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REVIEW

Beneficial Biofilm Formation by Industrial Bacteria *Bacillus subtilis* and Related Species

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Biofilms are densely packed multicellular communities of microorganisms attached to a surface or interface. Bacteria seem to initiate biofilm formation in response to specific environmental cues, such as nutrient and oxygen availability. Biofilms undergo dynamic changes during their transition from free-living organisms to sessile biofilm cells, including the specific production of secondary metabolites and a significant increase in the resistivity to biological, chemical, and physical assaults. *Bacillus subtilis* is an industrially important bacterium exhibiting developmental stages. It forms rough biofilms at the air-liquid interface rather than on the surface of a solid phase in a liquid, due to the aerotaxis of the cells. Biofilm formation by *B. subtilis* and related species permits the control of infection caused by plant pathogens, the reduction of mild steel corrosion, and the exploration of novel compounds. Although it is obviously important to control harmful biofilm formation, the exploitation of beneficial biofilms formed by such industrial bacteria may lead to a new biotechnology.

[**Key words:** biofilm, *Bacillus*, biocontrol, corrosion, bioreactor, bioremediation]

It is now widely recognized that in natural settings bacterial cells are most often found in close association with surfaces and interfaces, in the form of multicellular aggregates embedded in matricies commonly referred to as biofilms. Biofilms offer their member cells several benefits, among which protection from environmental insults and assaults is foremost (1). Some phenomena are postulated to contribute to the biofilm defence, including incomplete antimicrobial penetration, slow or no growth of some of the biofilm cells, and the expression of biofilm-specific phenotypes (2, 3). Biofilms are the source of persistent infections of many pathogenic microbes. They are responsible for dental caries and nosocominal infections, as well as a variety of other infections and diseases (4). Industrially, biofilms are also detrimental in many cases. For instance, natural biofilms can reduce heat transfer in heat exchangers and cooling towers (5), decompose reverse osmosis membranes (6), corrode metal surfaces, and contaminate food processing equipment (7). With the cells embedded in a polysaccharide matrix, biofilms are highly resistant to antibiotics and have higher genetic transformation frequencies than planktonic cells (8). Although planktonic cells are undetectable after treatment with antibiotics or chemicals, biofilm cells survive and are often responsible for recurring symptoms and medical treatment failure (9). Therefore, the control of harmful biofilms is an absolutely important issue clinically as well as industrially.

However, there are several successful examples of the positive use of biofilms that are called beneficial biofilms. The aim of this review is to highlight this beneficial biofilm formation by an industrial bacterium, *Bacillus subtilis* and related species. *B. subtilis* preferably produce biofilms at the air-liquid interface rather than on solid surfaces. Although this floating cellular community has long been called a pellicle, in this review a pellicle is considered as a type of biofilm (Fig. 1).

MOLECULAR MECHANISMS OF BIOFILM FORMATION BY *B. SUBTILIS*

Understanding the mechanisms of biofilm formation is undoubtedly important for exploring effective strategies to control harmful biofilm formation and promote beneficial biofilm formation. Because there are strict requirements to control biofilm formation by pathogenic bacteria, much of the research has been performed using clinically relevant bacteria. These bacteria include *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Streptococcus* sp., *Staphylococcus* sp., and *Candida* sp. (10–12). Excellent reviews based on what is known about these clinical bacteria can be referred to in order to address the fundamental questions of how and why bacteria form biofilms (13–15). Global gene expression profiles in the biofilm cells have been

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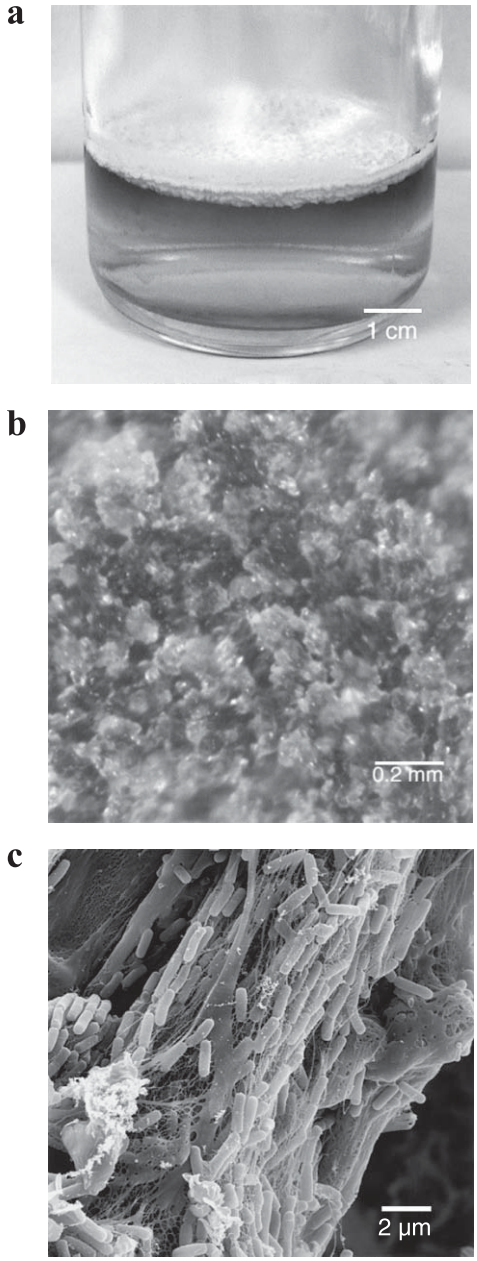

FIG. 1. Biofilm formed by *B. subtilis* wild type. (a) Side view; (b) optical microscopic observation; (c) scanning electron microscopic observation.

analyzed using DNA microarray technology and proteomic approaches (16, 17). DNA microarray analyses have suggested that there are common responses in biofilm formation, such as the repression of flagellar genes and the hyperexpression of genes for adhesion and ribosomal protein formation. Quorum sensing has been shown to be a crucial event in biofilm formation by *P. aeruginosa* (18).

B. subtilis and its related species, rod-shaped, spore-forming, gram-positive bacteria, have long been established as industrial bacteria for the production of various secretion enzymes, such as amylase, protease, pullulanase, chitinase, xylanase, lipase, among others (19). These enzymes are produced commercially and this enzyme production represents about 60% of the commercially produced industrial enzymes. *Bacillus* spp. have developed strategies for survival in unfavorable environments. Spore formation by *B. subtilis* has long been studied as a model for the cellular differentiation of bacteria, but it has been predominantly studied in a single cell. When it was analyzed within the context of biofilms, spore formation was discovered to have heretofore unsuspected spatial organization. A late-stage sporulation gene, *sspE*, has been shown to be expressed predominantly at the tip of the aerial projection of *B. subtilis* biofilms (20). Motile cells differentiate into aligned chains of attached cells that eventually produce aerial structures, or fruiting bodies, that serve as preferential sites for sporulation. Fruiting body formation depends on *spo0A*, a regulatory gene required early in sporulation, *spo0H*, a gene encoding starvation-activated transcription factor Sigma-H, *yveQ* and *yveR*, genes evidently needed for exopolysaccharide biosynthesis, and *sfp*, a phosphopantetheinyl transferase responsible for surfactin production. The role of Spo0A in this process is to repress the expression of AbrB (21). Spo0A binds and directly represses the *abrB* promoter and an *abrB* mutation restores biofilm formation to a *spo0A* mutant strain. Thus, AbrB is considered a negative regulator for the initiation of biofilm formation. Sigma-H may indirectly repress AbrB expression and stimulate the initiation of biofilm formation, as Sigma-H is known to activate the expression of *spo0A* (22). Further experiments have shown that genes encoding an alkaline protease, *aprE*, and a dipeptidyl permease, *dppA/B/C/D/E* are under the control of AbrB. Extracellular protease activity, including the activities of AprE (subtilisin) and NprE (neutral metalloprotease E), is reported to be essential for both swarming and biofilm formation (23). Strictly aerobic bacteria such as *B. subilis* tend to form biofilms at the air-liquid interface rather than on solid surfaces when grown in a standing culture. In a statically aerated culture system, *B. subtilis* grows in the liquid medium rather than forming floating cellular communities leaving a clear liquid phase (personal communication). This observation indicates that the depletion of dissolved oxygen triggers the formation of floating biofilms.

In structured microbial communities such as biofilms, the formation and maintenance of multicellular aggregates are mediated by an extracellular matrix that is predominantly composed of exopolysaccharides (EPS; 24). Mutation in *yveQ* or *yveR* gave rise to a strain generating thick but very fragile biofilms that usually split and sank to the bottom of the culture vessel. The surfaces of the biofilms and colonies formed by the mutants were smooth and lacked aerial structures. The 15 gene-long *yveK-yvF* operon was renamed the *eps A-O* operon. A DNA binding protein, SinR, was proposed to function as a master regulator in the assembly of *B. subtilis* cells into multicellular communities (25). A mutation in *sinR* causes the formation of rugose biofilms in which the cells constitutively grow in chains of nonmotile cells. In contrast, cells of a *sinI* mutant are routinely motile and do not form biofilms. SinR functions in this manner by repressing the transcription of the *eps* operon, which directs EPS production, as well as that of other unidentified target genes. Because SinI antagonizes the binding of SinR to DNA, SinI is probably required for the activation of the *eps*

operon. It has been reported that glucose was found to inhibit biofilm formation by *B. subtilis* through the catabolite control protein CcpA (26). The rapid metabolism of carbon under carbon-rich conditions does not seem to induce *B. subtilis* to undergo biofilm formation but allows cells to grow as free-living organisms.

Stanley and Lazazzera transferred the genetic determinants that control exopolymer production from a wild, exopolymer-positive strain to a domestic, exopolymer-negative strain (27). Mapping these genetic determinants led to the identification of gamma-poly-DL-glutamic acid (gamma-PGA) as an exopolymer that increases biofilm formation, possibly by enhancing cell-surface interactions. The production of gamma-PGA by *B. subtilis* depends on two-component regulator systems, ComP-ComA and DegS-DegU, and two regulator proteins, DegQ and SwrA. The inability of the domestic strain *B. subtilis* 168 to produce gamma-PGA was mapped to two base pairs; a single base pair change in the promoter region of *degQ* and a single base pair insertion in the coding region of *swrA*. The introduction of *degQ* and *swrA* alleles from the wild strain into the domestic strain was sufficient to allow gamma-PGA production. In addition to controlling gamma-PGA production, ComP/A and DegS/U have been shown to activate biofilm formation through an as yet undefined pathway. The identification of these regulators affecting biofilm formation suggests that the processes are regulated by osmolarity, high cell density and phase variation.

Gamma-poly-DL-glutamic acid appears to increase *B. subtilis* biofilm formation by enhancing cell-surface interactions. Most gamma-PGA produced by *B. subtilis* is secreted into the medium and is not physically attached to the cell. Salt ions, such as Mg^{2+} or Ca^{2+}, are known to be able to act as an intermediary between two negatively charged surfaces (28). Salt-bridges may form between the negatively charged gamma-PGA and the abiotic surface and between the gamma-PGA and the cell surface. As the density of negative charges may be higher on the gamma-PGA than on the abiotic surface, there will be more sites for cells to form salt-bridges when gamma-PGA has coated an abiotic surface. Consistent with this model, the addition of $MgSO_4$ to the biofilm growth medium induces efficient biofilm formation by *B. subtilis*. Salt-bridge bonds may also reflect the mechanism by which extracellular polymers, such as DNA, enhance biofilm formation (29). Another possible function of gamma-PGA in biofilm formation is to induce the transfer of the cells from a hydrophilic condition to a relatively hydrophobic air-water interphase. Hydrophobic interaction increases in solutions containing ionic substances, such as gamma-PGA. It has been reported that microorganisms attach more rapidly to hydrophobic and nonpolar surfaces such as Teflon, polypropyrene, and polystyrol, than to hydrophilic materials such as glass or metals (30, 31).

BIOFILMS AS BIOCONTROL AGENT

B. subtilis is often found in the plant rhizosphere, the area of soil surrounding a plant root system. In the rhizosphere, *B. subtilis* promotes plant growth and acts as a biocontrol agent (32). Recent studies have suggested that the biofilm mode is important for the bacterium's ability to act as a biocontrol agent (33). Among the first successful biocontrol agents used against insects and pathogens were members of the genus *Bacillus* (34, 35). Commercial strains of *B. subtilis* have been marketed as biocontrol agents for fungal diseases of crops. The commercial biofungicide, Serenade, whch contains a *B. subtilis* strain, is reported to be effective against a variety of pathogenic bacteria, including *Erwina*, *Pseudomonas*, and *Xanthomonas* strains (http://www.agraquest.com). The mechanism of this antibacterial effect is uncertain, however, it is known that *B. subtilis* can produce a variety of antibacterial agents. They include a broad spectrum of lipopeptides, such as surfactin that are potent biosurfactants and important for maintaining the aerial structure of biofilms (20). Asaka and Shoda showed that the half-life of surfactin in soil is longer than that of iturin A (36). This suggests a prolonged stable role of surfactin in the rhizosphere. Bais *et al*. have reported that the biocontrol of *P. syringae* by *B. subtilis* 6051 is related to surfactin formation on the surface of the root (33). Surfactin has a minimum inhibitory concentration (MIC) of approximately 25 μg/ml against *P. syringae*, which is relatively high for an antimicrobial agent but may be reasonable for the exigencies of rhizosphere settings (37). In the experiments with *Arabidopsis* roots that were precultured with *B. subtilis* 6051 forming biofilms, the levels of surfactin in rinsed roots were substantial (of the order of 151.6 μg/ml per 50 mg root fresh weight). Thus, it is possible that on the surfaces of the rinsed roots, the concentrations of dissolved surfactin are substantially higher than the MIC against *P. syringae*. It has been shown that surfactin inhibits the biofilm formation of *Salmonella enterica* at levels as low as 50 μg/ml and those of *E. coli* and *Proteus mirabilis* at higher levels *in vitro* (38). Taking these observations together, the presence of surfactin-producing *B. subtilis* 6051 biofilms is expected to prevent the planktonic cells of other microbes from colonizing biological surfaces, including plant roots. In fact, upon root colonization, *B. subtilis* 6051 forms a stable, extensive biofilm and secretes surfactin, which acts together to protect plants against infection by pathogenic bacteria (Fig. 2; 33).

It is known that the production of surfactin occurs in a cell density-dependent manner. The comQ/X/P/A quorum-sensing system of *B. subtilis* plays a key role in the development of genetic competence and other physiological systems when cells enter the stationary growth phase (39). The

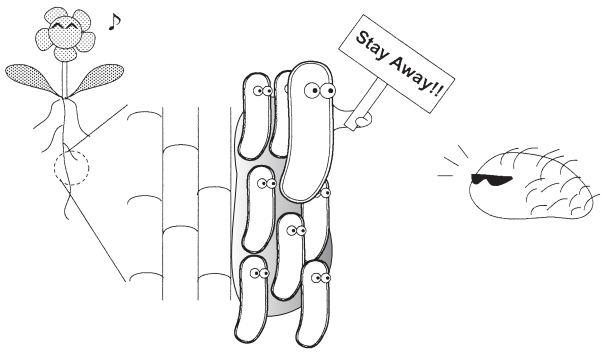

FIG. 2. Biofilm as biocontrol agent.

comQ/X/P/A quorum-sensing system controls various cell density-dependent phenotypes such as the production of degradative enzymes and antibiotics and the development of genetic competence. The *comX* gene of *B. subtilis* 168 encodes a 55-residue precursor of competence pheromone. The mature pheromone peptide of 10 amino acids is processed from the C-terminal of the *comX* product and is secreted into the medium with a concomitant modification at a tryptophan residue, probably by the ComQ function (40, 41). The molecular events involved in the processing, modification and secretion processes of the ComX pheromone remain to be elucidated. ComP is a sensor protein kinase of the ComP-ComA two-component system (42). The N-terminal sensor domain has eight possible transmembrane helices and appears to interact with the ComX pheromone (43). By analogy with other two-component systems, a signal generated upon interaction with the extracellular pheromone would be transduced to the cognate ComA by a phosphorelay. The phosphorylated form of ComA is a transcription activator of a set of genes that include *degQ*, *rapA*, *rapC* and *srfA* (41). The *degQ* gene encodes a positive regulator of the genes for degradative secretion enzymes, such as *aprE* and *sacB* (44). The *srf* operon encodes surfactin synthetases and includes the *comS* gene that encodes a 46-amino-acid polypeptide (45). ComS liberates ComK from a complex with MecA and ClpC so that ComK can function as a transcription activator for its own gene as well as for the late competence genes (46). Thus, the *comQ/X/P/A* operon controls the multiple genes that govern important traits expressed in the stationary phase cells.

Bacillus thuringiensis suppresses the quorum-sensing-dependent virulence of the plant pathogen *Erwinia carotovora* through a new form of microbial antagonism, signal interference. *E. carotovora* produces and responds to acyl-homoserine lactone (AHL) quorum-sensing signals to regulate antibiotic production and the expression of virulence genes, whereas *B. thuringiensis* strains possess AHL-lactonase, which is a potent AHL-degrading enzyme (47, 48). *B. thuringiensis* does not seem to interfere with the normal growth of *E. carotovora*; however, it abolishes the accumulation of the AHL signal when they are cocultured. In plants, *B. thuringiensis* significantly decreases the incidence of *E. carotovora* infection and symptom development of potato soft rot caused by the pathogen. Biocontrol efficiency should correlate with the ability of bacterial strains to produce AHL-lactonase. In recent years, other methods of microbial antagonistic mechanisms, that do not directly kill pathogens, have also been investigated. *Lactobacillus fermentum* RC-14, a probiotic bacterial isolate, inhibited acute *Staphylococcus aureus* infection (49). The probiotic bacterium does not appear to affect pathogen growth; however, it secretes cell-surface extracellular matrix-binding proteins and biosurfactant that somehow prevented pathogen adherence to surgical implants and inhibited *S. aureus* infection. These findings illustrate different potential microbial antagonistic mechanisms other than antibiotic production, such as signal interference, for the control and prevention of biofilm formation by pathogenic bacteria. Several natural and synthetic compounds that interfere with quorum sensing have also been reported. They interfere with both the AI-1 (AHLs) and AI-2 signaling systems. Hentzer *et al*. found that the synthetic compound 4-bromo-5-(bromomethylene)-2(5H)-furanone in *P. aeruginosa* PAO1 repressed 80% of the genes that were induced by AI-1 quorum sensing, and the natural brominated furanone, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone, from the marine alga *Delisea pulchra*, repressed 79% of the genes that were induced by AI-2 in *E. coli* without affecting cell growth (50). It is interesting that this brominated furanone was also found to inhibit growth, swarming, and biofilm formation of gram-positive bacteria, such as *B. subtilis*, because gram-positive bacteria do not have an AHL signaling system (51).

BIOFILM AS INHIBITOR OF MILD STEEL CORROSION

It is known that bacteria corrode metals. Sulfate-reducing bacteria (SRB) are responsible for the corrosion of cast iron, carbon steel, stainless steel, and some alloys (52). SRB corrosion damage in the United States results in losses of US $4–6 billion/year. Hydrogenase in SRB can utilize hydrogen as an electron donor to obtain energy and thus remove molecular hydrogen from the cathode, leading to cathodic depolarization of the metal surface (53). The corrosion product, iron sulfide, itself may deposit and become a cathode with a large surface area relative to the unreacted iron, accelerating the dissolution of the iron (54). The increase in iron sulfide concentration is accompanied by an increase in the corrosion rate of mild steel, which implicates SRB in its corrosion. In natural ecosystems, the SRB activity is not the only reason for microbiologically induced corrosion. As obligately anaerobic organisms, SRB growth is affected by the activities of other organisms in the same niche. The aerobes consume oxygen by respiration, creating a local anaerobic environment in the biofilm, as required by the SRB. Some fermentative facultative anaerobes provide organic electron donors for SRB to obtain energy. Moreover, the metal surface beneath the biofilms and mineral precipitates has a low redox potential and acts as the anode, resulting in dissolution of the metal (53). The iron-oxidizing bacterium *Leptothrix discophora* SP-6 is a gram-negative, sheath-forming, aerobic heterotroph growing at aerobic-anaerobic interfaces (55). This bacterium is often found growing at the periphery of a sulfate-reducing area containing black iron sulfide and may cause a blockage in water distribution systems. *L. discophora* SP-6 itself does not cause significant corrosion of mild steel. However, when *L. discophora* SP-6 was combined with another corrosion-causing bacterium, *Desulfosporosinus orientis*, in the presence of aerobic *Paenibacillus polymyxa* 10401, the corrosion rate of mild steel increased significantly compared with those caused by a mixture of *D. orientis* and *P. polymyxa* 10401. In addition, it was also reported that manganese oxide deposited by *L. discophora* might initiate pitting corrosion of stainless steel (56). Manganese oxide may act as a cathodic reactant, increasing the corrosion of the metal on which it is deposited.

Beneficial biofilms are capable of decreasing the above-described biological corrosion (57). A gramicidin-S-producing *Bacillus brevis* 18-3 biofilm has been shown to reduce the corrosion rates of mild steel by inhibiting both the sul-

fate-reducing bacterium *D. orientis* and the iron-oxidizing bacterium *L. discophora* SP-6. When *L. discophora* SP-6 was introduced together with *D. orientis* to a non-antimicrobial-producing control biofilm of *P. polymyxa* ATCC 10401, a corrosive synergy was created and mild steel coupons underwent severer corrosion than when only *D. orientis* was present, showing a 2.3-fold increase as demonstrated by electrochemical impedance spectroscopy (EIS) and a 1.8-fold difference as demonstrated by mass-loss measurements. However, when a gramicidin-S-producing, protective *B. brevis* 18-3 biofilm was established on mild steel, the metal coupons were protected against the simultaneous attacks of *D. orientis* and *L. discophora* SP-6. EIS data showed that the protective *B. brevis* 18-3 biofilm decreased the corrosion rate about 20-fold compared with the non-gramicidin-producing *P. polymyxa* ATCC 10401 biofilm. The mass loss for the protected mild steel coupons was also significantly lower than that for the unprotected ones (4-fold decrease). This protection achieved by the *B. brevis* 18-3 biofilm was not due to the removal of oxygen but due to the gramicidin-S production by the *B. brevis* 18-3 biofilm. Gramicidin-S-producing biofilms prevent *D. orientis* and *L. discophora* SP-6 from growing on the surface of mild steel.

BIOFILM AS BIOREACTOR

Bioreactors now play an important role in the biochemical industry, as the rate of reaction, ease, and length of reactor operation affect reactor productivities and hence process economics (58). There are two methods commonly used for increasing cell mass concentration inside the reactor; first, the use of a permeable membrane to retain cells, and the use of immobilized cell techniques. Membrane reactors allow the passage of liquid, substrate, and product out of the reactor while retaining the cells. Immobilized cell reactors fix cells on various supports by adsorption, entrapment, or covalent bond formation. Adsorption can be performed *in situ*, resulting in the economical operation of the reactor. Adsorbed cells form cell layers on the support, and the cell mass grows inside the reactor over time. These layers of cells form typical biofilms. These biofilm reactors have been successful in wastewater treatment and the production of fermented products such as vinegar and acetic acid. High cell concentrations can be achieved in biofilm reactors up to 70–80 g/*l*, maintaining a large surface area of the biofilm structure. This is one of the critical features which makes these reactors useful (59). Another advantage of the biofilm reactor is the different gene expression levels of the biofilm cells in comparison with free-living cells (Fig. 3). An example is given as follows.

Bacteria growing on the surfaces of marine algae and other organisms live in a highly competitive environment in which space and access to nutrients are limited (60). Previous studies have shown that a high percentage of marine epibiotic bacteria produce antimicrobial metabolites compared with the number of planktonic isolates that produce such metabolites (61). However, in the laboratory, most isolates stop producing antimicrobial compounds when they are continuously cultivated in shake flasks. Well-agitated suspension cultures in closed flasks represent artificial growth

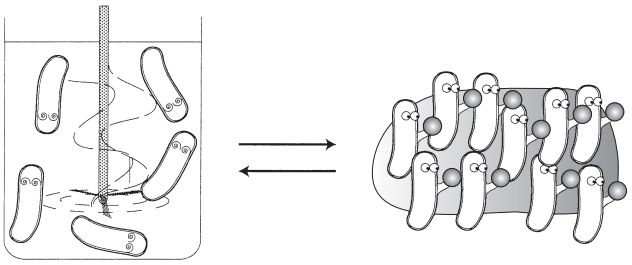

FIG. 3. Biofilm as bioreactor.

conditions that are very different from the natural environment. Previously, it was demonstrated that a modified roller bottle bioreactor, which mimicked the intertidal environment, could facilitate the production of antimicrobial compounds by two marine epiphytic isolates which had stopped producing them under planktonic growth conditions (62). Thus, new cultivation strategies, particularly strategies mimicking the natural habitats of microorganisms with niche-mimicking bioreactors, can be used to elicit the production of secondary metabolites by apparent nonproducers.

Yan *et al*. designed a biofilm reactor, namely an air-membrane surface (AMS) bioreactor, to allow bacteria to grow on semipermeable membranes as biofilms in contact with air (63). The membrane is in contact with medium on one side and with air on the other side where the cells are inoculated by swabbing. When *Bacillus licheniformis* EI-34-6, isolated from the surface of a marine alga, was grown in this reactor, cells produced antimicrobial compounds that they did not produce when they were grown in shake flask cultures. An unidentified red pigment was also produced by surface-grown cells but not by planktonically grown cells. Glycerol and ferric iron were important for the production of antimicrobial compounds and the red pigment. The release of these secondary metabolites was not due to the onset of sporulation. Cell-free spent medium recovered from beneath the reactor membrane induced the production of antimicrobial compounds and the red pigment in shake flask cultures. Neither glycerol nor ferric iron was required for the production of these inducer compounds. Spent medium from beneath the membrane of an AMS bioreactor culture of *B. subtilis* and *Bacillus pumilus* strains also induced the production of antimicrobial compounds and a red pigment in a *B. licheniformis* isolate EI-34-6 grown in shake flask cultures; however, the corresponding spent media from the shake flask cultures of *B. subtilis* and *B. pumilus* strains could not. These results suggest that there is a biofilm-specific cross-species signaling system that induces planktonically grown cells to behave as if they were in a biofilm by regulating the expression of pigments and antimicrobial compounds.

BIOFILM TECHNOLOGY FROM BIOREMEDIATION PERSPECTIVE

Bioremediation is the process of using *in situ* or *ex situ* microorganisms to clean up a contaminated site (64). Microorganisms decompose harmful compounds using enzymes, which are specific proteins that control reactions in living

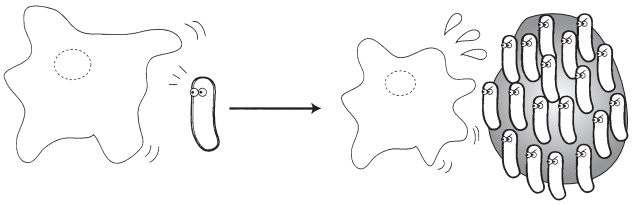

FIG. 4. Formation of inedible large biofilms in response to grazing.

cells. Microorganisms that produce enzymes capable of degrading petroleum are useful in cleaning up oil spills. Some common microorganisms that are capable of degrading oil are *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, *Azotobacter*, *Rhodococcus*, and *Bacillus*. We observed that the obligately aerobic and thermophilic *B. thermoleovorans*, which is capable of degrading long-chain alkanes at 80°C, did not grow in a shaking, biofilm-unfavorable culture system (65).

In the field of wastewater treatment, the effectiveness of adding a selected species to a complex ecosystem, called bioaugmentation, is under debate. Although several full-scale bioaugmentation experiments have been reported to be successful, other experiments conducted by independent laboratories have often shown that bioaugmentation had little effect on wastewater treatment (66). Problems concerning the misfixation of the inoculated microorganisms, the insufficiency of substrate, competition between the introduced species and the indigenous biomass, and grazing by protozoa, have been cited as possible reasons for the failure of the experiments. A recent study revealed that *P. aeruginosa* cells form inedible microcolonies in danger of flagellate predation at the onset of biofilm formation (Fig. 4; 67). An alginate exopolymer overproducing strain formed larger microcolonies in response to grazing. Grazing on biofilms by quorum sensing mutants (*lasR* and *lasR*/*rhlR*) also resulted in fewer and larger sizes of microcolonies than those by the wild type. These observations suggest that the formation of inedible large biofilms is a defensive strategy of bacteria to evade predators. Although microbial ecology issues are among the most important in bioaugmentation approaches, they are rarely addressed (68). The most direct and simple procedure, an enrichment culture, has been widely adopted for the selection of strains that express the required degradation ability under specific conditions of the enrichment culture. However, such enriched populations are not necessarily typical or representative of indigenous communities in the target habitat and could be equally, by chance, derived from transient populations. The problem is that enrichment culture is unlikely to have any influence on other traits that are also required for enriched strains to be competitive and effective in the target environment. These additional traits are required to survive prevailing, often fluctuating, environmental conditions (*e.g.*, moisture, nutrient, redox, pH and osmotic factor changes) and competition from indigenous microbial populations and predators, among many other stresses.

Understanding the microbial spatial communities in terms of mixed species biofilms present in the polluted site, and reintroducing these cooperative and inedible biofilms including pollutant-degrading bacteria to the polluted site would be crucial to render bioremediation, especially bioaugmentation, a more effective and practical technology with less environmental impacts.

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