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Research Article

Mass Spectrometry Identification of Outer Membrane Proteins of *Prevotella intermedia 17*, Interacting with Host Cells

Akentieva Natalia*

Department Kinetics of Chemical and of Biological studies, Institute of Problems of Chemical Physics Russian Academy of Sciences, Russia

***Corresponding Author:** Akentieva Natalia, Department Kinetics of Chemical and of Biological studies, Institute of Problems of Chemical Physics Russian Academy of Sciences, Russia.

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Abstract

Bacterial pathogens attach to the cell surface of host and then induce host cells or bacterial changes to gain entry to the host cells. An analysis of bacteria present in the mouth showed that some types of bacteria that lead to periodontal disease were associated with higher risk of esophageal cancer. Adhesin bacteria-host proteins interactions are necessary for invasion and bacterial colonization of host tissues. Adherence of *P. intermedia 17* to the host cells is the initial and crucial step in development of periodontal disease. Identification and characterization of outer membrane proteins (Omps) *P. intermedia 17* associated with cell surface host proteins is important step in investigation of molecular mechanism of host-bacteria interactions.

Here we used sensitive fluorescent labeling Omps of *P. intermedia 17*, followed incubation with host cells (oral epithelial and human fibroblasts), 1D gel separations and mass spectrometric identification (LC-MS/MS) of cell surface proteins. We identified 7 Omps of *P. intermedia 17*, interacting with oral epithelial cells. Two from them, Omp 41(PIN_A1455, immunoreactive 43 kDa antigen PG32, putative) and major Omp (PIN_A0102, immunoreactive 42 kDa antigen PG33, putative) were determined with high sequence coverage and top score of peptides match. Effect of heat on host-bacteria interaction has been studied. Similar native and denaturated Omps, bound with host cells, were identified by LC-MS/MS analysis. Denaturated Omps demonstrated the lower binding affinity in comparison with native. Nine Omps were detected, associated with human fibroblasts. Cell surface Omps bound to human fibroblasts were determined, using "shaving" approach. Hypothetical protein (PIN_A0324) has been identified as protein, interacting with cell surface proteins from human fibroblasts.

Keywords: Periodontal Disease; Cancer; Host Cells; Mass Spectrometry Analysis

Abbreviations

OMPs: Outer Membrane Proteins; HF: Human Fibroblasts; BHI: Brain and Heart Infusion; TLCK: Tosyl Lysyl Chloromethyl Ketone; Nano ESI MS/MS: Nano Electrospray Mass Spectrometry; LC-MS/MS: Liquid Chromatography Mass Spectrometry; DTT: Dithiothreitol; IAA: Iodoacetamide; OEC: Oral Epithelial Cells; HOEC: Human Oral Epithelial Cells

Introduction

Previous research has shown that periodontal disease caused by certain oral microbiota has been associated with several types of cancer, including oral and head, neck cancers and esophageal cancer. Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer death worldwide. Because the disease is often not discovered until it has reached an advanced stage, five-year survival rates range from about 15 to 25 percent worldwide. Certain bacteria types were associated with higher risk of esophageal cancer. For example, higher levels of the *Tannerella forsythia* bacteria were associated with a 21 percent increased risk of esophageal adenocarcinoma, which is more common in the developed world. The bacteria *Porphyromonas gingivalis* and *Prevotella intermedia 17* were associated with a higher risk of esophageal squamous-cell carcinoma, which is also more common in the developing world. Both species of bacteria are linked with common gum disease, periodontal disease.

Proteins that mediate the attachment of the bacteria to host, followed by invasion and colonization of the host cells, are present on the cell surface of the bacteria. Outer membrane proteins (Omps) of bacteria are integral membrane proteins, they span the membrane and contain the hydrophilic and hydrophobic domains, that interact with internal and external molecules. Functions of Omps, being exposed to the outside environment, can include cell-cell communications, surface recognition, signaling, enzymatic activity and others. Omps include different proteins and structures involved in cell adhesion. *P. intermedia 17* cell surface proteins (adhesins) mediate the attachment of the bacteria to host components. Adhesins and other membrane-associated proteins are important targets for vaccine development, because they are surface-located and readily accessible to antibodies. Additionally, Omps can be also the potential triggers of signal transduction leading to activation of host cells.

Therefore, the identification and characterization of cell surface proteins expressed by *P. intermedia 17* is a prerequisite for the development of vaccines designed to interfere with bacterial colonization of host tissues.

First step of bacterial colonization is attachment of bacteria to the epithelial cells.

Epithelial cells have two functions. From one site, they serve as a mechanical barrier to the aggression of microorganisms and as sensors of microbial infection. From another side, epithelial cells generate and transmit signals between bacteria and the adjacent and underlying immune cells in the periodontal tissues. Epithelial cells are the primarily target for pathogenic microorganisms. Attachment of bacteria to host is a complex process involving specific and nonspecific interactions. Nonspecific association is a temporary event and mediated by hydrophobic, lipophilic and electrostatic binding. Specific interactions involve the anchoring binding between lectin-like bacterial binding proteins (adhesins) and receptors or proteins on host tissues.

Pathogenic oral bacteria are causative agents of periodontal diseases. Interactions between oral bacteria and gingival epithelial cells are essential aspects of periodontal infections. Invasion of oral epithelial cells (OEC) by pathogenic oral bacteria may represent an important virulence factor in the progression of periodontal disease. Group of gram-negative anaerobic bacteria is frequently associated with periodontal diseases, including *Bacteroides forsythus, Campylobacter curvus, Eikenella corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis* and *Prevotella intermedia*.

Porphyromonas gingivalis (42%) and *Prevotella intermedia* (37%) were the most frequently detected species on and in epithelial cells derived from periodontal pockets and the gingival crevice of subjects with periodontitis [1].

Specific association of *P. gingivalis* has been reported to squamous human epithelial cells [2,3], epidermal carcinoma KB cells [4], HeLa cells (cervical carcinoma) [5] and gingival carcinoma (Ca 9-22 cells) [6].

A little known about interaction of *P. intermedia* and host cells. *P. intermedia 17* has been reported to bind to human epithelial cells [7]. It has been shown, that *P. intermedia 17* can invade human oral epithelial cells (HOEC) *in vitro*. Type C fimbriae and cytoskeletal rearrangement were required for invasion of oral epithelial cells (OEC).

P. intermedia 17 also possessed strong hemagglutinating activity for several mammalian erythrocytes (human, monkey, rabbit, sheep) [8,9]. Earlier studies demonstrated that clinical strains *P. intermedia* possessed various types of fimbria (surface appendages). The ability of these bacteria to agglutinate mammalian erythrocytes is attributed to surface structures, particular to the fimbriae or the fimbrial-associated components. Additionally, Omps and cell vesicles of *P. intermedia* demonstrated a hemolytic activity, because they were capable of liberating the hemoglobin from erythrocytes, thereby acquiring an essential nutrient, iron, for its metabolism [8].

However, a little information available about interaction between Omps of *P. intermedia 17* and whole human fibroblasts or OEC (cell line HN_4).

It was known, that association between *P. intermedia 17* and the host cells mediated by adhesins-host proteins interaction. The outer membrane (and its components) is the first key site of interaction between *P. intermedia* and host cells and thus is an important factor for entry and infection. It is known, that attachment of *P. intermedia* to the primary epithelial cells induces the formation of membrane invaginations that surround and engulf the bacteria, effecting their internalization [7].

Adhesins compounds of P. gingivalis, mediating attachment bacteria to host cells, were identified. It was shown, that they involve cell-surface and ECM components, including cysteine proteinases, gingipains [10], hemagglutinines, fimbiae [11-13], glycosyltransferase [14], lipopolysaccharides [15].

At the same time, no many data are available about adhesins components of *P. intermedia 17,* bound with OEC and human fibroblasts. There is no information about molecular mechanism of interaction Omps *P. intermedia 17* and host cells. This process can include binding Omps to cell surface proteins of host and also movement some Omps inside of host cells. The molecular mechanisms trough which *P. intermedia* attaches to and induces their internalization by host cells (epithelial) is still largely unknown. The Omps (cell surface proteins) of *P. intermedia 17*, which can be directly involved in attachment to host cells, are not identified yet. Omps and adhesins of *Prevotella intermedia 17* should be examine on their ability to adhere to and invade cultures of HOEC.

There have been several investigations about the effect of heat on host-bacteria association. It was demonstrated that heating of *E. coli* adhesin protein involved in diffuse adherence (AIDA-1) at 90^0C lead to the irreversible thermal denaturation of protein and reduce the adhesion [16]. Additionally, KB epithelial cells that had been incubated with heat-denaturated vesicles of *P. gingivalis* W83 did not contain adhesion peptides [16]. However, no information is available about thermal action on adherence *P. intermedia 17* to OEC or human fibroblasts.

Here we used a mass spectrometry approach in conjunction with traditional 1D separation of proteins to identify possible adhesins of *P. intermedia* 17, involved in association with OEC and human fibroblasts.

Using an in vitro tissue culture model, Omps of gram-negative anaerobic bacteria *Prevotella intermedia 17*, were examined for their ability to adhere to cultures of HOEC. The attachment of *P. intermedia 17* was also tested using OEC, line HN4. Association experiments performed on whole host cells and Omps *P. intermedia 17*, preliminary labeled with fluorescent compound Cy5. We identified 7 proteins, which were specifically bound by OEC and human fibroblasts. We demonstrated that binding Omps of *P. intermedia 17* to host cells was reduced by treatment of Omps with heat. The putative adhesins were identified as well-known *P. intermedia 17* OmpsA family proteins. Identification of Omps should improve our

understanding the molecular mechanism of the adherence of *P. intermedia 17* to host cells and role of Omps in progress periodontal disease.

Materials and Methods

Growth of *P. intermedia 17* cells

P. intermedia strain *17* was maintained anaerobically by routine weekly subculture on sheep blood agar (TSA 11,5% blood, BBL Cockeysville, MD). Cultures were grown in BHI broth media supplemented with 5 µg/mL hemin (Sigma) and 1 µg/mL menadione (Sigma) (enriched BHI) at 37^{0}C in an anaerobic chamber (N_2/H_2/CO_2, 80:10:10). Briefly, 3 day old, non-pigmented colonies from sheep blood agar plates were used to inoculate a 3 mL starter batch of enriched BHI medium. When turbid growth was observed, the starter culture was sub cultured into 10 mL of fresh enriched BHI, and grown overnight to a final OD_{660} of approximately 1.0. A quantity of 5 mL of the overnight culture was served to inoculate 300 mL of BHI broth containing menadione. Cells were harvested in late log phase at an OD_{660} of 1.0 by centrifugation at 15,000 x g for 30 minutes at 4^{0}C. Cell pellets were washed twice with 0.1M PBS (pH = 8.5), to minimize any carryover of proteins from the broth, and collected by centrifugation as described above.

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

Cell pellets (from 2L cell culture) washed with 0.1 M PBS (pH = 8.5) buffer twice and centrifuged at 15,000 x g, 20 minutes. Supernatants have been discarded and pellets have been used for cell surface protein labeling. As described previously [17], briefly, 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 CyDye™ (Cy5, Amersham Biosciences), the mixture was vortex for 2 minutes. The labeling reactions were incubated on ice in the dark for 1h. The labeling was terminated by the addition 20 µL of 10 mM L-lysine followed by incubation on ice for 20 minutes. Cell pellets have been kept in freezer (-80^{0}C) until isolation of outer membranes.

Preparation of *P. intermedia* outer membrane proteins, labeled with Cy5

All steps were carried out in dark. Cell pellets were resuspended in 10 ml 0.1M PBS buffer (pH = 8.5) and disrupted by cycled sonication on ice using a Branson 450 sonicator (15 s sonication each time with cooling on ice, four cycles). To degrade nucleic acids, DNase and RNase (40 µl/each tube, Promega) and 40 µl 1M $MgCl_2$/per tube were added to the cell lysate and incubated at room temperature for 1h in the presence of protease inhibitor (10 µl of TLCK, 1 mM final concentration) added to each tube. Samples were then centrifuged at 5,000 x g for 30 minutes and supernatant was collected for the Omps preparation according to the alkaline incubation method [18]. 60 ml of ice-cold 0.1 M Na_2 CO_3 (pH = 11) has been added to 10 ml of supernatant and stirred slowly in the cold room for 1 - 2 hours. Insoluble outer membrane proteins were precipitated by ultracentrifugation at 115,000 x g at 4^{0}C for 1 hour. Supernatants have been discarded. Pellets were washed in 2 ml of 0.1M PBS buffer (pH = 8.5) and harvested by ultracentrifugation as described above. Each pellet was resuspended in 300 µl of 0.1 M PBS buffer (pH = 8.5), covered with aluminium foil and kept at 40C until further use.

Culture of HN_4 cells

Originally the HN_4 cell line has been derived from primary SCC (human squamous cell carcinoma) of the tongue provided by Dr. Shushanov C.C. from FGBU " RONC of N.N. Blochin's" Russian Ministry of Health, Moscow, Russia.

HN_4 cells originated from a primary cancer of the squamous epithelial keratinized cells of the tongue from clinical patient extract. The HN_4 cells were grown on composed of DMEM media GIBCO (Invitrogen, Carlsbad, CA), (10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% heat-inactivated FBS, penicillin 100U/mL, streptomycin 100 mg/mL, 2.5 mg/mL fungizone) in an atmosphere of 90% air/10% CO_2 at 37°C. Cells were grown on 10 cm tissue culture plates with 10mL of cell medium. Adherent cells were expanded by washing with 4 - 6 mL of 0.1M PBS and then incubated with 0.1% Trypsin for 12 minutes at 37^{0}C. Cells were then removed from the plate into 50 mL tubes and centrifuged at 1100 rpm for 4 minutes at 4^{0}C. The supernatant was then poured off and discarded and the cell pellet was re-suspended and counted. Cells were then re-plated on 10 cm tissue culture plates at a confluence of about 2 X 10^6

Culture of human fibroblasts

Human fibroblasts (WI-38) have been grown on cell culture media (DMEM w/10% FBS, 50 U penicillin-streptomycin, 2 mM L-glutamine, 2.5 mg/mL fungizone, 10 mM HEPES buffer) in an atmosphere of 90% air/10% CO_2 at 37^{0}C. Cells were grown on 10 cm tissue culture plates with 10 mL of cell medium. Adherent cells were expanded by washing with 5 mL's of 0.1M PBS and then incubated with 0.1% Trypsin for 12 minutes at 370C. Following removal form plate cells were harvested by centrifugation (1100 rpm for 4 minutes at 4^{0}C) and resuspended in 0.1M PBS and cell number has determined. Finally, cells were re-plated on 10cm tissue culture plates at a confluence of about 80%.

Preparation samples of Omps, binding with cell surface proteins of host cells

200 µg of Cy5 labeled native Omps added to one plate host cells (HN_4 cells or human fibroblasts) and incubated overnight in the dark at room temperature. As a control, 200 µg Omps boiled at 100^{0}C, then added to one plate host cells (HN_4 cells or human fibroblasts), followed incubation overnight in the dark at room temperature.

As a negative controls, Cy5 labeled BSA (200 µg) and fetuin (200 µg) also incubated with host cells (HN_4 cells or human fibroblasts) overnight in the dark at room temperature. Unbound Omps washed out 3 times with 10 ml 0.1 M PBS (pH = 7.4).

Host cells, bound with Omps, scraped and transferred to Eppendorf tubes, followed centrifugation at 12,000 x g, 10 minutes. Samples solubilized with 2x Sample buffer and loaded for 1D SDS-PAGE analysis. Each set experiment has been done in triplicate.

Proteinase K-trypsin digestion of Omps, interacting with whole human fibroblasts ("shaving approach")

200 µg of Cy5 labeled native Omps added to one plate of human fibroblasts and incubated overnight in the dark at room temperature. As a control, 200 µg of Cy5 labeled Omps boiled at 100^0C, then added to one plate of human fibroblasts, followed incubation overnight in the dark at room temperature. Unbound Omps washed out 3 times with 10 ml 0.1M PBS (pH = 7.4). Further the Omps, bound with human fibroblasts, treated and digested with proteinase K (Promega) using a modified protocol [19]. Proteinase K was added in an enzyme: protein ration of 1:50, followed by incubation for 15h in a thermo mixer (37^0C). Then human fibroblasts scraped and transferred to Eppendorf tubes, followed centrifugation at 12,000 x g, 10 minutes.

Proteins (moved inside of cells) from pellet fraction were solubilized with 2x Sample buffer and loaded for 1D SDS-PAGE analysis. Proteins from supernatant fraction (containing Omps, bound on cell surface host cells) precipitated by methanol/chloroform extraction. Briefly, methanol/chloroform added to sample aliquot methanol: chloroform: sample ration of 4:1:4, vortex and then centrifuged at 12,000 x g, for 5 minutes at room temperature. The supernatant was carefully removed, and methanol (v/v) was added again, followed vortex and centrifugation at 12,000 x g, for 5 minutes at room temperature. The supernatant was discarded, the remaining methanol was evaporated. Then, protein pellet was re-suspended in 2x Sample buffer and loaded for 1D SDS-PAGE analysis.

Protein concentration

Protein concentration has been determined with the Bradford protein assay [20] using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) or by measuring UV absorbance at 280 nm with NanoDrop (ND-1000 Spectrophotometer).

Image acquisition and analysis

After separation in the 1D SDS-PAGE, gels were fixed for 30 minutes in fix solution (10% methanol, 7% acetic acid). Images of cyanine labeled proteins were acquired by scanning the 1D gel using the Molecular Imager System (Typhoon 8600). Wavelength laser of 635 was used for Cy5. Total proteins present in gel were visualized by staining with Gel code Blue Stain Reagent (Pierce, Rockford, IL, USA). Images of gels were used to excise the proteins bands/spots for mass spectrometry analysis.

Electrophoresis

The presence of proteins has been monitored after 1D gel separations. 1D SDS-PAGE electrophoresis was performed using 12% SDS-PAGE, under reducing conditions [21].

Mass spectrometry for protein identification

For mass spectrometric identification, gel spots were arbitrarily selected, excised, destained, and digested with sequencing grade trypsin (Promega, Madison, WI).

Proteins were identified by "*in-gel*" trypsin digestion and *"in-solution*" trypsin digestion followed LC-MS/MS analysis and matching of fingerprints with *P. intermedia 17* database. The "*in-gel*" digestion protocol consists of two parts. The first part, destaining the gel core/protein reduction and alkylation. The second part, "*in-gel*" digestion with trypsin and peptide extraction.

Destaining the gel core

Thus for "*in-gel*" trypsin digestion proteins bands were excised from gel and transferred to Eppendorf tubes. Gel cores (1 mm x 4 mm x 1 mm) were washed with 200 µl wash solution (50% CH_3CN, 50 mM NH_4HCO_3). The tubes were continuously vortex for 10 minutes at room temperature, then the liquid was removed and discarded. The procedure was repeated two additional times for a total of three washes. Then 200 µl 100% of acetonitrile (CH_3CN) was added to the gel core. The tubes were vortex briefly and incubated at room temperature for 5 minutes. The liquid was removed and discarded. The gel pieces were appeared shrunken and opaque at the end of treatment. The residual acetonitrile was removed from the destained gel core sample using a heated vacuum centrifugal concentrator for 5 minutes.

Protein reduction and alkylation

To reduce the protein sample, 100 µl freshly prepared dithiothreitol (DTT) solution (10 mM DTT in 50 mM NH_4HCO_3) was added to the dried gel core and incubated the tube at 55^0C for 1 hour. Then the liquid was removed and discarded.

To alkylate the protein sample, 100 µl of the freshly prepared iodoacetamide (IAA) solution (55 mM IAA in 50 mM NH_4HCO_3) was added to the sample tube and vortex to mix. Then the tube was incubated in the dark at room temperature for 45 minutes. The liquid was removed and discarded. Gel cores were washed with 200 µl of wash solution (50% CH_3CN, 50 mM NH_4HCO_3). The tubes were vortex for 10 minutes at room temperatures, then the liquid was removed and discarded. The procedure was repeated two additional times for a total of three washes. 200 µl of acetonitrile (CH_3CN) was added to the gel core. Then the tubes were vortex briefly and incubated at room temperature for 5 minutes. The liquid was removed and discarded. The residual acetonitrile was removed from the gel core sample using a heated vacuum centrifugal concentrator for 5 minutes.

"In-gel" digestion with trypsin and peptide extraction

The MS grade trypsin (Promega Gold mass-spectrometry grade) was reconstituted in 50 mM acetic acid at final concentration of 1 µg/µl and aliquot. For the current experiment, the 1 µg/µl trypsin was diluted an appropriate volume to 20 ng/µl in 50 mM NH_4HCO_3. Fifteen µl of the 20 ng/µl trypsin solution was added to the dried gel core. The samples incubated for 1 hour at 300C to allow gel rehydration. Then sufficient digestion buffer (50 mM NH_4HCO_3/10% CH_3CN) was added to the sample tube to completely cover the gel pieces. The tubes were incubated at 370 C overnight. 50 µl of ultrapure water (≥ 18 MΩ) was added to the "*in-gel*" digestion mixture. The tubes were vortex continuously for 10 minutes at room

temperature. The retain liquid was removed in a fresh, non-stick micro centrifuge tubes. Then 50 µl of 50% CH_3CN/5% (v/v) formic acid was added to the gel pieces. Tubes were incubated for 60 minutes at room temperature with frequent vortex. The tubes were spin briefly in a micro centrifuge and then the liquid was collected with a pipette. The liquid was added to the liquid collected in previous step. The volume of the pooled liquid was reduced using a centrifugal concentrator (at room temperature), until the appropriate volume (typically 10 - 20 µl) is reached.

"In-solution" trypsin digestion "traditional" protocol-urea denaturaion of protein with reduction and alkylation

Reduction: The protein sample is evaporated and re-suspended in 20 µl urea solution with DTT (8M urea/100 mM NH_4HCO_3/5 mM DTT) up to protein concentration~ 5 µg/µL, mixed carefully by drawing the solution into and out of the pipette. The tubes incubated 2h at 30^0C in a mixer. Then temperature was adjusted to room temperature and tubes were spin down.

Alkylation: 1.5 µl of alkylation reagent (200 mM IAA) was added to the reaction mixture. The samples were incubated 45 minutes at room temperature in the dark. Excess amount of IAA was neutralized with 1.5 µl 0f 200 mM DTT, the tubes were incubated ~ 20 minutes.

The urea concentration was reduced to 1M with 140 µl of 50 mM NH_4HCO_3/2 mM $CaCl_2$. Trypsin (1:40, w/w, Promega Gold mass-spectrometry grade) was added, vortex gently and the mixture was incubated at 37^0C overnight. Digestion was stopped by addition of 10% TFA solution to the digestion mixture and pH was adjusted to ~5.

The resulting tryptic peptides were loaded on C18 trap column (300 µm ID X 5 mm, Dionex Corp.) using Ultimate 3000 HPLC (Dionex Corp.), desalted and then separated on reverse phase in-house packed C18 (5 um Magic C18, Michrom BioResourses) column (75 µm ID X 150 mm) at flow rate of 200 nl/min water/acetonitrile/formic acid gradient. Eluted peptides were subjected to nanospray ESI and MS/MS analysis on LCQ Deca XP Plus (Thermo Electron Corp.). Mass spectrometer was operated in 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.

Database searching and protein identification

Proteins were identified by searching the MS/MS spectra against *P. intermedia 17* database available of www.tigr.org, using Bioworks v 3.2. Only peptides identified as possessing fully tryptic termini with X_{corr} greater than 1.9 for singly charged peptides, 2.3 for doubly charged peptides and 3.75 for triply charged peptides, 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.

Results and Discussion

Identification of Omps *P. intermedia 17* interacting with oral epithelial cells.

To identify *P. intermedia 17* cell surface components that interact with epithelial cells, we probed fluorescently labeled Omps in a binding assay, combined with LC-MS/MS analysis for the whole cells (OEC, line HN_4 and human fibroblasts). At first, we fluorescently labeled the Omps of *P. intermedia* with Cy5 (Figure1, lane 1). BSA and fetuin used as controls (Figure 1, lane 3 and 4, respectively). Aliquot of labeled Omps was denaturated by boiling at 100^0C (Figure 1, lane 2).

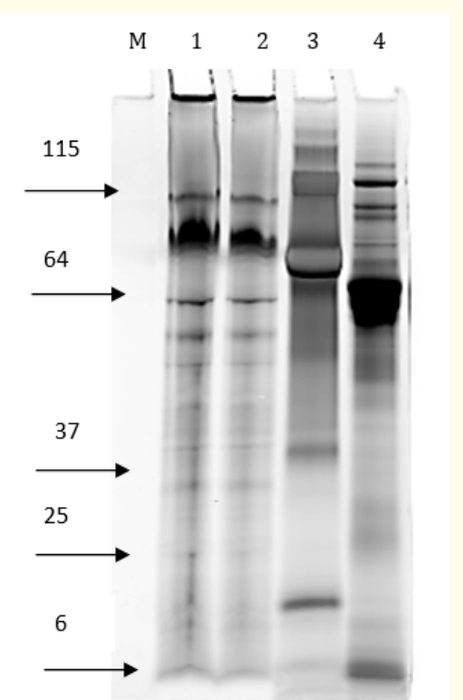

Figure 1: Images of 1D SDS-PAGE of OMPs from *P. intermedia 17.* Lanes: M: Molecular weight standard; 1: Native OMPs from Cy5-labelled cells (25 µg); 2: Denaturated OMPs (boiled at 1000C) from Cy5-labelled cells (25 µg); negative controls: 3: Cy5-labelled BSA (25 µg); 4, Cy5-labelled fetuin (25 µg). Protein samples were solubilized with 2x Sample buffer and separated on 1D SDS-PAGE gels (12%). Resolved protein bands were visualized using a Typhoon Imager at 635 nm for Cy5–labeled surface proteins.

Then whole HN_4 cells incubated with native, denaturated Omps, BSA and fetuin, washed out the unbound proteins and samples analyzed by 1D SDS PAGE. Figure 2 demonstrates the fluorescent image of Omps, bound with proteins from HN_4 cells.

Figure 2: Identification of OMPs *P. intermedia 17*, interacting with proteins from oral epithelial (HN4) cells. Lanes: M: Molecular weight standard; 1: Native OMPs incubated with whole human HN_4 cells; 2: Denaturated OMPs incubated with whole human HN_4 cells; negative controls: 3: BSA; 4: Fetuin incubated with whole human HN4 cells. 100 µg of fluorescently labeled OMPs incubated with whole human HN4 cells. Nonspecific binding proteins were washed with PBS buffer. OMPs of *P. intermedia* 17 were solubilized with 2x Sample buffer and separated on 1D SDS-PAGE gels (12%). Resolved protein bands were visualized using a Typhoon Imager at 635 nm for Cy5–labeled surface proteins. The gel was then stained with GBC to detect total proteins. Fluorescent proteins bands have been excised and submitted for mass spec analysis.

As illustrated in figure 2, epithelial proteins specifically recognize *P. intermedia* Omps with apparent M.W. of 64 kDa, 100 kDa and 180 kDa. These three proteins bands are major and most intensive, indicating strong binding capacity to oral cells. Less abundant bands detected at 82, 49, 40, 28, 20 and 15 kDa, showing weak and, probably, nonspecific association with host cells (Figure 2, lane 1). Profile of denaturated Omps is similar with native, however, the proteins bands are less intensive (Figure 2, lane 2). Surprisingly, the negative controls, BSA and fetuin also demonstrated the binding to OEC. We assume that BSA and fetuin are binding nonspecifically to host cells, by hydrophobic "sticking" to the cell-surface proteins.

The interest proteins bands were excised, "*in gel*" trypsin digested and analyzed by LC-MS/MS analysis. Table 1 lists the classes of identified Omps.

The total number identified Omps was seventeen, including five proteins with high sequence coverage ($X_{corr.}$ >10). Proteins identified include different classes of proteins, such as enzymes (ATP synthase, glutamate dehydrogenase, imidazolone propionase), outer membrane proteins, immunoreactive and conserved hypothetical proteins. The majority of identified proteins were described as membrane components, consistent with the isolation and separation method. Furthermore, each of the most abundant protein corresponded to Omps.

Our results showed that in case of native Omps the high binding capacity ($X_{corr.}$ >10) has been found for Omp 41 (putative immunoreactive 43 kDa antigen PG32, PIN_A1455), major Omp (putative immunoreactive 42 kDa antigen PG33, PIN_A0102), conserved hypothetical protein (PIN_A1050), RagA protein, putative (PIN_A0326). Omp 41 (PIN_A1455) produced a top scoring hit (score $X_{corr.}$ 90) that matched 14 peptides with a sequence coverage 4.1%. Conserved hypothetical protein (PIN_A1050) produced nine peptides masses with a 50.26 scoring hit that accounted for sequence coverage of 37.5%. Major Omp (PIN_A0102) demonstrated 17.10% of sequence coverage with a score hit 50.26 that matched with 7 peptides. Others proteins produced low scoring hits (X_{corr} = 10) matched only three or fewer peptides.

Protein[sa)]	*P. intermedia*17 database Accession no.[b)]	Predicted protein function	C_{y5} labeled	P(pro)[c)]	Coverage Delta Cn[d)]	Predicted mass	Peptide (hits)[e)] ions	Sf (final score)[f)]	Score Xcorr[g)]
Native OMPs	PIN_A1077	Hypothetical protein	Yes	7.30E-04	1.70	89351.0	1 (1 0 0 0 0)	0.63	10.12
	PIN_A1960	Imidazolonepropionase (hutI) [3.5.2.7]	Yes	1.84E-03	2.60	42383.3	3 (3 0 0 0 0)	0.33	10.13
	PIN_A1392	Hypothetical protein	Yes	2.55E-06	2.90	59878.6	2 (2 0 0 0 0)	0.95	10.19
	PIN_A1155	Glutamatedehydrogenase, NAD-specific (gdh) [1.4.1.2]	Yes	3.24E-03	2.50	48155.8	1 (1 0 0 0 0)	0.29	10.10
	PIN_A1455	Immunoreactive 43 kDa antigen PG32, putative	Yes	2.10E-11	4.10	43075.3	14 (14 0 0 0 0)	6.78	90.35
	PIN_A0102	Immunoreactive 42 kDa antigen PG33, putative	Yes	2.65E-12	17.10	41989.1	7 (7 0 0 0 0)	4.70	50.26
	PIN_A1852	ATP synthase F1, beta subunit (atpD) [3.6.3.14]	Yes	2.51E-03	3.30	46348.2	1 (1 0 0 0 0)	0.97	10.22
	PIN_A0500	Fumarate reductase, iron-sulfur protein (frdB) [1.3.99.1]	Yes	4.49E-03	6.80	27529.3	1 (1 0 0 0 0)	0.50	10.14
	PIN_A1050	Conserved hypothetical protein	Yes	3.06E-12	37.50	20097.1	9 (9 0 0 0 0)	4.74	50.29
	PIN_0093	Conserved hypothetical protein	Yes	1.36E-06	13.70	20443.6	2 (2 0 0 0 0)	1.49	20.27
	PIN_A0326	ragA protein, putative	Yes	1.71E-05	4.70	116737.6	5 (5 0 0 0 0)	2.54	30.19
	PIN_A2156	MotA/TolQ/ExbB proton channel family protein	Yes	4.11E-04	4.30	29676.3	1 (1 0 0 0 0)	0.95	10.19
	PIN_A1834	UpdY, putative	Yes	3.50E-03	7.00	22011.5	1 (1 0 0 0 0)	0.83	10.13
	PIN_0561	Conserved hypothetical protein	Yes	3.58E-05	8.30	14717.2	2 (2 0 0 0 0)	0.83	10.14
	PIN_A0324	Hypothetical protein	Yes	2.15E-07	3.10	55226.8	2 (2 0 0 0 0)	0.85	10.18
	PIN_A1392	Hypothetical protein	Yes	5.90E-06	2.90	59878.6	1 (1 0 0 0 0)	0.91	10.18
	PIN_0550	Hypothetical protein	Yes	2.96E-04	2.40	52612.4	2 (2 0 0 0 0)	0.45	10.10
Denatured OMPs	PIN_A1392	Hypothetical protein	Yes	6.64E-06	2.90	59878.6	3 (3 0 0 0 0)	0.95	10.20
	PIN_A1960	Imidazolonepropionase (hutI) [3.5.2.7]	Yes	9.89E-04	0.00	42383.3	1 (1 0 0 0 0)	0.77	10.15
	PIN_0550	Hypothetical protein	Yes	2.23E-03	2.40	52612.4	1 (1 0 0 0 0)	0.43	10.10
	PIN_A0184	Ribosomal protein S2 (rpsB)	Yes	4.23E-03	6.80	30773.0	1 (1 0 0 0 0)	0.81	10.17
	PIN_A1049	Ribosomal protein L17 (rplQ)	Yes	4.72E-03	10.00	17995.8	1 (1 0 0 0 0)	0.30	10.13
	PIN_0497	Conserved hypothetical protein	Yes	1.96E-03	3.20	40066.9	2 (2 0 0 0 0)	0.39	10.12
	PIN_A1852	ATP synthase F1, beta subunit (atpD) [3.6.3.14]	Yes	9.39E-05	3.30	46348.2	1 (1 0 0 0 0)	0.46	10.12
	PIN_A1050	Conserved hypothetical protein	Yes	2.98E-07	20.10	20097.1	2 (2 0 0 0 0)	1.95	20.25
	PIN_A0326	ragA protein, putative	Yes	2.40E-09	2.80	116737.6	2 (2 0 0 0 0)	1.86	20.24
	PIN_0484	Type IIS restriction enzyme R and M protein (ECO57IR)	Yes	3.85E-03	1.00	128506.8	1 (1 0 0 0 0)	0.83	10.13
	PIN_A1058	dnaK protein (dnaK	Yes	2.31E-07	2.50	67875.5	1 (1 0 0 0 0)	0.86	10.17
	PIN_A0157 (amyX)	Alpha-dextran endo-1,6-alpha-glucosidase (amyX)	Yes	1.07E-05	1.30	71229.1	1 (1 0 0 0 0)	0.46	10.11
	PIN_A1509	Hypothetical protein	Yes	6.12E-04	1.80	66703.1	1 (1 0 0 0 0)	0.76	10.12

Table 1: *P. intermedia 17* OMPs interacting with whole HN4 cells, identified by LC-MS/MS analysis "in-solution"/ "in-gel" trypsin digestion.

Foot notes:

OMPs used for binding with host cells; 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 is; d) Delta Correlation-the difference between the normalized XCorrs of the primary and secondary matches. The greater difference-the better match is. Delta Cn > 0.1 good match, Delta Cn > 0.3 great match; e) the ration number of ions observed/number of ions possible. Number of ions possible is the number of ions in MS/MS spectrum of this peptide. Acceptable number of observed peptide ions > 1; f) the protein Sf score is the sum of 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 cross correlation of the experimental MS/MS spectrum vs. candidate peptides in the database. The candidate producing the highest Xcorr value is chosen as #1 hit by Sequest.

A similar profile of proteins was obtained for denaturated Omps, interacting with OEC (Figure 2, lane 2). Thirteen proteins were identified based upon matches to sequence of *P. intermedia 17* databases. Proteins bands from denaturated sample produced similar peptide mass fingerprints, but containing fewer peptides (1-3) than the digests of bands from native sample (Figure 2, lane 1). Therefore, in case of denaturated Omps we detected the same proteins, such as imidazolone propionase, ATP synthase, Rag A, conserved hypothetical protein. These results indicate that denaturated Omps have the same binding motifs, as native Omps. However, the binding affinity was much lower in comparison with native Omps. In addition we were not be able identify the Omp 41 (PIN_A1455) and major Omp (PIN_A0102), indicating importance of structure for binding. Hence the lower binding capacity of denaturated Omps and low detection liability demonstrates that the third-dimensional structure of Omps might be important for their association to host cells.

Identification of Omps *P. intermedia 17* interacting with human fibroblasts

To study the interaction of cell surface proteins of human fibroblasts with Omps of *P. intermedia 17*, we incubated intact human fibroblasts with Omps (Cy5 labeled), then washed out human fibroblasts from unbound proteins and loaded samples for 1 D gel separation, followed mass spectrometry analysis. Omps associated with human fibroblasts were visualized as fluorescent labeled bands on 1D gel. Figure 3 (lane 1) illustrates the profiles bound proteins.

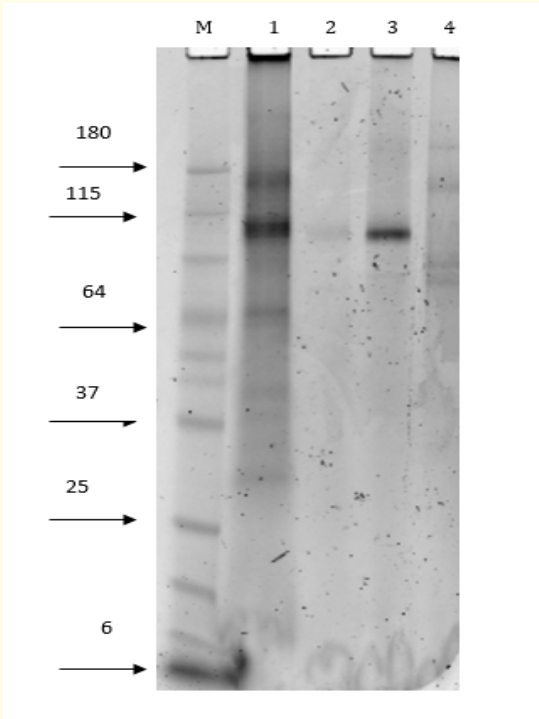

Figure 3: Identification of OMPs *P. intermedia 17*, interacting with proteins from human fibroblasts.

Lanes: M: Molecular Weight Standard; 1: Native OMPs incubated with whole human fibroblasts; 2: Denaturated OMPs incubated with whole human fibroblasts; negative controls: 3: BSA; 4: Fetuin incubated with whole human fibroblasts. Outer membrane proteins of *P. intermedia 17* analysed as described in figure 2.

1D gel of *P. intermedia 17* Omps yielded three dominant bands proteins at 64, 100 and 170 kDa. The relative intensity of the GBC (Gel Blue Code) stain revealed that these bands represented major of the Omps, bound to human fibroblasts. Interest bands were excised from the gel and digested with trypsin ("*in-gel*" digestion). In addition, an aliquot of eluent from the Dynabeads was used for *"in-solution"* trypsin digestion, followed by peptide mass fingerprinting analysis.

The peptides were analysed by LC-MS/MS analysis and the source proteins identified by peptide sequence searches against the *P. intermedia 17* databases, using the Bioworks v 3.2. program. Table 2 contains the Omps, associated with cell surface proteins of human fibroblasts.

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)]
Human fibroblasts	PIN_A0324	Hypothetical protein	Yes	1.57E-09	3.10	55 226	1(10 000)	0.98	10.28
	PIN_A0102	Immunoreactive 42 kD antigen PG33, putative	Yes	5.81E-09	21.80	41 989	9(90 000)	5.66	70.25
	PIN_A1455	Immunoreactive 43 kD antigen PG32, putative	Yes	4.00E-10	13.10	43 075	8(80 000)	3.92	50.33

Table 2: *P. intermedia 17* OMPs interacting with whole human fibroblasts, identified by LC-MS/MS analysis "in-gel" (1D gel) trypsin digestion. Values a), b), c), d), e), f) are the same, as described in table 1.

We identified three proteins, interacting with cell surface proteins of human fibroblasts. Two from them (major Omp, PIN_A0102 and Omp 41 precursor, PIN_A1455) demonstrated the high sequence coverage (Score Xcorr = 70.25 and 50.33, respectively). These proteins are also showed top peptide matches, 9 and 8 peptides, respectively. Notably, that these two proteins were also identified as proteins, interacting with OEC, they also demonstrated top sequence coverage with high scoring hits for peptides. The third protein, hypothetical protein, PIN_A0324 produced low scoring hit (Xcorr = 10) matched only one peptide.

Detection of Omps of *P. intermedia 17* binding with cell surface proteins of human fibroblasts

Omps of *P. intermedia 17* are interacting with cell surface proteins of human fibroblasts. We used "shaving" approach, to identify Omps, interacting with these cell surface proteins. Whole human fibroblasts incubated with Omps, unbound proteins were washed out with buffer and then human fibroblasts treated with proteinase K, to digest Omps proteins. Then Omps have been identified by 1D SDS-PAGE (Figure 4) and followed by MS analysis (Table 3).

Native Omps produced six major bands at 115, 82, 64 kDa and between 19-25 kDa (Figure 4, lane 1).

MS analysis identified 9 proteins, only one hypothetical protein (PIN_A0324) demonstrated score 20.25 matched one peptide (Table 3).

Five proteins demonstrated great match according to value Delta coverage, such as ribosomal protein L23 (PIN_A1024, Cn = 6.80), transcriptional regulator superfamily (PIN_A0954, Cn = 15.40), hypothetical protein (PIN_A0324, Cn = 5.60), major Omp (PIN_A0102, immunoreactive 42 kD antigen PG33, putative, Cn = 4.50), translation elongation factor Tu (tuf) (PIN_A1000, Cn = 4.0).

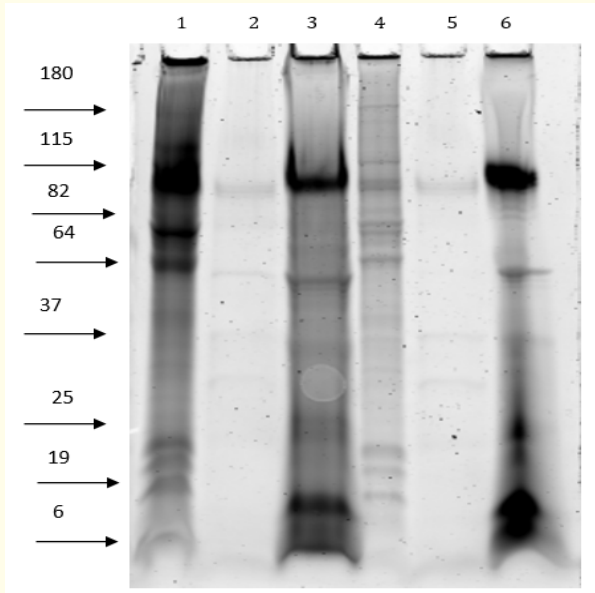

Figure 4: Identification of OMPs of *P. intermedia 17,* binding with cell surface proteins of human fibroblasts. Lanes: M: Molecular weight standard; 1: Native OMPs incubated with human fibroblasts (no "shaving"); 2: Native OMPs incubated with human fibroblasts (with "shaving"), supernatant fraction used for analysis; 3: Native OMPs incubated with human fibroblasts (with "shaving"), pellet fraction used for analysis; 4: Denaturated OMPs incubated with human fibroblasts (no "shaving"); 5: Denaturated OMPs incubated with human fibroblasts (with "shaving"), supernatant fraction used for analysis; 6: Denaturated OMPs incubated with human fibroblasts (with "shaving"), pellet fraction used for analysis. 100 µg of fluorescently labeled OMPs incubated with whole human fibroblasts. Non-specific binding proteins were washed with PBS buffer. Proteinase K was added in a enzyme: protein ratio of 1:50 for "shaved" samples, followed incubation for 15h in a thermomixer (370C). Then mixture of OMPs+human fibroblasts were centrifuged at 12,000 x g, 5 minutes. Supernatant and pellet fractions were analyzed by SDS-PAGE. Samples were solubilized with 2x Sample buffer and separated on 1D SDS-PAGE gels (12%). Resolved protein bands were visualized using a Typhoon Imager at 635 nm for Cy5–labeled surface proteins. The gel was then stained with GBC to detect total proteins. Fluorescent proteins bands have been excised and submitted for mass spec analysis.

Protein[sa)]	*P. intermedia 17* database Accession no.[b)]	Predicted protein function	C_{y5} labeled	P(pro)[c)]	Coverage Delta Cn[d)]	Predicted mass	Peptide (hits)[e)] ions	Sf (final score)[f)]	Score Xcorr[g)]
Native OMPs +HF, nonshaved	PIN_A0324	Hypothetical protein	Yes	1.85E-07	5.60	55226.8	2 (2 0 0 0 0)	1.80	20.25
	PIN_A0326	ragA protein, putative	Yes	7.13E-06	2.40	116737.6	1 (1 0 0 0 0)	0.90	10.17
	PIN_A1000	Translation elongation factor Tu (tuf)	Yes	8.49E-06	4.00	43888.2	1 (1 0 0 0 0)	0.96	10.20
	PIN_A0102	Immunoreactive 42kD antigen PG33, putative	Yes	4.10E-05	4.50	41989.1	1 (1 0 0 0 0)	0.84	10.14
	PIN_A0954	Transcriptional regulator superfamily	Yes	7.63E-04	15.40	12083.6	1 (0 1 0 0 0)	0.03	8.10
	PIN_A1024	Ribosomal protein L23 (rplW)	Yes	6.76E-04	6.80	16486.0	1 (1 0 0 0 0)	0.84	10.15
	PIN_A1058	dnaK protein (dnaK)	Yes	3.11E-11	2.50	67875.5	1 (1 0 0 0 0)	0.98	10.27
	PIN_A1392	Hypothetical protein	Yes	6.33E-05	2.90	59878.6	2 (2 0 0 0 0)	0.92	10.20
	PIN_A0160	Hypothetical protein	Yes	1.25E-04	3.80	28382.6	1 (1 0 0 0 0)	0.94	10.16
Native OMPs +HF, shaved, supernatant fracton	PIN_A0324	Hypothetical protein	Yes	2.66E-11	5.80	55226.8	2 (2 0 0 0 0)	1.96	20.25
Denaturated OMPs, nonshaved	PIN_A1058	dnaK protein (dnaK)	Yes	6.87E-06	2.50	67875.5	1 (1 0 0 0 0)	0.97	10.20
	PIN_A1392	Hypothetical protein	Yes	8.67E-05	2.90	59878.6	2 (2 0 0 0 0)	0.93	10.21
	PIN_A1845	ATP synthase F1, alpha subunit (atpA) [3.6.3.14]	Yes	1.53E-09	2.50	57697.7	1 (1 0 0 0 0)	0.97	10.22
	PIN_A1852	ATP synthase F1, beta subunit (atpD) [3.6.3.14]	Yes	7.44E-08	3.30	46348.2	2 (2 0 0 0 0)	0.99	10.29
	PIN_A0324	Hypothetical protein	Yes	1.78E-10	3.10	55226.8	1 (1 0 0 0 0)	0.97	10.25
Denaturated OMPs+HF, shaved, supernatant fraction	PIN_A1972	Conserved hypothetical protein	Yes	3.62E-04	15.20	13017.7	2 (1 1 0 0 0)	0.75	18.14
	PIN_A1024	Ribosomal protein L23 (rplW)	Yes	8.89E-04	6.80	16486.0	1 (1 0 0 0 0)	0.84	10.16
Denaturated OMPs +HF, shaved, pellet fraction	PIN_A0160	Hypothetical protein	Yes	4.37E-06	8.80	28382.6	2 (2 0 0 0 0)	1.35	20.12
	PIN_A0326	ragA protein, putative	Yes	3.36E-05	2.00	116737.6	2 (2 0 0 0 0)	1.59	20.17
	PIN_A1455	Immunoreactive 43 kDa antigen PG32, putative	Yes	3.44E-05	2.30	43075.3	1 (1 0 0 0 0)	0.95	10.14

Table 3: *P. intermedia 17* OMPs interacting with cell-surface human fibroblasts, identified by LC-MS/MS analysis "in-solution"/ "in-gel" trypsin digestion, using "shaving" approach.

The hypothetical protein (PIN_A0324) has been observed in non-"shaved" and in "shaved" human fibroblasts. After "shaving" with proteinase K, this protein was identified in supernatant fraction, containing the proteins associated with cell surface of human fibroblasts (Figure 4, lane 2). The sequence coverage was 20.25 with 2 match peptides, Cn = 5.80. These data indicate that this protein is binding directly with cell surface proteins of human fibroblasts. Total nine proteins have been detected in non-"shaved" fraction of proteins after association with human fibroblasts, but the low score correlation values indicate, that these proteins interact weakly with cell surface proteins of human fibroblasts. We used also denaturated Omps, to study their interaction with human fibroblasts. Profile of denaturated Omps, bound with human fibroblasts shown on figure4, lane 4. The pattern of proteins was similar with profile of native Omps (Figure 4, lane 1), but the affinity binding was much lower. The major bands were identified at 130, 115, 82, 64, 25, 19 kDa. We find out, that not so many proteins binding with human fibroblasts and, in addition to, such association has low binding capacity. Five proteins were identified in case, using the non-"shaved" denaturated Omps. They include ATP synthase F1, beta subunit [3.6.3.14], ATP synthase F1, alpha subunit [3.6.3.14], dnaK protein (dnaK) and two hypothetical proteins. All these proteins produced low scoring hits (X_{corr} = 10) matched only one or two peptides. Denaturated Omps demonstrated the low affinity binding to cell surface host proteins. These data indicate that third-dimensional

structure of proteins is important for protein-protein interactions. Boiling of Omps at high temperature produced nonreversible conformational changes in structure of proteins. However, the binding motifs are not destructed by boiling procedure and denaturated Omps showed the weak association with cell surface host proteins.

Human fibroblasts associated with denaturated Omps were "shaved" with proteinase K, followed separation "shaved" proteins on 1D SDS-PAGE (Figure 4, lane 5). Profile of proteins showed low abundant bands around 115, 49, 37 and 25 kDa (Figure 4, lane 5). After "shaving" of human fibroblasts, only two denaturated Omps have been detected as proteins, binding with cell surface proteins of human fibroblasts, such as conserved hypothetical protein (PIN_A1972) and ribosomal protein L23 (PIN_A1024) (rplW). Conserved hypothetical protein demonstrated high score correlation value (X_{corr} = 18.4), match with 2 peptides and Coverage Delta 15.20. Ribosomal protein L23 (PIN_A1024) (rplW) showed coverage 6.80, but low peptide match (1) and low score value (10), indicating low affinity binding. Others denaturated Omps were identified in pellet fraction, containing human fibroblasts proteins and Omps, which could moved inside the human fibroblasts or were not cleaved by proteinase K (Figure 4, lane 6). MS analysis identified total three proteins, such as hypothetical protein (PIN_A0160), RagA protein, putative (PIN_A0326) and immunoreactive 43 kDa antigen PG32, putative (PIN_A1455). Hypothetical protein (PIN_A0160) and RagA (PIN_A0326) both demonstrated a score hit 20.12 and 20.17, respectively, that matched with 2 peptides. These results indicate on high affinity binding these Omps proteins with intracellular human fibroblasts proteins. However, immunoreactive 43 kDa antigen PG32, putative (PIN_A1455) showed low score hit (10) and matched with 1 peptide. This indicates that probably, this protein is not so abundant protein or demonstrates lower binding affinity to human fibroblasts proteins.

Discussion

Association of *P. intermedia 17* with host cells and influence of bacteria on cell's function has been demonstrated in some papers. For example, four strains of *P. intermedia 17* were isolated from periodontally involved lesions [22] and effect on function of lymphocytes was studied. It was shown that all four strains inhibited lymphocyte functions and affected the early stages of cell activation.

Adhesion of the periodontitis-associated bacteria *Prevotella intermedia 17* to monolayers of fibroblasts, HEp-2, KB and HeLa cells was demonstrated using radiolabeled bacteria and microscopically with Giemsa-stained non-radioactive preparations [23]. It was reported that of *Prevotella intermedia,* strain 17 invaded a HOEC, line (KB) [24] and that the type C fimbriae and a cytoskeletal rearrangement are required for this invasion.

Species such as *Bacteroides forsythus* and *Prevotella intermedia* were more frequently detected in crevicular epithelial cells in elevated numbers in periodontally diseased sites [25]. *Prevotella intermedia* and *Porphyromonas gingivalis* were identified in rapidly progressive periodontitis biopsies using immuno-histological methods. These microorganisms were localized outside the epithelium and inside intercellular spaces [26]. It was shown that *Prevotella intermedia* have ability to adhere to and invade primary cultures of human gingival epithelial cells (HGEC) [27]. It was also demonstrated that *P. intermedia* species were frequently detected (37%) in epithelial cells obtained from periodontal pockets and the gingival crevice of subjects with periodontitis [1].

However, Omps of *P. intermedia* associated with oral epithelial cells were not identified yet. Our results provide direct evidence association of Omps *P. intermedia 17* with cell surface proteins of host cells. In our research we identified Omps, interacting with cell surface proteins of OEC and human fibroblasts. We demonstrated the similarity of Omps *P. intermedia 17*, bound with human fibroblasts and OEC. We detected the hypothetical protein (PIN_A0324), which is binding directly with cell surface proteins of human fibroblasts. We showed that both native and denaturated Omps *P. intermedia 17* bound to host cells, indicating that similar, conservative binding domains are involved in this host-bacteria interaction. However, we demonstrated that binding denaturated Omps *P. intermedia 17* to host cell was lower in comparison with native Omps. Therefore, our data demonstrated that third dimensional structure of proteins is important for protein-protein interactions. These results indicated that heat treatment lead to unfolding adhesins proteins ant disturb interaction between host cells and outer membrane proteins.

This observation fits well with experiments described by [28,29], demonstrating that binding of rickettsiae bacteria to host cells was greatly decreased when host cells or rickettsiae were treated with trypsin or heat. After heating at 55^0C for 15 minutes, rickettsiae losted more than 80% of their ability to attach to host cells. These results indicated that the bacteria binding structures are heat sensitive and likely to be surface proteins. Our data confirmed these observation and demonstrated that denaturated Omps have less affinity binding to host cells.

Conclusion

In this paper we suggest evidence that Omps of *P. intermedia 17* involved in adherence of these microorganisms to OEC. Recognition and binding to the host cells is a key step in the pathogenesis of many virulent bacterial strains, and identification of molecular basis of *P. intermedia* attachment to host cells remains an important objective. In this work, we used a global proteomic approach to identify *P. intermedia* ligands, involved in bacteria-host cell interactions. This study, which was carried out on *P. intermedia 17* and host cells (OEC, line HN4 and human fibroblasts), led to identification of putative *P. intermedia 17* adhesins recognized by host cells. In this paper, we identified five Omps of *P. intermedia 17*, demonstrating strong binding affinity to OEC (line HN_4). Two proteins, including (PIN_A0102) and (PIN_A1455), showed also strong binding capacity to human fibroblasts. All these proteins were belonged to bacterial outer membrane proteins. Future studies are needed, to synthesize the compounds, which will inhibit the activity of these OMPs and disturb the bacteria-host interactions. It will allow to block the development and progression of periodontal disease and decrease the risk of esophageal cancer.

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Conflict of Interest

No any financial interest or any conflict of interest exists.

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