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Deleya marina as a model organism for studies of bacterial colonization and biofilm formation

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***Deleya marina* was used as a model organism to identify cellular components essential to surface colonization, an important early step in biofouling. Model components include the test bacterium *D. marina*, its phenotypic variants, and a rapid microplate adhesion assay. Studies of *D. marina* exopolymer and motility properties are discussed in the context of colonization processes and model development. A testable hypothesis is presented which was formulated to explain events in surface colonization by *D. marina*.**

Keywords: biofilms; adhesion; motility; phenotype variability; *Deleya marina*; model organism

Introduction

Biofouling is the deposition and accumulation of cells and their metabolic products on equipment and structural surfaces [11]. Its occurrence is detrimental to the maritime, paper, electric power, and petroleum industries among others [8,11,19,26,28,40]. Fouling blocks flow, increases fuel consumption, conceals structural flaws, reduces heat/chill transfer, and adds weight or bulk that exceeds structural design limits. It may lead to microbial-induced corrosion (MIC) and structural failure [26,28,59], and can be a source of contaminating material that lowers the quality of a product such as paper [8]. Losses to industry are substantial, exceeding billions per year [11]. Decades of empirical efforts to find preventatives and treatments led to the use of toxic paints and claddings, poisons such as chlorine, and mechanical cleaning methods [11,12,19,26,28,35,63]. However, many of these methods are unsatisfactory due to high cost or toxic effects on the environment [12,28].

Fouling of aquatic surfaces generally follows a defined course that begins with primary surface colonization by bacteria and diatoms [11,15–17,34,35,63]. Once attached, cells multiply and produce exopolymers. A confluent biofilm forms as individual colonizations merge. The biofilm concentrates nutrients and may condition toxic surfaces, thus promoting secondary colonizations by other microorganisms, algae, invertebrates, and macroorganisms over a period of days or years [15,16,18,27,32,35]. Inhibition of primary colonization reduces the formation of a slime layer [13,28], and may reduce the occurrence and extent of secondary fouling [18,32,33]. A systematic examination of a representative bacterial model can elucidate specific processes involved in surface attachment, biofilm development, and cell dissemination. We postulate that identification and characterization of specific processes will facilitate the development of effective antifouling agents. Because these agents specifically target bacteria, they are less likely to have toxic effects on the environment. In this paper, we describe a model that uses *Deleya marina* as a test organism to identify essential components and fundamental processes in bacterial attachment to marine surfaces. From our studies, we formulated a hypothesis to explain events in *D. marina* surface colonization. This model can be tested using other adherent species to identify mechanisms common to bacterial fouling.

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The model

The model system consists of an adherent bacterium, its isogenic phenotype variants, and qualitative and quantitative assays for measuring cell adhesion under defined conditions. Fundamental to model development was the selection of an appropriate test organism and a precise picture of the phenotypic properties of the test strain and its variants. Just as essential was the modification of the adhesion assay to a rapid microplate format. Adhesion analyses performed under various environmental and cellular conditions allowed the identification of key influences on surface colonization.

The bacterium

The adherent marine bacterium *D. marina* was selected as the model test strain because it possesses both exopolymer and flagella [5,6,14,23]. Many acknowledge these bacterial features as probable adhesion factors [5,6,14,24,35,36,43,44,47,57]. *D. marina* produces a large amount of exopolysaccharide of known chemical composition [23], and most strains are motile, with 2–5 monopolar flagella [5,6]. This species was of additional interest because its biofilms reportedly influence secondary colonization by invertebrates and algae [27,32,33]. *D. marina* is a true halophile, requiring a concentration of 0.315 M Na^+ [1,5,6]. Conveniently, cultures grow rapidly at ambient temperatures [54], and a biochemical profile is available for easy differentiation between species and among genera [5,6].

In preparation for adhesion studies, we examined known cell features under a variety of conditions. These examin-

ations revealed previously unknown properties of *D. marina* and the *Deleya* genus. A prominent feature of *D. marina* is the formation of large, complex aggregates (Figures 1–3). Our results indicate that aggregation is dependent on exopolysaccharide [55]. The most interesting observation, however, was surface-associated motility. We discovered that *D. marina* and other *Deleya* species glide on semisolid medium when the agar concentration is between 0.15 and 0.35% (Figure 4a) [54]. This was the first report of gliding in any species known to have flagella [10,42,45]. Although flagella are not present on the gliding cells, they appear in subpopulations of gliding cell cultures [51,54]. The alternate appearance of swimming and gliding cells constitutes a phenotypic variation and is the first of this type to be reported. The mechanism controlling the appearance of the two motility phenotypes is not known.

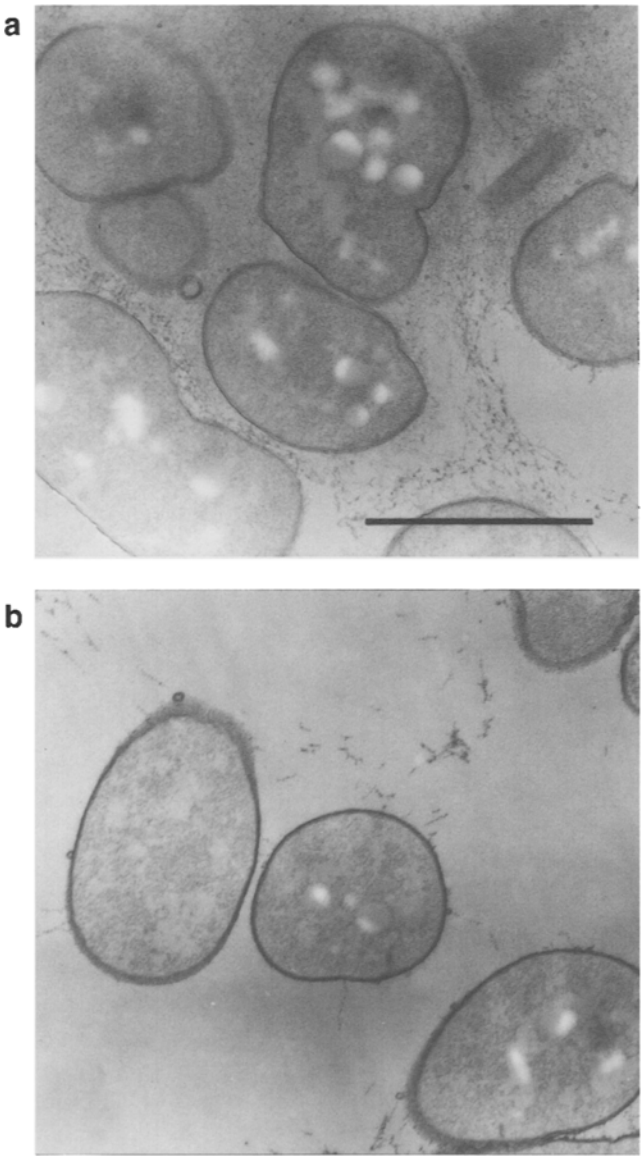

Figure 1 Transmission electron micrographs of ruthenium-red-stained exopolysaccharide associated with *D. marina* wild-type cells (a) and rough colony mutant cells (b). Bar = 1 μm. Reprinted from [55] with permission of Applied and Environmental Microbiology, American Society for Microbiology

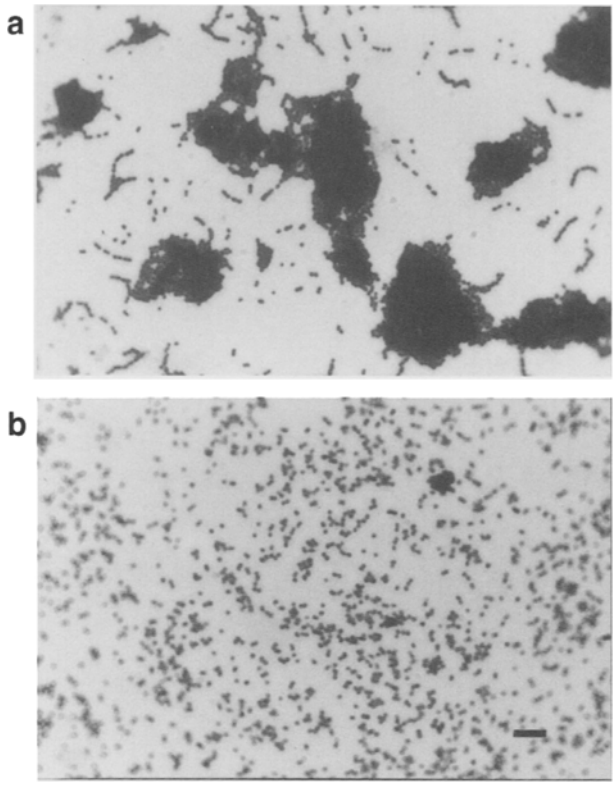

Figure 2 Photomicrographs of aggregates formed by *D. marina* wild-type cells (a) and DMR mutant cells (b). Cells were grown at 30° C and allowed to adhere to tissue-culture-treated glass slides and washed gently. Bar = 100 μm

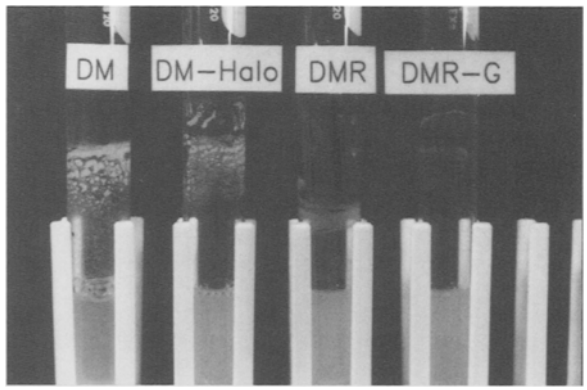

Figure 3 Abundant expolysaccharide is produced by wild-type *D. marina* cells DM and DM-Halo in overnight broth cultures, while mutant strains DMR and DMR-G show reduced production

The isolation and characterization of two categories of phenotype variants were essential to the identification of important colonization factors. Basic properties of the *D. marina* type strain and variant strains are presented in Table 1. The first category consists of stable spontaneous mutants deficient in exopolysaccharide and defective in cell aggregation, represented by *D. marina* R (DMR) (Figures 1–3) [55]. A second category contains strains that display an alternate motility phenotype in semisolid culture medium. The ATCC culture of the *D. marina* type strain is a non-flagellated variant. When inoculated into semisolid agar plates, this strain forms slime trails, a characteristic of glid-

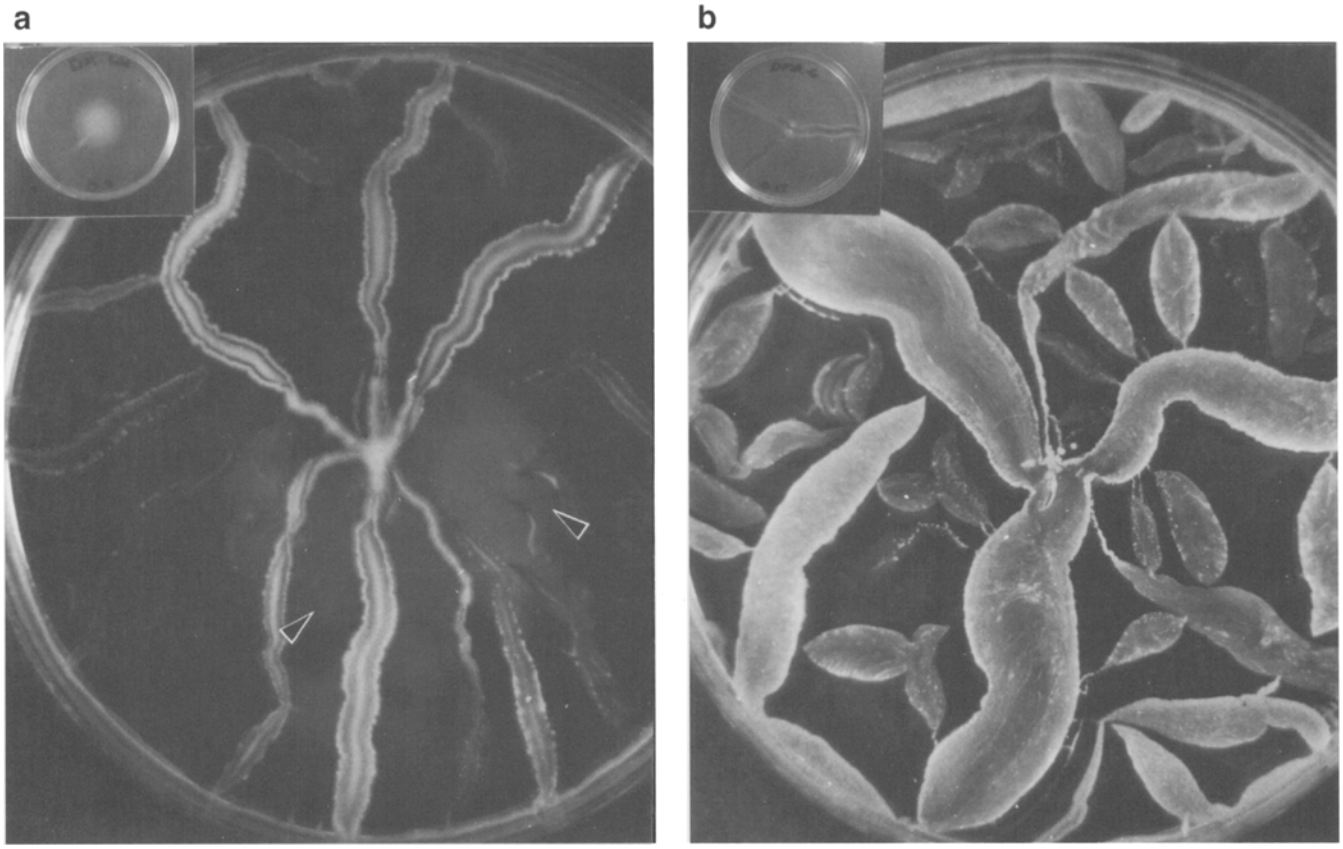

Figure 4 Motility patterns of *D. marina* strains on semisolid marine agar. (a) The nonflagellated wild type showing gliding trails and subpopulations of swimming cells (arrowhead) which appear upon extended incubation. Insert shows the wild-type swimmer DM-Halo with the initial chemotactic halo and recently developed gliding trails. (b) The exopolysaccharide-deficient mutant DMR showing gliding trails. Insert shows the mutant swimmer DMR-G with a small granular motility haze and trails of gliding cells. Reprinted from [51] with permission of Biofouling, Harwood Academic Publishers GmbH

Table 1 Properties of motility variants of wild type and exopolysaccharide-deficient *D. marina* strains [51,54,55]

Strain[a]	Exopolysaccharide	Aggregates	Motility phenotype	Flagellar bundle
D. marina (DM)	abundant	large	gliding	none
DM-Halo	abundant	large	swimming	1 subpolar
D. marina R (DMR)	deficient	none	gliding	none
DMR-G	deficient	3–7 cells	swimming	1 or more

[a]All strains utilize ribose, glucose, fructose, mannitol, galactose, and gluconate. Strains do not utilize maltose, sucrose, or sorbitol and are oxidase negative

ing motility (Figure 4a) [10,42,45]. As incubation continues, subpopulations of swimming cells appear in chemotactic halos among the trails of gliding cells. An isolate, *D. marina* DM-Halo, has a subpolar flagella bundle and resembles descriptions of the original swimming isolate of the *D. marina* type strain [14]. In semisolid medium, DM-Halo forms a chemotactic halo of swimming cells, and as incubation continues, produces subpopulations of gliding cells (Figure 4a, insert). Although variable in semisolid medium, each motility phenotype is stable in solid agar and broth cultures. Both flagellated and nonflagellated variants of the wild type strain produce abundant amounts of exopolysaccharide (Figure 3) [54].

The exopolysaccharide-deficient mutant DMR also has two motility phenotypes. Like the wild type, DMR retains the ability to glide and produces subpopulations of swimming cells (Figure 4b) [51]. However, swimming variants seldom appear in semisolid medium. Only 1 of 188 (0.53%) mutant colonies will yield a swimming variant in contrast to 1 in 13 (7.4%) wild type colonies [51,54]. The isolate *D. marina* R-G (DMR-G) represents a swimming variant of DMR (Table 1). DMR-G differs from DM-Halo in that flagella bundles are dispersed and appear inserted at more than one site. In semisolid medium, DMR-G produces a small granular haze of swimming cells at the inoculation site followed by the appearance of gliding trails (Figure 4b, insert) [51]. Like DMR, DMR-G is deficient in exopolysaccharide (Figure 3). Motility and exopolymer variants have different attachment properties (Table 2).

Table 2 Surface and environmental factors which influence the attachment of *D. marina* [51]

D. marina strains[a]	Surface preference	Attachment inhibited by			
		Turbulence	37° C	NaN_3	Tween 80
DM	Charged, hydrophilic	slightly	yes	yes	no
DM-Halo	Charged, hydrophilic	yes	yes	no	no
DMR	Hydrophobic[b]	yes	no	no	yes
DMR-G	Hydrophobic	yes	no	slightly	yes

[a]Nonflagellated (DM) and flagellated (DM-Halo) exopolysaccharide-producing wild type variants and nonflagellated (DMR) and flagellated (DMR-G) exopolymer-deficient mutant variants
[b]The mutant attaches less firmly to the hydrophilic substrate in turbulence assays

The assay

A model system requires a quantitative assay method to test generalizations and to define properties of the test organism. Adaptation of the adhesion assay to a microtiter format allows rapid measurement of many samples and replicates for statistical comparisons [52,55]. Selection of an appropriate assay for a specific bacterium requires consideration of assay sensitivity and the indicator method used, eg dye uptake. Some bacteria attach to surfaces at low cell concentrations, while others do not take up the indicator dye, resulting in signals at the lower limit of instrument sensitivity [52,53]. We found a crystal violet adhesion assay (Table 3) to be the most satisfactory for detecting the adhesion properties of *D. marina* [52,55]. In this assay, a spectrophotometric microplate reader measures the absorbances of crystal violet eluted from adherent, stained cells. The microplate format allows easy variation of environmental and cellular conditions. Parameters tested include temperature, substratum, cell concentration, cell growth condition, bacterial strain, turbulence, and the presence of inhibitors. Commercial test surfaces are available and include microplates of hydrophobic untreated-polystyrene (Dynatech, Chantilly, VA, USA) and hydrophilic, tissue-culture-treated (net negative charge) and Primaria-treated (net positive charge) polystyrene (Falcon, Becton Dickinson, Oxnard, CA, USA). ProBind microplates (Falcon) bind all cell types firmly, and provide a means to determine the absorbances of known cell numbers for quantitative adhesion measurements.

An important advancement in early adhesion studies was the concept of reversible and irreversible cell attachment [36,37]. This view altered assay methodology and interpretation. Generally, attachment is considered irreversible when cells resist removal by washing. In the microplate assay, a standardized wash procedure differentiates between irreversible and reversible attachments [52]. Differences in the effects of washing on *D. marina* wild type cells and exopolymer-deficient mutant cells formed the basis of the developed wash procedure. Initially, we applied the wash with a handheld multichannel micropipettor [52,55]. Proper application of the manual wash requires training and meticulous attention. Recent automation has eased performance and reproducibility of the assay. Figure 5 presents typical contrasting adhesion profiles of the wild type and mutant strains obtained with this assay. Drs Peter Angell and David C White (Center for Environmental Biotechnology, University of Tennessee) confirmed this difference in radial-flow cell experiments. The shear force required to remove wild type cells was ≥74 dynes, while removal of rough mutant cells required a force of only 4 dynes. The steps for the crystal violet microplate adhesion assay are summarized in Table 3.

Table 3 Crystal violet microplate adhesion assay [52]

A) Cell attachment[a]
1) Apply 100-μl volumes of bacterial cell suspensions to a 96-well microtiter plate
2) Centrifuge cells for 10 min at 215 × *g* using microplate carriers
3) Incubate cells in microplates at 25° C for 2 h with the supernatant phase in place

B) Automated wash to remove nonspecifically attached cells[a]
1) Aspirate fluid from the wells with a Dynatech Ultrawash II microplate washer
2) Wash cells by two automated cycles of a 180-μl wash (setting 4), 10-s soak (setting 1), and rapid aspiration (setting 1). The wash buffer is delivered from a tube located 12 mm above the top surface of microplates with wells 10–11 mm deep

C) Staining attached cells
1) Add 100 μl of 0.1% crystal violet aqueous dye to the wells for 5 min and aspirate off
2) Add 200 μl of deionized water and remove the rinse immediately by aspiration
3) Add 100 μl of 2% aqueous sodium desoxycholate and mix gently to release dye
4) Measure absorbances of the 100-μl detergent/dye solutions with a spectrophotometric microplate reader equipped with a filter in the range of 570–600 nm. Subtract the absorbance of a suspension medium control from sample absorbances

[a]The suspending medium and the wash solution used depend upon cell type and experimental goals, eg sterile artificial sea water was used for marine bacteria

Hypothesis

An hypothesis to explain events in *D. marina* colonization of an aquatic surface is illustrated in Figure 6 [51]. This hypothesis predicts that both flagella [36,51,57,58] and exopolysaccharide [4,21,24,43,44,51,52,55] have roles in cell attachment to surfaces. Our studies indicate that the exopolysaccharide of *D. marina* facilitates cell aggregation and preferentially mediates attachment to charged, hydrophilic surfaces [55]. Cells of this planktonic aggregate are depicted as flagellated since most *D. marina* isolates have flagella (top of figure) [5,6]. The contribution of flagella to attachment would depend on the size of the aggregate and

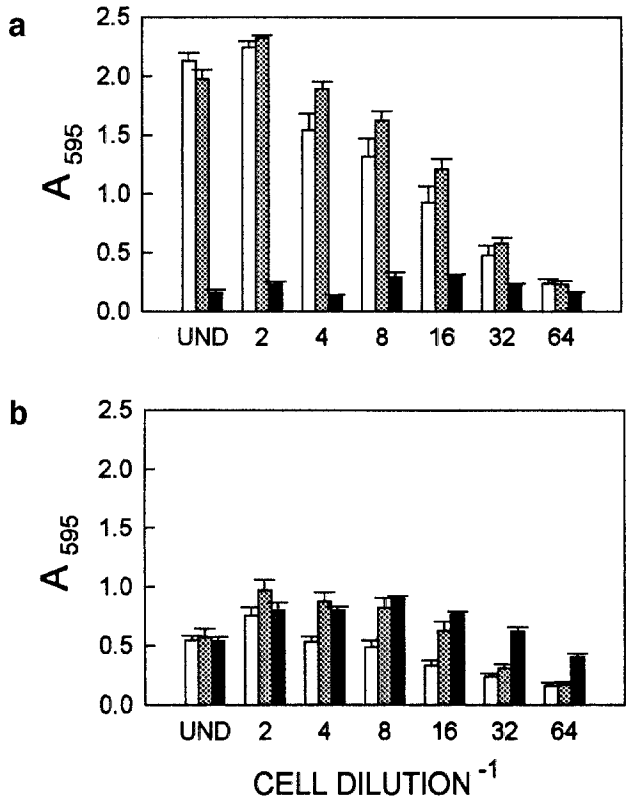

Figure 5 Adhesion profiles for the *D. marina* type strain (a) and its exopolysaccharide-deficient mutant (b) on three surfaces: tissue-culture-treated polystyrene (open bar), Primaria-treated polystyrene (shaded bar), and untreated polystyrene (solid bar). Firm attachments were differentiated from nonspecific associations by an automated wash. Bars represent mean and standard error of 2–4 replicates from each of three independent experiments. Cells were grown in marine broth culture at 25° C and harvested for assay in early logarithmic phase of growth

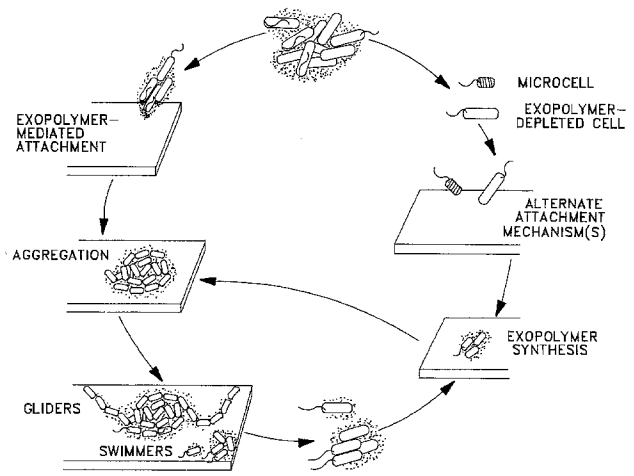

Figure 6 Hypothetical events leading to the colonization of a surface by *D. marina*. Experimental observations that support these hypothetical events include: (1) aggregates of swimming cells, (2) exopolysaccharide-mediated adsorption of cells, (3) large, surface-attached cell aggregates, (4) variable motility expression in semisolid medium, (5) surface-associated gliding motility, (6) a reduction in exopolysaccharide following nutrient deprivation, (7) persistence of swimming cells in starved cell cultures, and (8) colonization of a hydrophobic substratum by exopolysaccharide-deficient cells. Reprinted from [51] with permission of Biofouling, Harwood Academic Publishers GmbH

the degree to which flagellar function is inhibited by the glycocalyx. Exopolymer-mediated irreversible attachment (leftward direction in Figure 6) would occur quickly since exopolysaccharide is present initially [35,51]. A slime layer forms as attached cells multiply and elaborate exopolymer [20, 60]. Surface attachment, growth, or slime layer viscosity could lead to the appearance of subpopulations of cell types including both gliders and swimmers [2,4,20,39,49–51,54]. Gliding motility would facilitate expansion of the colony and dissemination of cells over the substratum. Swimmers probably break free of the slime layer as single cells or small groups and colonize new substrata [29,30,49,54]. Additional dispersal mechanisms may also exist [9,64].

Following nutrient deprivation, flagella may mediate reversible attachment to either hydrophilic or hydrophobic substrata as illustrated in the rightward direction in Figure 6 [29,30,36,43,51]. Results of turbulence assays indicate that the degree of attachment of the exopolymer mutant increased in the presence of flagella [51]. The data neither support nor disprove a requirement for flagella in irreversible attachment. Studies of nonflagellated, exopolymer-deficient cells indicate the presence of other cell adhesins [51]. Irreversible attachment of exopolysaccharide-deficient cells and starved microcells could be passive or active [35,51], and is probably mediated by a cellular adhesin. Exopolymer synthesis, initiated in the presence of surface-associated nutrients, would lead to cell aggregation and colony development.

Conclusion

D. marina is an excellent model organism for investigating surface colonization. Properties implicated in colonization of aquatic surfaces by adherent environmental isolates including exopolymer, flagella, and cellular adhesins [11,20,24,34–36,57,58] are present in *D. marina* [6,14, 23,51,54,55]. Relative contributions of cell properties to aggregation, motility, and attachment were identified through careful characterization. Crucial to this characterization was the development of a rapid microplate adhesion assay that allows easy testing of large numbers of samples under different environmental conditions. A variety of similar tests are now available [17,22,31,48,53,61,62]. The microplate assay and the availability of phenotype variants provide the means to study cell properties in relation to adhesion. We have used naturally-occurring variants to test these properties individually. Similar isolates are available for other model organisms [2,3,17,21,30,49,50], and genetic tools can be used to generate additional variants [7,38,41].

Our studies have shown that exopolysaccharide is the most effective adhesin in *D. marina* surface colonization [51,55]. Inhibition of exopolymer results in significant reduction in the numbers of attached cells [55]. Other adhesins, though less effective, do provide an attachment mechanism when the exopolymer is absent [55]. Gliding motility appears to function in the dissemination of cells over the colonized surface, and flagella contribute to cell positioning near a surface [51,54]. This systematic investigation of *D. marina* properties illustrates the remarkable

capacity of bacteria to adapt to a variety of environmental conditions ensuring survival, which must be linked to the colonization of substrate in adherent species. The gliding and swimming motility variation expressed in *Deleya* appears unique. However, these properties may have gone unnoticed in other species, since our studies indicate that to be detected motility must be examined under a broad range of conditions. Changes in phenotype, other than gliding and swimming motility have been reported [2,3,4,13,38,50,56] and in some instances linked to environmental or genetic regulatory signals [4,7,20,38,39,49,50].

Similarities in processes such as environmental or cellular controls on phenotype expression may provide a means to modify surface events. Our hypothesis can be tested in other species to identify common elements among isolates and determine the relative importance of various cell properties. The results would be the basis for identifying cell processes that can be targeted for inhibition. Identification of appropriate targets could lead to the development of viable alternatives to present day antifouling strategies. While our studies focused on the colonization of artificial marine surfaces, the model has application to other environments. Properties of *D. marina*, such as exopolymer and alternate motility modes, have been reported in clinical isolates [3,9,20] and may contribute to opportunistic colonization of prostheses and catheters [13,25,46]. This model can provide a framework for the systematic study of both environmental and clinical isolates to find practical solutions for problems associated with bacterial colonization of artificial surfaces.

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