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# *Streptomyces coelicolor* membrane vesicles: many messages to be decoded

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# 1. INTRODUCTION

## 1.1 Extracellular Vesicles: an overview

The release of spherical, membranous vesicles is a universally conserved cellular process among organisms of all three domains of life, Archaea, Bacteria and Eukarya (Deatherage and Cookson, 2012). Diverse terms have been employed to indicate extracellular vesicles (EVs), in general membrane vesicles (MVs) for archaea and Gram-positive bacteria, outer membrane vesicles (OMVs) for Gram-negative bacteria, and exosomes and ectosomes (also known as microvesicles) for mammalian cells (Kim *et al.,* 2013; Choi *et al.* 2015) (Fig. 1).

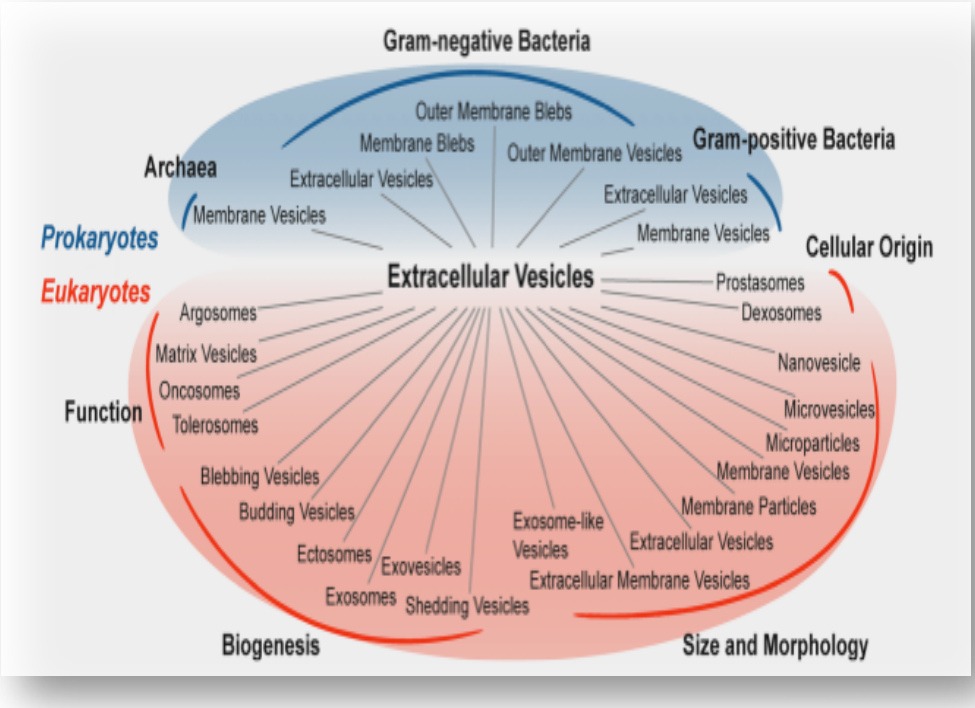



**Figure 1:** Extracellular vesicle-mediated intercellular communication is evolutionarily conserved from prokaryotes to eukaryotes (Choi *et al.* 2015).

EVs are broadly classified as: "particles naturally released from the cell that are delimitated by a lipid bilayer and cannot replicate" (Théry *et al*., 2018). EVs, with a nano-scale diameter between 20 and 1000 nm depending on the organisms, can transport various molecular cargoes (e.g., proteins, lipids, nucleic acids, sugars, metabolites) (Fig. 2) and delivery them to cells by fusion with the cytoplasmic membrane and/or by endocytosis in eukaryotes, modifying their physiology (Kim *et al.,* 2016; Gill *et al.,* 2019).

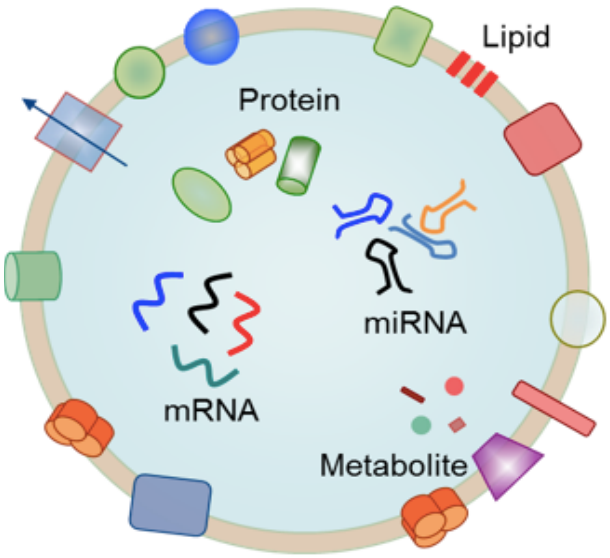



**Figure 2:** Structure and composition of EVs (http://evpedia.info).

The underlying molecular mechanisms of formation, cargo sorting, and release of vesicles are still largely unexplored. The importance of EV production as a major phenomenon in the living world was for a long time underestimated, with EVs being initially dismissed as artefacts of lipid self-aggregation or debris from lysed cells or waste products. EV-focused research over the past two decades has begun to reveal their significance in cell physiology and their diverse biological functions (Gill *et al.,* 2019; Coelho and Casadevall, 2019). In complex multicellular organisms or within populations of bacteria, the concentration of EV cargo offers protection against environmental degradation and are thus essential for exchange of RNA, proteins and other molecules that are prone to degradation. Moreover, EVs have the potential to facilitate the delivery of molecules that are generally excluded from entering target cells due to size, charge or hydrophobicity. Therefore, EVs offer an elegant solution to exchange biomolecules or to get rid of redundant and/or dangerous intracellular or membrane-associated compounds (Guerrero-Mandujano *et al.,* 2017; Yuana *et al.,* 2013; Yang *et al.,* 2018) (Fig. 3).

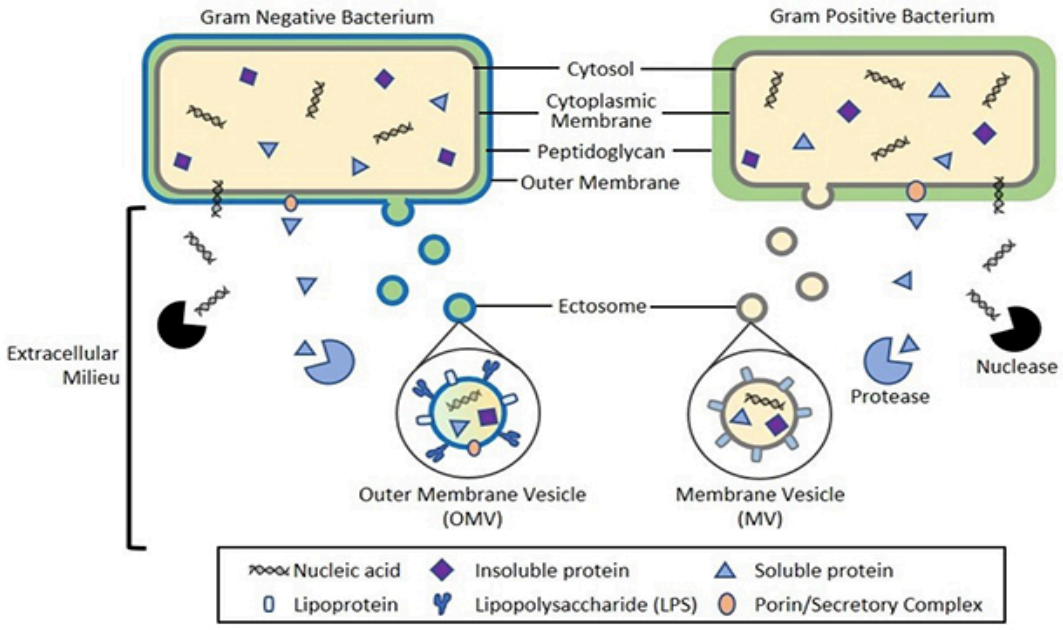



**Figure 3:** The lipid bilayer encasing the vesicular contents confers protection from extracellular degradation by nucleases and proteases, allowing distal transfer of bacterial EV cargo (Yang *et al.,* 2018).

## 1.2 Bacterial extracellular vesicles

Bacteria exhibit various types of cell envelopes that will impact on the nature of their vesicles (Gill *et al.,* 2019). Gram-negative bacteria (or diderm bacteria) envelope consists of two membranes (outer and inner membrane) that enclose a layer of peptidoglycan (Fig. 4a). Gram-positive bacteria (or monoderm bacteria) envelope consists in a single membrane covered by a thick layer of peptidoglycan (Fig. 4b).

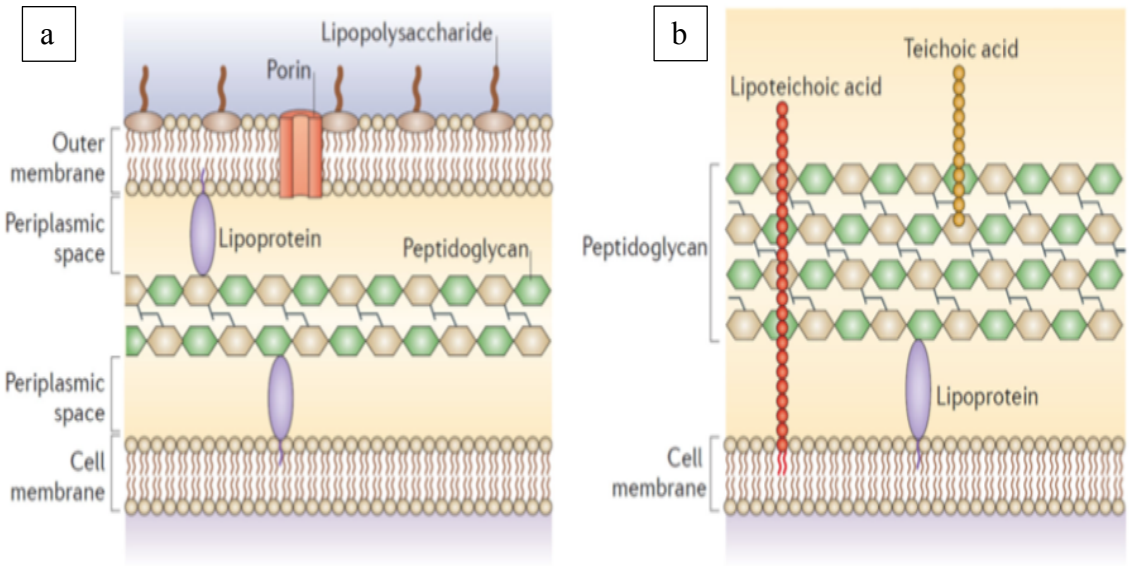



**Figure 4:** Cell wall structure of Gram-negative (a) and Gram-positive (b) bacteria (Brown *et al.,* 2015).

The majority of bacterial EV studies have been carried out on Gram-negative bacteria, where EVs originate from the outer membrane and are therefore often referred to as outer-membrane vesicles (OMVs) (Schwechheimer and Kuehn, 2015). Interestingly, some diderm bacteria EVs containing both outer and inner membranes have been identified and called outer-inner membrane vesicles (O-IMVs) (Pérez Cruz *et al.,* 2013, 2015). Gram-positive extracellular membrane vesicles (MVs) have attracted more attention only in recent years, because for a long time Gram-positive bacteria were considered not producing vesicles, because of the lack of an outer membrane layer and the presence of thick cell walls (Brown *et al.,* 2015; Kim *et al.,* 2015).

Several authors have proposed that vesicles should be considered as a new secretion system, the secretion system type zero (Fig. 5). In fact, vesicles can transport different macromolecules from the inside to the outside of the cell and also into host cells, regardless of the conventional secretion systems (Guerrero-Mandujano *et al.,* 2017).

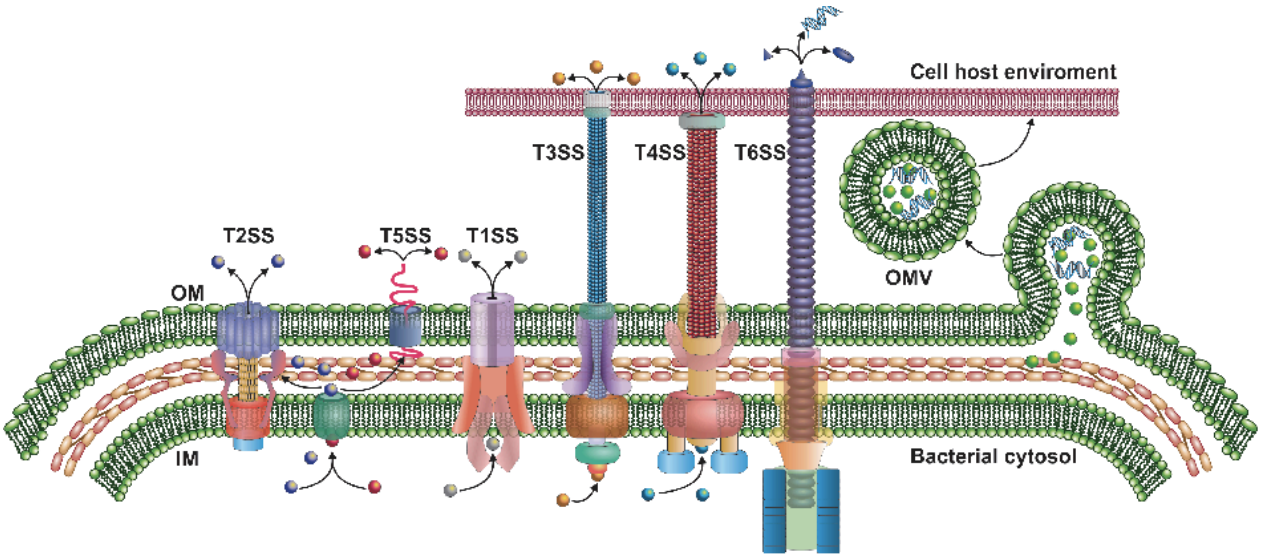



**Figure 5:** The six secretion systems and OMVs production as secretion system type zero (Guerrero-Mandujano *et al.,* 2017).

### 1.2.1 Gram-negative extracellular vesicles

OMVs were first observed in the 1960s, by electron microscopy studies of the bacterial structures (Knox *et al.,* 1966). They have an average size of 20-250 nm and carry lipopolysaccharides (LPS), outer membrane protein (OMPs), soluble proteins, phospholipids, glycolipids, genetic materials, virulence factor (Fig 6). The presence of LPS and OMPs was used to confirm that OMVs originate from outer membrane (Anand & Chaudhuri, 2016, Gill *et al.,* 2019, Kim *et al.,* 2015). Surprisingly, some proteomic studies of OMVs demonstrated the presence of cytoplasmic proteins although they were believed to be depleted in these vesicles (Pérez Cruz *et al.,* 2013, Berleman *et al.,* 2014). Further studies are necessary to understand the presence and the role of cytoplasmic proteins in the OMVs.

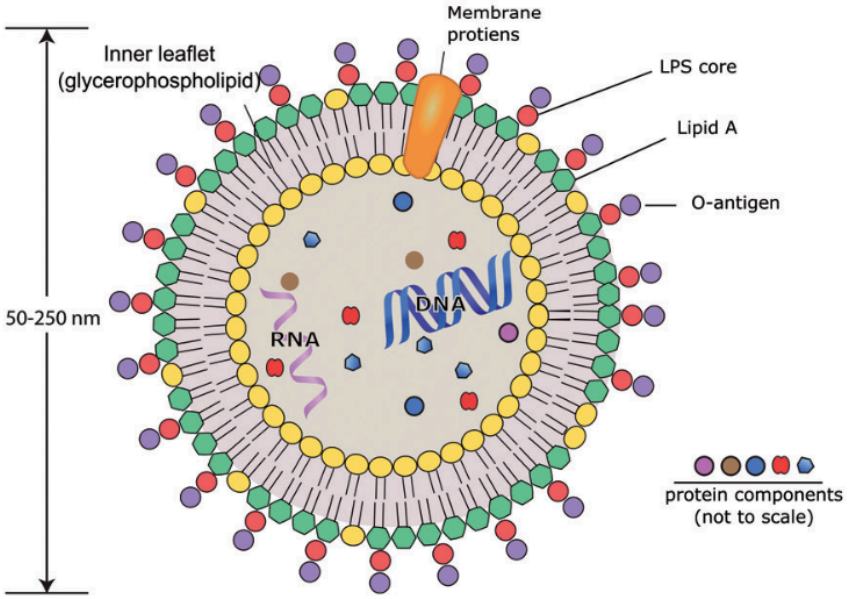



**Figure 6**: Architecture and composition of OMVs (Anand & Chaudhuri, 2016).

OMVs have been found *in vitro* experimental conditions (planktonic and biofilm cultures), in natural environments (e.g., water drains, soil, sewage, soil and house dust), in tissues and biological fluids (Kim *et al.,* 2015).

The process of biogenesis of OMVs is not fully understood. In general, OMVs are formed when a little portion of the membrane bulges out; this portion strangulates itself and it is released as a vesicle. The outer membrane composition and accumulation of specific molecules within the periplasm could be implicated in this process. The deformation of the OM is neither a spontaneous nor a random process, in fact the enrichment or depletion of OMV cargo compared to the cell suggests that is a regulated process (Schwechheimer and Kuehn, 2015; Deatherage and Cookson, 2012; Guerrero-Mandujano *et al.,* 2017).

Several models for OMV biogenesis have been proposed. Four main mechanisms are (Nevermann *et al*., 2019; Yu *et al.,* 2017) (Fig. 7):

a) dissociation of the outer membrane in specific zones lacking proper attachments to underlying structures (e.g., peptidoglycan);

b) a physical force induced by accumulation of envelope components and/or misfolded proteins pushes out OMVs;

c) a specific LPS enriched in some areas of outer membrane modulate OMV biogenesis, presumably by generating a differential curvature, fluidity, and/or charge in the outer membrane;

d) extracellular signals, for example *Pseudomonas* quinolone signal (PQS), stimulate the local curvature of outer membrane.

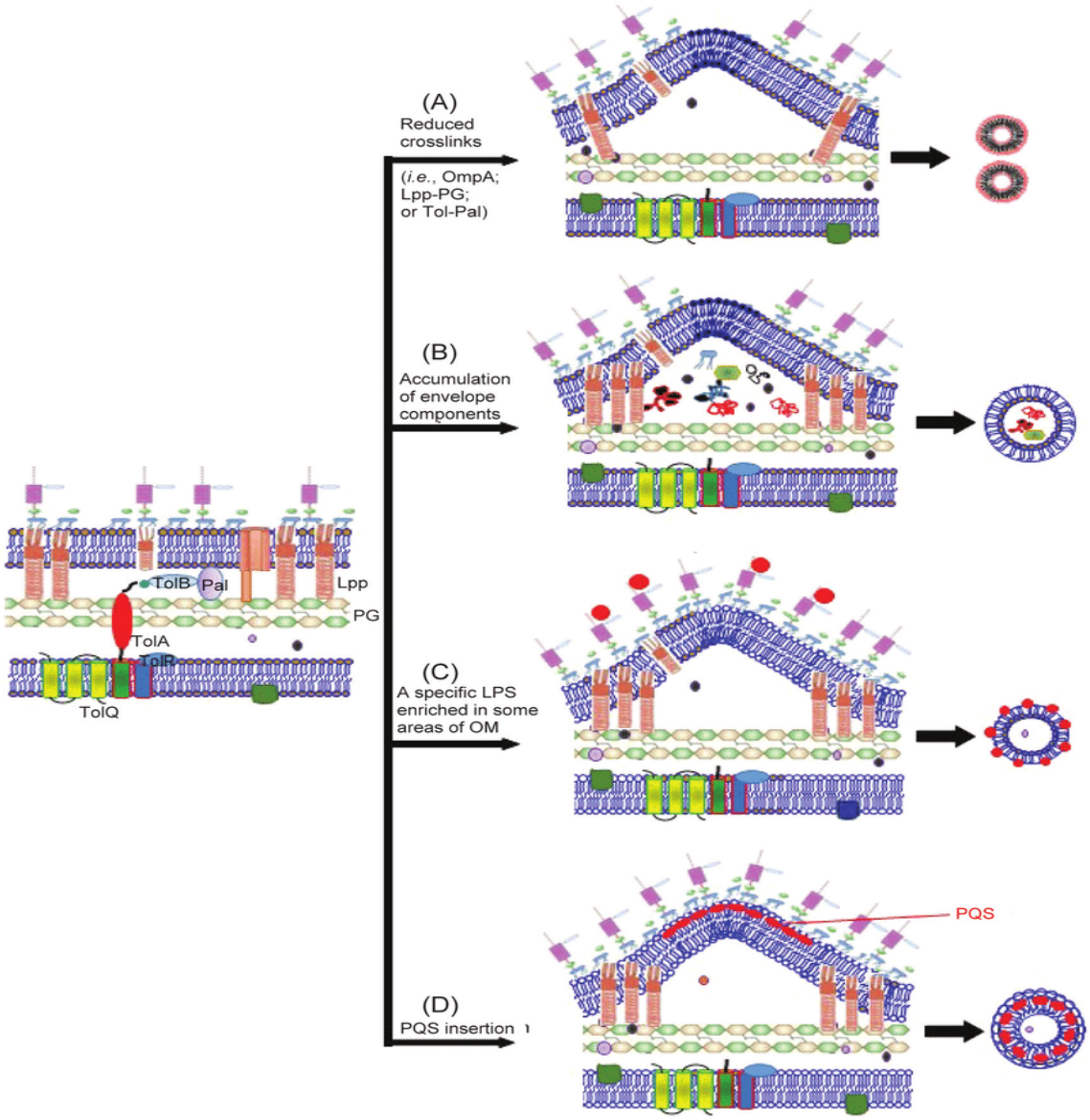



**Figure 7:** Models for the biogenesis of OMVs (Yu *et al.,* 2017).

OMVs have been suggested to play physiological and pathological functions, establishing inter- and intraspecies communication and also strengthen the interaction with the host (Jan, 2017). OMVs have been implicated in acquisition of nutrients, stress responses and delivery of toxins, adhesion and virulence factors to evade host defense system (Jan, 2017; Manning and Khuen, 2013). OMVs can deliver their cargo to eukaryotic cells and some of them have

been implicated in pathogenesis; these OMVs can contain biomolecules that enhance their invasive abilities and virulence such as the invasins IpaB, IpaC, and IpaD in OMVs of *Shigella flexneri;* OspA, OspB, OspD in *Borrelia burgdorferi*; the vacuolating cytotoxin VacA in *Helicobacter pylori;* Cif*,* b-lactamase, hemolytic phospholipase C, alkaline phosphatase, pro-elastase, hemolysin, and quorum sensing molecules, like N-(3-oxo-dodecanoyl) homoserine lactone and 2-heptyl-3-hydroxy-4-quinolone (PQS) in *Pseudomonas aeruginosa* OMV (Tab. 1). Furthmore, OMV associated toxins such as leukotoxin, LPS and the cytolysin A (ClyA) are more potent than their soluble forms (Jan, 2017; Ellis and Kuehn, 2010; Bomberger *et al.,* 2009).

| S. No | Bacterial species | Virulence factors as OMV component | Associated function | Reference |
|---|---|---|---|---|
| 1 | *Escherichia coli* [Enterotoxigenic *E. coli* (ETEC), Shiga toxin producing *E. coli* (STEC), Enterohemorrhagic *E. coli* (EHEC)] | Heat labile enterotoxin (LT), Shiga toxin, Cytolysin A (ClyA) | Pore forming ability, enterotoxic and vacuolating activity, cytotoxicity | Kolling and Matthews, 1999; Horstman and Kuehn, 2000; Yokoyama et al., 2000; Wai et al., 2003; Kuehn and Kesty, 2005; Kwon et al., 2009; Mendez et al., 2012; Jun et al., 2013 |
| 2 | *Helicobacter pylori* | Vacuolating toxin (VacA), Lewis antigen LPS, Helicobacter cysteine rich proteins (Hcp), Sialic acid binding adhesion (SabA) | Adherence, cytotoxic and vacuolating activity, cell proliferation activity | Fiocca et al., 1999; Keenan et al., 2000; Mullaney et al., 2009; Olofsson et al., 2010; Jun et al., 2013 |
| 3 | *Pseudomonas aeruginosa* | Alkaline phosphatase, Phospholipase C Protease, Hemolysin, Pseudomonas quinolone signal (PQS), Cif, hydrolases | *In vitro* enzyme activities, cytokine stimulation, bactericidal quinolines | Kadurugamuwa and Beveridge, 1995, 1996; Li et al., 1998; Mashburn and Whiteley, 2005; Mashburn-Warren et al., 2008; Bomberger et al., 2009; Ellis et al., 2010; Choi et al., 2011; Toyofuku et al., 2012 |
| 4 | *Borrelia burgdorferi* | Outer surface proteins (OspA, B, D) | Adherence to host cells | Dorward et al., 1991; Shoberg and Thomas, 1993, 1995 |
| 5 | *Shigella flexneri* | Invasion plasmid antigens (IpaB, C,D) | Invasion of host tissue | Kadurugamuwa and Beveridge, 1998 |
| 6 | *Shigella dysenteriae* | Shiga toxin (Stx) | Cytotoxicity, host cell apoptosis | Dutta et al., 2004 |
| 7 | *Salmonella typhi* | Outer membrane protein (OmpC), ClyA | Pore forming activity | Bergman et al., 2005 |
| 8 | *Treponema denticola* | Proteases, Dentilisin | Chymotryptic activity, disruption of tight junctions | Rosen et al., 1995; Chi et al., 2003 |
| 9 | *Neisseria meningitis* | NarE, NlpB, PorA, B | Cytokine production, fibrinolytic activity, adherence to host cells | Ferrari et al., 2006; Vipond et al., 2006; Massari et al., 2010; Van De Waterbeemd et al., 2013 |
| 10 | *Bordetella pertussis* | Pertussis toxin (Ptx), Adenylate cyclase hemolysin | Cytotoxicity | Hozbor et al., 1999 |
| 11 | *Burkholderia cepacia* | Phospholipas-N, Hemagglutinin | Enzyme activities | Allan et al., 2003 |
| 12 | *Vibrio cholera* | Rtx toxin, LPS | Depolymerising actin, stimulatory response | Bishop et al., 2010; Altindis et al., 2014 |
| 13 | *Xanthomonas campestris* | Type-3 secretion proteins, cellulase, xylosidae | Enzyme activity, insecticidal activity | Sidhu et al., 2008 |
| 14 | *Legionella pneumophila* | Acid phosphatase (Map), Protease (Msp), Chitinase (ChiA), Hsp60 | Adherence to ECM, enzyme activity | Fernandez-Moreira et al., 2006; Galka et al., 2008 |
| 15 | *Moraxella catarrhalis* | Ubiquitous surface protein (UspA1, A2) | Complement binding | Tan et al., 2007; Vidakovics et al., 2010 |
| 16 | *Acinetobacter baumannii* | Outer membrane protein (AbOmpA), PAMPS (LPS, flagellin),Proteases, Phospholipases, SOD, Catalase | Binding to host tissues, Immunomodulatory effect, enzyme activity | Kwon et al., 2009; Mendez et al., 2012; Moon et al., 2012; Jun et al., 2013 |
| 17 | *Campylobacter jejuni* | Cytolethal distending toxin (CDT) | Adhesion and invasion, immunomodulatory effect | Elmi et al., 2012; Jang et al., 2014 |
| 18 | *Porphyromonas gingivalis* | CTD family proteins such as gingipains (RgpA, RgpB, Kgp) | Adherence, host tissue Invasion, immune evasion | Veith et al., 2014 |
| 19 | *Yersinia pestis* | Adhesin Ail, Protease Pla, F1 outer fimbrial antigen | Complement binding, enzyme activity | Eddy et al., 2014 |
| 20 | *Cronobacter sp. [C. sakazakii, C. turicensis. C. malonaticus]* | Outer membrane protein (OmpA and OmpX) | Binding to host cell receptors | Kothary et al., 2017 |

**Table 1:** Gram-negative species showing OMV production (Jan, 2017).

OMVs play an important role in nutrient acquisition, in fact they can contain enzymes such as proteases and glycosidases but also proteins involved in iron acquisition. For example, OMVs from *Neisseria meningitidis* are enriched in iron acquisition proteins, such as the iron-transporter components FetA and FetB4 (Jan, 2017; Schwechheimer and Kuehn, 2015). OMVs are also known to play an important role in the biofilm formation. They were found to be involved in delivery of exopolysaccharides and the process of cell co-aggregation (Guerrero-Mandujano *et al.,* 2017)

The release of OMVs increases when bacteria are under physical and/or chemical stresses. This indicates that the OMV production plays an important role in survival mechanisms (Guerrero-Mandujano *et al.,* 2017; Haurat *et al.,* 2015; Lee *et al.,* 2008), for example, exposure of *Stenotrophomonas maltophilia* to the antibiotic ciprofloxacin resulted in an increased vesiculation (Devos *et al.,*2017).

### 1.2.2 Gram-positive extracellular vesicles

In 1990, the first hint of the presence of EVs in Gram-positive bacteria came from studies on *Bacillus* spp. where vesicle-like blebbing structures were observed on the bacterial surface (Dorward and Garon, 1990). Gram-positive membrane vesicles (MVs) have been drawing more attention in recent years and MV release into the surrounding environment of the cell has been observed for a wide range of bacterial species belonging to the phyla Firmicutes and Actinobacteria (Brown *et al.,* 2015; Liu *et al.,* 2018). The number of reports on Gram-positive vesicles is very limited in respect of OMVs, probably due to the low vesicle yield. In fact, only 200 μg (wet weight) of vesicles were obtained from 1L of *Staphylococcus aureus* culture, compared to the 1 mg (dry weight) of OMVs obtained from 10 ml of *Porphyromonas gingivalis* culture (Haurat *et al.,* 2015).

Little is known about the biogenesis of MVs, the release through the thick cell wall is still being disputed. The current evidence-supported three non-mutually exclusive hypotheses (Fig. 8) (Brown *et al.,* 2015; Liu *et al.,* 2018):

a) after release from the cytoplasmic membrane, MVs may be forced through the wall by turgor pressure. This mechanism may be regulated by cell wall pore size or thickness;

b) action of cell wall-modifying enzymes that weaken the peptidoglycan layer may facilitate the MV release;

c) protein channels or structural cables may guide MVs to the extracellular environment.

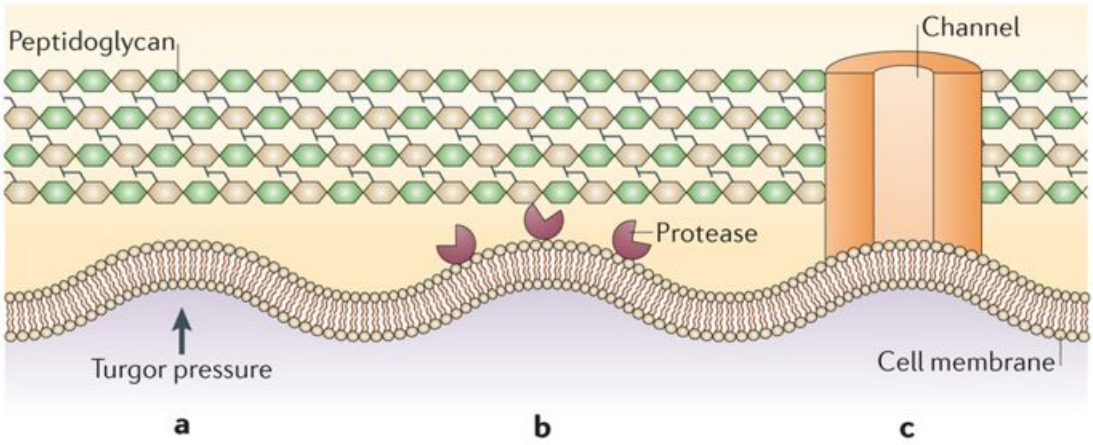



**Figure 8**: Models for the biogenesis of MVs (Brown *et al.,* 2015).

More recently, the biogenesis of vesicles is suggested to be associated with processes leading to cell death, as antibiotics targeting the cell wall or the action of phage-encoded endolysins (Toyofuku *et al.,* 2017; Toyofuku e*t al.,* 2019; Andreoni *et al.,* 2019).

The size range of MVs is between 20 and 400 nm (Brown *et al.,* 2015; Liu *et al.,* 2018) and similar to OMVs, Gram-positive MVs can carry a wide range of biomolecules: RNA, DNA, enzymes involved in peptidoglycan degradation and antibiotic degradation, virulence factors. However, these vesicles can still be distinguished from OMVs because they do not contain LPS (Kim *et al.,* 2015; Gill *et al.,* 2019; Liu *et al.,* 2018). The type of cargo determines the functions and the targets of MVs. Gram-positive MVs play pleiotropic roles, including in general metabolism, stress response, material exchange, virulence, horizontal gene transfer (Fig. 9) (MacDonald and Kuehn 2012; Lie *et al.,* 2018).

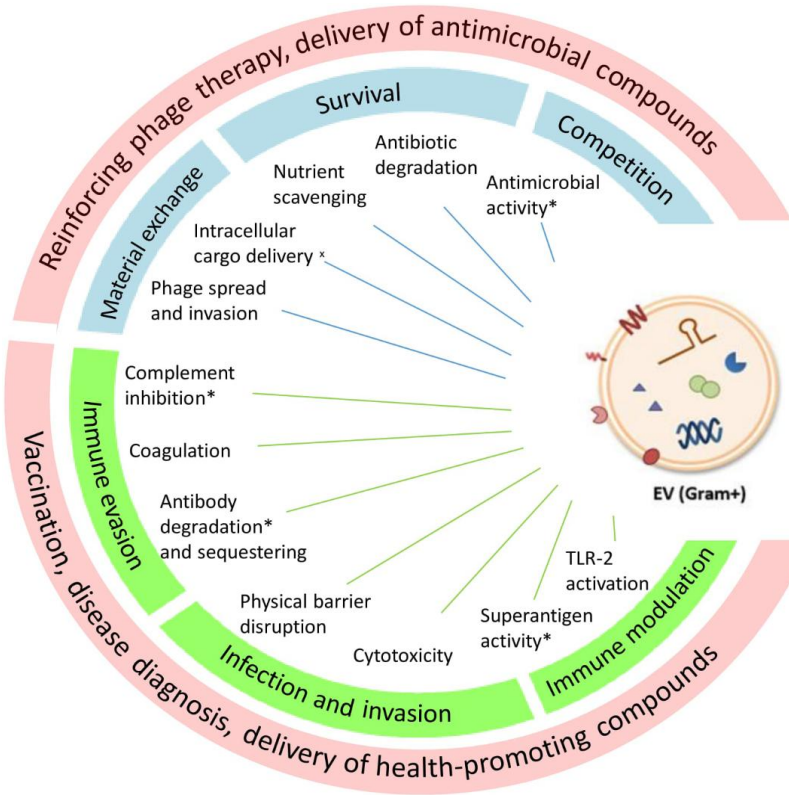



**Figure 9:** Proposed functions and potential biotechnology applications of MVs (Liu *et al.,* 2018).

MVs of *Ruminococcus* spp. mediate the transfer of short fragments of chromosomal DNA ranging from 23 to 90 kb. These fragments are resistant to digestion with *Eco*RI unlike chromosomal DNA, suggesting differences in the restriction/modification pattern in MV-associated DNA (Klieve *et al.,* 2005). MVs may also facilitate gene transfer by mediating bacteriophage infection (Tran and Boedicker, 2017).

MV cargo often include molecules involved in scavenging activities, contributing to microbial survival and competition. For example, *Staphylococcus aureus* MVs contain iron-binding factors that contribute to bacterial survival under iron-limited conditions (Lee *et al.,* 2009).

In the last years a strong interest was focused in the role of gram-positive MVs during host infection because their cargo can include various virulence factors that are important for drug resistance, immune system evasion, host cell invasion and pathogenesis (Lee *et al.,* 2013; Brown *et al.,* 2015). MVs produced by *Bacillus anthracis*, *Staphylococcus aureus,*

*Streptococcus pneumoniae, Streptococcus pyogenes, and Streptococcus agalactiae* were shown to carry a range of hemolysins and/or pore forming toxins (Liu *et al.,* 2018)

More recently, many studies evidenced MV production by probiotic bacteria such as *Bifidobacterium longum, Lactobacillus rhamnosus, Lactobacillus casei,* and *Lactobacillus plantarum.* These MVs carry molecules that are associated with the probiotic effects of the producing bacteria (Liu *et al.,* 2018; Dean *et al.,* 2019).

## 1.3 *Streptomyces*

The phylum Actinobacteria is composed of Gram-positive bacteria with high GC content in their genome, and is one of the largest taxonomic units among the 18 major lineages of bacteria (Ventura M. *et al.,* 2007). The most extensively studied genus of the Actinobacteria is *Streptomyces*. *Streptomyces* species are widely distributed in nature, in both terrestrial and marine environments (Fig. 10) and produce around half of the clinically used antibiotics and other pharmaceutically useful natural products such as anthelmintics, anticancer agents, and immunosuppressives (Mitchell, 2011).

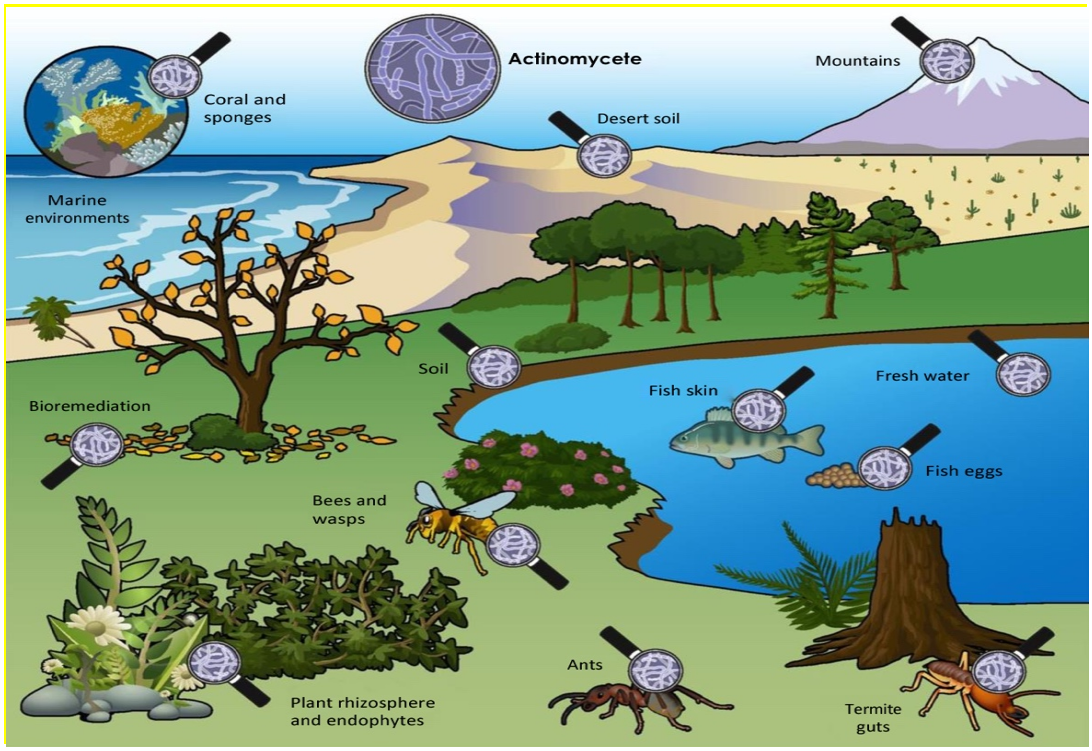



**Figure 10:** Actinomycete ecology **(**Van der Meij *et al.,* 2017).

The complex life cycle of *Streptomyces* starts when a spore senses a suitable source of nutrients. The germinating spore develops one or two germ tubes which elongate by apical tip extension. The hyphae branch regularly and occasionally form septal cross walls, without disaggregating the neighboring compartments. Thus, the multichromosomal filaments generate a substrate penetrating hyphal network, the "substrate mycelium" (Flardh and Buttner, 2009). Morphological differentiation, which coincides with the production of various secondary metabolites, including antibiotics, is initiated when partial nutrient limitation is encountered. Beside sensing of the nutritional situation, quorum sensing and other environmental stress signals are also involved and controlled by the hierarchical cascade of *bld* and *whi* regulatory genes (Chater, 1993; Flardh *et al.,* 2012). After partial lysis of hyphae of the substrate mycelium, there is the formation of specialized aerial hyphae (aerial mycelium). Finally, the aerial hyphae become septated into a chain of prespore compartments by the coordinated formation of up to 50 or even more septal cross walls, generating chains of spores (Flardh and Buttner, 2009; Sigle *et al.,* 2015) (Fig. 11).

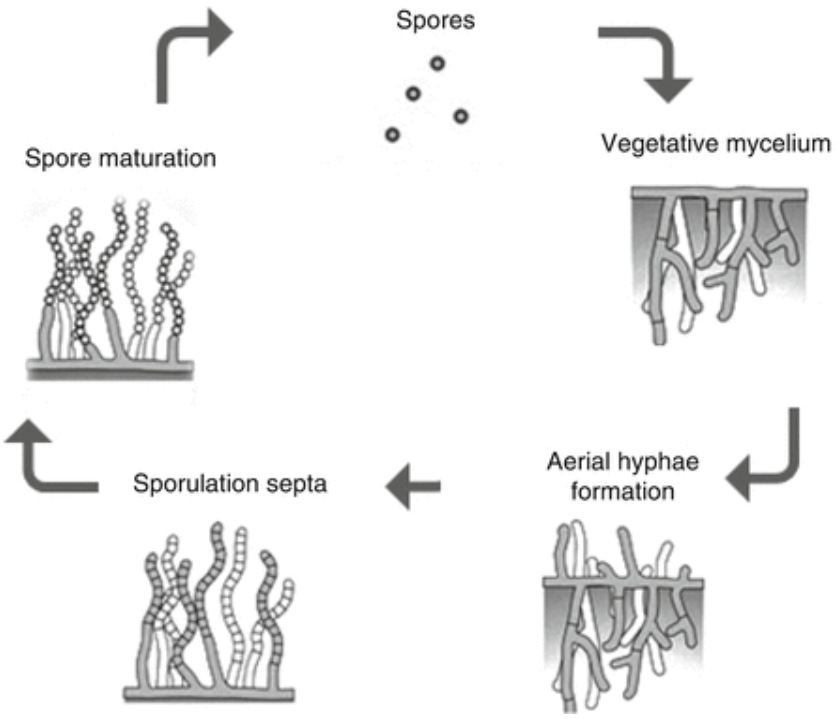



**Figure 11:***Streptomyces* life cycle (Hamedi *et al.,* 2017).

### 1.3.1 *Streptomyces* membrane vesicles

*Streptomyces coelicolor* and *Streptomyces lividans* produce pigmentated exudates that arise on the top of sporulated colonies known respectively as blue and red droplets (Rudd and Hopwood, 1979) (Fig. 12). Few years ago, it was demonstrated that these droplets contain membrane vesicles and antibiotics (Schrempft *et al.,* 2011, Schrempft *et al.,* 2015) (Fig. 13). In particular, blue droplets contain the polyketide antibiotic actinorhodin while red droplets contain alkaloid undecylprodigiosin.

Proteomic analysis of *S. coelicolor* M110 vesicle-containing droplets revealed the presence of proteins that are useful for survival and defence (Tab. 2) (Schrempft *et al.,* 2011).

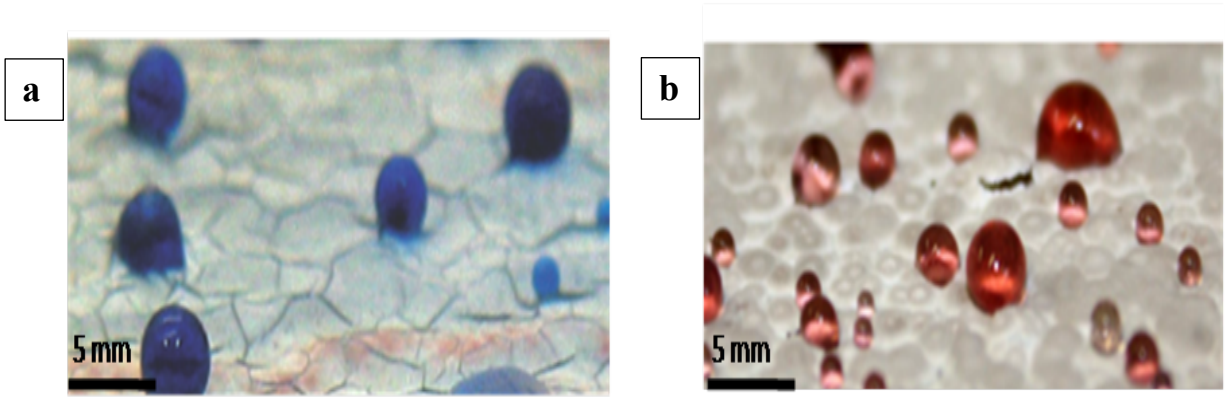



**Figure 12:** a) Blue droplets arising on the top of the sporulating *S. coelicolor* M110. b) Red droplets arising on the top of the sporulating *S. lividans* 66 (Schrempft *et al.,* 2011, Schrempft *et al.,* 2015).

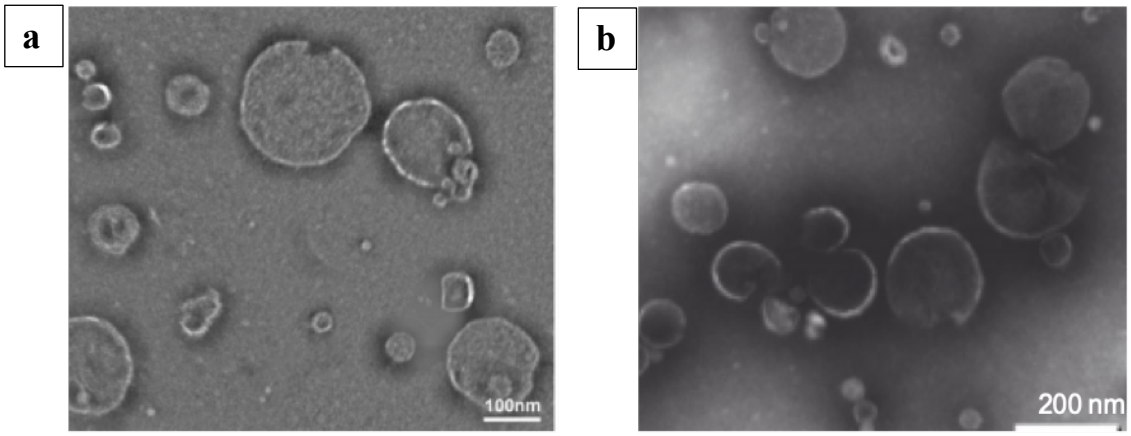



**Figure 13:** Transmission Electron Microscope (TEM) micrographs of vesicles detected in the blue droplets (a) and in red droplets (b) (Schrempft *et al.,* 2011, Schrempft *et al.,* 2015).

| SCO No. | MW | Deduced function | Remarks | | | |
|---|---|---|---|---|---|---|
| | | | N | T | S | L |
| 6691 | 75.667 | Phospholipase C | | T | | |
| 1230 | 58.499 | Tripeptidyl aminopeptidase | | T | | |
| 6281 | 58.486 | Oxidoreductase (FAD binding) | | T | | |
| 0379 | 55.082 | Catalase | N | | | |
| 2180 | 51.365 | Dihydrolipoamide dehydrogenase | N | | | |
| 3484 | 46.229 | Glycerol-3-phosphate-binding protein | | T | | L |
| 1169 | 42.979 | Xylose isomerase | N | | | |
| 1565 | 42.649 | Glycerophosphoryl-phosphodiesterase | | T | | |
| 4142 | 38.107 | Phosphate-binding protein | | T [SI] | S[Sc] | |
| 3471 | 35.142 | Agarase | | T | | |
| 1639 | 34.915 | Peptidyl-prolyl *cis–trans*-isomerase | | T | | L |
| 2770 | 34.281 | Agmatinase | N | | | |
| 6065 | 33.661 | Glycine-betaine-proline-binding protein | | T | | L |
| 1968 | 31.400 | Phospholipase C-like | | | S | |
| 5074 | 23.120 | Dehydratase | | T | | |
| 0999 | 23.585 | Fe/Mn superoxide dismutase | N | | | |
| 2633 | 23.382 | Fe/Mn superoxide dismutase | N | | | |
| 0852 | 21.087 | Aldolase-KHG type | N | | | |
| 2368 | 20.378 | TerD-like | N | | | |
| 0641 | 20.168 | TerD-like | N | | | |
| 2113 | 19.208 | Bacterio-ferritin-like protein | N | | | |
| 3767 | 16.595 | TerB-like | N | | | |

N, no predicted signal peptide; S, predicted Sec signal peptide; T, predicted Tat signal peptide; L, predicted lipoprotein.

**Table 2:** List of identified proteins in *S. coelicolor* M110 vesicle-containing droplets (Schrempft *et al.,* 2011).

*Streptomyces lividans* vesicles provoke pronounced damages in *Aspergillus proliferans* and *Verticellium dahlia* and induce clumping and distorsion of *Escherichia coli* (Schrempft *et al.,* 2015).

Hoefler *et al.,* (2017) isolated MVs that carry the linear polyketide linearmycins from *Streptomyces* Mg1 culture supernatants. Interestingly, a linearmycin-deficient strain of *Streptomyces* Mg1 is defective for production of MVs. This suggests a deep relationship between natural product biosynthesis and the physiology of cellular membranes.

Fröjd *et al.* (2019) observed the release of MVs from tips of vegetative hyphae of *Streptomyces venezuelae* (Fig. 14). Probably, there is a relationship between MV production and cell-wall weakness as the treatment with vancomycin to block peptidoglycan synthesis leads to a high frequency of vesicles extrusion.

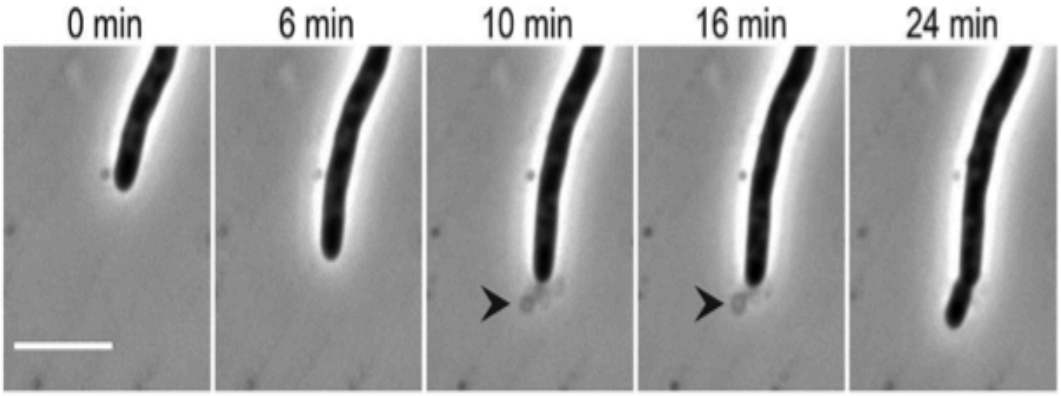



**Figure 14:** Images from time-laps movies of *Streptomyces venezuelae* hyphae undergoing growth arrest and vesicle release MVs indicated by arrowheads. Scale bar is 5 μm (Fröjd *et al.* 2019).

### 1.3.2 *Streptomyces coelicolor*

*Streptomyces coelicolor* is the model streptomycete organism for investigating the regulation of morphological differentiation and production of biologically active molecules, because of its predisposition to genetic analyses and because it produces different secondary metabolites such as, the red-pigmented prodiginine antibiotic undecylprodigiosin, the blue-pigmented polyketide actinorhodin, the nonribosomal peptide calcium- dependent antibiotic CDA and the cryptic polyketide with antibacterial activity (yellow pigment, Cpk). *S. coelicolor* was the first member of the genus to have its genome completely sequenced. It possesses a single linear chromosome (8,7 Mb with a G +C content of 72%), a linear plasmid (SCP1, 365 kb), and a circular plasmid (SCP2, 31 kb). The chromosome has a centrally located origin of replication (*oriC*) and the ends carrying terminal inverted repeats (TIRs) with covalently linked proteins (Fig. 15). Coding density is largely uniform across the chromosome, with only a slight decrease in the distal regions. The gene distribution reveals, however, a central core comprising approximately half the chromosome and a pair of chromosomal arms. Nearly all essential genes, such as those for cell division, DNA replication, transcription, translation and amino-acid biosynthesis, are located in the core region. In contrast, *loci* coding for probable non-essential functions, such as some secondary metabolites, hydrolytic exoenzymes, the conserved operons and ‘gas vesicle’ proteins, lie in the arms (Bentley *et al.,* 2002).

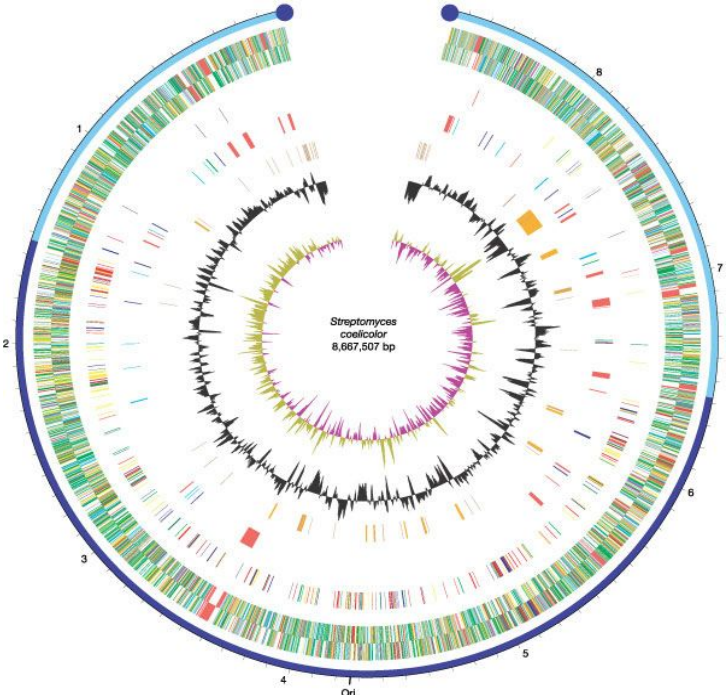



**Figure 15:** Circular representation of the *Streptomyces coelicolor* chromosome. In dark blue the core region and in light blue the arms (Bentley *et al.,* 2002).

The *S. coelicolor* growth in liquid medium, is characterized by four growth phases. During the first rapid growth phase (RG1) the cells divide quickly. The transition phase (T) is characterized by a growth arrest, transitional arrest of macromolecular biosynthesis and differentiation, beginning of secondary metabolite synthesis. During the second rapid growth phase (RG2) an increased secondary metabolite production occurs; in the stationary phase (S) cellular growth is stopped but the antibiotic production continues (Puglia *et al.,* 1995) (Fig. 16).

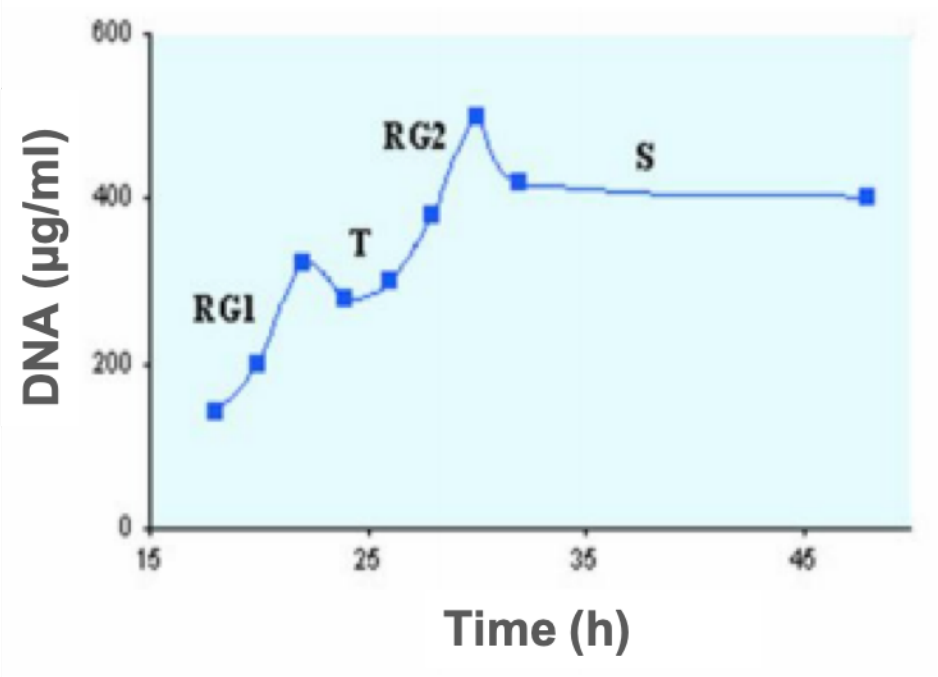



**Figure 16:** Growth curve of *S. coelicolor* in liquid medium (Puglia *et al.,* 1995).

# 2. AIM OF THE RESEARCH

The field of the extracellular vesicle biology is rapidly evolving and expanding. Isolation and analysis of MVs are prerequisites for understanding their biological roles and for their applications in medicine and in biotechnology.

*Streptomyces coelicolor* A(3)2 strain M145 is the best-known species of the genus *Streptomyces* at the genetic and molecular level and has been considered the model streptomycete for studying physiological and morphological differentiation and the antibiotic production. In 2011 Schrempft *et al.* demonstrated for the first time that *S. coelicolor* produces MVs when grown on complete solid medium.

Industrial biologically active molecule production of *Streptomyces* is mostly performed in liquid cultures, so it is interesting to study the production of MVs in liquid culture.

Thus, the aim of my PhD thesis was to isolate and characterize the MVs of *Streptomyces coelicolor* grown in liquid medium. In particular:

- to optimize protocol for the isolation and purification of MVs from minimal liquid culture;
- to characterize the macromolecule cargo composition (proteins nucleic acids and metabolites);
- to characterize the metabolic profile of MVs.

# 3. MATERIALS AND METHODS

## 3.1 Strain used in this study

*Streptomyces coelicolor* A3 (2) M145 SCP1$^-$ SCP2$^-$ (Kieser *et al.,* 2000)

## 3.2 Media

**Soya Flour Mannitol medium (SFM)** (Kieser *et al.,* 2000)

Agar…….20 g

Mannitol……...20 g

Soya flour…….20 g

Tap water to……1000 ml

Autoclave twice 115°C, 15 min

**J Medium (JM)** (Kieser et *al.,* 2000)

Sucrose…….……..100 g

Tryptone soya broth.……30 g

Yeast extract..……..10 g

$MgCl_2$*$6H_2O$……10 g

Distilled water to……1000 ml

Autoclave 121°C for 20 min

**Minimal Medium (MM)**

**Liquid Minimal Medium**:

$NaNO_3$ ……1 g

$MgSO_4$*$7H_2O$ ……..0,5 g

KCl ……..0,5 g

$KH_2PO_4$ ……..1 g

Trace Element solution……....1 ml

Distilled water to……1000 ml

Adjust pH to 7.0 with KOH

After autoclaving (121°C for 20 min) add 20ml of glucose 50%

**Solid Minimal Medium:**

$NaNO_3$ ……1 g

$MgSO_4{}^*7H_2O$ ……..0,5 g

KCl ……..0,5 g

$KH_2PO_4$ …….1 g

Trace Element solution……...1 ml

Agar……..15 g

Distilled water to……1000 ml

Adjust pH to 7.0 with KOH

After autoclaving (121°C for 20 min) add 20 ml of glucose 50% (w/v)

**Trace Element solution:**

$FeSO_4*7H_2O$……1 g

$ZnCl_2$……1 g

Biotin……0,1 g

Distilled water to……100 ml

## 3.3 Making a *Streptomyces coelicolor* spore suspension

The recovery of *S. coelicolor* spores from SFM plates was performed as described by Kieser *et al.,* 2000 with some modifications. The surface of the plate was scraped with a spatula after the addition of 3-6 ml of water. Spore suspension was rescued and then filtered in a plastic syringe containing a cotton wool. The suspension was centrifuged 10 min at 3'000 rpm, the supernatant poured off and the spore pellet was resuspended in an appropriate volume (0.2 ml to 1 ml) of glycerol solution 20% (v/v).

## 3.4 Isolation and purification of membrane vesicles (MVs)

(modified from Prados-Rosales *et al.,* 2014)

**Step 1: isolation of MVs**

1. Inoculate $10^8$ spores of *S. coelicolor* in 30 ml of JM for 30 h at 30 °C 200 rpm.
2. Harvest cells and wash twice with water. Suspend in 30 ml of water, inoculate 10 ml of this suspension in 500 ml of Minimal Medium and incubate at 30 °C at 180 rpm for 136 h.
3. Centrifuge the culture at 4000 rpm for 10 min at 4°C.
4. Filter the clarified supernatants through a 0.2 μm-pore size filter.
5. Concentrate the supernatant using an Amicon Ultrafiltration system with a 100-kDa-exclusion filter. Leave 10–15ml of liquid.
6. Rinse the filter with the remaining supernatant and recover the concentrate.
7. Sequentially centrifuge the concentrate and 15000 g (15 min, 4°C) to remove aggregates.
8. Ultracentrifuge the remaining supernatant at 100000 g (SW 40 Ti Rotor-Beckman Coulter) for 1 h at 4°C to obtain the membrane vesicle pellet.

**Step 2: purification of EVs by density gradient ultracentrifugation**

1. Discard the supernatant, suspend the pellet in DPBS (Dulbecco's phosphate buffered saline) and mix with OptiPrep solution (Sigma–Aldrich) to obtain a 35% (v/v) OptiPrep solution (final concentration).
2. Transfer to a 14 ml clear ultracentrifuge tube.
3. Overlay the vesicle sample with a series of OptiPrep gradient layers with Optiprep concentrations ranging from 10 to 35% (w/v).
4. Ultracentrifuge the gradient at 140.000 g for 16h at 4°C using a swinging rotor (SW 40 Ti Rotor-Beckman Coulter).
5. Take the several fractions from the top of the gradient to the bottom.
6. Dilute the fractions in DPBS and centrifuge at 38.400 g (SW 55 Ti Rotor-Beckman Coulter) for 2 h to remove the Optiprep.
7. Suspend the pellet in 0.2 ml of DPBS (0.22 μm filtered).

Aliquots of purified MVs were streaked on MS and MM plates to verify their sterility and then stored at -80°C until use.

## 3.5 Scanning Electron Microscopy (SEM) observations

Aliquots of 50 µl of 136h old liquid culture of M145 were placed on coverslips, incubate at room temperature (RT) for 30 min and then fixed using 4% v/v glutaraldehyde for 5 min. Upon removing of glutaraldehyde solution, samples were washed two times with 15% and 50% v/v ethanol and incubated at 65°C for 15 min. Samples were sputter-coated with gold (Sputtering Scancoat Six, Edwards) to avoid electrostatic charging under the electron beam and examined by Scanning Electronic Microscopy (SEM) (Quanta 200 ESEM, FEI).

## 3.6 Transmission Electron Microscopy (TEM) observations

Aliquots of 20 µl F3 and F4 MVs were pre-treated for 15 min with glutaraldehyde (0.25% v/v). Samples were placed onto carbon-covered Cu grids (300 mesh, Plano). After removal of the excess of liquid, neutralized phosphotungstic acid (3% w/v) was added for 1 min. Then, the grids were rinsed on drops of distilled water (Hegermannet *et al.,* 2006). Air-dried samples were analysed by TEM (JEOL Japan JEM-2100) operating at 120 and 200 kV.

## 3.7 Atomic Force Microscopy (AFM) observations

Aliquots of 20 µl F3 and F4 MVs (diluted 1:10 in $H_20$) were deposited on a mica substrate and then dried in vacuum (10 mbar). AFM measurements were carried out in air using a Bruker FAST-SCAN microscope.

## 3.8 Dynamic Light Scattering (DLS) measurements

The size distribution of MVs was determined with DLS using a Malvern NanoZS Zetasizer (Malvern Panalytical). Aliquots of 100 µl MVs (diluted 1:10 in DPBS) were loaded into disposable sizing cuvettes at 25°C, and the system was assigned with 1.330 dispersant refractive index and 0.8872 viscosity. The average diameter (Z-average) of MVs were analyzed with the Zetasizer software.

## 3.9 Protease accessibility assay

Aliquots of 40 µl F3 and F4 MVs were untreated or treated with 10 µg/ml proteinase K (PK) from *Tritirachium album* (Sigma-Aldrich), 0.02% (w/v) SDS, or proteinase k plus SDS for

1 h at RT. The proteinase activity was then inhibited by adding 0.1 mM phenylmethylsulfonyl fluoride (McCaig *et al.,* 2013). Samples were processed for SDS-PAGE and LC-MS/MS analysis.

## 3.10 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

MV protein profiles were determined by 12% (w/v) SDS-PAGE analysis using conditions described by Sambrook *et al.,* 2001. "SeeBlue Plus2 Pre-Stained Standard" (Invitrogen) was used as molecular weight marker.

## 3.11 Silver staining

SDS-PAGE gels were stained with silver staining method (Shevchenko *et al.,*1996). In particular, gels were incubated with 100 ml of fix solution containing methanol, acetic acid and deionized water (in 40:10:50 ratio) in orbital shaker for 30 min. After this step, four water wash steps were performed (for 45 min each). Gels were sensitized using 0.08% (w/v) of sodium thiosulfate solution for 5 min and rinsed with two wash in water (for 1 min each). Than the gels were incubated in 0.4% (w/v) silver nitrate solution for 1 h at 4°C. The gels were developed with a 0.04% (v/v) formaldehyde and 2% (w/v) sodium carbonate solution. When sufficient staining was obtained, the development was quenched with 1% (v/v) acetic acid.

## 3.12 Nucleic acid gel electrophoresis

Gel electrophoresis analyses of F3 and F4 MVs were performed as described by Sambrook *et al.,* 2001. The percentage of agarose used was 1% (w/v) in 1X Tris-Acetate-EDTA buffer (TAE). Ethidium bromide (EtBr) at concentration of 0.5 μg/ml was used as intercalating agent. "MassRuler DNA Ladder Mix, ready to use" (Thermo Scientific) was used as molecular weight marker. Voltage of 100 V was applied for the migration.

## 3.13 Treatment with DNase

Aliquots of 5 µl F3 and F4 MVs were treated with DNase I recombinant, RNase-free (Roche) following manufacturer's instructions. Then treatment, samples were analyzed by gel electrophoresis.

## 3.14 Treatment with RNase

Aliquots of 5 µl F3 and F4 MVs were treated with RNase A (Roche) following manufacturer's instructions. Then treatment, samples were analyzed by gel electrophoresis.

## 3.15 Polymerase chain reaction (PCR)

SCO7590 (*katA2*), SCO0560 (*cpeB*), SCO3671(*dnaK*), SCO5820 (*hrdB*) and SCOr06 (*rrnB*) genes were amplified from MV samples.

The amplification reaction mixture consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, forward and reverse primers (0.5 µM each) (Tab. 3), dNTPs (0.2 mM each), 1U of Taq DNA Polymerase, Recombinant (Invitrogen), and 1-500 ng/µl of template DNA. The PCR reaction was carried out in a "T Personal Thermocycler" (Biometra), the PCR were performed under the conditions described in Table 4

| | **Primer sequences (5' > 3')** | |
|---|---|---|
| **Gene name** | **Forward sequence** | **Reverse sequences** |
| SCO7590 (*katA2*) | AGGACCCGATGAAGTTCCAG | CATGTAGGTGTGGGAGGTGT |
| SCO0560 (*cpeB*) | AAGTACGGCCAGAACCTCTC | CCGGGTTGACGTAGATCAGA |
| SCO3671(*dnaK*) | CAAGAAGCTCGGGATGTTCG | GACGGTCATCTTCTGCTCCT |
| SCO5820 (*hrdB*) | CGTCGAGGGTCTTCGGCTC | CGCGAGCCCATCTCGCTGC |
| SCOr06 (*rrnB*) | AGAGTTTGATCMTGGCTCAG | TACGGYTACCTTGTTACGACTT |

**Table 3**:List of primers used for PCR of *S. coelicolor* genes.

| | | | |
|---|---|---|---|
| Initial denaturation | 95°C | 3' | 1 cycle |
| Denaturation | 95°C | 45" | 1 cycle |
| Annealing | 66°C | 30" | |
| Elongation | 72°C | 45" | |
| Denaturation | 95°C | 45" | 1 cycle |
| Annealing | 64°C | 30" | |
| Elongation | 72°C | 45" | |
| Denaturation | 95°C | 45" | 1 cycle |
| Annealing | 62°C | 30" | |
| Elongation | 72°C | 45" | |
| Denaturation | 95°C | 45" | 30 cycles |
| Annealing | 60°C | 30" | |
| Elongation | 72°C | 45" | |
| Final elongation | 72°C | 10' | 1 cycle |

**Table 4:** PCR program.

## 3.16 Proteomic analysis

The SDS-PAGE and LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) analysis was performed by the research group of Dott. Scaloni at the Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, Napoli.

Two biological replicates of untreated and PK-treated F3 and F4 vesicles were subjected to 12% SDS-PAGE. In particular, 40 µl aliquots were used for each sample and protein patterns were revealed by colloidal Coomassie blue staining. Each lane was cut in 16 slices, which were thoroughly triturated, in-gel reduced and *S*-alkylated, and finally digested with trypsin. Peptides were extracted from gel particles using 5% formic acid/acetonitrile (1:1 v/v) and digest solutions were concentrated and desalted before mass spectrometry analysis by using µZip TipC18 pipette tips (Millipore). Peptide mixtures were analyzed by LC-MS/MS in triplicate. The MS/MS raw data were subjected to protein database searches using Proteome

Discoverer vs 2.1 (Thermo Scientific), enabling the database search by Mascot engine vs 2.4.2 using the following criteria: UniProtKB protein database for *Streptomyces coelicolor* (downloaded in April 2018) including the most common protein contaminants such as the proteinase K from *Tritirachium album.* Carbamidomethylation of Cys as static modification and oxidation of Met as variable modification. Peptide mass tolerance was fixed to ± 10 ppm and fragment mass tolerance to ± 0.05 Da. Maximum number of missed cleavages was set to 2.

The results of MS identification were used to perform a gene ontology study. Metabolic pathways were reconstructed by the use of protein KEGG (Kyoto Encyclopedia of Genes and Genomes).

## 3.17 Targeted metabolomic analysis

The metabolomic profile of vesicle samples was carried by LC-MS/MS analysis in Multiple Reaction Monitoring (MRM) mode following protein precipitation and extraction of metabolites in organic solvents. All procedures reported below were carried out at Centro di Ingegneria Genetica (CEINGE) – Biotecnologie avanzate (Naples, Italy).

Aliquots of F3 and F4 EVs (two biological replicates) (100 mg) were lysed in $NH_4CO_3$ 10 mM, NaF 10 mM buffer containing 7M urea, 75 mM NaCl (250 µl) and the suspensions were homogenized by Ultra-Turrax. Cellular lysates were diluted 6-fold with cold methanol. The mixtures were sonicated for 10 min and centrifuged at 12000rpm for 10 min at 4°C. The volume of the supernatant was reduced to 200 µl. After centrifugation at 12000 rpm for 10 minutes, the supernatant was directly analyzed by LC-MS/MS. Quantitative analysis was performed by the external calibration method. Solutions of standard metabolites were prepared by dissolving 1.0 mg of each analyte in 1 ml of 5% acetonitrile (1000 ppm) whereas the stock solution of undecylprodigiosin was dissolved in 50% acetonitrile. Stock solutions were stored at −20°C until the analysis. Serial dilutions were prepared from stock solutions (1000 ppm) up to 0.01 ppb and used for calibration curves. Metabolites mixtures (6 µl) were analyzed by using a 4000 hybrid triple quadrupole Linear Ion Trap (LIT) system with a HPLC Eksigent operating in microLiters per minute (AB Sciex Framingham, MA, USA).

The analytes were separated by using a Halo C18 2.7 µm 90A 1x50 mm column at a temperature of 38°C. The eluent A was 0.1 % acetic acid and eluent B acetonitrile and 2-propanol 50% containing 0.1 % acetic acid. Elution gradient was from 0% to 95% B in 7 min at a flow rate of 40 µL/min.

The analysis was carried out in MRM mode, with an ionization source ESI. Precursor ion was scanned in negative for organic acid, monosaccharides and other molecules showing better ionization in such mode. Amino acids, vitamins and antibiotics were analysed in positive mode. The parameters for all the molecules monitored by MRM method e.g. precursor (Q1) and daughter ions (Q2) as well as Collision Energy (CE) as well as Declustering Potential (DP) were optimized.

## 3.18 Raman Spectroscopy analysis

Aliquots of 5 µl F3 and F4 MVs were placed on glass slides for Raman spectroscopy analysis. A Horiba HR-Evolution confocal Micro-Raman spectrometer equipped with a 633 nm laser line and a 600 l/mm grating with spectral resolution of 6 $cm^{-1}$ has been used. In all the measurements a minimum laser power (<8 mW) has been applied to avoid sample degradation and the long working distance (NA 0.50) objective with 50x magnification has been typically used to maximize the spatial resolution.

## 3.19 RNA sequencing

RNA extraction and RNA-sequencing were performed as a contract service by Genomix4life from two biological replicate of F4 MVs (aliquots of 150 µl). Yields and purity of vesicular RNA were determined by spectrophotometry and RNA integrity was assessed using a TapeStation (Agilent Technologies). Depletion of ribosomal RNA (rRNA) prior to RNA sequencing (RNA-Seq) was performed. The identification of the expressed genes in MV samples were carried out by RNA-Seq data obtained with Illumina NextSeq, sequenced in paired-end mode. Prior to further analysis, a quality check was performed on the sequencing data. All samples contained sequences 75 nucleotides long (75nt x 2). The samples were

mapped on reference *Streptomyces coelicolor* A3 ASM20383v1.45 (Provider European Nucleotide Archive | Taxonomy ID 100226) using the bioinformatics tool STAR (version 2.7.2b), with the standard parameters for paired reads.

# 4. RESULTS

## 4.1 Membrane vesicle isolation and purification from *Streptomyces coelicolor* liquid cultures

The *Streptomyces coelicolor* derived Membrane Vesicles were observed on the surface of hyphae when exanimated by Scanning Electron Microscopy, demonstrating that vesicles may bud off from the cells during growth in liquid medium (Fig. 17).

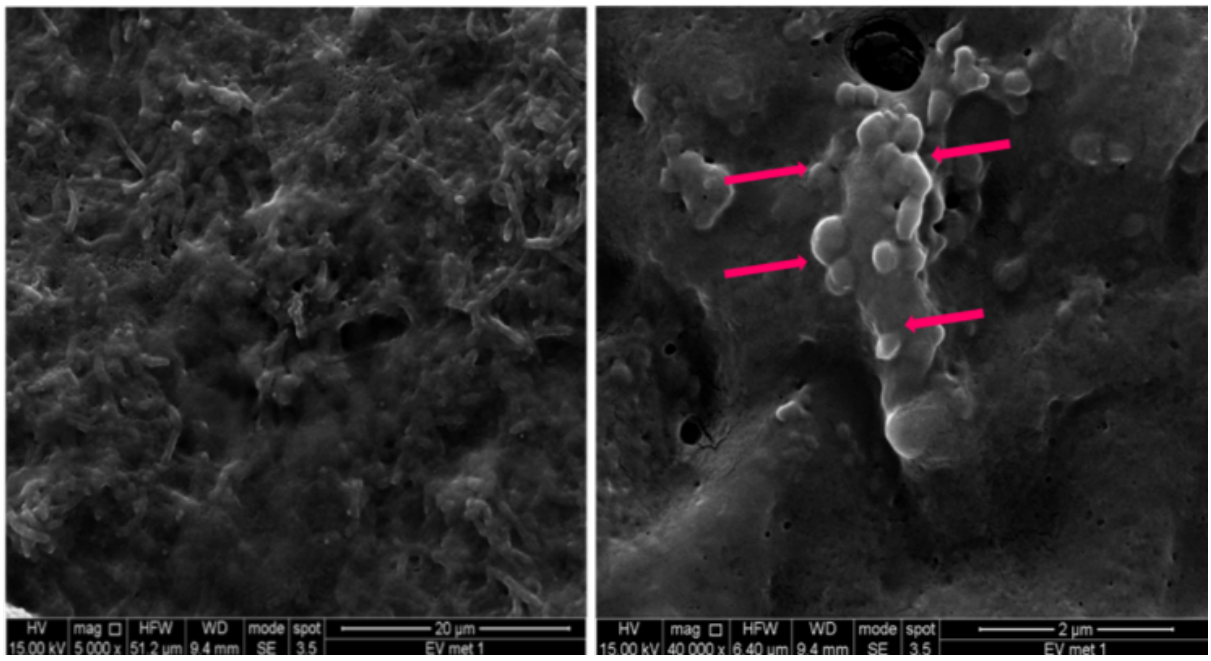



**Figure 17:** Scanning Electron Microscopy (SEM) images of *S. coelicolor* hyphae. Red arrows indicate MVs on the surface of hyphae.

The purification of the MVs is an essential step for the study and for the characterization of their cargo. MVs were isolated from the culture supernatant after 136 h of growth by filtration and centrifugation steps as described in 3.4 paragraph of Materials and Methods. To remove cell debris, protein and lipid aggregates and other non-MV associated material, crude MVs were subjected to Optiprep discontinuous density gradient ultracentrifugation. The gradient was composed of six fractions of Optiprep in DPBS with concentrations starting from 10% to 35% (v/v) with 5% increments (Fig.18).

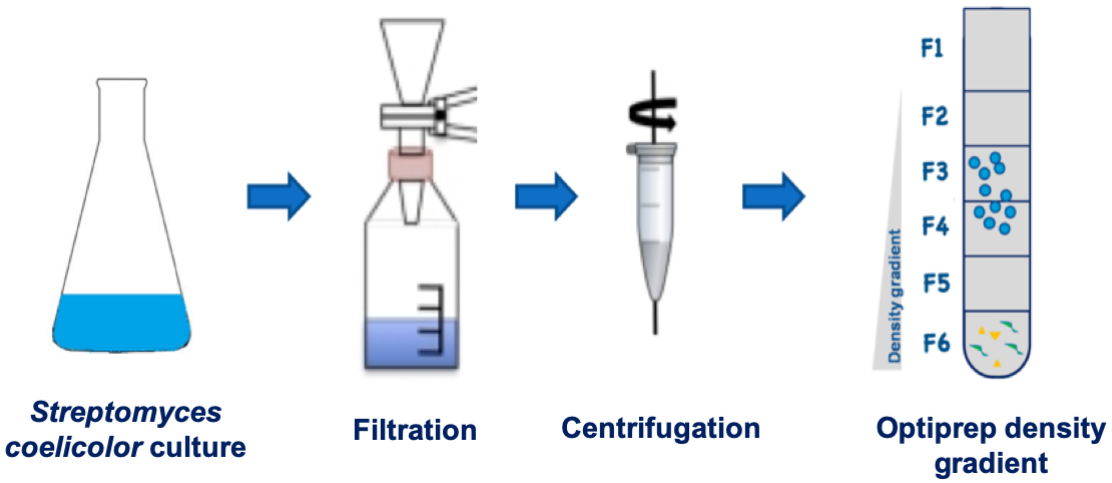



**Figure 18:** Schematic representation of MV isolation and purification procedures.

Ultracentrifugation at 140000 x g for 16 hours resulted in MVs reaching a density equilibrium point at gradient fractions 3 and 4 (20% and 25% v/v Optiprep, respectively), as determined by SDS-PAGE gel analysis (Fig 19).

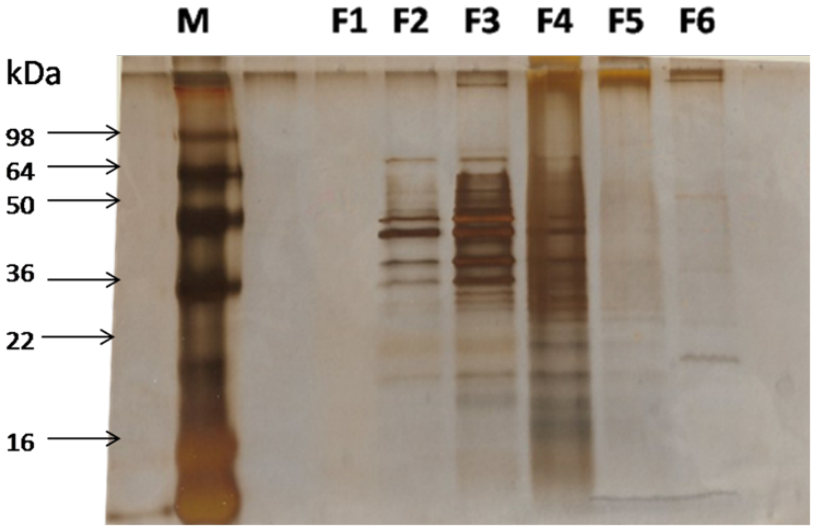



**Figure 19:** SDS-PAGE profile of Optiprep gradient fractions (F1-F6) of *S. coelicolor* supernatant.

F3 and F4 MVs were analyzed by Atomic Force Microscopy (AFM) to investigate their morphology (Witwer *et al.,* 2013). Topography of MVs revealed that they have a spherical shape and the 3D-reconstruction of AFM images confirmed this shape (Fig 20).

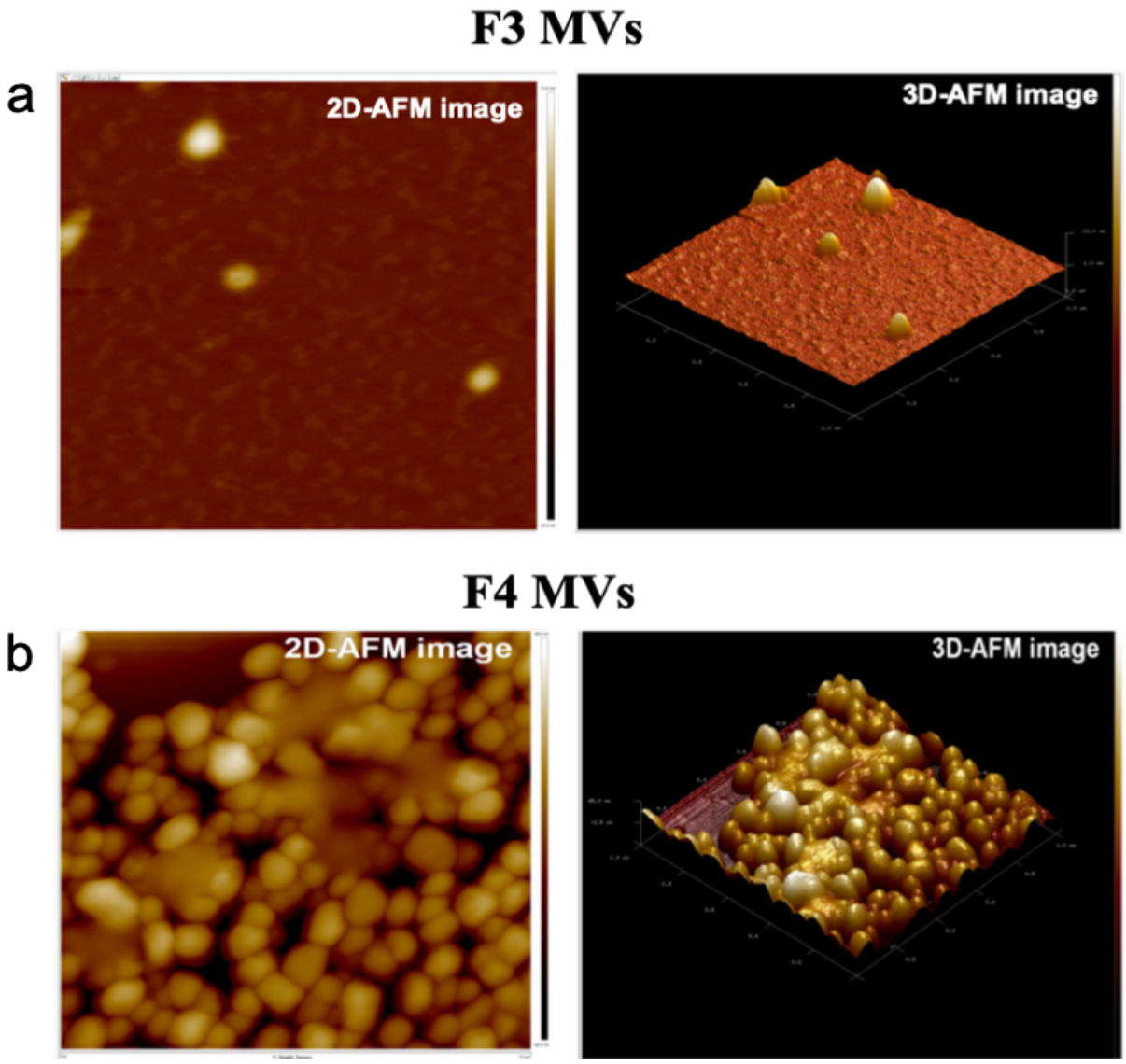



**Figure 20:** Atomic force microscopy (AFM) topography of *S. coelicolor* F3 (a) and F4 MVs (b).

Moreover, F3 and F4 MVs were analyzed by Transmission Electron Microscopy (TEM) to elucidate details of their shape and ultrastructure. TEM images of both MV fractions showed a spherical-shape and an electron-dense luminal content (Fig. 21). Electron-dense luminal content is consistent with the notion that vesicles contain proteins and nucleic acids (Domínguez Rubio *et al.,* 2017).

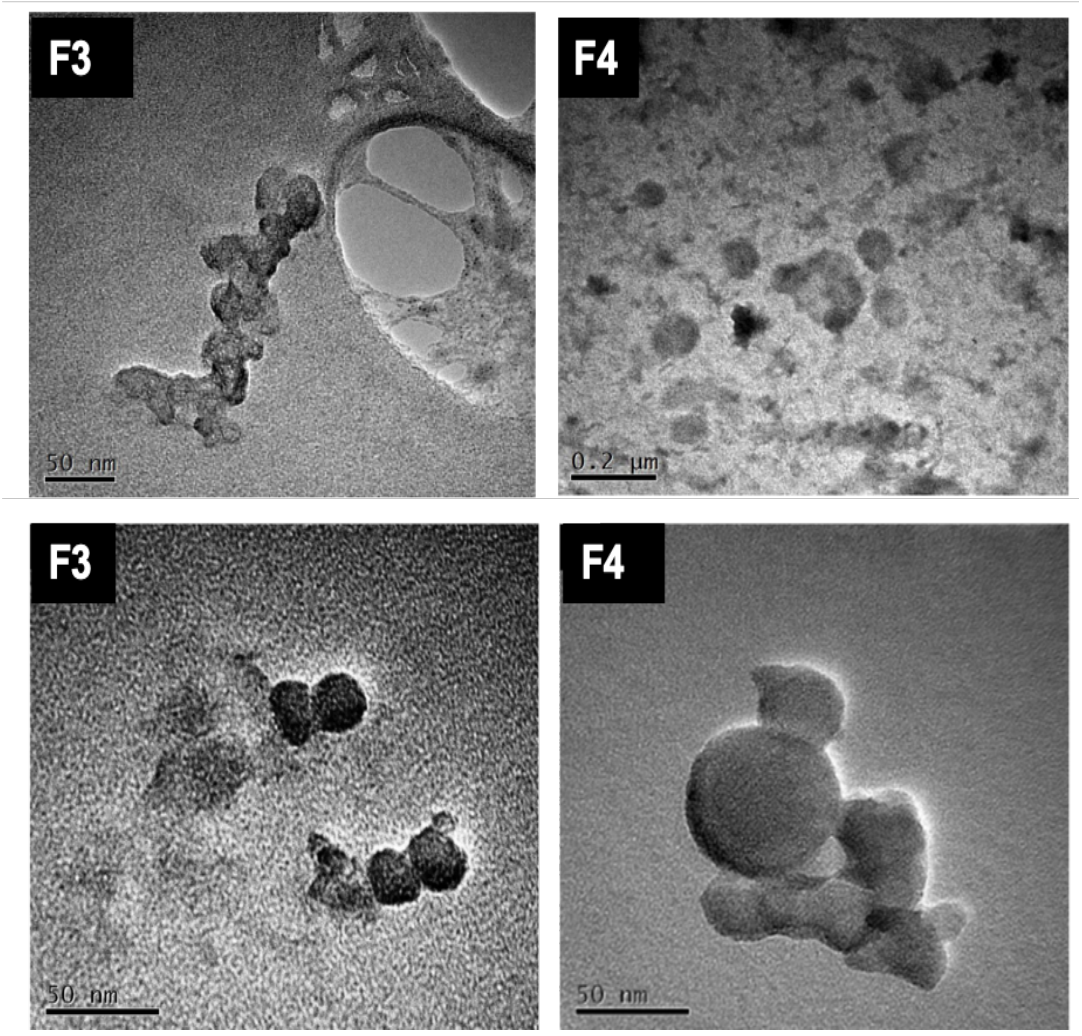



**Figure 21:** Transmission Electron Microscopy (TEM) micrographs of *S. coelicolor* F3 and F4 MVs.

To determine MV size, F3 and F4 MVs were examined by Dynamic Light Scattering (DLS). This analysis revealed that the two fractions of vesicles have different size, in particular F3 MVs have an average size of 100 nm and F4 MVs of 200 nm (Fig. 22).

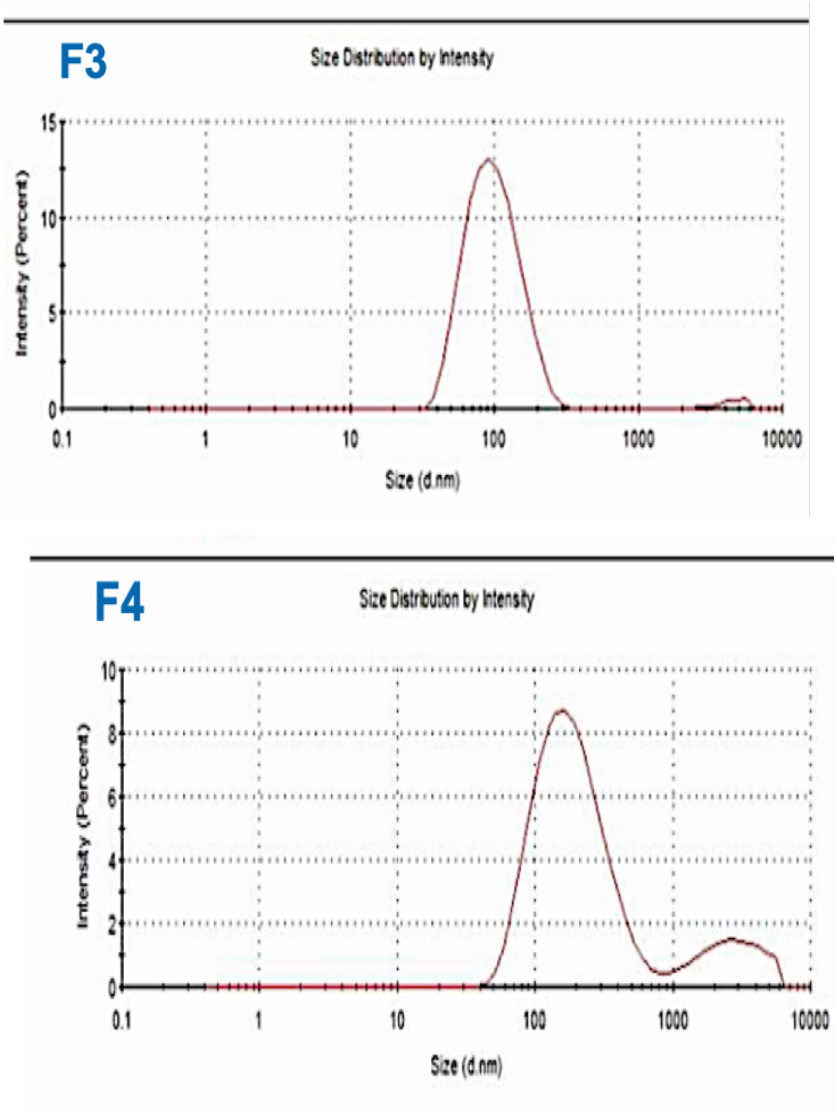



**Figure 22:** Dynamic Light Scattering (DLS) analysis of *S. coelicolor* MVs. F3 and F4 MVs have an average size of 100 nm and 200 nm respectively.

## 4.2 Strategy to identify luminal and surface proteome of *S. coelicolor* MVs

SDS-PAGE gel analysis revealed that F3 and F4 MVs were rich in proteins (Fig 19). In order to distinguish and compare the luminal protein content of MVs and the proteins associated to their surface, MVs were treated with Proteinase K (PK) to remove surface-accessible proteins. Since PK cannot penetrate through the membrane, it is most likely that proteins susceptible to PK are localized on the surface of MVs. DLS analysis revealed that PK treatment did not change the integrity of MVs (Fig. 23). Untreated and PK-treated F3 and F4 MVs were analyzed with 1-D-SDS-PAGE and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (Fig. 23) and bioinformatic tools were used to analyze the proteomic data.

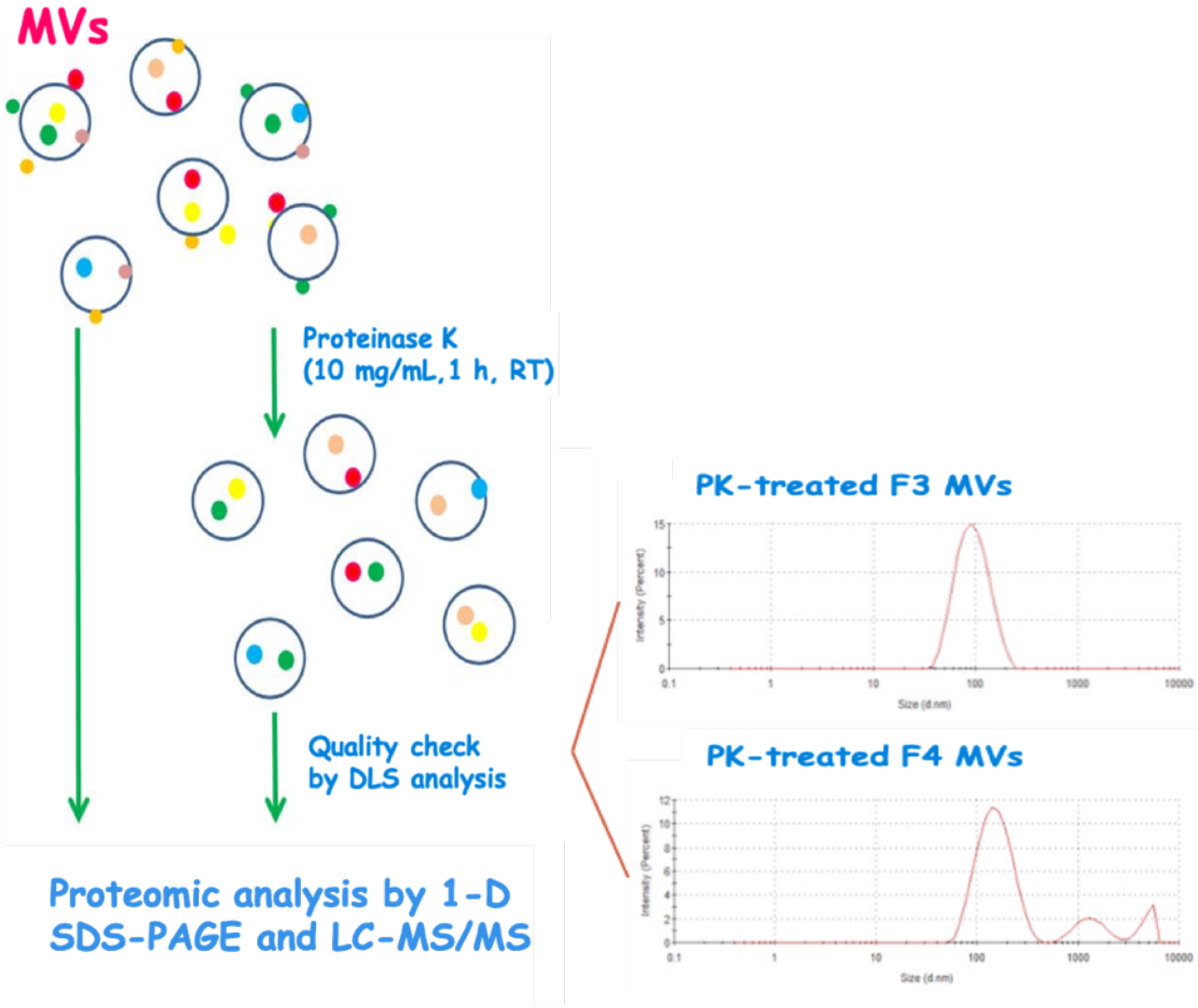



**Figure 23**: Diagram of the research strategy. F3 and F4 MVs were treated with proteinase K (PK). Untreated and PK-treated MVs were analyzed by 1-D SDS-PAGE and LC-MS/MS.

### 4.2.1 Protease accessibility assay

To define the luminal and the surface-accessible proteome of MVs, F3 and F4 MVs were incubated with PK (10 μg/ml) in the absence or presence of 0.02% SDS. Addition of SDS distrupted the integrity of MVs, allowing access of PK to the luminal content of the vesicles. Incubation of MVs with PK resulted in loss of surface-exposed proteins. Incubation of the MVs with SDS had no effect on the protein profile. The treatment with PK and SDS resulted in a dramatic loss of proteins bands (Fig. 24).

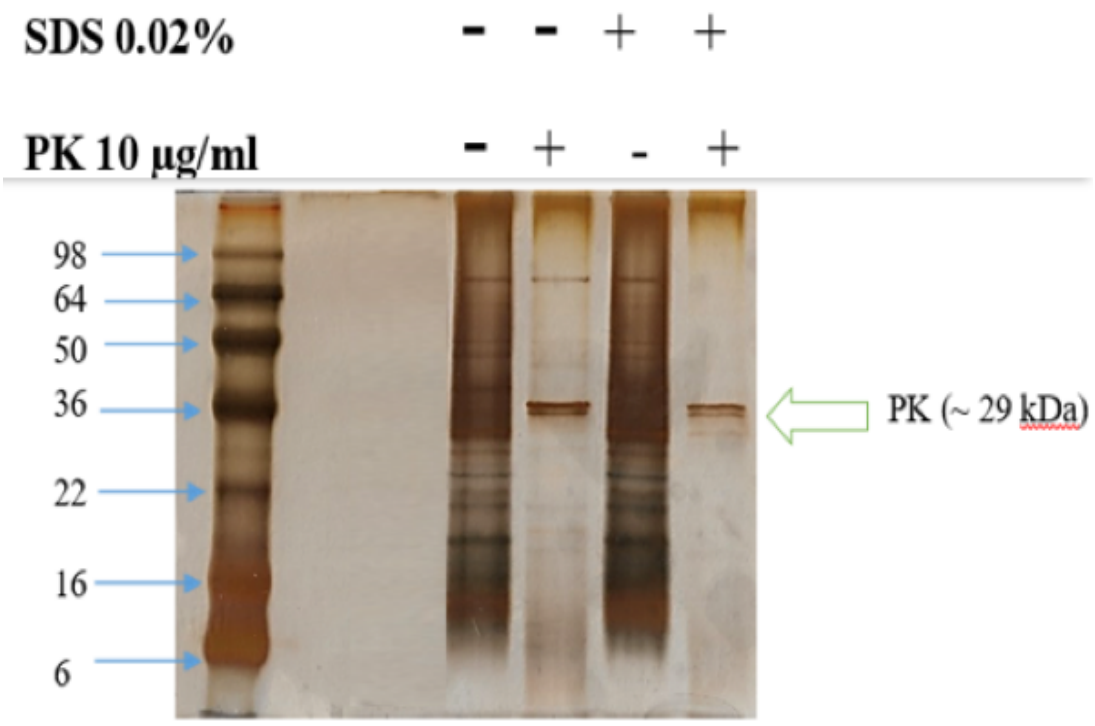



**Figure 24:** Protease accessibility assay. MVs were treated with PK in the presence or absence of SDS to distrupt vesicle integrity. MVs were subjected to SDS-PAGE and silver stained. The image is relative to F4 MV samples.

### 4.2.2 Proteome of *S. coelicolor* MVs

Duplicate untreated and PK-treated F3 and F4 MVs were separated on 12% SDS-PAGE gel. Each gel-lane was excised into 16 sections and analyzed by LC-MS/MS. A total of 166 protein species was identified (Tab. S1), 92 proteins (55%) were common to the two fractions, 51 (31%) were exclusively present in the F3 MVs and 23 (14%) were F4 specific (Fig. 25).

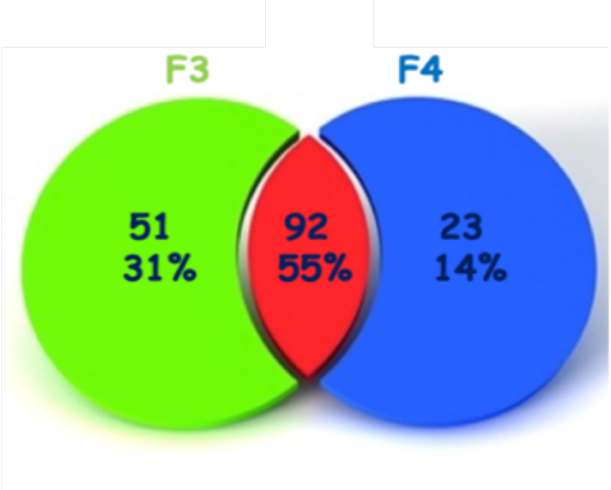



**Figure 25:** Number of proteins present in *S. coelicolor* F3 and F4 MVs identified by 1-SDS-PAGE and LC-MS/MS.

Most secreted proteins and surface proteins are known to have a signal peptide sequence in their N-terminal marking them for localization to the appropriate cellular location. The signal peptide predictor SignalP 5.0 was used to categorize the proteins identified in the MVs, only 3% of vesicle proteins contained a signal peptide (Fig. 26). For more detailed localization analysis, proteins were annotated by subcellular localization using "Subcellular Topology of Polypeptides in Streptomyces" (SToPSdb) database (Tsolis *et al.,* 2018). Proteins annotated as extracellular proteins were poorly represented while most proteins are cytosolic, ribosomal and membrane proteins (Fig. 27). This result agrees with previous studies of bacterial vesicles (Kim *et al.,* 2019; Domínguez Rubio *et al.,* 2017; Liu *et al.,* 2019*).* Moreover, many of these proteins were classified as moonlighting proteins such as Chaperone protein DnaK (DnaK), Enolase 1 (Eno1), 60 kDa chaperonin 1 (GroEL1), 60 kDa chaperonin 2 (GroEL2), Superoxide dismutase (SodF1) (Wanga and Jeffery, 2016; Jeffery, 2018).

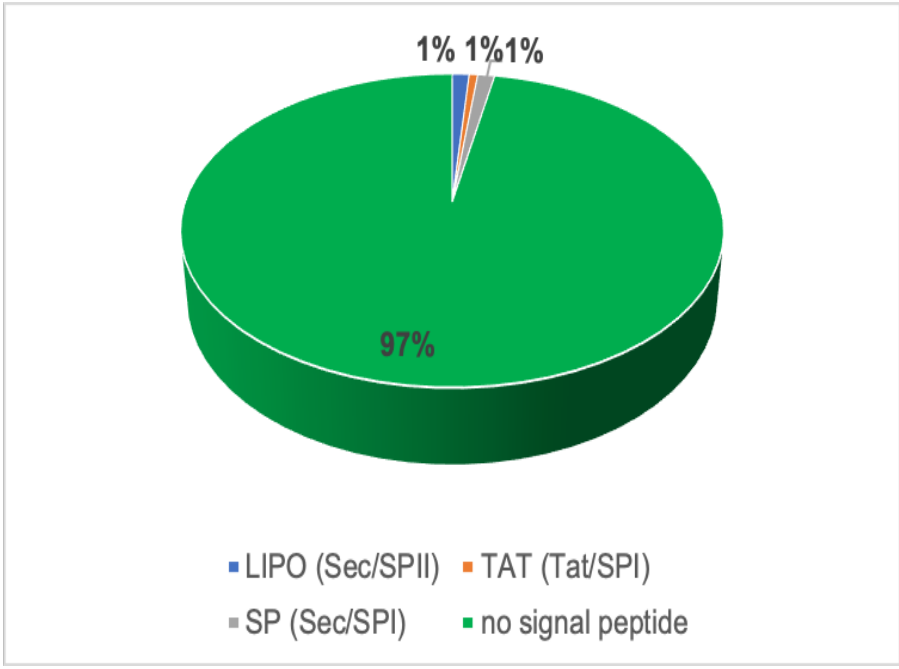



**Figure 26:** Prediction of the presence of signal peptide cleavage sites in MV proteins. Sec signal peptide (Sec/SPI), Lipoprotein signal peptide (Sec/SPII), Tat signal peptide (Tat/SPI) or No signal peptide at all.

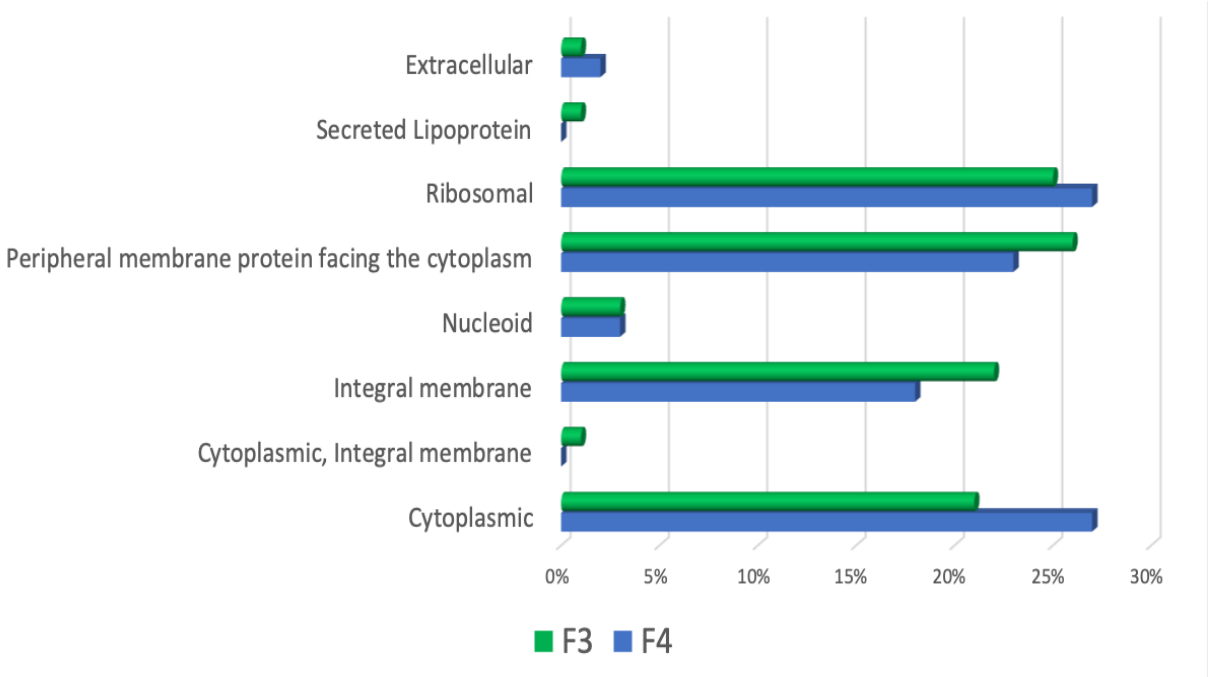



**Figure 27:** Subcellular localization of identified F3 and F4 MV proteins.

The functions of identified proteins were categorized according to KEGG metabolic database. Most proteins were involved in different function and this is in agreement with the presence of numerous moonlighting proteins. KEGG Mapper analysis tool was used to construct the pathways of vesicular proteins. Proteins involved in central carbohydrate metabolism, in energy metabolism and in oxidative phosphorylation were common to the two vesicle fractions. Proteins involved in peptidoglycan biosynthesis, fructose and mannose metabolism and biosynthesis of type II polyketide backbone were specific of F3 vesicles while some proteins involved in amino acid metabolism and purine and pyrimidine metabolism were exclusive of F4 vesicles (Fig. 28).

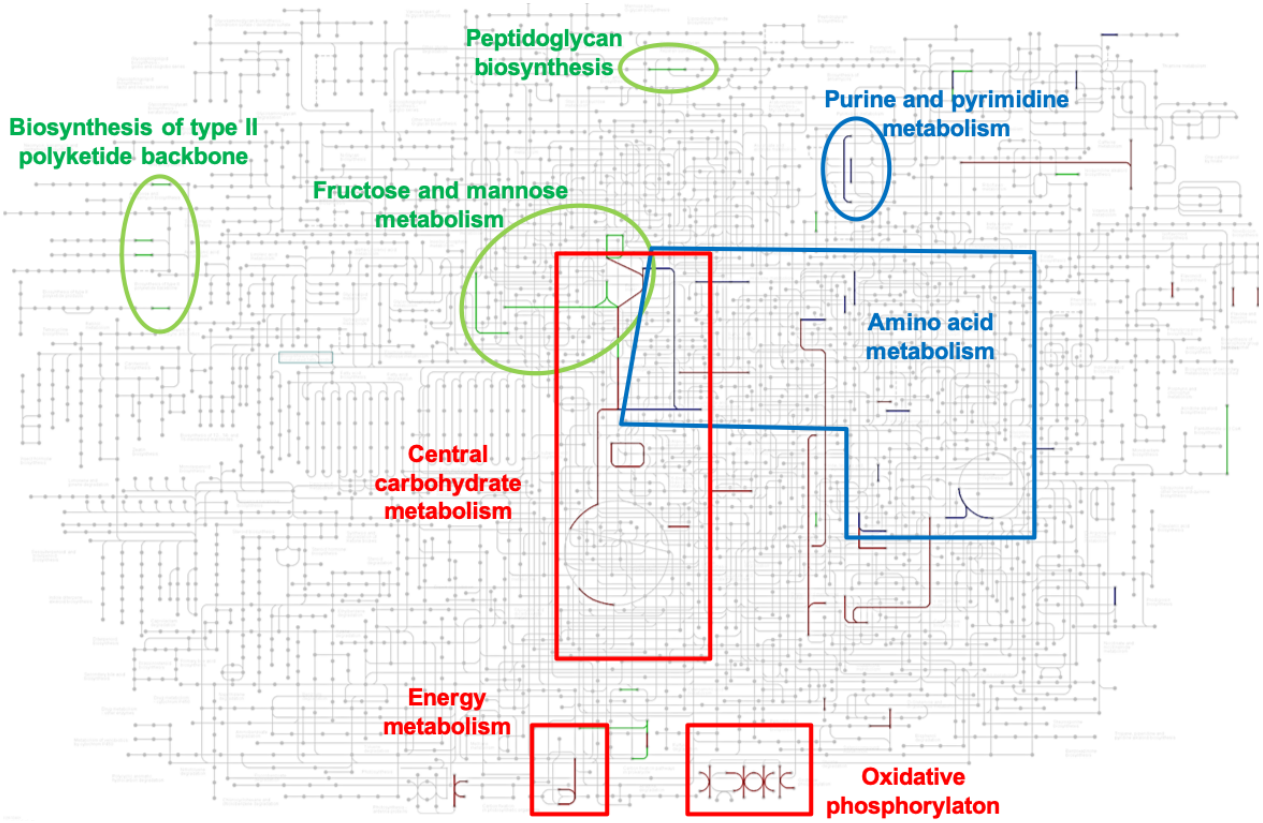



**Figure 28:** KEGG proteomic map of *S. coelicolor* MVs. F3 MV specific pathways are show in green, F4 MV specific pathways in blue and common pathways in red.

*S. coelicolor* MV proteome includes a set of stress response proteins. Some of these were in common with the two MV fractions, such as: DNA integrity scanning protein (DisA), Catalase-peroxidase (CpeB), Bacterioferritin (Bfr), Superoxide dismutase (SodF1), Chaperone protein DnaK, 60 kDa chaperonin 2 (GroEL2), 60 kDa chaperonin 1 (GroEL1), Pup-protein ligase (PafA), Polyribonucleotide nucleotidyltransferase (Pnp) Other stress response proteins were fraction specific. F3 MVs include ATP-dependent Clp protease proteolytic subunit 2 (ClpP2), Cold shock protein ScoF, Mycothiol acetyltransferase, Fe3+ ions import ATP-binding protein FbpC while F4 MVs include ATP-dependent Clp protease proteolytic subunit 1 (ClpP1). (Hesketh *et al.,* 2015; Busche *et al.,* 2016; Beites et al., 2015; Kim *et al.,* 2012; Puglia *et al.,* 1995; Bucca *et al.,* 1995; Compton *et al.,* 2015; Jones, 2018; de Crécy-Lagard *et al.,*1999; Kim *et al.,*2008; Thomas *et al.,* 2012). Moreover, many of them play a role in morpho-physiological differentiation such as ClpP1, ClpP2, Dnak, GroEL1, GroEL2, Polyribonucleotide nucleotidyltransferase (Pnp). Other proteins included in MV proteome with this role were S-adenosylmethionine synthase (MetK), Glutamine synthetase (GlnA), Aminopeptidase probable cytosol aminopeptidase (PepA), Anti-sigma-B factor antagonist (BldG) (de Crécy-Lagard *et al.,* 1999; Fink *et al.,* 2002; Song *et al.,* 2013; Okamoto *et al.,* 2003; Parashar *et al.,* 2009).

### 4.2.3 The luminal *S. coelicolor* MV cargo

To define the luminal MV cargo a comparison between untreated and PK-treated MVs using the Normalized Spectral Abundance Factor (NSAF) Ratio (Paoletti *et al.,* 2006) was carried out. Since large proteins tend to contribute more peptide/spectra than small ones, a normalized spectral abundance factor (NSAF) was defined to account for the effect of protein length on spectral count. NSAF is calculated as the number of spectral counts (SpC) identifying a protein, divided by the protein's length (L), divided by the sum of SpC/L for all proteins in the experiment. NSAF allows the comparison of abundance of individual proteins in multiple independent samples (Zhu *et al.,* 2009). The value of ratio between NSAF untreated MVs and NASF PK-treated MVs indicated the protein localization in the vesicles. Proteins with NSAF ratio >2 were categorized as proteins associated with vesicle-surface (out). When the value is < 1.5, proteins were categorized as internal proteins (in). For proteins with NSAF ratio between 1.5 and 2 it was not possible to assign a localization (unknown) (Tab. S1).

A total of 65 proteins were classified as internal proteins (Tab. S1). In particular, 19 proteins were common to the two MV fractions, other proteins were fraction specific (Fig. 29). Among the common proteins there were: bacterioferritin (Bfr), spore associated protein A (SapA), superoxide dismutase (SodF1), cytochromes (QcrB, QcrA, CtaD1, CtaC), sec system proteins (SecF, SecY).

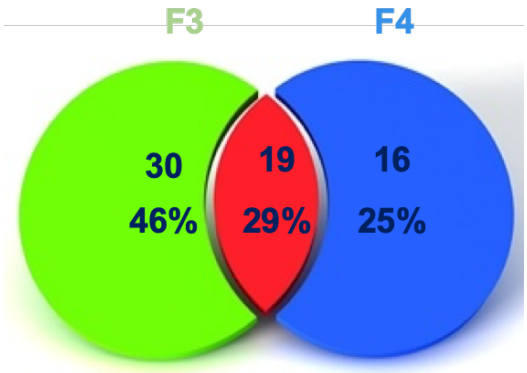



**Figure 29:** Number of internal proteins present in *S. coelicolor* F3 and F4 MVs.

## 4.3 Mass spectrometry analysis of metabolites in *S. coelicolor* MVs

A targeted metabolomic analysis was performed to identify metabolic profile of *S. coelicolor* MVs (contract service by Ceinge). A total of 99 metabolites (Tab. S2) was detected in both MV fractions but only 35 metabolites were present with an abundance greater than 0.05 ng/ml. Metabolites were categorized according to KEGG compound database in amino acids and amino acid precursors, vitamins, components of carbon metabolism, components of pyrimidine and purine metabolism, antibiotics and components of peptidoglycan biosynthesis (Fig. 30).

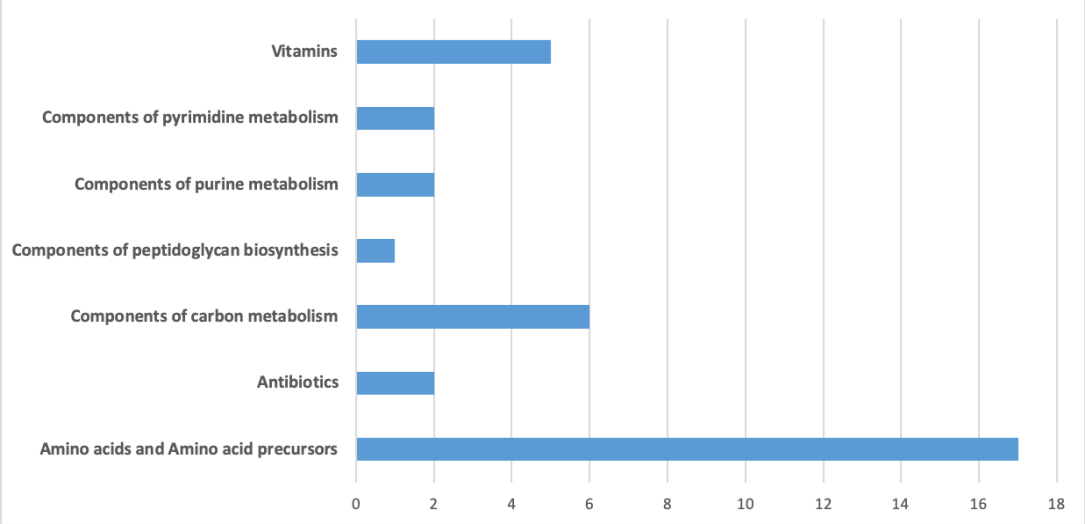



**Figure 30:** Qualitative analysis of metabolites identified in *S. coelicolor* F3 and F4 MVs.

Comparative quantitative metabolomic analysis showed that ten metabolites (Tab. 5) were more abundant (with relative abundance ≥ 1.3 fold) in F3 MVs compared F4 MVs. Other metabolites were present with the same profile in both fractions.

| Metabolites | Fold F3 vs F4 |
|---|---|
| Phenylalanine | +3.6 |
| Isoleucine | +1.5 |
| Leucine | +1.8 |
| Serine | +1.5 |
| Thryptophan | +1.4 |
| Actinorhodin | +2.1 |
| Undecyl-prodigiosin | +1.4 |
| Glucose-6-Phosphate | +1.3 |
| Folate | +2.3 |
| Vitamin B12 | +1.9 |

**Table 5:** Differentially abundant metabolites in *S. coelicolor* MVs.

In particular, the actinorhodin antibiotic was 2.1-fold higher than in F4 MVs and this result was supported by Raman Spectroscopy analysis. In fact, in F3 MVs a specific peak (1200 $cm^{-1}$) was detected corresponding the same peak that was detected in an actinorhodin extract (Fig. 31) Probably in F4 MVs the actinorhodin was below the threshold level to be detected by Raman Spectroscopy.

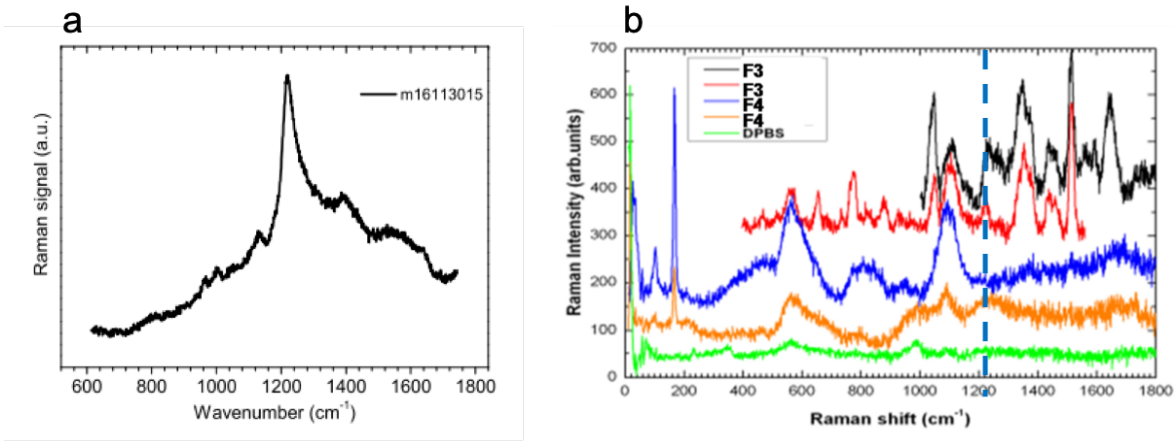



**Figure 31:** Raman spectre identifies the same signal in purified actinorhodin (a) and *S. coelicolor* F3 MVs (b).

Proteomic and metabolomic data were used to build comprehensive metabolic networks for *S. coelicolor* MVs, using the metabolic visualization software KEGG Mapper–Search&Color Pathway. Interestingly, many metabolites belong to the metabolic pathways associated with the identified vesicular proteins (Fig. 32).

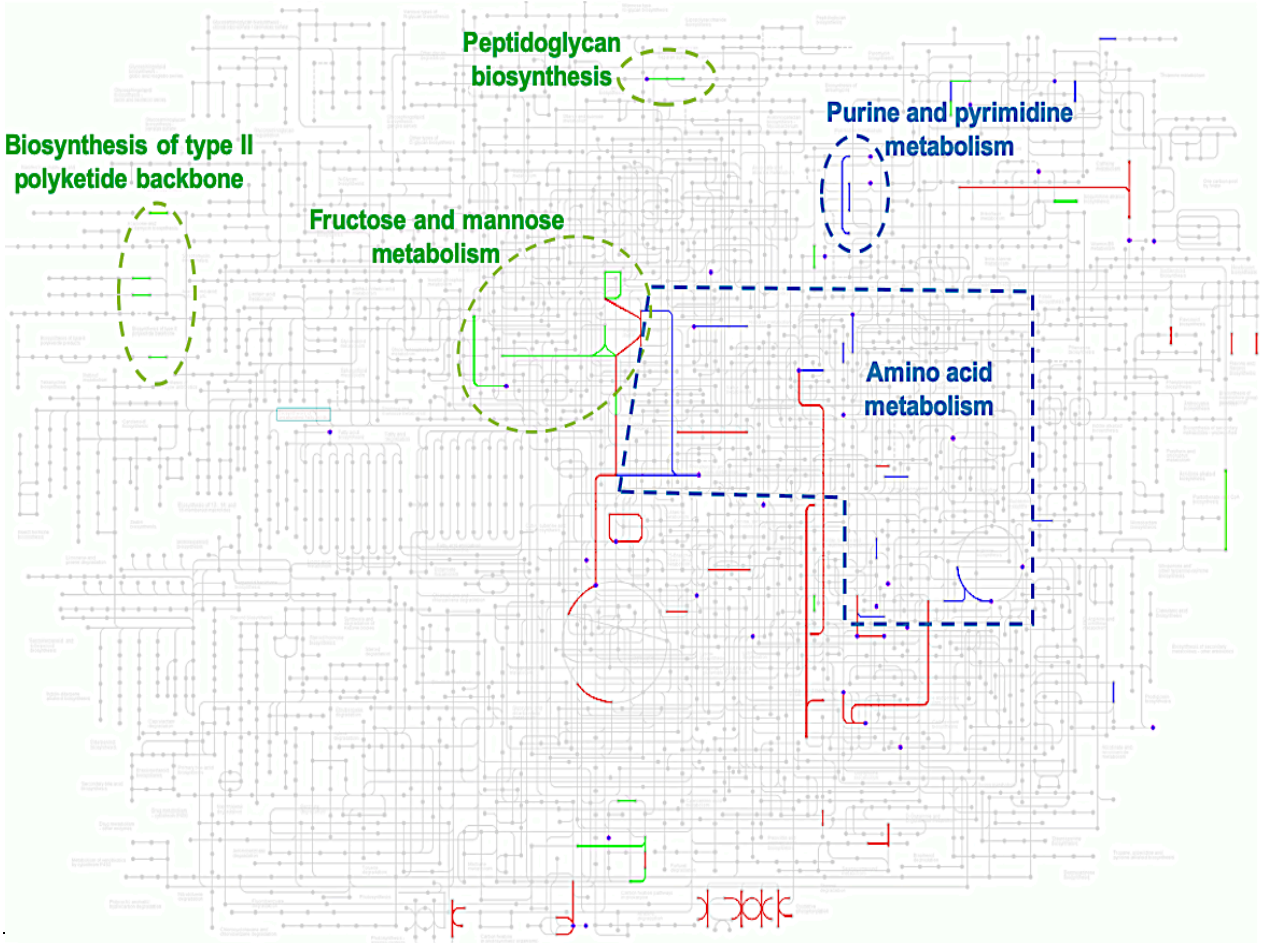



**Figure 32:** KEGG proteomic and metabolic map of *S. coelicolor* MVs. F3 MV specific pathways were showed in green, F4 MV specific pathways in blue and common pathways in red, metabolites in purple.

## 4.4 Vesicular nucleic acids of *S. coelicolor*

Agarose gel electrophoresis and ethidium bromide staining revealed the presence of three bands in both MV fractions: a high molecular weight band of approximately of 19 kb and two bands of ~ 900 bp and ~ 500 bp. Interestingly, the intensity of bands was different in MV fractions: the band of 19 kb was stronger in correspondence of F3 MVs while the smaller bands were more intense in F4 MVs (Fig. 33).

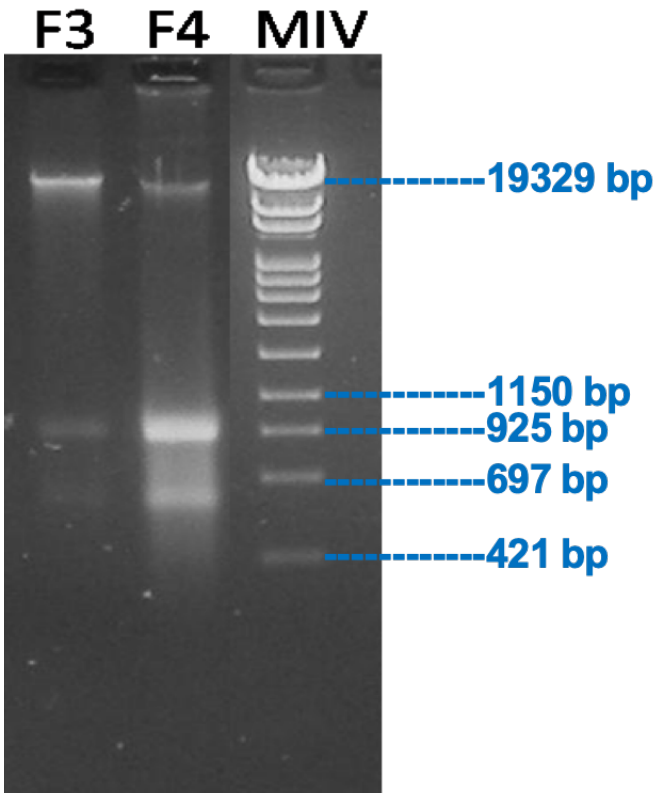



**Figure 33**: Electrophoresis analysis of nucleic acids of *S. coelicolor* MVs.

DNase-treatment demonstrated that the high-molecular weight nucleic acid was DNA whereas RNase treatment demonstrated that the two smaller bands corresponded to RNA (Fig. 34).

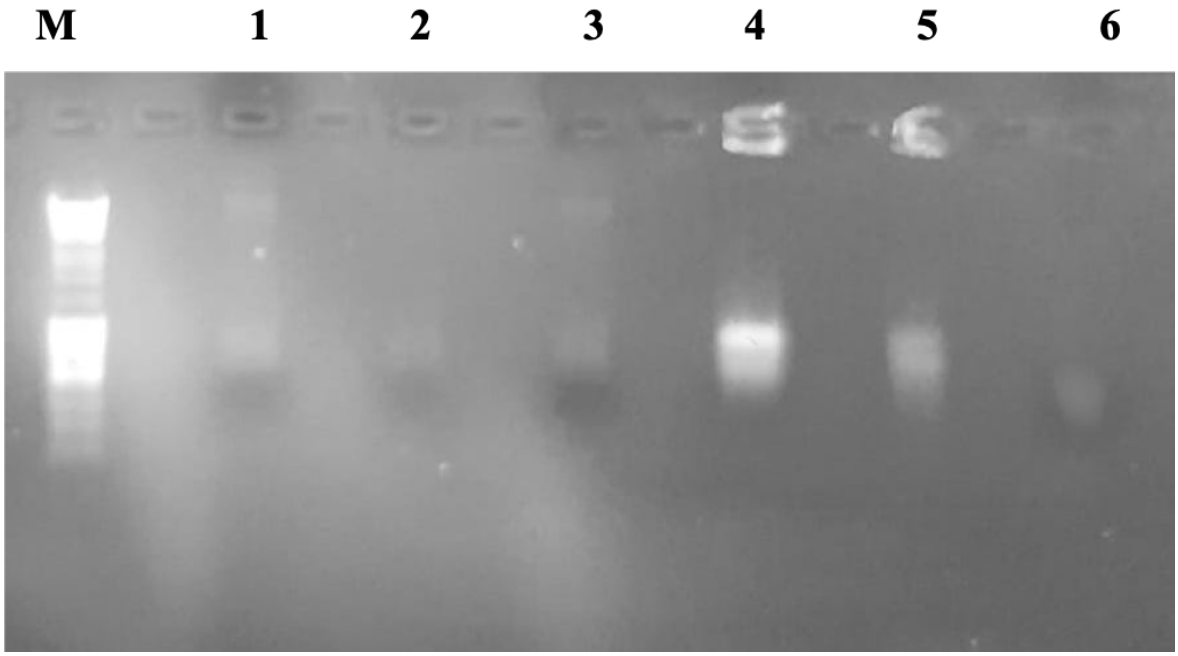



**Figure 34:** Nuclease treatment of *S. coelicolor* MVs. M: MassRuler DNA Ladder Mix, ready to use (Thermo Scientific); 1: F3 MVs; 2: F3 MVs treated with DNase; 3: F3 MVs treated with RNase; 4: F4 MVs; 5: F4 MVs treated with DNase; 6: F4 MVs treated with RNase.

Preliminary data suggested that the high-molecular weight band is consistent with genomic DNA. In fact, PCR analyses of F3 and F4 MV DNA demonstrated that 5 genes (*katA2, cpeB, dnaK, hrdB, rrnB*) mapping in different regions of *S. coelicolor* chromosome (Fig. 35) were present in the MVs (Fig. 36). The expected size of PCR product is: 241 bp for *cpeB,* 151 bp for *dnaK,* 197 bp for *katA2,* 1492 bp for *rrnB,* 220 bp for *hrdB.*

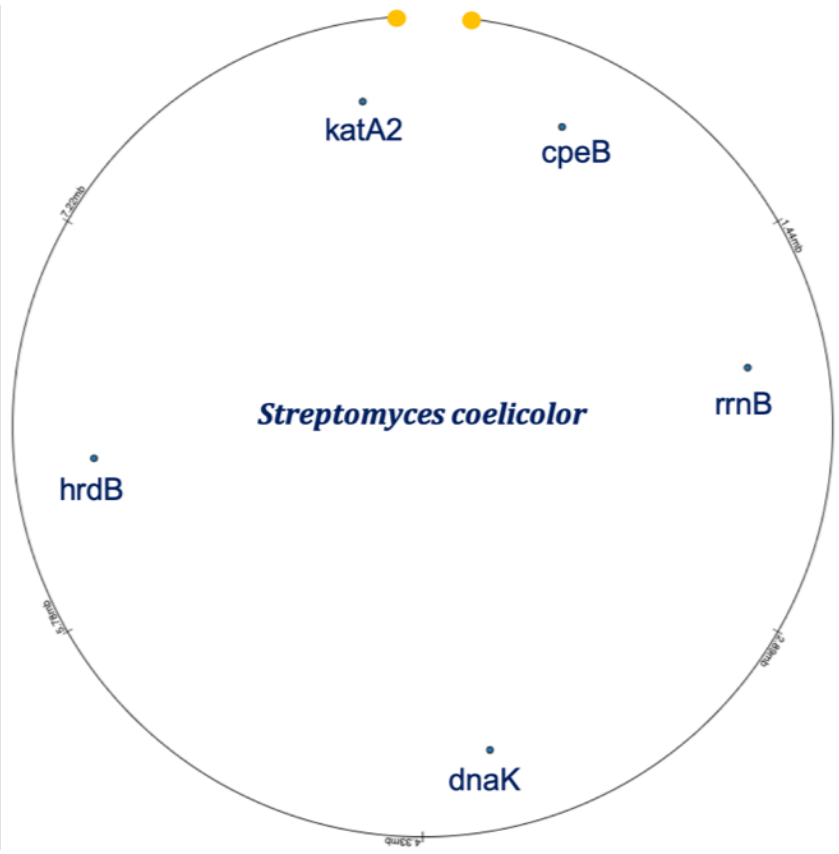
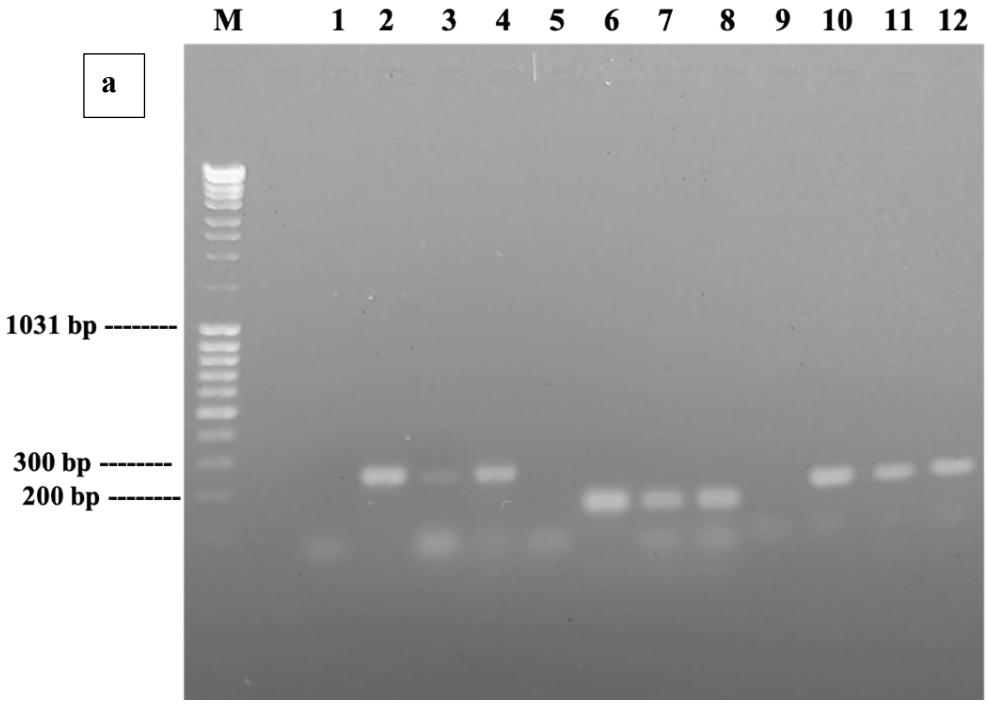



**Figure 35:** Localization of the five genes analyzed by PCR in *S. coelicolor* chromosome.



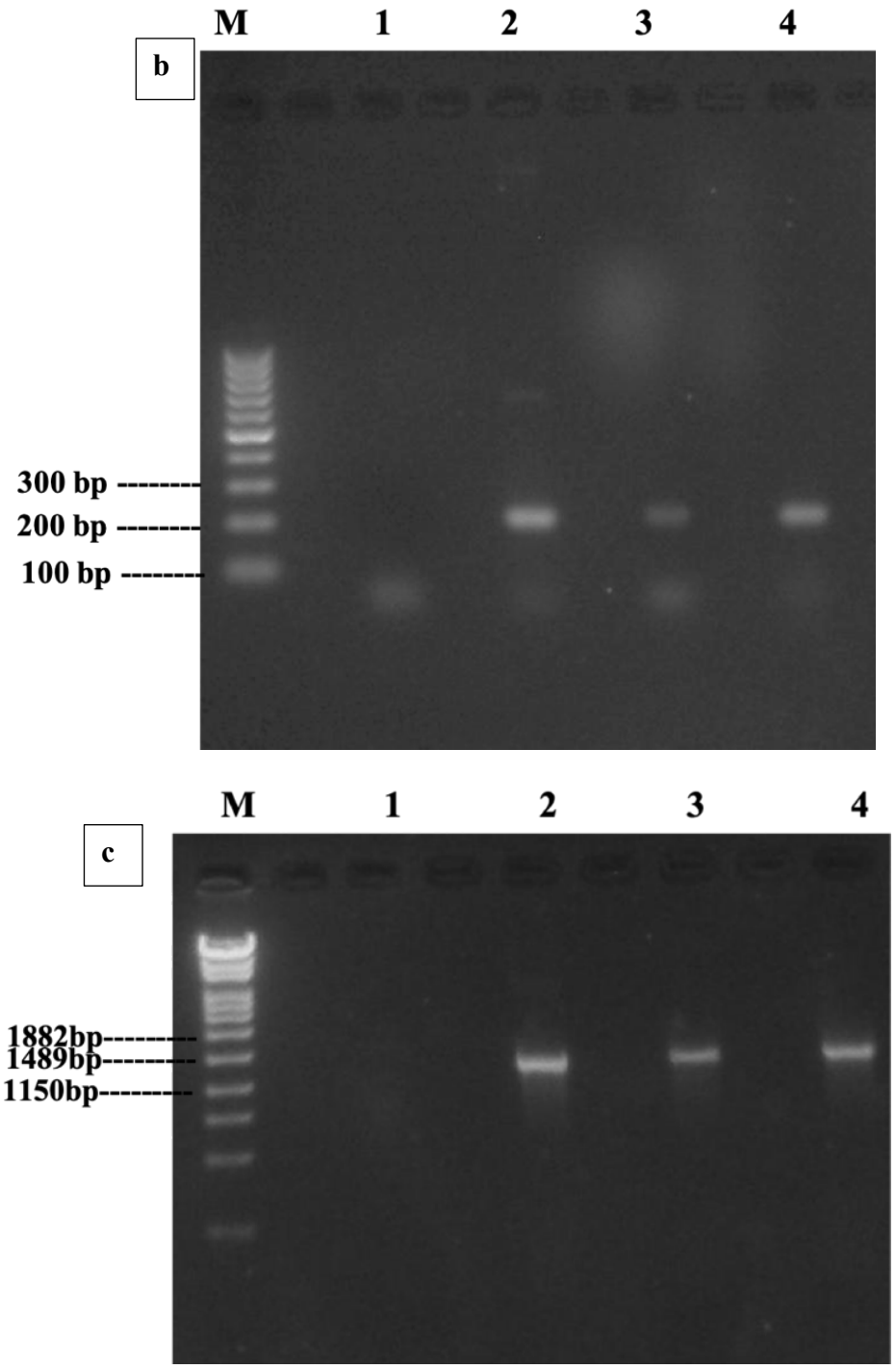



**Figure 36:** PCR analysis of *S. coelicolor* MVs. **a)** M: MassRuler DNA Ladder Mix, ready to use (Thermo Scientific); **1-4** PCR analysis of *cpeB* (SCO0560). 1: Negative control; 2: *S. coelicolor* genomic DNA; 3: F3 MVs; 4: F4 MVs. **5-8** PCR analysis of *dnaK* (SCO3671). 5: Negative control; 6: *S. coelicolor* genomic DNA; 7: F3 MVs; 8: F4 MVs. **9-12** PCR analysis of *katA2* (SCO7590). 9: Negative control; 10: *S. coelicolor* genomic DNA; 11: F3 MVs; 12: F4 MVs.

**b)** PCR analysis of *hrdB* (SCO5820). M: 100bp DNA ladder; 1: Negative control; 2: *S. coelicolor* genomic DNA; 3: F3 MVs; 4: F4 MVs.

**c)** PCR analysis of *rrnB* (SCOr06). M: DNA Molecular Weight Marker IV(Merck); 1: Negative control; 2: *S. coelicolor* genomic DNA; 3: F3 MVs; 4: F4 MVs.

### 4.4.1 RNA sequencing of *S. coelicolor* MVs

RNA extraction and RNA-sequencing were performed (contract service by Genomix4life) to analyze RNA profile of *S. coelicolor* MVs. The RNA concentration of F3 MV samples was outside of functional range for RNAseq, therefore only the RNA of F4 MVs was sequenced. Two F4 MV preparations showed variability in the RNA size patterns (Fig. 37); the same variability was observed in RNA preparations of uropathogenic *Escherichia coli* MVs. These authors of this paper suggested three possible factors to explain this variability: *i*) the contributions from the underlying natural variation caused during bacterial culture; *ii*) the likelihood of some unavoidable fragmentation of the isolated RNA during the lengthy MV processing; *iii*) variation in loading when handling small, dilute volumes (Blenkiron *et al.,* 2016). The sequencing of a third replicate is ongoing to investigate if this variability is a technical problem or a constant in *S. coelicolor* MV samples.

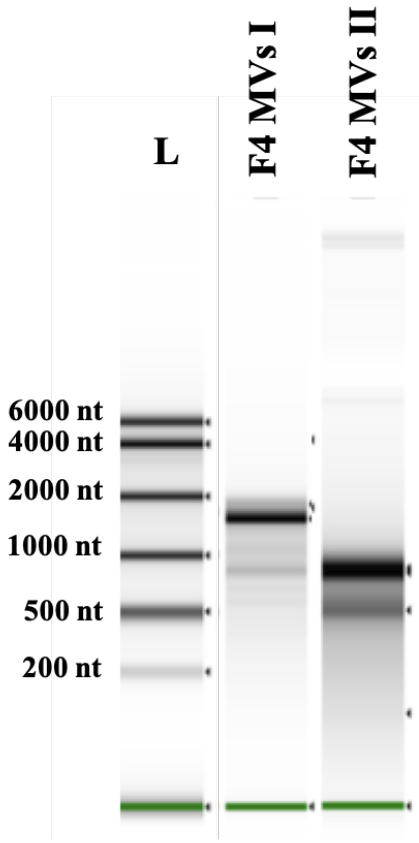



**Figure 37:** Agilent Tapestation gel for RNA from two replicate *S. coelicolor* F4 MVs. The green line marks the internal loading marker.

However, the two biological replicates have in common 16 mRNAs (Tab. 6) with a number of reads ≥ 0.01% of total reads. Interestingly, many of these mRNAs were transcripts of genes encoding of hypothetical proteins (Tab. 6).

| SCO | gene name | Description |
|---|---|---|
| SCO3088 | *SCE25.29c* | hypothetical protein |
| SCO3230 | *cdaPSI* | CDA peptide synthetase I |
| SCO3578 | *SCH17.12* | ion-transporting ATPase |
| SCO4186 | *SCD66.23* | hypothetical protein |
| SCO4443 | *SCD6.21c* | MerR family transcriptional regulator |
| SCO4567 | *nuoF* | NADH dehydrogenase subunit NuoF |
| SCO4632 | *SCD82.03* | ATP/GTP binding protein |
| SCO4674 | *SCD40A.20* | hypothetical protein |
| SCO4678 | *SCD31.03* | hypothetical protein |
| SCO4679 | *SCD31.04* | hypothetical protein |
| SCO4680 | *SCD31.05c* | DNA-binding protein |
| SCO4685 | *SCD31.10* | DEAD/DEAH box helicase |
| SCO4697 | *SCD31.22* | hypothetical protein |
| SCO5810 | *SC4H2.31c* | transmembrane efflux protein |
| SCO5861 | *SC2E9.02* | hypothetical protein |
| SCO6627 | *SC1F2.24* | hypothetical protein |

**Table 6:** mRNAs present in *S. coelicolor* F4 MVs.

# 5. DISCUSSION

The release of membrane vesicles from *Streptomyces coelicolor* in liquid culture was demonstrated in this work for the first time. SEM images showed hyphae with multiple protrusions, similar to those reported for other Gram-positive bacteria such as *Bacillus subtilis* and *Lactobacillus reueteri* (Brown *et al.,* 2014; Grande *et al.,* 2017). *S. coelicolor* MVs were characterized from a structural and biochemical point of view by microscopy, physical and -omics analyses.

Two main populations of MVs, F3 MVs and F4 MVs, were isolated and purified by using OptiPrep density gradient ultracentrifugation. The size of MVs was determined by DLS analysis; F3 MVs had an average size of 100 nm while F4 MVs were bigger with an average size of 200 nm. Diameter variations of isolated MVs may indicate that different pathways may be important in their biogenesis. Morphology of MVs was investigated by microscopy analyses. AFM images showed a spherical shape of vesicles and TEM analysis confirmed this shape and revealed an electron-dense luminal content suggesting a bioactive cargo such as proteins and nucleic acids. In fact, SDS-PAGE gel analysis and agarose gel electrophoresis of MVs revealed a cargo that includes proteins, DNA and RNA.

Proteomic analysis of F3 and F4 MVs disclosed a complex protein composition with a total of 166 identified protein species. The 55% of proteins identified was common to the two fractions and other proteins were specific of F3 or F4 MVs. Interestingly, bioinformatic analysis revealed that the 97% of proteins did not contain signal peptide for subcellular localization. *S. coelicolor* MVs contained an array of proteins localized in the cytosol, membrane and with a high abundance of ribosomal proteins, while proteins annotated as extracellular proteins were poorly represented. These results are in agreement with previous studies on Gram-positive vesicles. Some authors attribute the presence of cytosolic proteins to improper preparation of vesicle sample. Although this is possible, it seems unlikely to have occurred in all the rigorous studies published. A possible explanation for the presence of cytoplasmic proteins such as DnaK, GroEL1, GroEL2, Eno1, SodF1, is that some of them are highly conserved moonlighting proteins having multiple simultaneous locations and performing more than one biological function (Pérez-Cruz *et al.,* 2015; Tsolis *et al.,* 2018). Many of these moonlighting cytosolic proteins act as adhesins or invasins, promoting attachment to, or entry into, host cells (Ebner and Götz, 2019). For example, enolase from *Lactobacillus, Staphylococcus, Streptococcus* and several other species bind human plasminogen (Jeffery, 2018).

The functions of identified proteins were categorized according to the KEGG metabolic database and bioinformatic analyses were performed to construct the metabolic pathways of MV proteins. Interestingly, some metabolic pathways were common to the two MV populations (central carbohydrate metabolism, energy metabolism and oxidative phosphorylation) while some pathways were specific of F3 or F4 MVs. Proteins involved in peptidoglycan biosynthesis, fructose and mannose metabolism and biosynthesis of type II polyketide backbone were specific of F3 vesicles, while amino acid and purine and pyrimidine metabolism proteins were specific of F4 vesicles. These data suggest possible different functional roles of the two vesicle populations.
*S. coelicolor* vesicle proteome includes many stress response proteins such as GroEL1, GroEL2, DnaK, ClpP1, ClpP2 which also play an important role in *S. coelicolor* morpho-physiological differentiation. Many authors suggested that the production of vesicles is a bacterial stress response (Kulp and Kuehn, 2010; Baumgarten *et al.,* 2012; Macdonald and Kuehn, 2013) and in *S. coelicolor* is known that there is a strong correlation between stress and morphological differentiation (Puglia *et al.,* 1995; de Crécy-Lagard *et al.,*1999). In light of this consideration, it is very interesting to note that the vesicle proteome includes several stress proteins such as GroEL1, GroEL2, DnaK, ClpP1, ClpP2 which also play an important role in *S. coelicolor* morphological differentiation.

The treatment of MVs with Proteinase K allowed to identify 65 protein species that constitute the luminal proteome of *S. coelicolor* MVs. Bacterioferritin, spore associated protein A, superoxide dismutase, cytochromes and some sec system proteins were localized inside the vesicles.

In addition to proteomic results, metabolic analysis underlines a complex cargo of *S. coelicolor* MVs that includes amino acids and amino acid precursors, vitamins, components of purine and pyrimidine metabolism, components of carbon metabolism and antibiotics. In particular, the presence of the two antibiotics actinorhodin and undecylprodigiosin in *S. coelicolor* MVs suggested that the MV cargo reflects the “features” of the strain that produces them. Often MV cargo of pathogenic bacteria includes virulence factors, MV cargo of bacteria known to produces many hydrolytic enzymes includes these enzymes and likewise *S. coelicolor* known to produce an enormous repertoire of secondary metabolites produces MVs which reflects this feature.

Many studies have shown that extracellular nucleic acids can be secreted by bacteria via MVs which can serve as protected vehicles for nucleic acid transfer into host cells. *S. coelicolor* MVs contain nucleic acids and in particular F3 MVs are enriched with DNA and F4 vesicles with RNA. Preliminary data suggested the possibility that these MVs contained genomic DNA and this hypothesis could open up to the possibility that the MVs are involved in lateral gene transfer with important implications for bacterial evolution.
Bacteria secrete RNAs, some of which have effects on other cells and on other species. RNA sequencing showed that *S. coelicolor* F4 MVs carry mRNAs. Two biological replicates showed variability in the RNA size patterns, thus the sequencing of a third replicate will be able to clarify if this variability is a technical problem or a constant in *S. coelicolor* MV samples. Interestingly, most of the more abundant mRNAs present in both replicates are transcripts of genes encoding for hypothetical proteins.

In conclusion, this study demonstrates that *Streptomyces. coelicolor* produces, in liquid culture, two different populations of membrane vesicles that are different for size and cargo. The -omics analyses showed that *S. coelicolor* MV cargo is very complex and contain many "messages" to be decoded. In general, the results obtained in this thesis suggest that the MV cargo may play a key role in the induction of *S. coelicolor* morpho-physiological differentiation and gene transfer. This study could highlight not only the role of *S. coelicolor* MVs but also their potential biotechnological applications.

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# 7. SUPPLEMENTARY MATERIALS

**Table S1:** Description, functional classification and subcellular classification of MV proteins.

| | Uniprot accession number | Description | F3 | F4 | SCO | Gene | KEGG Orthology | Subcellular localization |
|---|---|---|---|---|---|---|---|---|
| 1 | P12690 | Spore-associated protein A | IN | IN | SCO0409 | *sapA* | Unknown | Extracellular |
| 2 | Q9RJM5 | NH(3)-dependent NAD(+) synthetase | - | OUT | SCO0506 | *nadE* | •Metabolism of cofactors and vitamins<br>Nicotinate and nicotinamide metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 3 | P48859 | Cold shock protein ScoF | OUT | - | SCO0527 | *scoF* | •Unclassified: genetic information processing<br>Transcription | Cytoplasmic |
| 4 | Q9RJH9 | Catalase-peroxidase | OUT | OUT | SCO0560 | *cpeB* | •Amino acid metabolism<br>Phenylalanine metabolism<br>Tryptophan metabolism | Cytoplasmic |
| 5 | Q9XA86 | Putative membrane protein SCO0839 | OUT | - | SCO0839 | / | Unknown | Integral membrane |
| 6 | Q93J59 | 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase | OUT | IN | SCO0985 | *metE* | •Amino acid metabolism<br>Cysteine and methionine metabolism<br>•Metabolism of other amino acids<br>Selenocompound metabolism | Cytoplasmic |
| 7 | Q9FCD7 | Tripeptidyl aminopeptidase | UNKNOWN | IN | SCO1230 | *tap* | Unknown | Extracellular; Vesicles |
| 8 | Q8CK28 | ATP phosphoribosyltransferase | - | OUT | SCO1438 | *hisG* | •Amino acid metabolism<br>Histidine metabolism | Cytoplasmic |
| 9 | Q9EWJ9 | 6,7-dimethyl-8-ribityllumazine synthase | OUT | UNKNOWN | SCO1440 | *ribH* | •Metabolism of cofactors and vitamins<br>Riboflavin metabolism | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 10 | Q9L0Y3 | S-adenosylmethionine synthase | OUT | OUT | SCO1476 | *metK* | •Amino acid metabolism<br>Cysteine and methionine metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 11 | Q9KXR7 | Dihydroorotate dehydrogenase (quinone) | IN | - | SCO1482 | *pyrD* | •Nucleotide metabolism<br>Pyrimidine metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 12 | Q9KXP5 | 30S ribosomal protein S4 | OUT | UNKNOWN | SCO1505 | *rpsD* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 13 | Q53956 | Protein translocase subunit SecF | IN | IN | SCO1515 | *secF* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Protein families: signaling and cellular processes<br>Secretion system | Integral membrane |
| 14 | Q53955 | Protein translocase subunit SecD | OUT | - | SCO1516 | *secD* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Protein families: signaling and cellular processes<br>Secretion system | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 15 | Q9L278 | Threonine--tRNA ligase | OUT | OUT | SCO1531 | *thrS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Peripheral inner membrane protein facing the cytoplasm |
| 16 | O88054 | Phenylalanine--tRNA ligase beta subunit | OUT | - | SCO1594 | *pheT* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Peripheral inner membrane protein facing the cytoplasm |
| 17 | O88058 | 50S ribosomal protein L20 | IN | UNKNOWN | SCO1598 | *rplT* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 18 | O88059 | 50S ribosomal protein L35 | OUT | - | SCO1599 | *rpmI* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 19 | O88060 | Translation initiation factor IF-3 | OUT | UNKNOWN | SCO1600 | *infC* | •Protein families: genetic information processing<br>Translation factors | Peripheral inner membrane protein facing the cytoplasm |

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| 20 | Q9RJ68 | Sec-independent protein translocase protein TatA | IN | - | SCO1633 | *tatA* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Protein families: signaling and cellular processes<br>Secretion system | Cytoplasmic, Integral membrane |
| 21 | Q9RJ61 | Pup--protein ligase | OUT | UNKNOWN | SCO1640 | *pafA* | •Protein families: genetic information processing<br>Proteasome | Cytoplasmic |
| 22 | P19255 | Probable glycerol uptake facilitator protein | UNKNOWN | IN | SCO1659 | *glpF* | •Protein families: signaling and cellular processes<br>Transporters | Integral membrane |
| 23 | Q9XAC0 | Transaldolase 2 | OUT | UNKNOWN | SCO1936 | *tal2* | •Carbohydrate metabolism<br>Pentose phosphate pathway | Cytoplasmic |
| 24 | Q9Z523 | Glucose-6-phosphate isomerase 2 | OUT | - | SCO1942 | *pgi2* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>Pentose phosphate pathway<br>Starch and sucrose metabolism<br>Amino sugar and nucleotide sugar metabolism<br>•Protein families: signaling and cellular processes<br>Exosome | Cytoplasmic |
| 25 | Q9Z520 | Triosephosphate isomerase | IN | - | SCO1945 | *tpiA* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>Fructose and mannose metabolism<br>Inositol phosphate metabolism<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |
| 26 | Q9Z519 | Phosphoglycerate kinase | IN | - | SCO1946 | *pgk* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 27 | Q9Z518 | Glyceraldehyde-3-phosphate dehydrogenase | OUT | UNKNOWN | SCO1947 | *gap* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>•Protein families: genetic information processing<br>Membrane trafficking<br>• Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |
| 28 | Q9Z513 | Nucleotide-binding protein SCO1952 | - | OUT | SCO1952 | / | •Protein families: genetic information processing<br>Messenger RNA biogenesis | Cytoplasmic |
| 29 | Q9S2U8 | Prolipoprotein diacylglyceryl transferase 1 | IN | IN | SCO2034 | *lgt1* | Unknown | Integral membrane |
| 30 | Q9S2X7 | Lipoprotein signal peptidase | IN | - | SCO2074 | *lspA* | •Folding, sorting and degradation<br>Protein export<br>•Protein families: metabolism<br>Peptidases | Integral membrane |
| 31 | Q9S2X5 | Isoleucine--tRNA ligase | - | IN | SCO2076 | *ileS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Cytoplasmic |
| 32 | Q9S2X2 | Cell division protein SepF 2 | IN | - | SCO2079 | *sepF2* | •Protein families: genetic information processing<br>Chromosome and associated proteins | Peripheral inner membrane protein facing the cytoplasm |

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| 33 | Q9ZBA5 | UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase | OUT | - | SCO2084 | *murG* | •Glycan biosynthesis and metabolism Peptidoglycan biosynthesis •Drug resistance: Antimicrobial Vancomycin resistance •Protein families: metabolism Peptidoglycan biosynthesis and degradation proteins | Peripheral inner membrane protein facing the cytoplasm |
| 34 | P56833 | Phospho-N-acetylmuramoyl-pentapeptide-transferase | IN | - | SCO2087 | *mraY* | •Glycan biosynthesis and metabolism Peptidoglycan biosynthesis •Drug resistance: Antimicrobial Vancomycin resistance •Protein families: metabolism Peptidoglycan biosynthesis and degradation proteins | Integral membrane |
| 35 | Q9S2N0 | Bacterioferritin | IN | IN | SCO2113 | *bfr* | •Metabolism of cofactors and vitamins Porphyrin and chlorophyll metabolism | Cytoplasmic |
| 36 | P80574 | Phospho-2-dehydro-3-deoxyheptonate aldolase | - | OUT | SCO2115 | *aroH* | •Amino acid metabolism Phenylalanine, tyrosine and tryptophan biosynthesis •Cellular community - prokaryotes Quorum sensing | Cytoplasmic |
| 37 | Q9X806 | Cytochrome bc1 complex cytochrome b subunit | IN | IN | SCO2148 | *qcrB* | •Energy metabolism Oxidative phosphorylation | Integral membrane |
| 38 | Q9X807 | Cytochrome bc1 complex Rieske iron-sulfur subunit | IN | IN | SCO2149 | *qcrA* | •Energy metabolism Oxidative phosphorylation | Integral membrane |
| 39 | Q9X813 | Probable cytochrome c oxidase subunit 1-alpha | IN | IN | SCO2155 | *ctaD1* | •Energy metabolism Oxidative phosphorylation | Integral membrane |
| 40 | Q9X814 | Probable cytochrome c oxidase subunit 2 | IN | IN | SCO2156 | *ctaC/cox* | •Energy metabolism Oxidative phosphorylation | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 41 | Q9S2R1 | Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase | OUT | - | SCO2175 | *cobT* | •Metabolism of cofactors and vitamins<br>Porphyrin and chlorophyll metabolism | Cytoplasmic |
| 42 | Q9S2Q7 | Probable cytosol aminopeptidase | OUT | IN | SCO2179 | *pepA* | •Metabolism of other amino acids<br>Glutathione metabolism<br>•Protein families: metabolism<br>Peptidases | Peripheral inner membrane protein facing the cytoplasm |
| 43 | P15106 | Glutamine synthetase | OUT | OUT | SCO2198 | *glnA* | •Carbohydrate metabolism<br>Glyoxylate and dicarboxylate metabolism<br>•Energy metabolism<br>Nitrogen metabolism<br>•Amino acid metabolism<br>Alanine, aspartate and glutamate metabolism<br>Arginine biosynthesis<br>•Signal transduction<br>Two-component system | Cytoplasmic |
| 44 | Q9L2H4 | Isoprenyl transferase 1 | IN | - | SCO2509 | *uppS1* | •Metabolism of terpenoids and polyketides<br>Terpenoid backbone biosynthesis<br>• Protein families: metabolism<br>Prenyltransferases | Cytoplasmic |
| 45 | Q9RDE2 | Tricorn protease homolog 1 | OUT | - | SCO2549 | *tri1* | •Protein families: metabolism<br>Peptidases | Cytoplasmic |
| 46 | Q9RDM3 | 30S ribosomal protein S20 | OUT | - | SCO2563 | *rpsT* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |

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|---|---|---|---|---|---|---|---|---|
| 47 | Q9L1I1 | 50S ribosomal protein L27 | OUT | OUT | SCO2596 | *rpmA* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 48 | Q9L1I0 | 50S ribosomal protein L21 | OUT | - | SCO2597 | *rplU* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 49 | O06851 | Valine--tRNA ligase | - | OUT | SCO2615 | *valS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Cytoplasmic |
| 50 | Q9ZH58 | ATP-dependent Clp protease proteolytic subunit 2 | OUT | - | SCO2618 | *clpP2* | •Protein families: metabolism<br>Peptidases | Cytoplasmic |
| 51 | Q9F315 | ATP-dependent Clp protease proteolytic subunit 1 | - | OUT | SCO2619 | *clpP1* | •Protein families: metabolism<br>Peptidases | Cytoplasmic |
| 52 | O51917 | Superoxide dismutase [Fe-Zn] 1 | IN | IN | SCO2633 | *sodF1* | Unknown | Cytoplasmic |
| 53 | Q9RDI1 | Ribose import ATP-binding protein RbsA 2 | OUT | - | SCO2746 | *rbsA2* | •Membrane transport<br>ABC transporters<br>•Protein families: signaling and cellular processes<br>Transporters | Peripheral inner membrane protein facing the cytoplasm |
| 54 | P54740 | Serine/threonine-protein kinase PkaB | OUT | - | SCO2973 | *pkaB* | •Protein families: metabolism<br>Protein kinases | Integral membrane |
| 55 | P54739 | Serine/threonine-protein kinase PkaA | IN | - | SCO2974 | *pkaA* | •Protein families: metabolism<br>Protein kinases | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 56 | P0A4G6 | Protein translocase subunit SecA | OUT | OUT | SCO3005 | *secA* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Cellular community - prokaryotes<br>Quorum sensing<br>•Protein families: signaling and cellular processes<br>Secretion system | Peripheral inner membrane protein facing the cytoplasm |
| 57 | Q9KZM1 | Adenosylhomocysteinase | - | OUT | SCO3023 | *ahcY* | •Amino acid metabolism<br>Cysteine and methionine metabolism<br>•Protein families: metabolism<br>Protein phosphatases and associated proteins<br>•Protein families: signaling and cellular processes<br>Exosome | Cytoplasmic |
| 58 | Q9F2Q3 | Enolase 1 | OUT | OUT | SCO3096 | *eno1* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>•Energy metabolism<br>Methane metabolism<br>•Folding, sorting and degradation<br>RNA degradation<br>•Protein families: genetic information processing<br>Messenger RNA biogenesis<br>•Protein families: signaling and cellular processes<br>Exosome | Cytoplasmic |

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|---|---|---|---|---|---|---|---|---|
| 59 | Q9K3U0 | Ribose-phosphate pyrophosphokinase | - | OUT | SCO3123 | *prs* | •Carbohydrate metabolism<br>Pentose phosphate pathway<br>•Nucleotide metabolism<br>Purine metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 60 | Q9RNU9 | Phosphoenolpyruvate carboxylase | UNKNOWN | UNKNOWN | SCO3127 | *ppc* | •Carbohydrate metabolism<br>Pyruvate metabolism<br>•Energy metabolism<br>Methane metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 61 | Q9RKA8 | Cyclic pyranopterin monophosphate synthase | - | IN | SCO3180 | *moaC* | •Metabolism of cofactors and vitamins<br>Folate biosynthesis<br>•Folding, sorting and degradation<br>Sulfur relay system | Cytoplasmic |
| 62 | Q9X895 | Lysine--tRNA ligase | - | OUT | SCO3303 | *lysS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Cytoplasmic |
| 63 | Q9WX29 | Arginine--tRNA ligase | OUT | UNKNOWN | SCO3304 | *argS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Cytoplasmic |
| 64 | Q9X8L6 | DNA integrity scanning protein DisA | OUT | OUT | SCO3352 | *disA* | Unknown | Cytoplasmic |
| 65 | Q9ZEP4 | Transcriptional regulatory protein CseB | - | OUT | SCO3358 | *cseB* | Unknown | Nucleoid |

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|---|---|---|---|---|---|---|---|---|
| 66 | Q9X913 | K(+)-insensitive pyrophosphate-energized proton pump | IN | IN | SCO3547 | *hppA* | •Protein families: signaling and cellular processes<br>Transporters | Integral membrane |
| 67 | Q9WVX8 | Anti-sigma-B factor antagonist | IN | OUT | SCO3549 | *rsbV/bldG* | •Protein families: genetic information processing<br>Transcription machinery | Cytoplasmic |
| 68 | Q9X928 | Acetyl-coenzyme A synthetase | OUT | OUT | SCO3563 | *acsA* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>Pyruvate metabolism<br>Glyoxylate and dicarboxylate metabolism<br>Propanoate metabolism<br>•Energy metabolism<br>Methane metabolism<br>•Protein families: metabolism<br>Lipid biosynthesis proteins | Peripheral inner membrane protein facing the cytoplasm |
| 69 | Q9X8R6 | Fructose-bisphosphate aldolase | UNKNOWN | - | SCO3649 | *fba* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>Pentose phosphate pathway<br>Fructose and mannose metabolism<br>•Energy metabolism<br>Methane metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 70 | Q05558 | Chaperone protein DnaK | UNKNOWN | OUT | SCO3671 | *dnaK* | •Folding, sorting and degradation<br>RNA degradation<br>•Protein families: genetic information processing<br>Messenger RNA biogenesis<br>Chaperones and folding catalysts<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 71 | Q9F2I9 | Methionine--tRNA ligase | OUT | OUT | SCO3792 | *metG* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Cytoplasmic |
| 72 | Q9F323 | Aspartate--tRNA ligase | OUT | UNKNOWN | SCO3795 | *aspS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Peripheral inner membrane protein facing the cytoplasm |
| 73 | Q9S2C0 | Serine/threonine-protein kinase PksC | UNKNOWN | - | SCO3821 | *pksC* | •Protein families: metabolism<br>Protein kinases | Integral membrane |
| 74 | Q9XA16 | Probable serine/threonine-protein kinase SCO3848 | IN | IN | SCO3848 | / | •Protein families: metabolism<br>Protein kinases | Integral membrane |
| 75 | P35885 | DNA gyrase subunit A | IN | UNKNOWN | SCO3873 | *gyrA* | •Protein families: genetic information processing<br>DNA replication proteins<br>DNA repair and recombination proteins | Cytoplasmic |
| 76 | P35886 | DNA gyrase subunit B | IN | OUT | SCO3874 | *gyrB* | •Protein families: genetic information processing<br>DNA replication proteins<br>DNA repair and recombination proteins | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 77 | P27903 | Beta sliding clamp | OUT | OUT | SCO3878 | *dnaN* | •Replication and repair<br>DNA replication<br>Mismatch repair<br>Homologous recombination<br>•Protein families: genetic information processing<br>DNA replication proteins<br>DNA repair and recombination proteins | Cytoplasmic |
| 78 | O54569 | Membrane protein insertase YidC | IN | IN | SCO3883 | *yidC* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Cellular community - prokaryotes<br>Quorum sensing | Integral membrane |
| 79 | Q9X8U3 | Single-stranded DNA-binding protein 2 | IN | IN | SCO3907 | *ssb2* | •Replication and repair<br>DNA replication<br>Mismatch repair<br>Homologous recombination<br>•Protein families: genetic information processing<br>DNA replication proteins<br>DNA repair and recombination proteins | Nucleoid |
| 80 | P66470 | 30S ribosomal protein S18 1 | UNKNOWN | - | SCO3908 | *rpsR1* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 81 | Q53868 | Uncharacterized protein SCO3922 | IN | - | SCO3922 | / | Unknown | Integral membrane |
| 82 | Q53867 | Uncharacterized protein SCO3923 | OUT | - | SCO3923 | / | Unknown | Cytoplasmic |

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|---|---|---|---|---|---|---|---|---|
| 83 | Q9ZBY8 | Putative phenylalanine aminotransferase | - | OUT | SCO3944 | *pat/hisC2* | •Amino acid metabolism<br>Histidine metabolism<br>Tyrosine metabolism<br>Phenylalanine metabolism<br>Phenylalanine, tyrosine and tryptophan biosynthesis<br>•Biosynthesis of other secondary metabolites<br>Novobiocin biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes | Cytoplasmic |
| 84 | Q9ZBX1 | Serine--tRNA ligase | - | OUT | SCO3961 | *serS* | •Translation<br>Aminoacyl-tRNA biosynthesis<br>•Protein families: metabolism<br>Amino acid related enzymes<br>•Protein families: genetic information processing<br>Transfer RNA biogenesis | Integral membrane |
| 85 | Q9KZV0 | Mycothiol acetyltransferase | IN | - | SCO4151 | *mshD* | Unknown | Cytoplasmic |
| 86 | P33158 | 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase | OUT | OUT | SCO4209 | *gpmA* | •Carbohydrate metabolism<br>Glycolysis / Gluconeogenesis<br>•Energy metabolism<br>Methane metabolism<br>•Amino acid metabolism<br>Glycine, serine and threonine metabolism<br>•Protein families: genetic information processing<br>Membrane trafficking<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 87 | Q9KXU5 | 60 kDa chaperonin 2 | OUT | OUT | SCO4296 | *groEL2* | •Folding, sorting and degradation<br>RNA degradation<br>•Protein families: genetic information processing<br>Messenger RNA biogenesis<br>Chaperones and folding catalysts<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |
| 88 | Q9XAQ7 | NADH-quinone oxidoreductase subunit D 2 | OUT | IN | SCO4565 | *nuoD2* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 89 | Q9XAQ9 | NADH-quinone oxidoreductase subunit F | IN | OUT | SCO4567 | *nuoF* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 90 | Q9XAR0 | NADH-quinone oxidoreductase subunit G | UNKNOWN | - | SCO4568 | *nuoG* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 91 | Q9XAR1 | NADH-quinone oxidoreductase subunit H | IN | - | SCO4569 | *nuoH* | •Energy metabolism<br>Oxidative phosphorylation | Integral membrane |
| 92 | Q9XAR5 | NADH-quinone oxidoreductase subunit L | IN | IN | SCO4573 | *nuoL* | •Energy metabolism<br>Oxidative phosphorylation | Integral membrane |
| 93 | Q9F2V2 | Protease HtpX homolog 2 | UNKNOWN | - | SCO4609 | *htpX2* | •Protein families: metabolism<br>Peptidases | Integral membrane |
| 94 | Q9F2U7 | UPF0234 protein SCO4614 | IN | - | SCO4614 | / | •Unclassified: signaling and cellular processes<br>Signaling proteins | Nucleoid |

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|---|---|---|---|---|---|---|---|---|
| 95 | P0A4G8 | Protein translocase subunit SecE | OUT | IN | SCO4646 | *secE* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Cellular community - prokaryotes<br>Quorum sensing<br>•Protein families: signaling and cellular processes<br>Secretion system | Integral membrane |
| 96 | P48950 | 50S ribosomal protein L1 | - | OUT | SCO4649 | *rplA* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 97 | P41109 | Putative lipoprotein SCO4651 | OUT | - | SCO4651 | / | Unknown | Secreted Lipoprotein |
| 98 | Q9L0L0 | DNA-directed RNA polymerase subunit beta | OUT | OUT | SCO4654 | *rpoB* | •Transcription<br>RNA polymerase<br>•Protein families: genetic information processing<br>Transcription machinery<br>DNA repair and recombination proteins | Peripheral inner membrane protein facing the cytoplasm |
| 99 | Q8CJT1 | DNA-directed RNA polymerase subunit beta' | OUT | UNKNOWN | SCO4655 | *rpoC* | •Transcription<br>RNA polymerase<br>•Protein families: genetic information processing<br>Transcription machinery<br>DNA repair and recombination proteins | Cytoplasmic |

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|---|---|---|---|---|---|---|---|---|
| 100 | P0A4A3 | 30S ribosomal protein S12 | UNKNOWN | OUT | SCO4659 | *rpsL* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 101 | Q9L0K4 | 30S ribosomal protein S7 | UNKNOWN | UNKNOWN | SCO4660 | *rspG* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 102 | P40173 | Elongation factor G 1 | - | OUT | SCO4661 | *fusA* | •Protein families: genetic information processing<br>Translation factors | Peripheral inner membrane protein facing the cytoplasm |
| 103 | P40174 | Elongation factor Tu-1 | UNKNOWN | UNKNOWN | SCO4662 | *tufI* | •Protein families: genetic information processing<br>Translation factors<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |
| 104 | P66337 | 30S ribosomal protein S10 | OUT | OUT | SCO4701 | *rpsJ* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 105 | Q9L0E0 | 50S ribosomal protein L3 | OUT | OUT | SCO4702 | *rplC* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |

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|---|---|---|---|---|---|---|---|---|
| 106 | Q9L0D9 | 50S ribosomal protein L4 | OUT | OUT | SCO4703 | *rplD* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 107 | Q9L0D7 | 50S ribosomal protein L2 | OUT | OUT | SCO4705 | *rplB* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 108 | Q9L0D6 | 30S ribosomal protein S19 | UNKNOWN | OUT | SCO4706 | *rpsS* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 109 | Q9L0D5 | 50S ribosomal protein L22 | OUT | OUT | SCO4707 | *rplV* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 110 | Q9L0D4 | 30S ribosomal protein S3 | UNKNOWN | UNKNOWN | SCO4708 | *rpsC* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 111 | Q9L0D3 | 50S ribosomal protein L16 | IN | OUT | SCO4709 | *rplP* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |

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|---|---|---|---|---|---|---|---|---|
| 112 | Q9L0D0 | 50S ribosomal protein L14 | OUT | UNKNOWN | SCO4712 | *rplN* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 113 | Q9L0C9 | 50S ribosomal protein L24 | - | UNKNOWN | SCO4713 | *rplX* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 114 | Q9L0C8 | 50S ribosomal protein L5 | UNKNOWN | UNKNOWN | SCO4714 | *rplE* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 115 | P66417 | 30S ribosomal protein S14 type Z | IN | IN | SCO4715 | *rpsZ* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 116 | P49399 | 30S ribosomal protein S8 | OUT | OUT | SCO4716 | *rpsH* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 117 | P46786 | 50S ribosomal protein L6 | OUT | IN | SCO4717 | *rplF* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |

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|---|---|---|---|---|---|---|---|---|
| 118 | P46788 | 50S ribosomal protein L18 | OUT | - | SCO4718 | *rplR* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 119 | P46790 | 30S ribosomal protein S5 | OUT | UNKNOWN | SCO4719 | *rpsE* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 120 | P46787 | 50S ribosomal protein L15 | OUT | OUT | SCO4721 | *rplO* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 121 | P46785 | Protein translocase subunit SecY | IN | IN | SCO4722 | *secY* | •Folding, sorting and degradation<br>Protein export<br>•Membrane transport<br>Bacterial secretion system<br>•Cellular community - prokaryotes<br>Quorum sensing | Integral membrane |
| 122 | P43414 | Adenylate kinase | OUT | - | SCO4723 | *adk* | •Nucleotide metabolism<br>Purine metabolism<br>•Metabolism of cofactors and vitamins<br>Thiamine metabolism<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 123 | O86773 | 30S ribosomal protein S13 | OUT | OUT | SCO4727 | *rpsM* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 124 | P72403 | 30S ribosomal protein S11 | OUT | OUT | SCO4728 | *rpsK* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 125 | P60312 | DNA-directed RNA polymerase subunit alpha | - | OUT | SCO4729 | *rpoA* | •Transcription<br>RNA polymerase<br>•Protein families: genetic information processing<br>Transcription machinery<br>DNA repair and recombination proteins | Peripheral inner membrane protein facing the cytoplasm, Nucleoid |
| 126 | O86775 | 50S ribosomal protein L17 | UNKNOWN | IN | SCO4730 | *rplQ* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 127 | Q53874 | 50S ribosomal protein L13 | UNKNOWN | UNKNOWN | SCO4734 | *rplM* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 128 | Q53875 | 30S ribosomal protein S9 | OUT | OUT | SCO4735 | *rpsI* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |

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|---|---|---|---|---|---|---|---|---|
| 129 | P40171 | 60 kDa chaperonin 1 | OUT | OUT | SCO4762 | *groEL1* | •Folding, sorting and degradation<br>RNA degradation<br>•Protein families: genetic information processing<br>Messenger RNA biogenesis<br>Chaperones and folding catalysts<br>•Protein families: signaling and cellular processes<br>Exosome | Peripheral inner membrane protein facing the cytoplasm |
| 130 | Q9KY56 | Succinate--CoA ligase [ADP-forming] subunit beta 1 | OUT | OUT | SCO4808 | *sucC1* | •Carbohydrate metabolism<br>Citrate cycle (TCA cycle)<br>Propanoate metabolism<br>C5-Branched dibasic acid metabolism | Peripheral inner membrane protein facing the cytoplasm |
| 131 | Q9K3J3 | Malate dehydrogenase | OUT | OUT | SCO4827 | *mdh* | •Carbohydrate metabolism<br>Citrate cycle (TCA cycle)<br>Pyruvate metabolism<br>Glyoxylate and dicarboxylate metabolism<br>•Energy metabolism<br>Methane metabolism<br>• Amino acid metabolism<br>Cysteine and methionine metabolism | Cytoplasmic |
| 132 | Q04943 | Signal transduction histidine-protein kinase AfsQ2 | OUT | - | SCO4906 | *afsQ2* | •Signal transduction<br>Two-component system<br>•Protein families: metabolism<br>Protein kinases<br>•Protein families: signaling and cellular processes<br>Two-component system | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 133 | Q04942 | Transcriptional regulatory protein AfsQ1 | OUT | OUT | SCO4907 | *afsQ1* | Unknown | Nucleoid |
| 134 | P46105 | Probable actinorhodin transporter | UNKNOWN | IN | SCO5083 | *actII-2* | Unknown | Integral membrane |
| 135 | Q53902 | Putative membrane protein ActII-3 | UNKNOWN | IN | SCO5084 | *actII-3* | Unknown | Integral membrane |
| 136 | P16544 | Putative ketoacyl reductase | OUT | - | SCO5086 | *actIII* | •Metabolism of terpenoids and polyketides<br>Biosynthesis of type II polyketide backbone<br>Tetracycline biosynthesis<br>•Protein families: metabolism<br>Lipid biosynthesis proteins<br>Polyketide biosynthesis proteins | Cytoplasmic |
| 137 | Q9FCI4 | UPF0182 protein SCO5204 | IN | IN | SCO5204 | / | Unknown | Integral membrane |
| 138 | Q7AKG9 | ECF RNA polymerase sigma factor SigR | IN | - | SCO5216 | *sigR* | •Protein families: genetic information processing<br>Transcription machinery | Cytoplasmic |
| 139 | P0A302 | ATP synthase protein I | - | IN | SCO5366 | *atpI* | •Protein families: metabolism<br>Photosynthesis proteins | Integral membrane |
| 140 | Q9K4D7 | ATP synthase subunit b | IN | UNKNOWN | SCO5369 | *atpF* | •Energy metabolism<br>Oxidative phosphorylation | Integral membrane |
| 141 | Q9K4D6 | ATP synthase subunit delta | IN | - | SCO5370 | *atpH* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 142 | Q9K4D5 | ATP synthase subunit alpha | IN | UNKNOWN | SCO5371 | *atpA* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |

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|---|---|---|---|---|---|---|---|---|
| 143 | Q9K4D4 | ATP synthase gamma chain | IN | - | SCO5372 | *atpG* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 144 | P0A300 | ATP synthase subunit beta | IN | UNKNOWN | SCO5373 | *atpD* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 145 | P0A2Z6 | ATP synthase epsilon chain | IN | - | SCO5374 | *atpC* | •Energy metabolism<br>Oxidative phosphorylation | Peripheral inner membrane protein facing the cytoplasm |
| 146 | Q9Z585 | DNA ligase 1 | OUT | - | SCO5494 | *ligA1* | •Replication and repair<br>DNA replication<br>Base excision repair<br>Nucleotide excision repair<br>Mismatch repair<br>•Protein families: genetic information processing<br>DNA replication proteins<br>DNA repair and recombination proteins | Cytoplasmic |
| 147 | Q9ZBR5 | 50S ribosomal protein L28-1 | UNKNOWN | UNKNOWN | SCO5564 | *rpmB1* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 148 | Q9ZBR1 | Phosphopantetheine adenylyltransferase | - | OUT | SCO5568 | *coaD* | •Metabolism of cofactors and vitamins<br>Pantothenate and CoA biosynthesis | Cytoplasmic |

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|---|---|---|---|---|---|---|---|---|
| 149 | Q9ZBQ8 | 50S ribosomal protein L32-1 | OUT | - | SCO5571 | *rpmF1* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 150 | O69879 | 30S ribosomal protein S16 | OUT | - | SCO5591 | *rpsP* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 151 | P0A477 | 50S ribosomal protein L19 | IN | IN | SCO5595 | *rplS* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 152 | O31212 | 30S ribosomal protein S2 | OUT | UNKNOWN | SCO5624 | *rpsB* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 153 | O86770 | Ribosome-recycling factor | UNKNOWN | - | SCO5627 | *frr* | •Protein families: genetic information processing<br>Translation factors | Peripheral inner membrane protein facing the cytoplasm |
| 154 | O86751 | Fe(3+) ions import ATP-binding protein FbpC | IN | - | SCO5648 | *fbpC* | •Membrane transport<br>ABC transporters<br>•Protein families: signaling and cellular processes<br>Transporters | Peripheral inner membrane protein facing the cytoplasm |
| 155 | Q9KYS0 | Putative zinc metalloprotease SCO5695 | UNKNOWN | IN | SCO5695 | / | Unknown | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 156 | Q8CJQ8 | Translation initiation factor IF-2 | OUT | - | SCO5706 | *infB* | •Protein families: genetic information processing<br>Translation factors | Cytoplasmic |
| 157 | O86655 | 30S ribosomal protein S15 | IN | IN | SCO5736 | *rpsO* | •Translation<br>Ribosome<br>•Protein families: genetic information processing<br>Ribosome | Ribosomal |
| 158 | Q8CJQ6 | Polyribonucleotide nucleotidyltransferase | OUT | OUT | SCO5737 | *pnp* | •Folding, sorting and degradation<br>RNA degradation<br>•Protein families: genetic information processing<br>Messenger RNA biogenesis<br>Transfer RNA biogenesis | Cytoplasmic |
| 159 | O86841 | 4-hydroxy-tetrahydrodipicolinate synthase 2 | OUT | - | SCO5744 | *dapA2* | •Amino acid metabolism<br>Lysine biosynthesis<br>•Biosynthesis of other secondary metabolites<br>Monobactam biosynthesis | Cytoplasmic |
| 160 | O69981 | Vitamin B12-dependent ribonucleotide reductase | - | OUT | SCO5805 | *nrdJ* | •Nucleotide metabolism<br>Purine metabolism<br>Pyrimidine metabolism<br>•Protein families: genetic information processing<br>DNA repair and recombination proteins | Cytoplasmic |
| 161 | P18183 | RNA polymerase principal sigma factor HrdB | IN | - | SCO5820 | *hrdB* | •Protein families: genetic information processing<br>Transcription machinery | Cytoplasmic |
| 162 | P0A4I1 | Transcriptional regulatory protein CutR | UNKNOWN | IN | SCO5862 | *cutR* | Unknown | Nucleoid |
| 163 | P0A4I7 | Sensor protein CutS | IN | IN | SCO5863 | *cutS* | Unknown | Integral membrane |

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|---|---|---|---|---|---|---|---|---|
| 164 | Q93JF1 | Ornithine carbamoyltransferase | - | OUT | SCO5976 | *argF/arcB* | •Amino acid metabolism<br>Arginine biosynthesis | Cytoplasmic |
| 165 | Q9FC36 | Undecaprenyl-diphosphatase 1 | IN | - | SCO7047 | *uppP1* | •Glycan biosynthesis and metabolism<br>Peptidoglycan biosynthesis<br>•Protein families: metabolism<br>Peptidoglycan biosynthesis and degradation proteins | Integral membrane |
| 166 | Q9FBT8 | Ketol-acid reductoisomerase (NADP(+)) 2 | - | OUT | SCO7154 | *ilvC2* | •Amino acid metabolism<br>Valine, leucine and isoleucine biosynthesis<br>•Metabolism of cofactors and vitamins<br>Pantothenate and CoA biosynthesis | Cytoplasmic |

**Table S2:** Description, abundance profile and functional classification of MV metabolites

| Metabolites | KEGG ID | Functional Class | F3 | F4 | F3 Vs F4 |
|---|---|---|---|---|---|
| Phenylalanine | C00079 | amino acids and amino acid precursors | 49 | 13.8 | +3.6 |
| 2-Dehydro-3-Deoxyphosphoheptonate | C04691 | amino acids and amino acid precursors | 128.05 | 122 | 1 |
| 6-Phosphogluconate | C04442 | components of carbon metabolism | 4.5 | 4.6 | 1 |
| Acetyl Coa | C00024 | components of carbon metabolism | 8.5 | 10.1 | -0.8 |
| Actinorhodin | C06691 | antibiotics | 159.9 | 77.2 | +2.1 |
| Adenosine | C00212 | components of purine metabolism | 206.05 | 257.95 | -0.8 |
| Arginine | C00062 | amino acids and amino acid precursors | 0.75 | 1.05 | -0.7 |
| Aspartic Acid | C00049 | amino acids and amino acid precursors | 91.7 | 85.35 | +1.1 |
| Biotin | C00120 | vitamins | 17 | 18.9 | -0.9 |
| CMP | C00055 | components of pyrimidine metabolism | 0.8 | 1.15 | -0.7 |
| D-Ribose 5-Phosphate | C00117 | components of carbon metabolism | 17.5 | 17.2 | 1 |
| Folate | C00504 | vitamins | 9.5 | 4.05 | +2.3 |
| Glucose 6 Phosphate | C00092 | components of carbon metabolism | 64.5 | 51.15 | +1.3 |
| Glutamine | C00064 | amino acids and amino acid precursors | 2.6 | 3..2 | -0.8 |

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|---|---|---|---|---|---|
| Glutammic Acid | C00025 | amino acids and amino acid precursors | 17 | 16.6 | 1 |
| GMP | C00144 | components of purine metabolism | 0.15 | 0.25 | -0.6 |
| Histidine | C00135 | amino acids and amino acid precursors | 3.5 | 4.6 | -0.8 |
| Isoleucine | C00407 | amino acids and amino acid precursors | 24.5 | 16 | +1.5 |
| Leucine | C00123 | amino acids and amino acid precursors | 18.3 | 10.25 | +1.8 |
| Lysine | C00047 | amino acids and amino acid precursors | 3.75 | 3.8 | 1 |
| Malonyl Coa | C00083 | components of carbon metabolism | 6.35 | 8.1 | -0.8 |
| Methionine | C00073 | amino acids and amino acid precursors | <0.5 | <0.5 | * |
| Ornithine | C00077 | amino acids and amino acid precursors | 4.15 | 3.5 | +1.2 |
| Oxalacetate | C00036 | components of carbon metabolism | 0.58 | 0.89 | -0.7 |
| Riboflavin | C00255 | vitamins | 11.25 | 15.15 | -0.7 |
| S-Adenosyl-Methionine | C00019 | amino acids and amino acid precursors | <0.5 | <0.5 | * |
| Serine | C00065 | amino acids and amino acid precursors | 4.4 | 2.85 | +1.5 |
| Thiamine | C00378 | vitamins | 549.75 | 592.35 | -0.9 |

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|---|---|---|---|---|---|
| Thryptophan | C00078 | amino acids and amino acid precursors | 15.25 | 10.85 | +1.4 |
| Tyrosine | C00082 | amino acids and amino acid precursors | <0.5 | <0.5 | * |
| UDP-N-Acetylmuramyl-Pentapeptide | C04702 | components of peptidoglycan biosynthesis | 0.9 | 0.9 | 1 |
| UMP | C00105 | components of pyrimidine metabolism | 0.95 | 1.1 | 0.9 |
| Undecyl Prodigiosin | C12023 | antibiotics | 7.05 | 5.15 | +1.4 |
| Valine | C00183 | amino acids and amino acid precursors | 4.45 | 4.25 | 1 |
| Vitamin B12 | C05776 | vitamins | 0.85 | 0.45 | +1.9 |
| 1,3-Bisphospho-D-Glycerate | C00236 | components of carbon metabolism | <0.01 | <0.01 | * |
| 2-Oxoglutarate | C00026 | components of carbon metabolism | <0.01 | <0.01 | * |
| 3-Phosphoglycerate | C00197 | components of carbon metabolism | <0.01 | <0.01 | * |
| 5-Phospho-Alpha-D-Ribose 1-Diphosphate | C00119 | components of carbon metabolism | <0.01 | <0.01 | * |
| Acetyl Phosphate | C00227 | components of carbon metabolism | <0.01 | <0.01 | * |
| Adenine | C00147 | components of purine metabolism | <0.01 | <0.01 | * |
| ADP | C00008 | components of purine metabolism | <0.01 | <0.01 | * |

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|---|---|---|---|---|---|
| Alanine | C00041 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| AMP | C00020 | components of purine metabolism | <0.01 | <0.01 | * |
| Anthranilate | C00108 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Calcium-Dependent Antibiotic | C12024 | antibiotics | <0.01 | <0.01 | * |
| Asparagine | C00152 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| ATP | C00002 | components of purine metabolism | <0.01 | <0.01 | * |
| Camp | C00575 | components of purine metabolism | <0.01 | <0.01 | * |
| Cgmp | C00942 | components of purine metabolism | <0.01 | <0.01 | * |
| Chorismate | C00251 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Cis-Aconitate | C00417 | components of carbon metabolism | <0.01 | <0.01 | * |
| Citrate | C00158 | components of carbon metabolism | <0.01 | <0.01 | * |
| Citrulline | C00327 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Coenzyme A | C00010 | components of carbon metabolism | <0.01 | <0.01 | * |
| CTP | C00063 | components of pyrimidine metabolism | <0.01 | <0.01 | * |

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|---|---|---|---|---|---|
| FAD | C00016 | coenzyme | <0.01 | <0.01 | * |
| D-Fructose 6-Phosphate | C00085 | components of carbon metabolism | <0.01 | <0.01 | * |
| D-Fructose 1,6-Bisphosphate | C00354 | components of carbon metabolism | <0.01 | <0.01 | * |
| Fumarate | C00122 | components of carbon metabolism | <0.01 | <0.01 | * |
| Gamma-Butyrolactone | C01770 | other | <0.01 | <0.01 | * |
| GDP | C00035 | components of purine metabolism | <0.01 | <0.01 | * |
| D-Glucosamine 6-Phosphate | C00352 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Glutaconate | C02214 | other | <0.01 | <0.01 | * |
| D-Glyceraldehyde 3-Phosphate | C00118 | components of carbon metabolism | <0.01 | <0.01 | * |
| Glycine | C00037 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| GTP | C00044 | components of purine metabolism | <0.01 | <0.01 | * |
| Guanine | C00242 | components of purine metabolism | <0.01 | <0.01 | * |
| Homocysteine | C00155 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| IMP | C00130 | components of purine metabolism | <0.01 | <0.01 | * |
| Inosine | C00294 | components of purine metabolism | <0.01 | <0.01 | * |

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|---|---|---|---|---|---|
| ITP | C00081 | components of purine metabolism | <0.01 | <0.01 | * |
| Malate | C00149 | components of carbon metabolism | <0.01 | <0.01 | * |
| Methylmalonyl Coa | C00083 | components of carbon metabolism | <0.01 | <0.01 | * |
| Methyl Malonate | C02170 | components of pyrimidine metabolism | <0.01 | <0.01 | * |
| N-Acetyl-D-Glucosamine | C00140 | components of peptidoglycan biosynthesis | <0.01 | <0.01 | * |
| N-Acetyl-Muramic | C02713 | components of peptidoglycan biosynthesis | <0.01 | <0.01 | * |
| NAD | C00003 | coenzyme | <0.01 | <0.01 | * |
| NADH | C00004 | coenzyme | <0.01 | <0.01 | * |
| NADPH | C00005 | coenzyme | <0.01 | <0.01 | * |
| Oxalate | C00209 | components of purine metabolism | <0.01 | <0.01 | * |
| Oxidized Glutathione | C00127 | other | <0.01 | <0.01 | * |
| Phenylpyruvate | C00166 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Phosphoenolpyruvate | C00074 | components of carbon metabolism | <0.01 | <0.01 | * |
| Polyphosphate | C00404 | other | <0.01 | <0.01 | * |
| Proline | C00148 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Propionyl_Coa | C00100 | other | <0.01 | <0.01 | * |
| Pyruvate | C00022 | components of carbon metabolism | <0.01 | <0.01 | * |

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|---|---|---|---|---|---|
| Reduced Glutathione | C00051 | other | <0.01 | <0.01 | * |
| S-Adenosyl-Homocysteine | C00021 | amino acids and amino acid precursors | <0.01 | <0.01 | * |
| Succinate | C00042 | components of carbon metabolism | <0.01 | <0.01 | * |
| Succinyl_Coa | C00091 | components of carbon metabolism | <0.01 | <0.01 | * |
| UDP | C00015 | components of pyrimidine metabolism | <0.01 | <0.01 | * |
| UDP-Glucuronate | C00167 | other | <0.01 | <0.01 | * |
| UDP-N-Acetylglucosamine | C00043 | components of peptidoglycan biosynthesis | <0.01 | <0.01 | * |
| Uracil | C00106 | components of pyrimidine metabolism | <0.01 | <0.01 | * |
| Uridine | C00299 | components of pyrimidine metabolism | <0.01 | <0.01 | * |
| UTP | C00075 | components of pyrimidine metabolism | <0.01 | <0.01 | * |
| Thymine | C00178 | components of pyrimidine metabolism | <0.01 | <0.01 | * |