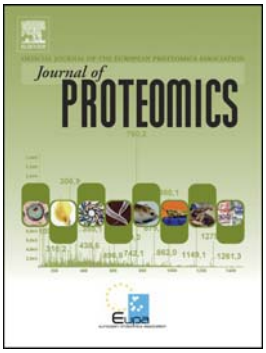

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Proteomic analysis of outer membrane proteins and vesicles of a clinical isolate and a collection strain of *Stenotrophomonas maltophilia*

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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.

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 resitance 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*.

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, 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 characterization 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.

Materials and Methods

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 550nm. 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.

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; 40K cell). The lysed cell preparation was centrifuged at 10000 x g for 10 min at 4°C to remove cell debris. The membranes were then collected by ultracentrifugation at 100000 x 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 100000 x 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).

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 x 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 20000 x g for 35 minutes, 40000 x g for 1 h and 150000 x g for 3 h at 4ºC. The final (150000 x 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 200000 x g for 20 h at 4ºC. 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 ten-fold with PBS and then centrifuged at 200000 x 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.

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 carried out as described by Görg *et al*. [38], with some minor modifications. To avoid potential degradation of proteins by proteolysis, a protease inhibitor was used following manufacturer indications (Protease Inhibitor Mix, GE Healthcare). First-dimension isoelectrofocusing was performed on immobilized pH gradient strips (24 cm, pH 3-10) using an Ettan IPGphor 3 System. Samples were applied near the basic end of the strips by cup-loading, after being incubated o/n in 450 μL of rehydration buffer (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 0.5% IPGs, pH 3–10, 100 mM DeStreak reagent). After focusing at 70 kVh strips were equilibrated, first for 15 min in 10 mL of reducing solution (6 M urea, 100 mM

Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 5 mg/mL dithiothreitol) and then in 10 mL of alkylating solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 22.5 mg/mL iodoacetamide) for 15 min on a rocking platform. Second dimension SDS-PAGE was performed by laying the strips on 12.5% isocratic Laemmli gels (24 cm × 20 cm) on an Ettan DALT Six system. Gels were run at 20 °C at a constant power of 2.5 W per gel for 1 h, followed by 17 W per gel until the bromophenol blue tracking front had run off the end of the gel. The sample amount loaded was 200 µg. Spot-volume, molecular weight and isoelectric point were experimentally determined with ImageMaster 5.0 software (GE Healthcare).

Gel staining and detection of proteins

All SDS-PAGEs were stained with 0.1% Coomassie blue R-250, while 2-D gels were silver stained as described elsewhere [39]. Briefly, the gels were fixed twice in 40% ethanol and 10% acetic acid for 30 min each. Sensitizing was carried out for 30 min in 0.02% (w/v) sodium thiosulfate. The gels were then washed three times for 5 min with distilled water and incubated in 0.1% (w/v) silver nitrate for 20 min. The gels were washed again twice for 1 min with distilled water and developed with 3% (w/v) sodium carbonate and 0.025% (v/v) formaldehyde until the desired contrast was reached. Reaction was stopped with 1.5% (w/v) EDTA-Na_2 for 45 min, after which the gels were washed twice with distilled water.

In-gel tryptic digestion

The Coomassie-stained lanes were cut into 10 equal bands, while the silver-stained protein spots were excised from the acrylamide gel with a cut tip and immediately destained and digested as described elsewhere [40]. Briefly, the Coomassie-stained lanes were washed twice with water for 20 min and distained with 200 µL of 50 mM ammonium bicarbonate/50% acetonitrile. Silver-stained spots were destained with 200 µL of 30 mM potassium ferricyanide

and 100 mM sodium thiosulfate (1:1) for 20 min in the dark, and spots were then washed with Milli-Q water until they were completely clear. Before tryptic digestion, reduction and alkylation with DTT/IAA was performed by incubating samples with 200 µL of 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 56°C, followed by alkylation with 200 µL of 55 mM IAA in 50 mM ammonium bicarbonate for 30 min at room temperature, protected from light.

Gel pieces were digested o/n with 6 ng/µL trypsin at 37 °C. The peptide extraction was carried out with three consecutive washes with 0.2% TFA for MALDI-TOF identifications or with 1% formic acid for ESI analysis. The eluted peptides were dried in a SpeedVac and stored at −20 °C until they were analysed by mass spectrometry.

Mass-spectrometry analysis

For MALDI analysis, 1 µL of sample was mixed with the same volume of a saturated solution of α-cyano-4-hydroxy-*trans*-cinnamic acid matrix (0.5 mg/mL in acetonitrile/water/TFA 1% 3:6:1) and spotted onto a MALDI target plate (Bruker). The drop was air-dried at room temperature. MALDI-mass spectra were recorded in the positive ion mode on an UltrafleXtreme™ time-of-flight instrument (Bruker). Ion acceleration was set to 25 kV. All mass spectra were externally calibrated using a standard peptide mixture containing angiotensin II (1046.54), angiotensin I (1296.68), substance P (1347.74), bombesin (1619.82), renin substrate (1758.93), adrenocorticotropic hormone 1-17 (2093.09), adrenocorticotropic hormone 18-39 (2465.20), and somatostatin 28 (3147.47). Calibration was considered good when a value below 1 ppm was obtained. For PMF analysis, the MASCOT search engine (Matrix Science) was used with the following parameters: one missed cleavage permission, 100 ppm measurement tolerance, and at least four matching peptide masses. Cysteine carbamidomethylation was set as fixed modification when appropriate, with methionine oxidation as the variable modification. Common contaminants were removed using the contaminants database available in the Mascot search

engine. Two different custom databases were created in order to perform the searches, containing the *S. maltophilia* ATCC13637 and M30 full protein sets as translated from the corresponding genomes ([41] and [42], respectively). Positive identifications were accepted with a Mascot score corresponding to a *p*-value ≤ 0.05.

For GeLC–MS/MS analysis, the digests of the SDS-PAGE lanes were analysed on an amaZon ETD Ion Trap mass spectrometer (Bruker), coupled to a nano-HPLC system (Proxeon). Peptide mixtures were first concentrated on a 300 µm i.d., 1 mm PepMap nanotrapping column and then loaded onto a 75 µm i.d., 15 cm PepMap nanoseparation column (LC Packings). Peptides were then eluted by a 0.1% formic acid/acetonitrile gradient (0-40% in 2 h; flow rate ca. 300 nL/min) through a nanoflow ESI Sprayer (Bruker) onto the nanospray ionization source of the Ion Trap mass spectrometer. MS/MS fragmentation (3 × 0.3 s, 100-2800 *m*/*z*) was performed on three of the most intense ions, as determined from a 0.8 s MS survey scan (310-1500 *m*/*z*), using a dynamic exclusion time of 1 min for precursor selection and excluding single-charged ions. An automated optimization of MS/MS fragmentation amplitude, starting from 0.60 V, was used. Proteins were identified using Mascot (Matrix Science) to search in the two custom databases, as done for MALDI analysis. Common contaminants were first removed using the contaminants database available in the Mascot search engine. MS/MS spectra were searched with a precursor mass tolerance of 0.4 Da, fragment tolerance of 0.7 Da, trypsin specificity with a maximum of one missed cleavage, and cysteine carbamidomethylation and methionine oxidation as variable modifications. Replicate analyses of all the LC-MS/MS analysis, using two independent biological replicas, showed ≈ 85% coincidence, indicating a high level of reproducibility. In order to ensure that the data are reliable a decoy database was used. In this sense the searches were repeated, using identical search parameters, against a database in which the sequences had been reversed or randomized [43]. In the Mascot search engine, during the search, every time a protein sequence from the target database is tested a decoy sequence of the same length is automatically generated and tested. The average amino-acid composition of the decoy

sequences is the same as the average composition of the target database. In addition, in order to compare the proteins from these two strains we have used *S.maltophilia* K279a as reference.

To accept the identification of a protein we applied the following cut-off: Proteins were only identified if at least two different peptides had been obtained, with a peptide match expectation value lower than 0.05. Furthermore proteins and peptides were included in the list only if they were present in both replicas.

Results and discussion

Analysis of *S. maltophilia* OMPs by 2-DE

OMPs were obtained by sarcosine fractionation and analysed by the combination of 2-DE and MS. Hobb *et al.* [35] had shown that, of nine examined methodologies for OMP extraction, extraction with N-lauroylsarcosine (Sarkosyl) produces the purest and most reproducible samples. In addition, Cao *et al.* had also reported sarkosyl extraction to be superior to carbonate extraction [44]. The 2-DE maps obtained for strains ATCC13637 and M30 showed an excellent resolution even for high-abundance spots (Figure 1).

A total of 48 spots (corresponding to 34 different proteins) and 52 spots (41 different proteins) were identified by PMF for the ATCC13637 and M30 strains, respectively (for a detailed report on identified spots see Supporting information Tables S1 and S2 for ATCC13637 and M30, respectively). A recognized drawback of 2-DE is the low representation of membrane proteins in the maps obtained. To overcome this limitation we have used sulfobetaine ASB-14 in sample preparation as well as during the isoelectrofocusing process. This is a detergent proven to increase the presence of membrane proteins in 2-DE maps [45, 46]. The results show a significant enrichment in membrane proteins as can be observed in the subcellular prediction

made by Psort [47] (Tables S1 and S2). Although we cannot completely exclude the possibility of contamination by cytoplasmic proteins, this must be clearly very low.

After spot-volume calculation, the most prominent spots on the 2-DE maps correspond to the putative quorum-sensing factor Ax21 encoded by *smlt0387* (locus name in the reference strain *S. maltophilia* K279a, spot 12), accounting for 28.48% of the total volume of all ATCC13637 spots and a corresponding 22.15% for M30, a newly identified Ax21 homolog [48] encoded by *smlt0184* (spot 1), with 19,45% of the total volume of all ATCC13637 spots and a corresponding 10.93% for M30, and the putative porin OmpW encoded by *smlt3805* (spot 33), with 9.79% of the total volume of all ATCC13637 spots and a corresponding 21.55% for M30. Note that spot volumes are not quantitatively comparable among the silver-stained gels for the two strains, especially under saturating conditions.

A deletion mutant of the Smlt0387 Ax21 homolog in *S. maltophilia* has been shown to affect various phenotypes leading to reduced motility, reduced tolerance to some antibiotics, reduced biofilm formation and reduced virulence [49]. The abundance of this Ax21 homolog has been also found to correlate with virulence in a zebrafish model of infection [33]. Both the *smlt0387* and *smlt0184* products have been very recently detected in OMVs of a clinical *S. maltophilia* isolate [48]. Stimulation with imipenem and with the quorum-sensing molecules DSF and BDSF led to large increases in OMV-associated secretion of these two Ax21 proteins, particularly of the newly identified Smlt0184 homolog. Interestingly, differential analysis of protein abundance in three clinical isolates with distinct virulence phenotypes, relative to the attenuated ATCC13637, showed significantly increased levels of Smlt0387 but not of Smlt0184 in the virulent strains [33], suggesting that the relative role of these two proteins in quorum-sensing/virulence-associated mechanisms may depend on strain phenotype and environment conditions. All in all, the high presence of these two proteins at the pathogen's surface, together with the observed implications in virulence and their up-regulation in response to antibiotics and

quorum-sensing signals suggests a potential target mechanism for antimicrobial-drug development. The third high-abundance protein, porin OmpW, has been linked to adaptive responses to various stress conditions [50, 51]. For example, it has been proposed to act as a receptor of colicins [52], a type of bacteriocin produced by and toxic to some strains of *E. coli*, which are released into the environment to reduce competition from other bacterial strains [53].

An advantage of 2-DE over LC-MS/MS based techniques is that it provides the molecular weight (MW) and isoelectric point (p*I*) of the proteins present in the gel. MW and p*I* of the identified spots were experimentally determined with ImageMaster 5.0 Software (GE Healthcare) and compared to gene-deduced MW and p*I* as obtained from MASCOT (Tables S1 and S2). A majority of gel-estimated values match the theoretical ones within the expected precision. Differences may be due to the presence of protein isoforms, post-translational processing, proteolysis, or binding of chemical groups. Remarkably, the Smlt0387 Ax21 homolog shows the highest variability, with five variants in ATCC13637 and six in M30. The Smlt0184 Ax21 homolog presents three identified variants in each strain. For both proteins, no differences in the peptides identified for the different variants have been found during the PMF analysis.

Some proteins have been identified in only one of the strains analysed. This is the case for 5 proteins from ATCC13637 and 11 proteins from M30. The absence of a protein in one of the samples should be, however, interpreted with care, as a number of different factors could contribute to it. Confirmation by LC-MS/MS analysis (below) is a necessary but neither sufficient condition.

Analysis of *S. maltophilia* OMPs by LC-MS/MS

The OMP samples used for 2DE analysis were later analysed by GeLC-MS/MS. GeLC-MS/MS resulted in the sequencing of a total of 1765 different peptides (Table S3, Supporting information), corresponding to 212 proteins (Table S4), for the ATCC13637 strain and 391

different peptides (Table S5), corresponding to 63 proteins (Table S6), for the M30 strain. In general, the proteins identified are those expected for the outer membrane subcellular fraction (e.g., flagellar components, secretins, efflux pumps and TonB-receptors). Two of the 212 proteins identified in the ATCC13637 strain and eight of the 63 proteins identified in the M30 strain have no clear homolog in K279a. Interestingly, some of these proteins could have implications in antibiotic resistance. For example, the protein SmeF (DF40_010335) belongs to a multi-drug efflux pump with three different components, *SmeD*, *SmeE* and *SmeF*. It has been described that hyper-expressed SmeF may function with additional multi-drug efflux components to promote multi-drug resistance in *S. maltophilia* [54]. The 55 proteins identified in M30 that have an ortholog in K279a have been also identified in the ATCC13637 strain. This is illustrated in Figure 2, which contains a four-set Venn diagram with ellipses showing common protein sets for the two strains analysed and the two experimental approaches used. Table S11 contains the information shown in this diagram at the protein level, i.e. proteins common or exclusive for each combination.

Two observations reinforce the idea that much is still unknown about the OMP subproteome of *S. maltophilia*. First, the difference in the number of proteins identified in the two strains (approximately 3.3x in ATCC13637). And second, the identification of many proteins of completely unknown function or annotated according to the predicted subcellular localisation.

Analysis of the native Outer membrane vesicles (OMVs) by LC-MS/MS

Native OMVs derived from strains ATCC13637 and M30 of *S. maltophilia* were purified from culture supernatants [24, 36, 37]. Examination of negatively stained OMVs by transmission electron microscopy (Figure 3) showed that almost all of them were closed vesicular forms, and neither membrane whorls nor pili were detected [19, 55]. Analysis of the vesicular proteins by SDS-PAGE (Figure 4) revealed that native OMVs contain many proteins with a wide range of

molecular weights and showed that ATCC and M30 OMVs have a distinct protein pattern. LC-MS/MS analysis of ATCC13637 OMVs resulted in the identification of 997 different sequenced peptides (Table S7), corresponding to 274 different proteins (6 of them with no ortholog in K279a, Table S8). For M30, a total of 638 different peptides were sequenced (Table S9), corresponding to 133 different proteins (8 of them with no ortholog in K279a, Table S10). Note that Devos *et al.* [48] identified 234 OMV proteins in the strain. One reason for this higher coverage is that they accepted protein identifications based on one single peptide (two in our case). In addition, the stimulation conditions under which they performed the analysis may affect OMV protein composition, as the presence of imipenem has been shown to increase the abundance of some proteins [56].

As shown in Figure 5A (see also Table S12), the OMP and OMV subproteomes of ATCC13637 share 132 proteins. For the M30 strain the number of shared proteins by the two subproteomes is 44 (Figure 5B). On the other hand, the OMV subproteomes of ATCC13637 and M30 have 108 proteins in common (Figure 5C). In addition, we have compared these protein sets with the proteins identified by Devos et al. in *S. maltophilia* 44/98 OMVs produced in the presence of the antibiotic imipenem [48] (Figure 5C). Thus, the ATCC13637 and M30 OMV protein sets share 131 and 84 proteins, respectively, with the 44/98 OMV protein set. Differences in the protein profiles observed for OMPs and OMVs in the same species (Figures 5A,B) strengthen the idea that specific protein-sorting mechanisms are involved in the biogenesis of OMVs [37]. In addition, interstrain differences in OMV protein profiles are sufficiently large that completely mask any potential differences due to the different growing conditions (primarily the absence or presence of imipenem) of ATCC13637 and M30 on the one hand and 44/98 on the other.

The protein sets shown in Tables S8 and S10 are consistent with previously reported results suggesting that vesicular proteins may be involved in OMV biogenesis, removal of toxic compounds and attacking phages, elimination of competing organisms, facilitation of the transfer

of genetic material and proteins to other bacteria, targeting of host cells and modulation of the host immune response [26, 28, 37, 57-61].

S. maltophilia is characterized by its high intrinsic resistance to a variety of structurally unrelated antimicrobials. Constitutive production of the families of class B L1 metallo- and class A L2 serine-β-lactamases is the major determinant for β-lactam (including carbapenem) resistance in this microorganism [14, 62-64]. Aminoglycoside-modifying enzymes, including *O*-nucleotidyltransferases and *N*-acetyltransferases, play also a key role in *S. maltophilia* aminoglycoside resistance [65-67]. Interestingly, we have identified proteins involved in the resistance to β-lactam only in ATCC13637 OMVs, despite we have previously demonstrated that *S. maltophilia* M30 is resistant to β-lactams and aminoglycosides [33]. It has been demonstrated that in some *S. maltophilia* strains β-lactamases are constitutively produced at very low levels until exposure to the antibiotic, at which time β-lactamase protein production increases dramatically [56]. This could explain the differences observed in the analysed strains. The potential lack of these enzymes in M30 OMVs could also indicate a distinct localisation distribution or the presence of yet undisclosed resistance mechanisms. We have also identified in the ATCC13637 strain two of the Sme multi-drug efflux pumps (Smlt3170/Smlt3171 and Smlt3924/Smlt3925). Only one of these resistance systems has been identified in M30 (Smlt3170/Smlt3171). These proteins, together with other identified proteins such as organic-solvent-tolerance factors, multi-drug-resistance efflux pumps and phage target receptors, may contribute to bacterial survival [58, 59].

Gram negative bacteria, including *Pseudomonas aeruginosa* and *Escherichia coli*, have been proposed to produce OMVs loaded with murein hydrolases to kill competing bacteria in polymicrobial populations [25, 26]. We have identified a murein hydrolase in the ATCC13637 strain (Smlt3434, Table S8) that might be involved in antimicrobial activity. Interestingly, this protein has not been found in the M30 strain. In contrast, a N-acetylmuramoyl-L-alanine amidase

(Smlt0154) involved in peptidoglycan catabolism has been identified in ATCC13637 and in the clinical isolate analysed by Devos *et al*. [48], but not in the M30 strain.

Several proteases have been also identified in each strain. The role of proteases in virulence has been widely discussed and it is accepted that they contribute to infection in at least three ways. First, they are part of the protein quality control machinery required for the turnover of unfolded proteins generated in an adverse host environment. Second, growing evidence supports a conserved role in specific and controlled proteolysis of regulatory proteins in response to temporal, spatial or environmental stimuli. Third and most direct, they may be secreted as exotoxins [68].

Among the proteins identified in OMVs, some are known to be present in the cytoplasm. Most of them are ribosomal or metabolic proteins. Nevertheless, other abundant cytoplasmic proteins, including pyruvate dehydrogenase or lactate dehydrogenase, were not detected in our OMVs preparations. Therefore the inclusion of particular proteins into OMVs does not appear to be a strict function of their abundance. Although OMVs have been suggested to consist of proteins and lipids of the outer membrane and periplasm, and not to contain inner membrane or cytoplasmic components [36], GeLC-MS/MS analysis of OMVs obtained from microorganisms like *E. coli* [28, 36, 37], *P. aeruginosa* [19, 27, 69] or *N. meningitidis* [29] contain many cytoplasmic proteins, as also observed in this study. Although we cannot completely exclude the possibility of cytoplasmic protein contamination, the presence of ribosomal proteins, chaperones or elongation factors in OMVs may not be surprising. The fact that vesicles carry DNA and RNA, and that translation of outer membrane proteins may occur simultaneously with their integration into the membrane suggests that transcriptional and ribosomal proteins can have a specific role into OMVs [69-72]. These findings strengthen previously published evidences [37] that vesiculation occurs at specific outer membrane sites with specific protein composition, or that specific mechanisms sort proteins into OMVs.

Conclusion

We have analysed 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. Most of the proteins identified in this work were previously known only from their ORFs, and their identification confirms the cognate genes are transcribed and translated. Our results show that the proteomic profiles of the OMP and OMV fractions are not equivalent, supporting previous evidence of the existence of specific mechanisms for the sorting of proteins into OMVs, and may vary largely for different strains of the same microorganism. The present study sets a first basis for the characterisation of the OMP subproteome of *S. maltophilia* as a function of phenotype and complements very recent work by Devos et al. [48] on the OMV subproteome of this species. In view of the variability observed, the characterisation of these subproteomes in additional, genotypically and phenotypically distinct strains and conditions will be required before general statements can be made for the OMP and OMV subproteomes of *S. maltophilia*.

Acknowledgements

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Figure Legends:

Figure 1. 2-DE maps of the OMPs from *S. maltophilia* ATCC13637 (**A**) and M30 (**B**). Information on each identified spot can be found in Supporting information Tables S1 (ATCC13637) and S2 (M30).

Figure 2. Four-set Venn diagram with ellipses to illustrate common protein sets for the two strains analysed and the two experimental approaches used. Table S11 contains this information at the protein level of detail.

Figure 3. Electron micrograph of purified native OMVs. Negative staining TEM of purified native OMVs after sucrose density gradient centrifugation. Left: OMVs obtained from the ATCC13637 strain. Right: OMVs obtained from the M30 strain.

Figure 4. Protein profile of purified native OMVs. Coomassie Brilliant Blue stained SDS-PAGE of OMVs from ATCC13637 and M30 as well as whole lysates of ATCC13637 and M30. Molecular weight standards are given on the left.

Figure 5. Venn diagrams showing common protein sets between: 5A: ATCC13637 OMPs and ATCC13637 OMVs; 5B: M30 OMPs and M30 OMVs; 5C: ATCC13637, M30 and 44/98 [48] OMVs. Table S12 contains this information at the protein level of detail.

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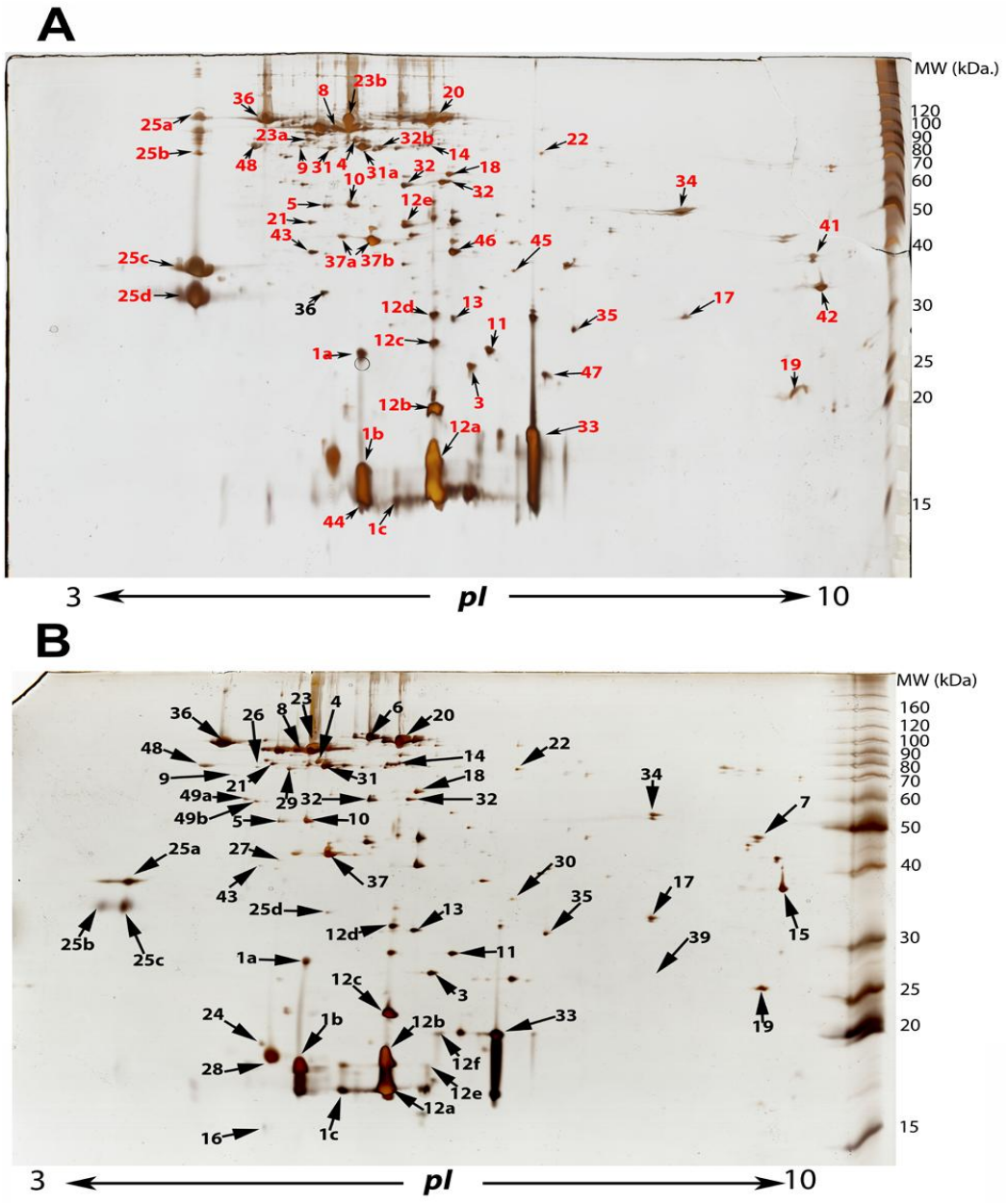


Figure 1

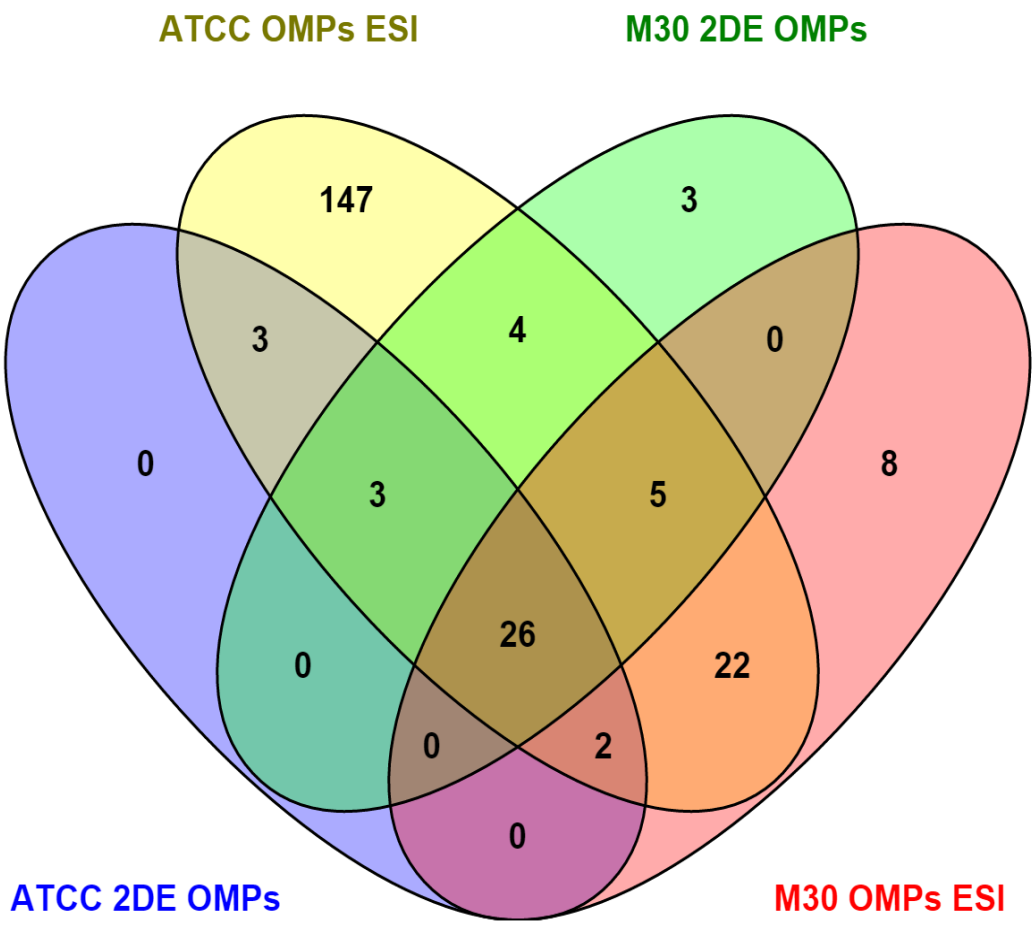


Figure 2

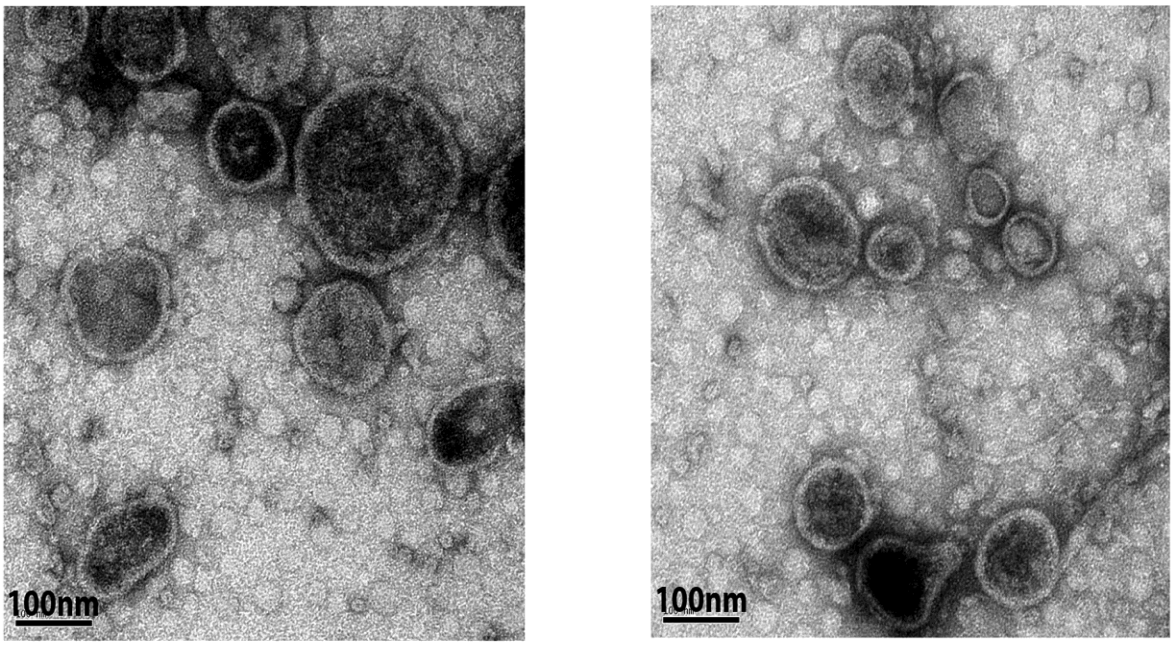


Figure 3

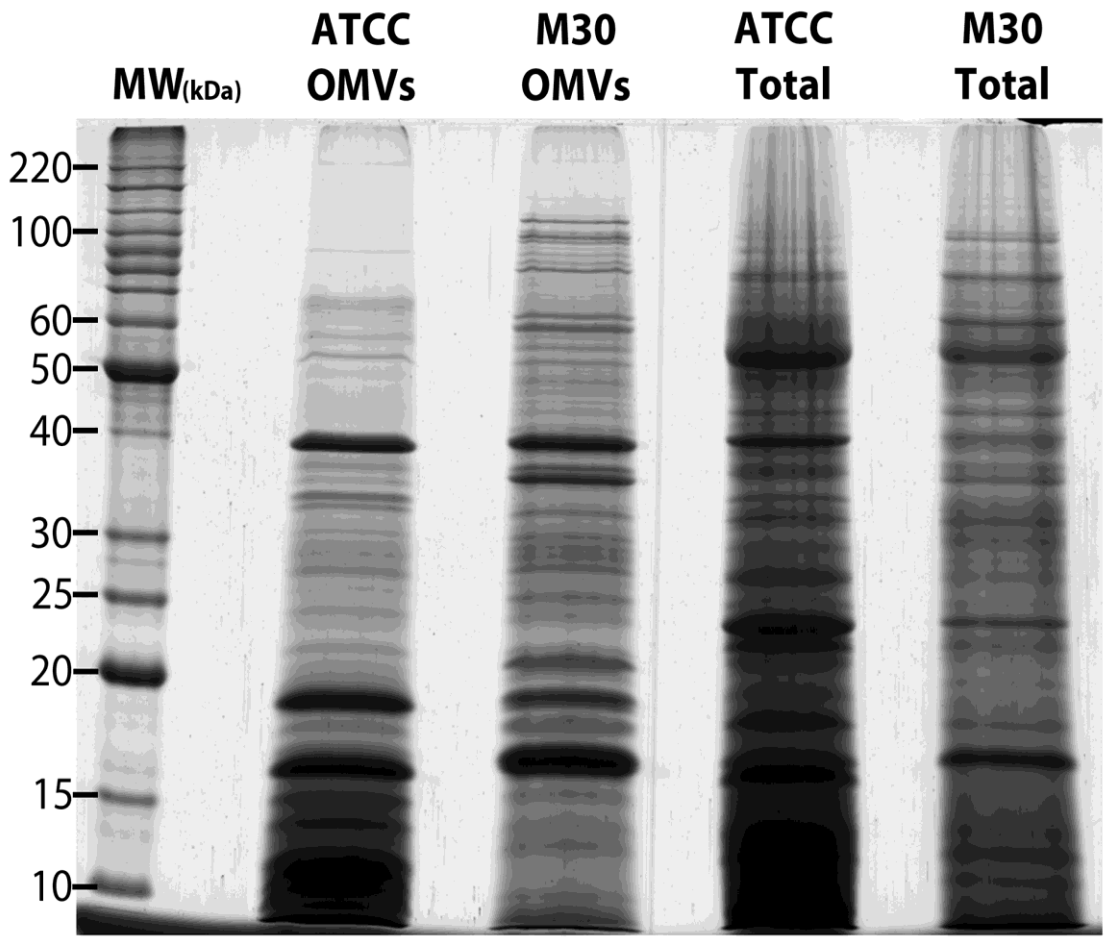


Figure 4

Figure 5A

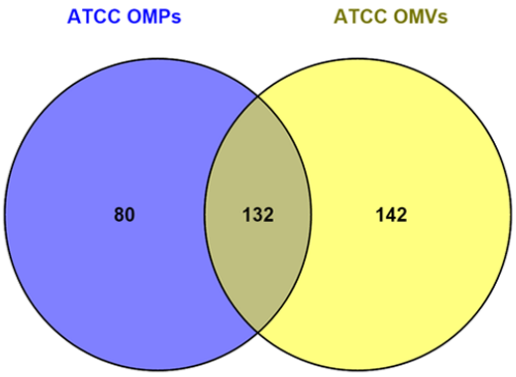


Figure 5B

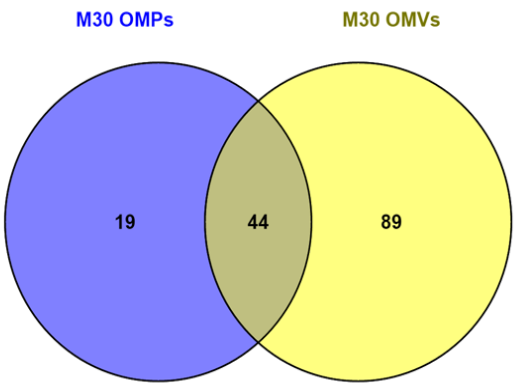
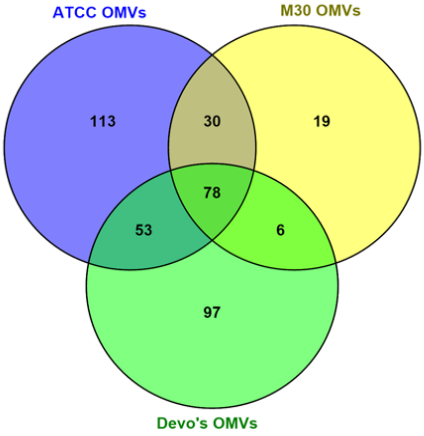
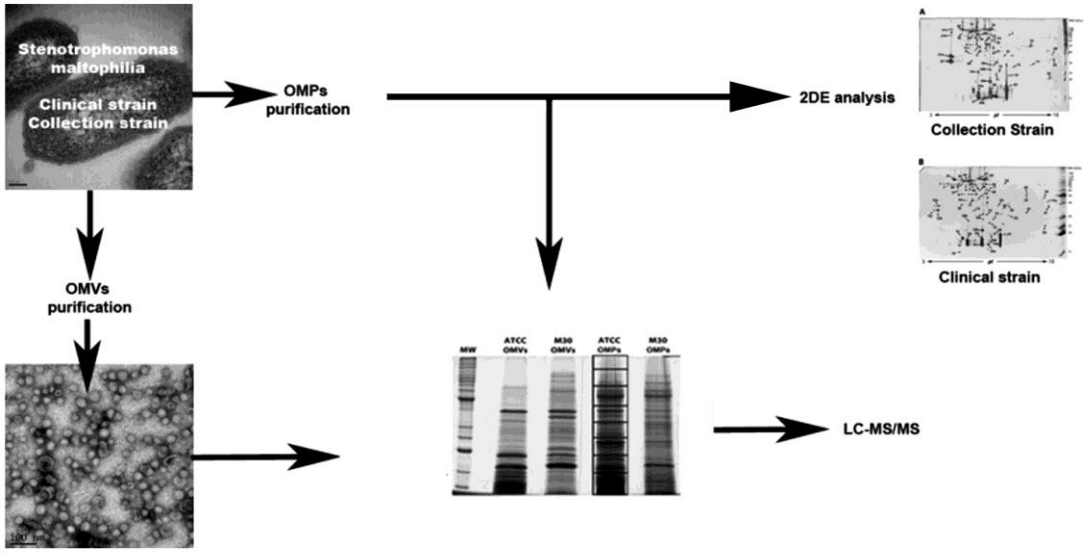


Figure 5C





Graphical abstract

HIGHLIGHTS

Characterization of OMPs of a clinical and a collection strain of *S. maltophilia*

Characterization of OMVs of a clinical and a collection strain of *S. maltophilia*

Different composition of OMVs and OMPs according to different infectivity capabilities