Published in final edited form as:

Mol Oral Microbiol. 2012 December ; 27(6): 420–435. doi:10.1111/j.2041-1014.2012.00661.x.

VimA – dependent modulation of the secretome in *Porphyromonas gingivalis*

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Abstract

The VimA protein of *Porphyromonas gingivalis* is a multifunctional protein involved in cell surface biogenesis. To further determine if its acetyl coenzyme A (acetyl-CoA) transfer and putative sorting functions can affect the secretome, its role in peptidoglycan biogenesis and effects on the extracellular proteins of *P. gingivalis* FLL92, a *vimA*-defective mutant, were evaluated. There were structural and compositional differences in the peptidoglycan of *P. gingivalis* FLL92 compared to the wild-type strain. Sixty-eight proteins were present only in the extracellular fraction of FLL92. Fifteen proteins present in the extracellular fraction of the parent strain were missing in the *vimA*-defective mutant. These proteins had protein sorting characteristics which included a C terminal motif with a common consensus Gly-Gly – Cterm pattern and polar tail consisting of aromatic amino acid residues. These observations suggest that the VimA protein is likely involved in peptidoglycan synthesis, and corroborates our previous report, which suggests a role in protein sorting.

Keywords

P. gingivalis secretome; Peptidoglycan sacculi; Protein sorting

INTRODUCTION

The secreted proteins of bacteria are important for their survival and sometimes play a vital role in the organism's interaction with the host during the disease process. These proteins, some of which can be derived from bacterial cell surface components, may function in nutrient acquisition and/or enhance the pathogenic potential by facilitating host tissue colonization or by modulating the host cell responses including the immune response (Lamont and Jenkinson 1998). *Porphyromonas gingivalis* an important Gram-negative periodontal pathogen is known to possess several outer membrane structures and secreted proteins including capsule, polysaccharides, proteases, hemagglutinin, lipopolysaccharides, fimbriae and major outer membrane proteins that contribute to cell adherence and virulence (Lamont and Jenkinson 1998; Yoshimura *et al.* 2008; Watanabe *et al.* 1992).

A key element in modulating the pathogenic potential of *P. gingivalis* is the post-translational modification of several of the major surface components. For example, the

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major proteases, called gingipains, consist of arginine-specific (Arg-gingipain [Rgp]) and lysine-specific (Lys-gingipain [Kgp]) proteases that are both extracellular and cell membrane associated. The maturation pathways of the gingipains are linked to the biosynthesis of surface carbohydrate moieties that are regulated by several proteins including the PorR (Shoji 2002), PorT (Nguyen *et al.* 2009; Sato 2005), Sov (Sato 2005) VimA, VimE and VimF (Vanterpool *et al.* 2004; Vanterpool *et al.* 2005; Vanterpool *et al.* 2006).

VimA is a 39 kDa protein which is encoded for by the *vimA* gene. This gene is part of the 6.15-kb *bcp-recA-vimA-vimE-vimF-aroG* locus. VimA plays a multifunctional role in *P. gingivalis*. In addition to the glycosylation and anchorage of several surface proteins including the gingipains, VimA can also modulate sialylation (Aruni *et al.* 2011). A recent report also documented a role in acetyl coenzyme A (acetyl-CoA) transfer. In these studies, VimA was shown to modulate lipid A and its associated proteins and may be involved in protein sorting and transport (Aruni *et al.* 2011).

The purified VimA protein in pull-down experiments interacted with several proteins (Aruni *et al.* 2011; Vanterpool *et al.* 2006). Common to many of these proteins were conserved secretory signals such as the TonB dependant receptor (PG0707), hypothetical protein (PG0410) (Aruni *et al.* 2011). The functional classifications of some of these proteins suggest an involvement in peptidoglycan and lipopolysaccharide synthesis. Several reports have suggested that an alteration or defect in the synthesis of the peptidoglycan layer and lipopolysaccharide can affect protein secretion (Bos and Tommassen 2004). While we cannot rule out that alteration in the cell surface may directly affect protein secretion, it remains unclear what role the *vimA* gene product may play in this process in *P. gingivalis*.

To further evaluate the role of VimA in protein sorting/trafficking in *P. gingivalis* we characterized the secretome of the *vimA*-defective mutant. The role of *vimA* in peptidoglycan synthesis was evaluated by assessing ultrastructure variations using atomic force microscopy, transmission electron microscopy and hydrolytic enzyme assays. Based on the data, the VimA protein is likely involved in peptidoglycan synthesis. In addition, a hypothesis that the *P. gingivalis* VimA protein is involved in protein post translational modification, anchorage and sorting needed for proper secretion of several extracellular proteins is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

P. gingivalis strains (W83, FLL92) were grown in either Brain Heart Infusion (BHI) broth (Difco Laboratories) supplemented with cysteine (0.1%), vitamin K (0.5 µg/ml) and hemin (5 µg/ml) (BHIKH) or in Trypticase Soy Broth containing menadione and hemin (TSBKH). Solid medium was prepared by supplementation with 1.5% agar and 5% defibrinated sheep blood (Hemostat laboratories). All cultures, unless otherwise stated, were incubated at 37°C in an anaerobic chamber (Coy Manufacturing) in 80% N₂, 10% H₂ and 10% CO₂. Growth rates were determined spectrophotometrically at 600 nm (optical density).

Complementation of *vimA*

PCR mediated gene replacement was used to complement the *vimA* defect. In brief, the ORF of *vimA* was amplified using primers specific for the *vimA* gene. The amplified fragment was purified by agarose gel electrophoresis, then electroporated into electro competent FLL92 cells grown to log phase (OD₆₀₀ of 0.6). Electroporated cells were incubated for 12 hours in 1 ml of broth followed by plating on BHIHK-blood plates. The plates were screened after 8 days for the presence of black-pigmented colonies. These

colonies were evaluated subsequently for the presence of the uninterrupted *vimA* gene using PCR.

Peptidoglycan isolation from W83 and FLL92

P. gingivalis peptidoglycan was prepared using the modified method previously reported by Isahii (Ishii *et al.* 2010). In brief, 200 ml of BHI broth cultures of *P. gingivalis* W83 and FLL92 were centrifuged (9000 ×g, 10mins at 10°C), and the precipitates resuspended in water. Trichloroacetic acid (10%) was added to the sample and the mixture was incubated at 4°C for 1 hour. The sample was centrifuged (9000×g, 5 min, at 10°C) then washed three times in water. The precipitate was incubated at 100°C for 1 hour after being suspended in 50mM sodium acetate buffer (pH 5.3) containing 8% SDS. This was followed by an overnight incubation at room temperature. After centrifugation (43,000×g, 1 hour, 12°C), the sample was washed three times in water and resuspended in Tris-HCL containing 2% SDS and proteinase K (50µg/ml) at 37°C for 12 hours.

Preparation of Murein sacculi

Murein sacculi were prepared from FLL92 and W83 using the method of Yao (Yao *et al.* 1999). Briefly, cells were grown to OD₆₀₀ of 0.7 representing log phase and 1.5–1.6 representing stationary phase in 2000 mls of BHIKH media. These cultures were then centrifuged at 6000×g for 30 minutes, washed twice in phosphate buffered saline (pH 7.0) containing 1mM magnesium chloride, and then the pellet was resuspended with the same buffer. The suspension was then boiled in 2% wt/vol SDS for 3 hours and left overnight at room temperature for 48 hours, after which it was centrifuged for 1 hour at 150,000×g (25°C) to pellet the sacculi. Sacculi were washed three times in deionized water at room temperature, then dialyzed overnight in one liter of deionized water.

Lytic Enzyme Assays

Several peptidoglycan hydrolytic enzymes, including muramidase (Sigma), and lysostaphin (Sigma) were used to determine the susceptibility of peptidoglycan and the murein sacculi to hydrolysis. In brief, peptidoglycan was suspended in 0.1 M potassium hydrogen phosphate (pH 7.3 to an approximate absorbance of 0.7 at 580 nm, in a 1cm light path. One hundred µg/ml of the enzyme was added to the peptidoglycan suspension at zero time and incubated at 37°C. The absorbance at 580 nm was followed for 24 hour and the percent hydrolysis was calculated employing the absorbance of an untreated, peptidoglycan control (Barnard and Holt 1985).

Microscopic examination

Atomic force microscopy (AFM) and transmission electron microscopy (TEM) were used to provide detailed visuals of the morphology of the murein sacculi. AFM was done using treated and untreated murein sacculi, isolated from W83 and FLL92 strains grown to stationary (OD 1.5–1.8) and exponential (OD 0.6 – 0.7) growth phase. Samples were washed twice in 0.1 M PBS buffer pH 7.4 and mounted on a cover slide, then allowed to air dry. Atomic force microscopy (AFM) images were generated with a Multimode 8 scanning probe microscope (Bruker, Santa Barbara CA) in the peak force™ tapping mode and using ScanAsyst™ (k = 0.4 Nm⁻¹, f = 70 kHz) air probes. Automated feedback parameter optimization was achieved using ScanAsyst™. The peak force™ tapping mode modulates the cantilever at ca 2 kHz at each pixel of the image where the feedback is based on the interaction force each time the tip taps the sample. Images were captured in the height mode or peak force error (Pratto *et al.* 2009).

Transmission Electron Microscopy was performed using the Philips Tecnai 12 TEM as per the method of Hyatt (Hyatt 1991). Briefly, sacculi were absorbed to 400- mesh carbon and Formvar coated EM grids. A drop of 2% (wt/vol) uranyl acetate was used to float the grid for 15 s to negatively stain the sample, this was followed by microscopy using a Philips Tecnai 12 operated under standard conditions with the cold trap in place (Yao *et al.* 1999).

Bioinformatics Analysis of VimA

The DNA and amino acid sequences were aligned using Bioedit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The signal peptide and potential cleavage sites were predicted using both Neural network and Hidden Markov Model (Johnson *et al.* 2010). Signal peptide prediction and cleavage site prediction were performed using Signal P 3.0 (Bendtsen *et al.* 2004). Transmembrane helices were predicted using the TMHMM server (Krogh *et al.* 2001). The post translational modification pattern motifs were identified using PPSearch tool (<http://www.ebi.ac.uk/Tools/ppsearch/>) (Kulikova *et al.* 2004),

Cell Fractionation

Extracellular fractions were prepared from cell-free culture fluid precipitated with 60% acetone (−20°C) (Vanterpool *et al.* 2006) from W83 and FLL92. The protein pellet was resuspended in 7 ml 100 mM Tris-HCL buffer (pH 7.4) in the presence of 1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), dialyzed for 48 hours against the same buffer then stored at −20°C until used.

Digestion of *P. gingivalis* wild-type and FLL92 proteins

Extracellular proteins were run on a 10% bis-Tris gel (Invitrogen, Carlsbad, CA) in 1X MOPS running buffer for 7.5 cm, then visualized by staining with SimplyBlue safe stain (Invitrogen) (Henry *et al.* 2008). After destaining in water, the gel was cut into 1–2 mm slices. Gel slices were subsequently dehydrated in acetonitrile and dried in a vacuum centrifuge for 30 minutes. The gel slices were incubated for 1 hour at 60°C in a solution containing 20 µl of 20 mM Dithiothreitol in 100mM NH₄HCO₃ (enough to cover the gel pieces). The Dithiothreitol solution was replaced with an alkylating solution (20 µl of 200 mM iodoacetamide in 100 mM NH₄HCO₃) after cooling the proteins to room temperature. Gel slices were further incubated at ambient temperature for 30 minutes in the dark, followed by two washes with 150 µl of 100 mM NH₄HCO₃, then finely minced with a flame sealed polypropylene pipette tip, dehydrated by the addition of acetonitrile, and vacuum dried. Following an overnight incubation of the gel pieces with 20 µl digestion buffer [1 µl of mass spectrometry (MS)-grade trypsin (www.promega.com) in 50 mM acetic acid with 1 µl of 100 mM NH₄HCO₃], the digestion reaction was stopped with 10 µl of 5% formic acid. After transferring the digest solution (aqueous extraction) to a 0.65 ml siliconized tube, 30 µl of 50% acetonitrile with 0.1% formic acid was added, the mixture was vortexed for 3 minutes, centrifuged and then sonicated for 5 minutes. The process was repeated and both extractions pooled and concentrated to 10 µl in a vacuum centrifuge. Peptide extraction was accomplished using standard C₁₈ ZipTip technology following the manufacturer's directions (Millipore, Bedford, MA).

MS and data analysis

An LCQ Deca XP Plus system (www.thermo.com) with nano-electrospray technology (www.newobjective.com) consisting of a reverse phase C₁₈ separation of peptides on a 10 cm by 75 µm capillary column using Microm Magic RP-18AQ resin (www.michrom.com) with direct electrospray injection was used to analyze the extracted peptides from each gel piece (Henry *et al.* 2008). A four part protocol was used for the MS and MS/MS analyses, this included one full MS analysis (from 450 to 1750 m/z) followed by three MS/MS events

using data-dependent acquisition, where the most intense ion from a given full MS scan was subjected to collision-induced dissociation, followed by the second and third most intense ions. The nanoflow buffer gradient was extended over 45 minutes in conjunction with the cycle repeating itself every 2 seconds, using a 0–60% acetonitrile gradient from buffer B (95% acetonitrile with 0.1% formic acid) developed against buffer A (2% acetonitrile with 0.1% formic acid) at a flow rate of 250 to 300 nl/min, with a final 5 minute 80% bump of buffer B before re-equilibration. In order to move the 20 µl sample from the autosampler to the nanospray unit, flow stream splitting (1:1000) and a Scivex 10 port automated valve (Upchurch Scientific, Oak Harbor, WA) together with a Michrom nanotrap column was used. The spray voltage and current were set at 2.2 kV and 5.0 µA, with a capillary voltage of 25 V in positive ion mode. 160°C was used as the spray temperature for peptides. Data collection was achieved using the Xcalibur software (Thermo Electron), then screened with Bioworks 3.1. MASCOT software (www.matrixscience.com) was used for each analysis to produce unfiltered data and output files. Statistical validation of peptide and protein findings was achieved using X TANDEM (www.thegmp.org) and SCAFFOLD 2 meta analysis software (www.proteomesoftware.com). The presence of two different peptides at a probability of at least 95% was required for consideration as being positively identified. Confirmation of individual peptide matches was achieved using the BLAST database (www.orolgen.lanl.gov). The experiments were repeated twice and the results were analysed.

RESULTS

Complementation of the *vimA* defect in *P. gingivalis* FLL92

Inactivation of the *vimA* gene in *P. gingivalis* W83 resulted in a non-black-pigmented isogenic mutant, designated *P. gingivalis* FLL92, which showed reduced levels of proteolytic, hemagglutinating, hemolytic and sialidase activities and decreased resistance to oxidative stress (Abaibou *et al.* 2001; Vanterpool *et al.* 2005; Vanterpool *et al.* 2006). This *vimA*-dependent phenotype was also observed in a different genetic background of *P. gingivalis* further supporting a possible multifunctional role for the VimA protein. To rule out the presence of other defects in *P. gingivalis* FLL92 that may contribute the observed phenotype, the inactivated *vimA* gene in this isogenic mutant was replaced with the wild-type gene. Following electroporation of *P. gingivalis* FLL92 with the *vimA* open reading frame and incubation on blood agar plates for 5–10 days, several black pigmented colonies (designated FLL92B, FLL92C, and FLL92D) were identified. *P. gingivalis* FLL92B and FLL92D were observed to have the similar characteristic phenotype of the wild-type W83 strain. *P. gingivalis* FLL92C became hemolytic and black pigmented much later than wild-type W83 (days) and autoaggregated when incubated in BHI broth. Notably, its gingipain activity was marginally higher than FLL92 in log phase. The *vimA* gene from this strain was sequenced in order to determine whether a mutation in the gene accounted for this phenotype. Two mutations were observed in FLL92C at positions 6 and 7, where CC was replaced by AG (results not shown). The translated product of this gene contained valine rather than Isoleucine at the third amino acid position.

The Murein Sacculi from FLL92 differs from W83

In silico analysis of the VimA protein predicted a structure that showed similarity to the Fem family of proteins which in Gram-positive bacteria are involved in cell envelope biogenesis, particularly in peptidoglycan formation (Brakstad and Maeland 1997; Mainardi *et al.* 2008; Stapleton and Taylor 2002). Furthermore, the increased sensitivity of *P. gingivalis* FLL92 could suggest an alteration in the peptidoglycan layer (Osbourne *et al.* 2010). TEM and AFM were used to determine the morphology and topography of the peptidoglycan sacculi. The sacculi from *P. gingivalis* FLL92 mutant when compared to the wild-type was distinctly

different, as it possessed a homogeneously uneven surface with numerous contours. Sacculi from *P. gingivalis* W83 were on average 100 nm longer than those observed in FLL92 (Figure 1 and Figure 2). Murein sacculi from both strains were subsequently treated with 15µg/ml of lysostaphin for ten minutes and imaged via AFM (Figure 3). Treated W83 were visibly different from the untreated control, as numerous contours were visible on the surface. In FLL92 mutant, the morphology of the treated sample was similar to the control.

Peptidoglycan from W83 is hydrolyzed faster than FLL92

Modifications of peptidoglycans can affect their sensitivity to peptidoglycan hydrolases (Osbourne *et al.* 2010). Several lytic enzymes including lysozyme and lysostaphin were used to determine whether the peptidoglycan of *P. gingivalis* FLL92 was chemically dissimilar to that of wild type W83. No significant change was observed between the wild-type and FLL92 using lysozyme; however, with the lysostaphin treated peptidoglycan, 75% of W83 peptidoglycan was hydrolyzed at 17 minutes, compared to 58% of FLL92 at the same time point (Figure 4).

VimA affects the secretion of several extracellular proteins

Mass spectrometric analysis was done to determine whether the differential protein profile observed previously in FLL92 (Osbourne *et al.* 2010), was unique to the membrane proteins or whether this mutation also affected the extracellular protein fraction. Mass Spectrometry of extracellular fractions of W83 and FLL92 identified 68 proteins that were present in the extracellular fraction of FLL92 (Table 1) but absent in W83. Fifteen proteins that were present in the extracellular fraction of *P. gingivalis* W83 were missing in FLL92 (Table 2). The majority of aberrantly expressed proteins were predicted to be involved in energy metabolism, while the remainder was predicted to be involved in proteolysis, protein binding and transport, DNA metabolism or were hypothetical. Of the fifteen proteins identified as missing, six were predicted to play a role in cell envelope biogenesis; while two - PG0468 and PG1357 were predicted to be involved in protein targeting and transport. Five of these proteins were hypothetical and two in amino acid metabolism. Seven extracellular proteins were identified in FLL92 as having spectral count values with negative fold changes (fc) of 1.2 or greater (Figure 5) - RgpA (PG1768, fc 1.2), Kgp (PG1605, fc 6.6), Carboxypeptidase D (PG0212, fc 28), Peptidylarginine deiminase (PG1249, fc 12.1), Hemagglutinin (PG1602, fc 22.3), TonB-dependent OM receptor (PG0170, fc 285), and a hypothetical protein (PG0554, fc 50.7).

VimA alters the secretome of *P. gingivalis* and could be involved in protein sorting

In silico analysis of the missing protein in the extracellular fraction of *P. gingivalis* FLL92 was carried out to identify common motifs in the C terminal of all the membrane proteins (both outer and inner membrane proteins). Out of the 15 missing proteins identified by mass spectrometry, 9 proteins were found to be localized in the membrane of *P. gingivalis* (Table - 3). The membrane proteins that were missing in *P. gingivalis* FLL92 were found to contain a strong N terminal signal peptide with a C terminal polar tail with aromatic residues at the end. Protein sequence alignment showed Gly-Gly – Cterm motif at positions 310 of all amino acids (Figure – 6 & 7), Figure – 1 (supplemental data). A glycine rich motif was noticed further down at positions 332 – 340. A common LxxxxG motif and DxGxTx motifs were present at the C terminal end (Figure - 6).

On further analysis of the 15 FLL92 missing proteins, several common domains were identified (Table 4). Casein Kinase II phosphorylation motifs, Protein Kinase C motifs and myristolation motifs were most abundant.

However, among the 68 aberrantly expressed proteins in FLL92, 14 were outer membrane proteins and 3 were periplasmic proteins. Only 7 out of the total membrane proteins had N terminal signal sequence. Multiple sequence alignment of these membrane proteins did not reveal any common consensus motif pattern except for the LxxxG motif (Figure-2 supplemental data). There were no Gly-Gly C-terminal motifs as noted in the FLL92 missing proteins.

DISCUSSION

In this report, we have further clarified the multifunctional role of the VimA protein in *P. gingivalis*. The ability to restore the wild-type phenotype in *P. gingivalis* FLL92 suggested that the observed multiple phenotype may result from a cascade of reactions or have a central mechanism. A non-black pigmented phenotype in *P. gingivalis* FLL92 could also be attributed to the missing Heme binding protein (PG0602) (Dashper *et al.* 2000) and its transport by the TonB dependant receptor HumY (Wojtowicz *et al.* 2009). In addition, Glyceraldehyde-3-phosphate dehydrogenase which is also one of the FLL92 missing proteins is important in the glycolytic pathway and could affect glycosylation which could be important to the activation and anchorage of the gingipains (Henderson and Martin 2011; S.S.Abdel-Meguid *et al.* 2008). Similar to the phenotype of *P. gingivalis* FLL92, multiple reports have documented the phenotypic effects of altered gingipain activities in *P. gingivalis* (Curtis *et al.* 1999; Curtis *et al.* 2001; Hasegawa *et al.* 2003; Kuboniwa *et al.* 2009; Lewis *et al.* 1999; Nakayama 2003; Potempa *et al.* 1998; Rangarajan *et al.* 2005; Roy *et al.* 2006; Vanterpool *et al.* 2010; Veith *et al.* 2002). While it is likely that VimA-dependent glycosylation of the gingipains maybe an important component in this process (Osbourne *et al.* 2010; Vanterpool *et al.* 2006), we cannot rule out other posttranslational modifications.

VimA *in silico* analysis, predicts several domains including one that is conserved among the acetyl-CoA N-acetyltransferase (Nat) superfamily and belongs to a broad family of proteins which includes the FemXAB (Schneider *et al.* 2004) (Figure -3a & b supplemental data). The Fem family of proteins (Osbourne 2010; Aruni *et al.* 2012) are known to be involved in cell envelope biogenesis, particularly peptidoglycan formation. Members of the FemABX family are novel ribosomal peptidyl transferases that are involved in the interchain peptide bridges of the peptidoglycan. Though primarily found in Gram positive bacteria they are also observed in a few Gram negative bacteria and spirochetes (Ghuysen 1968; Hegde and Shrader 2001). Our morphological studies showed variations in the sacculi of *P. gingivalis* FLL92 compared to the wild-type strain. The presence or absence of acetyl moieties on the amino sugars in the peptidoglycans of some organisms can determine their sensitivity or resistance to several peptidoglycan hydrolases. Due to the similar sensitivity of the lysozyme, it is unlikely that the variation observed in *P. gingivalis* FLL92 peptidoglycans is due to acetylation of the amino sugars in the peptidoglycans (Reith and Mayer 2011). Instead, the reduced ability of lysostaphin to hydrolyze the peptidoglycan from *P. gingivalis* FLL92 could indicate an alteration in the inter peptide bridges (Grundling *et al.* 2006). The amino acid composition of *P. gingivalis* tetrapeptide may contain Alanine, Diaminopimelic acid (DAP), Glycine and Lysine (Barnard and Holt 1985). While the amino acid composition of *P. gingivalis* FLL92 tetrapeptide is unknown, it is noteworthy that VimA has been shown to interact with Alanine t-RNA synthetase (PG1101) (Aruni *et al.* 2011; Vanterpool *et al.* 2006) which, in other strains, can play a role in peptidoglycan synthesis. The ability to transfer an acetyl group from the acetyl CoA to the amino group on the glucosamine-6 phosphate creating N-acetyl-glucosamine-6-phosphate is a major step in the synthesis of peptidoglycan (White .D 2007). VimA has acetyl CoA transfer function (Aruni *et al.* 2011) and could likely be directly involved in peptidoglycan synthesis. Confirmation of a specific mechanism in this process is under further investigation.

VimA can modulate several surface related structures including fimbriae, capsule and some outer membrane proteins (Osbourne 2010). In *P. gingivalis* some of these outer membrane surface proteins can become part of the secretome by a lipopolysaccharide directed sorting mechanism (Haurat *et al.* 2011). Results from this study have suggested that the secreted proteins are also altered in the *vimA*-defective mutant. Several proteins present in the extracellular fraction in FLL92 were missing from a similar *P. gingivalis* W83 fraction. It is noteworthy that PG1857 (Glyceraldehyde-3-phosphate dehydrogenase) which is involved in the colonization of *P. gingivalis* (Nagata *et al.* 2009), is the only protein that was found to be missing from both the membrane (Osbourne *et al.* 2010) and extracellular fractions of FLL92. This could suggest defects in other secretory pathway(s)/mechanism(s). Of interest, PG1496 - a conserved hypothetical protein predicted to be involved in transport, is aberrantly expressed in the outer membrane (Vanterpool *et al.* 2006) and extracellular fraction. Two other proteins with protein transport and binding functions – PG1497 and PG1504, were also aberrantly expressed. It is likely that these three proteins could be part of a novel transporter/cell sorting system in *P. gingivalis*, which when disrupted, results in alterations in secretion and anchorage of proteins. It is also noteworthy that several cytoplasmic proteins were found in the extracellular fraction of FLL92, including those involved in energy metabolism, translation and DNA metabolism. Though we cannot definitively rule out the possibility of cytoplasmic contamination, the absence of other periplasmic /cytoplasmic markers (e.g. HtrA) likely suggest that some of the proteins found in extracellular fraction of *P. gingivalis* FLL92 could be incorrectly targeted for secretion or their regulatory mechanism altered thus resulting in their secretion (Bohle *et al.* 2011).

Several secreted proteins have been found to have primary and secondary structure similarity to the C-terminal domain of RgpB. These proteins have been designated the CTD family; and members of this family are attached to the cell surface through cell envelope glycans, which are important for proper folding and processing in order to produce a fully functional enzyme (Seers *et al.* 2006). In contrast to the aberrantly expressed proteins, several of the missing extracellular proteins in *P. gingivalis* FLL92 did not conform to the C-terminal domain of RgpB CTD family but also carried an N terminal signal peptide, a common C terminal motif and a polar tail consisting of aromatic amino acids residues. Both the C terminal motif with its common consensus Gly-Gly – Cterm pattern and polar tail are known to have protein sorting characteristics in other organisms (Ton-That *et al.* 2004; Haft and Varghese 2011). Because the VimA protein is predicted to have sorting signals (Aruni *et al.* 2011), the unique C terminal domain (CTD) with a glycine rich region and a general sorting signal motif-like region (LxxxG) could likely be a general mechanism of VimA-mediated sorting for these missing proteins. It is unclear if there could be variation in VimA-mediated sorting. In a previous study (Aruni *et al.* 2011), proteins interacting with VimA were also observed to carry a unique CTD motif pattern which is different from those in this study. In addition, the Gly-Gly Cterm motif was not evident as noted in this study. However, both the study showed a common C terminal Glycine rich domain that encompassed the sorting motif previously reported (Aruni *et al.* 2011) and the Gly - Gly Cterm motif noted in this study. It is unclear if there could be differential sorting possibly based on the association of VimA with other protein(s). Confirmation of a mechanism(s) is being actively investigated in the laboratory.

Taken together, our results further support the hypothesis that the *P. gingivalis* VimA protein is involved in protein post translational modification, anchorage and sorting needed for proper secretion of several extracellular proteins. Preliminary observations in the laboratory suggest that VimA can modulate the acetylation profile in *P. gingivalis* (unpublished data). Acetylation is one of the most common post translational modifications in bacteria (Hu *et al.* 2010), and can affect protein sorting, cell-surface properties and morphology of important pathogens (Laaberki *et al.* 2011; Lee and Schneewind 2001;

Gabriel Waksman *et al.* 2005). The multifunctional VimA (Abaibou *et al.* 2001; Aruni *et al.* 2012; Osbourne 2010; Vanterpool *et al.* 2004; Vanterpool *et al.* 2005; Vanterpool *et al.* 2006) as a therapeutic target could have important implications for treatment strategies in controlling *P. gingivalis* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Loma Linda University and Public Health Grant DE13664 and DE019730 from NIDCR (to H.M.F).

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Figure 1. Muerin sacculus differs in FLL92 compared to wild type

Transmission Electron Microscopy (TEM) was used to visualize the peptidoglycan sacculi of *P. gingivalis* W83 and FLL92 strains, grown to 0.7 OD₆₀₀. Morphological differences were observed in FLL92, when compared to W83

Figure 2. Sacculi topography of W83 and FLL92

Atomic Force Microscopy (AFM) showing the peptidoglycan sacculi of *P. gingivalis* W83 and FLL92 strains, grown to 0.7 OD600. Morphological differences were observed in FLL92, when compared to W83.

A & B – *P. gingivalis* W83 sacculi, C & D - *P. gingivalis* FLL92 sacculi

Figure 3. Sacculi of W83 and FLL92 after 10 min treatment with 16 µg/ml of Lysostaphin
Atomic Force Microscopy (AFM) showing the peptidoglycan sacculi of *P. gingivalis* W83 and FLL92 strains, after 10 min of treatment with 16 µg/ml of Lysostaphin. A & B – *P. gingivalis* W83 sacculi after treatment, C & D – *P. gingivalis* FLL92 sacculi after treatment.

Figure 4. Peptidoglycan from *P. gingivalis* W83 is hydrolyzed faster than FLL92 by Lysostaphin
 Graph showing the hydrolysis of peptidoglycan sacculi of *P. gingivalis* W83 and FLL92 strains by lysostaphin. The test was performed in Biorad microplate reader on a continuous mode.

Figure 5. Extracellular proteins in *P. gingivalis* strains with different spectral count values
 Bar graph showing the variations in the spectral count of extracellular proteins between *P. gingivalis* FLL92 mutant and the wild type W83.

Figure 6. Multiple sequence alignment of the FLL92 missing proteins

Multiple sequence alignment of the FLL92 missing proteins using clustalW was performed with 9 out of 15 proteins which were membrane proteins and were found to have an N terminal signal sequence. All the proteins showed a LxxxxG domain, DxGxTx domain and a polar tail with aromatic amino acid residues at the end.

Figure 7. Sequence logo showing Gly-Gly-CTERM domain in the FLL92 missing proteins. The presence of the Gly-Gly CTERM domain is underlined in red.

Table 1

MS analysis of aberrantly expressed proteins in FLL92 extracellular fraction

Identified Proteins (68)	Accession #	Putative Role/Function	M.W.	W83 S.C.V	FLL92 S.C.V
Hypothetical protein	PG0027	Unknown	25 kDa	0	2
UDP-3-O-acetyl-GlcNAc deacetylase/(3R)-hydroxymyristoyl-aap-dehydratase	PG0060	Fatty acid biosynthesis	52 kDa	0	3
Nitrogen assimilation regulatory protein (immunoreactive 47 kD antigen PG120)	PG0136	Transcription	47 kDa	0	12
Endothelin converting enzyme/neprilysin (PepO)	PG0146	Proteolysis	79 kDa	0	2
NADH oxidase/peroxidase	PG0160	Energy metabolism	104 kDa	0	7
Probable outer membrane protein (Omp85 analog)	PG0175	Cell envelope	102 kDa	0	5
Uroporphyrinogen-III synthase	PG0185	Biosynthesis of cofactors	28 kDa	0	7
Hypothetical protein	PG0227	Unknown	11 kDa	0	4
Translation initiation factor IF-2	PG0230	Translation	108 kDa	0	2
Conserved hypothetical protein	PG0264	Unknown	39 kDa	0	4
Polyferredoxin	PG0276	Energy metabolism	30 kDa	0	2
Probable dipeptidyl peptidase	PG0291	Proteolysis	101 kDa	0	2
Conserved hypothetical protein	PG0293	Unknown	15 kDa	0	3
DNA-mismatch repair protein	PG0350	DNA metabolism	95 kDa	0	9
DNA-directed RNA polymerase subunit beta	PG0360	Transcription	142 kDa	0	2
ATP-dependent DNA helicase	PG0381	DNA metabolism	82 kDa	0	4
ATP-dependent ClpX-related protease	PG0382	Proteolysis	46 kDa	0	16
Ferredoxin oxidoreductase beta subunit	PG0394	Energy metabolism	37 kDa	0	2
Conserved hypothetical protein	PG0411	Unknown	52 kDa	0	2
Conserved hypothetical protein	PG0452	Unknown	54 kDa	0	49
Pyruvate ferredoxin/flavodoxin oxidoreductase	PG0498	Energy metabolism	132 kDa	0	2
Hypothetical protein	PG0510	Hypothetical	34 kDa	0	5
Beta-galactosidase	PG0598	Energy metabolism	127 kDa	0	11
DNA topoisomerase I	PG0680	DNA metabolism	90 kDa	0	17
Acyl-CoA dehydrogenase (coenzyme A dehydrogenase)	PG0696	Metabolic process	65 kDa	0	2
Probable Xaa-Pro dipeptidase	PG0795	Proteolysis	44 kDa	0	4

Identified Proteins (68)	Accession #	Pulative Role/Function	M.W.	W83 S.C.V	FLL92 S.C.V
Beta-galactosidase	PG0799	Energy metabolism	116 kDa	0	2
Translation elongation factor G protein	PG0832	Translation	80 kDa	0	2
Calcium ion-transporting ATPase	PG0838	Transport & binding	118 kDa	0	2
Threonyl-tRNA synthetase	PG0888	Translation	75 kDa	0	7
D-lysine 5,6-aminomutase alpha subunit	PG0955	Energy metabolism	57 kDa	0	6
Butyryl-CoA dehydrogenase	PG0958	Fatty acid metabolism	42 kDa	0	7
Alanine racemase; N-acetylmuramoylalanine-D-glutamate-2,6,-diaminopimelate-D-alanine-D-alanine ligase	PG0976	Cell envelope	92 kDa	0	2
Conserved hypothetical protein	PG0981	Unknown	107 kDa	0	9
Ribonucleotide reductase alpha subunit	PG1010	DNA replication	96 kDa	0	6
Probable long-chain fatty-acid-Coenzyme A ligase (long-chain acyl-CoA synthetase)	PG1028	Metabolic processing	69 kDa	0	3
Transferase protein	PG1029	Transposon functions	43 kDa	0	7
Xaa-Pro aminopeptidase	PG1071	Proteolysis	67 kDa	0	14
Hypothetical protein	PG1089	Unknown	80 kDa	0	17
GTP-binding protein (possible membrane protein)	PG1097	Signal transduction	66 kDa	0	2
Conserved hypothetical protein	PG1128	Unknown	47 kDa	0	5
Thiol protease (PrtT related)	PG1251	Proteolysis	93 kDa	0	2
O-succinylbenzoate--CoA ligase	PG1330	Biosynthesis of cofactors	40 kDa	0	9
Magnesium-protoporphyrin O-methyltransferase; cobalamin biosynthesis protein N	PG1359	Biosynthesis of cofactors	163 kDa	0	2
Aminomethyltransferase (glycine cleavage system T protein)	PG1364	Energy metabolism	40 kDa	0	16
Nicotinate-nucleotide pyrophosphorylase (quinolinate phosphoribosyltransferase)	PG1377	Biosynthesis of cofactors	30 kDa	0	4
Bacteroides aerotolerance operon protein, batD	PG1385	Adaptations to atypical conditions	67 kDa	0	2
Fumarate reductase/succinate dehydrogenase flavoprotein subunit	PG1413	Energy metabolism	72 kDa	0	7
Cell division protein (ATPase)	PG1430	Cell division	96 kDa	0	2
Hypothetical protein	PG1448	Unknown	15 kDa	0	21
Conserved hypothetical protein	PG1496	Transport	99 kDa	0	2
ABC transporter, ATP-binding protein, MsaA family; MSD-NBD fusion protein	PG1497	Protein transport & binding	70 kDa	0	4
Hypothetical protein	PG1504	Protein binding	53 kDa	0	2
Immunoreactive 46 kDa antigen PG99	PG1572	Unknown	46 kDa	0	6
Enolase (phosphopyruvate hydratase)(2-phosphoglycerate dehydratase) (laminin binding protein)	PG1593	Energy metabolism	46 kDa	0	22

Identified Proteins (68)	Accession #	Putative Role/Function	M.W.	W83 S.C.V	FLL92 S.C.V
Urocanate hydratase	PG1630	Energy metabolism	74 kDa	0	2
Na ⁺ H ⁺ -exchanging protein (Na ⁺ H ⁺ antiporter)	PG1634	Transport & binding	49 kDa	0	4
Polyphosphate kinase	PG1640	Polyphosphate biosynthesis	81 kDa	0	3
30S ribosomal protein S8	PG1677	Translation	15 kDa	0	4
30S ribosomal protein S3	PG1684	Translation	28 kDa	0	2
50S ribosomal protein L2	PG1687	Translation	30 kDa	0	3
30S ribosomal protein S7	PG1693	Translation	18 kDa	0	2
Hypothetical protein	PG1783	Cell redox, homeostasis	39 kDa	0	2
LPS-modified surface protein P59	PG1838	Cell envelope	61 kDa	0	38
Hypothetical protein	PG1867	Unknown	51 kDa	0	19
Hypothetical protein	PG1899	Hypothetical	27 kDa	0	6
Conserved hypothetical protein	PG1927	Unknown	214 kDa	0	3
Excinuclease ABC subunit A	PG1934	DNA metabolism	106 kDa	0	6

Table 2

MS analysis of missing proteins in FLL92 extracellular fraction showing the spectral count values (SCV)

Identified Proteins (15)	Accession #	Putative Role/Function	M.W.	W83 S.C.V	FLL92 S.C.V
Receptor antigen B	PG0171	Cell envelope	56 kDa	231	0
Conserved hypothetical protein	PG0375	Translation	144 kDa	5	0
Preprotein translocase subunit A protein	PG0468	Protein targeting	126 kDa	5	0
Glutamine-hydrolyzing carbamoyl-phosphate synthase large subunit	PG0484	Arginine biosynthesis	120 kDa	3	0
Hypothetical protein	PG0552	Unknown	37 kDa	10	0
Hypothetical protein	PG0592	Unknown	45 kDa	29	0
Heme-binding protein/peripheral outer membrane chelatase	PG0602	Cell envelope	33 kDa	10	0
Outer membrane protein	PG0626	Cell envelope	42 kDa	10	0
TonB-dependent receptor HmuY	PG1357	Transport & binding	16 kDa	9	0
Probable integral outer membrane protein P20	PG1592	Cell envelope	24 kDa	2	0
Glyceraldehyde 3 - phosphate dehydrogenase	PG1857	Energy Metabolism	36 kDa	2	0
Conserved hypothetical protein	PG1875	Unknown	123 kDa	3	0
Hypothetical protein	PG1894	Unknown	21 kDa	2	0
Outer membrane protein	PG1901	Cell envelope	32 kDa	7	0
Conserved hypothetical protein	PG1938	Unknown	61 kDa	7	0

Table 3
MS analysis of missing proteins in FLL92 extracellular fraction showing molecular characteristics of the proteins

Identified Proteins (15)	Unl Accession number	Putative Role/Function	Localization #	N terminal Signal peptide [*]	C terminal polar tail	M.W.	W83 S.C.V	FLL92 S.C.V
Receptor antigen B	PG0171	Cell envelope	OM	Yes	Yes	56 kDa	231	0
Conserved hypothetical protein	PG0375	Translation	OM	Yes	Yes	144 kDa	5	0
Preprotein translocase subunit A protein	PG0468	Protein targeting	IM	No	Yes	126 kDa	5	0
Glutamine-hydrolyzing carbamoyl-phosphate synthase large subunit	PG0484	Arginine biosynthesis	EC	No	No	120 kDa	3	0
Hypothetical protein	PG0552	Unknown	C	No	No	37 kDa	10	0
Hypothetical protein	PG0592	Unknown	OM	Yes	Yes	45 kDa	29	0
Heme-binding protein/peripheral outer membrane chelatase	PG0602	Cell envelope	PERI	Yes	Partial	33 kDa	10	0
Outer membrane protein	PG0626	Cell envelope	OM	Yes	Yes	42 kDa	10	0
TonB-dependent receptor HmuY	PG1357	Transport & binding	PERI			16 kDa	9	0
Probable integral outer membrane protein P20	PG1592	Cell envelope	OM	Yes	Yes	24 kDa	2	0
Glyceraldehyde 3-phosphate dehydrogenase	PG1857	Energy Metabolism	C	Yes	Yes	36 kDa	2	0
Conserved hypothetical protein	PG1875	Unknown	IM	Yes	Yes	123 kDa	3	0
Hypothetical protein	PG1894	Unknown	C	No		21 kDa	2	0
Outer membrane protein	PG1901	Cell envelope	OM	Yes	Yes	32 kDa	7	0
Conserved hypothetical protein	PG1938	Unknown	IM	Yes	Yes	61 kDa	7	0

[#]The subcellular localization was performed using Psort prediction server.

^{*}The N terminal signal sequence detection was performed using the iSort.

TABLE 4

Predicted post translational motifs in missing FLL92 extracellular proteins

Proteins	MYR	TYR_ PHOS	CK2_ PHOS	PKC_ PHOS	CAMP_ PHOS	ASN_ GLYC	Amidationon	Unique
PG0171	4	0	6	0	0	2	0	0
PG0375	18	0	18	15	0	22	0	PTS-HPR Rib S2
PG0468	7	0	20	23	3	4	4	ATP_GTP_A SECA
PG0484	15		17	7	1	3	0	CPSASE 1 CPSASE 2
PG0552	6	1	3	1	0	1	0	0
PG0592	5	0	0	5	1	3	0	0
PG0602	3	0	2	4	0	2	0	0
PG0626	8	1	5	5	0	0	0	OMPA_1
PG1357	2	0	1	6	0	0	1	ATP_GTP_A
PG1592	8	0	2	4	0	1	0	0
PG1615	4	0	5	3	2	2	0	0
PG1857	7	0	8	5	1	3	0	GAPDH
PG1875	16	0	17	19	2	7	0	RGD
PG1894	5	1	1	0	0	0	1	0
PG1901	1	0	3	5	1	2	1	0
PG1938	23	0	5	8	0	4	0	0

(MYR - N-myristoylation site, TYR_PHOS -Tyrosine kinase phosphorylation site, CK2_PHOS -Casein kinase II phosphorylation site, PKC_PHOS – Protein kinase C phosphorylation site, CAMP_PHOS - cAMP and cGMP-dependent protein kinase phosphorylation site, ASN_GLYC - N-glycosylation site, Amidation - Amidation site, PTS-HPR - Serine Phosphorylation site signature, Rib S2 - Ribosomal protein S2 signature, SECA - SecA Family signature, ATP_HPR - ATP/GTP-binding site motif A, CPSASE 1 - Carbamoyl-phosphate synthase subdomain signature 1, CPSASE 2 - Carbamoyl-phosphate synthase subdomain signature 2, OMPA_1 - OMPA-like domain, GAPDH - Glyceraldehyde 3-phosphate dehydrogenase active site, RGD – Cell attachment sequence)