ISSN 1330-9862
(FTB-3552)

minireview

Social Interactions and Biofilm Formation in *Bacillus subtilis*

Iztok Dogsa[§], *Anna Oslizlo*[§], *Polonca Stefanic and Ines Mandic-Mulec**

University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Chair of Microbiology, Večna pot 111, SI-1000 Ljubljana, Slovenia

Received: October 29, 2013
Accepted: April 16, 2014

Summary

Quorum sensing (QS) is a form of cooperative social behaviour which relies on extracellular signalling molecules that elicit the QS response across many cells and controls the development of many cooperative traits including biofilm formation. The main aim of this work is to review the published work on cooperative social behaviour of *Bacillus subtilis* and especially its QS system ComQXPA. This QS system involves four interacting components: the signal-processing enzyme ComQ, the ComX signal, the ComP receptor and the ComA transcriptional regulator. Phosphorylated ComA controls the transcription of many genes including those responsible for the production of surfactin and extracellular matrix, essential for biofilm formation. The ComQXPA QS shows a high degree of genetic polymorphism, which manifests itself in the separation of *Bacillus subtilis* strains into four different communication groups (pherotypes). The information exchange is possible between members of the same pherotype but not across pherotypes. We have recently suggested that this phenomenon is at least in part driven by the ecological divergence of strains, but may also be induced by frequency-dependent selection. The ComQXPA QS system controls the production of extracellular matrix (ECM) components: polysaccharides, proteins and nucleic acids. We will address the present understanding of the ECM structure-function relationships in *B. subtilis* biofilms and review published results on regulation, composition and distribution of ECM components. Despite many important recent discoveries on regulation of *B. subtilis* biofilm development, we know little about the molecular interactions in the ECM and the role they play in the QS and stability of the biofilm. Future research needs to address these questions better.

Key words: quorum sensing, *Bacillus subtilis*, biofilm formation, ComQXPA system, pherotype, extracellular matrix components, surfactin

Introduction

Social interactions are an inherent part of group living and are common at all levels of life, even in the microbial world. These interactions may be both beneficial and detrimental to the participants (*1*). However, recent reports suggest that beneficial cooperative behaviour is the social norm within microbial communities (*2*). Bacteria cooperate by secreting and sharing various compounds such as extracellular enzymes that assist in cross feeding (*3–5*), antibiotics that provide the means to outcompete the neighbours feasting on the same food source (*6*), extracellular polymeric substances that facilitate biofilm formation (*7*) and biosurfactants that enable cooperative movement (*8*). These compounds are considered public goods, which are shared and may directly benefit the entire community and even determine its survival (*1,3*). Secretion of public goods is typically regulated by quorum sensing (QS), a mechanism found in many bacterial and even fungal species that coordinates synthesis of public goods and other cooperative functions at the population level and in the cell-density-dependent manner (*9,10*).

*Corresponding author: Phone: +386 3203 409; Fax: +386 12 573 390; E-mail: ines.mandic@bf.uni-lj.si
[§]Both authors contributed equally to this manuscript and should be considered as first authors

In this short review we will focus on the role of QS in the social life of *Bacillus subtilis*, a Gram-positive soil-dwelling bacterium that is an excellent model organism to study group living. The emphasis will be given to the ComQXPA QS system, its role in surfactin synthesis and in biofilm formation. In addition, the striking polymorphism of this QS system associated with natural populations of *B. subtilis* and close relatives will be discussed. Finally, we will focus on the regulation, functional role, chemical composition and structure of extracellular matrix (ECM), a protective glue of biofilm communities.

Quorum Sensing Induces Diverse Social Behaviours in Bacteria

Since the discovery of QS in *Vibrio fisheri* (*9*), QS systems of Gram-negative species have been receiving much more attention compared to the QS systems of Gram-positive bacteria. Gram-negative bacteria communicate mostly by homoserine lactones (*9*,*11–13*), while Gram-positive bacteria mainly use peptide pheromones as QS signals (*14–16*).

For example, the model Gram-positive bacterium *B. subtilis* uses peptide ComX that is 5 to 10 amino acids long (*14*,*17*), which is unique among bacterial peptide signals, due to its posttranslational isoprenylation on the tryptophan residue. This modification is essential for the proper functioning of ComX (*17*) and exclusive among living organisms (*18*). Our recent bioinformatics study of bacterial genomes, however, revealed that other members of *Firmicutes* sp. may also produce isoprenylated signalling peptides (*19*). ComX of *B. subtilis* is synthesised as a prepeptide 55 amino acids long, which is processed and modified by the ComQ isoprenyl transferase before secretion into the extracellular medium (*20*). Upon reaching the threshold concentrations, the pheromone binds to the membrane-associated receptor ComP and induces its autophosphorylation. ComP-P then phosphorylates the response regulator ComA, which elicits the QS response (*21*). The ComQXPA QS system controls the expression of many genes including the *srfABCD* operon that encodes the lipopeptide antibiotic surfactin (*22*) and the *comS* gene, which controls competence development and is embedded within the *srfA-D* operon (*23*). Therefore, both surfactin production and competence development are strongly dependent on the active QS system (*24*).

B. subtilis, a soil, rhizosphere- and gut-dwelling bacterium (ecology of this bacterium is reviewed in *25*,*26*) is known as a master of differentiation (*27*). The ComQXPA QS system is the only one out of several QS systems used by this bacterium, which is known to generate different cell types that exhibit different kinds of social behaviours. The combination of QS signals and stochastic events can induce 'division of labour' strategies whereby the *B. subtilis* population differentiates into cell types that perform different functions within the population. These are extracellular food-degrading enzyme producers, antibiotic secretors, competent cells, extracellular matrix (ECM) producers, cannibals and finally the most differentiated, dormant spores (reviewed by Lopez and Kolter, *28*). We believe that the ability of the population to undergo differentiation into various cell types requires fully functional QS systems. For example, the *B. subtilis* PS-216 signal-deficient mutants (QSS^-) over-respond to exogenous QS signal and show a significant change in heterogeneity of *srfA* expression as compared to the QS^+ cells (Fig. 1), even when supplemented with the appropriate concentration of QS signal. We have recently shown that *B. subtilis* relays information provided by the intracellular production of ComX. This private link controls the QS response (*e.g.* surfactin production) of the signal producer and thus impacts the proportion of cells committed to the QS-controlled phenotype (*29*).

Years in the Laboratory Environment Make *Bacillus* Less Cooperative

Recently, McLoon *et al.* (*30*) have analysed the changes of *B. subtilis* strain 168 during more than a century of domestication in the lab. They identified mutations that make the domesticated strain (*B. subtilis* 168) different from its wild ancestors (*e.g. B. subtilis* NCIB 3610). Interestingly, the mutations mainly crippled the social behaviour of *B. subtilis* 168. The strain is not able to form a robust biofilm (mutation in *epsC*), secrete extracellular degrading enzymes (mutation in *degU*), produce surfactin (mutation in *sfp*, a surfactin synthetase-activating enzyme) and swarm (mutation in *swrA*) (*30*). It seems meaningful that a strain separated for years from multistrain/multispecies communities in the wild lost its crucial social skills.

The phenomena of domestication highlight a need for new tools to study bacterial social behaviour, like *B. subtilis* PS-216 (*31*), which has been recently sequenced (*32*). This strain isolated from the river bank soil in Slovenia has a great potential to replace or at least be complementary to more or less domesticated *B. subtilis* variants (like *B. subtilis* 168 and even NCIB 3610), because its social traits remain intact and it is naturally competent for genetic transformation (*31*,*32*).

Why Do Strains of a Bacterial Species Use Different QS 'Languages'?

Existence of distinct communication groups (pherotypes) that communicate efficiently within but not between groups of a single species is a striking feature of QS systems in certain Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae* (*33–35*), *B. subtilis* (*17*,*36–38*) and *B. cereus* (*39*). In *B. subtilis* the separation into different pherotypes is linked to genetic polymorphism in *comQ* and *comX* genes involved in signal production and the N-terminal end of *comP* gene that codes for the QS signal receptor. Sequencing of the three genes found in natural isolates revealed clustering into four distinct sequence similarity groups (*17*,*36–40*). Nucleotide sequence identity within this group was >93 % but <60 % between the gene groups if strains isolated from a soil aggregate were compared (*38*). However, *comQXP* genes that originate from strains of different geographical origin form common sequence clusters (*40*) and the three genes show congruence reflecting their coevolution (*17*,*36–40*). Sequence clusters correlate well with communication specificity groups (pherotypes). These

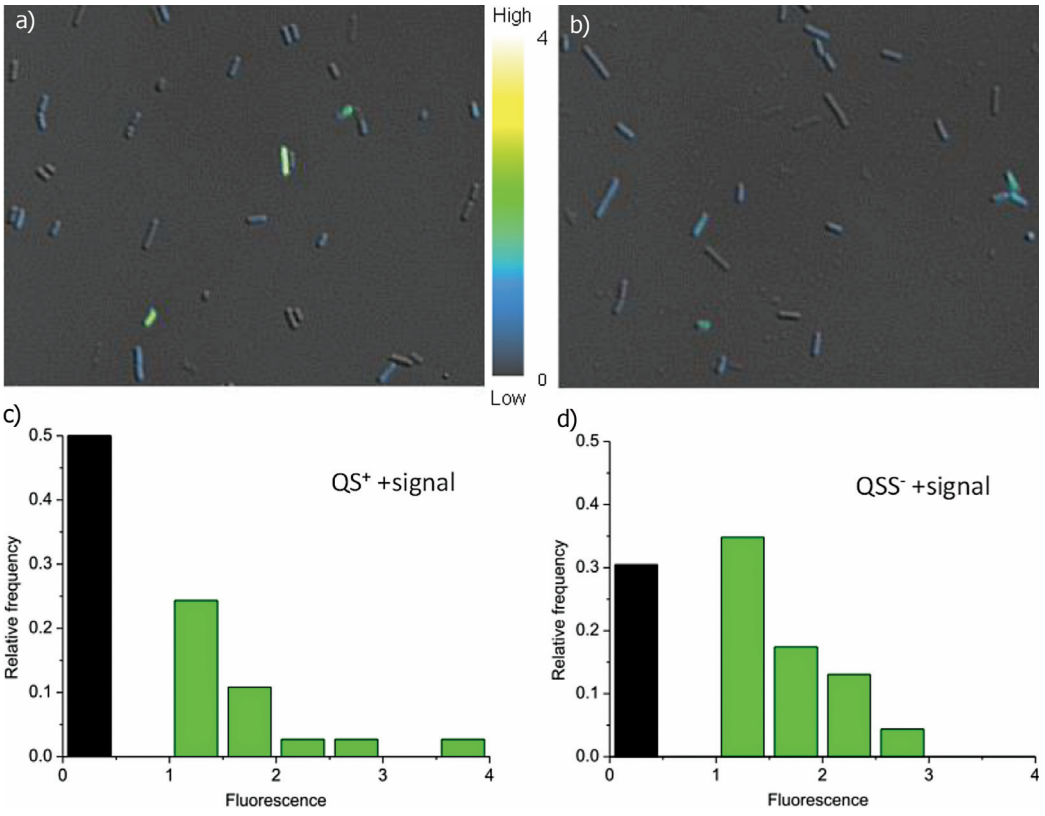

Fig. 1. The combined differential interference contrast (DIC) and fluorescence microscopy images of the *B. subtilis* PS-216 strain: a) quorum sensing and signal-proficient wild type (QS^+) and b) the signal-deficient mutant (QSS^-). Both strains were supplemented with saturating concentration (~10 nM) of the ComX signal purified from the ComX-expressing *E. coli* strain (for details see ref. *29*). Both strains are marked with *srfA-cfp* fusion and the cells expressing *srfA* (signal response) are coloured according to the fluorescence intensity scale (blue-green-yellow). In both populations, non-expressing cells are present; however, in signal-deficient mutants (QSS^-) the number of non-expressing cells is decreased. This can also be seen in histograms representing the distribution of *srfA-cfp* expression in the corresponding microscopy images of: c) QS^+ population, and d) QSS^- population. The relative frequency of non-induced cells is marked black. X-axes show fluorescence intensity corrected for the background and normalized to fluorescein standard. For representation of larger distributions of several combined microscopy images the reader is advised to check ref. *29*

were determined by cross-activation studies using pherotype-specific biosensor strains (*17,36–40*). Strains of the same pherotype can exchange signals and induce each other's QS response, but strains from different pherotypes cannot (*17,31,36–38*). It is even more striking that pherotypes are shared among *Bacillus* species (*17,40*), yet communication is restricted within species. It remains a mystery why pherotypes evolved, but we know that they coexist in soil even at a millimetre scale (*31*). Ansaldi and Dubnau (*41*) used two statistical tests (the ratio of synonymous and nonsynonymous substitution rates and the Tajima D test) to demonstrate that these polymorphic sequences evolved by diversifying selection rather than by neutral drift. Recently, Stefanic *et al.* (*40*) compared the abundance of pherotypes within a group of phylogenetically and ecologically related *B. subtilis* strains, also referred to as ecotypes. Ecotypes are defined as populations of genetically coherent and ecologically distinct cells that can be discovered and classified as DNA sequence clusters, determined on the basis of housekeeping gene sequences (*42,43*). Interestingly, strains that resided very close to each other in soil (at mm distances) diversified into three phylogenetic clusters or ecotypes with a different pherotype dominating each ecotype (*40*). This supports the hypothesis that pherotype diversity could be an adaptation to ecological diversity within *B. subtilis* (*40*). Although the majority of strains in one ecotype also shared a pherotype, we also detected one or more representatives that belonged to a different pherotype (*40*). This distribution could be the consequence of occasional horizontal gene transfer between ecotypes, which would result in a pherotype switch. This is supported by the 'rare' pherotype advantage hypothesis suggested by Stefanic *et al.* (*40*). It predicts that the rare pherotype has an advantage because it cannot induce its QS response due to low density and therefore low concentrations of the pherotype-specific signalling molecules. This situation may be temporarily advantageous for the rare pherotype A that is surrounded by relatives expressing a different pherotype B, as it is able to feast on the public goods (signals, enzymes, surfactants) provided by the pherotype B majority. This association may give the rare pherotype A a fitness benefit and support its spreading until it reaches the threshold density itself. At this point the rare pherotype A would stop being in minority and may even represent the majority that is now able to induce the quorum sensing response (*40*). It is possible to envision a continuous cycling of pherotype frequencies within an ecotype if horizontal gene transfer provided the mechanism of pherotype exchange. Finally,

a mechanism of continuous pherotype diversification was suggested with the modelling approach by Eldar (*44*). He hypothesized that a mutant deficient in the signal receptor would gain a fitness advantage over the QS wild type strain, which would have to invest in the production of public goods. This advantage would last only when the mutant was rare. At high frequency, the mutant would lose the advantage and in order to survive would need to accumulate additional mutations, changing it back to the QS-proficient strain. Both hypotheses, however, still require experimental confirmation.

Quorum Sensing Under Challenging Conditions and Its Evolutionary Stability

The problem of evolutionary stability of QS has been addressed before, mainly with reference to Gram-negative bacteria (*3,8*). It was shown that QS mutants that do not contribute to the community with signal production (QSS^-; signal deficient) or QS response (QSR^-; signal blind) may gain the advantage over the wild type strains under certain conditions (*3*). However, recent data suggest that both QS signalling (*45*) and QS response (*46*) remain evolutionary stable because they regulate many other traits that directly affect fitness. Our recent data also suggest that there is a strong selective pressure for QS signalling and that signal-deficient cells suffer from low fitness when surrounded by the wild type cells (*29*). Similar trends were observed for *Vibrio fisheri*, but the mechanism behind it was not elucidated (*45*). Decreased fitness of the *B. subtilis* QSS^- mutant surrounded by the wild type signal producers was associated with overproduction of public goods (*e.g.* surfactin) by the mutants and their increased sensitivity to surfactin (*29*). However, we do not know any set of environmental parameters or composition of growth medium in which QS would be required for growth of *B. subtilis* and this still needs to be established. It is known that QS enables bacteria to respond fast and in synchrony to environmental changes, however, little is known about how QS works in different environmental conditions. We have recently addressed this question using ComQXPA system of *B. subtilis*. It was noticed that the QS-regulated gene such as *srfA* changes the expression pattern when bacteria face osmotic stress and that the QS response decreases with increasing NaCl concentration and is strongly reduced in a medium containing 40 and 80 g/L of NaCl (Fig. 2).

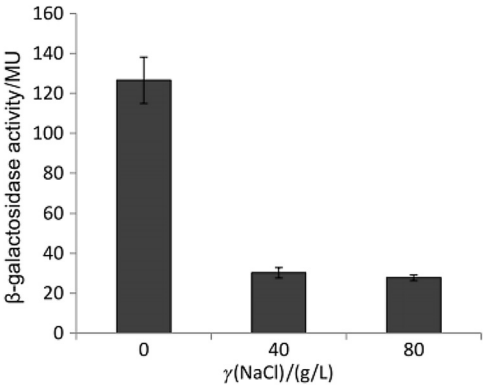

Fig. 2. Expression of *srfA* at T1 (one hour after transition into stationary phase) by β-galactosidase test (*srfA-lacZ* fusion) at three different NaCl concentrations in the medium containing *B. subtilis* BD2833 (*32*) grown in planktonic cultures. T1 was defined for each culture independently as the growth lag phase increased and growth rates decreased with increasing salt concentration in the medium. However, the final cell density achieved in each experimental variant was comparable. Data are presented as the mean of biological triplicates with standard errors indicated; MU=Miller units

B. subtilis Biofilms and Composition of Extracellular Matrix

Biofilms are surface-attached structured communities of cells embedded in a viscous extracellular matrix (ECM) where social interactions such as QS (*47*) and exchange of public goods (*e.g.* enzymes, plasmids, surfactants) are promoted by close physical association of the cells. The *B. subtilis* biofilm has been extensively studied as a model of biofilm group living in Gram-positive bacteria (*48*). Two types of *B. subtilis* biofilms have been the subject of intensive research: (*i*) floating pellicles that form on liquid-air interphases, and (*ii*) bacterial colonies that represent biofilms on solid surface-air interphases. The laboratory strain *B. subtilis* 168, which is most often used to address regulation of gene expression, does not form robust biofilms (*30,48*), and has been replaced by *B. subtilis* NCIB 3610 for biofilm studies (*49–55*). The subject has recently been extensively reviewed by Vlamakis *et al.* (*56*), while here we mainly focus on the molecular composition of ECM and how it is influenced by the composition of growth medium.

Studies on *B. subtilis* biofilms have most often been performed in minimal medium containing glycerol as the major carbon source (MSgg; *49*). Typically, pellicles develop after the standing culture reaches cell density of approx. $5 \cdot 10^7$ CFU/mL and at this point cells begin to migrate to the liquid-air interface where they form a floating biofilm. However, the final ratio of planktonic to biofilm-bound cells is dependent on the type of growth medium (*57*). In MSgg medium the majority of cells are gathered in pellicles, but in sucrose-rich medium cells are more evenly distributed between the medium and the floating pellicle (*57*). Cells are encased in ECM, which is composed of various exopolymers. Among these is the EpsA-O extracellular polysaccharide (synthesised by the components encoded by the 11-gene operon *epsA-O* (*eps* in continuation). EpsA-O is composed of glucose, galactose and *N*-acetylgalactosamine (*58*) and is presumably the dominant polysaccharide in biofilms grown in the glycerol-based MSgg medium (*49–51,57,58*) or in the medium based on plant root exudates (*59*). In a medium rich in sucrose, the predominant ECM component is the polysaccharide levan (*57*). This is a homopolymer, composed only of fructose. It is synthesised by levansucrase encoded by *sacB* (*60–66*) and it displays unusually low viscosity (*67*). The finding that levan can be the major constituent of *B. subtilis* biofilm emphasises its potential ecological importance. *Bacillus* biofilms form on plant roots (*6,59*), where sucrose is an important component of plant exudates. Therefore, levan might also be an ECM component in *B. subtilis* biofilms attached to plant root

surfaces, although a direct proof necessitates further studies. Apart from polysaccharides, ECM also contains proteins (*49*) and nucleic acids (*57*). TasA (*68*) is the major protein component, which forms amyloid fibres that mediate *Bacillus* cell-to-cell interactions and cell-to-surface adhesion in combination with other extracellular components (*69*). TasA is linked to the cell wall by TapA, which is a minor component of the ECM (*52*,*70*). TasA and EpsA-O polysaccharide are essential for the formation of the mature biofilms. Interestingly, however, the impact of the absence of TasA or EpsA-O on biofilm integrity is reduced when levan is a dominant component of the ECM (*57*). The microstructure and surface of *B. subtilis* biofilms are significantly impacted by the recently discovered protein BslA (*55*,*70*,*71*). This protein was identified as a major contributor to the surface repellence of *B. subtilis* biofilms (*70*,*71*). Natively synthesised and secreted BslA forms surface layers around the biofilm and has self-assembling properties (*72*). The homopolymer of glutamic acid, γ-polyglutamic acid (γ-PGA) is another *B. subtilis* ECM component. Its presence, however, is strain dependent and in pellicles formed by *B. subtilis* NCIB 3610 grown even in γ-PGA-stimulating medium, γ-PGA was not detected (*73*).

Role of QS Signal ComX and Surfactin in Synthesis of Extracellular Matrix

Regulation of ECM production is coupled with QS through ComQXPA (*28*), the QS system which through two signalling lipopeptides: ComX and surfactin (Fig. 3) positively regulates the synthesis of EpsA-O (*53*,*74*). It has been suggested that ComX and surfactin indirectly influence accumulation of Spo0A~P (*53*), which is a pleiotropic regulator that downregulates SinR, a repressor of biofilm formation (*75–77*). This is achieved indirectly as Spo0A~P induces expression of SinI (*78*), which then antagonises the repressor SinR (*79*). SinR is a pleiotropic DNA-binding regulator (*80*,*81*), a repressor of genes involved in matrix synthesis (*77*), but it also controls sporulation (*80*,*81*) and competence development. Surfactin expression shows a bimodal pattern, meaning that only some cells in the population synthesise this lipopeptide (*29*,*53*). Interestingly, it was suggested that these cells are unable to respond to surfactin, while nonproducing but surfactin-responsive cells commence matrix production. It is believed that matrix producers no longer responded to ComX and therefore cannot become surfactin producers (*53*). Unresponsiveness to ComX was related to the extracellular matrix genes, as mutant cells unable to make matrix responded to both ComX and surfactin. Moreover, it was also discovered that surfactin can act as a signal to induce cannibalism in the matrix-producing strains. Cannibals produce Skf and Sdp toxins, but also simultaneously express the resistance to them. Therefore, nutrients released by the cannibalised cells are presumably used by matrix-producing cells and consequently this subpopulation and ECM production are increased (*82*). This phenomenon might explain the high concentrations of nucleic acids found in the ECM of *B. subtilis* (*57*), although further experiments are needed to examine this phenomenon.

Additional Genetic Determinants of Biofilm Formation

The important role of surfactin in biofilm formation explains why *B. subtilis* 168, which carries mutation in *sfp* gene (surfactin synthetase-activating enzyme) needed for surfactin synthesis, forms morphologically less structured and robust biofilms (*74*). However, *sfp* revertants do not recover full biofilm morphology observed for the undomesticated NCIB 3610 strain, and revertants in *epsC* (putative UDP-sugar epimerase), *swrA* and *degQ* are necessary for the laboratory strain to produce robust biofilms (*30*). The morphology of the biofilm is also influenced by the plasmid-borne gene *rapP* present in NCIB 3610, but absent from domesticated strains. It is relatively straightforward to see how the exopolysaccharide-producing gene *epsC* influences biofilm formation, but it is less obvious how biofilm formation depends on *degQ* or *swrA*. SwrA is able to enhance the transcription of the *fla/che* operon through the action of the transcriptional activator DegU (*83*), as depicted in Fig. 3. The *fla/che* operon encodes the majority of flagellar and chemotaxis proteins including *sigD*, encoding the σ^D factor (*84*,*85*). The σ^D RNA polymerase transcribes genes for structural flagellar proteins, including flagellin, and stimulates the expression of *swrA* (*86*), which is also required for cell motility and thus essential for biofilm development. The pleitropic regulator DegU controls many genes including the one encoding hydrophobic protein BslA (*55*,*87*) and *sacB*-encoding levansucrase (*88–90*), which are both important for biofilm formation (Fig. 3). DegQ positively regulates DegU targets and, at least *in vitro*, DegQ stimulates phosphotransfer from DegS-P to DegU (*91*). Transcription of *degQ*, however, depends on ComA (*92*). This opens another interesting feature of the QS, namely its role in the regulation of biofilm formation. Indeed, all major ECM components are under QS control, including the synthesis of BslA and levan, controlled by the ComA/DegQ/DegU proteins and EpsA-

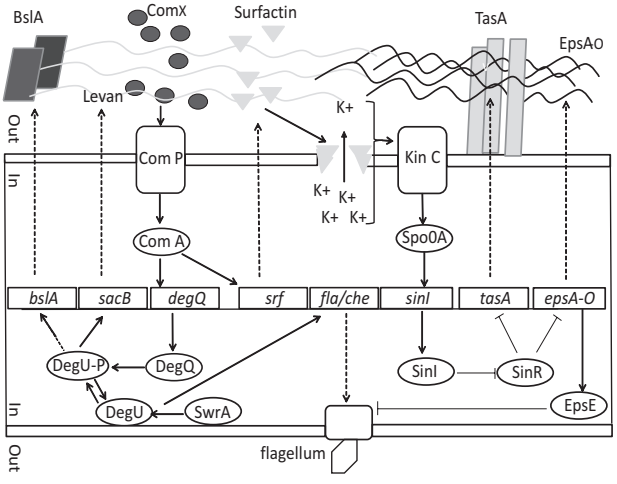

Fig. 3. The schematic representation of the ComQXPA quorum sensing system and its influence on the extracellular matrix (ECM) formation. The indirect products of genetic elements (blocks) are indicated by dashed arrows. Note that due to clarity not all phosphorylated states of the proteins (ellipses=cellular, and rounded rectangles=membrane proteins) are shown

-O, TasA and TapA, which are affected by the ComA/srfA pathway. Indirectly, this pathway may also influence the presence of nucleic acids in the ECM, as recently shown by Zafra *et al.* (*93*) and independently confirmed by our experiments (unpublished data).

Investigating Biofilm Structure at the Nanometre Scale – A Challenging Task

Biofilm structure can be directly investigated at the micrometre scale by light microscopy. For example, the fluorescence microscopy has been applied to study cell differentiation in *B. subtilis* biofilms (*28*,*94*) and transport of nutrient solution through biofilm channels recently described by Wilking *et al.* (*95*). At the nanometre scale, however, the biofilm is essentially an interplay between polymers. Elucidating structural information of these polymers and the matrices they form is not an easy task. One cannot directly observe and see the structure at the nanometre scale and since ECM does not form a regular lattice, the classical X-ray crystallography is not applicable to study these structures. Also, these high molecular mass polymers are unsuitable for analyses by NMR. Approaches involving atomic force microscopy (AFM), transmission electron microscopy (TEM) or scanning electron microscopy (SEM) are very powerful tools for analyses of biofilm surfaces. However, these approaches, although very powerful, may alter the native molecular structure of ECM and/or yield only structural information of the ECM surface. Thus, additional methods like small angle X-ray scattering (SAXS) combined with powerful 3D modelling are presently being applied in our group to study ECM spatial structure at 1–100 nm scale (*96–99*). To better simulate the native environment of *B. subtilis* ECM, samples under investigation with this method can either be aqueous or dry. Therefore, we hope that this method in combination with various microscopy techniques will give us better insight into the 3D structure of biofilm matrix and allow us to better address the role of ECM structure in signalling over distances at the 'bacterial' size scale.

Future Perspectives

An impressive amount of information regarding the genetics of *B. subtilis* biofilm formation has been gained over the past twenty years. The complex behaviour of *B. subtilis* cells at least in part associated with bimodal gene expression and phenotypic heterogeneity in biofilms has been well described. The studies were mostly performed on genetically homogeneous populations, using only one model strain. However, in natural environments, even at soil microscale, genotypes of one species are ecologically distinct and use diverged signalling systems (*40*). Furthermore, biofilms in natural settings are often composed of different species adding to the complexity of interactions (*100*). Therefore, in the future, we need to explore interactions in biofilms composed of genetically heterogeneous populations in order to understand the mechanisms behind their cooperative and antagonistic interactions that may influence biofilm function and evolution in natural settings. *B. subtilis* is a soil-dwelling bacterium, but also resides in the rhizosphere, on plant roots, in the intestinal tract and in a variety of aquatic ecosystems (*25,26*). We know very little about the *B. subtilis* cell--cell signalling and its biofilms in these environments. It will be exciting to see future developments addressing quorum sensing and biofilm formation in model systems that better resemble the natural habitats of *B. subtilis*, as well as to study the diversity of these phenomena in natural populations.

Furthermore, major components of *B. subtilis* ECM have been detected and their chemical composition well described. However, the information on spatial structural information of individual ECM components is still in its infancy, except for the TasA amyloid fibre structures, whose assembly has recently been elucidated (*101*, *102*). What is missing is spatial structural information on other ECM components at the molecular level. We would like to understand how these polymers interact to form matrices and affect diffusion process, which are especially interesting in relation to signalling and exchange of public goods in biofilms. The best of this era is yet to come.

References

1. S.A. West, C.E. Mouden, A. Gardner, Sixteen common misconceptions about the evolution of cooperation in humans, *Evol. Hum. Behav. 32* (2011) 231–262.
2. B. Xavier, W. Kim, K.R. Foster, A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*, *Mol. Microbiol. 79* (2011) 166–179.
3. S.P. Diggle, A.S. Griffin, G.S. Campbell, S. West, Cooperation and conflict in quorum-sensing bacterial populations, *Nature, 450* (2007) 411–414.
4. H.J. Folse, S.D. Allison, Cooperation, competition, and coalitions in enzyme-producing microbes: Social evolution and nutrient depolymerisation rates, *Front. Microbiol. 338* (2012) 1–10 (doi: 10.3389/fmicb.2012.00338).
5. J. Gore, H. Youk, A. van Oudenaarden A, Snowdrift game dynamics and facultative cheating in yeast, *Nature, 459* (2009) 253–256.
6. H.P. Bais, R. Fall, J.M. Vivanco, Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production, *Plant Physiol. 134* (2004) 307–319.
7. F. Harrison, A. Buckling, Siderophore production and biofilm formation as linked social traits, *ISME J. 3* (2009) 632–634.
8. J.B. Xavier, Social interaction in synthetic and natural microbial communities, *Mol. Syst. Biol. 7* (2011) 483 (doi: 10.1038/msb.2011.16).
9. M.B. Miller, B.L. Bassler, Quorum sensing in bacteria, *Annu. Rev. Microbiol. 55* (2001) 165–199.
10. C.M. Waters, B.L. Bassler, Quorum sensing: Cell-to-cell communication in bacteria, *Annu. Rev. Cell. Dev. Biol. 21* (2005) 319–346.
11. C. Fuqua, S.C. Winans, E.P. Greenberg, Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators, *Annu. Rev. Microbiol. 50* (1996) 727–751.
12. B.L. Bassler, How bacteria talk to each other: Regulation of gene expression by quorum sensing, *Curr. Opin. Microbiol. 2* (1999) 582–587.
13. T.R. de Kievit, B.H. Iglewski, Bacterial quorum sensing in pathogenic relationships, *Infect. Immunol. 68* (2000) 4839–4849.

14. R. Magnuson, J. Solomon, A.D. Grossman, Biochemical and genetic characterization of a competence pheromone from *B. subtilis*, *Cell, 77* (1994) 207–216.
15. W. Haas, B.D. Shepard, M.S. Gilmore, Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction, *Nature, 415* (2002) 84–87.
16. R.P. Novick, Autoinduction and signal transduction in the regulation of staphylococcal virulence, *Mol. Microbiol. 48* (2003) 1429–1449.
17. M. Ansaldi, D. Marolt, T. Stebe, I. Mandic-Mulec, D. Dubnau, Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants, *Mol. Microbiol. 44* (2002) 1561–573.
18. M. Okada, Post-translational isoprenylation of tryptophan, *Biosci. Biotechnol. Biochem. 75* (2011) 1413–1417.
19. I. Dogsa, K.S. Choudhary, Z. Marsetic, S. Hudaiberdiev, R. Vera, S. Pongor, I. Mandic-Mulec, ComQXPA quorum sensing systems may not be unique to *Bacillus subtilis*: A census in prokaryotic genomes, *PLoS ONE, 9* (2014) e96122 (doi: 10.1371/journal.pone.0096122).
20. K.B. Schneider, T.M. Palmer, A.D. Grossman, Characterization of *comQ* and *comX*, two genes required for production of ComX pheromone in *Bacillus subtilis*, *J. Bacteriol. 184* (2002) 410–419.
21. Y. Weinrauch, R. Penchev, E. Dubnau, I. Smith, D. Dubnau, A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems, *Genes Dev. 4* (1990) 860–872.
22. M.A. Marahiel, M.M. Nakano, P. Zuber, Regulation of peptide antibiotic production in *Bacillus*, *Mol. Microbiol. 7* (1993) 631–636.
23. D. Dubnau, Genetic competence in *Bacillus subtilis*, *Microbiol. Rev. 55* (1991) 395–424.
24. N. Comella, A.D. Grossman, Conservation of genes and processes controlled by the quorum response in bacteria: Characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*, *Mol. Microbiol. 57* (2005) 1159–1174.
25. I. Mandic-Mulec, J.I. Prosser: Diversity of Endospore-Forming Bacteria in Soil: Characterization and Driving Mechanisms. In: *Endospore: Forming Soil Bacteria*, N.A. Logan, P. de Vos (Eds.), Springer Verlag, Berlin, Germany (2011) pp. 31–59.
26. J.D van Elsas, I. Mandic-Mulec: Advanced Molecular Tools for Analysis of Bacterial Communities and Their Interactions in the Rhizosphere. In: *Molecular Microbial Ecology of the Rhizosphere*, F.J. de Bruijn (Ed.), Wiley-Blackwell, Hoboken, NJ, USA (2013) pp. 115–124.
27. D. Lopez, H. Vlamakis, R. Kolter, Generation of multiple cell types in *Bacillus subtilis*, *FEMS Microbiol. Rev. 33* (2009) 152–163.
28. D. Lopez, R. Kolter, Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*, *FEMS Microbiol. Rev. 34* (2010) 134–149.
29. A. Oslizlo, P. Stefanic, I. Dogsa, I. Mandic-Mulec, Private link between signal and response in *Bacillus subtilis* quorum sensing, *Proc. Natl. Acad. Sci. USA, 111* (2014) 1586–1591 (doi: 10.1073/pnas.1316283111).
30. A.L. McLoon, S.B. Guttenplan, D.B. Kearns, R. Kolter, R. Losick, Tracing the domestication of a biofilm-forming bacterium, *J. Bacteriol. 193* (2011) 2027–2034.
31. P. Stefanic, I. Mandic-Mulec, Social interactions and distribution of *Bacillus subtilis* pherotypes at microscale, *J. Bacteriol. 191* (2009) 1756–1764.
32. R. Durret, M. Miras, N. Mirouze, A. Narechania, I. Mandic-Mulec, D. Dubnau, Genome sequence of the Bacillus subtilis biofilm-forming transformable strain PS216, *Genome Announc. 1* (2013) e00288-13 (doi: 10.1128/genomeA.00288–13).
33. G. Pozzi, L. Masala, F. Iannelli, R. Manganelli, L.S. Havarstein, L. Piccoli, Competence for genetic transformation in encapsulated strains of *Streptococcus pneumoniae*: Two allelic variants of the peptide pheromone, *J. Bacteriol. 178* (1996) 6087–6090.
34. G. Ji, R. Beavis, R.P. Novick, Bacterial interference caused by autoinducing peptide variants, *Science, 276* (1997) 2027–2030.
35. A.M. Whatmore, V.A. Barcus, C.G. Dowson, Genetic diversity of the streptococcal competence (com) gene locus, *J. Bacteriol. 181* (1999) 3144–3154.
36. L.S. Tran, T. Nagai, Y. Itoh, Divergent structure of the ComQXPA quorum-sensing components: Molecular basis of strain-specific communication mechanism in *Bacillus subtilis*, *Mol. Microbiol. 37* (2000) 1159–1171.
37. P. Tortosa, L. Logsdon, B. Kraigher, Y. Itoh, I. Mandic-Mulec, D. Dubnau, Specificity and genetic polymorphism of the *Bacillus* competence quorum-sensing system, *J. Bacteriol. 183* (2001) 451–460.
38. I. Mandic-Mulec, B. Kraigher, U. Cepon, I. Mahne, Variability of the quorum sensing system in natural isolates of *Bacillus* sp., *Food Technol. Biotechnol. 41* (2003) 23–28.
39. L. Slamti, D. Lerecius, Specificity and polymorphism of the PlcR-PapR quorum-sensing system in the *Bacillus cereus* group, *J. Bacteriol. 187* (2005) 1182–1187.
40. P. Stefanic, F. Decorosi, C. Viti, J. Petito, F.M. Cohan, I. Mandic-Mulec, The quorum sensing diversity within and between ecotypes of *Bacillus subtilis*, *Environ. Microbiol. 14* (2012) 1378–1389.
41. M. Ansaldi, D. Dubnau, Diversifying selection at the *Bacillus* quorum-sensing locus and determinants of modification specificity during synthesis of the ComX pheromone, *J. Bacteriol. 186* (2004) 15–21.
42. F.M. Cohan, Bacterial species and speciation, *Syst. Biol. 50* (2001) 1671–1679.
43. F.M. Cohan, E.B. Perry, A systematics for discovering the fundamental units of bacterial diversity, *Curr. Biol. 17* (2007) 373–386.
44. A. Eldar, Social conflict drives the evolutionary divergence of quorum sensing, *Proc. Natl. Acad. Sci. USA, 108* (2011) 13635–13640.
45. G. Chong, O. Kimyon, M. Manefield, Quorum sensing signal synthesis may represent a selective advantage independent of its role in regulation of bioluminescence in *Vibrio fischeri*, *PLoS ONE, 8* (2013) e67443 (doi: 10.1371/journal.pone.0067443).
46. C.N. Wilder, S.P. Diggle, M. Schuster, Cooperation and cheating in *Pseudomonas aeruginosa*: The roles of the las, rhl and pqs quorum-sensing systems, *ISME J. 5* (2011) 1332–1343.
47. M.R. Parsek, E.P. Greenberg, Sociomicrobiology: The connections between quorum sensing and biofilms, *Trends. Microbiol. 13* (2005) 27–33.
48. K.P. Lemon, A.M. Earl, H.C. Vlamakis, C. Aguilar, R. Kolter, Biofilm development with an emphasis on *Bacillus subtilis*, *Curr. Top. Microbiol. Immunol. 322* (2008) 1–16.
49. S.S. Branda, J.E. Gonzalez-Pastor, S. Ben-Yehuda, R. Losick, R. Kolter, Fruiting body formation by *Bacillus subtilis*, *Proc. Natl. Acad. Sci. USA, 98* (2001) 11621–11626.
50. S.S. Branda, J.E. Gonzalez-Pastor, E. Dervyn, S.D. Ehrlich, R. Losick, R. Kolter, Genes involved in formation of structured multicellular communities by *Bacillus subtilis*, *J. Bacteriol. 186* (2004) 3970–3979.
51. S.S. Branda, F. Chu, D.B. Kearns, R. Losick, R. Kolter, A major protein component of the *Bacillus subtilis* biofilm matrix, *Mol. Microbiol. 59* (2006) 1229–1238.

52. A. Driks, Tapping into the biofilm: Insights into assembly and disassembly of a novel amyloid fibres in *Bacillus subtilis*, *Mol. Microbiol. 80* (2011) 1133–1136.
53. D. Lopez, H. Vlamakis, R. Losick, R. Kolter, Paracrine signalling in a bacterium, *Genes Dev. 23* (2009) 1631–1638.
54. D.B. Kearns, F. Chu, S.S. Branda, R. Kolter, R. Losick, A master regulator for biofilm formation by *Bacillus subtilis*, *Mol. Microbiol. 55* (2005) 739–749.
55. A. Ostrowski, A. Melhert, A. Prescott, T.B. Kiley, N.R. Stanley-Wall, YuaB functions synergistically with the exopolysaccharide and TasA amyloid fibers to allow biofilm formation by *Bacillus subtilis*, *J. Bacteriol. 193* (2011) 1821–1831.
56. H. Vlamakis, Y. Chai, P. Beauregard, R. Losick, R. Kolter, Sticking together: Building a biofilm the *Bacillus subtilis* way, *Nat. Rev. Microbiol. 11* (2013) 157–168.
57. I. Dogsa, M. Brloznik, D. Stopar, I. Mandic-Mulec, Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms, *PLoS ONE, 8* (2013) e62044 (doi: 10.1371/journal.pone.0062044).
58. Y. Chai, P.B. Beauregard, H. Vlamakis, R. Losick, R. Kolter, Galactose metabolism plays a crucial role in biofilm formation by *Bacillus subtilis*, *MBio, 3* (2012) e00184-00112 (doi: 10.1128/mBio.00184-12).
59. P.B. Beauregard, Y. Chai, H. Vlamakis, R. Losick, R. Kolter, *Bacillus subtilis* biofilm induction by plant polysaccharides, *Proc. Natl. Acad. Sci. USA, 110* (2013) e1621-1630 (doi: 10.1073/pnas.1218984110).
60. G. Meng, K. Fütterer, Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase, *Nat. Struct. Mol. Biol. 10* (2003) 935–941.
61. S.K. Rhee, K.B. Song, C.H. Kim, B.S. Park, E.K. Jang, K.H. Jang: Levan. In: *Biopolymers Online*, E. Vandamme, S.D. Baets, A. Steinbuchel (Eds.), Wiley-VCH Verlag GmbH, Weinheim, Germany (2002) pp. 351–377 (doi: 10.1002/3527600035.bpol5014).
62. C. Olvera, S. Centeno-Leija, P. Ruiz-Leyva, A. López-Munguía, Design of chimeric levansucrases with improved transglycosylation activity, *Appl. Environ. Microbiol. 78* (2012) 1820–1825.
63. J.A. Lepesant, F. Kunst, J. Lepesant-Kejzlarová, R. Dedonder, Chromosomal location of mutations affecting sucrose metabolism in *Bacillus subtilis* Marburg, *Mol. Gen. Genet. 118* (1972) 135–160.
64. H. Shimotsu, D.J. Henner, Modulation of *Bacillus subtilis* levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes, *J. Bacteriol. 168* (1986) 380–388.
65. Y. Pereira, M.F. Petit-Glatron, R. Chambert, *yveB*, encoding endolevanase LevB, is part of the *sacB–yveB–yveA* levansucrase tricistronic operon in *Bacillus subtilis*, *Microbiology, 147* (2001) 3413–3419.
66. M. Débarbouillé, V. Martin-Verstraete, M. Arnaud, A. Klier, G. Rapoport, Positive and negative regulation controlling expression of the *sac* genes in *Bacillus subtilis*, *Res. Microbiol. 142* (1991) 757–764.
67. S.A. Arvidson, B.T. Rinehart, F. Gadala-Maria, Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp., *Carbohydr. Polym. 65* (2006) 144–149.
68. A. Stover, A. Driks, Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein, *J. Bacteriol. 181* (1999) 1664–1672.
69. D. Romero, C. Aguilar, R. Losick, R. Kolter, Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms, *Proc. Natl. Acad. Sci. USA, 107* (2009) e2230-2234 (doi: 10.1073/pnas.0910560107).
70. A.T. Kovács, J. van Gestel, O.P. Kuipers, The protective layer of biofilm: A repellent function for a new class of amphiphilic proteins, *Mol. Microbiol. 85* (2012) 8–11.
71. K. Kobayashi, M. Iwano, BslA (YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms, *Mol. Microbiol. 85* (2012) 51–66.
72. L. Hobley, A. Ostrowski, F.V. Rao, K.M. Bromley, M. Porter, A.R. Prescott *et al.*, BslA is a self-assembling bacterial hydrophobin that coats the *Bacillus subtilis* biofilm, *Proc. Natl. Acad. Sci. USA, 110* (2013) 13600–13605.
73. M. Morikawa, S. Kagihiro, M. Haruki, K. Takano, S. Branda, R. Kolter *et al.*, Biofilm formation by a *Bacillus subtilis* strain that produces γ-polyglutamate, *Microbiology, 152* (2006) 2801–2807.
74. D. Lopez, M.A. Fischbach, F. Chu, R. Losick, R. Kolter, Structurally diverse natural products that cause potassium leakage trigger multicellularity in *Bacillus subtilis*, *Proc. Natl. Acad. Sci. USA, 106* (2009) 280–285.
75. M. Jing, W. Shao, M. Perego, J.A. Hoch, Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*, *Mol. Microbiol. 38* (2000) 535–542.
76. M.A. Hamon, B.A. Lazazzera, The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*, *Mol. Microbiol. 42* (2001) 1199–1209.
77. D.B. Kearns, F. Chu, S.S. Branda, R. Kolter, R. Losick, A master regulator for biofilm formation by *Bacillus subtilis*, *Mol. Microbiol. 55* (2005) 739–749.
78. S.H. Shafikhani, I. Mandic-Mulec, M.A. Strauch, I. Smith, T. Leighton, Postexponential regulation of *sin* operon expression in *Bacillus subtilis*, *J. Bacteriol. 184* (2002) 564–571.
79. U. Bai, I. Mandic-Mulec, I. Smith, SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein-protein interaction, *Genes Dev. 7* (1993) 139–148.
80. I. Mandic-Mulec, N. Gaur, U. Bai, I. Smith, Sin, a stage-specific repressor of cellular differentiation, *J. Bacteriol. 174* (1992) 3561–3569.
81. Mandic-Mulec, L. Doukhan, I. Smith, The *Bacillus subtilis* SinR protein is a repressor of the key sporulation gene *spo0A*, *J. Bacteriol. 177* (1995) 4619–4627.
82. D. Lopez, H. Vlamakis, R. Losick, R. Kolter, Cannibalism enhances biofilm development in *Bacillus subtilis*, *Mol. Microbiol. 74* (2009) 609–618.
83. M. Ogura, K. Tsukahara, SwrA regulates assembly of *Bacillus subtilis* DegU via its interaction with N-terminal domain of DegU, *J. Biochem. 151* (2012) 643–655.
84. D.B. Kearns, R. Losick, Cell population heterogeneity during growth of *Bacillus subtilis*, *Genes Dev. 19* (2005) 3083–3094.
85. J.E. Patrick, D.B. Kearns, Swarming motility and the control of master regulators of flagellar biosynthesis, *Mol. Microbiol. 83* (2012) 14–23.
86. C. Calvio, C. Osera, G. Amati, A. Galizzi, Autoregulation of swrAAand motility in *Bacillus subtilis*, *J. Bacteriol. 190* (2008) 5720–5728.
87. D.T. Verhamme, E.J. Murray, N.R. Stanley-Wall, DegU and Spo0A jointly control transcription of two loci required for complex colony development by *Bacillus subtilis*, *J. Bacteriol. 191* (2009) 100–108.
88. F. Kunst, G. Rapoport, Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*, *J. Bacteriol. 177* (1995) 2403–2407.
89. M. Débarbouillé, I. Martin-Verstraete, M. Arnaud, A. Klier, G. Rapoport, Positive and negative regulation controlling expression of the *sac* genes in *Bacillus subtilis*, *Res. Microbiol. 142* (1991) 757–764.
90. K. Tsukahara, M. Ogura, Promoter selectivity of the *Bacillus subtilis* response regulator DegU, a positive regulator of the *fla/che* operon and *sacB*, *BMC Microbiol. 15* (2008) 1–12.

91. K. Kobayashi, Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*, *Mol. Microbiol. 66* (2007) 395–409.

92. T. Msadek, F. Kunst, A. Klier, G. Rapoport, DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*, *J. Bacteriol. 173* (1991) 2366–2377.

93. O. Zafra, M. Lamprecht-Grandío, C.G. de Figueras, J.E. González-Pastor, Extracellular DNA release by undomesticated *Bacillus subtilis* is regulated by early competence, *PLoS ONE, 7* (2012) e48716 (doi: 10.1371/journal.pone.0048716).

94. H. Vlamakis, C. Aguilar, R. Losick, R. Kolter, Control of cell fate by the formation of an architectural complex bacterial community, *Genes Dev. 22* (2008) 945–953.

95. J.N. Wilking, V. Zaburdaev, M. De Volder, R. Losick, M.P. Brenner, D.A. Weitz, Liquid transport facilitated by channels in *Bacillus subtilis* biofilms, *Proc. Natl. Acad. Sci. USA, 110* (2013) 848–852.

96. I. Dogsa, M. Kriechbaum, D. Stopar, P. Laggner, Structure of bacterial extracellular polymeric substances at different pH values as determined by SAXS, *Biophys. J. 89* (2005) 2711–2720.

97. I. Dogsa, J. Štrancar, P. Laggner, D. Stopar, Efficient modelling of polysaccharide conformations based on small-angle X-ray scattering experimental data, *Polymer, 49* (2008) 1398–1406.

98. J. Orehek, I. Dogsa, M. Tomšič, A. Jamnik, D. Kočar, D. Stopar, Structural investigation of carboxymethyl cellulose biodeterioration by *Bacillus subtilis* subsp. *subtilis* NCIB 3610, *Int. Biodeter. Biodegr. 77* (2013) 10–17.

99. E. Benigar, I. Dogsa, D. Stopar, A. Jamnik, I. Kralj-Cigić, M. Tomšič, Structure and dynamics of a polysaccharide matrix: Aqueous solutions of bacterial levan, *Langmuir, 30* (2014) 4172–4182 (doi: 10.1021/la500830j).

100. M. Burmølle, D. Ren, T. Bjarnsholt, S. Sørensen, Interactions in multispecies biofilms: Do they actually matter?, *Trends. Microbiol. 22* (2014) 84–91.

101. D. Romero, H. Vlamakis, R. Losick, R. Kolter, An accessory protein required for anchoring and assembly of amyloid fibres in *B. subtilis* biofilms, *Mol. Microbiol. 80* (2011) 1155–1168.

102. L. Chai, D. Romero, C. Kayatekin, B. Akabayov, H. Vlamakis, R. Losick *et al.*, Isolation, characterization, and aggregation of a structured bacterial matrix precursor, *J. Biol. Chem. 288* (2013) 17559–17568.