Detection of Pigment Precursors in White Clinical Strains of *Serratia marcescens*

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Filter paper strips containing a pigment precursor extracted from *Serratia marcescens* strain 933 were used to determine whether white, clinical *S. marcescens* strains could form pigment syntrophically. In all, 114 strains (113 of clinical origin) were tested, and 99% were found to develop colors ranging from violets to pinks.

The presence of red pigment was an accepted characteristic of *Serratia marcescens* in the past. Today, it is estimated that more than 70% of both clinical and nonclinical isolates are nonpigmented (1, 2). Prodigiosin, 2-methyl-3-amyl-6-methoxy-5-(2-pyrrolyl)-2,2'-dipyrrolylmethene (6), the red pigment of *Serratia*, is formed by the conjugation of two colorless precursors, a volatile monopyrrole, MAP (2-methyl-3-amylpyrrole), and a nonvolatile, diffusible bipyrrole, MBC (4-methoxy-2,2'-bipyrrole-5-carboxyaldehyde) (5). A phenomenon known as syntrophic pigmentation exists (3, 9) which is most visible in the case of a white mutant which is unable to produce one of the precursors and can accept the missing one from another white mutant, forming the red pigment. In fact, both white strains can become red when mutual donation and acceptance occurs. The purpose of this study was to use syntrophic pigment assays to determine whether nonpigmented strains from clinical specimens are able to produce either MAP or MBC.

A total of 146 *Serratia* strains were received from various sources, including two major 600-plus-bed hospitals and the Kansas State Health Laboratory. An American Type Culture Collection (ATCC) strain (Rockville, Md.), ATCC 8100, which is white, was also obtained. Strains 933 and WF, two characterized syntrophic mutants, were donated by R. P. Williams of Baylor College of Medicine, Houston, Tex. The volatile MAP is synthesized in WF (3), and the diffusible MBC is found in 933 under certain growth conditions (5). The blocks responsible for the lack of MBC biosynthesis in WF and the lack of MAP in 933 are located at sites of unidentified molecules in each precursor pathway (10).

According to *Bergey's Manual of Determinative Bacteriology*, 8th ed., there is only one species of *Serratia*; however, Ewing et al. (4)

describe three. All 146 clinical *Serratia* strains received were tested biochemically to determine to which of the three species they belonged. One red strain was arabinose positive and hence was probably *S. rubidaea*; two white, arabinose-fermenting strains were probably *S. liquefaciens*. Of the 143 remaining strains, 29 were red, arabinose-negative *S. marcescens* strains; the other 114 *S. marcescens* produced no pigment and were used in the syntrophic assays with WF and 933 along with the strains of *S. liquefaciens*.

Control gram-positive and -negative species were obtained from the stock culture collection of the Division. Gram-positive organisms obtained were *Micrococcus lutea*, *Staphylococcus aureus*, *Bacillus cereus*, and *B. subtilis*. The gram negatives used as control strains were *Arizona arizonae*, *Citrobacter* sp., *Enterobacter aerogenes*, *E. cloacae*, *Escherichia coli*, *E. coli* C-10, *E. coli* ATCC 11303, *Klebsiella* sp., *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, and *P. maltophilia*.

Preliminary syntrophic pigment assays were done on Trypticase soy agar (Baltimore Biological Laboratory) and on 0.5% peptone (Difco)-1.0% glycerol-1.5% agar (Difco) (PGA) agars. Half of each plate was heavily inoculated with an experimental strain, and the other half was inoculated with one of the two characterized mutants, WF or 933. The plates were incubated for 36 h at room temperature (22 to 24°C) and observed for color formation in either the clinical or the known mutant strain.

When 933 and WF were on the same plate, both cultures exhibited red color formation. There were no color changes either in the clinical strains or in WF when they were tested against WF. Therefore, none of the clinical strains was able to donate MBC to WF or accept MAP from WF. With 933 on half of the agar as the MBC donor, color changes were observed in the clinical

cal isolates. PG agar proved more sensitive than Trypticase soy agar, showing colors in 94 out of 114, or 82% of the white clinical strains tested. Trypticase soy agar only allowed color to form in 25 out of 48, or 52% of the strains tested. These results indicated that medium influences pigmentation in *Serratia* as previously reported (5) and that the majority of the clinical isolates were able to conjugate the donated MBC.

The colors observed on these plates were only light pinks and purples, not the strong vivid reds of the normal red pigment or the pigment formed syntrophically between 933 and WF. To enhance the color formation, a more concentrated application technique for MBC was developed. The source of MBC was 933, but in these experiments, living cells were not used. Strain 933 was grown with vigorous shaking in 200 ml of PG broth at room temperature for 3 days.

The flasks of 933 were autoclaved at 121°C for 15 min and cooled to 4°C and extracted with 100 ml of chloroform for each 200 ml of culture fluid used. The chloroform extract from each flask was used to impregnate 10 strips (7.6 by 1.2 cm) of Whatman no. 1 filter paper. The MBC-impregnated filter paper strips were evaporated to dryness, foil wrapped, and then autoclaved.

Quality controls and experiments with the test strips were done by using strain WF on a modified Mueller-Hinton medium which substituted 3 g of beef extract (Difco) for the beef infusion and 5 g of tryptone (Difco) for the Casamino Acids. A single inoculum line of WF from a turbid suspension was put on each plate. After air drying for a few minutes, an MBC-impregnated strip from each 933 flask was then placed perpendicular to WF and incubated at room temperature for 24 to 48 h. The development of color on the control strips when WF was growing on the plate indicated that the formation of prodigiosin had occurred from the inherent MAP of WF and the donated MBC of 933. Only those flasks whose control strips showed prodigiosin formation were used in testing the clinical strains. Duplicate platings of clinical strains were done against strips from at least two different flasks.

The colors developed by the clinical strains in these strip assays were not the intense red of the syntrophic pigment seen with WF. The majority of strains developed light and dark pink colors, with a few producing purples and a few others showing purple or pinkish grays. Most colors could be classified by Ridgway's *Color Standards and Color Nomenclature* (8) and are listed in Table 1. Of the 114 clinical white strains tested, all but one, or 99%, formed visible colors. The two colorless *S. liquefaciens* strains, as well

TABLE 1. Hue frequency observed on MBC-impregnated paper strips with clinical strains

Color	No. of strains ^a
Purples/violets	
Pale lobelia violet	22, 8
Light vinaceous purple	1
Pale amparo purple	1, 1
Pale rose purple	12, 3
Rose purple	4
Dull lavender purple	1
Lavenders	
Vinaceous lavender	16, 6
Dull lavender	10, 5
Deep vinaceous lavender	1
Deep dull lavender	2
Lilacs	
Light pink lilac	9, 2
Light pale lilac	2
Light vinaceous lilac	2
Pale vinaceous lilac	13, 2
Pale lilac	6
Vinaceous lilac	2
Pinks	
Light pink ^b	6, 5
Light livid pink ^b	2
Livid pink	19, 6
Very light purple-pink ^b	1
Mallow pink	1
Rosalane pink	2
Cameo pink	5, 4
Pale amaranth pink	2, 1
Pale vinaceous	26, 6
Mauvette	1
Grays	
Light plumbago gray	1
Light purple gray	1
Pale Varley's gray	1
Heliotrope gray	1
Pale gray vinaceous	1
Pallid purple drab	1
Pale purple drab	1
Vinaceous gray	1
Light Quaker drab	1

^a The first number, or the only number if a single number appears, denotes the number of strains giving that color on one of the two test strips. The second number denotes the number of strains giving the same color on both strips.

^b Signifies a color not illustrated in Ridgway's *Color Standards*.

as all gram-negative and gram-positive control species, did not form colors. These results indicate that the ability of MBC acceptance may be species specific in white *S. marcescens*.

Several technical concerns exist with the extraction technique. Occasionally, a 933 PG-grown flask developed a purple tinge, and it has been reported that 933 can do this under certain

growth conditions (7). Also, occasionally strips made from an untinged flask would develop a slight degree of some purple color during preparation. If such a purple color appeared on strips, they were rinsed with 1 to 2 ml of fresh chloroform. If this failed to clear them, they were not used. Cleared strips reacted like white, uncleared strips in quality control and experimental replicates. In addition, there was a bluish discoloration in the chloroform extracts if they aged for more than 24 h. Best strips were obtained by using 1- to 2-h-old chloroform extracts. If a commercial source of MBC was available, it would eliminate these concerns and be a suitable taxonomic tool to identify white, clinical *Serratia* isolates. Outside of the precautions mentioned, however, pigmentation of white *S. marcescens* isolates is easily performed and frequent in occurrence when a source of MBC is provided.

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