MINIREVIEW

Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis

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Abstract

Bacterial exopolymeric substances (EPS) are molecules released in response to the physiological stress encountered in the natural environment. EPS are structural components of the extracellular matrix in which cells are embedded during biofilm development. The chemical nature and functions of these EPS are dependent on the genetic expression of the cells within each biofilm. Although some bacterial matrices have been characterized, understanding of the function of the EPS is relatively limited, particularly within the *Bacillus* genus. Similar gaps of knowledge exist with respect to the chemical composition and specific roles of the macromolecules secreted by *Bacillus subtilis* in its natural environment. In this review, the different EPS from *B. subtilis* were classified into four main functional categories: structural (neutral polymers), sorptive (charged polymers), surface-active and active polymers. In addition, current information regarding the genetic expression, production and function of the main polymers secreted by *B. subtilis* strains, particularly those related to biofilm formation and its architecture, has been compiled. Further characterization of these EPS from *B. subtilis* remains a challenge.

Introduction

Microbial exopolymeric substances (EPS) include a wide diversity of molecules released by microorganisms in their natural environment as well as under laboratory conditions (Flemming *et al.*, 2004; Dupraz & Visscher, 2005; Aguilar *et al.*, 2007). Although initially the term EPS was used to describe extracellular polysaccharides, recent studies have revealed that these matrixes are more complex, including lipopolysaccharides, glycolipids, lipids, proteins or peptides and nucleic acids (Wingender *et al.*, 1999; Decho, 2000). This complex structure comprises the exopolymeric matrix in which cells are embedded, and is also referred to as the biofilm (O'Toole & Ghannoum, 2004). The chemical composition of the EPS depends on the genetics of the microbial cells and the physicochemical environment in which the biofilm matrix develops (Sutherland, 2001a). Consequently, environmental conditions ultimately dictate the key properties of the biofilms such as porosity, density, water content, charge, sorption and ion exchange properties, hydrophobicity and mechanical stability (Wingender *et al.*, 1999).

Substances associated with exopolymeric matrices have multiple functions. Some serve as signaling molecules or messengers and others are energy and nutrient reserves with an important role in polymer degradation and surface adhesion (O'Toole & Ghannoum, 2004; Decho *et al.*, 2010). Recently, the polyelectrolytic nature of some of these molecules has been described with concomitant use in the fabrication of nanowires (Dobrynin, 2008; Lovley, 2008). Although EPS are common to bacteria and critical in cell survival, they are relatively poorly studied, especially with respect to the matrix composition in natural environments (Davey & O'Toole, 2000). In this review, some of the current information on the EPS of *Bacillus subtilis* is compiled. The role of these molecules within natural environment is also discussed. The focus is on *B. subtilis* because it is ubiquitous, present in almost all ecosystems and the EPS produced by this organism have significant ecological relevance with respect to cell survival and differentiation within a biofilm (Earl *et al.*, 2008). As shown in Supporting Information, Table S1, a wide variety of EPS are secreted by *B. subtilis* strains. In the review process, the lack of classification of the

FEMS MICROBIOLOGY LETTERS

main EPS from *B. subtilis* was noticed. It is often unclear whether a particular polymer under investigation is produced by all wild-type strains of *B. subtilis* or is unique to a particular isolate. Several hundred wild-type *B. subtilis* strains have been collected to date, only some of which have the potential to produce different types of EPS. One caveat in these studies is that strains able to secrete polymeric substances are not genetically characterized and those genetically characterized are defective in EPS production. For example, *B. subtilis* 168 is the most studied type strain, is used in many laboratories and industrial processes and is an excellent candidate for genetic studies. It is easy to transform, it grows under planktonic conditions, its genome has been sequenced (Kunst *et al.*, 1997) and its proteome has been characterized (Wolff *et al.*, 2007). Unfortunately, *B. subtilis* 168 produces only a few antibiotics and it is defective or attenuated in EPS production (Stein *et al.*, 2004; Aguilar *et al.*, 2007). Several of the biosynthetic pathways are not functional because of the domestication processes (i.e. mutations that allow easier genetic manipulations coupled with repeated growth under artificial settings). The *B. subtilis* 168 strain derives from X-ray mutations of the original Marburg strain (Burkholder & Giles, 1947; Chu *et al.*, 2006; Earl *et al.*, 2007). In contrast, various other *B. subtilis* wild-type strains produce numerous peptide antibiotics as well as abundant EPS (Stein, 2005). In this review, EPS described are specifically matched with the actual *Bacillus* strains responsible for its production (Table S1).

Main characteristics of EPS by *B. subtilis*

EPS produced by wild-type *B. subtilis* strains grown under controlled laboratory conditions exhibit a wide range of sizes (with molecular weights ranging from 0.57 to 128 kDa) and chemical compositions (i.e. neutral polysaccharides, charged polymers, amphiphilic molecules and proteins) (Priest, 1977; Lin *et al.*, 1999; Omoike & Chorover, 2004). Fourier-transformed infrared spectroscopy studies of cell-bound and 'free' EPS (in aqueous phase) from *B. subtilis* ATCC7003 grown in Luria broth showed that the composition of the functional groups of the matrix depends on the cell growth phase (e.g. exponential vs. stationary) (Omoike & Chorover, 2004). Greater amounts of free EPS (relative to cell-bound EPS) are produced during the stationary phase. Quantification of the types of macromolecules within these matrices indicated that proteins and carbohydrates are the major constituents of EPS by mass, with protein levels increasing in free EPS as growth proceeded from the exponential to the stationary phase (Omoike & Chorover, 2004). More detailed investigations are needed to explore differences in the abundance and composition of the proteins, acidic groups and sugars of the biofilms of *Bacillus* grown under specific conditions. Additional knowledge of the chemical composition and three-dimensional architecture of the biofilms will aid in solving practical problems in industrial and medical applications and will also help in the classification of EPS based on function.

Categories of EPS produced by *B. subtilis* according to function

Flemming *et al.* (2007) proposed seven categories of EPS: structural, sorptive, surface-active, active, informative, redox-active and nutritive EPS. However, only four of these classes occur in molecules identified in *B. subtilis*: the categories include structural, sorptive, surface-active and active EPS (Table S1). Structural EPS refer to molecules such as neutral polysaccharides, which serve as architectural components in the matrix, facilitating water retention and cell protection. Sorptive EPS are composed of charged polymers, whose function is sorption to other charged molecules involved in cell–surface interactions. Surface-active EPS are molecules with an amphiphilic behavior. These molecules, with different chemical structures and surface properties, are involved in biofilm formation and sometimes possess antibacterial or antifungal activities. The active EPS group is the most diverse group and includes all extracellular proteins produced by *B. subtilis*. Only those enzymes required for biofilm formation and architecture are discussed.

Structural EPS (neutral polysaccharides)

Structural EPS are mainly composed of neutral polysaccharides that lend structure to the exopolymeric matrix. These exopolysaccharides are formed in the biofilm matrix of many bacterial species for example *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Morikawa *et al.*, 2006; Ryder *et al.*, 2007). However, only a few studies report the isolation and identification of exopolysaccharides from *B. subtilis*.

The best-studied exopolysaccharide produced by *B. subtilis* is levan type I and II. Levan type I consists of β-2,6-linked D-fructose units, whereas type II is a fructose polymer with a glucose residue linked to the terminal fructose by α-glycoside bond. Levan can be synthesized outside the cell following the extrusion of the extracellular enzyme levansucrase (Abdel-Fattah *et al.*, 2005; El-Refai *et al.*, 2009). Further details on levansucrase extrusion and induction are included in the section describing active EPS. Levan is widely distributed and produced by various plants and microorganisms including *B. subtilis* strains 327UH, ISS3119, QB112 and *Pseudomonas* sp. (Yamamoto *et al.*, 1985; Pereira *et al.*, 2001; Shida *et al.*, 2002). In *Pseudomonas*, it has been suggested that levan forms a capsule protecting against the attack of bacteriophages and also helps prevent cell

desiccation (Paton, 1960). Capsule formation draws nutrients by attracting solutes and creating an osmotic gradient until equilibrium is reached (Paton, 1960). Another ecological role of levan has been described for *Paenibacillus* (formerly *Bacillus*) *polymyxa* CF43, where this polysaccharide facilitates the aggregation of root-adhering soil on wheat plants (Bezzate *et al.*, 2001).

Different exopolysaccharides have been reported in other *B. subtilis* strains such as strain FT-3 (Morita *et al.*, 1979). Although specific roles for these polysaccharides have not been proposed, they are known to be comprised of glucose, galactose, fucose, glucuronic acid and *O*-acetyl groups in an approximate molar ratio of 2 : 2 : 1 : 1 : 1.5 (Morita *et al.*, 1979). Information regarding the genes encoding the proteins that make these exopolysaccharides is also limited.

yhxB is a gene related to the synthesis of an uncharacterized exopolysaccharide component of the *B. subtilis* biofilm matrix and putatively encodes an α-phosphoglucomutase and/or phosphomannomutase (Branda *et al.*, 2004). In *B. subtilis* 3610, a deletion in *yhxB* is responsible for the production of a fragile surface pellicle when grown in a liquid culture and flat undifferentiated colonies when grown on solid media. On the contrary, the *B. subtilis* wild-type strain shows a robust pellicle in liquid culture and colonies on plates with web-like structures (i.e. bundled structures).

Other genes important in matrix structure and biofilm architecture include the 16 genes of the *eps* operon (*yveK-yvfF*) involved in polysaccharide biosynthesis, modification and export (Branda *et al.*, 2001). From sequence comparisons, two genes belonging to the *eps* operon, named *epsG* (*yveQ*) and *epsH* (*yveR*), may be involved in the synthesis of exopolysaccharides. *epsG* encodes a protein that is presumably involved in EPS polymerization, while *epsH* encodes a glycosyl-transferase (Branda *et al.*, 2001). *eps* mutants in *B. subtilis* 3610 show a reduction in the carbohydrate content and complexity of biofilm pellicle (Branda *et al.*, 2006). Blair *et al.* (2008) have recently demonstrated that another member of this *eps* operon, the EpsE protein, is an inhibitor of cell motility. Despite the extensive study of the *eps* operon and its role, the structure and function of the polysaccharides resulting from the expression of these genes remain unknown. Characterization of this polysaccharide and its regulation awaits further investigations.

Sorptive EPS

The second category of EPS secreted by *B. subtilis* includes a polymer, which plays a role in the sorption of ions and/or charged molecules. Poly-γ-glutamate (γ-PGA) produced by *B. subtilis* strain IFO3336 is a well-characterized anionic, nontoxic and biodegradable viscous polymer of D- and L-monomers with a molecular mass of over 10 000 kDa. The γ-PGA of *B. subtilis* (*natto*) is composed 50–80% of D- and 20–50% of L-glutamate (Ashiuchi *et al.*, 1999; Morikawa *et al.*, 2006; Inbaraj *et al.*, 2008). γ-PGA is synthesized by several *Bacillus* species, especially wild-type isolates, including *B. subtilis* strains IFO3336, IFO3335, TAM-4, F-2-01, B-1 (mucoid-type colonies), ZJU-7, *B. subtilis* (*natto*) and *Bacillus anthracis* (Kubota *et al.*, 1993; Kunioka, 1995; Ito *et al.*, 1996; Shi *et al.*, 2006).

The *pgsBCA* genes are responsible for the synthesis of γ-PGA. The PgsBCA system is the sole machinery for γ-PGA synthesis, whose *in vitro* polymerization reaction is given by the equation:

$$[\gamma\text{-PGA}]_n + \text{Glutamate} + \text{ATP} \xrightarrow{\text{PgsBCA complex}} [\gamma\text{-PGA}]_{n+1} + \text{ADP} + \text{P}_\text{i}$$

Ashiuchi *et al.* (2001) showed that the reaction is dependent on the presence of membrane fractions of recombinant *E. coli* carrying *B. subtilis pgsBCA* genes. No γ-PGA was produced if cytosolic or other extracellular fractions were used in the *in vitro* assay, indicating that a membrane association was required. The enzyme complex remains attached to the cell membrane while γ-PGA is secreted by the cell. The PgsA protein can function as a γ-PGA transporter, indicating an important role in the elongation of the γ-PGA polymer (Ashiuchi *et al.*, 2001). The production of γ-PGA was repressed by the sporulation-specific transcription factor Spo0A. Even though the *pgsBCA* operon is highly regulated, γ-PGA is not essential for cell growth and biofilm formation (Branda *et al.*, 2006). The sequences of *pgsBCA* genes have been found to be similar to those of the *ywsC* and *ywtAB* genes of *B. subtilis* 168 (Urushibata *et al.*, 2002).

As described, the synthesis of γ-PGA requires energy, posing an interesting question: what is the advantage to the cell? Stanley & Lazazzera (2005) proposed that γ-PGA is involved in biofilm formation to enhance cell–surface interactions through salt bridges (e.g. Ca^{2+} or Mg^{2+}) as intermediaries between negative-charged cell surfaces. The *in vitro* production of γ-PGA could also be activated during biofilm formation in response to an increase in the salinity and osmolarity of the medium resulting from evaporation of water during a long duration of incubation. In *B. anthracis* the production of γ-PGA results in the formation of a capsule and is correlated to the virulence of the strain (Candela & Fouet, 2006). However, in spite of some detailed studies, the specific role of γ-PGA in natural environments needs to be further clarified and investigations are needed to assess the presence of other sorptive EPS.

Surface-active EPS

The third category of EPS includes surface-active lipopeptides, such as surfactin, which are among the most-studied molecules produced by *B. subtilis* (Flemming *et al.*, 2007).

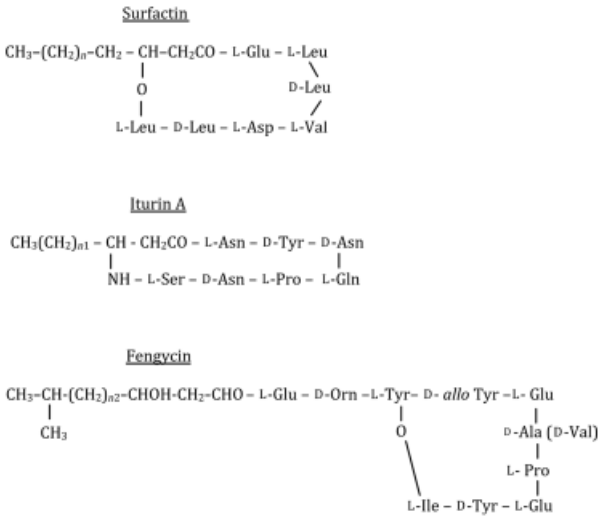

Fig. 1. Schematic structure of surface-active EPS: surfactin, iturin A and fengycin. Carbon chain length of the fatty acid in iturin A. n, 8–10; n_1 is generally 14 (myristate); n_2, 16–19.

On the basis of the structural relationships, lipopeptides have been classified into three groups: the surfactin group, the iturin group and the plipastatin–fengycin group (Tsuge *et al.*, 2001) (Fig. 1). Although these surfactants are not large polymeric compounds, they play a very important role in solubilizing substrates that otherwise would be inaccessible to the bacteria (Neu, 1996; Sutherland, 2001b). Synthesis of lipopeptides does not occur on ribosomes, but is catalyzed by large complex peptide synthetases protein structures (Lin *et al.*, 1999). Even though surfactants exist in nature in both low- and high-molecular-weight forms, only the low-molecular-weight forms are found in *B. subtilis* (Ron & Rosenberg, 2001).

The lipopeptide surfactins are the most important surfactants studied in *B. subtilis* (Fig. 1). Surfactin and a peptide signal ComX are involved in biofilm formation in *Bacillus* (Connelly *et al.*, 2004; Hofemeister *et al.*, 2004; López *et al.*, 2009). ComX is a quorum-sensing peptide pheromone that triggers the production of surfactin. The lipopeptide is then involved in a paracrine signaling pathway that triggers a subpopulation of cells to produce an extracellular matrix. Interestingly, the surfactin-producing cells do not produce a matrix themselves, but upstream activation of *com*X is needed for biofilm production (Magnuson *et al.*, 1994; López *et al.*, 2009). It is still unclear how ComX-producing cells activate surfactin synthesis and how surfactin can then trigger matrix production.

In *B. subtilis* 168 strains, single-base duplications in *sfp* genes cause impairment in surfactin production (Zeigler *et al.*, 2008). This mutation also produces losses of swarming and affects the speed of colonization (Julkowska *et al.*, 2005). *sfp* encodes a phosphopantetheinyl transferase that activates the peptidyl carrier protein domain of the first three subunits (SrfABC) of surfactin synthetase (Quadri *et al.*, 1998). Microorganisms, which require the activation of carrier proteins involved in secondary metabolic pathways, such as nonribosomal peptide synthetase or polyketide synthase pathways, require the activity of these Sfp-like proteins (Copp *et al.*, 2007). Consequently, in the absence of the Sfp enzyme, *B. subtilis* cannot synthesize compounds such as surfactin, which are dependent on nonribosomal peptide synthetase or polyketide synthase-type mechanisms.

Bacillus subtilis strain 3610 that carries the intact *sfp* gene swarms rapidly in symmetrical concentric waves, forming branched dendritic patterns. This observation was confirmed by Debois *et al.* (2008), who reported that surfactin molecules with a specific chain length play an important role in the swarming of communities on the agar surface.

Although the specific mechanisms of surfactant secretion are unknown, lipopeptide secretion provides a powerful competitive advantage for any species during surface colonization and during competition for resources (Ron & Rosenberg, 2001). For example, surfactin produced by *B. subtilis* inhibits *Streptomyces coelicolor* aerial development and causes altered expression of developmental genes (Straight *et al.*, 2006). It has also been established that surfactin is required for the formation of aerial structures on *B. subtilis* biofilm (Branda *et al.*, 2001). The ecological role of the aerial structures is to increase the spore dispersal capacity.

The second and third groups of surfactants produced by *B. subtilis* are peptides belonging to the iturin and plipastatin–fengycin groups, respectively (Fig. 1). Using HPLC, Ahimou *et al.* (2000) reported considerable variations in the lipopeptide content of seven *B. subtilis* strains. Among the three types of lipopeptides, only iturin A was produced by all seven *B. subtilis* strains. Indeed, more studies are needed for a complete understanding of the role of surfactants, including iturin and plipastatin–fengycin groups during biofilm formation, particularly in wild-type strains.

Active EPS

By definition, extracellular enzymes are proteins completely dissociated from the cell and found free in the surrounding medium or within the exopolymeric matrix (Priest, 1977). At least 200 proteins compose the *B. subtilis* 'secretome,' which also includes the proteins responsible for the secretion of extracellular enzymes (Tjalsma *et al.*, 2000; Antelmann *et al.*, 2001). Three distinct pathways for protein export from the cytoplasm to the surrounding environment have been identified in *B. subtilis*. Most protein export follows the Sec-SRP pathway that secretes proteins directly into the growth medium. A smaller number of proteins are secreted via twin-arginine translocation pathway or ABC

transporters in *B. subtilis* (Ling Lin *et al.*, 2007). Some extracellular enzymatic activities have been demonstrated while others have not due to the difficult task of distinguishing free enzymes from those associated to the cell wall. According to Tjalsma *et al.* (2004), the secretome also includes peptides with antibiotic functions. *Bacillus subtilis* produce a wide variety of antibiotics, with peptide antibiotics representing the dominant class. These peptide antibiotics exhibit a rigid structure, are resistant to hydrolysis by peptidases and proteases and can have amphipathic (discussed in Surface-active EPS) or nonamphipathic properties. Peptide antibiotics are reviewed by Stein (2005), and a description of the secretome has been summarized (e.g. Priest, 1977; Simonen & Palva, 1993; Antelmann *et al.*, 2001). Both subjects are beyond the scope of this review, which focuses on extracellular proteins involved in the architecture and chemical modification of the exopolymeric matrix.

Active EPS on polysaccharides

In this initial category enzymes involved in the chemical modification of polysaccharides are discussed, with two main examples. The first is levansucrase (2,6-β-D-fructan-6-β-D-fructosyl-transferase) encoded by *sacB* and involved in the synthesis of levan. Levansucrase is an exoenzyme, whose synthesis is highly inducible by sucrose. When sucrose is used as a substrate, levansucrase transfers the fructose residue to the acceptor levan (Shida *et al.*, 2002; Castillo & Lopez-Munguia, 2004). Levansucrase is secreted by the SecA pathway and increased levels of SecA result in an elevated production of exogenous levansucrase (Leloup *et al.*, 1999), indicating a strict control for its regulation. The second enzyme active on polysaccharides is levanase (β-D-fructofuranosidase) encoded by *sacC* and responsible for levan degradation (Gay *et al.*, 1983; Wanker *et al.*, 1995). SacC acts in single-chain mode, is active on levan, inulin and sucrose (Wanker *et al.*, 1995; Shida *et al.*, 2002) and is induced by low concentrations of fructose (Martin *et al.*, 1989). Inactivation of SacC results in an increase in levan polymerization possibly due to the loss of the degradative activity of the SacC protein (Shida *et al.*, 2002). *Bacillus subtilis* also produces a series of enzymes involved in the hydrolysis of carbohydrates that are commonly found in its natural environment. Pectate lyases, amylases and xylanases are examples of probably the most ubiquitous hydrolytic enzymes secreted by *Bacillus* species (Priest, 1977; Tjalsma *et al.*, 2004).

Active EPS on proteins

Bacillus subtilis secretes at least seven different exoproteases including two major proteases (subtilisin and neutral metalloprotease E) and five minor proteases (bacillopeptidase F, Mpr, Epr Npr and Vpr) (Pero & Sloma, 1993, Table S1). These exoproteases digest proteins present in the environment, a response that is induced by low levels of available nitrogen (Hata *et al.*, 2001). Wild-type strains of *B. subtilis* that are deficient in the production of these extracellular proteolytic activities are also unable to swarm or form biofilms (Pero & Sloma, 1993; Connelly *et al.*, 2004).

Other active EPS

The other active EPS category includes proteins that interact with substrates of different chemical nature that can be secreted during nutrient deprivation. *Bacillus subtilis* strains secrete many proteins involved in the degradation of a variety of molecules such as lipids, glutathione, phytic acid and extracellular nucleic acids to cope with conditions of low nitrogen (Priest, 1977; Tjalsma *et al.*, 2004).

Among the proteins active in the formation of the exopolymeric matrix, special attention needs to be drawn to the recently identified TasA protein. This protein is encoded by *tasA*, a gene expressed at the onset of sporulation in *B. subtilis* (Branda *et al.*, 2006). TasA is required for the structural integrity of the matrix as well as biofilm development: it has been proposed that TasA forms amyloid fibers that bind cells together in the biofilm (Romero *et al.*, 2010). TasA localization within the exopolymeric matrix is dependent on a functional *yqxM* gene, but the role of YqxM in biofilm development is still unknown, another area that requires further investigation (Branda *et al.*, 2006).

Extracellular DNA

The presence and role of extracellular DNA in *B. subtilis* strains is another topic that is poorly understood. In the close relative *Bacillus cereus*, biofilm formation requires DNA as part of the extracellular polymeric matrix (Vilain *et al.*, 2009). DNA in biofilms may be involved in events of recombination that take place in natural environments (Spoering & Gilmore, 2006). Further studies on extracellular DNA in *B. subtilis* biofilms will help elucidate its role in natural environments.

EPS beyond biofilm formation in *B. subtilis*

Microorganisms in nature are subject to sudden changes in the environmental conditions such as nutrient deprivation, desiccation, osmotic stress, action of antibiotic molecules released by other microorganisms, UV radiation and temperature variations. *Bacillus subtilis* can survive these environmental fluctuations, which are typical for soils, through several defense mechanisms (Setlow, 1992). Although spore formation is the main mechanism for long-term survival for *B. subtilis*, the secretion of EPS during vegetative growth also

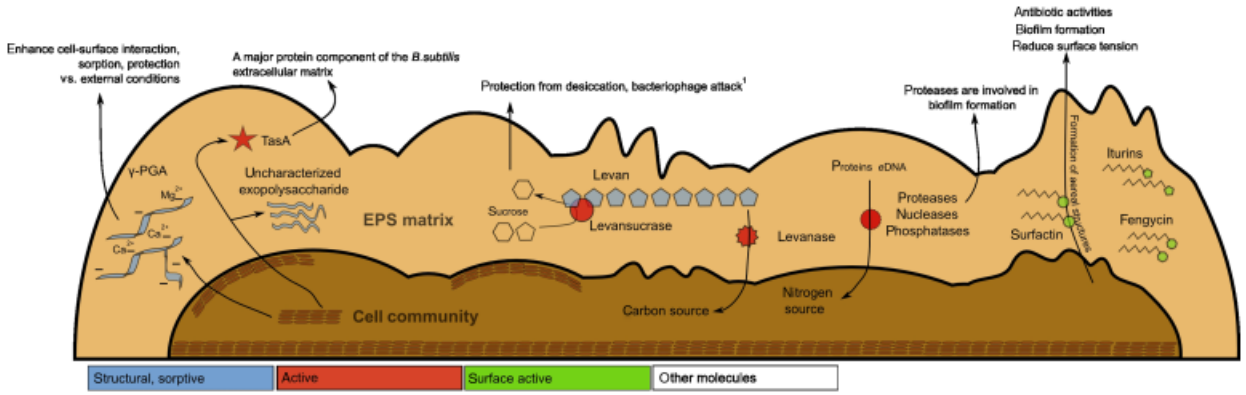

Fig. 2. Diagram of a surface-attached *Bacillus subtilis* community embedded in an exopolymeric matrix depicting some of the EPS discussed in the review including γ-PGA, TasA and levan. [1]Paton, 1960.

plays an important role in community survival. However, the nature and genetic controls of the production of these polymeric substances remain poorly understood.

In this review different genes and proteins related to the production of EPS are addressed. EPS are an integral part of the survival strategy of the individual cells and well as the entire community (see Fig. 2 for a summary of such molecules and their functions).

In addition to surviving environmental fluctuations, microorganisms in nature also adopt social skills such as communication, organization, compartmentalization, competence and defense (Earl *et al.*, 2008). There are many levels of regulation for the production of EPS; some are specific, while others are general, but all are tightly regulated. For example, during the early stages of biofilm formation, only a subpopulation of cells express genes of the *eps* operon as well as the *yqxM* gene (involved in the proper localization of TasA) for the entire community (Chai *et al.*, 2008). As the production of the EPS requires copious amounts of energy, regulatory controls are important.

It has been proposed that *B. subtilis* biofilms can be viewed as a multicellular organism (Aguilar *et al.*, 2007). When bacterial biofilms behave as multicellular communities, they exhibit various degrees of compartmentalization. For example, during staphylococcal biofilm formation, at least four distinct cellular states are represented: cells growing aerobically, cells growing fermentatively, dormant cells and dead cells (Rani *et al.*, 2007). In *B. subtilis*, motile cells transit to matrix-producing cells and ultimately to sporulating cells localized in distinct regions of the biofilm (Vlamakis *et al.*, 2008). The exopolymeric matrix is shared by the different cells types and complementation of matrix components may take place among bacterial mutants (Branda *et al.*, 2006; Chai *et al.*, 2008). Interestingly, recent findings by López *et al.* (2009) suggest that the exopolymeric matrix does not serve only to hold different *B. subtilis* cell types together, but also acts as a timing mechanism. Once cells begin to produce an exopolymeric matrix as a result of surfactin signaling development, the surfactin production stops or is arrested (López *et al.*, 2009). The concept of bacterial multicellularity within *B. subtilis* biofilms is likely to continue to develop novel insights.

As pointed out above, the wide heterogeneity of *B. subtilis* wild-type strains used to characterize or study EPS (Table S1) and the lack of genetic information concerning such strains complicate understanding of the development, role and function of the exopolymeric matrix. Indeed, a future challenge is to focus studies on a single reference strain, for example *B. subtilis* strain 3610 as a model organism. The sequencing of its entire genome will be useful for comparisons with the genome of strain 168.

It should be noted that care needs to be exercised when drawing conclusions regarding the function of EPS in domesticated or wild-type strains grown under controlled or laboratory conditions. Previous studies have shown that EPS synthesis was affected in domesticated strains (Aguilar *et al.*, 2007) and studies conducted with wild-type strains are usually conducted *in vitro* using synthetic media that do not mimic environmental conditions. The role of EPS still requires future investigations, particularly with respect to the genetic expression underlying its properties and production in natural environments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. EPS produced by *Bacillus subtilis* according to their function.

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