Flagellar Variation in *Serratia marcescens* Is Associated with Color Variation

DURGA K. PARUCHURI AND RASIKA M. HARSHEY*

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

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Color variation in *Serratia marcescens* was noted as early as 1888 (26). Its inheritance pattern was studied by Bunting (1). In strain 274, she noted the occurrence of variants that were deep red, light red, bright pink, pale pink, and white. The variations were distinct, sequential, and reversible and ranged generally from $10^{-3}$ to $10^{-6}$ per bacterium per generation. She suggested that a phenomenon other than spontaneous gene mutation could be responsible for the high frequency of color variation. A strain showing very high color instability is HY, in which every colony can be seen to sector (Fig. 1). This high degree of genetic instability is reminiscent of DNA rearrangements such as transpositions, deletions, or inversions which occur during various biological processes (see references 4, 8, 9, 17, and 21).

The red color of *S. marcescens* is due to the pigment prodigiosin (25). Prodigiosin, a linear tripyrrole, is synthesized in a bifurcated pathway, in which mono- and bipyrrole precursors are synthesized separately and then coupled to form pigment (15). Genes encoding parts of the biosynthetic pathway have recently been cloned in *Escherichia coli* (3). The terminal step in prodigiosin biosynthesis (condensing of mono- and bipyrrole moieties) is temperature sensitive (24).

The function of this water-insoluble pigment in *S. marcescens* is not clear. It was shown to be associated with the cell envelope (19) and to be excreted as a water-soluble form composed of prodigiosin, carbohydrate, and protein (27). More recently, prodigiosin has been shown to be a component of a receptor complex for the *Serratia* bacteriophage kappa (16).

We reasoned that color variation could be caused by the variation of a cell surface component associated with the pigment and decided to follow the fate of a pigment-associated component in the different color variants. Since phage kappa was shown earlier to use one such component as a receptor (16), phage binding was used as one of the assays for isolating a pigment-bound cell component.

**MATERIALS AND METHODS**

**Wild-type strains and culture conditions.** Strain 274 was obtained from the American Type Culture Collection, New Haven, Conn. Strain HY was from R. Redfield, Stanford University, Stanford, Calif. Strain Nima was from S. Malik, Maharaja Sayajirao University, Baroda, India. Strains were grown on peptone-glycerol agar plates (2).

**Color variants from *S. marcescens*, 274.** The light-red strain arose spontaneously. The red revertant arose at a frequency of $10^{-6}$. Pink and white strains were isolated from the light-red strain at a frequency of $10^{-3}$ when the latter was grown on medium containing 30 μg of kanamycin per ml.

**Phage binding.** Phage kappa was from S. Malik, Maharaja Sayajirao University, Baroda, India. Phage growth was monitored both by spot tests and by scoring PFUs on bacterial lawns. Phage binding was assayed as follows. Cells were suspended in buffer (0.2 M NaCl, 0.02 M Tris hydrochloride, 0.001 M CaCl₂, 0.025 M MgCl₂), and phage were added at a multiplicity of infection of 3. After a 30-min incubation at 4°C, cells were pelleted by centrifugation. Bound phage were determined by scoring the supernatant for unadsorbed PFUs. Cell-free fractions were tested for phage receptor activity by mixing them with phage lysate, incubating for 1 h at 4°C, and plating the mixture on indicator wild-type 274 bacteria (11).

**Purification of pigment-protein complex.** Cells growing on solid media show profuse pigmentation, as compared with those growing in liquid medium. Cells were therefore routinely grown on peptone-glycerol agar plates, collected by scraping the surface into buffer A (50 mM Tris hydrochloride [pH 7.5], 100 mM NaCl), vortexed, and pelleted by centrifugation. The supernatant is referred to as the culture supernatant. A 30 to 60% ammonium sulfate fraction of this supernatant had most of the bound pigment. The ammonium sulfate fraction was dialyzed against buffer A and eluted from a DEAE-Sephael column with a linear salt gradient (0.1 to 0.5 M NaCl). Pigment-protein complex, assayed by determining the A₅₇₅, eluted at 0.15 to 0.2 M NaCl. The complex was concentrated by dialysis against polyvinylpyrrolidone and size fractionated on a Sepharose CL-6B column.

**Antisera to pigment-protein complex.** Rabbits were immunized with the 39-kilodalton (kDa) protein purified from strain 274. The first two injections were against protein that was eluted from a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel. The subsequent two injections, includ-
ing the booster, were against the purified pigment-protein complex eluted from Sepharose CL-6B.

**Western blotting.** Culture supernatants were prepared as described above. Protein (20 μg) was electrophoresed on an 8% SDS-polyacrylamide gel and blotted onto nitrocellulose (23). Blots were blocked for 1 h with buffer B (50 mM Tris hydrochloride [pH 8], 100 mM NaCl) containing 5% nonfat dry milk. A 3-h incubation with antisera (1:200 dilution) in the same buffer was followed by two 10-min washes in buffer B containing 1% Triton X-100 and three washes in buffer B alone. Antibody binding was detected by incubation of the blot with 125I-labeled protein A (106 cpm/ml) in buffer B, which was subsequently washed and exposed to film.

**Immunoelectron microscopy.** Cells were deposited onto 300-mesh Parlodion- and carbon-coated nickel grids, incubated with purified immunoglobulin G (20 μg/ml) for 1 h at room temperature, and washed three times in buffer A with 1% bovine serum albumin. Grids were then incubated with goat anti-rabbit immunoglobulin G conjugated to 4-nm colloidal gold particles (prepared by reducing hydrochloroauric acid with sodium borohydride; gift from Cheng-Ming Chang). After 1 h, grids were washed three times with buffer A and negatively stained with a 2% solution of neutralized uranyl acetate.

**TABLE 1. Relative efficiency of plating (EOP) of phage kappa on S. marcescens 274**

| Phage incubated with: | EOP |
|-----------------------|-----|
| **S. marcescens 274** |     |
| Dark red (wild type)  | <10⁻⁵ |
| Light red             | 1   |
| Dark red (revertant from light red) | <10⁻⁵ |
| Pink                  | 1   |
| White                 | 1   |

| **E. coli K-12** | 1 |

* Relative efficiency of plating is the ratio: phage titer after incubation/phage titer before incubation on tester strain. Incubations to test phage binding were performed as described in Materials and Methods.

**RESULTS**

**Change in susceptibility to phage kappa with change in color.** Phage kappa could grow only on the wild-type dark-red strain 274. At temperatures above 37°C, when pigment synthesis is blocked (25), the strain became phage resistant. A spontaneously occurring light-red derivative was also unable to support phage growth. Reversion from light red to dark red resulted in the restoration of kappa-plating ability. Pink and white strains isolated from the light-red strain remained resistant to phage infection. The inability of phage kappa to grow on the light strain was due to an inability of the phage to bind these cells (Table 1).

**Kappa-binding activity of a pigment-protein complex found in culture medium.** Phage kappa binding was used as an assay for purification of the pigment-associated component from the dark-red strain 274. In initial experiments, this activity was purified from the outer membrane. The purification enriched a 39-kDa protein as determined by SDS-polyacrylamide gel electrophoresis. We subsequently ob-

**TABLE 2. Purification of pigment-bound protein**

| Purification fractions | Amt of protein (mg)* | Amt of pigment (μg/mg of protein)* | Relative EOP of phage kappa* |
|------------------------|----------------------|------------------------------------|-------------------------------|
| 274 culture supernatant | 15.22                | 646                                | <10⁻⁵                         |
| 30 to 60% pellet       | 11.25                | 150                                | <10⁻⁵                         |
| DEAE-Sephadex (pooled 575-nm-absorbing peak fractions) | 4.56                | 130                                | <10⁻⁵                         |
| Sepharose CL-6B (pooled 575-nm-absorbing peak fractions) | 3.1                 | 121                                | <10⁻⁵                         |

* Starting material was obtained after scraping cells from 10 plates.

b Amount of pigment is calculated as follows: a difference in absorbance of 1.0 optical density unit between 534 and 655 nm is equivalent to 19.3 μg of prodigiosin (7).

* Relative efficiency of plating (EOP) (see Table 1, footnote a) of phage kappa after incubation with cell-free fractions. Relative efficiency of plating after incubation of culture supernatants from light-red, pink, and white derivatives of strain 274 was 1. Phage-binding activity followed the 575-nm-absorption profile.
observed that this pigment-bound protein is a major component of the culture supernatant. The protein was therefore purified from such a supernatant (Table 2; Fig. 2). Pigment was associated with the protein through all stages of purification. On gel filtration columns, the complex aggregated and eluted over a broad size range with a peak observed at 2 × 10^6 daltons. The pigment is noncovalently associated with the protein, since it could be released from it with SDS.

Pigment was extracted from the different color variants of strain 274, and absorption spectra were determined from 250 to 650 nm (25). All variants gave an identical absorption spectrum (data not shown). However, the amounts of pigment were different in each variant, with the dark-red strain having the most, followed by light red and pink, with no detectable pigment in the white strain. Analysis of pigment in the pigment-protein complex of culture supernatants gave a similar result. Therefore, it appears that in these color variants, the different hues of red are due to different levels of pigment.

Color variants make a variant protein. When used as a probe in Western blots, antiserum to the purified 39-kDa protein reacted with a protein that shows variation in different color variants of strain 274, as well as other strains of S. marcescens (Fig. 3). The light-red kappa-resistant derivative of 274 contained a smaller, 36.5-kDa protein (lane 2). The pink and white strains derived from the light-red one had 36- and 36.5-kDa proteins, respectively (lanes 3 and 4). This latter white strain is leaky and turns gradually red. Concomitant with this change in color, we observed the presence of a second 39-kDa protein species in this strain (lane 4). The kappa-sensitive dark-red revertant isolated from the light-red strain was also seen to have regained a 39-kDa protein (lane 5). Treatment of the proteins from the dark- and light-red strains of 274 (lanes 1 and 2) with endo-β-N-acetylglucosaminidase H (endo H) or endo F, enzymes that cleave sugar residues in glycoproteins (6, 12), did not produce any shift in their mobility. In a second wild-type red strain, Nima, the antiserum cross-reacted with a 38-kDa protein (lane 8). A third wild-type strain, HY, switched between white and red cell types at a high frequency (Fig. 1).

Although we have no stable derivatives of this strain, we analyzed proteins from a predominantly red or white culture. Both showed the presence of a 37-kDa protein (lanes 6 and 7).

The 39-kDa protein is a component of bacterial flagella. S. marcescens 274 cells were reacted with anti-39-kDa protein antibody followed by a second antibody coupled to colloidal gold and examined by electron microscopy. Electron-dense gold particles were specifically bound to the surface of flagella (Fig. 4A). These structures dissociated in acid pH and reassembled into longer filaments in alkaline pH, a property observed earlier for bacterial flagella (10, 22). The colloidal gold-tagged antibody labeled these structures at both pHs. Their diameter (13 nm) is consistent with their being flagella rather than pil. The purified 39-kDa protein showed a similar pH-dependent polymerization (Fig. 4B). Purified flagella from strain 274 showed a single 39-kDa protein (Fig. 2, lane 5) which also cross-reacted with the antiserum (data not shown). We therefore conclude that this protein is the flagellin.

Flagellin synthesis has been observed to be temperature sensitive in other enterobacteria (10, 22). We found that the 39-kDa protein is not synthesized above 37°C. A test for motility (assayed by stabbing a 0.35% agar slant with bacteria and observing whether bacterial growth was diffuse or occurred only along the length of the stab) showed that the bacteria are nonmotile above 37°C. No pigment synthesis was observed at the higher temperatures.

**DISCUSSION**

We demonstrated that flagellar variation in S. marcescens is closely associated with color variation. Although variation of flagellar antigens has been previously observed in S. marcescens (20, 28), no correlation with color variation has been reported. We found that not only are changes in the flagellar protein coupled to changes in pigment levels but that even in a normally flagellated and pigmented strain, flagellin and pigment synthesis are coregulated, both being shut off at temperatures above 37°C.

We do not know the biochemical basis for flagellar variation. The mobility shifts we observed could represent additions, deletions, or substitutions of amino acid residues. Also, mobility shifts on SDS-polyacrylamide gels could be caused by chemical modification of amino acids. A preliminary test for differential glycosylation in the variant proteins was negative.

![FIG. 2. SDS-polyacrylamide gel electrophoresis of pigment-bound 39-kDa (kd) protein purification fractions from S. marcescens 274. An 8% SDS-polyacrylamide gel was run (13) and stained with Coomassie brilliant blue. Lanes: 0, molecular weight standards; 1, culture supernatant; 2, ammonium sulfate fraction; 3, pooled fraction after DEAE-Sepahel chromatography; 4, pooled fraction after Sepharose CL-6B chromatography; 5, purified flagella from 274, prepared according to the method in reference 5.](image-url)

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![FIG. 3. Western blot of proteins from culture supernatants of color variants. Protein (20 μg) was electrophoresed on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-39-kDa protein serum. Lanes: 1, dark-red strain 274; 2, light-red 274; 3, pink 274; 4, white 274; 5, dark-red revertant 274; 6, red HY; 7, white HY; 8, red Nima.](image-url)
Is the rapid color change in strain HY (Fig. 1) due to switching of flagellar antigens? Although red and white cells of this strain have a flagellar protein of the same size, it is possible that the difference between the proteins from the two cell types is a substitution that does not result in a visible alteration in protein size. We are currently isolating stable red or white derivatives of this strain to facilitate a study of the color switch.

There are reports of defects in prodigiosin formation by penicillin-induced L-forms of *S. marcescens* (18), which lack an intact cell wall. In the course of this study, we observed the occurrence of white mutants of *S. marcescens* that are extremely slow growing. Some of these revert to fast-growing reds. These mutants may have lost a portion of the membrane that houses the enzymes for pigment biosynthesis. Alternatively, they may be indirectly affected in flagellar assembly. We suggest that mutations that perturb the membrane will cause variation in pigment levels.

Some bacteriophages attack only motile strains of either gram-positive or gram-negative species (14). They use the flagellum as a primary attachment site, move down the flagellar filament, and inject their DNA near the base of the structure. Phage kappa may use a similar mechanism for infection. Resistance to kappa should prove useful in isolating flagellar mutants in *S. marcescens*.

Many systems show a variation of surface antigens (4, 8, 9, 17, 21), the best characterized procaryotic system being the phase variation of *Salmonella* flagellar antigens (see reference 21). A specific rearrangement of genetic information is responsible for switching between two antigenic types. In *S. marcescens*, there appear to be many different flagellar types between which the bacteria can switch. *S. marcescens* is an opportunistic pathogen and causes serious, even fatal, nosocomial infections. Variation of surface antigens is a phenomenon associated with pathogenesis and escape of a pathogen from the immune surveillance system of its host. Association of flagellar variation with color variation provides us with a powerful tool for the investigation of this phenomenon. We are currently isolating the gene(s) for this flagellar protein so that this fascinating phenomenon can be studied at the genetic level.

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