Putative Role of a 70 kDa Outer-surface Protein in Promoting Cell-surface Hydrophobicity of *Serratia marcescens* RZ

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Serratia marcescens RZ has been previously shown to possess pronounced cell-surface hydrophobicity, as evidenced by its affinity for hydrocarbons and polystyrene. The present report suggests the involvement of a 70 kDa protein, serraphobin, in this phenomenon. The 70 kDa protein was recovered from both the cell surface and culture supernatant of hydrophobic wild-type cells, but was either totally absent or present in minor quantities in hydrophobicity-deficient mutants. Similarly, loss of hydrophobicity of RZ cells following growth at 39 °C was accompanied by loss of the protein. Serraphobin was capable of binding to hexadecane droplets following a brief mixing procedure, and could be desorbed by solidifying and melting the hexadecane phase.

INTRODUCTION

Serratia marcescens is a micro-organism of both ecological and medical importance (Yu, 1979; Sleight, 1983). *Serratia* strains adhere avidly to a variety of hydrophobic surfaces such as air/water and oil/water interfaces (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1982; Kefford *et al.*, 1982; Syzdek, 1982) and to various non-wettable plastics (Rosenberg, 1981). Hydrophobicity of *Serratia* has been suggested as a survival mechanism in the open environment (Kefford *et al.*, 1982). Similarly, in a recent study of the adhesion properties of 14 clinical *S. marcescens* isolates, all were capable of adhering to hydrocarbons and polystyrene, suggesting a possible role for cell-surface hydrophobicity in initial host colonization (Rosenberg *et al.*, 1986a).

Previous investigations have reported correlations between hydrophobicity in *Serratia* and production of the pigment prodigiosin (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1979, 1982; Kefford *et al.*, 1982; Syzdek, 1982). However, non-pigmented clinical strains (Rosenberg *et al.*, 1986a) and mutants (Rosenberg, 1984) may also exhibit intermediate or pronounced hydrophobic surface properties, suggesting the participation of additional factor(s).

In the present study, correlations between hydrophobicity of *S. marcescens* RZ and the presence and level of a major 70 kDa surface protein (which we have named serraphobin), are reported.

METHODS

Bacterial strains and growth conditions. *S. marcescens* strain RZ was originally obtained from R. Zack, Tel Aviv University. Mutant 3162, exhibiting intermediate cell-surface hydrophobicity, and mutant 3164, almost completely devoid of cell-surface hydrophobicity were derived from strain RZ as previously reported (Rosenberg, 1984). The bacteria were maintained on brain heart infusion (BHI) agar (Difco) at 4 °C and transferred every month. Starter cultures were prepared by inoculating bacteria into tubes containing 2 ml PG broth [5 g Bactopeptone, (Difco); 10 ml glycerol, distilled water to one litre] or BHI broth. The tubes were incubated for 24 h with shaking at 30 or 39 °C. Bacteria were then inoculated into flasks containing 20 ml of PG or BHI broth to an

initial cell concentration of 5×10^5 c.f.u. ml⁻¹. Following 72 h incubation at 30 or 39 °C in a G76 gyratory shaker (New Brunswick Scientific) at 300 r.p.m., samples were taken for c.f.u. determination. Bacteria were harvested by centrifugation at 10000 g, and wet weights were determined.

Spent culture medium. The culture supernatants were sterilized by filtration and stored at -20 °C for hexadecane extraction and SDS-PAGE.

Cell pellet extractions. The cell pellets were extracted by 2% (w/v) SDS/5% (v/v) β -mercaptoethanol as described by McBride *et al.* (1985). Pellets were washed twice in 0.15 M-NaCl by centrifugation for 20 min at 10000 g. Pellets were weighed and subsequently suspended 1:1 (w/v) in 2% SDS/5% β -mercaptoethanol, vigorously agitated on a vortex mixer and boiled for 30 min. The mixtures were then centrifuged for 10 min at 6000 g. The supernatants were stored at -20 °C for SDS-PAGE.

SDS-PAGE. This was performed according to the method of Laemmli (1970). Samples of spent culture medium were diluted in water, correcting for differences in wet weights of cell pellets following growth. Samples of outer-surface protein extractions were diluted either 1:4 or 1:8 in distilled water. Sample buffer (1 vol.) containing 40 mmol l⁻¹ dithiothreitol (DTT) was added to 3 vols of sample, and the mixture was boiled for 2 min. Samples (40 μ l) and standards (12 μ l; molecular mass markers, 14.2–66 kDa, Sigma) were layered on to 7.5% or 10% polyacrylamide gels, as indicated. Following electrophoresis at 200 V, protein bands were visualized by staining with Coomassie blue. Since the presence of pigment precluded protein measurement by standard spectrophotometric techniques, the total amounts of protein in each sample were estimated by densitometric comparisons of each lane with that of the molecular mass marker proteins, using a CAMAG TLC Scanner II (Camag, Switzerland); the percentage of serraphobin with respect to total protein was similarly estimated densitometrically.

Hexadecane extraction. Hexadecane extraction of spent culture fluids was carried out essentially as previously described (Rosenberg *et al.*, 1986b). Hexadecane (0.5 ml) was added to 0.5 ml culture supernatant supplemented with 0.5 ml phosphate buffer (44.4 g K₂HPO₄, 14.52 g KH₂PO₄, distilled water to one litre, pH 7.1). The aqueous hydrocarbon mixtures were then vortexed for 2 min on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuque, Iowa, USA) at room temperature. Following phase separation, the lower aqueous phase was removed carefully using a Pasteur pipette to another test tube containing an additional 0.5 ml hexadecane, and the procedure was repeated. Following four such extraction cycles the final lower aqueous phase was stored at -20 °C for SDS-PAGE. Half-strength phosphate buffer (0.25 ml) was added to the hexadecane portions. The mixtures were cooled to 0 °C until solidification of the hexadecane was complete. The test tubes were then incubated at 30 °C until the hexadecane melted. The test tubes were cooled and heated in this manner three consecutive times; the final mixture was stored at -20 °C for at least 18 h. Prior to SDS-PAGE, the test tubes were allowed to reach room temperature, and samples for SDS-PAGE were removed from the lower aqueous phase.

Cell-surface hydrophobicity. Bacterial hydrophobicity was determined by adhesion to hexadecane (Rosenberg, 1981, 1984). To 1.4 ml bacterial suspension, washed and suspended in half-strength phosphate buffer, 0.2 ml hexadecane was added and the mixtures vortexed in semi-micro polystyrene disposable cuvettes for 60 s. Following phase separation, the absorbance of the lower aqueous phase at 400 nm was determined. Adhesion is expressed as the percentage decrease in turbidity of the lower aqueous phase following mixing, compared to that of the initial cell suspension (Rosenberg, 1984).

RESULTS

Hydrophobic *Serratia marcescens* RZ cells, grown at 30 °C on PG or BHI medium, produced an extracellular protein (serraphobin) with a molecular mass of approximately 70 kDa, which was one of the major proteins (25% of total supernatant protein) recovered from the culture supernatant (Fig. 1, lane 1). Serraphobin appeared to be totally absent in spent culture media of non-hydrophobic mutant 3164 (Fig. 1, lane 2) and non-pigmented mutant 3162 (Fig. 1, lane 3). A second supernatant protein of about 50 kDa was present in wild-type RZ cells and mutant 3164 cells (Fig. 1, lanes 1, 2), but was absent from spent culture media of mutant 3162 (Fig. 1, lane 3). The absence of this protein may be related to loss of pigmentation in mutant 3162.

In order to determine whether serraphobin is located on the outer surface of RZ cells, treatment with 2% SDS/5% β -mercaptoethanol, as described by McBride *et al.* (1985), was employed (Fig. 1, lanes 4–6). Serraphobin was extracted in relatively large amounts (5% of total protein, lane 4) from wild-type RZ cells, as opposed to no appreciable amounts in non-hydrophobic 3164 cells (lane 5), and minimal quantities (0.4% of total protein) in partially hydrophobic 3162 cells (lane 6). Serraphobin was also extracted from wild-type RZ cells following 1% SDS treatment (data not shown), as described by Jenkinson (1986). Thus, this protein appears to be a cell-surface component of the outer surface of RZ.

Fig. 1. SDS-PAGE of culture supernatants and outer-surface protein extracts following growth at 30 °C in BHI broth. Culture supernatant samples were diluted with water to correct the differences in wet weights of pellets following growth: RZ, 46.9 mg ml⁻¹; mutant 3164, 29.1 mg ml⁻¹; mutant 3162, 40.6 mg ml⁻¹. About 2 µg of protein of each sample was loaded onto a 7.5% polyacrylamide gel. Outer-surface proteins were extracted from washed cell suspensions, as described in Methods. Samples were diluted 2.5-fold in water. Approximately 250 µg of protein of each sample was loaded onto the gel. Protein bands were visualized by Coomassie blue staining. Adhesion to hexadecane was 76% for RZ cells, 0% for 3164 cells, and 28% for strain 3162. Lanes: culture supernatants of RZ (lane 1), 3164 (lane 2) and 3162 (lane 3); outer surface protein extracts of RZ (lane 4), 3164 (lane 5) and 3162 (lane 6). Serraphobin is indicated by the arrow. Molecular mass standards: bovine serum albumin (66 kDa); ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20 kDa).

Serraphobin, released from the cell surface, exhibited affinity for hexadecane droplets (Fig. 2). Mixing with hexadecane removed 92% of the protein from the aqueous phase (lane 2). Its partitioning to the oil/water interface could be demonstrated by subsequent desorption (lane 3) following solidification of the hexadecane droplets at below 18 °C, a technique recently proposed for separation of amphipathic proteins (Rosenberg *et al.*, 1986*b*). Another major culture supernatant protein of about 42 kDa was also eluted by hexadecane. This 42 kDa protein was also present in the non-hydrophobic mutant 3164, and remained following growth at 39 °C.

Previous studies have shown that cell-surface hydrophobicity in strain RZ and other *S. marcescens* isolates is lost when the cells are grown at temperatures above 38 °C (Rosenberg *et al.*, 1986*a*). Fig. 3 compares the proteins extracted from the cell surfaces of RZ, 3162 and 3164 grown at 39 °C. Serraphobin was found neither in cell-surface extracts (Fig. 3, lanes 2–4), nor in the spent culture medium (not shown) of any of the strains.

DISCUSSION

The present study suggests the involvement of a 70 kDa surface protein, serraphobin, in mediating cell-surface hydrophobicity in *S. marcescens* RZ. Serraphobin was recovered from

Fig. 2

Fig. 3

Fig. 2. SDS-PAGE of culture supernatant of *S. marcescens* RZ following growth at 30 °C on PG medium, before and after hexadecane extraction and desorption. Samples were applied onto a 10% gel. Protein bands were visualized by Coomassie blue staining. Adhesion of washed cells to hexadecane was 71%. Lanes: 1, RZ culture supernatant prior to hexadecane extraction (about 10 µg protein was loaded onto the gel); 2, RZ culture supernatant following 8 min mixing with hexadecane; 3, desorption following solidification and melting of the upper phase following mixing with hexadecane. Twenty-five percent of the adsorbed serraphobin was recovered by the desorption procedure, as determined by densitometry. Serraphobin is indicated by the arrow. Molecular mass standards, see Fig. 1.

Fig. 3. SDS-PAGE of cell-surface extracts following growth at 39 °C. Samples of cell-surface extracts following growth at 39 °C (approx. 20 µg protein per sample) were applied to a 7.5% gel: lane 2, RZ; lane 3, 3164; lane 4, 3162. As control, culture supernatant of RZ cells grown at 30 °C (2 µg protein) was applied to lane 1. Protein bands were visualized by Coomassie blue staining. Adhesion to hexadecane was less than 10% for all strains. Serraphobin is indicated by the arrow. Molecular mass standards, see Fig. 1.

both the cell surface and culture supernatant of hydrophobic wild-type cells, but was absent in non-hydrophobic mutant 3164, and present in minor quantities on the surface of partially hydrophobic 3162 mutant cells. Similarly, loss of hydrophobicity of RZ cells following growth at 39 °C was accompanied by loss of serraphobin. Serraphobin was capable of binding to the hexadecane/water interface following a brief mixing procedure, and could be desorbed by solidifying the hexadecane phase. Preliminary data (not shown) show a correlation between serraphobin production and cell-surface hydrophobicity in several clinical *S. marcescens* isolates.

Recent studies have shown that cell-surface hydrophobicity in *Serratia* strains is more complicated than previously anticipated, involving an interplay of various 'hydrophobins' (hydrophobicity-promoting factors) and 'hydrophilins' (hydrophobicity-reducing components). For example, whereas earlier investigations of hydrophobicity in *S. marcescens* suggest that prodigiosin is chiefly responsible for this phenomenon, more recent studies have demonstrated

that non-pigmented isolates and mutants can also exhibit hydrophobic properties (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1979, 1982; Rosenberg, 1981, 1984), suggesting that additional hydrophobins are involved. Based on the present data, serraphobin appears to be a hydrophobin in RZ cells. Conversely, the potential role of cell-surface components which reduce hydrophobicity must also be considered. For example, serratamolide, an amphipathic cell-surface component of some *S. marcescens* strains (including RZ) acts as a hydrophilin (Bar-Ness *et al.*, 1988).

Investigations are presently under way to further characterize serraphobin, its distribution in clinical *Serratia* strains, and its role in promoting adhesion of *S. marcescens* to hydrophobic surfaces.

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