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Growth phase-dependent composition of the *Helicobacter pylori* exoproteome

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ABSTRACT

Helicobacter pylori colonizes the human stomach and is associated with an increased risk of gastric cancer and peptic ulcer disease. Analysis of *H. pylori* protein secretion is complicated by the occurrence of bacterial autolysis. In this study, we analyzed the exoproteome of *H. pylori* at multiple phases of bacterial growth and identified 74 proteins that are selectively released into the extracellular space. These include proteins known to cause alterations in host cells, antigenic proteins, and additional proteins that have not yet been studied in any detail. The composition of the *H. pylori* exoproteome is dependent on the phase of bacterial growth. For example, the proportional abundance of the vacuolating toxin VacA in culture supernatant is higher during late growth phases than early growth phases, whereas the proportional abundance of many other proteins is higher during early growth phases. We detected marked variation in the subcellular localization of putative secreted proteins within soluble and membrane fractions derived from intact bacteria. By providing a comprehensive view of the *H. pylori* exoproteome, these results provide new insights into the array of secreted *H. pylori* proteins that may cause alterations in the gastric environment.

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1. Introduction

Helicobacter pylori colonizes the gastric mucosa of humans and is present in about 50% of the population worldwide. A humoral immune response and a gastric mucosal inflammatory response are consistently detectable in *H. pylori*-infected persons [1,2], but the bacteria resist clearance by these host defenses [3]. Most *H. pylori*-infected persons remain asymptomatic, but have an increased risk for development of peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma [4,5].

H. pylori is localized to the gastric mucus layer overlying gastric epithelial cells and typically does not invade host cells. Therefore, there is considerable interest in identifying proteins released by the bacteria into the extracellular space that can cause alterations in host cells or alterations in the gastric environment [6]. The identification of proteins specifically secreted by *H. pylori* is complicated by the occurrence of bacterial autolysis (resulting in non-selective release of intracellular proteins into the extracellular space) [7–10], the release of membrane vesicles into the extracellular space [11,12], and a requirement of protein-rich culture medium of undefined composition to support growth of the bacteria to high optical densities.

Three previous studies have utilized proteomic methods to analyze the *H. pylori* exoproteome [8–10]. Two studies used two-dimensional gel electrophoresis methods and evaluated the specificity of *H. pylori* protein release in comparison to UreB (an abundant cytoplasmic protein that is released into the culture supernatant) [8,9]. One of these studies reported the detection of 33 protein spots when the culture supernatant was analyzed, and identified 26 distinct proteins that were selectively released into the supernatant [8]. The second study identified 16 proteins that were selectively released into the supernatant [9]. Another study used direct LC-MS/MS methods to analyze the exoproteome of two *H. pylori* strains [10]. Among 130 *H. pylori* proteins detected in the culture supernatant, 45 were considered to be enriched in the supernatant of one or both strains (based on a >1.5 ratio in the number of unique peptides detected in supernatant compared to the numbers of unique peptides detected in a soluble cell-associated sample). Only four proteins (the vacuolating toxin VacA; "cell binding factor 2" or HP0175; hypothetical protein HP1286; and thioredoxin TrxC or HP1458) were reported to be selectively released into the extracellular space in all three of the previous proteomic studies.

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The previous proteomic studies provided valuable insights into the exoproteome of *H. pylori*, but there are several limitations of the previous studies. For example, two-dimensional gel electrophoresis is dependent on successful resolution of proteins in 2-D gels, is not optimal for identification of low-abundance proteins, and is not well-suited for analyzing large numbers of replicate samples. In addition, the methods used in the previous studies for evaluating the selectivity of protein release were based on measuring gel spot densities and the use of a single protein (UreB) as a reference [8,9]. The use of this protein as a reference may not be optimal, since the findings of one study suggested that UreB is specifically secreted by *H. pylori* [13]. All of the previous studies analyzed protein release at a single time point, and therefore, it was not possible to evaluate growth phase-dependent variations in protein release. Finally, many of the putative secreted proteins identified in the previous studies are orthologs of proteins localized to the cytoplasm, periplasm, or inner membrane in other bacterial species. Thus, the composition of the *H. pylori* exoproteome is not yet well established, and the mechanisms underlying release of most *H. pylori* proteins into the extracellular space are not understood.

In this study, we sought to define more clearly the set of *H. pylori* proteins that are selectively released into the extracellular space. To do this, we undertook a comprehensive analysis of the *H. pylori* exoproteome, using direct mass spectrometry-based methods to analyze the protein composition of *H. pylori* broth culture supernatants in comparison to subcellular bacteria fractions derived from intact bacteria. This approach allowed a high level of sensitivity for protein detection, as well as a quantitative means for assessing the selectivity of protein release into the culture supernatant. To facilitate these experiments, we used a culture medium that was optimized to have low protein content (compatible with direct mass spectrometric analysis), while still supporting robust bacterial growth. In total, we identified 74 proteins that are enriched in the culture supernatant compared to a subcellular fraction derived from intact bacteria, thereby indicating selective release of these proteins into the extracellular space. Analysis of the *H. pylori* exoproteome at multiple phases of bacterial growth allowed us to detect growth phase-dependent differences in the composition of the exoproteome. In addition, we show that there is considerable variation in the subcellular localization of selectively released proteins within soluble and membrane fractions derived from intact bacteria, suggesting that there are multiple mechanisms for the selective release of *H. pylori* proteins into the extracellular space. By providing a comprehensive view of the *H. pylori* exoproteome, these results provide new insights into the array of extracellular *H. pylori* proteins that may cause alterations in the gastric environment.

2. Methods

2.1. Bacterial strains and culture conditions

All experiments were performed with *H. pylori* strain 26695, which was originally isolated from a patient with gastritis [14]. *H. pylori* was grown at 37 °C in room air supplemented with 5% CO_2. The bacteria were routinely passaged on Trypticase soy agar plates containing 5% sheep blood. Liquid cultures were grown in a modified form of sulfite-free Brucella-cholesterol broth, termed “Brucella broth filtrate”. This medium was prepared by passing sulfite-free Brucella broth [15] through a 3 kDa cut-off ultrafiltration membrane (Amicon Ultra-15; EMD Millipore). The resulting filtrate was supplemented with 1× cholesterol (Gibco) [16]. The use of this serum-free medium, which had a reduced content of high molecular mass proteins normally present in Brucella broth, facilitated subsequent mass spectrometry analysis.

Bacteria were harvested from blood agar plates after one day of growth, and were inoculated into seed cultures with a starting inoculum of OD_{600} 0.02. Following growth of the seed cultures overnight with shaking at 160 rpm, larger volume cultures (250 ml for most experiments) were inoculated with the seed culture at the same initial density (OD_{600} 0.02). Aliquots (each about 50 ml) were removed at serial time points (12, 24, 30, 36, and 48 h post-inoculation). The samples were centrifuged at 4500×*g* at 4 °C for 10 min, yielding broth culture supernatants and bacterial pellets.

2.2. Processing of broth culture supernatants

Supernatants were passed through a 0.22 μm filter to remove any remaining bacteria, and filtered supernatants were concentrated to a final volume of 1 ml using a 10 kDa cut-off centrifugal filter ultrafiltration unit (Amicon Ultra-15; EMD Millipore). Remaining Brucella broth proteins that could potentially interfere with mass spectrometry analysis were removed by buffer exchange with Tris-buffered saline (20 mM Tris, 136 mM NaCl, pH 7.4). Concentrated culture supernatants were centrifuged at 100,000×*g* for 2 h at 4 °C to remove outer membrane vesicles or other insoluble components [12]. The resulting supernatants were removed and further concentrated to 50 μL by ultrafiltration with a 10 kDa cut-off unit. Protein concentration was quantified by bicinchoninic acid assay (Thermo Scientific).

2.3. Bacterial subcellular fractionation

Bacterial subcellular fractions were prepared as described previously [17]. Bacterial pellets were washed twice in TNKCM buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 27 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) and resuspended in resuspension and lysis buffer (50 mM Tris [pH 7.4] and 1 mM $MgCl_2$ with EDTA-free protease inhibitor cocktail [Roche]). Cells were lysed by sonication (5 pulses, 20 s on/40 s off, 20% amplitude) and lysates were centrifuged for 10 min at 4500×*g* at 4 °C. Supernatants (predicted to be enriched in cytoplasmic and periplasmic proteins) were removed and centrifuged at 100,000×*g* at 4 °C for 2 h to pellet insoluble proteins. The pellets (predicted to be enriched in membrane proteins) were solubilized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (U.S. Biologicals), 0.25% sodium deoxycholate) containing protease inhibitor cocktail (Roche). The solubilized pellet contents and supernatants were concentrated, and protein concentration was quantified by bicinchoninic acid assay (Thermo Scientific).

2.4. Mass spectronomic analysis of samples

Protein preparations were run about 2 cm into a 10% Bis-Tris NuPAGE gel, stained with colloidal Coomassie blue, and then subjected to in-gel trypsin digestion [18]. Samples were then analyzed by either single dimensional LC-MS/MS or multidimensional protein identification technology (MudPIT). Single dimensional LC-MS/MS (1D analysis) was performed using ThermoFisher LTQ equipped with a nanoelectrospray source and attached to a Nanoacuity (Waters) HPLC unit with an autosampler. Peptides were resolved via reversed phase separation on a 20 cm by 100 μm column packed emitter tip using an aqueous to organic gradient (2–45%). Peptide MS/MS spectra were acquired data-dependently with one full scan MS followed by 5 MS/MS scans. Analysis by 8-step MudPIT was performed essentially as described previously [17] and using same instrumentation. Acidified peptides were loaded onto a 150-μm-inner-diameter (ID) biphasic trapping column comprised of 4-cm strong cation exchange resin (Luna [5-μm particle size]; Phenomenex) followed by 4-cm reverse-phase resin (Jupiter [5-μm particle size, 300-Å pore size]). After offline loading of samples, the trapping column was then attached to a 20-cm-long (Jupiter [3-μm particle size, 300-Å pore size]) 100-μm-ID fused silica analytical column packed into a pulled nanospray tip. Aliquots of ammonium acetate (5 μl) at concentrations of 0, 100, 150, 200, 300, 500, 750, and 1000 mM were injected by autosampler. After each salt injection, peptides were separated using a 105-min aqueous-to-organic gradient (2% to 35% acetonitrile for all but the last step, which went to 98%). Peptide MS/MS spectra were queried using SEQUEST (full tryptic