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Mini Review

Excretion of cytosolic proteins (ECP) in bacteria

Friedrich Götz*, Wenqi Yu, Linda Dube, Marcel Prax, Patrick Ebner

Microbial Genetics, Interfaculty Institute for Microbiology and Infection Medicine Tübingen (IMIT), University of Tübingen, 72076 Tübingen, Germany

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ABSTRACT

Excretion of cytosolic proteins (ECP) has been reported in bacteria and eukaryotes. As none of the classical signal peptide (SP) dependent or SP-independent pathways could be associated with ECP, it has been also referred to as 'non-classical protein export'. When microbiologists first began to study this subject in 1990, mainly singular cytoplasmic proteins were investigated, such as GAPDH at the cell surface and in the supernatant of pathogenic streptococci or glutamine synthetase (GlnA) as a major extracellular protein in pathogenic mycobacteria. Later, with the rising popularity of proteomics, it became obvious that the secretome of most bacteria contained a copious amount of cytosolic proteins. In particular ancient proteins such as glycolytic enzymes, chaperones, translation factors or enzymes involved in detoxification of reactive oxygen were found in the supernatants. As the excreted proteins do not possess a common motive, the most widespread opinion is that ECP is due to cell lysis. Indeed, upregulation of autolysins or distortion of the murein structure increased ECP, suggesting that enhanced ECP is some sort of survival strategy to counteract osmotic stress. However, in the meantime there are mounting evidences and hints that speak against cell lysis as a primary mechanism for ECP. Very likely, ECP belongs to the normal life cycle of bacteria and involves a programmed process. This review provides a brief overview of the 'non-classical protein export'.

Introduction

The cytoplasmic membrane (CM) is the most dynamic structure of bacterial cells. Its main function is the formation of a selective permeability barrier that regulates the passage of substances into and out of the cell. It allows the undirected transition of water and uncharged molecules up to MW of about 100 Da, but does not allow the passage of larger molecules or any charged substances except by means of special transport systems. Proteome analysis of the *Staphylococcus aureus* membrane revealed that exponential growing cells contain at least 270 integral proteins (Becher et al., 2009) and approximately 30% of the encoded proteome (±2600 proteins) could be secreted (Kusch and Engelmann, 2014). Normally, proteins that are translocated over the cytoplasmic membrane are distinguished by appropriate signal peptides and are translocated by defined transport systems. However, there is an increasing number of typical cytosolic proteins described which do not have a signal sequence and are still found extracellularly. It is hotly debated whether the release of such proteins is due to cell lysis or whether they are exported by a so far unknown mechanism (Wang et al., 2013).

In eukaryotes such proteins were secreted distinct from the classical ER-Golgi route and the pathway was referred to as 'non-classical protein export' (Muesch et al., 1990). At least four distinct types of nonclassical export were distinguished (Nickel, 2003): (a) for IL-1β, En2 (transcription factor engrailed homeoprotein isoform 2) and HMGB1 (intra-nuclear factor that mediates the assembly of site-specific DNA-binding proteins within chromatin), export involves import into intracellular vesicles, which are probably endosomal sub-compartments (Rubartelli et al., 1992, 1990); (b) FGF-1 and 2 (fibroblast growth factor 1 and 2) probably reach the extracellular space by direct translocation across the plasma membrane; (c) the *Leishmania* cell surface protein HASPB is also translocated directly across the plasma membrane via dual acylation at the N-terminus and using a flip-flop mechanism to localize the protein in the outer leaflet of the plasma membrane; (d) the final postulated pathway of non-classical export involves exosomal vesicles formed on the outer surface of the cell in a process known as membrane blebbing. Exosomes are labile structures that release their contents into extracellular space. It has been suggested that this pathway may be used by galectins (Nickel, 2003). One possible

Abbreviations: ECP, excretion of cytosolic proteins; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGN, peptidoglycan; SP, signal peptide; *S.*, *Staphylococcus*; Sec, major secretion system; WTA, wall teichoic acid.

* Corresponding author at: Mikrobielle Genetik, Universität Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany. Tel.: +49 7071 2974636; fax: +49 7071 295937.
E-mail address: friedrich.goetz@uni-tuebingen.de (F. Götz).

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benefit of the 'non-classical protein export' in eukaryotes could be that this export system is a way to clear unfolded proteins from the cytoplasm (Sloan et al., 1994).

For ECP in eukaryotes, the term '*moonlighting proteins*' has also been coined (Jeffery, 1999). Moonlighting refers to a single protein that has multiple functions. For example the mammalian thymidine phosphorylase catalyzes the intracellular dephosphorylation of thymidine but acts outside as a platelet-derived endothelial cell growth factor, which stimulates endothelial cell growth and chemotaxis (Jeffery, 1999). Most moonlighting proteins represent evolutionarily conserved (ancient) enzymes. The glycolytic enzymes, GAPDH and enolase and the cell stress proteins chaperonin 60, Hsp70 and peptidyl prolyl isomerase, are among the most common of the bacterial moonlighting proteins. They play a role in bacterial virulence, since they are involved in adhesion and modulation of cell signaling processes. An overview of moonlighting proteins deriving from bacteria and their role in bacterial virulence is given by (Henderson and Martin, 2013).

Early observation of excretion of cytosolic proteins (ECP) in bacteria

One of the first reports that typical cytosolic proteins are found on bacterial cell surface came from Vincent Fischetti's group. They found that the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is present in large amounts on the cell surface of pathogenic streptococcal groups (Pancholi and Fischetti, 1992) and also in the supernatant of several bacteria, fungi and even protozoans (Pancholi and Chhatwal, 2003). Interestingly, this GAPDH functions also as an ADP-ribosylating enzyme catalyzing the NAD-dependent, auto-ADP-ribosylation at its cysteine residue via a thioglycosidic linkage (Pancholi and Fischetti, 1993), a modification that is stimulated by nitric oxide (NO). GAPDH is a very 'sticky' protein as it binds to various human proteins, including plasmin(ogen) (D'Costa and Boyle, 2000; Lottenberg et al., 1992; Winram and Lottenberg, 1996), lysozyme, myosin, actin, fibronectin (Pancholi and Fischetti, 1992) and PAR/CD87 on pharyngeal cells (Jin et al., 2005). GAPDH also stimulates B-lymphocytes and induces an early IL-10 production that facilitates host colonization (Madureira et al., 2007). In group B streptococci (GBS) GAPDH acts as an inducer of apoptosis of murine macrophages (Oliveira et al., 2012). Moreover, in enterohemorrhagic and enteropathogenic *E. coli* GAPDH was exposed on surface where it binds to human plasminogen and fibrinogen, suggesting a role in pathogenesis (Egea et al., 2007).

Besides GAPDH, a number of other cytosolic proteins have been found on the surface or to be excreted, such as α-enolase (Pancholi and Fischetti, 1998), glucose-6-phosphate isomerase (Hughes et al., 2002), glutamine synthetase (Suvorov et al., 1997), ornithine carbamoyltransferase (Hughes et al., 2002), fibrinogen-binding protein A of *Listeria monocytogenes* (Dramsi et al., 2004) or Fbp54 of *Streptococcus pyogenes* (Courtney et al., 1996). All these proteins do not possess a traditional signal peptide and appear to be typical 'moonlighting' proteins with different intra- and extracellular activities.

Secretome analysis revealed much more cytosolic proteins

Release of typical cytosolic proteins into the culture supernatant is not restricted to individual species; as it has been observed in Gram-positive and Gram-negative bacteria such as staphylococci, streptococci, *Bacillus subtilis*, *Listeria monocytogenes* or *E. coli*. In particular, glycolytic enzymes, chaperones, translation factors or enzymes involved in detoxification of reactive oxygen species were found in the supernatants by secretome analysis (Li et al., 2004; Sibbald et al., 2006; Tjalsma et al., 2004; Trost et al., 2005; Xia et al., 2008).

Proteome analyses were carried out in staphylococci to study the expression of cytosolic proteins under biofilm and anoxic growth conditions (Fuchs et al., 2007; Resch et al., 2006), of growing and non-growing cells (Kohler et al., 2005) or of global regulator mutants such as *agr*, sigmaB and *clpC* (Chatterjee et al., 2009; Ziebandt et al., 2004, 2001). Only later, secretome studies were also conducted to show that a number of typical cytosolic proteins were present in the culture supernatants of *B. subtilis* and *S. aureus* (Sibbald et al., 2010; Tjalsma et al., 2004; Ziebandt et al., 2004). A recent study compared the exoproteomes of three different *S. epidermidis* strains (Siljamaki et al., 2014). Approximately 80% of the proteins identified in their analysis belonged to the cytoplasmic fraction. Interestingly, strain specificity with respect to the protein composition could be made, hypothesizing a possible correlation of pathogenicity and the level of ECP.

Only certain cytosolic proteins are excreted

A comparative proteomic analysis of cytosolic and culture supernatant proteins demonstrated that in mid-exponential culture of *S. aureus* SA113, quite a number of cytosolic proteins were found in the secretome, while many other cytosolic proteins were missing (Pasztor et al., 2010). Using a 2D-PAGE gel, cytosolic proteins of SA113 were separated and proteins found also in the secretome were labeled blue, while those solely found in the cytoplasm were labeled red (Fig. 1, adapted from Pasztor et al., 2010). Notably, highly expressed cytosolic proteins such as Fhs, GuaB, SA0802 (Ndh-2), EF-TS, GlnA, PdhD, SucC were *not* found in the secretome. Table 1 lists excreted and non-excreted proteins. Two conclusions can be drawn from this observation: (a) there is no correlation between the quantity and the excretion level of cytosolic proteins and (b) a specific selection procedure in the excretion of cytosolic proteins has to exist.

In this context an interesting observation has been made with the glycolytic enolase of *E. coli*. Like in other bacteria, the enolase from *E. coli* is also excreted. The enzymatic reaction involves a transient covalent binding of the substrate 2-phosphoglycerate (2-PG) to the active site Lys341. Replacement of Lys341 with other amino acids not only prevented the automodification but also the export of enolase (Boel et al., 2004). One of the enolase mutants (K341E) was almost as active as the wild-type enzyme and still was not exported, suggesting that the enolase export was correlated with the loss of modification and not the loss of glycolytic activity. This is one of the strongest examples that excretion of cytosolic proteins is selective; this selectivity speaks against the indiscriminate excretion by cell lysis. The unsolved question is however, via which transport system cytosolic proteins could be excreted. For this reason the known transport systems in *S. aureus* are briefly addressed.

Specific protein transport systems in Gram-positive bacteria

The main protein/peptide transport systems can be grouped in signal peptide dependent and signal peptide independent systems (Fig. 2). The signal peptide dependent systems can be subdivided into the Sec translocation system, representing the major secretion system, and the twin-arginine translocation system (Tat). Proteins are translocated through this pathway in a more or less unfolded state (Driessen and Nouwen, 2008) and are targeted to the Sec translocon via their N-terminal signal peptide (von Heijne, 1990). The Sec translocon is also used for the translocation of lipoproteins, which are distinguished by their own signal peptide (Babu et al., 2006). To answer the question whether the Sec pathway is involved in ECP, a *secA*-temperature sensitive *B. subtilis* mutant was used to

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