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Identification and Characterization of Surface Adhesive Proteins of *Prevotella Intermedia* that Bind to Saliva and Extracellular Matrix Proteins

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Abstract: Adhesin proteins *Prevotella intermedia* mediate the attachment of bacteria to host cells and thereby induce the development of periodontal disease. These adhesin proteins are important targets for developing vaccines for treating periodontal disease. This study used two groups of host proteins to capture potential adhesins: salivary proteins and host extracellular matrix proteins (fibrinogen, fibronectin, laminin, collagen I, and collagen IV). We identified 17 adhesin candidates using this approach, including outer membrane proteins, proteases, hemagglutinins, lipoproteins, and porins. Two adhesins (PIN_A0102 and PIN_A1455) were expressed as recombinant proteins and purified, and their binding to ECM proteins was analyzed by the ELISA method. The outer membrane protein (PIN_A0102) was shown to bind to ECM proteins (fibrinogen and fibronectin). Another protein, the protease/hemagglutinin PrT (PIN_A1373), binds to saliva proteins, fibrinogen, and lectin. Thus, *P. intermedia 17* adhesin proteins have been identified that effectively bind to host proteins and are potential candidates for developing vaccines against periodontal disease.

Keywords: adhesins; mass spectrometry; ECM; human fibroblasts; saliva; *P. intermedia 17*.

1. Introduction

Adhesin proteins mediate the attachment of pathogenic bacteria to host cell components and affect cell invasion and host tissue colonization [1-5]. Adhesin proteins are known to target bacteria to host epithelial cells and attach to the surface of the host's oral cavity [6-8]. It has been established that adhesin proteins are a group of proteins associated with the cell surface; these are outer membrane proteins with agglutinating, hemagglutinating, hemolytic, and proteolytic activities [9-11]. These adhesin proteins are the most promising candidates for the development of anti-adhesin strategies and antibiotic therapy and are also potential triggers for signal transduction leading to host cell activation [12-14]. Recently, interest has been growing in studying adhesin proteins involved in the development and progression of periodontal disease [15-18].

P.gingivalis and *P.intermedia* are the main inducers of periodontal disease [19, 20]. These bacteria have adhesive proteins on their cell surface. It is known that *P.intermedia 17* is a black-pigmented gram-negative microorganism and one of the etiological agents of periodontal disease [21]. Attachment of adhesion molecules to human oral epithelial cells is

essential for the survival of this bacterium since *P. intermedia 17* obtains nutrition for its growth from the host environment and tissues. It has been established that hemin and hemoglobin are important nutrients for *P. intermedia 17*, and their presence in the nutrient medium of the body is necessary for penetration into the dentinal tubes [22]. Adhesion of *P.intermedia 17* to host cells is the initial and key stage in developing infectious processes in the human body. For efficient colonization of the host, *P. intermedia 17* produces many pathogenic factors, such as lipopolysaccharides, protein adhesins, proteinases, hemin-binding proteins, hemagglutinins, peptidases, collagenases, fimbriae, hemolysin [23-28].

However, previous research in the field of identification of adhesive proteins has focused on the bacterium *P. gingivalis*, which is the main causative agent of periodontal disease. Adhesin proteins of *P. gingivalis* and their role in the development of periodontal disease have been extensively studied. First reported that the surface components of *P. gingivalis* have hemagglutinating activity [29]. Considerable attention has been paid to the characterization of these bacteria's surface-associated proteins and the study of their contribution to virulence. In particular, the properties of cysteine proteinases with C-terminal adhesins have been extensively studied [30, 31]. Proteinases (gingipains) have been found to have multiple functions. They can cleave proteins into peptides and provide adhesion substrates for bacterial cells [32]. Proteinases are also directly involved in the invasiveness and destruction of tissues by bacteria, as well as in joint aggregation with other bacteria and in the modulation of the host's protective immune system [33-35]. O'Brian *et al.* [36] recently overexpressed and purified various recombinant domains of adhesin proteins derived from the HGP44B protein region of *P. gingivalis.* This HGP44B protein has been shown to have the ability to agglutinate erythrocytes, and it can also bind to host ECM proteins such as fibronectin, fibrinogen, laminin, and collagen V [37]. In addition, about 40 different proteins with proteolytic activity have been isolated from the bacterium *P. gingivalis*, most of which are called "trypsin-like" enzymes [38, 39]. It is assumed that most of the proteolytic, hemagglutinating, and hemoglobin-binding activity can be associated with the expression of the three main genes of cysteine proteinases and partly of the hag genes [40]. More recently, three cysteine proteinases have been isolated from *P. gingivalis*, two of which are capable of hydrolyzing peptide bonds at Arg-X residues (Arg-gingipain or RGP) and one with specificity for Lys-X (Lys-gingipain or KGP) [41, 42]. *P. gingivalis* cysteine proteases have been shown to be important virulence agents in periodontal diseases [43, 44]. In addition, several other proteins associated with the surface of *P. gingivalis* have also been investigated, including hemagglutinins and putative TonB-coupled receptors designated as Tlr, HmuR, and RagA [45-49]. However, identifying *P. gingivalis* adhesin proteins using proteomic approaches has been limited. For example, only two major outer membrane proteins (Omp) of *P. gingivalis*, Omp40, and Omp41, have been identified using 2D SDS-PAGE and peptide mass fingerprinting. It has been suggested that these proteins form a unique heterodimeric porin [50, 51].

There is little information in the literature on *P. intermedia* adhesin proteins. It was previously reported that *P.intermedia* has the ability to bind to human oral cavity epithelial cells, coronary artery cells, and atherosclerotic plaques [52-56]. However, *P. intermedia* OMPs that bind to cell surface proteins of epithelial cells have not been identified. The molecular mechanism of interaction between *P. intermedia* bacteria and host cells also remains unclear. Some studies show that *P. intermedia* can bind to host ECM proteins such as fibrinogen, fibronectin, laminin, type 1 collagen, and lectin [57-59]. Binding these matrix proteins is also important for the invasiveness of *P. intermedia* into host cells and tissues. *P. intermedia* outer

membrane adhesin proteins are known to interact with extracellular matrix proteins. However, little is known about these adhesive proteins. For example, only one protein, AdpB, from the cell surface of *P. intermedia* has been identified and characterized using proteomic approaches [60]. It has been shown that AdpB has a broad spectrum of binding activity for ECM proteins, in particular fibrinogen, fibronectin, and laminin. However, there are no data in the literature on other *P. intermedia* adhesive proteins interacting with ECM proteins. It should also be noted that there is little information in the literature on the study of the agglutinating, hemagglutinating, and proteolytic activity of *P. intermedia*. For example, it was found that *P.intermedia* has a pronounced agglutinating activity against several mammalian erythrocytes (human, monkey, rabbit, sheep) and hemagglutinating activity [61, 62]. In addition, several proteases have been identified in *P. intermedia*, including "trypsin-like" serine proteases, dipeptidyl peptidase IV, and CP [63, 64]. However, there is currently no information on the molecular mechanism of the interaction of *P. intermedia* proteases with host cells.

It has been previously shown that salivary components can promote or inhibit the colonization of host cells [65]. A number of studies have shown that individual saliva molecules have an antibacterial effect on *P. gingivalis*, inhibit bacterial growth, disrupt adhesive-hemagglutination interactions, and suppress hemagglutination and proteolytic activity [66-68]. It is known that the bacteria *P.gingivalis* and *P.intermedia* interact with saliva proteins when they enter the human oral cavity. However, research efforts have focused on *P. gingivalis* rather than on *P. intermedia*, and these demonstrated that *P. gingivalis* attached to experimental salivary pellicles formed on mineral surfaces [69]. Also, adsorbed proline-rich acidic salivary proteins have been found to promote the attachment of *P. gingivalis* to hydroxyapatite surfaces [70]. Salivary components that mediated attachment of other oral bacteria, such as *Actinomyces viscosus* (associated with gingivitis and periodontal disease) have also been identified. It was determined that the acidic proline-rich proteins and statherin also served as adhesin receptors [71]. A number of salivary components, such as mucins, proline-rich proteins, histatins, lysozyme, fibronectin, and secretory immunoglobulin A (sIgA), have been found to interact with *Streptococcus mutans* [72, 73]. Proline-rich proteins, proline-rich glycoproteins, and mucins are also shown to promote the adherence of *Streptococcus gordonii* strain HG 222 [74]. In addition to directly mediating bacterial adhesion, binding to salivary proteins enhanced oral bacteria co-adhesion, i.e., saliva enhanced the binding of *S. mutans* to *A. naeslundii, S. sanguinis*, and *S. mitis* [76].

In addition, salivary components contributed to colonization by fungal organisms, e.g., *Candida albicans* was shown to specifically attach to immobilized basic, proline-rich salivary proteins [77], and saliva promoted the binding of *C. albicans* to immobilized streptococcal cells [78].

However, no studies have been conducted on the interaction of *P. intermedia* outer membrane proteins with saliva proteins. Thus, it was of interest to identify *P. intermedia* surface proteins that bind to salivary proteins. Therefore, we hypothesized that saliva might play a major role in establishing *P. intermedia* infections.

The sequence of *P. intermedia* strain 17 is available, allowing for the application of genome-wide approaches to identify potential adhesins. The most widely used approaches are bioinformatic analyses used to identify putative adhesins based on sequence similarities to adhesins identified in other organisms. However, since *P. intermedia* is distantly related to most well-characterized organisms (such as *Escherichia coli*), many candidate adhesins could be missed using this approach. We, therefore, designed a functional approach to detect and

identify putative *P. intermedia* adhesins. In addition, using bacteria with labeled cell surface proteins for our studies further ensured that the proteins identified were surface exposed.

This work aimed to identify *P. intermedia 17* cell surface proteins mediating bacterial colonization in the oral cavity. We designed an adhesin capture assay to screen *P. intermedia 17* OMPs for their potential to bind ECM and salivary proteins, which were identified by nanospray ESI LC-MS/MS analysis and characterized using functional binding assays.

2. Materials and Methods

2.1. Materials.

For the culture of the *Prevotella intermedia strain 17*, we used sheep blood agar (TSA II, 5% blood, BBL, Cockeysville, MD). Cultures were grown in media (BHI, Difco Laboratories, Detroit, USA) supplemented with 5 µg/mL hemin and 1 µg/mL menadione (Sigma-Aldrich, St. Louis, MO, USA). Monoclonal anti-laminin rabbit, anti-fibrinogen rabbit pAB, anti-collagen pAB (type I mouse pAB), or anti-collagen pAB were purchased from Sigma-Aldrich (St. Louis, USA). Goat anti-rabbit IgG alkaline phosphatase-conjugated and goat anti-mouse IgG alkaline phosphatase-conjugated were purchased from Promega (Madison, USA). Trypsin (mass-spectrometry grade) was purchased from Promega Gold (USA). Plastic laboratory glassware, including various culture flasks, ELISA plates, test tubes, and disposable pipettes for cultivating bacteria, was purchased from Corning-Costar (NY, USA). CyDyeTM (Cy5) was purchased from GE Healthcare (Piscataway, NJ, USA). Dynabeads were purchased from Invitrogen (Dynal AS, Oslo, Norway).

2.2. Growth of Prevotella intermedia 17 cells.

The *Prevotella intermedia strain 17* was used in this study since it is genetically well-defined. This strain was initially isolated and described by H. Fukushima [79]. The *P. intermedia strain 17* bacteria were maintained anaerobically ($N_2/H_2/CO_2$, 80:10:10) at 37°C on sheep blood agar (TSA II, 5% blood) (BBL, Cockeysville, MD, USA). Cultures were grown in brain heart infusion broth media (BHI) (Difco Laboratories, Detroit, USA) supplemented with 5 µg/mL hemin and 1 µg/mL menadione (Sigma-Aldrich, St. Louis, MO, USA). This media, when supplemented, was called enriched BHI (eBHI). To start broth cultures, 3-day-old, non-pigmented colonies grown on sheep blood agar plates were used to inoculate a 3 mL starter batch of eBHI medium. When turbid growth was observed, the starter culture was subcultured into 10 mL of fresh eBHI and grown overnight to a final density of 1.0 at an OD_{660}. 5 mL of the overnight culture was then used to inoculate 300 mL of eBHI. Cells were harvested at the late log phase at an OD_{660} of 1.0 and centrifuged at 15,000 x g for 30 min at 4°C. Cell pellets were washed twice with 0.1 M PBS (pH 8.5) to minimize any carryover of proteins from the broth and collected by centrifugation as described above.

2.3. Dot-blot assay.

P. intermedia 17 whole cell lysates and outer membrane proteins were tested for fibrinogen, collagen I, collagen IV, or laminin binding activities using dot blot assays as described previously [80]. Briefly, *P. intermedia 17* cell lysates were prepared by lysing cells in 2% sodium dodecyl sulfate (SDS) in denaturing conditions (20 mM Tris-HCl, pH 7.4, 8 M urea). *P. intermedia* OMPs were solubilized in 2% SDS under denaturing conditions. *P.*

gingivalis W83 OMPs were prepared in BHI medium as described below (section 2.5.) using cells from 18 hr broth cultures and then solubilized in 2% SDS under denaturing conditions. The *P. gingivalis* OMPs were demonstrated to bind to ECM proteins [81] and were used as a positive control. We used as a negative control bovine serum albumin (BSA), which is known to not bind ECM proteins. Aliquots (5 and 1 µl) of bacterial cell lysates (0.2 µg/mL), OMPs (0.2 µg/mL), and BSA (0.2 µg/mL) were spotted onto nitrocellulose membranes and air-dried. The membranes were blocked with 3% BSA in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 1 hr at room temperature. The membranes were then probed with 1 µg/mL ECM proteins: fibrinogen (membrane A, isolated from human plasma), collagen I (membrane B, isolated from human skin), collagen IV (membrane C, isolated from the human placenta) or laminin (membrane D, isolated from *Engelbreth-Holm-Swarm murine sarcoma*) for 2 hr as described above. Unbound proteins were removed by incubation in TBS containing 0.05% Tween-20 (6 x 15 min). A sandwich assay was performed to detect bound anti-laminin, fibrinogen, collagen I, and collagen IV. Briefly, the membranes were incubated in a 1:2,000 dilution of monoclonal anti-laminin rabbit, anti-fibrinogen rabbit pAB, anti-collagen pAB (type I mouse pAB), or anti-collagen pAB (type IV mouse pAB). Bound laminin and fibrinogen were subsequently detected with a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG. Bound collagen I and collagen IV were detected using a 1: 2,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG. Membranes were washed (3 x 15 min), and bound proteins were visualized with an alkaline phosphatase substrate (Bio-Rad, USA).

2.4. Fluorescent labeling of P. intermedia 17 cell surface proteins.

Cell pellets (from 2 L cell cultures) were washed twice with 0.1 M phosphate-buffered saline (PBS, pH 8.5) and centrifuged at 15 000 x g for 20 min. Supernatants were discarded, and pellets were used for cell surface protein labeling as described previously [80]. Briefly, the cell pellet was resuspended in 1 mL of 0.1 M PBS (pH 8.5) containing 1 mM TLCK and 17 µl of 1 mM CyDyeTM. After vortex for 2 min, the reactions were incubated on ice in the dark for 1 hr. Labeling was terminated by adding 20 µL of 10 mM L-lysine, followed by incubation on ice for 20 min. Cell pellets were stored at -80°C until further use.

2.5. Preparation of P. intermedia 17 and P. gingivalis OMPs.

To protect the fluorescently labeled surface proteins from light, all steps were carried out in the dark. Cell pellets were resuspended in 10 mL of 0.1 M PBS buffer (pH 8.5) and disrupted by cycled sonication on ice using a *Branson 450* sonicator (15 s sonication with cooling on ice four times). To degrade nucleic acids, DNase and RNase (40 µL/10 mL) plus 40 µL of 1 nM $MgCl_2$ per 10 mL were added to the cell lysates and incubated at room temperature for 1 hr in the presence of protease inhibitors (10 µL of TLCK, 1 mM final concentration). Samples were then centrifuged at 5,000 x g for 30 min, and the supernatants were collected for the OMP preparation according to the alkaline incubation method [82]. Sixty mL of ice-cold 0.1 M Na_2CO_3 (pH 11) was added to 10 mL of supernatants and stirred slowly in at 4°C for 1-2 hr. Insoluble outer membrane proteins were harvested by ultracentrifugation at 115 000 x g at 4°C for 1 hr and washed in 2 mL of 0.1 M PBS buffer (pH 8.5). Each pellet was resuspended in 300 µL of 0.1 M PBS buffer (pH 8.5) and kept in the dark at 4°C until further use.

2.6. Preparation of salivary proteins.

Saliva was collected by expectoration from female volunteers according to Collaborative Institutional Training Initiative regulations. Saliva was collected into a pre-chilled container, and protease inhibitors (Protease Inhibitor Cocktail, Sigma, USA) were added to prevent proteolysis of the collected proteins. The saliva samples were centrifuged at 5,000 x g for 10 min to remove insoluble materials present in saliva and then dialyzed against PBS supplemented with protease inhibitors. Salivary proteins were immobilized on surface-activated beads (Dynabeads) according to the manufacturer's protocol described below.

2.7. Adhesin capture assay using M-270 epoxy Dyna beads.

The adhesin capture assay is depicted in Figure 1. M-270 Epoxy Dynabeads (60 mg) were suspended in 4 mL of 0.1 M PBS (pH 7.4) to a final concentration of approximately 109 beads per mL. The beads were washed two times with 1 mL of 0.1 M PBS (pH 7.4) and collected using a magnetic device (Invitrogen Dynal AS, USA). Ligands (fibronectin, fibrinogen, laminin, collagen I and IV, salivary proteins, BSA, fetuin) were prepared in 0.1 M PBS, pH 7.4, at a concentration of 1 mg/mL. Sixty µL (60 µg) of each ligand was then added to 60 µl of washed beads. As a negative control, we added 60 µl of 0.1 M PBS (pH 7.4) to uncoated beads. 60 µL of a 3 M stock-solution (1 M final concentration) of ammonium sulfate was used to enhance binding during coating. Following incubation for 24 hr at 37°C in a water bath with slow tilt rotation, the coated beads were harvested. The supernatants were removed, and the coated beads were washed four times with 0.1 M PBS (pH 7.4). Physically absorbed ligands were removed by washing for 10 min in 10.5% Tween-20 (dissolved in 0.1 M PBS, pH 7.4). Coated beads were then resuspended in 60 µl of 0.1 M PBS (pH 7.4) and used for the isolation of target molecules.

Then 110 µg of OMPs (5.5 mg/ml), derived from fluorescently cell surface-labeled bacteria, were solubilized with 0.1 M PBS (pH 8.5), Zwittergent 3-14 (1% final concentration) and incubated for 1 hr at 4^{o}C and added to coated beads. The mixture was incubated for 2 hr with rotation at 4°C to capture target OMPs. Beads were then collected using the magnetic device and washed in PBS. To elute proteins, 60 µl of 2 x SDS-Sample buffer (125 mM Tris HCl, pH 6.8, 4 % SDS, 100 mM DTT, 20% glycerol [v/v], 0.004% bromphenol blue) was added to the mixture and boiled for 10 min. Beads were then collected using the magnetic device, and the supernatant proteins were subjected to SDS-PAGE. Each set of experiments was done in triplicate.

2.8. Electrophoresis.

The presence and integrity of proteins were monitored using 12% SDS-PAGE under reducing conditions [83].

2.9. Image acquisition and analysis.

Following SDS-PAGE, the gels were fixed for 30 min in fixative (10% methanol, 7% acetic acid), and images of cyanine (Cy5)-labeled proteins were acquired using the Molecular Imager System (Typhoon 8600). A laser set to a wavelength of 635 nm was used to detect Cy5-labeled proteins. Total proteins present in the gel were visualized by staining with Gel Code Blue Stain Reagent (Pierce, Rockford, IL, USA) that uses Coomassie Blue as a basis for protein

stain. Gel images were used to excise protein bands for MS analysis.

2.10. Protein concentration determination.

Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, USA) or by measuring UV absorbance at 280 nm using an ND-1000 NanoDrop Spectrophotometer (Nanodrop Technologies, Thermo Scientific, Wilmington, USA).

2.11. Nanospray ESI LC-MS/MS analysis ("in-solution" and "in-gel" trypsin digestion).

"In-gel" trypsin digestion and *"in-solution"* trypsin digestion followed by LC-MS/MS analysis were used to fingerprint targets to the *P. intermedia 17* database in order to identify the captured OMPs. The *"in-gel"* digestion protocol consisted of two parts: 1) destaining gel slices followed by protein reduction and alkylation and 2) "*in-gel"* trypsin digestion followed by peptide extraction. Specifically, gel slices (1 mm x 4 mm x 1 mm) were washed three times with 200 µL of wash solution (50% CH_3CN, 50 mM NH_4HCO_3) with continuous agitation for 10 min at room temperature and then rinsed with 200 µL of 100% of acetonitrile (CH_3CN) for 5 min at room temperature. To reduce proteins, 100 µl of freshly prepared dithiothreitol (DTT) solution (10 mM DTT in 50 mM NH_4HCO_3) was added to the gel slices, and the mixtures were incubated for 1 hr at 55°C. Following the removal of the reducing liquid, the proteins present in the gel slices were alkylated with 100 µL of freshly prepared iodoacetamide (IAA) solution (55 mM IAA in 50 mM NH_4HCO_3) for 45 min at room temperature. The gel slices were then washed three times with 200 µL of wash solution (50% CH_3CN, 50 mM NH_4HCO_3) and rinsed with 200 µl of acetonitrile (CH_3CN for 5 min) at room temperature. MS grade trypsin (Promega Gold mass-spectrometry grade, USA), reconstituted in 50 mM acetic acid to a final concentration of 1 µg/µl, was diluted to 20 – 32 ng/µl in 50 mM NH_4HCO_3. Then 15 µl of the 20 ng/µL trypsin solution was added to the gel slices, and the samples were incubated for 1 hr at 30°C to allow gel rehydration. The mixture was then supplemented with additional digestion buffer (50 mM NH_4HCO_3/10% CH_3CN) to cover the gel pieces completely and incubated at 37°C overnight. Fifty µl of ultrapure water (≥18 M) was then added to the *"in-gel"* digestion mixture. Following the vortex for 10 min at room temperature, 50 µL of 50% CH_3CN/5% (v/v) formic acid was added to the gel pieces to terminate the digestion reaction. Mixtures were incubated for 60 min at room temperature with frequent vortex, and then supernatants were removed from the mixtures following brief microcentrifugation. The volume of the pooled liquid was reduced using a centrifugal concentrator (at room temperature) until the appropriate volume (typically 10-20 µL) was reached.

"In-solution" trypsin digestion was performed using the traditional method involving protein denaturation with urea followed by protein reduction and alkylation. The protein sample was then suspended in 20 µl urea/DTT solution (8 M urea/100 mM NH_4HCO_3/5 mM DTT), resulting in a final protein concentration of 5 µg/µL and the mixture incubated for 2 hr at 30°C with constant agitation. To alkylate the samples, 1.5 µL of 200 mM IAA was added, and the samples were incubated for 45 min at room temperature. Excess IAA was then neutralized with 1.5 µL of 200 mM DTT for ~20 min. Following reduction with 1 M urea plus 140 µl of 50 mM NH_4HCO_3/2mM $CaCl_2$, trypsin (1:40 w/w, final concentration 20 ng/µL) was added and the mixture incubated at 37°C overnight. Digestion was terminated by adding 10% TFA. The resulting tryptic peptides were loaded onto a C18 trap column (300 µm ID X 5 mm) (Dionex Corp., Sunnyvale, USA) using an Ultimate 3000 HPLC (Dionex Corp., USA),

desalted and then separated on a reverse phase in-house packed C18 (5 µm Magic C18) (Michrom Bioresources, Auburn, USA) column (75 µm ID X 150 mm) at a flow rate of 200 nL/min on a water/acetonitrile/formic acid gradient. Eluted peptides were subjected to nanospray ESI and MS/MS analysis on the LCQ Deca XP Plus (Thermo Scientific, Waltham, USA). The mass spectrometer was operated in the data-dependent mode. The four most abundant ions in each MS spectrum were selected and fragmented to produce tandem mass spectra. The MS/MS spectra were recorded in the profile mode.

2.12. Proteins identification.

Proteins were identified by searching and analyzing MS/MS spectra in the *P. intermedia 17* database available at *www.tigr.org* using *Bioworks v3.2.*

.Only peptides identified as possessing fully tryptic termini with an Xcorr greater than 1.9 for singly charged peptides, 2.3 for doubly charged peptides, and 3.75 for triply charged peptides, as well as delta-correlation scores (ΔCn) greater than 0.3, were used for protein identification. In addition, we set our filter for protein probability to be less than 0.0001 and the number of peptides for the protein to be greater than 2.

2.13. Expression of OmpA 41 (PIN_A1455) and mOmpA (PIN_A0102) in E. coli.

The oligonucleotides 5'-CACCATGGTATTAGCATTTGCTGGTGT-3' and 5'-GTCTGTAACCTGAACAGTTGCAC-3' were designed to amplify an 1143 base pair (bp) DNA fragment encoding the major outer membrane protein (mOmpA, PIN_A0102). The oligonucleotides 5'-CACCTTGCGTAATTTTTTATTACTTTGCA-3' and 5'-CTTAACGTAAACAACTGCAAGGC-3' were designed to amplify an 1170 bp DNA fragment encoding the OmpA 41 precursor (PIN_A1455). The *P. intermedia* genomic DNA was used as a template. The PCR products were cloned into the pEXP5-CT/TOPO expression vector (pEXP5-NT/TOPO and pEXP5-CT/TOPO TA Expression kit (Invitrogen, USA). Recombinant plasmids were transformed into competent *E. coli* One Shot TOP10, and then positive transformants were plated on *Luria-Bertani* (LB) agar plates containing ampicillin (100 µg/ml).

The sequence of the cloned DNA isolated from the positive transformants was verified by DNA sequencing. Recombinant proteins encoded by PIN_A1455 and PIN_A0102 were isolated from the *E. coli BL21 (D3)* strain carrying the recombinant plasmid pEXP5-CT/TOPO-PIN_A0102.

The bacteria were grown overnight in LB medium containing ampicillin (100 µg/mL). This overnight culture was diluted with fresh LB medium and grown at 37°C to mid-log phase. Gene expression was induced by adding 1 mM IPTG for 4 hours at 37°C, then the bacteria were centrifuged at 10,000 g for 20 minutes, and the bacteria pellet was used for further experiments.

The 6xHis-tagged recombinant PIN_1455 and PIN_A0102 proteins were purified using Ni^{2+}-NTA agarose beads under denaturing conditions according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). The purity of the isolated proteins was verified by SDS-PAGE. To renaturate the recombinant proteins, dialysis was performed against 50 mM PBS containing 0.5 M NaCl, pH 8.0.

2.14. Binding of OmpA 41 (PIN_A1455) and mOmpA (PIN_A0102) to ECM proteins.

ELISA-based assays were carried out to test the ability of recombinant OMPs to adhere to various ECM proteins. Proteins: OmpA 41 (immunoreactive 43 kDa antigen PG32), putative PIN_A1455, and the mOmpA (immunoreactive 42 kDa antigen PG33) putative PIN_A0102 protein were 2-fold serially diluted in 0.1 M PBS (pH 7.4) starting at a concentration of 300 µg/mL. Then 100 µL of each dilution (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) was immobilized on 96-well plates resulting in the following final protein concentrations: 30, 15, 7.5, 3.75, 1.87, 0.94 and 0.48 µg/well, respectively. ELISA plates were covered with an adhesive plastic and incubated overnight at 4°C. Following three washes with 100 µL/well of PBS-Tween 20 (0.05%), any remaining protein-binding sites present on the plate wells were blocked by adding 100 µL/well of blocking buffer (PBS, 1% nonfat dry milk)/well. The plates were covered with an adhesive plastic and incubated for 3 hr at 37°C. Following three washes described above, 100 µL/well of ECM proteins (fibrinogen, fibronectin, laminin, collagen I or collagen IV) (20 µg/ml in PBS) were added and incubated for 2 hr at 37°C. Plates were washed again as above, and 100 µl/well of the primary antibodies were added. Murine monoclonal anti-collagen (type I and IV), rabbit polyclonal anti-fibrinogen, rabbit polyclonal anti-fibronectin, rabbit polyclonal anti-laminin prepared at a dilution of 1:2,500 in blocking solution were used to detect respective ligands. Plates were incubated overnight at 4°C and then washed as above. 100 µL/well of alkaline-phosphatase-conjugated IgG was then added, and the plates were incubated overnight at 4°C. We used a 1:1,000 dilution of alkaline-conjugated anti-rabbit IgG to detect fibrinogen and fibronectin and alkaline-conjugated anti-mouse IgG to detect collagen and laminin. Following washes, the bound proteins were visualized with the alkaline phosphatase substrate-pNPP (p-Nitrophenyl-phosphate) (Alkaline Phosphate Substrate kit, BioRad, USA). The yellow color of nitrophenol was measured at 410 nm using the FLUO-STAR Galaxy microplate reader (BMG, Labtech GmbH, Offenburg, Germany).

2.15. Bioinformatics analysis.

Amino acid sequences of the identified proteins, cellular location, MW, pI, functional class characterization, and alignments were determined using either the NCBI or the Oral Pathogen sequence databases (BLASTP program). Conserved domains, related proteins, and structures were predicted using the Conserved Domain Architecture Retrieval Tool (NCBI), LipoP 1.0 Server, or CELLO program.

2.16. Statistical analysis.

To ensure the reproducibility of our results, all experiments were performed three times. An unpaired t-test was used to determine statistical differences.

3. Results and Discussion

3.1. Adhesion of P. intermedia 17 to ECM proteins.

We first examined the ECM-binding capacity of *P. intermedia 17* by dot blotting. Cell lysates and outer membrane proteins (OMPs) isolated from *P. intermedia 17* and *P. gingivalis W83* were used for this study. Lysates prepared from the bacterium *P. intermedia 17* showed a high binding affinity for fibrinogen, collagen I, collagen IV, and laminin (Figure 1, a-d, lanes 1 and 2, rows a and b).

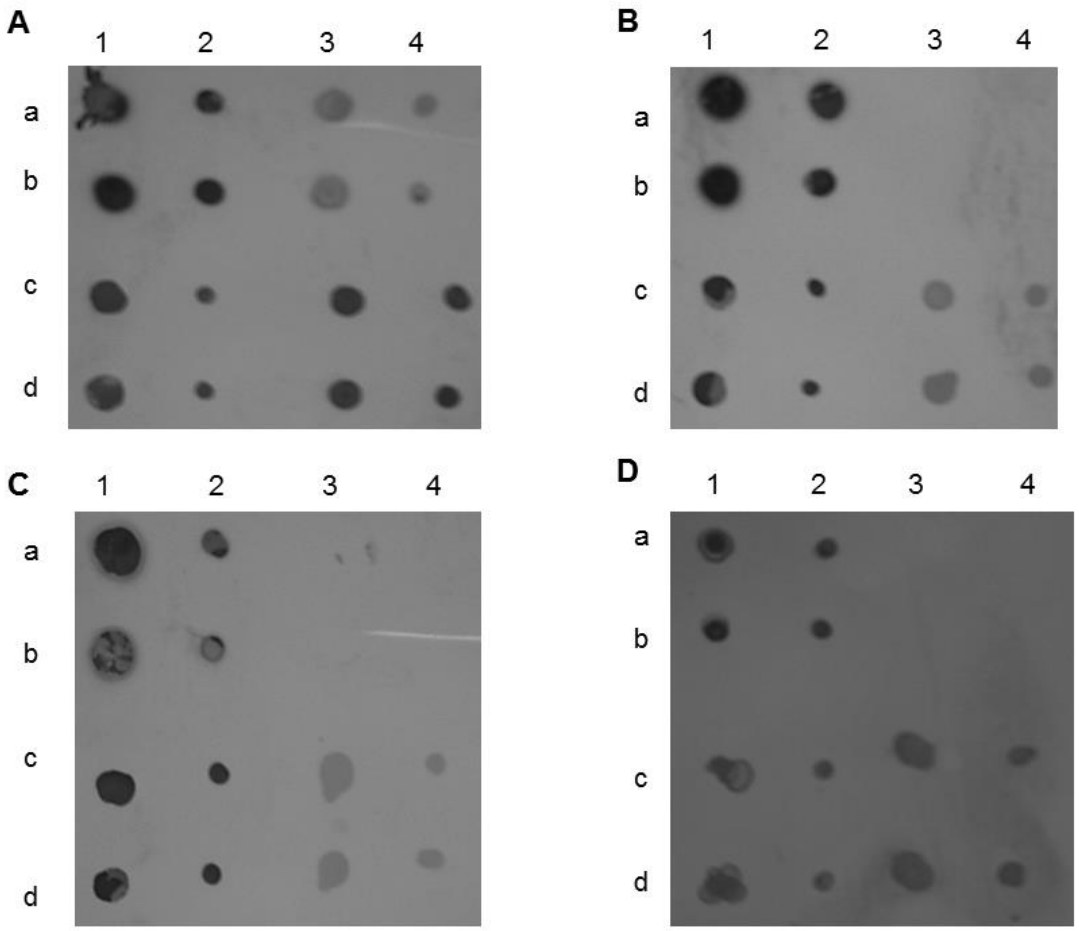


Figure 1. Dot-blot assays of adherence. Lysates from the bacterium *P. intermedia 17* (lanes 1 and 2, rows **(a)** and **(b)**, OMPs of *P. intermedia 17* (lanes 1 and 2, rows **(c)** and **(d)**, OMPs of *P. gingivalis W83* (lanes 3 and 4, rows **(c)** and **(d)** or BSA (control, lanes 3 and 4, rows **(a)** and **(b)** were applied to nitrocellulose membranes and tested for binding to **(A)** fibrinogen**, (B)** collagen I, **(C)** collagen IV or (**D**) laminin.

A similar binding intensity was observed for these ECM proteins when the assay was performed using OMPs derived from *P. intermedia 17* (Figure 1, A-D, lanes 1 and 2, rows c and d). As expected, high fibrinogen and laminin-binding activity was also found for OMPs isolated from *P. gingivalis W83* (positive control, Figure 1 A, D, lanes 3 and 4, rows c and d). However, *P. gingivalis*-derived OMPs had weaker binding to collagen I and collagen IV (Figure 1, B, C, lanes 3 and 4, rows c and d). The ability of various bacterial fractions to bind ECM depended on their concentration, which indicates the specificity of the interaction. Most ECM proteins did not bind to BSA (negative control). Only weak binding was observed between fibrinogen and BSA (Figure 1 A, lanes 3 and 4, rows a and b), indicating a non-specific interaction. Taken together, these results indicate that *P. intermedia 17* outer membrane proteins are able to bind to ECM proteins specifically and that bacterial OMPs mediate this interaction.

3.2. Identification of P. intermedia 17 OMPs that bind to the ECM.

We developed the Dynabead capture method to investigate the binding of *P. intermedia 17* OMPs to extracellular matrix proteins and salivary proteins by immobilizing the extracellular matrix proteins on the beads (Figure 2).

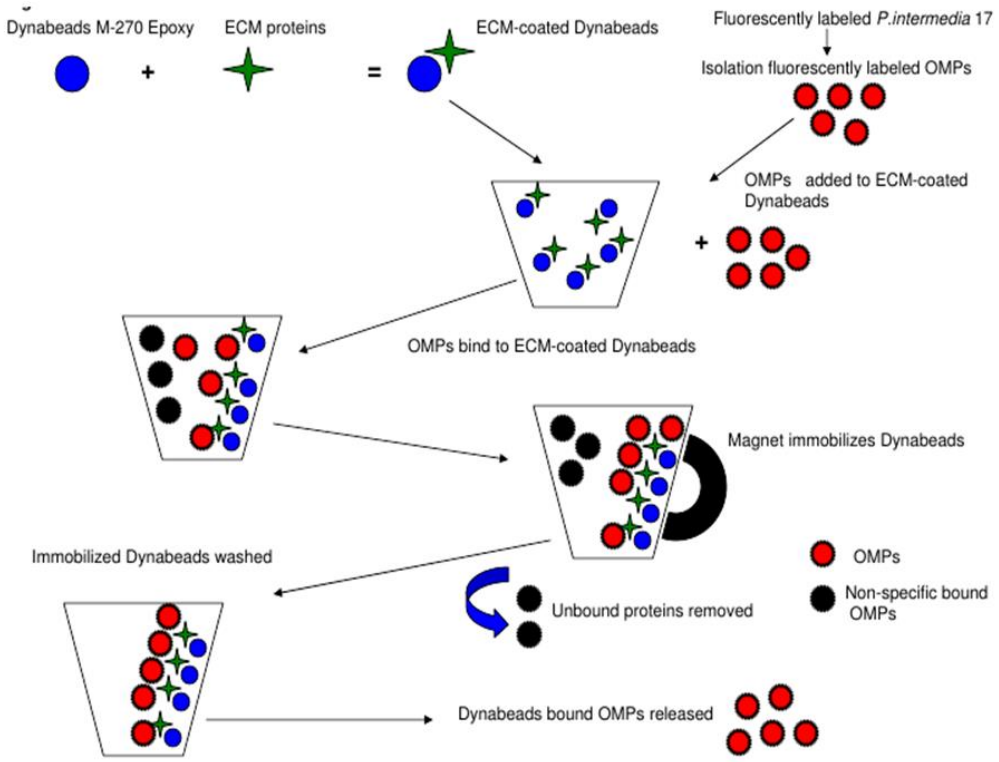


Figure 2. Schematic depicting the design of the M-270 Dynabead epoxy capture assay developed to identify *P. intermedia 17* OMPs adhesins.

Cy5-labeled OMPs profiles were eluted from ECM-conjugated Dynabeads and examined by SDS-PAGE (Figures 3 and 4). OMPs that bind to fibronectin are shown in Figure 3 (track 1).

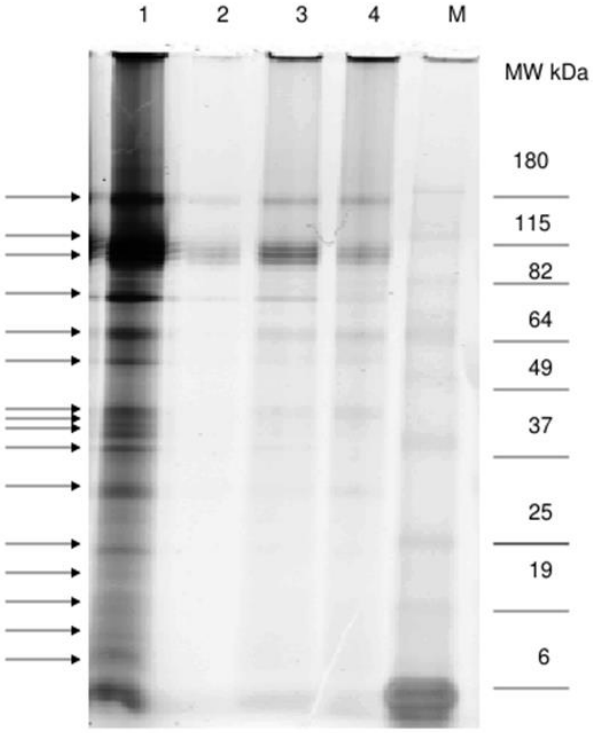


Figure 3. *P. intermedia 17* OMPs binding fibronectin. The following proteins were used to coat Dynabead: fibronectin (lane 1), BSA (lane 2), fetuin (lane 3), or uncoated beads (lane 4), M (MW marker). Cy5-labeled OMPs (100 µg) derived from bacteria were added to coated aliquots of Dynabead. Bound OMPs were eluted with Laemmli sample buffer and separated on a 12% SDS-PAGE gel. The resolved bands of the Cy5-labeled OMPs were visualized using a Typhoon Imager at 635 nm. Arrows indicate protein bands excised and presented for MS analysis.

Protein bands corresponding to the bands seen in the fluorescence image were excised from the Coomassie blue stained gel, and MS. Several dominant protein bands identified the proteins were observed at MW of approximately 180, 115-82, 70, 64-49, 45-37 and 27 kDa. In addition, many other bands of lower intensity were observed, which were also suitable for MS analysis. OMPs interacting with other ECM proteins (collagen I, collagen IV, fibrinogen) are shown in Figure 4A (lanes 1–3, 5), and laminin is shown in Figure 4B (lane 7).

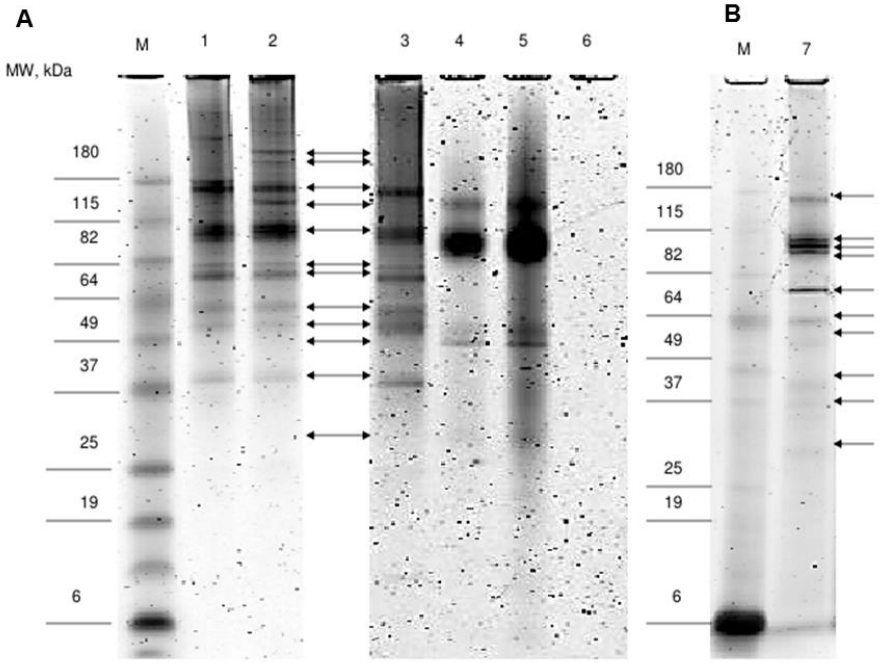


Figure 4. *P. intermedia 17* OMPs binding ECM: **(A)** Dynabeads were coated with either collagen I (lane 1), collagen IV (lane 2), fibrinogen (lane 3), BSA (lane 4), fetuin (lane 5), or uncoated beads (lane 6), M (MW marker); **(B)** M (MW marker) and laminin (lane 7). Associated with ECM proteins, *P. intermedia 17* OMP proteins were analyzed by 12% SDS-PAGE, and images were obtained using the Typhoon Imager. Arrows indicate protein bands excised and presented for MS analysis.

The most common proteins identified had a strong binding affinity for collagen and fibrinogen and had high molecular weights (100, 84, 64, and 49 kDa). The binding profile of OMP to laminin was different from that of collagen, fibronectin, and fibrinogen. Three major bands of laminin-binding proteins were found with molecular weights of 180, 115-82, and 67 kDa. Diffuse signals have also been found for lower molecular weight proteins. Negative controls: BSA and uncoated beads showed little binding to OMP (Figure 3, lanes 2-4, and Figure 4, lanes 4-6). The binding profiles of OMPs to collagen I and collagen IV were similar. However, the fibrinogen-binding OMPs differed from the binding profiles of OMPs to collagen I and collagen IV, reflecting the binding specificity of the respective OMPs to ECM proteins.

To identify OMPs interacting with ECM proteins, protein fluorescent bands were excised from SDS-PAGE gels, and then the proteins were digested *"in-gel"* with trypsin and analyzed by MS. In addition, an aliquot of the OMPs eluent captured by the ECM-conjugated Dynabeads was used for *"in-solution"* trypsin digestion, followed by peptide mass fingerprinting analysis. Table 1 lists the identified OMPs that bind to ECM proteins.

Table 1 presents data obtained from two experiments, the digestion of proteins with trypsin *"in-gel"* and *"in-solution"*. Using OMPs isolated with immobilized fibrinogen, we identified nine proteins; six demonstrated high sequence coverage (≥20) based on Delta correlation value and protein and peptide ion probability scores (hit) (Table 1). According to their predicted function, these proteins may be *P. intermedia 17* adhesin proteins.

Table 1. *P. intermedia 17* OMPs interacting with different ECM proteins, identified by LC-MS/MS analysis" *in-solution*" & *"in-gel"* trypsin digestion.

Ligand[a)]	***P.intermedia 17* database accession no.**[b)]	**Predicted protein function**	**C_{y5} labeled**	**P(pro)**[c)]	**Delta Cn**[d)]	**Predicted mass**	**Peptide (hits)**[e)] **ions**	**Sf (final score)**[f)]	**Score Xcorr**[g)]
Fibrinogen	PIN_A0160	Hypothetical protein	Yes	2.01E-05	13.70	28 382	3(30 000)	1.39	30.15
	PIN_A0324	Hb binding protein	Yes	6.57E-07	7.40	55 226	3(30 000)	2.34	30.14
	PIN_A1509	Hypothetical protein	Yes	2.7E-09	8.90	66 703	3(30 000)	2.40	30.17
	PIN_A0102	FN-binding protein, similar to *P.gingivalis* immunoreactive 42 kDa antigen PG33, putative	Yes	2.22E-15	19.40	41 989	5(50 000)	3.07	40.29
	***PIN_A1455**	OmpA 41 precursor	Yes	4.16E-12	9.70	43 075	3(30 000)	1.96	20.32
	***PIN_A0326**	TonB-dependent outer membrane receptor protein, similar to *P.gingivalis* Rag A protein, putative	Yes	1.87E-07	7.80	116 737	4(40 000)	3.36	40.18
	PIN_A1050	Conserved hypothetical protein	Yes	1.99E-04	17.40	20 097	2(20 000)	1.13	20.15
Fibronectin	PIN_A0380	Hypothetical protein	Yes	8.82E-04	6.60	32 651	2(20 000)	1.38	20.16
	PIN_A0160	Hypothetical protein	Yes	1.4E-08	32.10	28 382	13(130 000)	5.56	80.27
	PIN_A0324	Hb binding protein	Yes	4.77E-05	7.60	55 226	2(20 000)	1.48	20.19
	PIN_0633	Hypothetical protein	Yes	3.36E-03	7.00	28 888	3(30 000)	0.8	20.14
	PIN_A1184	Outer membrane lipoprotein, putative	Yes	7.24E-04	12.90	31 273	3(30 000)	2.49	30.25
	PIN_A1318	Ribosomal protein L7/L12 C-terminal	Yes	2.33E-08	10.80	62 532	5(50 000)	3.74	40.32

Ligand[a)]	*P.intermedia 17* database accession no.[b)]	Predicted protein function	C_{y5} labeled	P(pro)[c)]	Delta Cn[d)]	Predicted mass	Peptide (hits)[e)] ions	Sf (final score)[f)]	Score Xcorr[g)]
		domain protein							
	PIN_1509	Hypothetical protein	Yes	3.01E-08	10.40	66 703	7(70 000)	4.21	50.20
	PIN_A1050	Conserved hypothetical protein	Yes	3.52E-12	29.30	20 097	6(60 000)	2.90	30.28
	PIN_A0326	Rag A protein, putative	Yes	2.16E-07	5.4	116 737	5(50 000)	3.24	40.24
	PIN_A0102	FN-binding protein, similar to *P.gingivalis*, immunoreactive 42 kDa antigen PG33	Yes	6.48E-05	11.0	41 989	5(50 000)	2.98	40.22
	PIN_A1455	OmpA 41 precursor	Yes	7.98E-11	23.80	43 075	7(70 000)	5.95	70.34
Lectin	PIN_A1373	Hypothetical protein, similar to peptidase C10 family of proteins	Yes	2.9E-05	3.8	112 567	2(20000)	1.73	20.20
	PIN_A1164	Lipoprotein, putative	Yes	9.55E-04	11.30	16 282	4(40000)	0.99	20.18
	PIN_A0324	Hb binding protein	Yes	9.42E-12	13.20	43 075	9(90 000)	5.33	60.29
	PIN_A0160	Hypothetical protein	Yes	1.19E-07	14.90	28 382	6(60 000)	3.02	40.21
	PIN_0228	Hypothetical protein	Yes	4.20E-06	11.60	15 547	3 30 000)	1.82	20.17
	PIN_A1184	Outer membrane lipoprotein,putative	Yes	7.61E-06	17.60	31 273	4(40 000)	2.57	40.17
	PIN_0093	Conserved hypothetical protein	Yes	1.63E-04	13.20	20 443	3(30 000)	1.39	20.19
	PIN_A1509	Hypothetical protein	Yes	3.93E-05	5.20	66 703	2(20 000)	1.70	20.15
	PIN_0633	Hypothetical protein	Yes	5.44E-04	4.0	28 888	5(50 000)	0.92	20.13
Collagen 1	PIN_A0160	Hypothetical protein	Yes	4.71E-11	6.10	28 382	2(20 000)	0.96	10.20

Ligand[a)]	*P.intermedia 17* database accession no.[b)]	Predicted protein function	C_{y5} labeled	P(pro)[c)]	Delta Cn[d)]	Predicted mass	Peptide (hits)[e)] ions	Sf (final score)[f)]	Score Xcorr[g)]
	PIN_0228	Hypothetical protein	Yes	2.67E-09	15.20	15 547	1(10 000)	0.82	10.13
	***PIN_A1509**	Hypothetical protein	Yes	1.48E-04	3.7	66 703	1(10 000)	0.94	10.18
	***PIN_A0324**	Hb binding protein	Yes	2.43E-03	2.5	55 226	1(10 000)	0.95	10.17
	PIN_A1050	Conserved hypothetical protein	Yes	1.28E-06	23.90	20 097	3(30 000)	1.8	20.18
Collagen 1V	**PIN_A1509**	Hypothetical protein	Yes	5.99E-06	3.40	66 703	1(10 0000	0.97	10.24
	PIN_A0324	Hypothetical protein	Yes	6.64E-04	2.50	55 226	1(10 000)	0.89	10.14
	PIN_A0326	Rag A protein, putative	Yes	4.15E-07	1.90	116 737	1(10 000)	0.79	10.12
	PIN_A1050	Conserved hypothetical protein	Yes	1.81E-06	12.0	20 097	1(10 000)	0.92	10.18
Laminin	***PIN_0228**	Hypothetical protein	Yes	3.98E-04	21.70	15 547	3(30 000)	1.24	20.12
	***PIN_A0324**	Hb binding protein	Yes	1.86E-08	5.80	55 226	2(20 000)	1.48	20.27
	PIN_A0160	Hypothetical protein	Yes	3.35E-11	12.60	28 382	5(50 000)	1.18	20.24
	PIN_0004	Conserved hypothetical protein	Yes	1.29E-07	11.70	17 222	2(20 000)	0.97	10.24
	PIN_A1373	Hypothetical protein, similar to peptidase C10 family of proteins	Yes	6.65E-05	2.50	112 567	1(10 000)	0.77	10.14
	PIN_A0990	Fibronectin type 111 domain protein	Yes	4.77E-14	1.30	117 715	2(20 000)	0.95	10.20
	PIN_A1455	OmpA 41 precursor	Yes	3.56E-10	4.10	43 075	3(30 000)	0.99	10.35
	PIN_A0102	Immunoreactive 42 kDa antigen PG33, putative	Yes	2.81	3.90	41 989	8(80 000)	0.79	10.17
	PIN_A0326	Rag A protein, putative	Yes	1.92E-05	1.10	116 737	3(30 000)	0.68	10.16

Ligand[a)]	*P.intermedia 17* database accession no.[b)]	Predicted protein function	C_{y5} labeled	P(pro)[c)]	Delta Cn[d)]	Predicted mass	Peptide (hits)[e)] ions	Sf (final score)[f)]	Score Xcorr[g)]
Uncoated beads	***PIN_A1050***	Conserved hypothetical protein	Yes	3.07E-08	12.00	20 097	1(10 000)	0.89	10.16
	PIN_A0102	Immunoreactive 42 kDa antigen PG33, putative	Yes	1.75E-05	6.30	41 989	1(10 000)	0.98	10.27
	PIN_A0326	Rag A protein, putative	Yes	2.31E-04	2.10	116 737	1(10 000)	0.7	10.12
	PIN_0235	Hypothetical protein	Yes	4.91E-03	1.90	121 516	1(10 000)	0.59	10.13
Fetuin	***PIN_A1455***	OmpA 41 precursor	Yes	6.14E-05	2.60	43 075	1(10 000)	0.7	10.10
	PIN_A0102	Immunoreactive 42 kDa antigen PG33, putative	Yes	1.94E-03	6.30	41 989	1(10 000)	0.68	10.12
	PIN_A0326	Rag A protein, putative	Yes	2.7E-03	0.80	116 737	1(10 000)	0.79	10.11
BSA	***PIN_A1455***	OmpA 41 precursor	Yes	4.12E-10	9.00	43 075	5(50 000)	2.58	30.34
	PIN_A1050	Conserved hypothetical protein	Yes	4.64E-06	17.40	20 097	2(20 000)	1.84	20.18
	PIN_A1642	Sporulation related repeat family	Yes	3.71E-05	5.00	19 534	1(10 000)	0.86	10.12

Foot notes: (a) ECM proteins used for the binding assay on Dynabeads; (b) Accession numbers from the Comprehensive Microbial Resource (CMR) at TIGR; (c) The protein probability score is the best probability score of peptides associated with the protein. The lower the score, the better match it is; (d) Delta Correlation - the difference between the normalized XCorrs of the primary and secondary matches. The greater the difference - the better match it is. Delta Cn>0.1 is a good match, and Delta Cn>0.3 is a great match; (e) the ratio of the number of ions observed/number of ions possible. The number of possible ions is the number of ions in the MS/MS spectrum of this peptide. The acceptable number of observed peptide ions is >1; (f) the protein Sf score is the sum of the peptide Sf scores for all the peptides associated with that protein. The higher the value of the Sf score, the better the protein match. Numbers above 0.7 are considered good; g) Xcorr - the cross-correlation value computed from the cross-correlation of the experimental MS/MS spectrum vs. candidate peptides in the database. The candidate producing the highest Xcorr value is chosen as the #1 hit by Sequest. Bold shows the proteins identified in "*in-gel"* trypsin digestion. * shows the proteins identified *"in-gel"* and "*in-solution*" trypsin digestion. Italics show the proteins identified in negative controls.

For example, PIN_A0324 has been identified as a hemoglobin-binding protein, PIN_A0102 is a fibrinogen-binding protein similar to *P. gingivalis* immunoreactive antigen PG33 (42 kDa), PIN_A1455 is an OMP similar to *P. gingivalis* immunoreactive antigen PG32 (43 kDa), and PIN_A0326 is a putative TonB-dependent Om receptor protein similar to the *P. gingivalis* Rag A protein (database *www.tigr.org*). MS analysis of fibronectin-binding OMPs demonstrated high sequence homology (range 40 to 80%) for several proteins, including

PIN_A0160, PIN_A0326, PIN_A0102, and PIN_A1455 (Table 1). Nine proteins were identified based on matches between 3-13 *P. intermedia 17* peptides with approximately 20-80% sequence homology. The most relevant proteins were identified as the hypothetical PIN_A1060 protein (80% homology) and the putative OMP homologous to *P. gingivalis* immunoreactive antigen PG32 (43 kDa) (PIN_A1455, 70% homology). Five proteins (PIN_A0102, PIN_A1455, PIN_A0326, PIN_A0160, and PIN_A0324) have also been identified as potential fibrinogen-binding proteins, indicating that these proteins may have the potential to bind to a wide range of ECM proteins (Table 1). Of note, collagen I and collagen IV interacted with several Omps (Table 1). However, sequence coverage was very low for all proteins found (<20).

We also identified laminin-binding OMPs, which are a group of nine proteins (Table 1). Some proteins, such as PIN_0228 (hypothetical protein), PIN_A0324 (Hb binding protein), and PIN_A0160 (hypothetical protein), had a Delta Cn of 20 (Table 1). Other proteins had a Delta Cn <20 (Table 1). Based on fewer bands observed on the SDS-PAGE gel (Figure 4B, lane 7) and MS data, *P. intermedia 17* OMPs appear to have less affinity for laminin compared to other ECM proteins. Collectively, similar profiles of fibrinogen-, fibronectin-, laminin-, and collagen-binding OMPs were observed on SDS-PAGE gels, consistent with mass spectrometry data that also identified fibrinogen-, fibronectin-, and laminin-binding OMPs. However, these OMPs showed a low binding affinity for collagen I and collagen IV. Negative controls (uncoated beads, BSA, and fetuin) showed either no binding or low binding to ECM proteins. These results indicated that specific interactions were observed between *P. intermedia 17* OMPs and host ECM proteins.

3.3. Identification of salivary protein-binding OMPs.

The profiles of OMPs isolated using immobilized saliva proteins are shown in Figure 5. Strong fluorescent signals were found for protein bands at 115, 80, and 64 kDa (Figure 5, lane 1). OMPs with a molecular weight of about 37-25 kDa also showed binding to salivary proteins. However, the binding affinity was lower, as evidenced by lower-intensity signals. When negative controls (BSA, fetuin, or uncoated beads) were used, no OMP binding was observed, indicating binding specificity to salivary proteins.

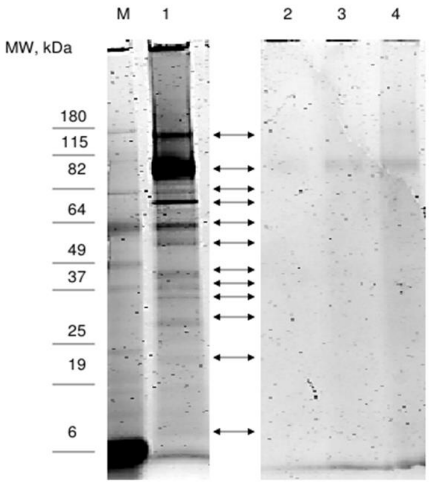


Figure 5. *P. intermedia 17* OMPs profiles binding to salivary proteins. Cy-5 labeled proteins were eluted from Dynabeads coated with either saliva proteins (lane 1), BSA (lane 2), fetuin (lane 3), or uncoated beads (lane 4). BSA, fetuin, and uncoated beads were used as negative controls. Captured *P. intermedia 17* OMPs were analyzed by SDS-PAGE, and Cy5 labeled proteins were visualized with a Typhoon Imager. Arrows indicate bands of proteins excised and presented for MS analysis.

Protein bands showing strong signals on SDS-PAGE were excised, and proteins were identified by MS (Table 2). High sequence coverage was obtained for proteins such as the *PrtT* protease/hemagglutinin (PIN_A1373) and the OmpA family protein (PIN_A0261), indicating that they may be possible adhesin proteins.

Bioinformatics analysis showed that the *PrtT* protease/hemagglutinin (PIN_A1373) has significant similarities with *P. gingivalis W83* proteins PG1356 (*PrtT* protease/hemagglutinin) and PG1251 (thiol protease (*PrtT*-related)), as well as with *Bacteroides thetaiotaomicron VPI-5482* proteins, BT2451, BT2217, and BT2220.

Table 2. Identification of *P. intermedia 17* salivary protein-binding OMPs by LC-MS/MS.
Values a), b), c), d), e), and f) are the same as described in Table 1. Proteins identified in negative controls are shown in italics.

Ligand[a)]	***P.intermedia 17* database Accession no.**[b)]	**Predicted protein function**	**C_{y5} labeled**	**P(pro)**[c)]	**Delta Cn**[d)]	**Predicted mass, M.W., D**	**Peptide (hits)**[e)] **ions**	**Sf (final score)**[f)]	**Score Xcorr**[g)]
Saliva proteins	PIN_A1960	Imidazolonepropionase (hutI) [3.5.2.7.]	Yes	4.86E-05	2.60	42 383	1(10 000)	0.35	10.10
	PIN_A1049	Ribosomal protein L17(rplQ)	Yes	1.57E-12	11.90	17 995	2(20 000)	1.07	20.32
	PIN_A0261	OmpA family protein	Yes	5.86E-07	20.20	30 026	4(40 000)	2.50	30.19
	PIN_A1740	Hystidyl-tRNA synthetase(hisS) [6.1.1.21]	Yes	9.68E-06	2.90	46 260	1(10 000)	0.60	10.11
	PIN_A1373	Hypothetical protein, similar to peptidase C10 family of proteins	Yes	4.70E-05	4.50	112 567	6(60 000)	1.58	30.17
	PIN_A1184	Lipoprotein, putative	Yes	6.82E-05	5.00	31 273	2(20 000)	0.91	10.19
	PIN_A0990	Fibronectin type 111 domain protein	Yes	9.54E-05	1.40	117 715	1(10 000)	0.68	10.14
	PIN_A0184	Ribosomal protein S2(rpsB)	Yes	2.37E-04	8.90	30 773	2(20 000)	0.66	10.16
	PIN_A0437	Lipoprotein, putative	Yes	6.58E-04	3.20	39 456	1(10 000)	0.55	10.11

However, significant block overlaps (4/6 blocks) place the *PrtT* protease/ hemagglutinin (PIN_A1373) in the *Streptopain* (C10) cysteine protease family.

The search for protein domains showed that residues 43-381 correspond to PD:PD004169, which was described as the thiol precursor of *PrtT*, hemagglutinin-exotoxin-protease-*Streptococcal* peptidase-cysteine. In addition, residues 1-381 place PIN_1373 (PI0811) in the C10 peptidase family (PF01640). *PrtT* protease/hemagglutinin (PIN_A1373) was paralogically linked to PI1993 (thiol protease/hemagglutinin, *PrtT* precursor), pPI0032 (cysteine protease), PI0493 (BspA surface antigen), PI2050 (hypothetical protein), PI1937 (hypothetical protein), and PI0137 (lectin-like adhesin precursor). The search for a conserved domain showed that the C10 peptidase family contains a YopT-type cysteine protease domain. YopT is a *Yersinia pestis* virulence effector protein that cleaves and releases the host cell's Rho-GTPase from the membrane, thereby destroying the actin cytoskeleton. The catalytic domain of peptidase C10 is a triad of Cys, His, and Asp. This domain has been described in

proteins of plant pathogens (*Pseudomonas syringae*) and intracellular animal pathogens (e.g., *Y. pestis, Haemophilus ducreyi, Pasteurella mulcodia, Chlamydia trachomatis*).

We also performed an analysis of a putative adhesin protein (PIN_A0261). The search for conserved domains and comparative analysis showed that OmpA (PIN_A0261) contains a conserved MotB domain found at the C-terminus of OmpA proteins. In addition, OmpA (PIN_A0261) was found to have significant similarity to the W83 lipoprotein from *P. gingivalis* (PG1793) and had a 19.5% similarity to the PDB:1OAP Mad structure of the *E. coli* Pal protein periplasmic domain. Table 2 shows saliva-associated OMPs including possible flagellar motor proteins, protease/hemagglutinin proteins, and hemoglobin/fibrinogen binding proteins.

3.4. Bioinformatic analysis of some P. intermedia 17 proteins.

Based on the MS data, proteins with a high X-score correlation were selected to confirm their adhesin uptake results. We chose two candidates: a major outer membrane protein (mOmpA, PIN_A0102), which binds to fibrinogen and fibronectin, and a precursor to outer membrane protein 41 (OmpA 41, PIN_A1455), which also interacts with fibrinogen and fibronectin. It is known that mOmpA (PIN_A0102) belongs to the family of flagellar motor proteins and shares a number of characteristics with proteins of the OmpA family found in other bacterial species. According to the block search, the proteins belonged to the IPB001145 family, which is described as a "bacterial outer membrane protein." The search for the protein domain showed that residues 1-200 correspond to the sequence in PD:PD35998, which is described as a membrane signal precursor for *P. gingivalis W83* OmpA, Omp41, Omp40, basic PG32 and PG33. It should be noted that amino acid residues 283 to 373 of mOmpA (PIN_A0102) were identical to the OmpA-like motif (Figure 6a), which is characteristic of the OmpA family of membrane proteins, lipoproteins, and porins.

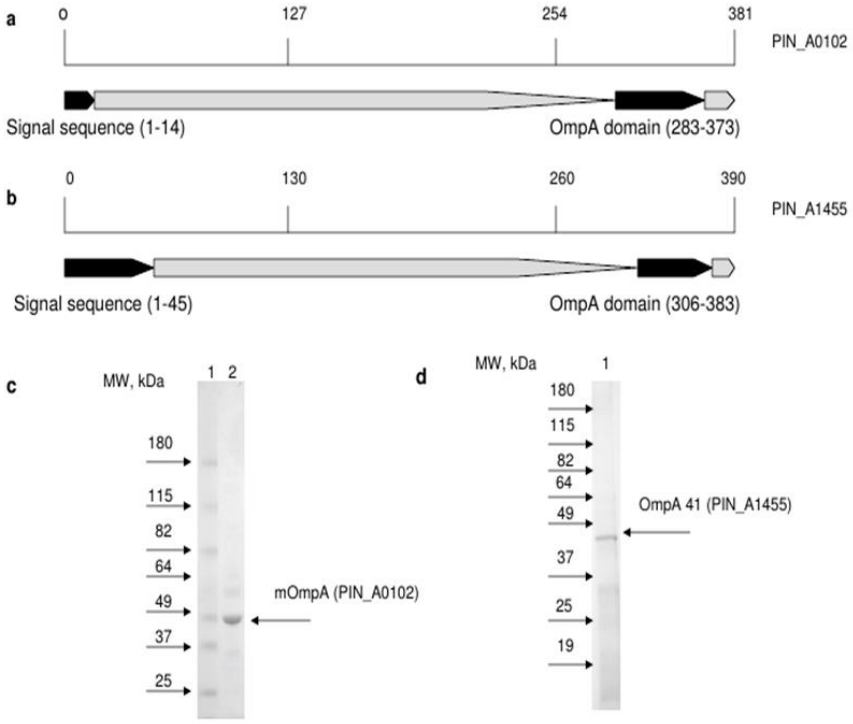


Figure 6. Characteristics of the recombinant proteins PIN_A0102 and PIN_A1455: schematic representation of the protein domains encoded by (**a**) PIN_A0102 or (**b**) PIN_A1455; recombinant proteins (**c**) PIN_A0102 and (**d**) PIN_A1455 were subjected to SDS-PAGE and visualized by Coomassie staining.

Bioinformatic analysis has shown that mOmpA (PIN_A0102) has a similar architecture of the OmpA/MotB domain (residues 125-256) with proteins from *Proteobacteria* (OmpA/MotB domain), *Mycobacterium* (OmpA), *Rhizobiaceae* (OmpA/MotB domain), *Bacteroidetes* (OmpA), *Alphaproteobacteria* (hypothetical protein OmpA/MotB domain), *Burkholderiales* (cytochrome c oxidase), *Acidobacteria, Actinobacteria*, *Cyanobacteria*, and *Spirochetes*. In addition, mOmpA (PIN_A0102) was found to have similarities with the OmpA/MotB domain protein of the *Pseudomonas putida W616* protein, *Mycobacterium tuberculosis* OmpA protein, *Neisseria meningitides* Rmpm protein, and *Haemophillus influenza* lipoprotein. Finally, the **176**AEAGWNRYEGDLGDYDQYYG**195** sequence was shown to be homologous to the putative *Bacteroides fragilis NCTC 9343* protein and *B. fragilis* erythrocyte hemolysin lysis protein *YCH46*.

The results showed that the recombinant OmpA 41 (PIN_A1455, PI0893) had a molecular weight of 43 kDa, which is consistent with our calculated mass (Figure 6**d**) and likely corresponds to the 43 kDa protein shown in Figure 3 (lane 1) and Figure 4 (lane 3).

Thus, we identified OmpA 41 (PIN_A1455) in the outer membrane fraction of *P. intermedia 17*, which is consistent with the predicted extracellular location of the protein in the outer membrane. OmpA 41 (PIN_A1455) showed a significant hit in 2/4 blocks of the IPB001145 family, which describes this protein as a "bacterial outer membrane protein". OmpA 41 (PIN_A1455) is conserved between residues 306-383 characteristics of the OmpA (PF00691) family of proteins (Figure 6**b**). In addition, OmpA 41 (PIN_A1455, PI0893) is paralogically associated with mOmpA (PIN_A0102, PI 1717) and PI1070 (peptidoglycan-associated lipoprotein). This protein showed significant similarity to the *P. gingivalis W83* immunoreactive antigen PG32 (43 kDa) and putative proteins *PG0627* (outer membrane protein) and *PG0626* (outer membrane protein). In addition, homology with proteins of the *B. thetaiotaomicron VPI-5482* strain was revealed: BT1391, BT0066 and BT3852, BT0418, BT1791, and BT4481.

3.5. Adhesion of mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) to ECM proteins.

mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) proteins were cloned and expressed in *E. coli*. The purity of mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) was confirmed by SDS-PAGE. One of the protein fractions corresponded to the protein band at 42 kDa after Coomassie staining (Figure 6c, lane 2). The predicted molecular weight of mOmpA (*www.tigr.org*, PIN_A0102) was 42 kDa and thus corresponded to the band size of the identified protein (Figure 6c, lane 2). The OmpA 41 protein (PIN_A1455) was prepared in a similar manner, and SDS-PAGE analysis showed a single protein band at 43 kDa, which matched the predicted size of OmpA 41 (*www.tigr.org*) (Figure 6d, lane 1).

To investigate the binding affinity of mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) to ECM proteins, we performed an ELISA assay. ELISA analysis was performed as described in the "Materials and Methods" section. Microtiter plates were coated with mOmpA (PIN_A0102) or OmpA 41 (PIN_A1455) and then probed with fibrinogen, fibronectin, collagen I, collagen IV, or laminin. This assay showed that mOmpA (PIN_A0102) binds fibrinogen and fibronectin with a high binding affinity (Figure 7), which correlates with the results obtained using our capture assay (Figures 3-4 and Table 1).

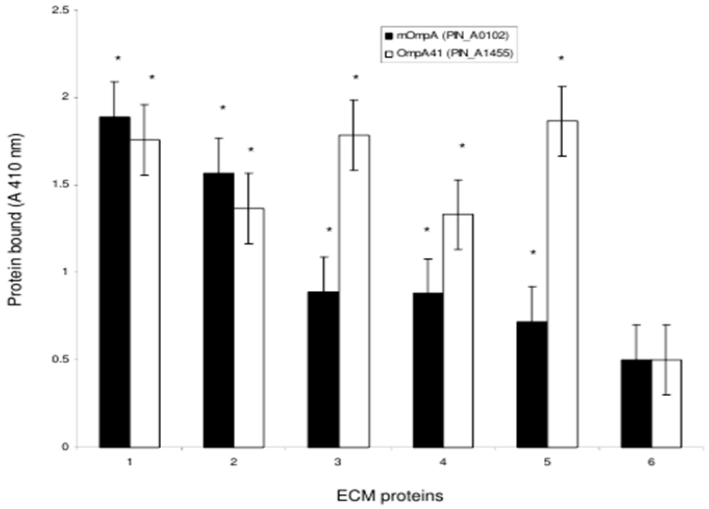
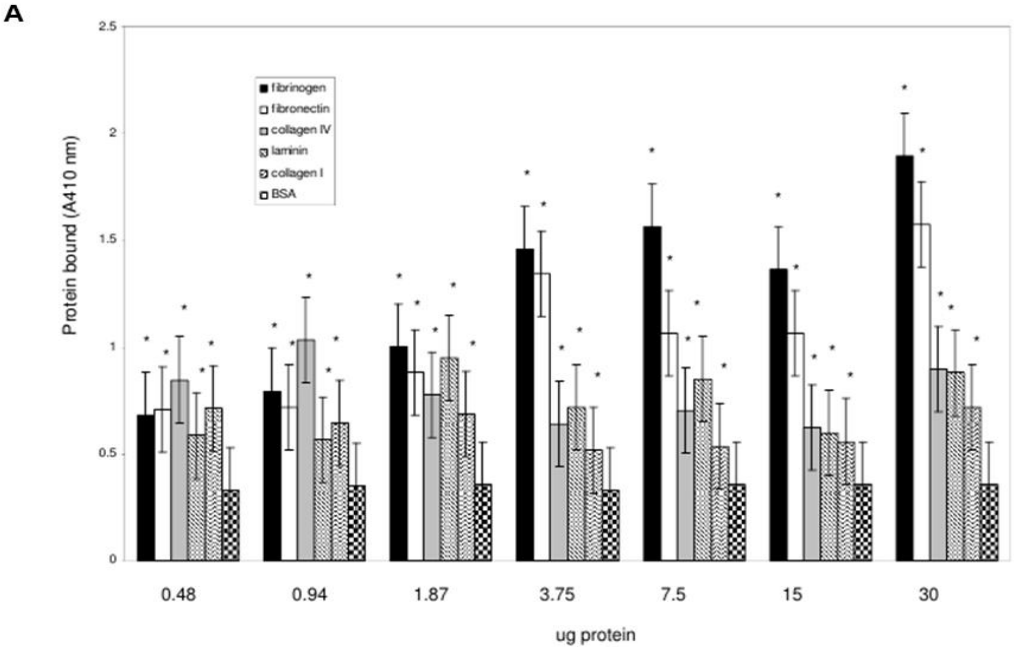


Figure 7. Binding of mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) to ECM proteins. Microtiter plates were coated with recombinant mOmpA or OmpA 41 proteins at an initial concentration of 300 µg/ml and incubated with 30 µg/ml of the respective ECM proteins: 1-fibrinogen, 2-fibronectin, 3-collagen IV, 4-laminin, 5-collagen I or 6-BSA. The amount of bound ECM proteins was determined spectrophotometrically at 410 nm. Data are presented as the mean ± standard deviation of analyzes performed in triplicate. Statistical significance was determined using an unpaired t-test. Asterisks denote P values ≤ 0.0001 compared to BSA.

ELISA analysis also showed a lower binding affinity of mOmpA (PIN_A0102) for collagen I, collagen IV, and laminin. As expected, no binding was observed in the BSA-coated wells. These data showed that mOmpA PIN_A0102 has a broad binding profile to ECM proteins and that differences in the binding capacity to different ECM proteins (combined with no significant binding to collagen or laminin) suggest that interactions between mOmpA and other ECM proteins were specific. Binding specificity was further analyzed using increasing amounts of mOmpA (PIN_A0102).

As shown in Figure 8a, increasing the concentration of recombinant mOmpA resulted in increased binding to ECM proteins, consistent with saturation kinetics and further confirming the specificity of observed binding.



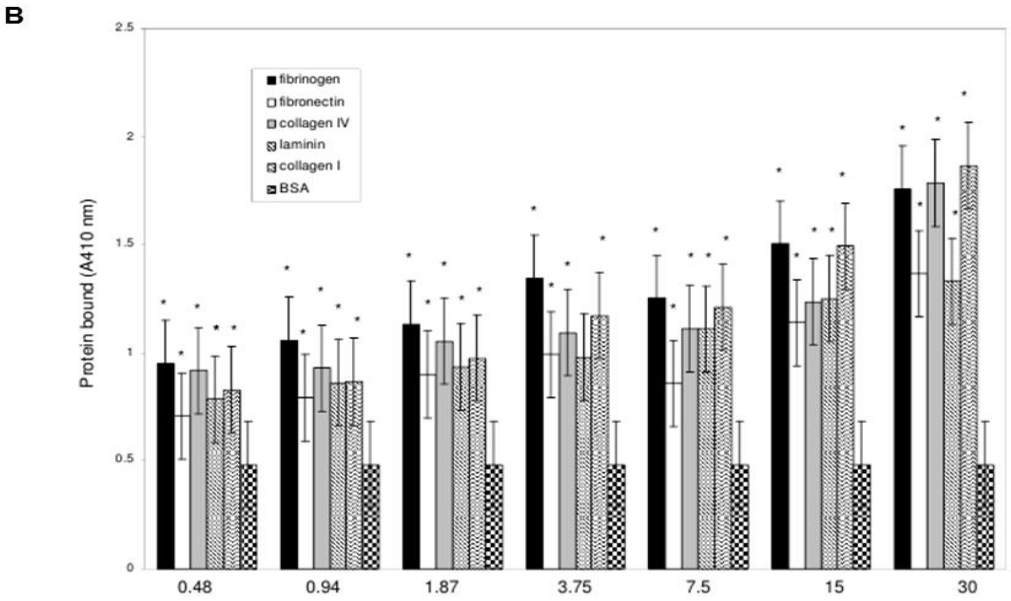


Figure 8. Binding characteristics of mOmpA (PIN_A0102) and OmpA 41 (PIN_A1455) to ECM proteins. Microtiter plates were loaded with increasing amounts of either (a) mOmpA or (b) OmpA 41 and then probed with 20 μg/ml of the respective ECM proteins (fibrinogen, fibronectin, collagen IV, laminin, collagen I, or BSA). The number of bound proteins was determined spectrophotometrically at 410 nm. Data are presented as the mean ± standard deviation of analyzes performed in triplicate. Statistical significance was determined using an unpaired t-test. Asterisks denote P values ≤ 0.0001 compared to BSA.

In parallel experiments, the binding of OmpA 41 (PIN_A1455) to fibrinogen, fibronectin, collagen I, collagen IV, and laminin was investigated. OmpA 41 (PIN_A1455) was identified using immobilized fibrinogen and fibronectin (Table 1), which also correlated with adhesion capture assay results. In addition, ELISA analysis showed that OmpA 41 also has specificity for collagen and laminin. This was expected, given the adhesion profiles shown in Figure 4 (lanes 1-3, 7).

The binding profile of OmpA 41 to the tested ECM proteins also showed a dose-dependent saturable adhesion consistent with specific interactions (Figure 8b). Taken together, a comparison of the adhesion profiles of both proteins showed that OmpA 41 (PIN_A1455) has a broader spectrum of binding to ECM proteins compared to mOmpA (PIN_A0102), which specifically binds only to fibrinogen and fibronectin. As expected, both recombinant proteins did not interact with immobilized BSA. Thus, we conclude that our ELISA approach confirmed our adhesion capture method's specificity and further defined the binding profiles of novel adhesin proteins.

4. Conclusions

This study aimed to identify novel *P. intermedia 17* adhesin proteins using proteomic approaches coupled with an adhesion capture assay. Although it has previously been shown that *P. intermedia* can bind to various ECM proteins [53, 54, 58], the strains used for these studies differed from the *P. intermedia* strain 17 used here, for which it was only reported that this strain is able to bind to fibronectin [80]. Therefore, in this work, the binding of bacterial lysates of *P. intermedia 17*, OMPs to various ECM proteins was studied. The results of the study showed that *P. intermedia* strain 17 lysates have a strong binding affinity for fibrinogen, laminin, collagen I, and collagen IV. Of note, *P. intermedia 17* OMPs also showed a high binding affinity for fibrinogen, collagen I, collagen IV, and laminin. These data confirmed that

P. intermedia strain 17 could bind to various host ECM proteins and that this interaction between ECM components and *P. intermedia* strain 17 occurs on the outer bacterial membrane, suggesting that surface proteins mediate this binding.

Currently, several methods are used to identify protein-protein interactions; in particular, bioinformatic approaches are used to identify and classify membrane proteins based on the presence of a signal peptide [84, 85]. However, the disadvantage of this approach is that surface proteins secreted by mechanisms independent of the signal sequence are missed [86]. Other functional approaches that use isolation of the entire outer membrane to identify adhesin proteins make it possible to isolate proteins located in the membrane but not exposed on the surface [80, 87]. We developed a novel adhesin uptake assay to identify all bacterial proteins involved in recognition and binding to host components. Identifying bacterial adhesins using Cy5-labeled bacteria before isolation of outer membrane proteins was previously described, which were then separated using SDS-PAGE and detected using an imager capable of detecting fluorescently labeled proteins [80, 87-89].

The strategy described in our work combines labeling surface proteins with the capture of *P. intermedia* adhesins using ECM-coated magnetic beads. We have demonstrated that this new approach is both sensitive and efficient for the identification of novel bacterial adhesin proteins. Unlike other approaches, this method can be used to identify proteins located on the cell surface and proteins secreted by atypical mechanisms. The magnetic separation technology used in this study is simple and reproducible. All protein capture steps occur in a single tube for maximum sensitivity and minimum loss of target proteins. Dynabeads have a mono-size and surface chemistry optimized for minimal binding of non-specific proteins. OMPs isolated with magnetic Dynabeads were effective in studying binding to extracellular matrix proteins and to studying binding to salivary proteins, which were used as ligands for protein affinity capture. In addition, Cy5-labeling of samples greatly increased the sensitivity of protein detection, and the use of mass spectrometry was sufficient to identify putative adhesin proteins. We identified ECM protein-binding *P. intermedia 17* OMPs using nano-ESI MS/MS analysis. This method makes it possible to detect up to 50 attomoles of a protein [89- 91]. Before mass spectrometric sequencing of OMPs, we used two proteolytic methods for digesting proteins with trypsin (*"in-gel*" and *"in-solution*"). The *"in-gel"* digest method is a classic approach to proteomic studies because it is possible to identify proteins from corresponding bands on an SDS-PAGE gel quickly, and we have identified several proteins from major protein bands using this approach (Table 1).

However, for some experiments, it was preferable to use trypsin digestion *"in- solution"* instead, as this method can identify a greater variety of protein targets compared to trypsin digestion *"in-gel"* (Table 1), which increases the detection threshold for proteins present in low concentrations. The increased sensitivity results from the fact that the components are not diluted during additional procedures during and after the electrophoresis steps. An additional benefit is also time savings by reducing the number of mass spectrometric experiments to a single experiment. Although the *"in-solution"* trypsin digestion method does not separate Cy5-tagged proteins from unlabeled ones, the surface effect of most proteins has been confirmed based on the fact that the same proteins have been identified by the *"in-gel"* digestion method (see Table 1). Only three additional proteins, PIN_A0160, PIN_0380, and PIN_0633, were identified using trypsin digestion *"in-solution*" rather than *"in-gel"* digestion. According to the *P. intermedia* database available at *www.tigr.org,* PIN_A0160 and PIN_A0633 are hypothetical proteins, and their function, as well as localization, is unknown. However,

PIN_A0389 is annotated as a lipoprotein; thus, we assume it is a surface protein. In general, it can be concluded that in our study, mainly surface proteins were identified.

The fluorescent profiles of captured proteins using different ECM/bead conjugates were similar, especially for OMPs binding to fibrinogen, collagen I, and IV. These data are also consistent with our MS analysis, which identified the same proteins from different capture assays. This indicates that the identified adhesin proteins have a wide range of binding affinities. These observations were expected since many adhesin proteins have previously been shown to bind multiple substrates. For example, *S. aureus* extracellular adhesion protein (Eap), iron-regulated adhesin (IsdA), and protein Emp have been shown to bind to various ECM proteins, and *P. gingivalis* surface proteinase-adhesin complexes (RgpA and KgpA complexes) have shown a broad spectrum of binding activity. with ECM proteins, including fibrinogen, fibronectin, hemoglobin, type V collagen, and laminin [89, 92-95].

In our study, the proteins that bind to laminin and fibronectin exhibited very different patterns, i.e., several protein bands were common across the gels. However, the overall patterns were different, indicating the specificity of our adhesin uptake assay. In addition, the profiles of proteins binding to the extracellular matrix and saliva proteins were completely different. The results of the adhesin uptake assay were ultimately validated using ELISA-based assays. These proteins were found to bind to the proteins used to *"capture"* them, i.e. fibrinogen and fibronectin, confirming the specificity of our adhesin capture assay. We identified seventeen OMPs that bind to ECM proteins based on X-correlation data. These proteins included hemoglobin binding protein (PIN_A0324), basic outer membrane protein (PIN_A0102), TonB-dependent outer membrane receptor protein (encoded by PIN_A0326) similar to *P. gingivalis* Rag A protein, hypothetical protein (PIN_A0160), outer membrane protein 41 (PIN_A1455) and a conserved hypothetical protein (PIN_A1509). Only one of these proteins, the *P. intermedia* hemoglobin-binding outer membrane protein encoded by PIN_A0324, has been previously isolated and characterized [96]. This protein (M.W.=60 kDa) had a pH-dependent hemoglobin binding activity with a dissociation constant of approximately $1.48x10^{-8}$ M as measured by surface plasmon resonance. These results suggest that *P. intermedia* specifically binds to hemoglobin via a 60 kDa outer membrane protein. TonB-dependent receptors are well known for their role in iron uptake [97]. For example, *H. ducreyi* contains a Ton-B-dependent receptor (hemoglobin HgbA receptor), which is essential for iron absorption and virulence in humans [97]. *P. gingivalis* RagA was originally identified as an immuno-dominant surface antigen recognized by serum antibodies in patients with periodontal disease [98]. Protein RagA shares homology with TonB-coupled outer membrane receptors involved in the recognition and active transport of specific outer ligands by a wide range of Gram-negative species [99]. For example, *P. gingivalis* Rag A functions as an active carrier of large protein degradation products [100]. However, to date, there is no information on the role of TonB-dependent *P. intermedia* outer membrane receptors, including PIN_A0326, identified in this study. Interestingly, the iron acquisition transporters identified in this study can bind ECM proteins. The major outer membrane protein (PIN_A0102) and the outer membrane protein precursor 41 (PIN_A1455) are paralogically related since they both contain conserved domains characteristic of the OmpA family. The hypothetical protein (PIN_A0160) and the conserved hypothetical protein (PIN_A1509) do not have significant similarities to the *P. gingivalis* W83 protein or the *B. thetaiotaomicron* VPI-5482 protein, and their functions remain unknown. However, the hypothetical PIN_A0160 protein is similar to the hypothetical *P. intermedia* PI1774, PI0855, and PI0856 proteins. The conserved hypothetical protein PIN_A1509 is

paralogically related to PI0426 (conservative hypothetical protein, probable outer membrane lipoprotein), PI0765 (probable surface protein), and PI1498 (probable membrane-associated lipoprotein). Until now, the biological role of these proteins remains uncertain. However, based on their strong affinity for ECM proteins, these proteins may be promising adhesin proteins. Among OMPs that bind to salivary proteins, we identified lipoproteins and enzymes. In particular, enzymes such as histidyl-tRNA synthetase, imidazolonepropionase, and protease/hemagglutinin *PrtT* have been identified. In addition, the flagellar motor protein Omp A (PIN_A0261) and the protease/hemagglutinin *PrtT* of the streptopain family proteins (PIN_A1373) also showed high binding affinity to salivary proteins. While bioinformatic analysis of OmpA (PIN_A0261) showed that this protein could be located in the outer membrane, inner membrane, or periplasm, our studies clearly showed that it is located in the outer membrane and on the exposed surface of the bacterium. In addition, bioinformatics analysis has shown that it is similar to a family of bacterial outer membrane proteins that are similar to integral porin membrane proteins and small molecular weight lipid-anchored proteins (such as Pal).

These proteins are present in the outer membrane of many Gram-negative organisms.

Previously, trypsin-like serine proteases (dipeptidyl peptidase IV and CP) were identified in *P. intermedia* [63, 101, 102], and our study, we identified the protease/hemagglutinin *PrtT* (PIN_A1373), which also interacts with saliva proteins. Bacterial proteases degrade proteins, peptides, and glycopeptides; therefore, we hypothesize that protease/hemagglutinin *PrtT* (PIN_A1373) can promote the binding of *P. intermedia* to host cell surfaces and its protease properties facilitate penetration of bacteria into the oral cavity. In addition, by binding to various saliva proteins, this enzyme can cause their degradation.

Thus, using the adhesin uptake assay developed in this study, we identified 17 putative *P. intermedia 17* adhesin proteins. These include outer membrane proteins, proteases, lipoproteins, hypothetical proteins, and flagellar motor proteins. Further studies are needed to determine the specific domain(s) and individual amino acids of the OMPs responsible for the adhesion of *P. intermedia 17* to the extracellular matrix and/or proteins of the host's saliva. Importantly, further study of their role in adherence and host-pathogen interactions will contribute to the understanding of the molecular mechanisms of oral bacterial virulence. These data will also provide valuable information that can be used to develop new antibacterial drugs and more effective treatments for periodontal disease.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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