer may also serve as a cell surface storage of iron and PPIX<sup>(70)</sup>. During haem-limitation depletion of this source of iron PPIX was shown to result in an increased susceptibility to oxidative stress<sup>(64)</sup>. The substantial increase in abundance of alkyl hydroperoxide reductase during haem-  
10 limitation could therefore be in response to the increased oxidative stress caused by the absence/reduction of the  $\mu$ -oxo bishaem layer. OxyR an oxygen sensitive transcriptional activator also plays a role in the expression of alkyl hydroperoxide during anaerobic growth<sup>(71)</sup> where a *P. gingivalis* OxyR<sup>-</sup> mutant shows decrease of 16 fold in gene expression. More recently Duran-Pinedo and co-workers also demonstrated the positive regulation of  
15 *aphC* expression by the RprY response regulator. The substantial increase in abundance thus suggests haem availability may have a role in RprY and OxyR-controlled gene expression. Interestingly the very high Codon Adaption Index (CAI) value of this protein (0.838) suggests this protein is able to be highly expressed in the cell for rapid induction in response to such stress.

20

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.

25 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  
30 invention as it existed anywhere 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.

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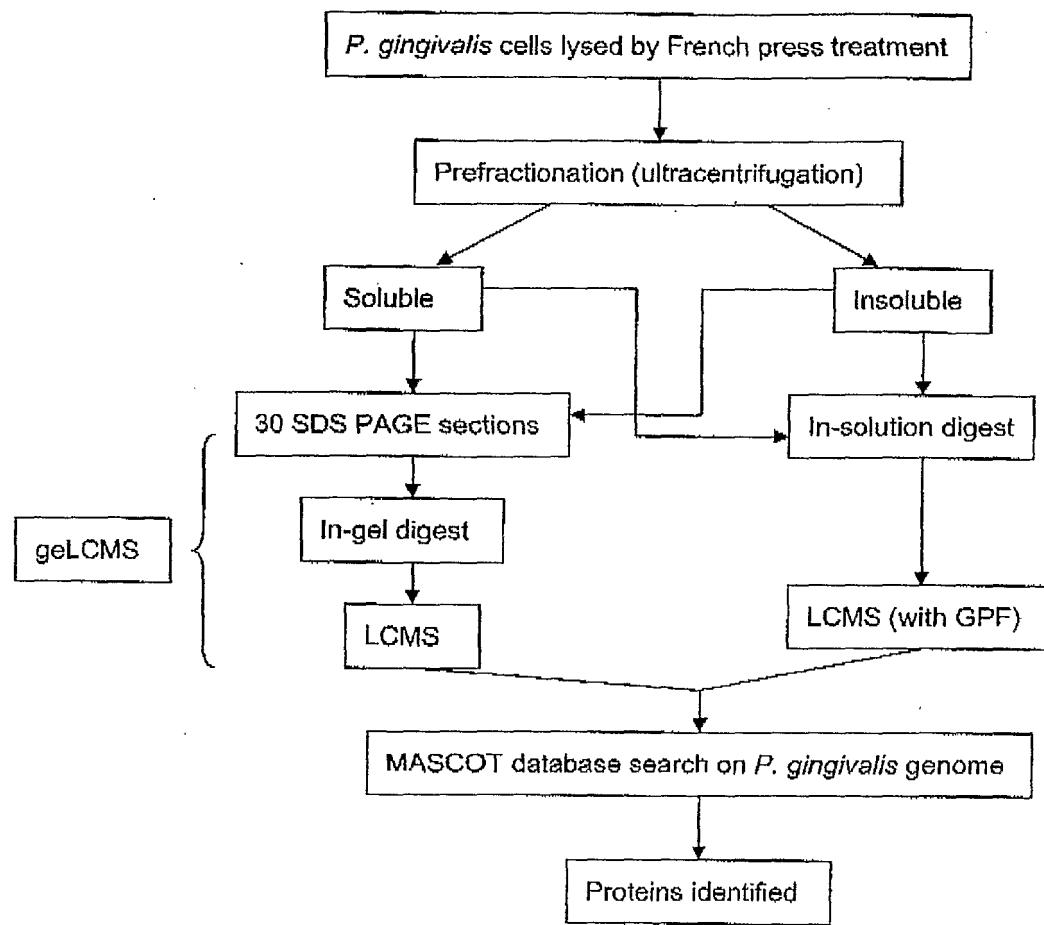
## CLAIMS

1. An isolated antigenic *P. gingivalis* polypeptide, the polypeptide being selected from the group consisting of:
  - (i) the PG0350 protein having the amino acid sequence of SEQ ID NO:1;
  - 5 (ii) the PG1374 protein having the amino acid sequence of SEQ ID NO:2;
  - (iii) the PG1019 protein having the amino acid sequence of SEQ ID NO:3;
  - (iv) the PG0618 protein having the amino acid sequence of SEQ ID NO:4;
  - (v) an amino acid sequence at least 85% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4; or
- 10 (vi) an amino acid sequence comprising at least 10 amino acids identical to a contiguous amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.
2. A polypeptide as claimed in claim 1 in which the polypeptide is 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.
- 15 3. A polypeptide as claimed in claim 1 in which the polypeptide comprises a contiguous sequence of at least 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids which is identical to a contiguous amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4.
- 20 4. A vaccine 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 according to any one of claims 1 to 3 and a pharmaceutically acceptable carrier.
- 25 5. A method of preventing or treating a subject for periodontal disease comprising administering to the subject a vaccine composition according to claim 4.

6. Use of at least one polypeptide according to anyone of claims 1 to 3 in the manufacture of a medicament for the treatment of periodontal disease in a subject.
7. A method as claimed in claim 9 in which the subject is selected from the group consisting of humans, sheep, cattle, horses, bovine, pigs, poultry, dogs and cats.
- 5 8. A method as claimed in claim 10 in which the subject is a human.
9. An antibody which binds specifically to a polypeptide according to any one of claims 1 to 3.
10. An antibody of claim 9 which is a polyclonal antibody.
11. An antibody of claim 9 which is a monoclonal antibody.
- 10 12. A composition useful in the prevention or treatment of periodontal disease, the composition comprising an antibody according to any one of claims 9 to 11 and a pharmaceutically acceptable carrier.
13. A method of identifying a *P. gingivalis* polypeptide involved in the progression of periodontal disease, wherein the method comprises the steps of:
  - 15 a) determining the relative amount of a polypeptide or peptide thereof produced by *P. gingivalis* grown under haem limited conditions; and
  - b) determining the relative amount of the polypeptide or peptide thereof produced by *P. gingivalis* grown under higher haem conditions than step a);

wherein an increase in the amount of the polypeptide or peptide fragment thereof detected in step a) compared to step b) indicates that the polypeptide is involved in the progression of periodontal disease.
- 20 14. A method as claimed in claim 13 in which the haemin concentration in the haem limited conditions of step (a) is about 0.1 µg/mL to about 0.5 µg/mL.

15. A method as claimed in either claim 13 or 14 in which the haemin concentration in the higher haem conditions of step (b) is above 5 µg/mL.
16. A method as claimed in any one of claims 13 to 15 in which the relative amounts of a polypeptide in the haem limited of step (a) and higher haem conditions of step (b) are determined using a qualitative proteomic approach.
- 5 17. A method as claimed in claim 16 in which the amount of a polypeptide is determined using either a combined strategy of in-solution and in-gel digestion and LC-MS/MS, or by analysis using stable isotope labelling strategies (ICAT) in combination with MS.
- 10 18. 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 SEQ ID NO: 5 or SEQ ID NO: 6 or SEQ ID NO: 7 or SEQ ID NO: 8.
19. An interfering RNA molecule of claim 18 wherein 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.
- 15 20. A method of treating a subject for periodontal disease comprising administering to the subject at least one interfering RNA molecule according to claim 18 or claim 19.

**FIGURE 1**

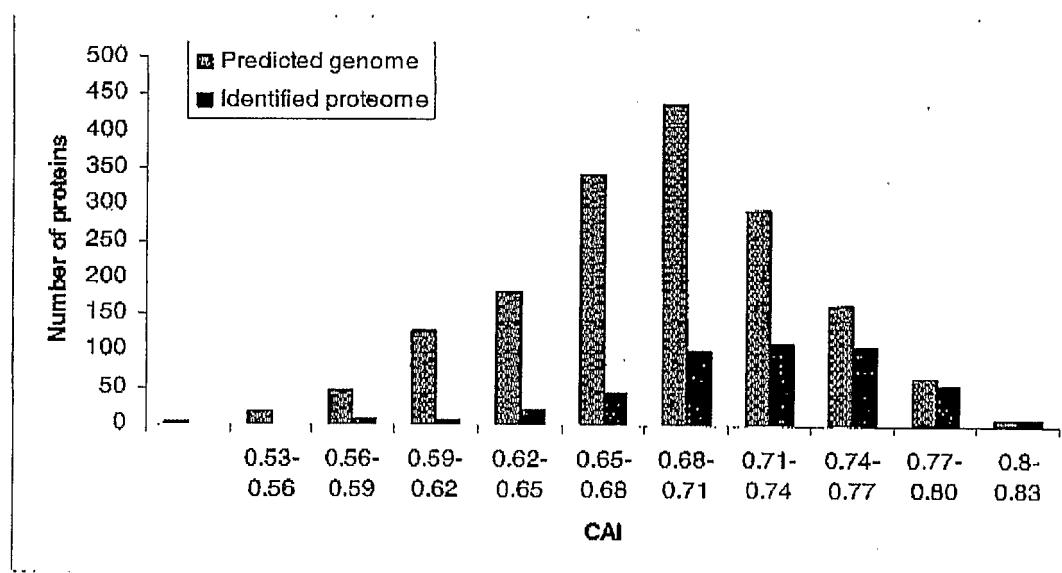
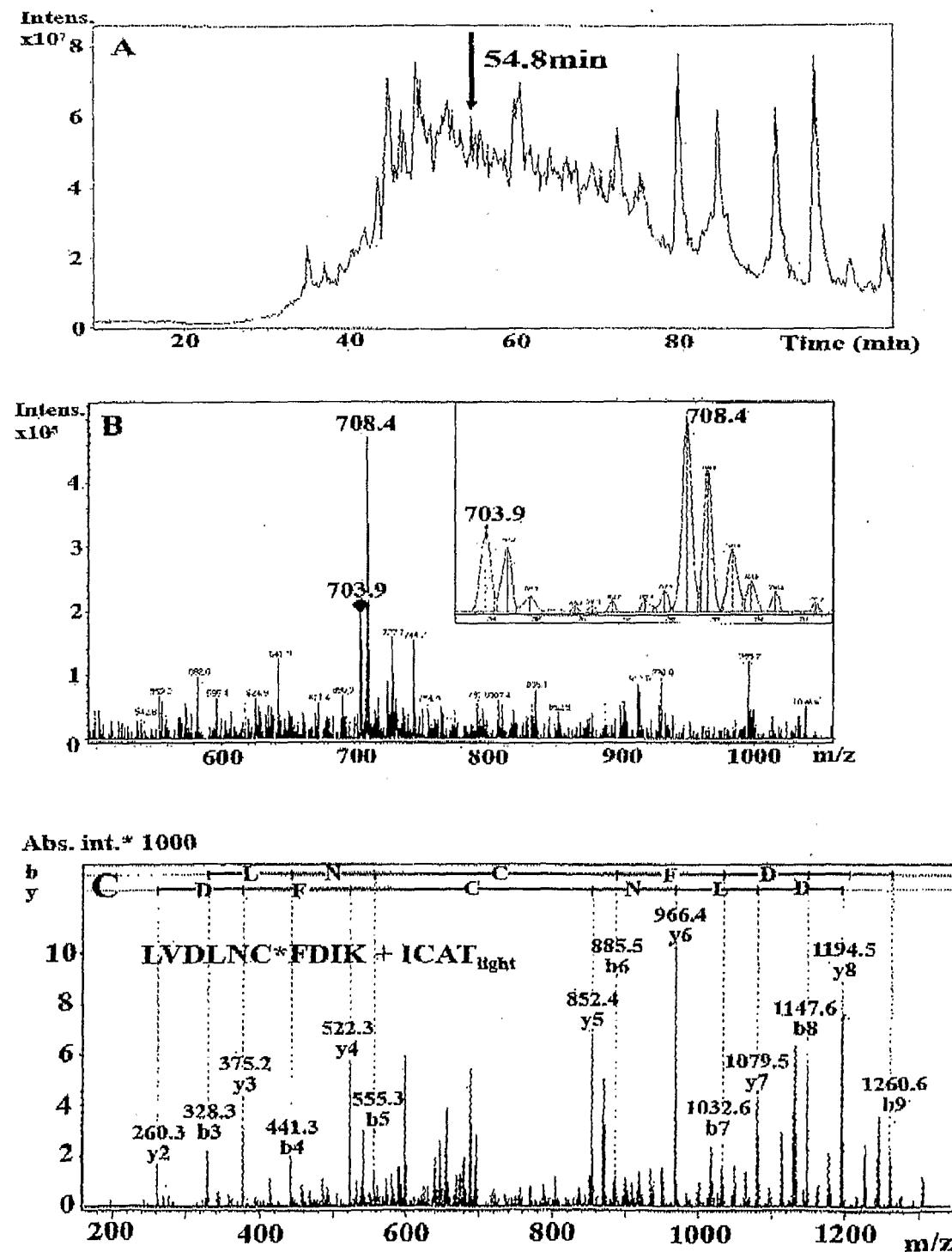
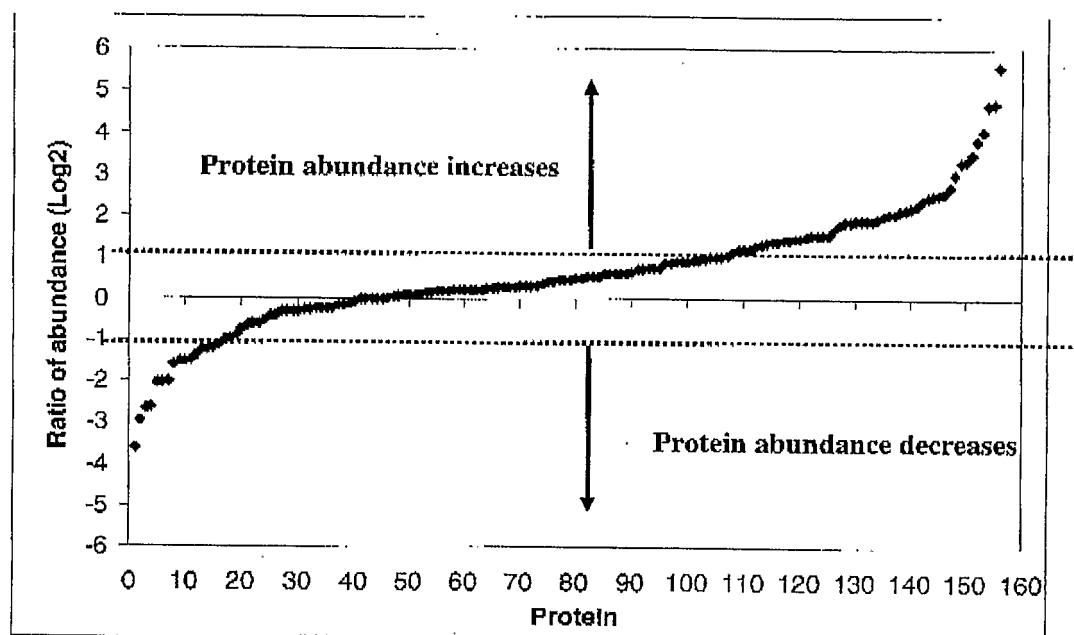
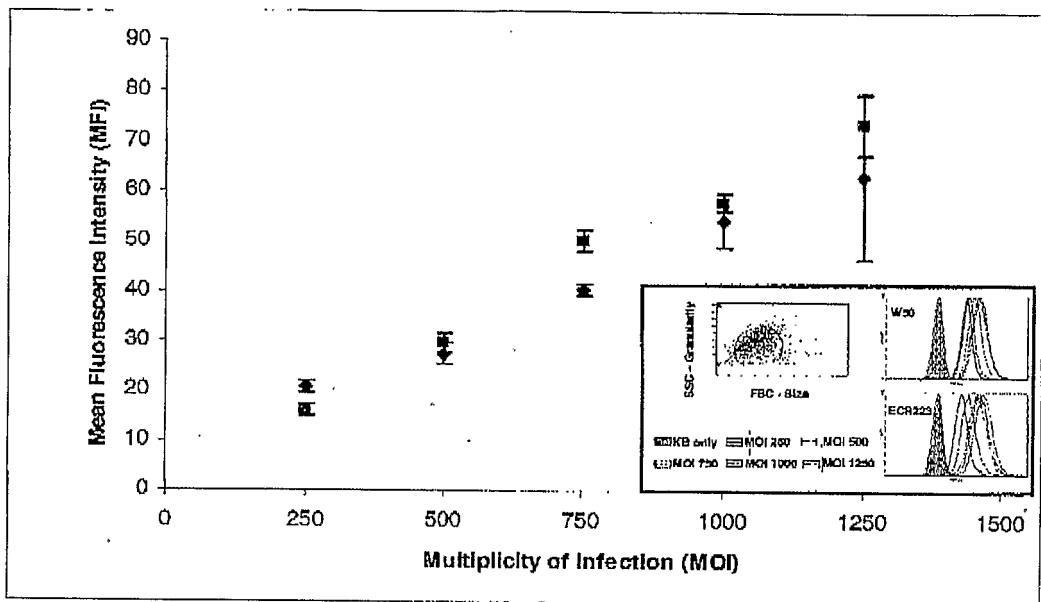
**FIGURE 2**

FIGURE 3



**FIGURE 4**

**FIGURE 5**

**FIGURE 6****SEQ ID NO: 1 - Amino Acid sequence of PG0350**

MKRKPLFSALVILSGFFGSVHPASAQKVPAVDGERIIMELSEADVECTI  
KIEAEDGYANDIWADLNGNGKYDSGERLDSGEFRDVEFRQTKAIVYGKMA  
KFLFRGSSAGDYGATFIDISNCTGLTAFDCFANLLTELDLSKANGLTFVN  
CGKNQLTKLDL PANADIELNCSKNKITSNLSTYTKLKELYVGDNGLTA  
LDLSANTLLEELVYSNNEVTTINLSANTNLKSLYCINNKMTGLDVAANKE  
LKILHCNNNQLTALNLSANTKLTTLSFFNNELTNIDLSNTALEWLFCNG  
NKLTKLDVSANANLIALQCSNNQLTALDLSKTPKLTTLNCYSNRIKDTAM  
RALIESLPTITEGEGRFVPYNDDEGGEEENVCTTEHVEMAKAKNWKVLTS  
WGEPPFGITALISIEGESEYSVYAQDGILYLSGMEQGLPVQVYTVGGSMM  
YSSVASGSAMEIQLPRGAAYVVRIGSHAIKTAMP

**FIGURE 7****SEQ ID NO: 2 – Amino Acid sequence of PG1374**

MKLSSKKILAIALLTMGHAVQAQFVPAPTTGIRMSVTTKAVGEKIELL  
VHSIEKKGIWIDLNGDATYQQGEEITVFDAYHEYTIGTQLTIYGNTTR  
LGCRSTGATAVDVTKNPNLTYLACPKNNLKSLDLTQNPKLLRVWCDSNEI  
ESLDLSGNPALIILGCDRNKLTELKTDNNPKLASLWCSDNNLTEL  
PRLNDLWCFCGNRITKLDLSANPLLVTLWCSDNELSTLDLSKNSDVAYLWC  
SSNKLTSLNLSGVKGGLSVLVCHSNQIAGEEMTKVVNALPTLSPGAGAQSK  
FVVVDLKDTDEKNICTVKDVEAKSKNWRFDFNGDSDNMLPYEGSPTSN  
LAVDAPTVRIYPNPVGRYALVEIPESLLGQEAALYDMNGVKVYSAVESL  
RQNIDLTHLPDGTYFFRLDNYTTKLIKQ

**FIGURE 8****SEQ ID NO: 3 - Amino Acid sequence of PG1019**

MKKNFLFFSLVLAAIMSLLSSCAKDTDAPEQYAITIRAKLPEGSTIESLAGIAIEFLDL  
RTQQKVEKQLDKAGVCSLSDLASVYTITIRGEIGNNSIVAIKENYSIAENTTLELPLIVT  
KIRPSGLLFKEVFFNGETNNGQMMHPDQYFVIYNNSDKVVYADGVAFGLAAHANVT  
GEDAFTEELTKNNRIVLSMIYTIPGNGSQYPIQPGGQLVIAGTAINHHDAEHPNSVDLS  
GADLEVYEPDQPANFGQDVDPNVPNMVKIFNRFGVFMMHPRGFIPPVLFEIDEPIET  
FLAKNQFEYTNNDGENIMLYAVPVENVLDGIETANTGNMKVKSLPVTVDKSMIGVP  
GCHRGILILRKTEEKNGRTYMIDTNDSENDCIARQGQNSFPARF

**FIGURE 9****SEQ ID NO: 4 - Amino Acid sequence of PG0618**

MTPILNTVFPEFKLNAYHNGEFKVITNEDLKGKWSLVVFYPGDFTFVCPTTELEDLANK  
YEEFKQLGVEVYSCSCDTHFVHKAWADASPAIKKVQYPMLADPSGALTRDLGILIDD  
VHMAYRGSFVINPEGIINKIVELDNSVGRDAEEILRKIKAAQYVAAHDGQVCPAKWRE  
GQQTLKPSIDLVGKI

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000890

## A. CLASSIFICATION OF SUBJECT MATTER

### Int. Cl.

**C07K 14/195** (2006.01)    **A61K 39/40** (2006.01)    **C12N 15/31** (2006.01)  
**A61K 38/16** (2006.01)    **A61P 1/02** (2006.01)  
**A61K 39/02** (2006.01)    **C07K 16/12** (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**, sequence search, ID numbers 1-4. Database: STN, File: CA, Biosis, Medline, WPIDS, Keywords: porphyromonas, gingivalis, heme, haem.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/0288866 A1 (G. SACHDEVA et al) 29 December 2005 & Database Ref. Seq. accession number NP_904662, submission date 8 April 2002. See sequence id 0177.	1-12, 18-20
X	WO 1999/029870 A1 (CSL LIMITED) 17 June 1999 & Database Ref. Seq. accession number NP_905539, submission date 8 April 2002. See sequence id 0526.	1-12, 18-20

Further documents are listed in the continuation of Box C

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	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 09 August 2007	Date of mailing of the international search report 17 AUG 2007
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Name and mailing address of the ISA/AU <b>AUSTRALIAN PATENT OFFICE</b> PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer <b>FRANCES RODEN</b> AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6283 2239
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/AU2007/000890

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	K. E. NELSON et al, Journal of Bacteriology, vol. 185, no. 18, 2003, pages 5591-5601, "Complete Genome Sequence of the Oral Pathogenic Bacterium <i>Porphyromonas gingivalis</i> Strain W83." & Database Ref. Seq. accessions numbers NC_002950.2 (for sequence id no. 3) and NP_904903 (for sequence id no. 4), submission date 8 April 2002.	1-20
A	US 6962706 B1 (THE UNIVERSITY OF MELBOURNE) 8 November 2005. See entire document.	1-20
A	US 6528038 B1 (THE UNIVERSITY OF MELBOURNE) 4 March 2003. See entire document.	1-20
A	WO 2001/047961 A1 (CSL LIMITED) 5 July 2001. See entire document.	1-20
A	S. G. DASHPER et al, Journal of Bacteriology, vol. 182, no. 22, 2000, pages 6456-6462, "Characterization of a Novel Outer Membrane Hemin-Binding Protein of <i>Porphyromonas gingivalis</i> ." See entire document.	1-20

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/AU2007/000890

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			
US	2005/288866	EP	1721283	KR	2007002021	WO	2005076010
WO	1999/029870	AU	16487/99	CA	2313823	EP	1037999
		EP	1681350	NZ	504811	US	6444799
		US	2002172976	ZA	9811333		
US	6962706	AU	27867/00	EP	1157033	NZ	514555
		US	2005169853	WO	0052041		
US	6528038	AU	21452/97	CA	2249746	EP	0954529
		NZ	331557	WO	9736923	ZA	9702757
WO	2001/047961	AU	23314/01	CA	2395423	EP	1240191
		NZ	519365	US	2003232022		

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

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