Bacterial behavior at surfaces

Gill G Geesey

Population level studies demonstrate that bacterial colonization of surfaces and subsequent biofilm architecture are controlled by a variety of factors that include the hydrodynamics, surface chemistry and genotype of the cell. New molecular tools now extend our ability to investigate among bacterial cells within a surface-associated population subtle phenotypic differences that do not involve changes in genotype. Such resolution has led to new discoveries in relationships between bacterial cells and their environment.

Addresses
Department of Microbiology and Center for Biofilm Engineering, PO Box 173520, Montana State University, Bozeman, Montana 59717-3520, USA; e-mail: gill_g@erc.montana.edu

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Abbreviations
GFP green fluorescent protein

Introduction

Surfaces promote novel behavior in bacterial cells. Since submerged surfaces sorb solutes and small particles such as bacteria from the bulk aqueous phase, sharp, highly localized three-dimensional chemical gradients are formed at the liquid–solid interface. The nature of the substratum often determines which solutes are adsorbed from the bulk aqueous phase, the orientation of the sorbed molecules and the strength of their interaction with the substratum. The metabolic activities of those bacteria that become associated with a surface cause these interfacial chemical gradients to evolve over time and space, creating conditions not normally encountered in the bulk aqueous phase. The deposition of extracellular polymeric substances by the surface-associated microbial cells transforms the surface into a highly heterogeneous milieu, the complexity of which is only now being characterized.

It has been known for some time that certain aspects of bacterial cell behavior vary depending on whether the cell is associated with a surface or suspended as an individual in the bulk aqueous phase. Since the comprehensive review on this subject by Lawrence *et al.* in 1995 [1], significant advances have occurred in this field. The development of molecular tools over the past two decades has expanded our appreciation of the range and genetic basis of surface-induced phenotypes [2,3]. In many instances, the behavior of cells at surfaces exerts a significant impact on system ecology and performance. This review highlights some of the recent significant discoveries in this area.

Control of mineral transformation by surface-associated bacteria and bulk aqueous phase chemistry

Thiobacillus ferrooxidans is the microorganism primarily associated with the oxidation of sulfide minerals. This process has been exploited in the extraction of gold, nickel, copper and cobalt from sulfide ores. Bacterial interaction with sulfide minerals is also associated with acid mine drainage and habitat destruction. Fowler and Crundwell [4] employed a shrinking core model based on experimental data to show that the chemical leaching of zinc sulfide (sphalerite) is controlled by the diffusion of Fe^{2+} ions from the bulk aqueous phase through a sulfur product layer that forms on the mineral surface. Their results further demonstrated that diffusion through the product layer does not limit the rate of dissolution when bacteria have colonized the mineral surface. They suggest that by oxidizing the sulfur formed on the mineral surface, the surface-associated bacteria remove the barrier to diffusion by Fe^{2+} ions, allowing the reaction to proceed at a faster rate than can be achieved abiotically at high Fe^{2+} ion concentrations. Thus, bacteria enhance the rate of sphalerite dissolution under conditions in which diffusion through the sulfur layer controls the overall rate of chemical leaching. The results suggest that the transformation of minerals is modulated by complex interactions between bulk aqueous phase chemistry and metabolic activities of bacteria associated with the mineral surface. This information should be useful in developing more efficient commercial mineral leaching operations, and more effective remediation strategies for acid mine drainage.

Influence of substratum surface properties on bacterial adhesion and surface colonization

Food products contaminated by pathogenic microorganisms during processing are a major source of foodborne infection. Rapid, efficient methods are needed to assess product contamination at various stages of processing. Siragusa *et al.* [5] demonstrated the usefulness of a bioluminescent reporter to study the attachment and colonization of beef carcasses by *Escherichia coli* O157:H7. This approach circumvented the need to exogenously sample the tissue surface. After inoculating carcass tissue surfaces with an *E. coli* O157:H7 strain that carried a plasmid containing the complete *lux* operon, *luxCDABE*, from *Photorhabdus luminescens*, the efficiency of carcass washing was macroscopically evaluated in a nondestructive manner in real time. The results demonstrated that there was differential adherence of bacterial cells to the various surface types. After washing, the leaner muscle tissue retained a greater fraction of the bacterial inoculum than the adipose tissue. This molecular photonic approach can be used to develop more efficient washing and disinfection procedures for carcasses as well as for food preparation surfaces.

The approach can be easily adapted to detect expression of genotypic traits that influence pathogen survival in animals, such as those that determine the minimum infection dose, as well as contaminant transfer from the animal or gastrointestinal tract to the carcass during processing.

Dalton *et al.* [6] exploited the sensitivity and response of surface-associated bacteria to substratum surface properties to develop a novel 'bioprobe' that was sensitive to microscale changes in surface free energy. The morphology of the attached cells reported changes in antifouling properties of the surface of a substratum submerged in water over the long term. This is one of the first examples of a surface analytical technique that exploits the response of bacteria to subtle, small-scale changes in substratum surface properties.

Phenotypic variability within surface-associated populations

Over the past two decades, much of the research on surface-associated bacteria has focused on differences in behavior relative to free-living cells suspended in the bulk aqueous phase [7]. However, the sharp physical and chemical gradients that have been demonstrated to exist within a biofilm also promote differences in behavior among surface-associated cells that are as great as those observed between surface-associated and free-living cells [8]. Nevertheless, evaluation of surface-associated bacterial behavior, for the most part, remains focused at the population rather than at the level of the individual cell [9–13]. Although a population level evaluation reveals the 'average' behavior of the cells, it offers little insight to the range of behavior displayed by individuals in the population.

Single-cell activity on surfaces

Even though it has been known for some time that cells within specific microbial populations display the ability to differentiate morphologically in response to environmental cues, only in the last several years have we begun to explore less obvious differences in cell behavior within a single population. More recently, molecular approaches have revealed subtle differences in the activities of cells within a population. Sternberg *et al.* [14••] used a novel reporter for nondestructively monitoring in real time the overall cell activity at the individual cell level. The reporter consisted of a transposon cassette carrying fusions of the growth-rate-regulating *rrnBP1* promoter for rRNA gene expression in *E. coli* and a modified *gfp* gene, which encodes a green fluorescent protein (GFP) that is susceptible to protease attack within the cell. In chemostats, free-living cells, replicating at fixed rates controlled by nutrient dilution rate, expressed fluorescence intensities that correlated with dilution rate and ribosome content of the cell. When incorporated into the chromosome of a toluene-degrading strain of *Pseudomonas putida*, the cassette reported fluorescence intensities that differed among individual cells growing on a surface. Early colonizers of a substratum were highly fluorescent, indicating that the cells had a relatively high rRNA content. As the biofilm developed and microcolony size increased, cells in the center of microcolonies displayed weaker fluorescence intensities than cells at the border, revealing the heterogeneity of overall cell activity among cells within a single microcolony. On the basis of these results, it was suggested that initial colonizers of a surface display relatively high growth rates, and that cells at the edge of microcolonies subsequently formed at the surface replicate faster than those at the center of the colony.

Caution should be exercised, however, when applying relationships inferred from free-living populations to surface-associated populations. The relationship between rRNA synthesis and growth rate, obtained in free-living bacterial populations by Sternberg *et al.* [14••], may not apply to surface-associated populations. For example, upon attachment to surfaces, cells synthesize products, such as exopolysaccharide, not produced during their residence in the overlying bulk aqueous phase [15,16]. Exopolysaccharide synthesis, in many instances, is independent of cell growth rate. More recently, Baty *et al.* [17] showed that exoenzyme synthesis was uncoupled from the growth rate of surface-associated cells. Thus, a portion of the rRNA produced by the initial surface colonizers in the study by Sternberg *et al.* [14••] may have been involved in the synthesis of growth-rate-independent products that are not produced by free-living cells exposed to the same bulk aqueous phase. Nevertheless, the demonstration of a reporter of rRNA synthesis that can be used in diverse groups of bacteria to nondestructively quantify overall activity at the single-cell level in real time offers the opportunity to explore complex cell behavior at a level never before achieved.

Ramos *et al.* [18••] used the approach of Sternberg *et al.* [14••] to gain a better understanding of the physiological status of bacterial cells in plant–microbe systems. Successful implementation of microbial inoculants for biological pest control, promotion of plant growth, and plant-assisted elimination of pollutants from soils requires efficient colonization of the root system and a high likelihood that the intended activity of the introduced microorganisms is expressed in the field.

When sterile barley seedlings coated with 10^4 colony-forming units of *P. putida* carrying the gene cassette that encodes stable GFP had been incubated for 1–3 days, green fluorescent cells and microcolonies of *P. putida* were observed throughout the root system. When the same experiment was carried out with a cell inoculum similar to that described above but carrying a gene cassette encoding the protease-sensitive GFP, green fluorescence was observed only in bacterial cells on the sloughing root sheath cells after 2 days. As in an earlier study, cells at the center of some microcolonies displayed reduced fluorescence compared to cells at the periphery of the colony (see also Update). The molecular approach described above offers a useful way of nondestructively assessing the activity and dissemination of individual cells of a population in

a variety of engineered systems designed to study the behavior of populations of bacteria in diverse habitats.

Cell–cell interactions

The ability of each individual cell to receive, interpret and respond to information from its neighbors allows bacteria attached to surfaces to collaborate on a variety of essential cellular activities ([19]; see also Update). Using a combination of molecular approaches with resolution at the single cell level, Baty *et al.* [17] detected two subpopulations of cells of the marine bacterium S91 during colonization of a chitin surface. One subpopulation expressed chitin-degrading genes and produced active extracellular chitinase enzyme, and the other did not produce chitinase but used for cell replication the extracellular chitin hydrolysis products generated by the chitinase enzymes of neighboring cells. Cell densities and substratum properties were not significantly different for the two subpopulations, suggesting that communication between the subpopulations occurred via a mechanism other than quorum sensing.

It has been proposed that nongenetic variability in microbial populations occurs under conditions in which gene induction by all members of a population may not be relevant or useful. Tolker-Nielsen *et al.* [20] suggested that this may be the case when an abrupt but subsequently reversible detrimental environmental change occurs so rapidly that there is not enough time for induction and expression of stress response genes. Upon return to normal conditions, the few surviving nongenetic variants would still be well adapted to the normal conditions and form a new population, whereas any mutant survivors would be less well-adapted to the normal condition.

Baty *et al.* [17] proposed that the nongenetic variability detected in a chitin-surface-associated population of the marine bacterium S91 offers a more efficient means of converting energy in the form of insoluble chitin into bacterial cell biomass for dispersal and colonization of other particles. The replication and detachment of daughter cells of S91 in some ways resemble the behavior of *Caulobacter crescentus* [21], although asymmetric cell division is not evident in the former. In the open ocean, nutrients are concentrated on detrital particles that are present at very low densities. Degradation of these particles and survival of the heterotrophic bacterial populations mediating particle degradation likely depends on surface-associated intra- and interpopulation cell–cell interaction and cooperation. In these examples, heterogeneous behavior among members of a population imparts efficiency to the population as a whole, promoting survival in the absence of genetic mutation.

Structure–function relationships

Biofilms have been described as highly organized microbial communities [22,23]. However, most of the structural information presented to date has been qualitative in nature. Yang *et al.* [24] recently described an image analysis approach to quantifying biofilm structure. This approach, and the biofilm modeling approach of Picorenau *et al.* [25,26], permits the assignment of significance to structure–function relationships within surface-associated bacterial populations.

Using a complement of molecular-based reporters, Moller *et al.* [27] tested the hypothesis that a defined mixed population of cells growing as a biofilm in a laboratory-based flow chamber forms highly organized communities during benzyl alcohol degradation. One member of the bacterial consortium, *P. putida*, carried a chromosomally-inserted transposon containing either the promoter (*Pu*) and regulator gene (*xylR*) of the upper pathway, or the promoter (*Pm*) and regulator gene (*xylS*) of the meta pathway, the two pathways together controlling the degradation of toluene and related hydrocarbons. Through an elegant set of studies, it was shown that the *P. putida* variant carrying the *Pm* promoter required an association with cells of another population of bacteria, *Acinetobacter* sp. strain C6, for maximum activity. In contrast, activity of the *Pu* promoter did not require an association between this variant of *P. putida* and cells from the other populations present in the biofilm. Because promoter activity could be assessed nondestructively in real time, the extent of promoter activity in *P. putida* cells could be related to the density and proximity of cells of the other population. By performing *in situ* hybridization with fluorescence-labeled 16S-rRNA-targeting probes at the end of the experiment, the association between these specific members of the seven-member biofilm consortium was confirmed. It was further demonstrated that although structural interactions in the benzyl-alcohol-degrading biofilm appeared rather loose, architectural features were observed. For example, *Acinetobacter* sp. congregated at the base of the biofilm, whereas *P. putida* concentrated in the outermost layers of the biofilm. Thus, some community level processes are not restricted to highly organized microbial communities.

Community level processes did appear to be highly organized in nitrifying aggregates in a continuous-upflow reactor [28•]. Hybridization with fluorescence-labeled 16S-rRNA-targeting probes specific for certain NH_4^+-oxidizing and NO_2^--oxidizing bacteria was employed along with a series of microsensors that measure O_2, NH_4^+, NO_2^-, and NO_3^- concentration to produce microprofiles of the aggregates. From these microprofiles, volumetric reaction rates were calculated and related to cell numbers of nitrifying bacteria in specific regions of the aggregates. Whereas nitrification occurred in a narrow zone on the surface of the aggregates, the central zone of the aggregates appeared inactive. Through this approach, it was possible for the first time to estimate the cell-specific activity of *Nitrosospira* spp. and previously uncultured *Nitrospira*-like bacteria *in situ*. Such microscale information should facilitate optimization of reactor design and operation for biological nitrification in industrial wastewater treatment processes.

Using the same approach as that described by Schramm *et al.* [28•], microbiologically-mediated nitrification was

also shown to occur in highly structured biofilms formed on rotating disk reactors submerged in domestic wastewater [29]. In contrast to the findings of Schramm *et al.* [28•], NO_2^- oxidation occurred within the inner parts of the biofilm in areas occupied by *Nitrospira*-like bacteria, whereas NH_4^+ oxidation occurred throughout the biofilm. Furthermore, *Nitrosomonas* spp. appeared to be the numerically dominant NH_4^+-oxidizing bacteria in the biofilm. Differences in the hydrodynamics of the different reactors employed in these studies may have accounted for the different structure–function relationships observed.

Even though microelectrodes do provide the opportunity to determine specific metabolic activities at high spatial resolution, the measurements are generally obtained under a hydrodynamic regime different from that present in the system being investigated. Furthermore, the intrusive nature of microsensors makes it difficult to assess their influence on some structure–function relationships of the microbial communities under study. Fluorescence lifetime imaging is a nonintrusive approach that has been used to define chemical gradients in microbial biofilms at high spatial resolution [30]. pH gradients in both axial and lateral directions were defined within 140 μm-thick biofilms in real time by quantifying fluorescence decay of carboxyfluorescein introduced to the biofilm from the overlying bulk aqueous phase using two-photon excitation microscopy. Fluorescent reporters, however, have not yet been developed for all of the useful chemical species currently measured by microsensors.

The structure of microcolonies within biofilms as well as biofilm architecture is controlled by flagellar motility of the surface-associated bacterial cells. Delpin *et al.* [31] found that a flagellum-negative mutant of a marine *Vibrio* sp. did not form colonies like the parent strain: mature biofilms of the flagellum-negative mutant did not contain tower structures of cells like biofilms of the parent strain did. These observations support a growing body of evidence that shows that flagella and other cell surface appendages exert significant control over cell behavior at a surface.

Recent advances have been made in digital image processing using exhaustive photon reassignment deconvolution algorithm and three-dimensional rendering software [32]. For image acquisition and processing, Metamorph Imaging Software (Universal Imaging Corporation, West Chater, Pennsylvania) was used. EPR-deconvolution software (Scanalytica, Billerica, Massachusetts) was used to enhance optical resolution of stacked optical sections. This approach has several advantages over confocal scanning laser microscopy. Application of the algorithm on digital image data obtained from widefield epifluorescence microscopy after *in situ* hybridization with fluorescence-labeled 16S-rRNA-targeting probes resolved the alignment of *Desulfovibrionaceae* cells with micropores of the mediterranean sponge *Chondrosia reniformis*.

Conclusions

Surfaces provide a niche that promotes the evolution of complex interactions between bacterial cells. The biological complexity of a system is defined by intra- as well as interpopulation cell behavior. Full appreciation of the range of interactions between different populations of organisms at surfaces and within biofilms therefore requires an understanding of the interactions and behavior of individual cells within each population. Whereas population level evaluation has been convenient for identifying genes expressed by cells only at surfaces, evaluation at the single cell level reveals the influence of highly localized physical and chemical gradients on gene expression and gene product activity. Phenotype resolution at the single cell level should facilitate detection of novel communication systems that operate within the steep chemical and hydrodynamic gradients occurring in biofilms, and promote a better understanding of the subtle features that make biofilms such attractive structures for microbe inhabitation in the biosphere.

Update

Miller *et al.* [33] validated the use of whole-cell biosensors, using transcriptional fusions of a sucrose-sensing promoter and the GFP reporter to measure sucrose concentrations on a leaf at high spatial resolution. When cells were inoculated onto bean leaves, whole-cell ice nucleation and *gfp*-based biosensors for sucrose both indicated an average concentration of 20 mM across the moist leaf surface.

DeKievit *et al.* [34] showed that when glucose is used as a carbon source, the structure of a *Pseudomonas aeruginosa* biofilm and twitching motility of cells under static conditions are controlled by quorum sensing molecules, the synthesis of which is controlled by the *lasI* and *rhlI* genes. However, when cells are cultured under static conditions on a citrate-based medium, twitching motility is inhibited and biofilm formation is independent of quorum sensing molecules. Under hydrodynamic flow conditions, the influence of the medium observed under static conditions was no longer apparent. Transcriptional fusions of the *lasI* promoter and a *gfp* gene encoding an unstable GFP molecule ($t_{1/2}$ = 40 minutes) were used to follow synthesis of quorum sensing molecules in cells during biofilm development. Qualitative evaluation indicated that a larger fraction of the total cell population expressed *lasI* in four- to six-day-old biofilms than in eight-day-old biofilms. The majority of cells expressing *lasI* were in the bottom 10 μm of a 30 μm-thick biofilm. Transcriptional activity of *rhlI* was much less variable than *lasI* in cells during biofilm maturation and at different depths in the biofilm. These studies reveal just how important environmental conditions are in controlling expression of quorum sensing genes and consequent biofilm structure.

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