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Biofilm formation and dispersal in Gram-positive bacteria

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Biofilms are structured communities of bacteria, which are adhered to a surface and embedded in a self-produced matrix of extracellular polymeric substances. Since biofilms are very resistant to antimicrobial agents, they are at the basis of a range of problems, including quality and safety issues in food industry. Recently, major advances have been made in elucidating the different structural components of the biofilm matrix, the regulatory pathways involved in biofilm formation, and signaling molecules involved in biofilm formation and dispersal, which provide opportunities for prevention and control of these biofilms in the food industry.

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Introduction

Biofilms are complex microbial communities established on a wide range of surfaces that are generally encapsulated by an extracellular protective matrix composed of various types of biopolymers. Since biofilms are very difficult to eradicate, the ability of bacteria to form biofilms poses a major problem in various industrial and medical settings, being a persistent source of (re)contamination and/or infection, respectively. Mechanisms that have been proposed to explain the observed increased resistance of biofilms to antimicrobial agents are the impenetrable character of the biofilm, the slow growth rate of organisms, and the induction of resistance mechanisms [1]. The molecular mechanisms and factors involved in biofilm formation and subsequent dispersal of bacteria from the biofilm are starting to be unraveled

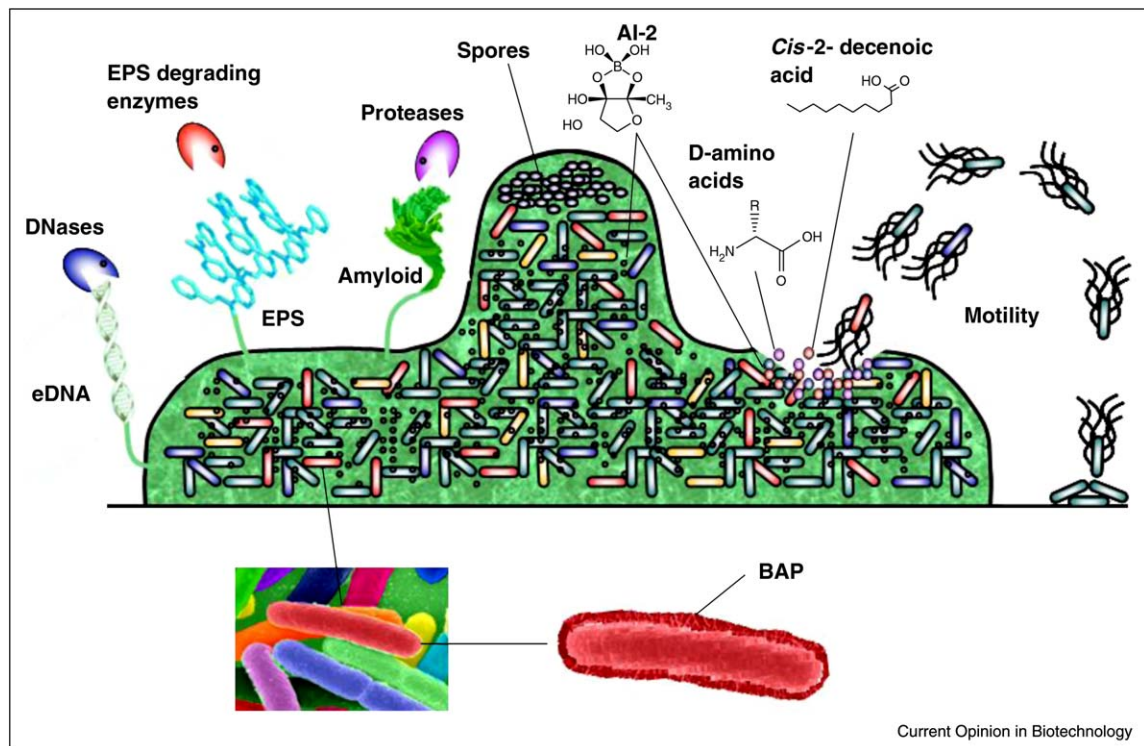
(Figure 1). This review provides an overview of the recent insights in the factors and mechanisms involved in biofilm formation and dispersal focusing on a selection of Gram-positive bacteria including *Bacillus* spp, *Listeria monocytogenes*, *Staphylococcus* spp, and lactic acid bacteria (LAB).

Diversity in biofilms

Depending on the specific systems investigated and the nature of the microorganism, biofilms can display a wide range of phenotypes. Biofilms of *L. monocytogenes*, grown under static conditions, generally consist of a homogeneous layer of cells and/or microcolonies, with the biofilm cells displaying a morphology similar to that of planktonic cells. By contrast, *L. monocytogenes* biofilms grown under continuous flow conditions consist of spherically shaped microcolonies that are surrounded by a network of knitted chains composed of elongated cells [2]. Interestingly, the formation of this type of *L. monocytogenes* biofilms is dependent on the activation of the SOS response factor YneA, which is responsible for cell elongation [3^{*}]. *Staphylococcus aureus* biofilms grown under both static and continuous flow conditions consist of a dense layer of cells with an elaborate matrix harboring various types of polymers. Biofilm formation and maturation in *S. aureus* is dependent on the interplay between various regulators including those encoded by *sarA*, *agr*, *ica*, and *sigB* [4–6]. Also, static and continuous flow biofilms of LAB such as *Lactobacillus plantarum* and *Lactococcus lactis* consist of a dense layer of cells that are frequently embedded in polymeric substances [7^{*},8]. Biofilms of *Bacilli* are generally studied at the air–liquid interface where they form structured pellicles floating on culture media or as architecturally complex colonies on solid agar media [9–11]. The latter type of biofilms develops aerial structures called ‘fruiting bodies’ that serve as sites for sporulation [9]. The formation of these fruiting bodies is dependent on the temporal and spatial regulation of several distinctive pathways. In addition, *Bacilli* can form submersed biofilms both under static and continuous flow conditions [10,11].

In most conditions bacteria will generally grow on surfaces in competition with other microorganisms in a mixed species biofilm. Mixed species biofilm formation of several *S. aureus* strains with *L. monocytogenes* has been demonstrated and generally the number of cells in the mixed species biofilm is similar to the number of cells in single species biofilms [12]. A study on mixed biofilm formation of *L. monocytogenes* in combination with various secondary species showed that mixed species biofilms were established and depending on the specific combination, they

Figure 1



Schematic presentation showing mechanisms and components involved in biofilm formation and dispersal. Biofilms can contain various extracellular biopolymers like extracellular DNA (eDNA), extracellular polysaccharides, amyloid fibers, and biofilm-associated proteins (BAP). These matrix components might be good targets for (combinations of) putative enzymes such as DNases, proteases, and extracellular polysaccharide degrading enzymes to prevent formation of biofilms or to stimulate dispersal of already formed biofilms. Communication between cells during biofilm formation and dispersal of biofilms is dependent on quorum sensing systems and molecules like autoinducer 2 (Al-2), D-amino acids, and *cis*-2-decenoic acid. Furthermore, motility is an important factor in the establishment of new biofilms and the dispersal of cells from mature biofilms. Also, aerial structures of the biofilm serve as specific sites for the generation of spores (see text for details and corresponding references).

showed increased, reduced, or no effect on the number of *L. monocytogenes* cells in the biofilm [13]. Furthermore, an elaborate study on the formation of mixed species biofilms of *L. monocytogenes* and *L. plantarum* showed that mixed species biofilms have the capacity to be more resistant against disinfectant treatments than single species biofilm or planktonic cells [14]. Another interesting study on the adherence of *L. monocytogenes* to preformed *L. lactis* biofilms with different architectures, matrices, and cell surface properties showed that *L. monocytogenes* biofilm formation can be influenced by resident biofilms [7^{*}]. The impact of secondary species on *L. monocytogenes* settlement, biofilm formation and persistence in food processing environments remains to be characterized.

The biofilm matrix

When forming a biofilm, bacteria produce various biopolymers mediating cell-to-cell and cell-to-surface attachments. However, diverse polymers are used by different species or strains of the same species. These extracellular polymeric substances (EPS) are mainly polysaccharides, proteins, nucleic acids, and lipids, that provide on the one

hand mechanical stability of biofilms and adhesion to surfaces, and on the other hand form a scaffold for the three-dimensional architecture that interconnects and immobilizes biofilm cells [15]. For example, *Bacillus subtilis* NCIB3610 produces an exopolysaccharide [16], while *B. subtilis* RO-FF-1 synthesizes poly- γ -D,L-glutamate [17] as matrix component. *S. aureus* biofilms contain the polymer poly-N-acetyl glucosamine (PNAG), which is also referred to as polysaccharide intercellular adhesion (PIA), and is synthesized by enzymes encoded on the *ica*-operon [18]. A functional biofilm matrix often also requires single protein or several protein components. The *B. subtilis* matrix contains amyloid fibers of the protein TasA [19^{*}], while for *L. monocytogenes* the presence of a biofilm-associated protein, BapL, in the matrix has been described [20]. BAP proteins are also involved in biofilm formation in various *S. aureus* strains [21–23], but in this species several other extracellular biofilm promoting proteins have also been described [24,25]. Recently, an extracellular protein, Maba, was described for *Lactobacillus rhamnosus*, which appeared to be very important for biofilm formation

[26]. Furthermore, a function for extracellular DNA (eDNA) in cell adhesion and biofilm formation was recently shown for *Bacillus cereus* [27], *S. aureus* [28,29], and *L. monocytogenes* [30]. The release of genomic DNA from a subpopulation of cells of *S. aureus* during biofilm development is functionally analogous to the role of apoptosis in eukaryotic development [31]. Although the Cid/Lrg system was revealed to be involved in the activation of programmed cell death (PCD) of *S. aureus*, and although it is not known how PCD is induced only in a subpopulation of the cells, it provides an intriguing example of differentiation in bacterial biofilms. The identification of these novel matrix components may provide clues to the identification and application of matrix-degrading enzymes that prevent formation and/or activate dispersal of biofilms [32,33] (Figure 1).

Involvement of flagella and motility in biofilm formation

Biofilms generally consist of cells that are not motile. However, for several bacteria motility has been indicated to be an important factor during biofilm formation or attachment of cells to a surface (Figure 1). For *B. subtilis*, flagella and swarming motility was important, but not essential for normal progression of pellicle type biofilm formation [34]. For *L. monocytogenes* static biofilm formation, flagellar based motility appeared to be essential to propel cells towards the surface before attachment [35], while under continuous flowing conditions loss of flagellar motility resulted in lower initial cell attachment but increased biofilm formation [36]. Similar observations were made for the role of flagella-based motility in *B. cereus* biofilm formation [10]. Motility appeared to be important for static biofilm formation in this organism but not for biofilm formation under continuous flow conditions. These results show that it is important for bacteria to control the transition between the motile planktonic mode of growth and the biofilm mode of growth. For *L. monocytogenes*, it has been shown that this transition was under control of the DegU response regulator, that appeared not to be associated with a cognate histidine protein kinase, and its phosphorylation status seems to be directly controlled by Acetyl-phosphate levels. This may connect biofilm formation to the metabolic state of cells, which may be highly relevant for biofilm formation control [37–39]. DegU also plays an important role in biofilm formation in *B. subtilis*. Gradual phosphorylation of DegU determines whether cells activate swarming (low DegU~P level), biofilm development (medium DegU~P level), or exoprotease production (high DegU~P level) [40,41]. Furthermore, a very sophisticated mechanism to control motility of *B. subtilis* cells in biofilms was recently identified. EpsE, which is encoded within an operon required for biofilm matrix synthesis, is capable of arresting flagellar rotation in biofilm cells by disengaging the motor force generating elements [42].

Spore formation in biofilms: intertwinement of regulatory pathways

Control of sporeformers and resultant spores is of eminent importance for food quality and safety, owing to the resistance of spores against processing regimes such as heating. Notably, biofilms provide an optimal environment for sporulation of *Bacilli*. Developmental processes, like motility, biofilm formation, and sporulation are strictly connected and share global regulators. Furthermore, these processes are spatio-temporally coordinated, while genes facilitating motility are first expressed, matrix production is activated thereafter, and finally spores appear on the upper aerial structures of the biofilm (Figure 1) [43]. This complex coordination of developmental processes is achieved by an interplay of several regulators. Also the activity states of various regulators are important for proper modulation of phenotypic traits. The coordination of the formation of different cell types is achieved through cell–cell communication. In case of high cell density, quorum sensing molecules accumulate and induce the production of surfactin in a subpopulation of cells [44]. The produced surfactin is in turn sensed by distinct cells, but not by the surfactin producing cells themselves, and triggers potassium leakage and subsequent activation of the KinC sensor kinase localized in membrane microdomains analogous to lipid rafts in eukaryotic cells [45,46]. In spite of differences in the regulatory networks that control biofilm formation in *B. subtilis* and *S. aureus*, compounds that inhibit the formation of lipid rafts such as zaragozic acid, also inhibited the development of *S. aureus* biofilm [46]. The generation of a signal by some cells to which only certain target cells respond presents a novel paracrine signaling pathway that requires a proper distribution of signal producing and sensing cell types. Activated KinC modulates activity of the master regulator Spo0A through a phosphorelay pathway. Intermediate levels of Spo0A~P activate the production of SinI, a small protein that antagonizes SinR, the repressor of several biofilm operons [47]. Upon alleviating the inhibitory effect of SinR, the operons involved in matrix production are expressed. As Spo0A phosphorylation and expression of matrix operons are activated only in a subpopulation of cells, biofilm components are produced by part of the population as a division of labor [48]. Recently, the new regulator SlrR was identified that plays a role in biofilm formation by repressing *sinR* and by forming a SinR–SlrR complex. It titrates SinR and prevents it from repressing *slrR* itself [49]. Furthermore, this complex represses autolysin and motility genes. Thus, this epigenetic switch controls cell separation and helps the formation of long chains of cells that is a prerequisite for biofilm development. Further increase of the Spo0A~P level drives a subpopulation of cells in the maturing biofilm to the formation of spores. However, in matrix-deficient mutants the KinD membrane sensor protein acts rather as a phosphatase than a kinase and inhibits the activity of Spo0A that results in a delay of

sporulation [50]. Notably, most of these regulators that assist in *B. subtilis* biofilm formation are conserved in *Bacilli*, but their role in biofilm formation and regulatory cascades involved, remain to be elucidated.

In *B. cereus* the production of virulence factors, including enterotoxins, is regulated by PlcR, a pleiotropic transcriptional regulator that downregulates biofilm formation presumably via altering production of biosurfactants [51]. Interestingly, master regulator Spo0A was shown to downregulate *plcR* expression [52], suggesting that enterotoxin production is hampered in biofilms. By contrast, the putative cell wall peptidase, CwpFM, was shown to contribute to virulence and biofilm formation [53]. The cells and spores accumulated in biofilms may disperse and recontaminate foods, where they are delivered to the gastrointestinal (GI) tract after consumption, followed by germination, in case of spores, growth and production of enterotoxins [54].

Communication to build and break biofilms

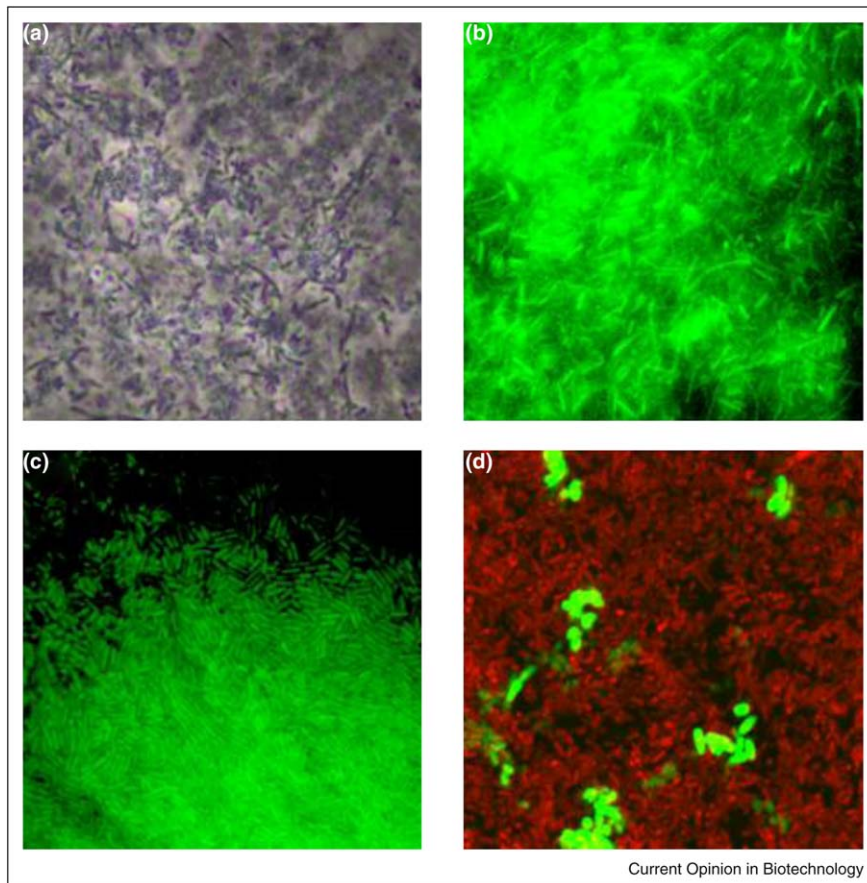
Single and mixed species biofilm development, maturation, survival of different physicochemical conditions encountered in the range of micro niches in biofilms, and the release of cells from mature biofilms, require complex sequential mechanisms for which cross-species cell to cell communication and/or interspecies quorum sensing (QS) might be important factors (Figure 1). Although the specific QS molecules fill an important role in affecting the developmental regulatory networks like sporulation and competence, there are examples of broadly conserved QS systems that control biofilm formation. One of these systems involves the LuxS enzyme that synthesizes autoinducer-2 (AI-2). For several Gram-positive bacteria it has recently been shown that a functional *luxS* gene and AI-2 signal were present and that this system is involved in biofilm formation [55]. For *L. monocytogenes*, it was shown that inactivation of *luxS* resulted in increased biofilm formation [56,57]. However, addition of *in vitro* synthesized AI-2 did not restore wild-type biofilm levels. Instead, biofilm formation appeared to be increased after addition of S-ribosyl homocysteine (SRH), which is one of the precursors in the AI-2 pathway [56]. In *B. cereus*, the addition of *in vitro* synthesized AI-2 inhibited biofilm formation and stimulated the release of cells from a mature biofilm [58]. Increased biofilm formation for a *luxS* mutant was also shown in *Lactobacillus reuteri* [59] and *Staphylococcus epidermidis* [60]. However, in *S. epidermidis* the *luxS* mutant showed increased expression of the *ica*-operon and increased production of biofilm polysaccharide PIA, which could explain the observed biofilm phenotype. By contrast, biofilm formation of the *luxS* mutant was reduced in *L. rhamnosus* [61] and *B. subtilis* [62]. In *B. subtilis*, AI-2 production was furthermore regulated by the sporulation and biofilm regulators Spo0A and SinR and spatial activity of LuxS appeared to be important for the development of specific biofilm

structures [62]. The other major quorum sensing system is the peptide-mediated accessory gene regulator (Agr) system, which consists of an operon containing four genes (*agrBDCA*). In *L. monocytogenes* a specific role for the *agr*-system in the positive regulation of adherence and biofilm formation was identified by mutant analyses and expression studies [63,64]. Similar observations were made for *L. plantarum*, although interestingly, this organism contains two homologous Agr systems. However, only the regulatory two-component part of the four-gene module is duplicated, while only a single copy of the peptide generating gene-set is present in this species. The corresponding peptide is proposed to communicate via both two-component regulatory Agr-like systems mediating control of cell adherence and biofilm formation [65]. By contrast, *S. aureus* biofilm formation was shown to be negatively regulated by the Agr-system, since its activation stimulated biofilm dispersal [66]. Other communication molecules that are involved in dispersal of biofilm cells have also been identified (Figure 1). The fatty acid *cis*-2-decenoic acid, which is produced by *Pseudomonas aeruginosa*, induced dispersal in various bacteria, including *B. subtilis* and *S. aureus* [67]. Another communication or dispersal molecule was identified in *B. subtilis* biofilms. As *B. subtilis* biofilms age, D-amino acids are produced just before its disassembly. Addition of individual or combinations of D-amino acids (e.g. D-leucine, D-methionine, D-tyrosine, and D-tryptophan) before biofilm formation inhibits the functional assembly of the matrix [68**]. The D-amino acids modify the connection between the TasA protein component and the cell wall, acting through the YqxM protein. Particular strains harboring mutations in the protein sequence of YqxM that is required for the association of TasA are resistant to the presence of D-amino acids and delayed in disassembly. It is hypothesized that a domain near the C terminus of YqxM could trigger the release of TasA in response to the presence of D-amino acids in the cell wall. Interestingly, D-amino acids are effective against biofilms of *S. aureus* and *P. aeruginosa*, in spite of the lack of TasA and YqxM homologs in these species, suggesting a general strategy for biofilm disassembly. The production of D-amino acids by bacteria can be a common signal for biofilm disassembly and may provide clues for the search and development of effective inhibitors of biofilm formation in food processing environments.

Tools in biofilm analysis

Advancements in imaging techniques for studying the formation of single and mixed species biofilms have contributed significantly to the current status of the biofilm research community. These new techniques, which include laser scanning microscopy, scanning transmission X-ray microscopy, and magnetic resonance imaging, made it possible to study the formation and dynamics of biofilms *in situ* (reviewed in [69]). In particular, imaging techniques that use fluorescent reporters have proven to be an excellent tool to study activation of specific genes in biofilms, to

Figure 2



Application of fluorescent reporters to study the expression of specific genes in biofilms (a,b) and to investigate the formation of single and mixed species biofilms (c,d). Phase contrast (a) and fluorescent microscopy images (b) of a *L. monocytogenes* continuous flow biofilm expressing EGFP from the *yneA* promoter. Fluorescent microscopy image of *L. monocytogenes* constitutively expressing EGFP in a static biofilm on stainless steel (c), and confocal microscopy image of *L. monocytogenes* constitutively expressing DsRed and *L. plantarum* constitutively expressing EGFP in a mixed dual species biofilm (d).

investigate biofilm formation on surfaces that are not penetrable by light, or to study the distribution of organisms in mixed species biofilms (Figure 2). Fluorescent imaging furthermore proved very useful for investigating the dynamics in biofilm matrix structures. This is highlighted with the example of programmed cell death in a subpopulation of the *S. aureus* biofilm cells, which resulted in the release of genomic DNA that subsequently becomes an important component for biofilm maturation [28,29]. Future tool developments in biofilm research will most probably focus on capturing the role and fate of single cells in biofilm formation and maturation.

Concluding remarks

The majority of bacteria are able to form biofilms displaying a large variety in architecture, phenotypes, and matrix components. Novel insights include factors contributing to phenotypic heterogeneity within biofilms, the identification and characterization of a range of matrix building

blocks such as extracellular polysaccharides, eDNA, and amyloid fibers, and the identification of components that activate dispersal such as D-amino acids. Notably, most information has been obtained from studies with single species biofilms, and there is an urgent need to extend our knowledge on mixed species biofilms, since these presumably also develop in food processing environments displaying features different from that of the respective single species biofilms. The advances in our understanding of the different factors and mechanisms involved in biofilm formation and dispersal of various Gram-positive bacteria might provide clues and stimulate developments in the search for (natural) compounds and combinations thereof, for prevention and control of spoilage and pathogenic bacteria in industrial settings.

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