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Review

Bacterial Excretion of Cytoplasmic Proteins (ECP): Occurrence, Mechanism, and Function

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The excretion of cytoplasmic and signal-peptide-less proteins (ECP) by microorganisms and eukaryotes remains a fascinating topic. In principle, it appears to be a waste of energy. However, it turns out that – extracellularly – some cytoplasmic proteins (CPs) exert a completely different function such as contributing to pathogenicity or evasion of the immune system. Such CPs have been referred to as 'moonlighting' proteins. ECP is boosted by many endogenous or external factors that impair the membrane or cell wall structure. There are also differences regarding their mode of release. In some microorganisms they appear to be released directly, while in others they are embedded in membrane vesicles, or bound to the cell envelope. Some CPs might be promising candidates for vaccine developments against major bacterial pathogens.

Highlights

Cytoplasmic proteins (CPs) maintain the metabolism in living cells and therefore fulfill their main function inside the cell. Although they lack a signal peptide, they are found in the supernatants of prokaryotes and eukaryotes.

The release of CPs is promoted by many factors, such as mutations, environmental changes, membrane distortion, cell-wall integrity or osmotic balance.

The CPs may be released either directly into the environment or embedded in membrane vesicles.

Some released CPs are immunogenic, and vaccination trials with selected antigens have yielded promising results.

Some CPs show extracellular activity which differs from their intracellular activity – for example, by acting as adherence factors, killing host cells, contributing to biofilm formation, or immune evasion.

What Is Excretion of CPs (ECP)?

CPs fulfill versatile roles in bacterial physiology. They are involved in central anabolic and catabolic metabolism, protein biosynthesis and folding, DNA replication, and many more functions. However, some CPs are also released into the supernatant. This has been shown by many studies which identified CPs in the secretome of both Gram-positive and Gram-negative bacteria [1–8] as well as in that of eukaryotes [9,10]. At first glance, the release of CPs seems to be a waste of energy and resources since their physiological function is based in the cytoplasm. Whether this release is caused accidentally by cell lysis within a bacterial population, or whether CPs are actively secreted, remains hotly debated [11]. However, the search for mechanisms allowing the release of CPs and their potential extracellular roles and functions has gained increasing attention in recent years. In this review we describe and discuss the advances and new insights gained on this topic. We focus on mechanisms by which bacteria release CPs to the extracellular milieu and on their 'moonlighting' function, which they may exert extracellularly.

Extracellular CPs – How Do They Get There?

Cell Lysis, Autolysins, and Cell-Wall Integrity as Contributing Factors for the Release of CPs

Different mechanisms for the release of CPs (excretion) have been described, and cell lysis is an often-discussed reason for such release [11]. In Group B streptococci (*Streptococcus agalactiae*) the amount of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) on the cell surface and in the supernatant was strongly decreased in pilus-deficient *pilB*$^{-}$ and *pilA/C*$^{-}$ mutants [12,13]. These pilus-deficient mutants showed a lower level of cell lysis, which seems to be responsible for the decreased amount of extracellular GAPDH. In general, it can be concluded that the induction of bacterial cell lysis increases the release of GAPDH in streptococci [12]. A similar effect was shown for *Streptococcus pneumoniae*, where a portion of the GAPDH was released by cell lysis and

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bound to the cell surface by direct interaction of GAPDH with peptidoglycan (PGN) [14] (Figure 1A). Cell lysis or defective cell division seems to play a certain role in the release of CPs by *Staphylococcus aureus* as well. In addition, in an *atlA* (major autolysin) mutant in which the release of GAPDH and FbaA (fructose-bisphosphate aldolase) was severely affected [15,16] the expression analysis showed no altered expression of GAPDH, meaning that the decreased release was not due to decreased expression [16]. Not only increased cell wall hydrolase activities enhanced

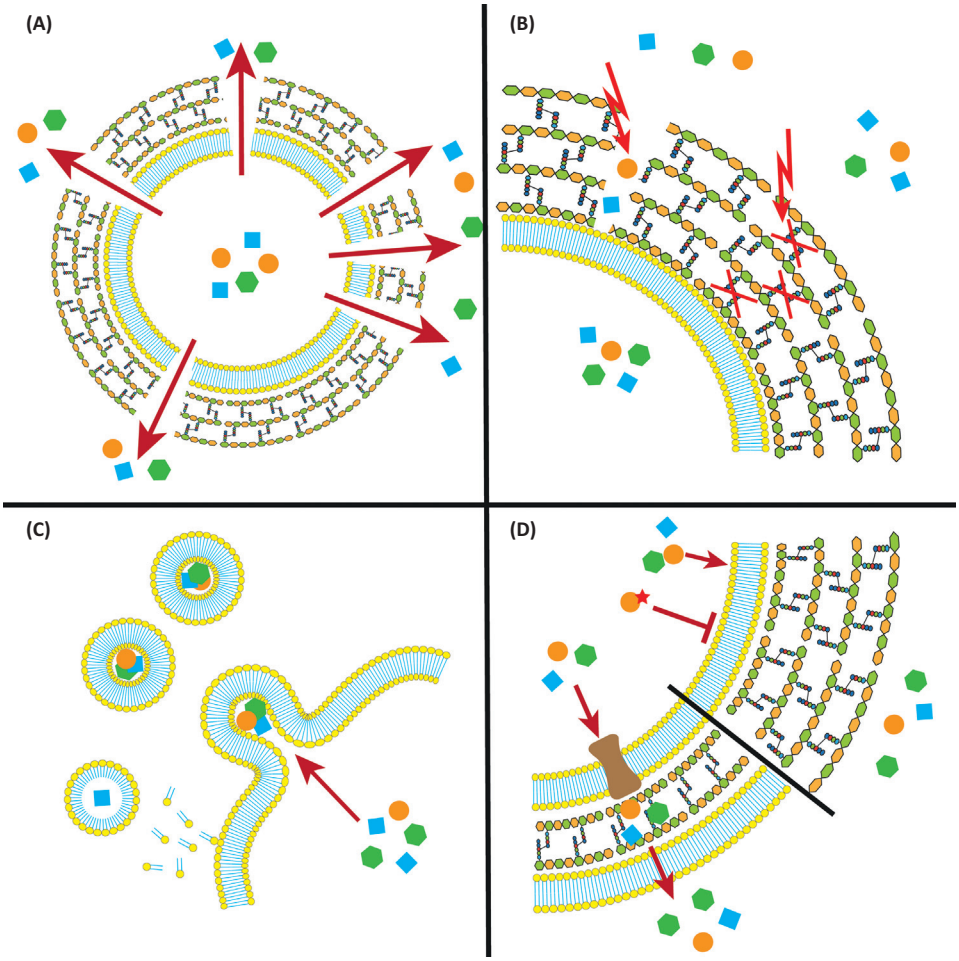

Figure 1. Excretion and Release Routes for Cytoplasmic Proteins (CPs). (A) Cell lysis is a major contributor to the release of CPs in many bacteria. Cell lysis can be due to either environmental or cellular factors – which then consequently cause the excretion or release of cytoplasmic compounds such as proteins and DNA. (B) Weakening of the cytoplasmic membrane or cell wall by autolysins or membrane-acting agents – either produced endogenously or applied exogenously – can lead to decreased cell integrity and consequently the release of CPs. (C) Membrane- or outer-membrane-derived vesicle formation is a contributing factor for the excretion of CPs. The biogenesis of outer-membrane vesicles (OMVs) occurs by different mechanisms. However, a common feature is that OMVs often contain a large amount of protein, including a large number of CPs. (D) Biochemical properties – such as hydrophobicity, or structural features like intramolecular helices – can influence the excretion behavior of CPs (upper part). Translational and osmotic stress – overexpression of recombinant proteins or treatment with antibiotics – can lead to activation of mechanosensitive channels and permeabilization of the membranes, thus leading to the release of CPs.

excretion of CPs but also cell wall integrity itself plays an important role. This has been shown in a *femB* mutant of *Staphylococcus carnosus*, which has a shortened glycine bridge between the stem-peptides of the staphylococcal PGN. Deletion of *femB* consequently results in decreased PGN crosslinking, thus decreasing the compactness of the murein sacculus [16]. We assume that any maceration of the cell wall causes the cytoplasmic membrane to be hypersusceptible to osmotic changes. Most likely the cell membrane cannot resist the usually hypotonic environment and releases osmolytes together with proteins in order to keep up the osmotic balance (Figure 1B). Cell wall hydrolases and cell wall integrity also play a role in *Pseudomonas aeruginosa* in which the bacteriophage-like endolysin Lys is responsible for a mechanism named 'explosive cell lysis'. This mechanism leads to the formation of membrane vesicles (MVs) in which DNA (eDNA) and CPs are embedded. The deletion of *lys* reduced cell lysis markedly [17]. Additionally, a comparable mechanism was described for the endolysin PBSX in *Bacillus subtilis*. The prophage-encoded endolysin generates holes in the PGN in a subpopulation of cells, through which membrane bubbles protrude to form MVs containing CPs, DNA, and RNA. This mechanism, however, does not include exploding *B. subtilis* cells [18], which might be caused by the remaining integrity of the thicker PGN layer in *B. subtilis* compared to that in *P. aeruginosa*.

Membrane Integrity and (Outer)-Membrane-Derived Vesicles (OMVs)

Besides cell wall integrity and cell lysis, the integrity of the cytoplasmic membrane has been shown to play an important role in the release of CPs. In *S. aureus*, small cytolytic peptide toxins, the phenol-soluble modulins (PSMs) play a crucial role in CP release [15] and lipoprotein mobilization [7]. Furthermore, the α-type, but not the β-type, PSMs or δ-toxin are responsible for the nonspecific release of CPs, which agrees with other studies identifying α-type PSMs as the most cytolytic PSMs [19]. The release of cytoplasmic compounds, such as proteins and ATP, was indeed due to membrane perturbance and leakiness [15]. Recently, PSMs were shown – together with cell-wall integrity – to play a role in the formation of MVs in *S. aureus*; such MVs contained up to 165 proteins, including several CPs [20], making the PSMs a major contributor to CP release in *S. aureus*. However, not only endogenously produced PSMs but also antibiotic treatment or mutants impaired in cell-wall biosynthesis promote production of MVs [20–24].

In addition to *S. aureus*, MVs occur in other Gram-positive pathogens, such as *Listeria monocytogenes, Bacillus anthracis*, and *Streptococcus* spp., and they fulfill versatile roles in infections. However, a common feature of all of these MVs was the occurrence of incorporated CPs [20,25–27]. In *L. monocytogenes* hemolytic MVs contain listeriolysin (LLO) and phosphatidylcholine-specific phospholipase (PC-PLC), which represent Sec-secreted proteins. Beside those virulence factors, many CPs were found associated with MVs [25,28]. Interestingly, the formation and protein content of MVs was strongly influenced by the alternative sigma factor SigB. A *sigB* deletion mutant formed nine times less MVs with only 89 proteins associated, while the wild-type MVs contained up to 130 proteins [28]. Both MVs share 80 commonly found proteins [28], including several CPs [25,28]. In streptococci, MVs have been identified for *S. agalactiae* [26], *S. pneumoniae* [29], and *Streptococcus pyogenes* [30]. In *S. agalactiae*, MVs consist of DNA and proteins. They are loaded with virulence factors, including extracellular matrix-degrading proteases and pore-forming toxins. As a consequence, they are toxic to both mouse fetal and maternal cells. *S. agalactiae*-derived MVs cause a disruption of the connective tissue of the fetal membrane, reducing its mechanical strength, which can cause premature rupture of the amniotic sac. Protein analysis of these MVs revealed 22 individual protein bands on SDS-PAGE from which 8 were further analyzed. Proteins located in the cell membrane, secreted and one cytoplasmic protein, and GroEL (a chaperonin that is found in a large number of bacteria, where it is involved in proper folding of many proteins, and an often-

found released CP), have been identified by mass spectrometry [26]. MVs of *S. pneumoniae* contained different numbers (43 up to 153) of CPs [29,31]. Some of the CPs were immunogenic, and immunization studies using isolated MVs showed a protective effect against *S. pneumoniae* infections in mice [29,31]. The numbers of MVs depended on an intact two-component system, *covRS*. CovRS negatively regulates the production of MVs; an astonishingly high number of 905 proteins were associated with such MVs. Most of the identified proteins (>50%) were CPs involved in nucleotide metabolism, glycolysis, and the TCA/PP pathway [30].

In contrast to Gram-positive bacteria, many Gram-negative bacteria form (outer)-membrane-derived vesicles (OMVs) which frequently contain CPs [20,32–35]. For instance, OMVs from *Cronobacter sakazakii* can be incorporated by eukaryotic cells. They induce cell proliferation and an innate immune response without exerting a cytotoxic effect. In total, 18 proteins have been identified in such OMVs, which can be grouped in classically sec-dependent secreted proteins, outer-membrane proteins, and CPs (such as GroEL, DnaK, and EF-Tu) [35,36]. Accordingly, an analysis of OMVs of pathogenic (ETBF) and non-toxic (NTBF) strains of *Bacteroides fragilis* revealed a high content of CPs that were involved in carbohydrate and amino acid metabolism, the citrate cycle, and cell division. Although the pattern of CPs was different, the OMVs of both strains contained about 30 CPs in common [32] (Figure 1C). Another interesting example of a Gram-negative bacterial CP is the nonclassically secreted cytotoxic necrotizing factor 1 (CNF1) of *Escherichia coli*, which has no signal peptide but is first translocated to the periplasm and subsequently released by OMVs [37–39]. Transposon mutagenesis revealed a gene encoding ferredoxin (*fdx*). In a *fdx* deletion mutant no CNF1 was found in the periplasm, suggesting an important role for ferredoxin in the translocation of CNF1 across the cytoplasmic membrane [38]. The subsequent release of CNF1 is then accomplished by the release of CNF1 containing OMVs [39], and this release is positively influenced by the periplasmic protein YgfZ [37].

Additional Factors Contributing to the Release of CPs

Many other factors influence or contribute to the release of CPs. For example, in *E. coli* under conditions of osmotic and translation stress, caused by recombinant protein overexpression or antibiotic treatment, there is enhanced release of CPs. Those stress factors caused activation of the mechanosensitive channel (MscL), which leads to an increased permeability of both the inner and outer membrane, thus allowing release of CPs without affecting cell viability (Figure 1D) [40]. These results agree with the observation that, upon a sudden drop in osmolarity, *E. coli* cells with a permeabilized outer membrane release certain CPs into the surrounding medium – for example, elongation factor Tu [EF-Tu], thioredoxin, and DnaK (a molecular chaperone). Interestingly, proteins larger than 100 kDa were retained, whereas smaller proteins were selectively released, suggesting a sieve effect of the PGN layer [41]. It is therefore not surprising that antibiotics targeting the cell wall or the membrane promote ECP [42–44]. However, ECP can be boosted not only by the external exposure of bacteria to antimicrobials, it can also be boosted by endogenously produced membrane- or cell-wall-active compounds. The staphylococcal lantibiotic producers, *Staphylococcus epidermidis* and *Staphylococcus gallinarum,* which produce epidermin and gallidermin respectively, have been shown to be sensitive to their own product. Mutants with a deletion in the structural gene (*epiA* or *gdmA*) showed a significantly lower release of CPs than the corresponding parent strains [45].

In addition to cellular effects playing a role in the release of CPs, the biochemical properties of the CPs themselves also seem to be of importance. In *B. subtillis*, Est55 (a carboxylesterase

from *Geobacillus stearothermophilus*), GroEL, DnaK, enolase, pyruvate dehydrogenase subunits PdhB and PdhD, and SodA (a superoxide dismutase, which destroys superoxide anion radicals) have been identified in the supernatant. This study concluded that cell lysis was not the major reason for the release of CPs since not all CPs were found in the supernatant; additionally, there was no effect on the release of the CPs in mutants lacking autolysins [46]. Interestingly, SodA of *Rhizobium leguminosarum* bv. *viciae 3841* was reported to be secreted to the periplasm of *E. coli* in a SecA-dependent manner, despite lacking an N terminal signal peptide [47]. However, a hydrophobic α-helical domain within enolase turned out to be important and contributed to its release [46]; mutations within its hydrophobic α-helical domains resulted in the loss of release. On the other hand, fusion of the hydrophobic helix domains with green fluorescence protein (GFP) was not sufficient as an export signal; a minimum of 140 aa were necessary for mediating excretion [48].

Another interesting finding was observed in *B. subtilis.* When d-psicose-3-epimerase (RDPE) from *Ruminococcus* sp. was overexpressed it folded into a multimeric protein complex important for its release; such release was mostly independent of cell lysis. By using alanine scanning mutagenesis, the C and N terminus and two hydrophobic domains were identified as necessary motifs for structural stability and excretion; in this example, multimerization was important for the excretion of RDPE [49]. This mechanism of excretion could be used for heterologous protein expression and production in *B. subtilis*. Some fusion proteins of RDPE and proteins from other bacteria, and proteins from eukaryotic organisms, were successfully secreted into the extracellular milieu [50].

Overall, many CPs have been reported to be excreted by several different excretion routes; however, more proteins and excretion routes might be discovered in the future. The roles of those proteins, after excretion, are discussed in the following section.

The Roles of Excreted Cytoplasmic Proteins

As described above, CPs can reach the extracellular milieu by different excretion routes; however, a common feature of many excreted CPs is the different functions that they exert in the extracellular environment as compared to the intracellular environment. Therefore, released CPs are often referred to as having 'moonlighting' protein functions [51–57]. Many released CPs act as adhesins or invasins, promoting attachment to, or entry into, host cells. Some support the establishment or maintenance of biofilms, contributing to immune evasion, and some are immunogenic. Known moonlighting functions for different proteins from various bacterial species can be found in the MoonProt database available at moonlightingproteins.org [58,59].

CPs as Adhesins

CPs that act as adhesins are described mainly for Gram-positive bacteria but also for some Gram-negatives, where CPs are both excreted and bound to the bacterial cell surface. CPs in Gram-positive species have been reported in *S. aureus* [60–62], *Streptococcus* spp. [12,14,63,64], *Listeria monocytogenes* [65], *Mycobacterium tuberculosis* [66,67], *Enterococcus faecalis* [68], and *Acinetobacter baumanii* [69]. Particularly surface-exposed CPs – such as GAPDH, aldolase, enolase, DnaK, EF-Tu, Cpn60, and others – were reported to bind to host cells or matrix proteins [70] (Figure 2A). For example, the surface-exposed GAPDH of *M. tuberculosis* acts as a receptor for host-cell lactoferrin (Lf), which facilitates the sequestering of human Lf. Interestingly, GAPDH activity was not necessary for the binding to Lf [67]. Other CPs of *M. tuberculosis* are surface-associated, especially glycolytic enzymes such as enolase; enolase not only binds to plasminogen it also converts plasminogen to plasmin [66]. Similarly, in

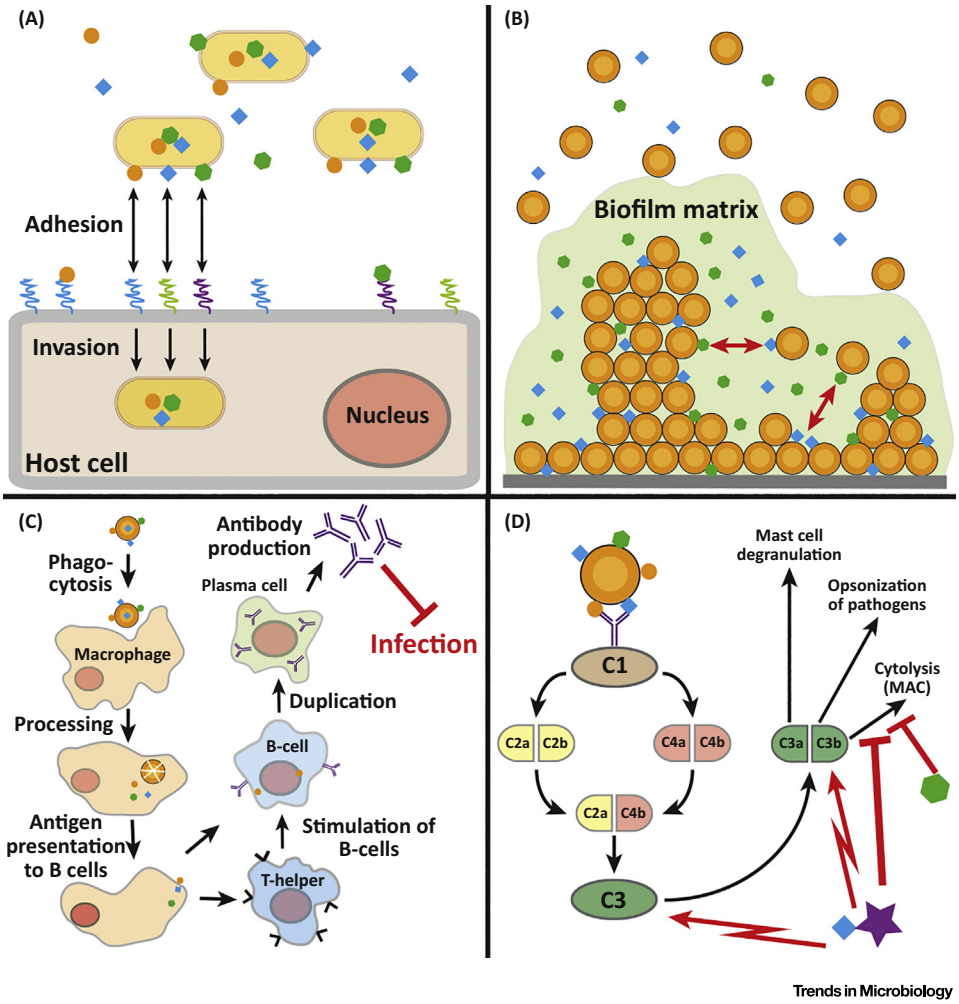

Figure 2. The Roles of Excreted Cytoplasmic Proteins (CPs). (A) Excreted CPs can often be found bound to the bacterial surface, exhibiting adhesion- or invasion-promoting functions by binding to the host cell matrix proteins or by direct binding to host cell surfaces. (B) Extracellular CPs can be frequently found embedded in bacterial biofilm matrices. They are thought to mediate cell–cell contact and thereby strengthen the biofilms' integrity and robustness against environmental changes. (C) CPs are often immunogenic, and antibodies against several CPs can be found to be present in human serum. Vaccination experiments and some of these anti-CP antibodies have been shown to have a protective effect against bacterial infections. (D) Some CPs have been reported to have complement-inhibitory effects – for example, inhibition of the MAC formation, which then leads to an increased survival of the bacteria in the presence of human serum.

S. aureus, GAPDH and aldolase are also surface-associated; they can bind to host-matrix proteins, such as plasminogen and fibrinogen, and enhance the adhesion of *S. aureus* cells to host cells in a concentration-dependent manner [60]. In streptococci, the endopeptidase PepO shows surface exposure [71] and is a receptor for plasminogen and fibronectin, promoting bacterial adhesion to host cells [72]. Consequently, a *pepO*-mutant was impaired in adherence and invasion of host cells [72]. In *Streptococcus suis* approximately 20 surface-associated CPs have been identified to interact with laminin or fibrinogen and they contribute to binding to Hep-2 cells; among those were OppA, EF-Tu, enolase, LDH, aldolase (FBA), and GAPDH [73]. Another frequently found moonlighting protein is EF-Tu, which is released by numerous bacteria, fulfilling versatile roles. EF-Tu from the human pathogens *S. aureus* (SaEF-Tu),

Mycoplasma pneumoniae (MpnEF-Tu), and the porcine pathogen *Mycoplasma hyopneumoniae* (MhpEF-Tu), is surface-exposed in all of these bacteria, and it undergoes several processing steps on the surface. Recombinant MpnEF-Tu bound to a diverse range of host molecules with high affinity, and when bound to plasminogen it converted plasminogen (in the presence of activators) to plasmin [74].

Most moonlighting proteins represent evolutionarily conserved (ancient) proteins, such as the glycolytic enzymes, GAPDH and enolase, and the cell-stress proteins chaperonin 60, heat-shock protein (Hsp70), peptidyl prolyl isomerase, or EF-Tu; it was therefore speculated that these ancient proteins had to fulfill originally diverse tasks [70,75,76].

Released CPs as Part of Bacterial Biofilms

Chronic bacterial infections often go along with biofilm formation, explaining the interest in understanding their architecture and physiology. Both Gram-positive and Gram–negative organisms can form robust biofilms, surrounded in a matrix comprising exopolysaccharides, DNA, and proteins [77–80]. The fact that treatment with protease and DNase reduced biofilm formation suggests an important role for both macromolecules in biofilm formation or maintenance. Interestingly, protease treatment showed a stronger effect, leading to the conclusion that proteins fulfill a crucial role in biofilms [61]. Surprisingly, released CPs, but not secreted or surface-anchored proteins, were found largely embedded in the biofilm matrix [61,81]. For example, in *S. aureus* the surface-associated GAPDH and enolase are thought to mediate cell–cell contact, which contributes to the formation of a stronger and more resistant biofilm [61] (Figure 2B). In *Propionibacterium acnes*, enolase alongside the 30S ribosomal protein S4, and DNA-binding protein HU, were also identified as part of the biofilm matrix. However, protease treatment of these biofilms showed sensitivity of some, but not all, biofilms of different strains, leading to the hypothesis that not all isolates depend on CPs in establishing robust biofilms [82].

Gram-negative bacteria also release CPs that assemble with the biofilm matrix [17,83–86]. In *P. aeruginosa* a portion of CPs is embedded in OMVs, and these are also part of the biofilm matrix. CPs may be released from the OMVs and then enter the biofilm matrix [83,86], an event which might be caused by mechanical destruction or enzymatic degradation of the OMVs [17]. The role of CPs in the biofilm matrix of *P. aeruginosa* is not fully clear; however, it is assumed that they bind antibiotics from the environment and hence lower their effective concentration. In particular, ribosomal proteins, targets of many translation-inhibiting antibiotics, and their excreted counterparts could neutralize such antibiotics outside the cells, making them less harmful for the cells [84]. CPs also comprise a major component in the oleolytic biofilm of the marine bacterium *Marinobacter hydrocarbonoclasticus*, and the addition of proteinase K at the beginning of growth partly impaired and delayed biofilm development [87]. This hypothesis is supported by the loss of structural integrity and mechanical strength observed upon protease treatment. The release of CPs to the biofilm matrix is most likely due to regulated autolysis, which is supported by the increased permeability of biofilm-embedded cells as shown by Live/Dead staining [87].

Many CPs Are Immunogenic – But Are They Potential Vaccine Candidates?

The versatile roles of released CPs involve not only bacterial physiology but also the host immune system. Many moonlighting CPs have been shown to be immunogenic (Figure 2C). It is therefore not astonishing that vaccination experiments, using CPs as antigens, have shown protection against bacterial infections [88–93]. For example, pooled human immunoglobulins contained antibodies against surface-exposed proteins of *S. pyogenes*, including DnaK, enolase, GAPDH, aldolase, or EF-Tu. Consequently, treatment with purified antistreptococcal

antibodies resulted in promoting opsonophagocytic killing of *S. pyogenes in vitro*, and vaccination induced a passive immunity in mice [94]. These findings were in line with two studies investigating the usage of recombinant-FbaA (rFbA) of *S. pneumoniae* and recombinant-GAPDH (rGAPDH) of group B streptococci as potential vaccine candidates [95,96]. Indeed, mice vaccinated with rGAPDH, and subsequently challenged with group B streptococci, showed lower bacterial loads in their organs (brain, blood, heart, and liver) and a decreased mortality compared to the nonimmunized animals [95]. Similar results were obtained with vaccination with rFbA, which showed a decreased mortality after challenge with *S. pneumoniae*. In general, animals pretreated with anti-rFbA antibodies or vaccinated with rFbA antigen survived significantly longer than the control animals. This could be due to an increased proliferation of $CD4^+$ T cells and an increased production of Th1-, Th2-, and Th17-type cytokines [96]. A similar protecting effect was observed after immunization of mice with recombinant Eno from *M. tuberculosis* which protected mice from infections and decreased CFU counts in the lung [66]. Another frequently found released CP – EF-Tu from *Borrelia burgdorferi* – has also been shown to be highly immunogenic in mice, and, in addition, antibodies against EF-Tu could be detected in patients suffering from lyme disease. However, immunization with *B. burgdorferi*-specific EF-Tu antibodies showed no protective effect against *B. burgdorferi* infections, questioning the role of EF-Tu during Lyme disease caused by *B. burgdorferi*; nevertheless, EF-Tu antibody detection might be used as a serological marker for Lyme disease [90]. In Gram-negative bacteria, immunogenic CPs are released as well. The heat-shock protein, Hsp60, of *E. coli*, *Histophilus somni*, *Pasteurella multocida*, and *Salmonella enteritidis*, stimulated the immune system and could therefore be a promising component in a multiple-antigen vaccine to protect from infections caused by Gram-negative bacteria [92].

Immune Evasion Mediated by Released CPs

Besides their immunogenicity, several CPs have been shown to help bacteria to evade the host's immune system. CPs have anti-inflammatory activities [97], suppress macrophage activation [98], and inhibit or interact with parts of the complement system [99–101]. The complement system is an important part of the innate immune system that combats infections caused by pathogens. However, bacteria have evolved factors that can inhibit this system, including classically secreted virulence factors as well as released CPs. One example of the latter is the cytoplasmic hydrogen peroxide-neutralizing enzyme catalase (KatA), which moonlights on the surface of *Helicobacter pylori* by binding to vitronectin (Vn). This interaction protects *H. pylori* from complement-mediated killing, as indicated by an increased killing of a *katA*-deficient mutant when exposed to human serum. Furthermore, the depletion of Vn from the serum resulted in a similar killing pattern for both wild-type and *katA* mutant, and deletion of *katA* increased the deposition of the membrane attack complex (MAC) [99]. This is a consequence of the interference of Vn with MAC formation, which is due to inhibition of the C5b–C7 complex and polymerization of C9. All of this results in preventing cell lysis by MAC, which particularly lyses Gram-negative bacteria [99,102]. A comparable moonlighting function was identified for the phosphoglycerate kinase (PGK) from *S. pneumoniae*. PGK is like KatA; it is surface-exposed and binds plasminogen and the tissue plasminogen activator tPA. It interacts with the components C5, C7, and C9 of MAC, leading to a decreased cytolytic activity of human serum. Moreover, the complex – consisting of plasmin(ogen) and PGK – was able to cleave the complement protein C3b, thereby counteracting the complement attack [103]. However, *S. pneumoniae* harbors an additional moonlighting CP, EF-Tu, that interferes with the host's complement system. Similar to PGK, it is surface-exposed and is capable of binding to human complement inhibitors Factor H, FHL-1, CFHR1, and plasminogen. Factor H and FHL-1, bound to EF-Tu, were still able to regulate complement activities, and the plasminogen–EF-Tu complex was accessible to the activator uPA. Activated plasmin cleaved the natural

substrates fibrinogen and the complement proteins C3 and C3b. Altogether, EF-Tu is a multifunctional moonlighting protein that helps *S. pneumoniae* to escape the complement attack [104]. A similar complement-escape function was described for the surface-bound EF-Tu of *Leptospira* [100] (Figure 2D).

However, complement inhibition is not the only immune system modulating the moonlighting function of CPs. For instance, *S. aureus* releases a lipoylated metabolic protein, lipoyl-E2-PDH, which suppresses macrophage activation and thereby acts as a macrophage immunosuppressant. This is caused by the reduction of Toll-like receptor 1/2 (TLR1/2) activation by bacterial lipopeptides. A LipA-deficient mutant induced an increased production of proinflammatory cytokines and restricted macrophage activation via TLR2. Furthermore, the release of Lipoyl-E2-PDH happened to coincide with the observed macrophage suppression, and further evidence was given that Lipoyl-E2-PDH is sufficient for this activation. Additionally, mutants lacking LipA were attenuated in virulence [98], speaking in favor of an important role for LipA during infections.

Concluding Remarks

Almost 30 years have passed since the first description of the extracellular occurrence of glyceraldehyde-3-phosphate-dehydrogenase in certain streptococci [105]. At that time it was thought that this phenomenon is an accidental and unique feature of *S. pyogenes*. Today, we know that ECP is found in almost all microorganisms, Gram-positive and Gram-negative bacteria, and fungi. Increased ECP is observed when microorganisms are exposed to stressors that act on the membrane or cell wall, thus interfering with osmotic balance. Stressors can be exogenous compounds such as membrane- and cell-wall-active antibiotics; they can also be endogenously expressed membrane-active peptides such as PSMs or cell-wall-active antibiotics. Upregulation of autolysins, or any kind of mutation that impairs cell-wall structure, increases ECP. However, even without such endogenous and exogenous stressors we believe that microorganisms undergo phases during cell division in which it is difficult for the cells to maintain an osmotic balance – particularly if membrane and cell wall biosynthesis is not yet fully completed (see Outstanding Questions). In order to keep the osmotic balance, they release small solutes up to the size of ATP as well as lipids and proteins. This would explain why, in a non-PSM-producing *S. aureus* strain, an *agr* mutant, the highest ECP was observed in the mid-exponential phase with the highest cell division rate [106]. It looks as if this kind of ECP – together with the release of small solutes – is an immanent feature of all growing microorganisms, similar to the vital feature of breathing. It is unlikely that ECP is programmed to survive better during an infection or during other unfavorable environmental changes. But there is mounting evidence that the 'moonlighting' function of some excreted CPs contribute to survival.

Outstanding Questions

Is the release of CPs a controlled mechanism or does it happen accidentally during the life cycle of microorganisms?

Is it only the increased membrane permeability or are there specific mechanisms that cause a selective release of CPs?

In various studies it was shown that CPs were not randomly excreted; are those CPs, particularly highly charged solutes, better able to counteract osmotic stress?

Is ECP an immanent feature of all microorganisms, like breathing in animals?

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