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Proteomic profiling of the surface-exposed cell envelope proteins of *Caulobacter crescentus*☆

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ABSTRACT

Biotinylation of intact cells, avidin enrichment of derivatized peptides, and shotgun proteomics were employed to reveal the composition of the surface-exposed proteome of the aquatic bacterium, *Caulobacter crescentus*. Ninety-one unique proteins were identified with the majority originating from the outer membrane, periplasm, and inner membrane, subcellular regions that comprise the Gram-negative bacterium cell envelope. Many of these proteins were described as 'conserved hypothetical protein' or 'hypothetical protein'; and so, the actual expression of these gene products was confirmed. Others did not have any known function or lacked annotation. However, this investigation of the *Caulobacter* surfaceome did reveal the unanticipated presence of a number of enzymes involved in protein degradation.

Biological significance
The results presented here can provide a starting point for hypothesis-driven research projects focused on this bacterium in particular and centered on understanding Gram-negative cell architecture and outer membrane biogenesis broadly. The detected protein degradation enzymes anchored on or located within the outer membrane suggest that *Caulobacter* has nutrient sources larger than small molecules and/or further processes surface proteins once secreted to this location. Additionally, confirmation of outer membrane residency of those proteins predicted to be periplasmic or whose location prediction was not definitive could potentially elucidate the identities of Gram-negative specific anchorless surface proteins.
This article is part of a Special Issue entitled: Trends in Microbial Proteomics.

1. Introduction

Definition of the surface-exposed proteome has shed light on the complexity of biological processes taking place at the interface between bacteria and their external milieu. These environment-accessible proteins are not only involved in the intake of food/fuel and the release of waste [1], they also facilitate physical interactions of bacteria with living/non-living surfaces [2,3] and serve as sensors for extracellular signals [4,5]. Understanding the surface proteome (or surfaceome [6]) of pathogenic bacteria is of special importance since these microorganisms exploit their proteic cell surface activities in ways that allow them to invade host cells, thrive within the particular physiological niche, and thwart immune responses [3,7–9]. Although disease-supporting surface proteins can be taken advantage of by researchers as well – leading to the creation of new targeted

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antibiotics or vaccines [10,11] – continued investigation is called for due to the rapid pace of antimicrobial resistance to current therapeutics [12].

A number of methods have been devised to make the large-scale study of the surface proteome possible [13,14]. The choice of technique varies depending upon whether Gram-positive or Gram-negative cells are of interest. For Gram-negative bacteria, which have an outer membrane (OM) and inner cytoplasmic membrane (IM) separated by the periplasmic (PERI) space, only two techniques have been reproducibly successful: isolation of outer membranes [15–18] and labeling of the cell surface [6,19,20]. It is unknown if these two procedures lead to the identification of complementary surface-exposed proteins when applied to the same microorganism. Much of what we understand in regards to Gram-negative bacteria architecture is based upon research on a limited number of model organisms, particularly the γ-proteobacterium *Escherichia coli*. And so, this type of research remains essential in order to fully appreciate how broadly applicable certain surface-accessible processes are and to tease out species-dependent surface biology.

In this work, we describe our efforts to characterize the cell surface proteome of the α-proteobacterium *Caulobacter crescentus*. *Caulobacter* is an aquatic bacterium that can exist as an immature, mobile, flagellated cell or as a reproducing, sessile stalked cell [21]. It is also a very important model organism whose proteome has been studied in great detail within the context of investigating its cell cycle [22], essential genome [23], growth under starvation conditions [24], and intracellular polar positioning of proteins [25–27]. As an extension of our research mapping the protein composition of its outer membrane, we used a surface-layer (S-layer) deficient strain of *Caulobacter* in combination with biotinylation labeling of intact cells and high resolution LC-MS/MS analysis to identify 91 putative surface-exposed proteins, 28 of which were unique to this study.

2. Materials and methods

2.1. Culture of bacterial cells

Two biological replicates of RsaA-negative (*RsaA*-) *Caulobacter* cells [28] were grown to the late exponential phase in 10 mL peptone yeast extract (PYE) medium. Then, 200 mL fresh PYE medium was inoculated with 10 mL of bacterial cells. The cells were cultured at 30 °C with constant shaking at 100 rpm until an OD_{600} of 0.6 was reached. Cells were harvested by centrifugation (4000 *g*, 10 min, 4 °C) and washed three times with ice-cold PBS (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 138 mM NaCl, 2.7 mM KCl) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were resuspended to an OD_{600} of 2.0 using the same ice-cold PBS. After resuspension, each cell culture was split into two aliquots: the first half was used to biotinylate the surface proteins of intact cells whereas the other half served as a negative control.

2.2. Biotinylation of cell surface proteins

Exposed proteins on the surface of intact bacterial cells were labeled with EZ-link™ Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL) essentially as described before [19]. The experimental aliquots of cells, hereafter referred to as 'biotinylated' cells, were incubated with Sulfo-NHS-LC-LC biotin (0.2 mM final concentration) for 1 h at room temperature with gentle rotation. Excess Sulfo-NHS-LC-LC biotin was quenched by a 10 min incubation of the reaction mixture with Tris–HCl (pH 7.5) to a final concentration of 50 mM. Cells were washed three times with 50 mM Tris–HCl (pH 7.5) and resuspended in 50 mM AMBIC (pH 7.5) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The 'negative control' cells were incubated with Nanopure water instead of the biotin reagent and underwent the same procedure. Cell lysates were generated from both biotinylated and control cells by subjecting the cells to repeated intermittent sonic oscillation (20 × 5 s). Cellular debris was removed by centrifugation (10,000 *g*, 10 min) and supernatants were collected and stored for further analysis. The Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL) was used to determine protein concentrations. Streptavidin Western blotting was conducted to visualize biotinylated proteins as described before [18].

2.3. In-solution tryptic digestion

Proteins were reduced with 5 mM Tris(2-carboxyethyl) phosphine (TCEP; Thermo Fisher Scientific) for 1 h at room temperature and then alkylated with fresh iodoacetamide (IAA) at 10 mM final concentration in the dark for 30 min at room temperature. Residual IAA was quenched by a subsequent addition of 5 mM TCEP for 30 min at room temperature. Proteins were digested (37 °C, overnight) with sequencing-grade modified-trypsin (Promega, Madison, WI) at an enzyme: substrate ratio of 1: 50 (w/w).

2.4. Peptide-level enrichment of biotinylated samples

Biotinylated peptides (100 μg) were purified using an avidin cartridge column (ICAT™ kit, AB SCIEX, Framingham, MA) according to vendor instructions. Purified samples were reduced to near dryness via vacuum centrifugation. Labeled peptides were reconstituted with 0.1% acetic acid (50 μL) for LC-MS/MS analysis.

2.5. nanoLC-MS/MS analyses

LC-MS/MS analysis was performed using both a QSTAR Elite (AB SCIEX, Foster City, CA) equipped with a Tempo nanoLC system and a LTQ-Orbitrap Velos (Thermo Scientific, West Palm Beach, FL) coupled to a 1200 Binary LC system (Agilent Technologies, Santa Clara, CA). Sample was loaded onto home-made pre-columns (75 μm ID × 3 cm) packed with 5 μm Monitor C_{18} particles (Column Engineering, Ontario, CA) and eluted at flow rates of 100 nL/min (Tempo nanoLC) or 200 nL/min (Agilent 1200 LC system) onto analytical columns (75 μm ID × 10 cm) of Column Engineering Inc. 3 μm Monitor 100 Å-Spherical Silica C_{18}. The gradient was as follows: 0%–30% B in 60 min; 30%–60% B in 40 min; 60%–70% B in 20 min; and 70% B for 10 min. Solvents A and B consisted of 0.1% formic acid in water and 0.1% formic acid/2% water in ACN, respectively. For both MS instrument, ionization voltage was set at 1.8 kV, MS full scan range was *m/z* 400–2000, and MS/MS spectra were acquired using the automatic