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Full Length Research Paper

Biofilm formation and sporulation in *Bacillus subtilis*

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Biofilms are architecturally complex communities of microorganisms in which the cells are held together by an extracellular matrix, typically containing exopolysaccharides (EPSs), proteins and some nucleic acids. Our understanding of the molecular mechanisms involved in biofilm formation has increased tremendously in recent years. However, information about biofilm formation and sporulation is still limited. EPS is hallmark for biofilm formation and is linked with sporulation through the Spo0A signaling pathway. An attractive test organism in which to identify, and investigate the link between biofilm formation and endospore development is *Bacillus subtilis.* Phosphorylated form of the master transcriptional regulator Spo0A (Spo0A~P) regulates both processes in this organism. Its low availability results in delayed expression of sporulation specific gene in matrix mutants. This is due to the presence of KinD, a component of Spo0A~P, which plays a significant role in it's control and regulation. It's deletion enable the bacteria to sense matrix production, causing defect in sporulation while overproduction results in delayed sporulation. In summary, understanding the governing linkages between biofilm formation and sporulation is necessary to discover new antagonists for combating biofilm associated infections in a variety of environmental and medical settings and is exhaustively discussed.

Key words: Biofilm, sporulation, *Bacillus subtilis*, EPS, Spo0A.

INTRODUCTION

Biofilms are assemblages of microrgansims in which the cells are held together by an extracellular binding matrix typically called exopolysaccharides (EPS) and attached to a hydrated surface [Branda et al., 2005; Lisa, 2006]. Bacterial Biofilm formation is carried out in three basic steps: an initial adhesion phase, followed by proliferation and then detachment. The creation of a viable biofilm requires channels through which nutrients can diffuse into deeper biofilm layers. Accordingly, additional factors are involved that may cause disruption of the cell to cell interactions [Watrick and Kolter, 2000]. These factors can result in detachment of cells and cell clusters from the biofilm and thus control biofilm architecture including its thickness and proliferation. Biofilm detachment plays a critical role during biofilm-associated infections since it permits the cells to spread through blood and other body fluids to new infection sites.

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[Periasam et al., 2012]. Biofilm formation is influenced by a number of factors e.g. physical shearing forces, biological factors and prevailing environmental conditions. Variable gene expression observed in dispersed clonal populations of bacteria lead to phenotypic heterogeneity, a characteristic feature of biofilms [Dubnau and Losick, 2006]. These processes may render the biofilm-associated cells more capable and responsive to sudden changes in external cues, eventually leading to the observed spatiotemporal organization of cells within the biofilm. Similar to eukaryotic development, a complex interplay between various gene expression and environmental conditions generate the morphologically complex and highly organized structures observed in biofilms.

EPS production and sporulation

The EPS matrix (made up of polysaccharides, proteins and in some cases nucleic acids) is a key element in the development of a three-dimensional, organized complex biofilm. Interestingly, the processes leading to the

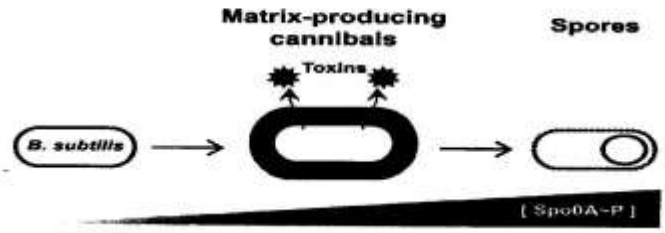

Fig. 1. Diagram illustrating the development of *B. subtilis* into matrix-producing cannibals and then spores. At low Spo0A~P levels *B. subtilis* activates genes required for the production of matrix and produces two cannibalism toxins. At high Spo0A~P levels sporulation begins.

(From Shank et al, 2011)

production of the matrix differ significantly from bacterium to bacterium, suggesting that production of microbial communities arose independently [Branda et al., 2006]. Apart from matrix production and motility there are many other key elements that are critical for biofilm formation in most bacteria. For example, in some bacteria transition from motile phase to sessile matrix producing phase leads to formation of spores (sporulation) [Branda et al., 2001].

Spores are highly resistant forms of vegetative cells that are able to withstand extremes in environmental conditions such as high temperatures, pressure, radiations and changing chemicals exposure. Once environmental conditions become favorable, spores are converted into actively growing vegetative cells, hence enabling the respective species to survive under adverse conditions but also produce disease in other species. If spores are produced on medical equipment or food processing plants, then economic loss caused by them have serious adverse effects [Elhariry, 2008]. Usually, predominant sites for sporulation were observed to be the aerial structures in filamentous bacteria and fungi. Likewise, in myxobacteria, aerial projections were found to be sites for bearing fruiting bodies containing spores [Branda et al., 2001]. Depending on the strain and culture conditions, spores constitute up to 90% of the total biofilm and are important for function such as a nidus for sporulation. The major genera of endospore forming bacteria are *Bacillus, Clostridium, Helicobacter, Heliophilum, Anaerobacter and Sporosarcina* [Wolska et al., 2007].

Sporulation is regulated during biofilm growth, likely triggered by unfavorable growth conditions including nutrient limitation and environmental stress. For example, in *B. subtilis* ~ 60% of the genes are sporulation genes expressed during biofilm formation [Branda et al., 2004]. Hence it is be of utmost importance to understand the link between biofilm formation and sporulation in this organism [Sonenshein, 2000]. Chai et al. [2008] found that the activity of the master regulator, phosphorylated Spo0A is necessary for both biofilm formation and sporulation. According to their study, expression of genes involved in matrix production serves as a preceding event followed by sporulation. In this regard, members of the Firmicute family are highly accessible to manipulation by the techniques of classical and molecular genetics. In *B. cereus*, for example, cells associated with biofilm formation [Rasko et al., 2005; Auger et al., 2006] produce multiple granules of polyhydroxyalkonate granules, that later progress to sporulate. Spores once produced are confined in EPS [Vilain et al., 2009].

B. subtilis as a model organism linking sporulation and Biofilm formation

The ability to differentiate into many distinct cell types is a prominent characteristic of the soil-associated Gram +ve bacterium *B. subtilis*. As an example, in nutrient-limited conditions and highg population densities, fraction of the cells differentiate into spores [Sonenshein, 2000, Piggot and Hilbert, 2004]. However, spore development is an energy intensive process and once initiated the cells may not exit this state for prolonged periods. Accordingly, this bacterium has evolved strategies to delay entry into sporulation as long as possible. It accomplished this goad by directing a subpopulation of cells to differentiate into becoming “Cannibal cells”[Lopez et al., 2008]. Cannibal cells are resistant to two toxins Skf and Sdp that they secrete to kill a fraction of their siblings. As a result of this, nutrients are released from the dead cells and hence the cannibal cells of *B. subtilis* are able to overcome nutritional limitation and delay the on set of sporulation. It should be noted here that all the three development pathways for *B. subtilis*, i.e. sporulation, cannibalism and biofilm formation (matrix production) are strongly inter connected and activated by the same master regulator,

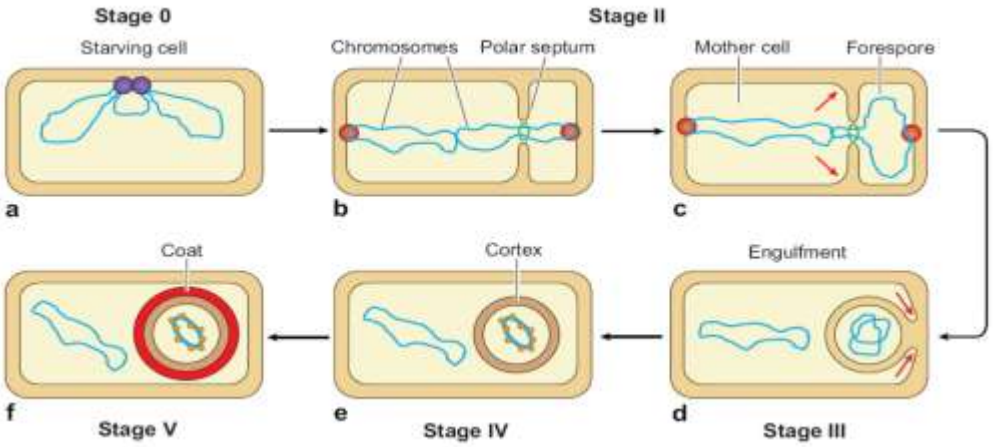

Figure 2. Morphological events during spore formation. © Kroos [2007], Annu Rev Genet.

Spo0A [Shank et al., 2011] (Figure 1).
At the molecular level, the regulation of *B. subtilis* endospore formation is probably the best understood microbial developmental process. During the sporulation process, a cell undergoes an asymmetric division producing a mother cell and a forespore.
Forespore is engulfed by the mother cell followed by cortex development and coat formation (Figure. 2). Finally, the mother cell is lysed releasing dormant and environmentally resistant spores. Mature spores can remain dormant for many years and become vegetative once appropriate environmental conditions become available [Kroos, 2007].

Competence Vs Sporulation

In addition to sporulation, *B. subtilis* possesses another developmental pathway known as competence by which the cells can bind and take up exogenous DNA. Genetic pathways of sporulation and competence have been studied thoroughly and a number of associated genes have been identified [over 125 genes for sporulation and 40 genes for competence [Grossman, 1995, Lazazzera and Grossman, 1998]. In this regard, Ren et al. [2004] observed strong links between sporulation, competence development, and biofilm formation. Using DNA microarrays, they observed that sporulation and biofilm formation are related processes. Compared with the large number of sporulation genes induced in the wild-type biofilm, only 1.5% of the biofilm cells were found as heat resistant spores. Similar biofilms of *B. subtilis* have also been studied in batch culture, and more than 50% of the biofilm cells formed spores 96 h after inoculation. The lower percentage of spores in the biofilm may be due to continuous addition of nutrients (LB, 8 mL/h), delaying the completion of sporulation, though not completely eliminating it. However, sporulation is the main feature of wild type biofilm due to the presence of a large number of sporulation genes induced (74 genes), with some cells in the wild type biofilm found to be competent [Ren et al., 2004]. Although both processes share some regulatory proteins such as Spo0A, Spo0B, Spo0F, Spo0H, and Spo0K, however activation of one leads to the inhibition of the other. Environmental and physiological signals determine the activation of either factor [Grossman, 1995].Cell density, a very important determining factor is primarily expressed through quorum sensing system [Ren et al., 2004].

Quorum sensing regulation of sporulation

Quorum sensing is a process which occurs both in Gram-negative and Gram-positive organisms [Liaqat et al., 2008; 2010] and has been found to control many different bacterial phenotypes, such as bioluminescence, swarming motility, biofilm formation [Liaqat et al., 2008], and virulence factor production [Zhu et al., 2002]. *B. subtilis* has two major quorum-sensing signals, the ComX pheromone and the competence and sporulation factor (CSF), produced during cell growth and secreted into the environment [Lazazzera and Grossman, 1998]. When the cell density is high, ComX activates the histidine protein kinase ComP (sensor of ComX) under high cell density. Through a series of reactions, it encodes transcription factor of competence. Compared to ComX, CSF has more functions to control.
It's low concentrations activates competence development, while high concentrations inhibits

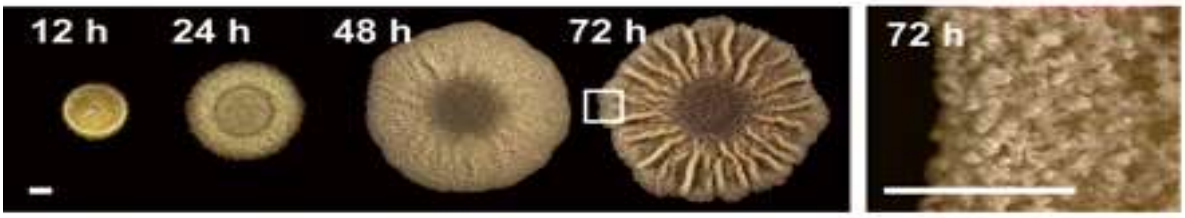

Figure. 3. Top view of biofilm development over time. The *right* panel is magnified to highlight the aerial structures observed at 72 h. Bars, 1mm. © Vlamakis et al. [2008] Genes and Dev.

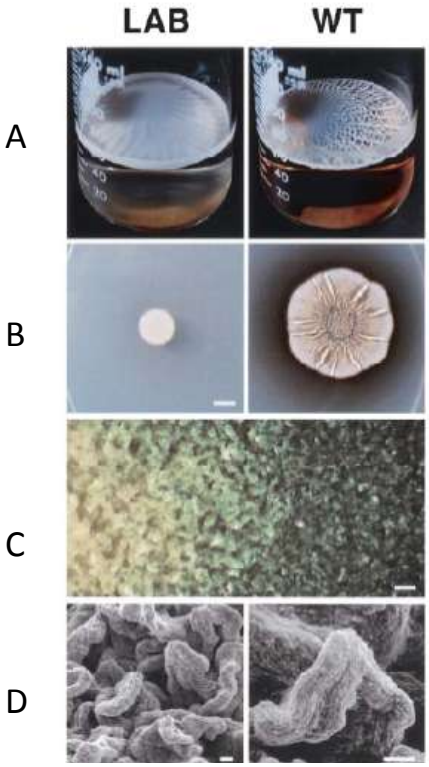

Figure. 4) [7].

competence resulting in sporulation [Bassler 1999; Ren et al., 2004]. Additionaly, *B. subtilis* produces and secretes some other signaling peptides that belong to the Phr family (CSF, encoded by phrC, also belongs to this family), including PhrA, PhrE, PhrF, PhrG, PhrI, and PhrK. These peptides sense the cell density and inhibit several sporulation inhibitors [Lazazzera, 2001].

Regulation of biofilm formation

In *B. subtilis* biofilm studies, research has been focused more on the development of complex, wrinkled colonies and development of pellicles at air-liquid interface, although some studies have been conducted on solid surface-associated biofilms [Stanley et al., 2003, Hamon et al., 2004]. Biofilm formation by *B. subtilis* occurs in a distinct developmental pathway [Branda et al., 2001]. After inoculation of standing cultures in a defined minimal medium containing glycerol [Branda et al., 2001], motile cells proliferate throughout the liquid as planktonic cells until they reach a density of approximately $5x10^7$ cfu/ml after 1 day at 25^oc. At that point, most of the cells begin to migrate to the air-liquid interface, forming pellicle or a floating biofilm on the surface of the medium.

The pellicle is readily visible but flat after 3 days. However, few remaining planktonic cells (10^5 cfu/ml) retain their motility and do not sporulate. While cells in pellicle undergo differentiation and proliferate, they become nonmotile and form long chains that are aligned in parallel. With the passage of time (5 days after), the cell mass increases, the pellicle begins to wrinkle, and some groups of cells begin to grow as aerial projections within the wrinkles. Fruiting bodies are produced that extend from the surface of the pellicle and form spores at their tips [Vlamakis et al., 2008] (Figure. 3).

It is noteworthy to mention, that structured communities and their high degree of cellular differentiation are only apparent when wild isolates (*B. subtilis*: NCIB3610, X5, 1431, 1440) are analyzed. Most of the laboratory domesticated strains (*B. subtilis*: 168, PY79, 168, JH642) do not display such robust community structure (Figure. 4) [Branda et al., 2001].

Molecular mechanisms regulating sporulation and matrix production

Through a combination of genetic and biochemical approaches, researchers have begun to identify the

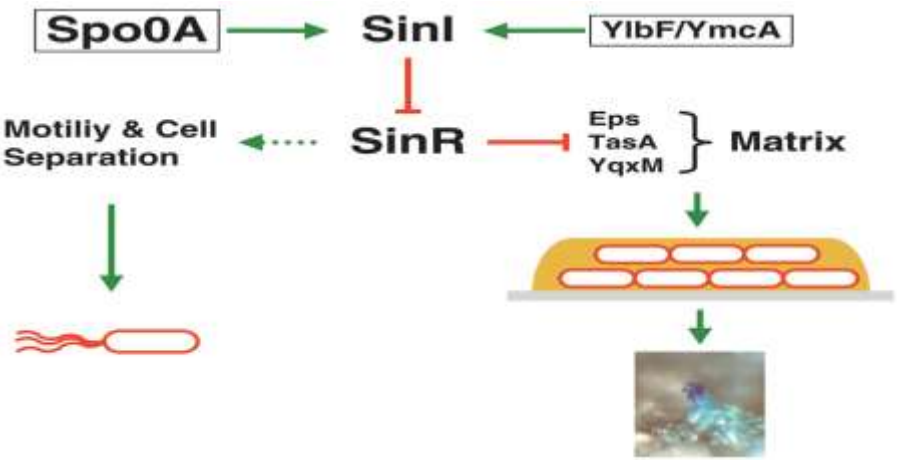

Figure. 5. Simplified view of the genetic circuitry governing *B. subtilis*'s lifestyle switch from nomadic to a sedentary existence. © Lemon et al. [2008] Curr Top Microbiol Immunol.

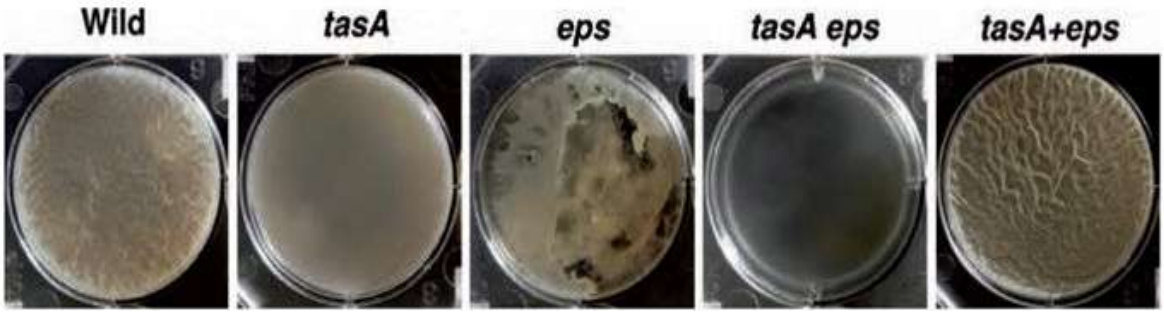

Figure 6. Phenotype of tasA, eps, and tasAeps mutants and extracellular complementation in tasA+eps co-culture. © Lemon et al. [2008] Curr Top Microbiol Immunol.

molecular regulatory circuitry that governs the transition from motile cells to matrix- producing cells of the sporulating *Bacillus* sp. Lemon et al. [2008] reported SinR, as the master regulator controlling production of exopolysaccharide matrix, necessary for biofilm formation and sporulation (Figure. 5), and SinI as its antagonizer. SinR controlled repression of genes involved in matrix production and positively influence motility and cell separation. During vegetative growth, cells swim, are? unit length, and do not produce extracellular matrix. However its activity antagonized once conditions become unfavorable. 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 chains, become enclosed in a self-produced extracellular matrix, and do not produce flagella [Lemon et al., 2008]. Synthesis of the matrix renders the cells able to attain a high degree of spatiotemporal organization, producing spores at the tips of aerial projections [Veening et al., 2006]. In addition to the EPS component of the matrix, three proteins, encoded in the three-gene operon *yqxM-sipW-tasA*, were identified to be involved in matrix assembly [Branda et al., 2001, 2004]. In-frame deletion mutations in any of the genes of operon *yqxM-sipW-tasA* causes 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 (Figure. 6). TasA is present in the biofilm's extracellular matrix. When pellicles are separated from the culture medium, no TasA is detected in the medium [Branda et al., 2001]. Vlamakis et al. [2008], linked production of spores to the

formation of biofilm. Extracellular matrix necessary for biofilm formation consists primarily of an exopolysaccharide (EPS) and a protein, TasA 5). Mutants lacking either of the EPS or TasA will be unable to assemble into biofilm in *B. subtilis*. Only after production of matrix, the community develops a high degree of spatiotemporal organization resulting in sporulation at the tips of aerial structures. Furthermore, observation of sporulation defect only in the context of a biofilm and not in dispersed culture indicates a correlation between the two processes. It can be explained by the fact that energy required for the matrix production creates local regions (microenvironments) within biofilm structure with severely depleted nutrients.

Spo0A signaling pathway controls the link between matrix production and sporulation

Nutrient depleted areas activates Spo0A to high levels triggering spore formation. Also, the generation of local regions of nutrient depletion may serve as a developmental checkpoint that ties an early step in sporulation (Spo0A activation) to the successful production of an extracellular matrix during biofilm formation. In the absence of EPS production, no sporulation would be observed [Veening et al., 2006]. It was observed that the delay in sporulation is not due to significant depletion of energy resources of the matrix-deficient mutant. Instead, Spo0A signaling pathway controls the link between matrix production and sporulation. It is the phosphorylated form of the master transcriptional regulator Spo0A that governs both processes. Matrix genes are expressed at low levels of phosphorylated Spo0A (Spo0A~P) in cells; however, sporulation commences at higher levels of Spo0A~P. They reported that delayed expression of sporulation-specific genes in the matrix-deficient mutant are due to continuous low levels of Spo0A~P. This is controlled by one of the components of the Spo0A phosphotransfer network, KinD. Sporulation defect of matrix mutants is suppressed with deletion of *kinD*, while its overproduction delays sporulation. Hence, *KinD* plays a dual role as a phosphatase or a kinase and that its activity is linked to the presence of extracellular matrix in the biofilms. In other words, *KinD* plays a novel role in biofilms as a checkpoint protein that regulates the onset of sporulation by inhibiting the activity of Spo0A until matrix, or a component therein, is sensed [Vlamakis et al., 2008].

Other regulators of sporulation and matrix production

Among other regulators, the protein kinase PrkC, which is similar to the eukaryotic sensor Ser/Thr and to the Tyr kinases from *B. subtilis,* also appears to regulate biofilms. Mutants with *prkC* deletions have decreased sporulation efficiency and a reduced capacity to form biofilms. Additionally, the gene *prpC*, which encodes a PPM phosphatase that is cotranscribed with *prkC*, is also required for normal biofilm and endospore formation. Veening et al. [2006] also emphasized the fact that multicellular structure formation and sporulation are intertwined by the action of Spo0A in *B. subtilis* by proposing the role of epigenetic inheritance in the formation of biofilms as well as fruiting bodies.

Link between sporulation and biofilm formation is a universal feature

There are other microorganisms also where the association of sporulation with multicellular development has been observed. For example, in filamentous bacterium, *Streptomyces coelicolor,* spore formation is normally dependent on the formation of the upwardly projecting hyphae. It produces spores in raised structures known as aerial mycelia [Chater, 2001]. Another multicellular bacterium, *Myxococcus Xanthus* sporulates under starved conditions, and when cells aggregate into macroscopic fruiting bodies [Sogaard-Andersen et al., 2003]. Likewise, in *B. cereus* biofilm formation is associated with nutrient depletion and in *B. anthracis* biofilm formation is a response to environmental stress (Lee et al., 2007). Thus, mechanisms connecting sporulation to the formation of intricate multicellular communities seem to be universally shared features of otherwise unrelated developmental processes. This is not only present in prokaryotes; but also featured in Basidiomycetes such as *Coprinus cinereus*, fruiting bodies consisting of raised mushroom structures harboring many differential cell types including fungal sexual spores [Kues, 2000].

Future perspectives and prognostications

Much more work is still needed if we are to comprehensively describe the physiological changes that occur during biofilm formation and sporulation. The present review discusses sporulation mechanisms in association with biofilm formation from the *B. subtilis* perspective. It is evident from this study that sporulation which was considered a characteristic feature of cells living planktonically is closely associated with a multicellular mode of survival. Hence, it may be assumed that many other microbial developmental processes still considered to be unicellular may exhibit multicellular features in biofilm mode. The information from the present study has applications in two areas. First, it may help find the conditions favorable for biofilm formation in endopsore forming species and therefore improve those

applications involving beneficial biofilms, such as those used in corrosion prevention, antibiotic production and enzymes secretion. Second, drug screening with these sporulation genes controlling biofilm formation as targets may find new antagonists for biofilm formation and therefore help finding novel therapies for biofilm caused infections in food industry and hospitals. The Center for Disease Control and Prevention (CDC) has estimated that 70% of all bacterial infections involve biofilm formation in cases of dental caries, catheter infections, middle ear infections, cystic fibrosis and endocarditis. In the future, we can expect the combination of genetics, microscopy and biochemistry to increase our understanding of molecular mechanisms involved in biofilm formation and sporulation. Analysis of endospore forming microbial activities in biofilm mode will continue to provide new insights into the astonishing array of microbial diversity. It will also enable us, to unravel the important information relevant to ecology and distribution of many divergent species of microbes that inhabit a variety of differing environments, the vast majority of which have yet to be properly identified and studied.

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