Biofilm Development with an Emphasis on *Bacillus subtilis*

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Abstract Our understanding of the molecular mechanisms involved in biofilm formation has increased tremendously in recent years. From research on diverse bacteria, a general model of bacterial biofilm development has emerged. This model can be adjusted to fit either of two common modes of unicellular existence: nonmotile and motile. Here we provide a detailed review of what is currently known about biofilm formation by the motile bacterium *Bacillus subtilis*. While the ability of bacteria to form a biofilm appears to be almost universal and overarching themes apply, the combination of molecular events necessary varies widely, and this is reflected in the other chapters of this book.

In most natural settings, bacteria are found predominantly in biofilms (Henrici 1933; Costerton et al. 1999; Hall-Stoodley et al. 2004). The widespread recognition that biofilms impact myriad environments, from water pipes to indwelling devices in hospital patients, led to an increased interest in investigating the molecular mechanisms underlying the formation and maintenance of these communities. As a consequence, we have recently witnessed much growth in our knowledge of biofilms. The ability to form biofilms, once considered the domain of a few species, is now seen as a nearly universal attribute of microorganisms. It has also become evident that the pathways utilized by bacteria to build biofilms are extremely

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diverse, varying enormously among different species and under different environmental conditions. There are, however, several common features among all biofilms examined to date:

1. Constituent cells are held together by an extracellular matrix composed of exopolysaccharides (EPS), proteins, and sometimes nucleic acids (Whitchurch et al. 2002; Branda et al. 2005; Lasa 2006).
2. Biofilm development occurs in response to extracellular signals, both environmental and self-produced (Kolter and Greenberg 2006; Spoering and Gilmore 2006).
3. Biofilms afford bacteria with protection from a wide array of environmental insults, as diverse as antibiotics (Mah and O'Toole 2001), predators (Kadouri et al. 2007), and the human immune system (Singh et al. 2000; Fedtke et al. 2004; Leid et al. 2005).

Initial studies on bacterial biofilms were predominantly descriptive. By applying novel microscopic approaches, most notably laser scanning confocal microscopy, a whole new universe of biofilm architecture became apparent. These approaches, coupled with time-lapse video microscopy and microsensors, have given us a more complete view of the complex structure of biofilms. These descriptive advances have been followed in the last decade by an outburst in the number of molecular genetic analyses carried out in biofilms. Today, investigators are applying a wide range of molecular biological approaches to the study of the regulatory processes that underlie biofilms. As is made clear in the chapters of this book, there are many examples where molecular genetics has had great impact on biofilm research. For almost every organism that has been investigated, we can now draw working genetic models for the steps in the pathways of biofilm development. There are genes expressed during each step of development that can serve as reporters of that stage and there are genes whose functions are essential for each particular step to be completed successfully. In spite of these advances, one can argue that biofilm genetics is still in its infancy. Investigators continue to identify novel genes that are either essential for, or expressed during, biofilm formation in many different organisms. Even among genes that have been previously identified, we have only recently begun to elucidate exactly how some of these genes' products contribute to biofilm development, maintenance, and dissolution.

1 How Do We Study Biofilms in the Laboratory?

Pipelines, catheters, teeth, plant roots, and the lungs of cystic fibrosis patients are but a few of the most widely recognized surfaces where the effects of biofilms are readily apparent. The biofilms that form on such surfaces almost invariably house a complex mixture of species, rendering them not particularly amenable to molecular genetic studies. To be able to address questions regarding the molecular basis of biofilm formation, investigators have developed artificial biofilm model systems that are easy to control and reproducible from laboratory to laboratory.

While there are numerous laboratory conditions that favor biofilm formation, investigators have routinely utilized four general systems for the study of biofilms. First among these systems is the flow cell (Christensen et al. 1999; Branda et al. 2005). Flow cells are small chambers with transparent surfaces where submerged biofilms can form and be continually fed fresh nutrients. The submerged biofilms that form on flow cells are particularly amenable to observation through confocal scanning laser microscopy. This allows for the capture of images of biofilm development in real time. The results obtained using flow cells have provided us with the familiar images of submerged biofilms consisting of mushroom-like structures separated by water-filled channels (Christensen et al. 1999; Branda et al. 2005). However, flow cells can be cumbersome and are not easily adapted for high throughput mutant screens. Submerged biofilms can also be studied in batch culture under conditions of no flow in microtiter dishes (O'Toole and Kolter 1998b; O'Toole et al. 1999). In this system, large numbers of samples can be quickly analyzed. Using the microtiter dish assay system, many investigators have carried out high-throughput screens and identified genes involved in biofilm formation and maintenance in numerous bacterial species (O'Toole and Kolter 1998a, 1998b; Pratt and Kolter 1998; Watnick and Kolter 1999; Watnick et al. 2001; Valle et al. 2003). The floating pellicles that form at the liquid-air interface of standing cultures represent another form of biofilm that is easily studied and adaptable for mutant screens (Guvener and McCarter 2003; Friedman and Kolter 2004; Enos-Berlage et al. 2005). Finally, the colonies that grow on the surface of agar dishes and demonstrate macroscopically complex architecture are now widely recognized as a form of biofilm (reviewed in Branda et al. 2005). This complex colony morphology correlates with production of extracellular matrix and the morphological variation observed in colonies often correlates with cells' ability to form robust biofilms in other assays. Like pellicles and the biofilms that form on the walls of microtiter dish wells, colonies are amenable to high-throughput screens to identify genes involved in biofilm formation and maintenance.

While all four systems for studying biofilm formation have been successful in broadening our understanding of biofilm development among diverse microorganisms, it is important to note that there can be variation among the phenotypes observed as one moves between systems. For example, mutants that exhibit a biofilm defect in one system may have imperceptible or no phenotype in another (O'Toole and Kolter 1998b). The converse also holds true; there are classes of biofilm mutants that do have a reproducible phenotype across all systems, for example, mutants defective for extracellular matrix production (Friedman and Kolter 2004). Ultimately, of these four general systems, no single one stands out as clearly superior; rather, the methods complement each other. Analyses of the phenotypic changes expressed by different mutants using combinations of several, or all four, of these systems can greatly aid our understanding of the role that different gene products play in biofilm development.

Individual species of bacteria vary greatly with regards to the environmental conditions under which they will produce maximal amounts of biofilm. These optimal conditions may, in fact, be telling us something about the biology and/or

ecology of the organism. We have also noted that many commonly used laboratory strains produce only frail or weak biofilms when compared to wild strains of the same species. In a number of instances, it has been possible to show that this stems from laboratory strains having accumulated numerous mutations over years of passaging through liquid cultures in a process we refer to as domestication (Branda et al. 2001; Valle et al. 2003). In working with liquid cultures of dispersed populations of cells, we appear to have unwittingly enriched for strains that have lost some of their potential to form structured multicellular communities while growing rapidly in liquid culture.

2 A General Model for Biofilm Development

Biofilm formation is a developmental process in which bacteria undergo a regulated lifestyle switch from a nomadic unicellular state to a sedentary multicellular state where subsequent growth results in structured communities and cellular differentiation. Results of prior work by many groups allow the construction of a hypothetical developmental model for biofilm formation that can be generalized for many different bacterial species. This model can be adjusted to fit either of two general modes of unicellular lifestyle: nonmotile and motile.

In the case of non-motile species (Fig. 1), when conditions are propitious for biofilm formation, individual bacteria appear to increase the expression of adhesins on their outer surface, i.e., they increase their "stickiness". This increased stickiness promotes both cell-cell adherence and cell-surface adherence when these bacteria encounter a surface (Gotz 2002). For example, in the case of some strains of staphylococcal species, surface-expressed proteins, including Bap, promote cell-cell interaction and contribute to the extracellular matrix (Lasa and Penades 2006). Many other species, both nonmotile and motile, harbor homologs of Bap. A unifying feature of these large extracellular proteins is the presence of repeated domains. At the level of the *bap* gene, these repeats have been shown to be recombinogenic, resulting in the production of proteins of variable length within a biofilm population (Latasa et al. 2006). Yet, the significance of this variability in the size of Bap proteins within a biofilm remains unknown. Nonmotile species also produce exopolysaccharides (EPS) that form an integral part of the extracellular matrix. One example of this is the PIA or PNAG EPS, produced by the gene products of the *ica* operon of staphylococcal species. Thus, in nonmotile bacteria, changes in cell surface proteins, along with the production of EPS, play a critical role in the initiation of biofilm formation (Gotz 2002; Latasa et al. 2005).

In the case of motile species (Fig. 2), when conditions favor biofilm formation, individual bacteria localize to a surface and initiate a dramatic lifestyle switch. Motility is lost and bacteria begin to produce an extracellular matrix that holds the cells together. For a number of motile organisms, the dominant role for flagella in initiation of biofilm formation is to provide motility as flagella-minus and paralyzed flagella mutants are comparably defective in biofilm formation (Pratt and Kolter

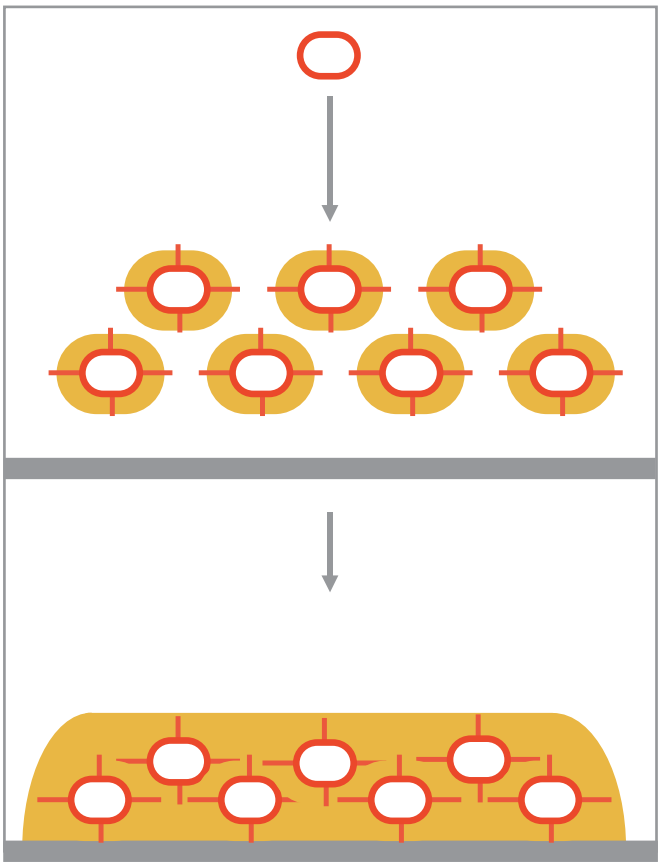

Fig. 1 General model for biofilm formation by nonmotile bacteria. Thick gray lines represent surfaces. (Top panel) To initiate biofilm formation nonmotile cells increase expression of adhesins. (Bottom panel) This results in surface-adhered, matrix-enclosed cells in a biofilm

1998; Watnick and Kolter 1999; Lemon et al. 2007). In fact, in *Listeria monocytogenes* supplying exogenous cell movement directed toward the surface via centrifugation restores wild type levels of initial surface adhesion to nonmotile mutants (Lemon et al. 2007). In these cases, it appears that motility is the driving force that overcomes repulsive forces between the bacteria and the surface. Initial encounters with a surface usually lead to transient adherence. This transient adherence can result in either a stable surface association, and a subsequent switch to biofilm development, or in a return to planktonic existence.

The first strides in understanding the molecular mechanisms of biofilm formation were made in Gram-negative Proteobacteria, especially *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa*, using surface-adhered biofilm assays (O'Toole and Kolter 1998a, 1998b; Pratt and Kolter 1998; Watnick and Kolter 1999; Watnick et al. 2001). Based on mutant phenotypes from these organisms, biofilm formation can be divided into five genetically distinct stages:

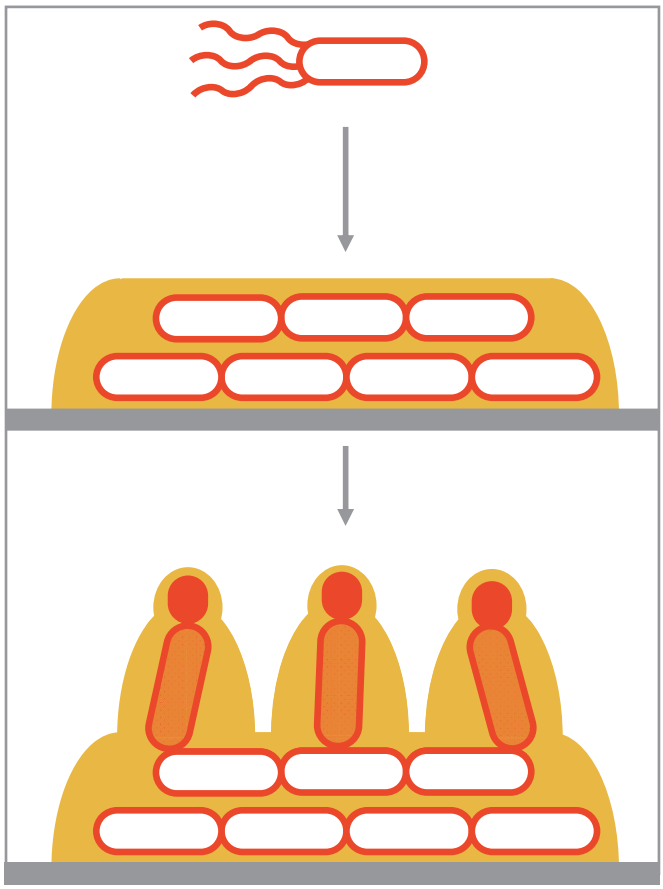

Fig. 2 General model for biofilm formation by motile bacteria. Thick gray lines represent surfaces. (Top panel) Motile, planktonic cells transition to nonmotile, matrix-producing, surface-adhered cells in a biofilm. (Bottom panel) Subsequently, cells differentiate within the biofilm

1. Initial surface attachment
2. Monolayer formation
3. Migration to form multilayered microcolonies
4. Production of extracellular matrix
5. Biofilm maturation with characteristic three-dimensional architecture (O'Toole et al. 2000)

These general stages provide a paradigm for studying biofilms formed by motile bacteria, although the precise details vis-à-vis regulation of this process do vary greatly from species to species. While initial surface attachment is dependent on flagella-mediated motility in a wide variety of motile bacteria, in some Gram-negative bacteria, microcolony formation and final three-dimensional architecture are also dependent on type IV pili-associated surface motility, which is notably absent in

Gram-positive bacteria (with the exception of Clostridia ssp.; Varga et al. 2006; O'Toole et al. 2000). After cells have adhered, matrix production begins; the extracellular matrix serves as an organizing principle that permits the building of structured communities within which there can be extensive cellular differentiation, shown as cells of different shapes and shades in Fig. 2. While these genetically defined stages of biofilm formation correlate with the temporal progression of biofilm development, it should be noted that the production of extracellular matrix appears to overlap with all stages that occur after initial surface adhesion. Also, while cellular differentiation within a biofilm is illustrated in our model for biofilm formation by a motile bacterium (see Figs. 1 and 2), we postulate that many bacteria, both motile and nonmotile, undergo comparable processes during biofilm formation.

While it is generally agreed upon that matrix production and motility are mutually exclusive, much remains to be learned regarding the molecular mechanisms that underlie this lifestyle switch for most bacterial species. Through a combination of genetic and biochemical approaches, our group, in close collaboration with the group of Richard Losick, has begun to identify the molecular regulatory circuitry that governs the transition from motile cells to matrix-producing cells of the Gram-positive soil bacterium *Bacillus subtilis*.

3 *Bacillus subtilis* as a Model System for Studying Biofilm Formation

B. subtilis, a Gram-positive motile rod-shaped bacterium, is best known for its ability to become competent and undergo sporulation in response to starvation and high population densities (Grossman 1995). The regulatory processes controlling *B. subtilis* sporulation and competence have been extensively characterized (Sonenshein et al. 2002; Piggot and Hilbert 2004). At the molecular level, the regulation of *B. subtilis* endospore formation is probably the best understood microbial developmental process; however, until very recently sporulation has been analyzed almost exclusively from the perspective of a single cell and not as a process occurring within a spatially organized community.

Most of the *B. subtilis* biofilm data come from studies on the development of complex, wrinkled colonies and from the development of pellicles at an air-liquid interface, although some studies have focused on solid surface-associated biofilms (Hamon and Lazazzera 2001; Stanley et al. 2003; Hamon et al. 2004). Biofilm formation by *B. subtilis* follows a distinct developmental pathway (Branda et al. 2001) (Fig. 3). After inoculation of standing cultures in a defined minimal medium containing glycerol as the major carbon source (MSgg; Branda et al. 2001), motile cells proliferate throughout the liquid as planktonic cells until they reach a density of approximately 5×10^7 cfu/ml after 1 day at room temperature (Fig. 3a). At that point, the vast majority of the cells begin to migrate to the air-liquid interface, where they form a floating biofilm or pellicle on the surface of the medium. The

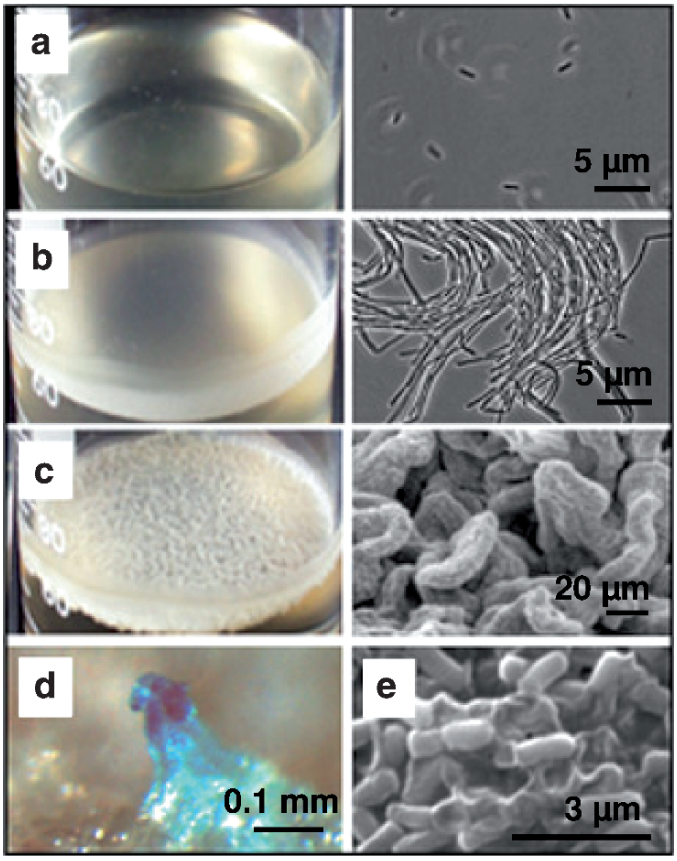

Fig. 3 Development of a *B. subtilis* biofilm. Panels **a-c** show pellicles on the *left* and microscopic images on the *right*. **a** and **b** are phase-contrast images, **c** and **e** are SEM images. **d** is from a dissecting microscope

pellicle is readily apparent but flat after 3 days (Fig. 3b). At this and subsequent times, the few remaining planktonic cells ($<10^5$ cfu/ml) retain their motility and do not sporulate. In contrast, cells within the pellicle undergo dramatic differentiation as they continue to proliferate. Cells become nonmotile and form long chains that are aligned in parallel (Fig. 3b). After 5 days of incubation, and as the cell mass increases, the pellicle begins to wrinkle, and, within the wrinkles, some groups of cells begin to grow as aerial projections (Fig. 3c). The tips of these projections serve as preferential sites of sporulation, as evidenced by the localized expression of the sporulation-specific gene *sspE* fused to *lacZ* (Branda et al. 2001) (Fig. 3d). Because a similar spatial organization of sporulation is characteristic of myxobacterial fruiting bodies, we refer to the *B. subtilis* aerial structures as fruiting body-like structures. Aerial structures indistinguishable from those observed in pellicles form at the edges of colonies grown on agar plates. To achieve such spatiotemporal

organization, the cells rely on an extracellular matrix to hold them together; scanning electron microscopy of cells from a 5-day-old colony reveals that they are indeed enclosed in such a matrix (Fig. 3e).

It is important to note that these structured communities and their high degree of cellular differentiation are only apparent when wild isolates are analyzed. Most of the standard laboratory strains, derivatives of strain *B. subtilis* 168, do not display such robust community structure, presumably as a result of domestication. Thus, most studies have focused primarily on the wild strain *B. subtilis* NCIB3610 (henceforth referred to as wild or 3610).

4 The Genetic Circuitry of *Bacillus subtilis* Biofilm Formation

During biofilm development, *B. subtilis* switches from being flagellated, motile single cells to growing in long chains of nonmotile cells that form parallel bundles (Branda et al. 2001). Figure 4 is a simplified view of the key players in this lifestyle switch. The transcriptional regulator SinR serves as the master regulator governing this switch (Kearns et al. 2005). In motile cells, SinR represses the transcription of genes responsible for matrix production and indirectly promotes cell separation and motility (Branda et al. 2006). SinR is constitutively produced, and when conditions become favorable for biofilm formation, SinR activity is antagonized. SinI and two newly identified proteins, YlbF and YmcA, all serve to directly and/or indirectly

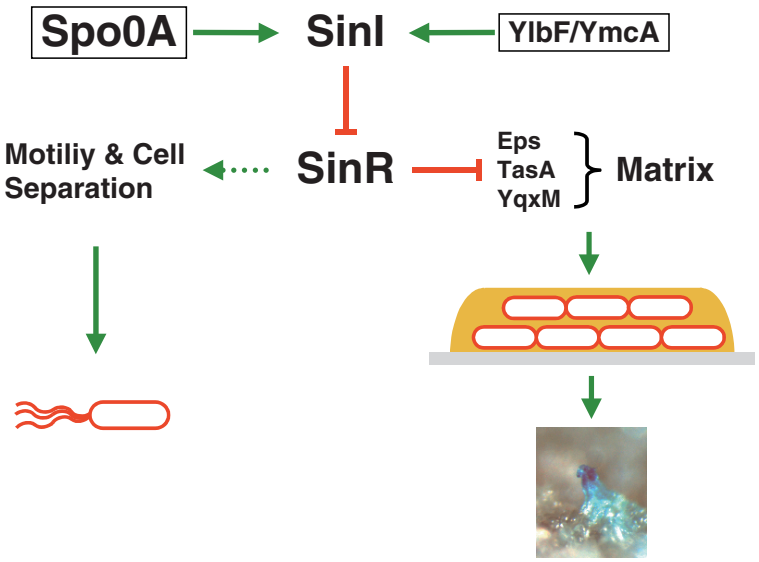

Fig. 4 Simplified view of the genetic circuitry governing *B. subtilis*'s lifestyle switch from nomadic to a sedentary existence

antagonize SinR activity. Lowered SinR activity results in loss of motility, cell chain formation, and matrix production. The extracellular matrix responsible for proper biofilm development in 3610 consists primarily of an exopolysaccharide (EPS) and a protein, TasA (Branda et al. 2006). Once this matrix is produced, the community develops a high degree of spatiotemporal organization culminating with sporulation occurring preferentially at the tips of aerial structures.

Prior to the discovery of SinR as the master regulator of biofilm formation in *B. subtilis,* Spo0A and σ^H were identified as transcriptional factors involved in biofilm development (Branda et al. 2001; Hamon and Lazazzera 2001). Two transcriptional profiling studies had identified members of the Spo0A and σ^H regulons (Fawcett et al. 2000; Britton et al. 2002). One fifteen-gene operon designated as *yveK-T yvfA-F*, later renamed *epsA-O*, under control of both Spo0A and σ^H, was predicted to encode products likely to be involved in EPS synthesis and export (Branda et al. 2001). EpsA and B are similar to enzymes that regulate EPS chain length, EpsC is similar to nucleotide sugar synthesizing enzymes, EpsD, E, F, H, J, L, and M are all predicted to be glycosyl transferases, EpsK is similar to proteins involved in saccharide export, and EpsG is similar to proteins involved in polymerization of EPS repeating units. Mutants lacking EpsG and EpsH, as well a mutant lacking the entire *eps* operon, all produce flat colonies and extremely fragile pellicles. Microscopic examination of these mutants revealed that the product(s) of these genes is important for structuring the community. Phase-contrast microscopic analyses made it clear that *eps* mutants still proliferate as long chains, but these chains no longer align, nor they are bound together (Fig. 5) (Branda et al. 2001). Scanning electron microscopy (SEM) also revealed bare cells with only small amounts of extracellular material remaining.

In addition to the EPS component of the matrix, three proteins, encoded in the three-gene operon *yqxM-sipW-tasA*, were identified as involved in matrix assembly in a transposon mutant screen for genes involved in biofilm formation (Branda et al. 2004). In-frame deletion mutations in any of the genes of the three-gene operon *yqxM-sipW-tasA* result in defective pellicle formation and defective colony architecture. Microscopic analyses demonstrate that, like the *eps* mutants, *tasA* and *yqxM* mutants produce cell chains that are not held together and are defective for extracellular matrix production. The *tasA* and *yqxM* mutants alone or in combination, as well as a mutant deleted for the entire operon, have similar phenotypes, suggesting that TasA and YqxM act via the same mechanism. The *yqxM* and *tasA* genes encode preproteins that are converted to their mature, secreted forms by the product of *sipW*, a dedicated signal peptidase (Stover and Driks 1999a, 1999b). Previous to these findings, relatively little was known about the function of YqxM and TasA. YqxM was detected in culture supernatants, but only in the presence of high salt, suggesting that it is a cell-surface-associated protein (Stover and Driks 1999a). TasA was detected in the supernatant as well as associated with both cells and spores, and has been reported to have a poorly characterized antimicrobial activity (Serrano et al. 1999; Stover and Driks 1999b).

TasA is present in the biofilm's extracellular matrix. When pellicles were separated from the culture medium, no TasA was detected in the medium (Branda

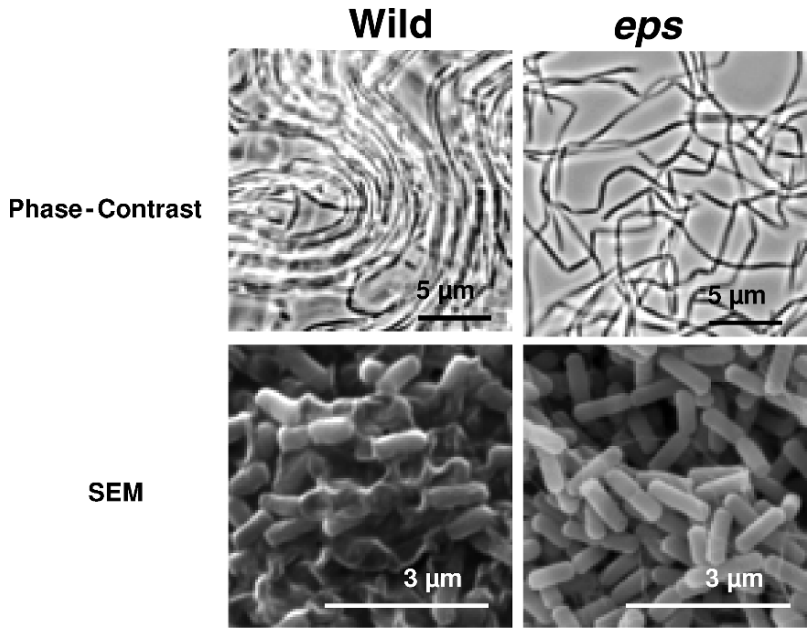

Fig. 5 Phenotype of *eps* mutant

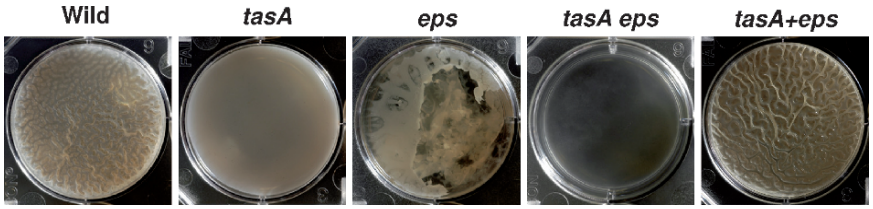

Fig. 6 Phenotype of *tasA*, *eps*, and *tasAeps* mutants and extracellular complementation in *tasA+eps* co-culture

et al. 2006). When mild sonication of the pellicle was used to separate cells from the matrix material, most of the TasA was shown to be present in the matrix fraction. Quite interestingly, TasA remains cell-associated and is not delivered to the matrix fraction when cells lack YqxM, leading to the hypothesis that YqxM is involved in delivering TasA to the matrix (Branda et al. 2006).

While single *eps* or *tasA* mutants still produce weak, unstructured pellicles, an *eps tasA* double mutant produces no pellicle whatsoever, suggesting that the products of these two operons represent the major structural components of the matrix (Fig. 6). Quite strikingly, when an *eps* mutant is co-cultured with a *tasA* mutant, there is restoration of the wild pellicle phenotype, suggesting that these components exert their function outside of the cell. In contrast, it was not possible to restore the

wild pellicle phenotype by co-culturing *tasA* and *yqxM* mutants, consistent with the idea that YqxM is needed to deliver TasA to the matrix. Poly-γ-glutamate has also been shown to be an extracellular polymer important for biofilm formation in a different wild strain of *B. subtilis* (Stanley and Lazazzera 2005). However, mutants unable to produce poly-γ-glutamate display a wild type biofilm phenotype in *B. subtilis* 3610 (Branda et al. 2006).

Mutants lacking SinR or SinI greatly affect biofilm development (Kearns et al. 2005). In the absence of SinI, no pellicle forms and colonies are flat, while the lack of SinR results in extremely wrinkled pellicles and colonies (Fig. 7). *eps* mutations are epistatic to *sinR*, i.e., the *eps* flat colony phenotype is retained in a *sinR eps* double mutant. DNA footprinting and gel shift analyses using purified SinR revealed that SinR binds directly to the promoter regions of both the *eps* (Kearns et al. 2005) and *yqxM-sipW-tasA* operons (Chu et al. 2006). Also, SinR binding to the *eps* regulatory region is inhibited if purified SinR protein is complexed with purified SinI prior to mixing with DNA (Kearns et al. 2005). Thus, SinR acts as a transcriptional repressor of the genes involved in producing the extracellular matrix, and SinI can antagonize its action.

The involvement of SinR and SinI in the regulation of *epsA-O* and *yqxM-sipW-tasA* explains the indirect effects of Spo0A and σ^H on extracellular matrix synthesis. The *sinI* and *sinR* genes are adjacent to each other, with *sinI* lying upstream. The *sinR* gene is transcribed primarily from a constitutive promoter dependent on the major housekeeping sigma factor σ^A, while *sinI* is transcribed from two σ^A-dependent promoters, the major one also being dependent on Spo0A~P (Shafikhani et al. 2002). The σ^H effect is probably due to the fact that *spo0A* itself contains a σ^H-dependent promoter (Predich et al. 1992). Therefore, mutants lacking Spo0A or σ^H will express *sinI* at a lower level, so that the negative effects of SinR on matrix synthesis will not be antagonized, resulting in defects in biofilm development (Fig. 3). Another regulatory protein known to control *B. subtilis* biofilm formation is AbrB (Hamon and Lazazzera 2001). However, just exactly how AbrB acts is not yet known.

Spo0A is not the only signal transducer feeding into the pathway regulating extracellular matrix synthesis. Two genes, *ylbF* and *ymcA*, when mutated lead to

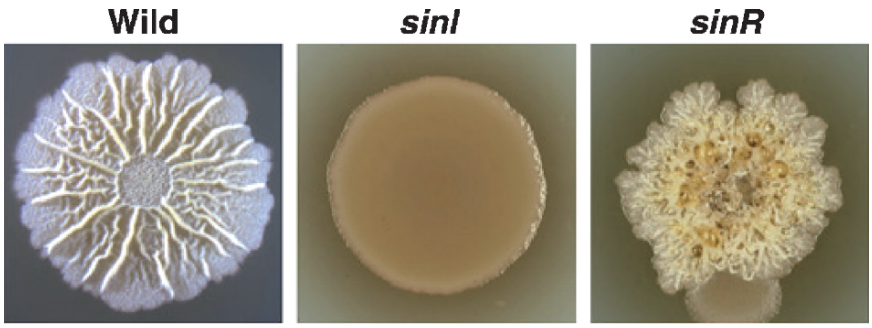

Fig. 7 Colony phenotype of *sinI* and *sinR*

flat colonies and no pellicles (Branda et al. 2004). In mutants lacking YlbF or YmcA, suppressor mutants take over the surface of the culture and form late-arising pellicles (Kearns et al. 2005). These suppressors that produce hyperwrinkled colonies do, indeed, harbor suppressor mutations in their *sinR* genes (Kearns et al. 2005). Thus, it appears that YlbF and YmcA function upstream of SinR. Because the expression of *ylbF* and *ymcA* does not appear to be regulated by either Spo0A or σ^H (Britton et al. 2002), we posit that YlbF and YmcA feed into the SinI-SinR circuitry via a different pathway (Fig. 3).

SinR functions as a master regulator of the lifestyle switch in *B. subtilis* (Fig. 4). In the model, SinR acts as a direct repressor of the genes involved in extracellular matrix production (*epsA-O* and *yqxM-sipW-tasA*). At the same time and through a mechanism that remains largely unknown, SinR acts positively to influence motility and cell separation. During vegetative growth, cells swim, are unit length, and do not produce extracellular matrix. When nutrient limitation is sensed, presumably through both the Spo0A/σH and the YlbF/YmcA pathways, SinI activity increases and SinR is antagonized. In the absence of SinR the expression of matrix components is de-repressed and cell separation and the assembly of motility machinery ceases. As a result, the cells switch to a mode of life where they form chains, become enclosed in a self-produced extracellular matrix, and stop making flagella. Synthesis of the matrix renders the cells able to attain a high degree of spatiotemporal organization, culminating in the production of spores at the tips of aerial projections.

5 The Future of Biofilm Development Research

Elucidation of the genes, proteins, and molecular mechanisms involved in *B. subtilis* biofilm formation continues and, though much progress has been made in the past 5 years, much remains to be done. Among Gram-positive bacteria, the molecular mechanisms of biofilm formation appear to be species-specific. For example, the master regulators of biofilm formation in *B. subtilis* (the transcriptional repressor SinR; Kearns et al. 2005), *Staphylococcus* (the transcriptional activator SarA; Beenken et al. 2003; Valle et al. 2003; Tormo et al. 2005) and *Enterococcus* (the response-regulator FsrA; Hancock and Perego 2004) are not homologs of each other. In the future, we can expect the combination of genetics, biochemistry, and microscopy to yield an ever-increasing understanding of the molecular mechanisms of biofilm formation unique to many bacteria. Invariably, microbes carry out fascinating, and often unexpected, processes when presented with the greater organizing potential afforded by a surface. Once on a surface, microbial cells can begin long-term relationships with each other; therein lies the transition from unicellularity to multicellularity. Analyses of microbial activities on surfaces will continue to provide new insights into the marvelous and astounding diversity of the microbial world.

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