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Droplet Enrichment Factors of Pigmented and Nonpigmented *Serratia marcescens*: Possible Selective Function for Prodigiosin

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Drops produced by bursting bubbles provide a mechanism for the water-to-air transfer and concentration of matter. Bacteria can adsorb to air bubbles rising through bacterial suspensions and enrich the drops formed by the bubbles upon breaking, creating atmospheric biosols which function in dispersal. This bacterial enrichment can be quantified as an enrichment factor (EF), calculated as the ratio of the concentration of bacteria in the drop to that of the bulk bacterial suspension. Bubbles were produced in suspensions of pigmented (prodigiosin-producing) and nonpigmented cultures of *Serratia marcescens*. EFs for pigmented cultures were greater than EFs for nonpigmented cells. Pigmented cells appeared hydrophobic based on their partitioning in two-phase systems of polyethylene glycol 6000 and dextran T500. The surface hydrophobicity of pigmented cells may result from the hydrophobic nature of prodigiosin and could account for the greater ability of these bacteria to adsorb to air bubbles and enrich airborne droplets. Enhancement of the aerosolization of *S. marcescens* may be a selective function of the bacterial secondary metabolite prodigiosin.

Serratia marcescens is a gram-negative bacterium classified within the family *Enterobacteriaceae*. Most wild-type strains of *S. marcescens* produce a characteristic secondary metabolite, prodigiosin, that causes a bright red pigmentation. Colonies of these red bacteria are easily seen with the naked eye, with the result that both the bacterium and its secondary metabolite have been described since antiquity. During medieval times, the deep red color of prodigiosin was viewed as a sign of the blood of Christ, causing proclamations of holy miracles and even inspiring riots by superstitious mobs (19, 39). More recently, the distinctive red pigment of the organism and its presumed lack of pathogenicity have made *S. marcescens* a popular biological marker. The microorganism has been used in germ warfare experiments, infectious disease research, and countless experiments on airborne transmission of disease. *S. marcescens* is now recognized as an opportunistic human pathogen (15, 19, 37, 38); nevertheless, it remains a favorite organism for studies on bacterial aerosolization.

Bacteria can be carried into the air by drops from bursting bubbles. These drops are of two kinds: the larger, less numerous jet drops travel directly upward from the center of the collapsing bubble cavity, while the more plentiful, minute film drops, formed from the rupture of the bubble film, are explosively dispersed in all directions. The concentration of bacteria in the drops can far exceed that of the bacterial suspension through which the bubbles rose (5–7, 10, 11). Different species of bacteria differ in their ability to be aerosolized; i.e., *S. marcescens*, *Serratia marinorubra*, and *Micrococcus euryhalis* show greater concentrations in bursting bubbles than *Escherichia coli* or *Pseudomonas bathycetes* (20). Moreover, there is a preliminary report that nonpigmented strains of *S. marcescens* are less likely than pigmented strains to attach to air bubbles (9).

Although the structure of prodigiosin, a tripyrrole, has been elucidated (30) and the biosynthetic pathway has been extensively studied (32, 35, 36), the function of prodigiosin, like that of most secondary metabolites, remains speculative (2, 3, 12, 16, 34). If the presence of prodigiosin enhances the ability of bacteria to become aerosolized in the drops produced by bursting bubbles, this secondary metabolite might increase the geographical distribution of pigmented strains, a possible selective advantage.

Bacterial enrichment of drops from bursting bubbles can be quantified as an enrichment factor (EF), the ratio of bacteria in a drop to the bacteria in the suspension through which the bubbles rose. We have extended the studies of Blanchard and Syzdek (9) by determining the comparative EFs of a pigmented and a nonpigmented strain of *S. marcescens* under carefully controlled conditions. In addition, to determine whether prodigiosin, known to be hydrophobic when pure (38), also renders the surfaces of pigmented cells hydrophobic, pigmented and nonpigmented strains of *S. marcescens* were tested for partitioning in a two-phase system of polyethylene glycol 6000 (PEG) and dextran T500 (D).

(A preliminary presentation of this work has been given [S. R. Burger and J. W. Bennett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q 103, p. 277].)

MATERIALS AND METHODS

Strains and culture conditions. The prodigiosin-producing strain of *S. marcescens* (Nima) was generously provided by R. P. Williams, Baylor College of Medicine, Waco, Tex. The nonpigmented strain (ATCC 8100) was obtained from the American Type Culture Collection, Rockville, Md.

Difco nutrient agar (Difco Laboratories, Detroit, Mich.) was used to maintain stock cultures and obtain viable counts. Stationary cultures (20 h) grown in Difco nutrient broth were used for experiments involving drop collection; 40-h cultures grown in Difco tryptic soy broth were used for cell separation experiments with two-phase systems. All experiments were maintained at 27°C in stationary culture.

Drop production, collection, and quantification. Bubbles were produced in bulk bacterial suspensions by the method of Blanchard and Syzdek (8) by forcing air through an upright, submerged glass capillary tip in a glass tube (Fig. 1). Bubbles of 0.66- to 0.89-mm diameter were used, with a rise

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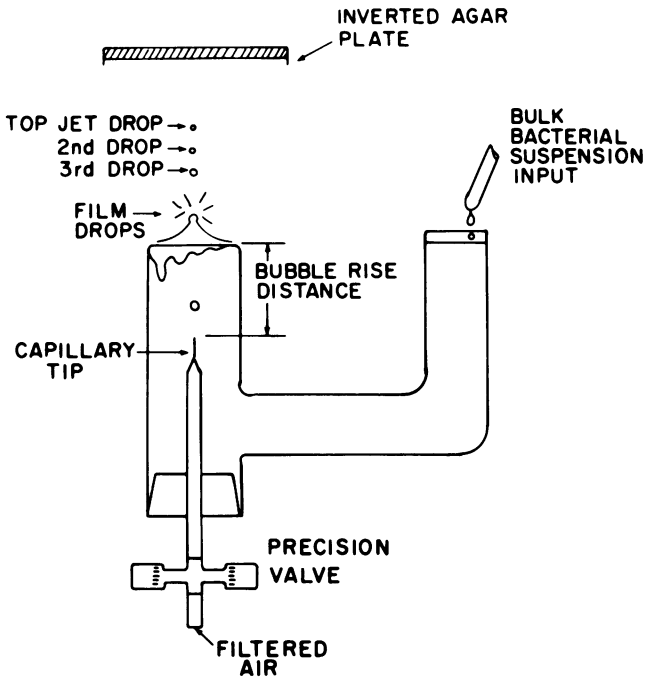

FIG. 1. Schematic diagram of bubbling apparatus. The agar surface is about 0.5 cm below the apogee of the top jet drop. (Diagram not to scale.)

distance of 6.5 cm from the tip to the surface. The liquid surface at which the bubbles burst was cleaned periodically by overflowing with additional bacterial bulk suspension added to the input arm of the tube. This overflowing removed surface active materials that otherwise would accumulate at the water-air interface.

The top jet drop from each bursting bubble was collected on an inverted nutrient agar plate placed over the suspension surface at a height such that only the top drop would impact on the agar. The drop was then spread with a sterile glass rod to disperse the cells. One top drop per plate was collected; approximately 100 plates were collected for each experiment.

At the end of each experiment, 20 to 25 drops were collected on a magnesium oxide-coated glass microscope slide for determination of the drop diameter. Drop impact craters in the magnesium oxide layer were sized microscopically, and the crater diameter was multiplied by a spread factor given by May (28). This computation gave the true drop diameter, allowing calculation of the drop volume as $4/3\ \pi r^2$.

Calculation of EFs. Bacterial bulk suspensions (10^6 to 10^7 bacteria per ml) were produced by appropriately diluting a sample of 20-h culture with 0.7% sterile saline. Viable cell concentration of the bacterial bulk suspension was determined by serial dilution plating at the close of each experiment.

The bacterial concentration of the drop was calculated by dividing the mean number of bacteria per drop by the drop volume. The EF was calculated by dividing the concentration of bacteria in the top jet drop by the concentration of bacteria in the bulk suspension. EF was called concentration factor in earlier work (9, 20).

Two-phase systems. Cell-surface hydrophobicity or hydrophilicity was determined by partitioning cells in two-phase PEG-D systems. Cell separation in the two-phase system was quantified as the partition coefficient, K, calculated as the ratio of the bacterial concentration in the PEG-rich phase to that of the D-rich phase.

The two-phase isotonic (0.7% [wt/wt] saline) solution of 5% D (average molecular weight, 510,000; Sigma Chemical Co., St. Louis, Mo.) and 4% PEG (J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared by weighing out appropriate amounts of D, PEG, NaCl, and sterile water and combining these in a sterile test tube (31). All solids dissolved within 10 min with agitation. The system was heated in a water bath at 95°C for 20 min to prevent contamination.

S. marcescens Nima and ATCC 8100 were grown separately, then harvested by centrifugation at 12,000 rpm for 10 min, and washed in sterile 0.7% saline. Upon cooling to room temperature, 10 ml of the system, composed of equal volumes of D and PEG, were thoroughly mixed with 0.1 ml of the washed cells. The system was allowed to stand undisturbed for 25 min, allowing distinct phases of D and PEG to form, whereupon samples were taken of each phase for determination of viable cell concentration. Mean Ks and their ranges were calculated for both strains of *S. marcescens* used.

RESULTS AND DISCUSSION

Jet drop diameters as measured on magnesium oxide-coated slides showed little variation (118.3 to 122.0 μm), yielding a calculated range of drop volumes from 8.6×10^{-7} to 9.5×10^{-7} ml. Mean EFs for four trials for both the pigmented strain Nima and the nonpigmented strain ATCC 8100 are presented in Table 1. Pigmented cells had mean EFs of 2.1 to 14.0, while nonpigmented cells had mean EFs of 0.2 to 0.6. Many factors can affect EF, especially bubble rise distance and bulk concentration of bacteria (4, 6, 20); hence we were careful to keep these parameters tightly controlled.

These data indicate that the pigmented cells enrich jet drops more than the nonpigmented cells. The exceptionally small EFs of strain ATCC 8100 are, however, surprising. Blanchard and Syzdek (9) reported an EF of 0.3 in one trial for nonpigmented *S. marcescens* but felt their report should be treated with caution since it was based on only two white cells showing up in 84 drops. Our data are based on over 2,800 colonies in over 240 drops collected. Hejkal et al. (20) reported EFs of less than unity for *P. bathycetes* at drop diameters of 61, 99, and 125 μm and hypothesized that these low EFs were due to migration of the bacteria away from the surface of the liquid.

TABLE 1. EF for pigmented (Nima) and nonpigmented (ATCC 8100) *S. marcescens* strains

Strain	Mean concn (cells/ml) in:		EF[a]
	Bulk suspension	Drop	
Nima	1.03×10^6	3.03×10^6	2.94 ± 2.2
Nima	2.10×10^5	4.39×10^5	2.09 ± 1.51
Nima	4.26×10^6	4.96×10^7	11.64 ± 0.79
Nima	4.40×10^6	6.16×10^7	14.00 ± 2.11
ATCC 8100	2.55×10^6	1.52×10^6	0.60 ± 0.47
ATCC 8100	3.43×10^7	7.10×10^6	0.21 ± 0.07
ATCC 8100	5.20×10^7	2.95×10^7	0.57 ± 0.27
ATCC 8100	5.10×10^7	3.16×10^7	0.62 ± 0.27

[a] EF = (mean concentration in drop)/(mean concentration in bulk suspension). Values are mean ± standard deviation.

TABLE 2. Mean K for pigmented (Nima) and nonpigmented (ATCC 8100) *S. marcescens* strains in two-phase system of PEG and D

Strain	Mean concn (cells/ml) in:		K^a	K range
	PEG	D		
Nima	1.06×10^9	1.16×10^8	9.14	8.13–10.00
Nima	2.14×10^6	5.55×10^5	3.86	3.09–4.71
ATCC 8100	9.25×10^4	1.87×10^6	0.05	0.04–0.07
ATCC 8100	3.83×10^4	2.98×10^6	0.01	0.01–0.02

[a] K = (mean concentration in PEG)/(mean concentration in D).

Air bubbles rising through bacterial suspensions collect bacteria by interception (11, 33). It is essential that the bacteria adsorb to the bubble surface upon colliding for subsequent drop enrichment to occur. Therefore, the surface nature of the bacteria must permit adhesion to air-water surfaces. A variety of factors can control bacterial attachment to interfaces (1, 14, 17, 18, 22, 26). In particular, cell-surface hydrophobicity may enhance the ability to adsorb to air bubbles in water and then become concentrated in jet and film drops (14, 25, 26). Kjelleberg et al. (24) have reported correlation of pigmentation in certain strains of *S. marcescens* with increased cell surface hydrophobicity, and Hermansson et al. (21) have postulated that prodigiosin contributes to the hydrophobic interaction, perhaps by affecting the electrical charge on the bacterial surface. In discussing the differential attachment of different species and strains of bacteria to air bubbles, Blanchard and Syzdek (9) concurred that the more hydrophobic the surface of a bacterium, the more easily it will become attached. Curiously, they considered it unlikely that prodigiosin itself was responsible for the greater aerosolization of pigmented *S. marcescens*.

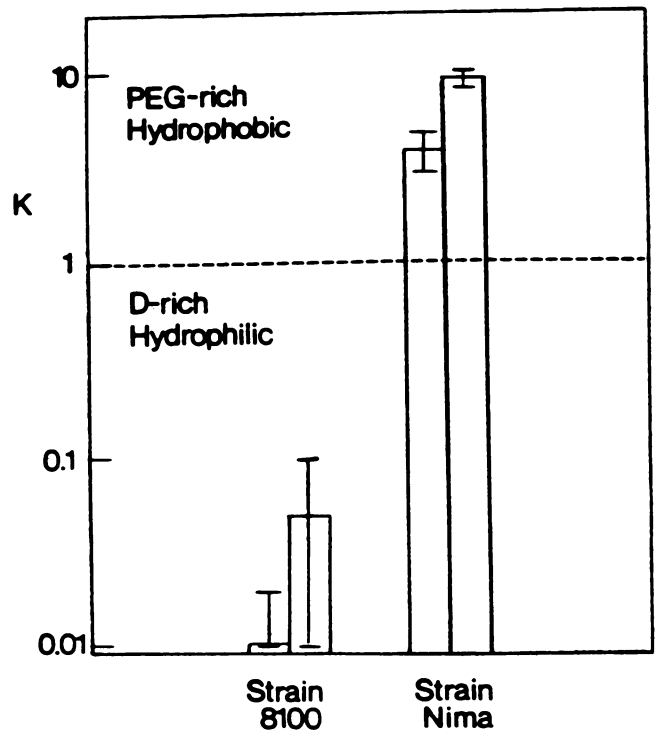

FIG. 2. Mean Ks for pigmented (Nima) and nonpigmented (ATCC 8100) *S. marcescens* strains in two-phase systems of 5% D (D-rich) and 4% PEG (PEG-rich). The dotted horizontal line specifies the interface between the systems. The bars represent two separate trials for each strain.

To compare hydrophobicity of pigmented and nonpigmented *S. marcescens* strains, we determined Ks for strains Nima and ATCC 8100 in a two-phase system of 5% D and 4% PEG. These results are presented in Table 2 and Fig. 2. Each error bar in Fig. 2 represents the range of Ks possible, based on serial dilution plate counts of 500 to 700 colonies per experiment. The pigmented strain Nima displayed mean Ks of 3.9 to 9.1, characteristic of hydrophobic cells, while the nonpigmented strain ATCC 8100 displayed mean Ks of 0.01 to 0.05, characteristic of hydrophilic cells. Using hydrophobic interaction chromatography and other strains of *S. marcescens*, Humphrey et al. (22) and Kjelleberg et al. (24) also determined that pigmented strains are hydrophobic, while the nonpigmented strains are hydrophilic. We suggest that the hydrophobic nature of the pigmented cells is due to the presence of prodigiosin. Prodigiosin is hydrophobic when pure (39) and located within the cell envelope of the bacterium (29).

Most of the research on bacterial aerosolization has been interpreted within the context of pure research on the mechanics of bacterial adsorption to bubbles (4–6, 17, 18, 22) or on the potential health hazards associated with microbial pollution of the atmosphere (20, 23). Indeed, since *S. marcescens* is an occasional human pathogen, the higher EFs reported for pigmented strains may have clinical significance in the generation of potentially pathogenic aerosols.

We believe this research also has important theoretical implications for the general class of compounds with which prodigiosin is classified, secondary metabolites. Secondary metabolism remains an evolutionary enigma. Functions for secondary metabolism have been debated extensively in the literature and numerous theories have been proposed as to the evolutionary advantage of secondary metabolism (3, 12, 13, 16, 27, 34, 38). This plenitude of theory and dearth of proof prompted Demain (16) to write: "analysis of the problem of function would probably proceed faster if we stopped attempting to formulate a grand and glorious unified proposal to explain the function of all secondary metabolites. It would be satisfying simply to elucidate the function of a single antibiotic."

Prodigiosin production by *S. marcescens* is a model for studying secondary metabolism. Therefore, we feel it appropriate that research on microbial aerosolization has serendipitously suggested a possible function for a secondary metabolite with such a long and distinguished history (19, 39). Bacteria possessing the ability to adsorb to air bubbles have a selective advantage in aerial dispersal over those that cannot. The exceptionally small EFs displayed by nonpigmented *S. marcescens* suggest that these bacteria are less likely than pigmented strains to be dispersed in aerosols. We postulate that prodigiosin increases the hydrophobicity of the cell surface and hence increases adhesion to bubbles that burst at the surface of bacterial suspensions, creating atmospheric aerosols which aid in dispersal of prodigiosin-producing strains.

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ADDENDUM IN PROOF

Syzdek (L. D. Syzdek, Appl. Environ. Microbiol. **49**:173–178, 1985) has demonstrated that cultures of *S. marcescens* that produce increased concentrations of prodigiosin show similarly increased enrichments of drops from bursting bubbles. Duplicate nonpigmented cultures do not show these increased enrichments.

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