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Isolation of Pigmented and Nonpigmented Mutants of *Serratia marcescens* with Reduced Cell Surface Hydrophobicity

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Enrichment for nonhydrophobic mutants of *Serratia marcescens* yielded two types: (i) a nonpigmented mutant which exhibited partial hydrophobic characteristics compared with the wild type, as determined by adherence to hexadecane and polystyrene; and (ii) a pigmented, nonhydrophobic mutant whose colonies were translucent with respect to those of the wild type. The data suggest that the pronounced cell surface hydrophobicity of the wild type is mediated by a combination of several surface factors.

Bacterial hydrophobicity is currently recognized as an important factor in mediating bacterial adherence to a wide variety of surfaces, including marine sediment (6), oil (10, 16), nonwettable plastics (14), phagocytes (20), epithelial cells (12), teeth (15), and one another (3).

The hydrophobic surface properties of *Serratia marcescens* and other *Serratia* species are of considerable interest, both from an ecological and medical point of view. Cell surface hydrophobicity plays a major role in determining the ability of *Serratia* cells to adhere at and colonize the air-water interface (1, 2, 7, 19), as well as in scavenging of organic materials at solid-liquid interfaces (8). Surface hydrophobicity may also be an important factor in adherence and subsequent colonization of hosts by clinical *Serratia* isolates (M. Rosenberg and Y. Blumberger, manuscript in preparation).

The spontaneous occurrence in pigmented *Serratia* strains of nonpigmented mutants which are deficient in hydrophobic surface characteristics (1, 7) has led investigators to assume that the pigment prodigiosin (11, 21) is responsible for the hydrophobic surface properties of these strains. This premise was further supported by physiological correlations between cell surface hydrophobicity and pigment synthesis (9, 13, 14). The purpose of the present study was to determine whether enrichment for nonhydrophobic mutants in a pigmented strain of *S. marcescens* yields exclusively nonpigmented mutants.

The hydophobic adherence properties of the *S. marcescens* strain employed in this study has been studied previously (13). Enrichment and selection of mutants deficient in their ability to adhere to hydrocarbons were carried out essentially as previously described (16). Wild-type cells were inoculated 1:200 and grown for 18 h at 30°C with shaking in brain heart infusion broth (Difco Laboratories), conditions which maximize their adherence properties. The same growth conditions were used in subsequent adherence experiments and hexosamine determinations. Cells from 50 ml of broth were washed twice and suspended in 5 ml of PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, and distilled water to 1,000 ml [pH 7.1]) to which 1 ml of hexadecane was then added. After 5 min of agitation, the bottom aqueous phase was removed to a second test tube and again mixed with hexadecane. This process was continued until cells in the aqueous phase were too few to be observed microscopically. The bottom phase was then inoculated into 50 ml of fresh broth and grown as previously described. Cells were then washed and again partitioned with hexadecane. After three such growth and partition cycles, considerable turbidity remained in the aqueous phase, even after repeated mixing in the presence of hexadecane. Appropriate dilutions of the lower aqueous phase were spread onto brain heart infusion agar (Difco). After 24 h of growth at 30°C under aerobic conditions, three colony types were observed: (i) pigmented, opaque colonies resembling those of wild-type cells; (ii) white, nonpigmented colonies; and (iii) translucent, pigmented colonies.

For adherence to polystyrene, cells were grown as described above, harvested, washed twice, and suspended in PUM buffer to an optical density of 2.0 at 400 nm. Samples (50 μl) of each cell suspension were added to 24 wells in a flat-bottomed microtiter plate (nontreated polystyrene; NUNC, Roskilde, Denmark). After 20 min of incubation at room temperature, wells were washed copiously with tap water (14), stained with Gentian violet, and allowed to dry.

To determine the hexosamine content of hydrolyzed cells, cells were grown as described above, harvested, washed, and suspended in cold distilled water. Hexosamines were determined by the indole-nitrite method (5) after hydrolysis in 5 N HCl at 100°C for 20 min and neutralization with NaOH (17), with galactosamine as the standard.

Several nonpigmented colonies were transferred and found to be similar to one another in adherence properties. One strain, designated 3162, was further investigated. Translucent colonies were also similar to one another, and one strain (3164) was chosen for further study. Colonies with wild-type morphology resembled wild-type cells in adherence characteristics.

Cell surface hydrophobicity of wild-type and mutant strains was determined by adherence to hexadecane (13). Results are summarized in Fig. 1. Whereas wild-type cells adhered with high affinity to hexadecane, pigmented, translucent mutant 3164 was nonadherent. Nonpigmented 3162 cells adhered with intermediate affinity to the test hydrocarbon. Adherence of washed cell suspensions to the hydrophobic solid surface polystyrene revealed results similar to those obtained with hexadecane. Results (expressed as relative absorbance at 570 nm ± standard deviation, measured in a Dynatech MR600 microplate reader) were as follows: wild type, 1.00 ± 0.26; 3162, 0.60 ± 0.05, 3164, 0.09 ± 0.03.

The above data strongly suggest that cell surface hydro-

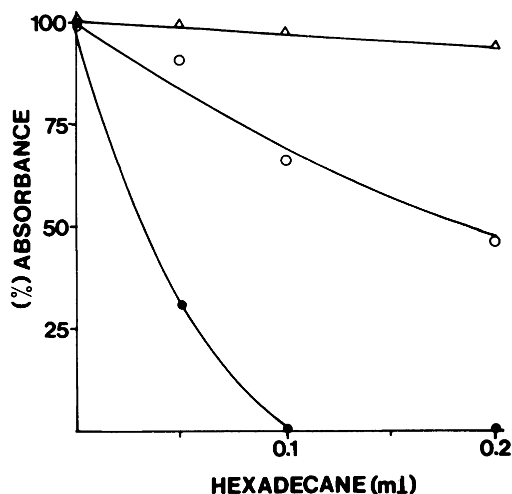

FIG. 1. Adherence to hexadecane. Cells were grown for 18 h in brain heart infusion broth (Difco) with shaking at 30°C, harvested by centrifugation, washed twice, and suspended in PUM buffer to an initial optical absorbance at 400 nm of 1.27 to 1.35 as measured in a Uvikon 710 Kontron spectrophotometer. Adherence of wild-type (●), 3162 (○), and 3164 (△) cells to hexadecane was carried out by vortexing 1.2 ml of bacterial suspension with various volumes of hexadecane under controlled conditions (13) for 120 s. After phase separation, the absorbance of the lower aqueous phase was measured at 400 nm. Results are shown as the percentage of cell-mediated turbidity remaining in the lower aqueous phase after the mixing procedure (13).

phobicity in the wild-type cells, as determined by adherence to hydrocarbon (14) and to polystyrene (13), is not solely a function of the presence or absence of pigmentation because (i) nonpigmented mutant 3162 still maintained partial adherence properties, despite the loss of pigment; and (ii) pigmented mutant 3164 was nonhydrophobic despite the presence of pigment. It should be emphasized that acidic and alkaline ethanolic extracts of both wild-type and 3164 cells yielded coloration indicative of prodigiosin (4); no color was observed in similar extracts of strain 3162.

Of particular interest is the apparent relationship between loss of colony opacity and concomitant loss of hydrophobicity in translucent mutant 3164. In another hydrophobic gram-negative bacterium, *Acinetobacter calcoaceticus* RAG-1, colony opacity is due to the presence of a hexosamine-containing polysaccharide on the cell surface (18). A hexosamine-containing capsule has been reported to be intimately related with surface prodigiosin in *S. marcescens* (11). However, the results of this study show that the wild-type and both mutant strains were similar in hexosamine content, indicating that a hexosamine-containing capsule is not involved. The strains contained the following amounts of hexosamine (in micrograms per milligram [dry weight] of cells): wild type, 22.9; 3162, 22.9; 3164, 24.0. Moreover, microscopic observation of India ink-stained preparations of the wild-type and mutant strains, grown under the conditions stated, did not reveal the presence of a capsule. It is tempting to speculate that the opacity-conferring factor(s) which is absent in translucent mutant 3164 is the same component(s) which contributed to the intermediate cell surface hydrophobicity of nonpigmented mutant 3162. Since the sole presence of pigment does not result in the expression of surface hydrophobicity in the translucent mutant, pigment may have to be intimately associated with such factor(s) to contribute to the pronounced hydrophobicity observed in wild-type cells.

The results presented here appear to be in agreement with a recent study of cell surface hydrophobicity in clinical *Serratia* isolates (Rosenberg and Blumberger, in preparation); intermediate cell surface hydrophobicity was observed in several strains which were not pigmented, whereas pigmented strains exhibited pronounced cell surface hydrophobicity.

In summary, the data indicate that the pronounced cell surface hydrophobicity of wild-type cells may be due to the simultaneous presence of several components on the outmost bacterial cell surface. The results presented here, taken together with other studies of hydrophobicity in *Serratia* strains (1, 2, 7–9, 13, 14, 19), suggest that these components include prodigiosin, as well as the factor(s) responsible for the opacity of wild-type colonies.

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