Separation of Prodigiosenes and Identification as Prodigiosin Syntrophic Pigment from Mutant Pairs of *Serratia marcescens*¹

WALTER R. HEARN, ROBERT H. WILLIAMS, ROGER C. BURGUS, AND ROBERT P. WILLIAMS

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010, and Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025

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Countercurrent distribution is capable of resolving mixtures of closely related prodigiosene pigments. Syntrophic pigment produced by several pairs of *Serratia marcescens* color mutants was identified as prodigiosin (2-methyl-3-ethyl-6-methoxyprodigiosene) by countercurrent distribution, soda lime pyrolysis, and other techniques. The metabolic block of mutant strain H-462, derived from parent strain HY, was located between the blocks of mutant strains OF and WF, both derived from parent strain Nima.

The name prodigiosene (10) has been given to the unsubstituted nucleus of prodigiosin, red pigment of wild-type strains of *Serratia marcescens* (Fig. 1). Eight naturally occurring prodigiosene pigments have now been isolated, from strains of *S. marcescens* (12, 18), *Streptomyces longisporus ruber* (8, 20, 21), *Actinomadura* (Nocardia) madurae (4, 5), and *A. pelletieri* (4, 6).

The availability in our laboratories of two additional biosynthetic derivatives, 2,4-dimethyl-6-methoxyprodigiosene and 2,4-dimethyl-3-ethyl-6-methoxyprodigiosene (10), gave us an opportunity to test the resolving power of several analytical techniques for compounds closely related to prodigiosin. With the aid of these techniques, we identified the syntrophic pigment formed by pairs of *S. marcescens* color mutants and located the metabolic block in a new mutant, strain H-462.

**MATERIALS AND METHODS**

**Mutants.** The wild-type strains HY and Nima of *S. marcescens* and the interaction of various mutants were described in detail by Williams and Hearn (23). Orange donor mutant OF and the nonpigmented acceptor mutant WF (WCF in earlier papers) of *S. marcescens* strain Nima have been studied (7, 22). Mutant H-462 was one of several hundred mutants isolated after ultraviolet irradiation of wild-type strain HY. It was normally nonpigmented, appearing white to dull gray on surface culture and faintly pink in vigorously aerated liquid culture. When streaked on agar, H-462 formed red pigment in the growth adjacent to mutant OF, which remained orange. When streaked next to mutant WF, H-462 remained nonpigmented, but red pigment was produced by WF. Except for abnormal pigmentation, these mutants were identical to the parent strains in morphology, biochemical characteristics, and growth rates, as measured by viable counts or protein determinations. They were stable when preserved frozen.

**Prodigiosene derivatives.** A reference sample of prodigiosin (2-methyl-3-ethyl-6-methoxyprodigiosene) isolated from *S. marcescens* strain Nima was available as the free base and as crystalline hydrochloride and perchlorate salts. The isolation of 2,4-dimethyl-6-methoxyprodigiosine and 2,4-dimethyl-3-ethyl-6-methoxyprodigiosine from *S. marcescens* mutant 933 after supplementing the growth medium with 2,4-dimethylpyrrole and 2,4-dimethyl-3-ethylpyrrole, respectively, has been described (10).

**Countercurrent distribution (CCD).** An all glass micro-distribution apparatus (1) was constructed with 60 tubes of 1-ml capacity. With the buffered solvent system of Fig. 2, a microgram sample of prodigiosin gave the same distribution pattern when added to the first tube as hydrochloride, perchlorate, or free base. After the distribution, a drop of 3 N HCl and 4 ml of acetone (to give a single phase) were added to each tube. Absorbance at 537 nm (Bausch & Lomb Spectronic 20 colorimeter) was plotted versus tube number. The approximate distribution coefficient K was calculated from experimental plots of absorbance versus tube number, using the expression K = N/(n - N), where N = number (not necessarily integral) of the peak tube, and n = total number of transfers.

**Soda lime pyrolysis.** The pyrolysis procedure was modified from that of Wrede and Rothhaas (25). A
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1
FIG. 2. CCD pattern of (A) 2,4-dimethyl-6-meth oxyprodigiosene; (B) 2,4-dimethyl-3-ethyl-6-meth oxyprodigiosene; and (C) 2-methyl-3-amyl-6-meth oxyprodigiosene (prodigiosin). Solvent system: ligroin (bp 65 to 67 C); 2-methoxyethanol; 0.01 m sodium phosphate buffer, pH 7.2 (4:3:1). First 60 tubes of 100-transfer distribution at 25 C. Values for K calculated from these data according to the formula given in Materials and Methods.
sample of 30 mg of pigment (base form) was finely ground with 120 mg of soda lime. A thick-walled borosilicate glass capillary tube (1 by 15 cm) was drawn out at one end and bent at a 90° angle. The tube was charged with the pigment-soda lime mixture between 3-cm sections of powdered soda lime. Ends of the tube extended through holes in a small metal box containing a heating lamp and a thermometer adjacent to the tube. The drawn-out end was submerged in diethyl ether in a test tube cooled in ice. A stream of hydrogen was passed through the tube while the temperature was gradually raised to 250 C. After 5 min at 250 C, the tube was broken, and condensate still on the walls was washed into the distillate with ether. The Na2SO4-dried ether solution was analyzed by vapor-phase chromatography (VPC) with an Aerograph model A-90-P2 gas chromatograph, using a thermal conductivity detector. The partitioning liquid phase was silicone fluid GE SF-96 (Varian Aerograph, Inc.) on diatomaceous earth (column temperature, 158 C; detector, 219 C; injector, 220 C; He flow rate, 110 ml/min). The ether-distillate samples were also compared by thin-layer chromatography (TLC) on Silica Gel G in benzene solvent. Developed plates were sprayed with Ehrlich reagent (1% methanolic p-dimethyl-amino benzaldehyde diluted 1:5 with 1 n HCl) for pyroles.
Production and purification of syntrophic pigment. The OF/WF pair was grown together in surface culture (7) on a large scale in glass baking dishes (9 by 14 inches) (13). Submerged culture in Harned medium (9) was more convenient and was used for growing the pairs OF/H-462 and H-462/WF. Inocula for culture flasks were tubes of 5 ml of Harned's broth inoculated by needle from a thawed stock culture and grown for 24 hr at 26 C. Separate 2-liter flasks containing 400 ml of sterile broth were each inoculated with one mutant of the pair and incubated for 24 hr. The content of one of the flasks was then added to the other, and the mixed culture was incubated another 24 hr. Incubations were carried out in the dark at 28 C on a horizontal gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The cells were centrifuged and used at once, or lyophilized and stored at 5 C.

Pigment was extracted from NaOH-treated cells into lignin (bp 65 to 67 C; Skellysolve B, Skelly Oil Co.), precipitated as the hydrochloride, and converted to the perchlorate salt (24). In a typical experiment, six flasks (4.8 liters) of mixed culture yielded 21 to 25 g of lyophilized cells, from which could be prepared 120 to 150 mg of perchlorate salt, twice crystallized from hot 95% ethanol containing a little 5% aqueous perchloric acid. For further purification the perchlorate was converted to the free base and chromatographed over a column of diatomaceous earth (Hy Flo Super-cel, Johns Manville Co.). Development with ligroin left a magenta band at the top but moved an orange major component trailing into a red fraction. The orange and red fractions were eluted by 0.25% methanol-ligroin, combined, and evaporated to dryness. The pigment residue was dissolved in hot 95% ethanol and treated dropwise with aqueous 5% perchloric acid. Yield of crystalline perchlorate after chromatography was 30 mg.

RESULTS
Resolving power of CCD and pyrolysis techniques. In our CCD system at 25 C, 2,4-dimethyl-6-methoxyprodigiosene had K = 0.1 and 2,4-dimethyl-3-ethyl-6-methoxyprodigiosene had K = 0.2, in comparison with K = 0.8 for prodigiosin (12). A 20-transfer distribution of the two analogues was sufficient to show distinct peaks but insufficient for separation. In a 100-transfer distribution, a mixture of the three prodigiosene derivatives was clearly resolved (Fig. 2), although prodigiosin appeared to be held back to K = 0.7 by the

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presence of the large amounts of other components. Traces of material at both extremes of the distribution pattern (Fig. 3) were present in CCD of highly purified prodigiosin and probably represent decomposition products. The CCD peak of a single prodigiosene pigment generally gave a close fit to the theoretical curve with only minor skewing.

The two prodigiosin analogues were also distinguishable by soda lime pyrolysis. The dimethylethyl analogue gave reproducibly a single VPC peak of retention time 2.1 to 2.2 min, indistinguishable from the peak given by an authentic sample of 2,4-dimethyl-3-ethylpyrrole. TLC of the pyrolysis distillate showed a single red-purple Ehrlich-positive spot with the \( R_f \) of dimethylethyl pyrrole. The VPC pattern of the dimethyl analogue pyrolysate was less reproducible, but had a central major peak corresponding to the position of authentic 2,4-dimethylypyrrole. The VPC pattern of prodigiosin pyrolysis products showed two minor peaks at 0.8 min and 1.4 to 1.6 min, in addition to the major 2-methyl-3-amylpyrrole peak at 5.8 min. TLC showed a red Ehrlich-positive spot at \( R_f \) 0.68 corresponding to methylamylpyrrole, plus four other distinct spots at \( R_f \) 0.05, green; 0.15, yellow; 0.27, gray; and 0.80, pink.

Identification of syntrophic pigment as prodigiosin. The perchlorate salts from each mutant pair (OF/WF, OF/H-462, and H-462/WF) were compared directly with prodigiosin perchlorate obtained from wild-type strain Nima. On a hot stage under a polarizing microscope, the red crystals sintered around 205 C and melted with decomposition (loss of birefringence) at about 222 C (lit., 226-228 C [2, 24]). TLC behavior on Silica Gel in diethyl ether, ethyl acetate, or methanol was identical to that of authentic prodigiosin perchlorate; in ethyl acetate, \( R_f = 0.72 \). Infrared spectra obtained with a Perkin-Elmer model 20 spectrometer (KBr) were identical to the published spectrum of prodigiosin (2). Electronic spectra obtained with a Cary model 14 spectrometer were identical to published spectra (2, 13, 15) for both acid (95% ethanol, 0.01 N HCl) and base (95% ethanol, 0.01 N NaOH). The maximum in acid was at 537 nm, with \( E_{1%} \), (6 \( \mu \)M) = 10,800 (lit., 11,200 [2]). Microanalyses were performed by Ilse Beetz Mikroanalytisches Laboratorium, Kronach, West Germany, (calculated for \( C_{10}H_{14}N_2O\cdotHClO_4 \); C, 56.67; H, 6.18; N, 9.91; Cl, 8.36. Found: [OF/WF pigment] C, 56.36; H, 6.31; N, 9.74. [OF/H-462 pigment] C, 57.35; H, 6.17; N, 9.73. [H-462/WF pigment] C, 57.17; H, 6.31; N, 9.75. [prodigiosin from Nima] C, 57.03; H, 6.20; N, 9.75; Cl, 8.38).

A 20-transfer distribution of syntrophic pigment (as in Fig. 3) from each of the three mutant pairs was essentially superimposable on the CCD pattern of prodigiosin. Soda lime pyrolysis of the syntrophic pigments gave a mixture of products that was complex but identical to the pattern of prodigiosin pyrolysis products.

DISCUSSION

The natural occurrence of variously substituted prodigiosene pigments requires that unequivocal identification be made before biological conclusions are drawn. The criteria of chromatographic behavior and ultraviolet-visible spectra frequently reported by microbiologists working in this field are insufficient to distinguish between closely related prodigiosene derivatives. Thus the identification of a pigment isolated from a Streptomyces strain as prodigiosin (17) was premature without supplementary data; the pigment was possibly one of the \( C_{12} \) prodigiosene derivatives since identified (20, 21) primarily through nuclear magnetic resonance and mass spectrometry.
Where expensive analytical techniques are unavailable, TLC can often be counted on to distinguish between closely related derivatives, especially if reference samples are available (4). Analytical-scale CCD takes longer than TLC and requires an apparatus fabricated by a glassblower, but gives reproducible distribution coefficients. Like TLC, it requires only a microgram sample and has high resolving power. We conclude that CCD is a useful technique for identifying prodigiosene pigments. Soda lime pyrolysis, however, in spite of its classical use in elucidating the structure of prodigiosin (25), was not sufficiently reproducible as an analytical technique, and required too much sample.

More than one prodigiosene derivative may be present in a single organism (4). Multiplicity sometimes may be truly biogenetic but at other times may be due to artifacts of isolation. Pigment extracts of S. marcescens always show a number of bands when chromatographed on diatomaceous earth, alumina (12, 14, 15), or sucrose (2). However, highly purified prodigiosin itself continues to show a multiplicity of minor bands on chromatography or CCD. Differences in molecular association might be expected in solvents of different polarity (3). Finally, even synthetic prodigiosene derivatives have been found to rearrange chemically under relatively mild conditions (10).

When highly purified prodigiosin is chromatographed as the free base on diatomaceous earth, the red and orange bands eluted in that order by ethanol-ligroin seem to be the acid and base forms, the acid band arising from interaction of the base with some acid in the solvent, adsorbent, or atmosphere (11). The magenta to blue bands at the top of the column appear to be decomposition products which do not give rise to reproducible faster moving bands on chromatography. In an experiment in which 70 mg of combined orange-red fractions of prodigiosin were chromatographed on diatomaceous earth, protected from light, the eluted fractions were red, 3 mg; orange, 57 mg; and magenta, 2 mg. An additional 3 mg of magenta material was eluted by absolute ethanol, leaving a faint blue band at the top of the column. Even minor components are easily visible because of the relatively high absorbance of prodigiosene derivatives.

Analytical results on chromatographed and recrystallized perchlorate salts establish that the pigment produced syntrophically by pairs of our mutant strains of S. marcescens is prodigiosin. The yield of syntrophic prodigiosin was approximately the same as the yield of prodigiosin obtained from a culture of wild-type strain Nima under the same conditions. When mutant OF served as the donor, the culture had already produced enough norprodigiosin, 2-methyl-3-amyl-6-hydroxyprodigiosene (12), to appear bright orange when mixed with the acceptor mutant. Prodigiosin perchlorate from the mixed culture was not contaminated by norprodigiosin, however. Insolubility of the prodigiosin hydrochloride in ligroin facilitated separation from the more soluble norprodigiosin salt prior to chromatography.

Unequivocal identification of syntrophic prodigiosin served to locate (Fig. 4) the new mutant H-462 on the biosynthetic route to 4-methoxy-2,2'-bipyrole-5-carboxaldehyde (MBC), the immediate bipyrole precursor of prodigiosin (16, 23). The block in mutant H-462 must occur farther along the pathway than the unknown block of mutant WF and before that of mutant OF, which produces 4-hydroxy-2,2'-bipyrole-5-carboxaldehyde (HBC) but is unable to methylate it to MBC (W. R. Hearn et al., Fed. Proc., p. 354, 1963). All of these strains produce the immediate monopyrrole precursor of prodigiosin, 2-methyl-3-amylpyrrole, since they are both donors and acceptors to MBC-producing mutant 933 (19). Mutant H-462 was of great interest to us as a source of a precursor of HBC in the bipyrole pathway, but our attempts to isolate such a precursor have been unsuccessful.

![Fig. 4. Bioysynthetic pathway for prodigiosin as now conceived. Location of mutants described in the text is shown, as are the structures of the known intermediates MAP, MBC, and norprodigiosin.](image-url)
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