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Proteomic analysis of outer membrane proteins and vesicles of a clinical isolate and a collection strain of *Stenotrophomonas maltophilia*

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ABSTRACT

Stenotrophomonas maltophilia is a Gram-negative pathogen with emerging nosocomial incidence that displays a high genomic diversity, complicating the study of its pathogenicity, virulence and resistance factors. The interaction of bacterial pathogens with host cells is largely mediated by outer membrane proteins (OMPs). Indeed, several OMPs of Gram-negative bacteria have been recognized as important virulence factors and targets for host immune recognition or to be involved in mechanisms of resistance to antimicrobials. OMPs are also present in outer membrane vesicles (OMVs), which bacteria constitutively secrete to the extracellular milieu and are essential for bacterial survival and pathogenesis. Here, we report the characterization of the OMP and native OMV subproteomes of a clinical isolate (M30) and a collection strain (ATCC13637) of *S. maltophilia*. We had previously shown that the ATCC13637 strain has an attenuated phenotype in a zebrafish model of infection, as well as a distinct susceptibility profile against a panel of antimicrobials. The protein profiles of the OMP and OMV subproteomes of these two strains and their differences consequently point at pathogenesis, virulence or resistance proteins, such as two variants of the quorum-sensing factor Ax21 that are found to be highly abundant in the OMP fraction and exported to OMVs.

Biological significance: Stenotrophomonas maltophilia is rapidly climbing positions in the ranking of multidrug-resistant pathogens that are frequently isolated in hospital environments. Being an emerging human pathogen, the knowledge on the factors determining the pathogenicity, virulence and resistance traits of this microorganism is still scarce. Outer membrane proteins (OMPs) and vesicles (OMVs) are key elements for the interaction of Gram-negative bacteria with their environment — including the host–and have fundamental roles in both infection and resistance processes. The present study sets a first basis for a phenotype-dependent characterisation of the OMP subproteome of *S. maltophilia* and complements very recent work on the OMV subproteome of this species. The variability found among even two strains demonstrates once more that the analysis of genotypically and phenotypically distinct isolates under various conditions will be required before we can draw a significant picture of the OMP and OMV subproteomes of *S. maltophilia*.

1. Introduction

Stenotrophomonas maltophilia is a non-fermentative Gram-negative bacterium with an increasing incidence in hospital environments [1,2]. Although this obligate aerobic bacterium can be found in almost any aquatic or humid environment, including drinking-water supplies [3], it is now recognized as an emerging nosocomial pathogen and has been associated with respiratory infections, septicemia, biliary sepsis, endocarditis, conjunctivitis, meningitis, urinary tract infections and various wound infections in immunocompromised patients as well as in cystic fibrosis (CF) patients [1,4,5]. Currently, *S. maltophilia* is isolated from the lungs of approximately 10% of the CF patients in USA and up to 25% of those in Europe [2] and displays significant morbidity and mortality rates among debilitated patients [1,4,6–8].

S. maltophilia exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics, including β-lactams, quinolones, aminoglycosides, tetracycline, disinfectants and heavy metals [4,9–14]. Intrinsic resistance may be due to reduced outer-membrane permeability, changes in LPS structure, production of multidrug efflux pumps and the presence of integrons for site-specific insertion of resistance gene cassettes [15,16]. The production of melanin-like pigments and biofilm has been also linked to antimicrobial resistance [16]. Thus, the adhesion of *S. maltophilia* to medical implants, catheters and epithelial cells,

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leading to the formation of biofilm, confers to this bacterium natural protection against different antimicrobial agents and host immune defences. In this regard, the development of therapies against *S. maltophilia* infection represents a significant challenge for both clinicians and microbiologists. In addition, there is very limited knowledge of the virulence factors of this bacterium, beyond those established by homology relations, and there is still considerable uncertainty about the route(s) of infection of *S. maltophilia*.

Among a wide spectrum of potential factors, outer membrane proteins (OMPs) are critical for bacterial interactions and survival in different environments. Many macromolecules in the outer membrane of bacterial pathogens, especially proteins exposed on the cell surface, are important virulence factors and targets for host immune recognition. Identification of abundant and/or novel OMPs and characterisation of their roles in pathogen physiology, pathogenesis, and defence against the host, is an important preliminary step in development of diagnostics, vaccines, and therapeutics. Until recently, OMPs were identified using two-dimensional gel electrophoresis (2-DE) of solubilized outer membranes (OMs) followed by peptide-mass-fingerprint (PMF) identification of single spots. However, advances in liquid chromatography coupled to tandem mass spectrometry allow the identification of a larger number of proteins from whole cells or subcellular fractions in one go. The OM is an excellent subcellular fraction to target for shotgun proteomics since its protein complexity is relatively low. On the other hand, its purification to homogeneity free of inner membrane, cell wall components, and cytoplasmic proteins is challenging [17].

Protein secretion to the extracellular milieu is an essential process for communication, sensing and invasion, both in prokaryotes and eukaryotes. In addition to releasing soluble proteins and mediators, cells also release proteins in association with membrane vesicles. Membrane vesicles from Gram-negative bacteria are known as outer membrane vesicles (OMVs), spherical blebs of average diameter 10–300 nm that are naturally released to the environment [18]. Although the budding mechanisms are unclear, it has been shown that OMVs are continuously produced during growth of various Gram-negative bacteria, including *Eschericia coli*, *Helicobacter pylori*, *Vibrio cholerae* or *Pseudomonas aeruginosa*, to cite some [19–22]. OMVs are known to contain lipopolysaccharides, lipoproteins, outer membrane, periplasmic, and cytoplasmic proteins as well as DNA and RNA [18,19,23,24], and have been suggested to be involved in exclusion of competing bacteria, conveyance of proteins or genetic material to other bacteria and presentation/delivery of virulence factors to host [18]. It has been also reported that OMVs contain several secreted virulence factors targeting host cells or other bacteria such as phospholipase C, alkaline phosphatase, proelastase, hemolysin [25], and antibacterial factors including murein hydrolases [26]. In addition, OMVs are involved in transferring antibiotic resistance [27] and trafficking signals via packaging of quorum sensing molecules [28]. Despite these important roles, a limited number of OMV proteins have been identified. Furthermore, although recent progress in this area has revealed some functions for OMVs, most studies have been performed using detergent-extracted OMVs [29–32], produced under artificial conditions, instead of native OMVs. A global proteomic profiling of native *S. maltophilia* OMVs, along with OMPs, should provide a basis for the understanding of OMV biogenesis and function in microorganism. Here, we present an accurate and comprehensive analysis of the OMP and native OMV subproteomes of a recent clinical isolate (M30) and a collection strain (ATCC13637) of *S. maltophilia*. The two subcellular fractions have been analysed by GeLC-MS/MS. In addition, OMPs have been also analysed by 2-DE to obtain a global picture of this subproteome. We had previously shown that the ATCC13637 strain has an attenuated phenotype in a zebrafish model of infection, as well as a distinct susceptibility profile against a panel of antimicrobials [33]. Differences between the protein profiles of the OMP and OMV subproteomes of these two strains may therefore point at factors involved in the pathogenesis, virulence or resistance mechanisms of this pathogen.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Two different strains of *S. maltophilia* have been used, the collection strain ATCC13637, isolated in 1960 from the oropharyngeal region of a patient with mouth cancer [34], and the clinical strain M30, isolated from a decubitus ulcer at the Hospital Municipal de Badalona (Barcelona, Spain) during the year 2009 [33]. Species identification was confirmed biochemically using the API NE system (bioMérieux). *S. maltophilia* strains were routinely cultured o/n in Luria-Bertani (LB) media at 37 °C and 250 rpm unless otherwise stated, and growth curves were monitored following the optical density at 550 nm. A single colony was transferred to 5 mL of LB broth and incubated in an orbital shaking incubator for 8 h at 150 rpm and 37 °C. Then, 500 mL of LB broth were inoculated with 1/100 dilutions of 8 h culture and grown for approximately 12 h at 37 °C with shaking (250 rpm), until an OD of 1 was reached.

2.2. Purification of OMPs

Purification of OMPs from *S. maltophilia* was carried out as described by Hobb et al. [35] Briefly, cells from 250 mL culture of *S. maltophilia* ATCC13637 or M30 were resuspended in 7 mL of 10 mM HEPES, pH 7.4, and lysed by passing the culture twice through a French Press (Thermo Electron Corporation) at 1000 p.s.i. (6.9 MPa; 40 K cell). The lysed cell preparation was centrifuged at 10,000 ×*g* for 10 min at 4 °C to remove cell debris. The membranes were then collected by ultracentrifugation at 100,000 ×*g* for 1 h at 4 °C (Beckman Ti70.1 rotor). The pellet was then resuspended in 2 mL 10 mM HEPES, pH 7.4, and washed in a total volume of 10 mL HEPES 10 mM, pH 7.4 and collected again by ultracentrifugation as described previously. The final pellet was resuspended in 5 mL 1% (w/v) *N*-laurylsarcosine (Sigma Aldrich) in 10 mM HEPES, pH 7.4, and incubated at 37 °C for 30 min with gentle shaking. The sarkosyl-treated membranes were then collected at 100,000 ×*g* for 1 h at 4 °C (Beckman, Ti70.1 rotor) and the resulting pellet was washed with 10 mL of HEPES 10 mM, pH 7.4. Following the final ultracentrifugation, the pellet was resuspended in 500 μL of lysis solution (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 0.5% pharmalytes, pH 3–10 and traces of bromophenol blue).

2.3. Purification of OMVs

OMVs were purified from culture supernatants, as described previously, with some minor modifications [24,36,37]. Briefly, the cells were pelleted at 6000×*g* for 15 min. The supernatant fraction was then filtered through a 0.45 μm syringe-driven filter to remove any remaining cell. The resulting filtrate was subjected to serial centrifugation at 20,000 ×*g* for 35 min, 40,000 ×*g* for 1 h and 150,000×*g* for 3 h at 4 °C. The final (150,000 ×*g*) pellets were resuspended in 1.25 mL of PBS, layered over a sucrose gradient (1.25 mL at each of 2.5, 1.6 and 0.6 M sucrose), and centrifuged at 200,000 ×*g* for 20 h at 4 °C. Finally, protein concentration was estimated using the 2D-Quant kit (GE, Healthcare).

For Transmission electron microscopy (TEM) analysis, fractions obtained after sucrose density gradient centrifugation were diluted tenfold with PBS and then centrifuged at 200,000 ×*g* for 3 h. The pelleted vesicles were resuspended in PBS, applied to 400-mesh copper grids, and stained with 2% uranyl acetate. Electron micrographs were recorded with a JEM1400 microscope (JEOL, Japan) at 120 kV acceleration voltage.

2.4. Electrophoresis

For GeLC–MS/MS analysis, 10% acrylamide SDS-PAGE was performed with approximately 75 μg of protein loaded in each analysis. Two-dimensional electrophoresis with immobilized pH gradients was