Prodigiosin Synthesis in Mutants of *Serratia marcescens*

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ABSTRACT

MORRISON, D. A. (Harvard College, Cambridge, Mass.). Prodigiosin synthesis in mutants of *Serratia marcescens*. J. Bacteriol. 91:1509-1604. 1966.—Exchange of biosynthetic intermediates through the culture medium was used to characterize several hundred new color mutants of *Serratia marcescens*. The general scheme of prodigiosin synthesis as a bifurcated pathway, in which monopyrrole and bipyrrrole precursors are synthesized separately and then coupled to form pigment, was confirmed and extended. Mutants of one new class excreted a product likely to be a new intermediate in monopyrrole synthesis, those of a second excreted a new product in the bipyrrrole pathway, and those of a third were blocked at early steps in both pathways. Two novel classes of mutants were isolated, in each of which a lack of some product present in *Serratia* and *Escherichia* cultures resulted in loss of all steps in prodigiosin biosynthesis.

The analysis of syntrophic interactions among nonred color mutants of *Serratia marcescens* has provided information about the late steps in the pathway of prodigiosin biosynthesis. In one of the early studies using this technique, Santer (Ph.D. Thesis, Yale Univ., New Haven, Conn., 1958) induced mutations affecting pigmentation in strain HY with ultraviolet radiation; selected pink, white, and orange mutants; and established relative positions for their biosynthetic blocks on a proposed bifurcated pathway of prodigiosin synthesis. Each of the mutants she described was located at one of four points (M1, B1, B3, and C in Fig. 2) on this pathway: one in the synthesis of a volatile monopyrrole precursor, 2-methyl-3-n-amylyl-pyrrole (MAP); two in the synthesis of a bipyrrrole precursor, 4-methoxy-2,2'-bipyrrrole-5-carboxaldehyde (MBC); and another in the coupling of the two terminal precursors to form pigment (7). Two of the precursors, MBC and 4-hydroxy-2,2'-bipyrrrole-5-carboxaldehyde (HBC), produced by these mutants have since been characterized (6, 8; Burgus, Ph.D. Thesis, Iowa State Univ., Ames), and the structure of another (MAP) has been inferred (8). Prodigiosin, the principal red pigment of wild-type *Serratia*, was characterized as a result of the structural work with its precursors (3, 4, 8). Hearn et al. (2) showed that the orange pigment of certain mutants blocked late in the synthesis of MBC is a hydroxy analogue of prodigiosin.

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The present work was undertaken to determine whether this approach could yield further information about the pathway. Several hundred mutants were isolated and examined with the cross-feeding techniques developed by Santer (Ph.D. Thesis, Yale Univ., New Haven, Conn., 1958) and Rizki (5). Six clearly defined new mutant classes were found, two of which excrete new intermediates in prodigiosin biosynthesis.

MATERIALS AND METHODS

Media. PG broth contained 5 g per liter of peptone (Difco) and 10 ml per liter of glycerol in distilled water, with 20 g per liter of agar added for solid media. It was found that this low phosphate medium gave better pigmentation and precursor production than did the minimal media used by Burgus (Ph.D. Thesis, Iowa State Univ., Ames, 1962) and others (2, 9). The minimal medium used was that described by Bunting (1). Unless otherwise noted, all cultures were grown at 24 to 27 C in PG medium. The volatile precursor MAP was provided to cultures on agar plates by inverting them over plates streaked with the B1 strain WF. For MBC-supplemented plates, the supernatant fluid from a low-speed centrifugation of a 3-day shake culture of the M1 mutant 9-3-3 was supplemented with 3 g of peptone per liter, 5 ml of glycerol per liter, and 20 g of agar per liter; the mixture was autoclaved and poured in 15-ml portions onto plates containing 20 to 25 ml of PG agar.

Strains. The HY strain of *S. marcescens* used by Santer (Ph.D. Thesis, Yale Univ., New Haven, Conn., 1958) and Rizki (5), which yielded color mutants more readily than other strains available (274, XII, Nima), was the source of most of the mutants studied. Mutants from HY were selected from the 5 to 10%

color mutants present in populations that had been irradiated to 0.01 to 0.1% survival under a germicidal lamp. Mutants of strain XII induced with various mutagens were obtained from Lydia Butler. Mutants 9-3-3 and P18 were grown from lyophilized stocks of the strains used by Santer. Samples of the Nima mutants WF and OF were kindly provided by R. P. Williams.

Feeding tests. Syntrophic interactions (feeding, as defined by Santer) were determined with the agar streak method described by Rizki (5) and Santer. The technique was occasionally adapted, especially in preliminary screening tests, for use with many samples by spotting adjacent colonies in place of larger streaks. Cultures were tested for volatile feeding by streaking them on separate plates and inverting one over the other for the 2- to 3-day incubation period. In general, new mutants were tested first against members of known classes (M1, B1, B3, C) and then among themselves to establish homogeneity of new classes. Particular caution was necessary when testing MAP⁻ mutants for MBC excretion, because a supply of MAP during incubation would allow pigmentation and prevent MBC excretion.

Selection of revertants. The ease with which red mutants could be detected in white confluent growth on plates allowed recovery of rare pigmented revertants from mutant 5-3. Broth cultures that had been irradiated to 5 to 10% survival were spread on agar plates in 0.1-ml portions containing 10⁴ to 10⁷ viable cells. Revertants were found and counted as small red flecks in the white confluent growth. These could be picked with a needle under the dissecting microscope for further characterization. Partial revertants (MAP⁺MBC⁻ or MAP⁻MBC⁺) were selected on plates supplemented with MBC or MAP as described above, and were distinguished from the complete revertants on those plates by their inability to pigment when restreaked on unsupplemented plates.

RESULTS

Several hundred color mutants obtained from HY after ultraviolet irradiation were examined for syntrophic pigmentation interactions. The mutants that gave clear feeding patterns could all be placed in 10 distinct classes. The interaction patterns defining these classes are shown in Fig. 1, and specific members of each class are given in Table 1. Members of the four classes described by Santer (here termed C, M1, B1, and B3) were among the new mutants, as well as mutants representing six new classes.

To estimate the relative abundance of the several classes, 73 mutants picked randomly from one set of postirradiation plates were all characterized. The relative frequency of the classes in that set of mutants is given in Table 1. Although about two-thirds of those mutants were members of the classes discussed in this paper, many of them exhibited ambiguous or complex patterns of syntrophy, as described below. In most other ex-

		DONOR CLASS									
		C	B1	B2	B3	M1	M2	X	Y	Z	I
ACCEPTOR CLASS	C	\	-	-	-	-	-	-	-	-	-
	B1	S	/	S	S	S	S	-	-	-	-
	B2	S	-	/	S	S	S	-	-	-	-
	B3	S	-	-	/	S	S	-	-	-	-
	M1	V	V	V	V	/	V	-	-	-	-
	M2	V	V	V	V	-	/	+	-	-	-
	X	VS	-	VS	VS	-	VS	/	-	-	-
	Y	S	S	S	S	S	S	S	/	S	S
	Z	S	S	S	S	S	S	S	S	/	S
	I	-	-	-	-	-	-	-	-	-	/

FIG. 1. Syntrophic pigmentation pattern among certain color mutant classes. Red pigmentation in the acceptor strain is induced by a volatile product (V) or a soluble product (S), or requires both soluble and volatile products (VS) from the donor strain. One interaction (+) is interpreted as a reverse feeding, in which the X mutant converts the MAP precursor from the M2 feeder into MAP and then behaves like other MBC⁻ mutants, feeding the M2 culture with MAP; -, no induced pigmentation.

periments, selection for specific feeding patterns was done early in the characterization process.

Certain mutants, not discussed in detail in this paper, behaved as though they had blocks at several steps of the pathway. In several such mutants there was a clear distinction between blocked and unaffected steps, whereas in many more there was a range of partial activities among the affected steps. However, all such mutants could be mapped on the pathway outlined in Fig. 2. It was observed that among these mutants those lacking all the steps in one line generally had also lost an early step in the other line.

When irradiated cultures of 5-3 were plated so as to produce confluent growth, three types of revertants were regularly found. Fully pigmented mutants appeared on all media; revertants (MAP⁻MBC⁺ and MAP⁺MBC⁻) that could synthesize just one of the terminal precursors were isolated on media supplemented with the other terminal precursor. The MAP⁻MBC⁺ mutants behaved just like 9-3-3 in feeding tests; the MAP⁺MBC⁻ mutants were all slightly pink and behaved just like WF in feeding tests. The relative frequencies of these three reversion types are shown in Table 2.

The two classes Y and Z exhibited a novel type of feeding pattern. They responded to every

Serratia strain tested, and they showed a bright-red reaction to these cultures just within a constant small distance from the donor. With P18, for instance, the Y mutant 7-23 was red only within 2

mm of the donor, whereas B, X, and M mutants were all pigmented 2 to 3 cm away. Members of classes Y and Z were pigmented in the same characteristic way when grown next to *Escherichia coli* cultures. Mutant 7-23 was also tested with several other species and did not react to *Pseudomonas fluorescens*, *Bacillus subtilis*, or *Mycobacterium butyricum* cultures. It was noted that, although mutant 7-23 grew well on minimal medium, the one Z mutant, HY-4, did not grow at all on minimal agar.

TABLE 1. Characteristic members of mutant classes

Class	Mutant	Strain of origin	Isolated by*	Color on PG medium	Frequency†
C	P18	HY	MTR	Pink	10.9
M1	9-3-3	HY	UVS	Light-pink	2.7
	114	XII	LRB	Blue	
M2	20	XII	LRB	Pink	0
	319		RPW	White	
B1	W1	HY	MTR	White	39.7
	WF	Nima	RPW	Yellow	
	2-2	HY	DAM	White	
B2	3-14	HY	DAM	Pink	4.1
	1023		RPW	Pink	
	64	XII	LRB	White	
B3	03	HY	MTR	Orange	5.5
	OF	Nima	RPW	Orange	
	7-6	HY	DAM	Orange	
X	5-3	HY	DAM	White	2.7
	IXA-2	274	DAJ	Red‡	
Y	7-23	HY	DAM	Light-pink	4.1
Z	4	HY	DAM	White	0
I	6-13	HY	DAM	White	1.4

* LRB, Butler; DAJ, Jackson; DAM, Morrison; MTR, Rizki; UVS, Santer; RPW, Williams.

† The per cent of mutants in each class among a total of 73 color mutants from one experiment.

‡ PO_4^{3-} -sensitive.

DISCUSSION

The mutants obtained in this study include members of the four classes reported by Santer (Ph.D. Thesis, Yale Univ., New Haven, Conn., 1958), as well as mutants defining six new classes. The data in Fig. 1 allow the new classes B2 and M2 to be mapped at positions in the scheme for prodigiosin biosynthesis intermediate between previously reported classes, as shown below and diagrammed in Fig. 2. With these mutants, as with Santer's, a block in the synthesis of one terminal precursor did not interfere with the synthesis and excretion of the other.

The positive reaction of members of class M2 to sources of monopyrrole precursor shows that they can couple the terminal precursors, and their feeding of B3 mutants shows that they produce MBC; hence, their block must be in the MAP pathway. Since M2 mutants produce a volatile compound that allows pigmentation in M1

FIG. 2. Location of mutant blocks on the pathway of prodigiosin biosynthesis.

TABLE 2. Occurrence of partial and complete reversion in irradiated* cultures of the X mutant 5-3

Selection medium	No. of plates	Revertant types	Avg no. per plate†	Absolute frequency
PG	2	MAP ⁺ MBC ⁺	3	3×10^{-6}
PG + MBC	8	MAP ⁺ MBC ⁺	2.7	3×10^{-6}
		MAP ⁺ MBC ⁻	0.3	3×10^{-6}
PG + MAP	8	MAP ⁺ MBC ⁺	3.8	4×10^{-6}
		MAP ⁻ MBC ⁺	34.2	4×10^{-4}

* In this experiment a suspension of 9×10^8 cells per milliliter was irradiated to 10% survival and plated at a dilution of 10^{-3} to obtain 9×10^4 cells per plate.

† Twenty mutants from each set of plates were transferred to PG agar to measure the fraction of partial revertants.

mutants, they can be mapped after M1 on the MAP pathway.

Class B2 presents a similar case on the MBC pathway. Mutants of this class excrete a volatile product, presumably MAP, that allows pigmentation in M1 and M2 mutants. They pigment when provided with MBC or HBC and, thus, can couple and can perform the methylation of HBC. Since they also excrete a product that allows pigmentation in the B1 mutant WF, these mutants can be mapped between B1 and B3 on the MBC pathway.

The block in each of these new classes results in the excretion of a new compound which is likely to be an intermediate in the biosynthesis of prodigiosin. The M2 product is volatile and, since it reacts to give a red product with Ehrlich reagent, may be a pyrrole. The B2 product can be detected with B1 mutants in filtrates of 3-14 cultures, but it appears to be unstable.

Mutants of class I were mentioned only in the first paper of Rizki (5), but they may well have been observed by others and discarded. The lack of reaction to class C testers indicates that class I mutants cannot perform the coupling step. That they do not induce pigmentation in M2 mutants indicates that the M2 step is inactive (compare with the ability of X mutants to allow pigmentation of M2 mutants described in Fig. 1). Since class I mutants do not act as donors to M1 mutants, they must be blocked in at least one earlier step in MAP synthesis as well. Similar arguments applied to the behavior of class I mutants with B1, B2, and B3 testers lead to the conclusion that class I mutants have also lost activity at the B2 and B3 steps and in at least one earlier step in MBC synthesis. The existence of such mutants indicates that the various steps of prodigiosin synthesis may be subject to a common control, since a single mutation affecting an overall control system is more likely than the many coincident mutations required if the steps are considered independent.

Two sets of mutants possess the feeding pattern of class X: the nonconditional color mutants isolated in these studies with HY, and the type of conditional color mutant called phosphate-sensitive. Phosphate-sensitive strains, which are defined (Jackson, Bunting, and Morrison, *Bacteriol. Proc.*, p. 53, 1963) as lacking pigment only when grown on media containing more than 150 $\mu\text{g/ml}$ of PO_4^{3-} , have been found to exhibit the complete feeding pattern of class X mutants (D. A. Jackson, *personal communication*).

Class X requires a more elaborate interpretation than the other classes. The ability of the mutants of this class to perform the steps lost by classes M2, C, B3, and B2 is shown by their positive feeding reactions to mutants of those classes; however, when not given such precursors, they are white and do not produce any of the late intermediates in either part of the pathway. They must be mapped as having early blocks in both MAP and MBC synthesis.

The structural similarity between MAP and MBC led to the suggestion that there could be an early step common to both pathways (Santer, Ph.D. Thesis, Yale Univ., New Haven, Conn., 1958). The possibility that class X mutants were blocked at such an early common step was tested by isolating revertants from the X mutant 5-3. The recovery of both MAP⁺MBC⁻ and MAP⁻MBC⁺ as well as of fully pigmented revertants showed that two distinct steps in prodigiosin synthesis had been affected in 5-3. The behavior of the two 5-3 blocks with respect to MAP and MBC synthesis requires that they be classed formally as M1 and B1 blocks. The only reason for hesitating to assign them to these steps is that these two blocks appear to be the result of a single mutation.

Examination of reversion rates provided evidence that the two blocks in 5-3 result from a single mutation. Since a model of two separate mutations undergoing independent reversion

would predict that the frequencies of complete reversions should be no larger than the product of the frequencies of individual reversions, the relative rates of appearance of complete and partial revertants were measured (Table 2). The result was that complete reversion occurred more often than MAP⁺ reversion and less often than MBC⁺ reversion. Since these revertants all appeared at a frequency of about 10^{-5} per cell, the model of independent mutations is not satisfactory. The two blocks are clearly interdependent in that there are mutations possible which restore activity at both steps simultaneously, and, indeed, such reversions occur as frequently as mutations restoring activity at either step alone.

Systems which would allow such mutations include multifunctional enzymes and poly-operonic control mechanisms. Elucidation of the exact mechanism involved, however, must await either development of a system of genetic exchange in *Serratia* or identification of the relevant biosynthetic steps and characterization of the enzymes responsible for them. That some sort of coordination between early steps in the MAP and MBC pathways should exist is reasonable, since the only known final product of these pathways is prodigiosin.

Mutants of classes Y and Z are distinguished from the others described here in that the feeding reactions they exhibit do not appear to involve biosynthetic intermediates directly. Members of class Y have the unusual characteristic of reacting only in an area very near (2 mm) the donor culture, a pattern that is not seen with known intermediates. It was found that any HY or XII strain at all, including the wild type, would act as a donor in this way. Members of class Z react to the same wide distribution of strains, but do so up to 15 mm from the donor.

Since these mutants do not induce pigmentation in 9-3-3 or WF, they must have at least one block at, or prior to, the B1 step, and another at, or prior to, the M1 point. Since these mutants do not allow pigmentation in XII-20, it is also seen that they have lost the M2 step. It is clear from the reaction pattern on P18 plates that these mutants cannot couple MAP and MBC unless also supplied with some other "factor." Although many, and probably all, steps in prodigiosin synthesis are inactive in these mutants, addition of the proper "factor" removes all of these blocks in pigment synthesis. Since this factor can be provided by strains not expected to excrete prodigiosin precursors (*E. coli*, HY, X, and I mutants), and since the terminal precursors cannot substitute for it, the factor appears to be a mate-

rial or condition required for pigment synthesis but not directly involved in it as an intermediate. That there are two distinct such factors is clearly shown by the existence of two classes, each of which produces the factor required by the other.

Two kinds of mutations affecting prodigiosin synthesis have been encountered in this study: those for which a block in the biosynthetic sequence can be mapped and which have probably lost activity at only one step, and those which result in loss of activity at all steps. The single blocks observed include those described by Santer and several new ones which fit consistently into her biosynthetic scheme. The new blocks result in excretion of products whose characterization should indicate the nature of earlier steps in prodigiosin biosynthesis. The second type of mutation, in which all steps are inactivated, seems quite likely to be affecting some part of the system controlling pigment biosynthesis. The case of classes Y and Z is particularly interesting, because they appear to reveal the dependence of prodigiosin synthesis on the presence of two compounds normally present in *Serratia* cultures, but not essential for growth; this case may provide a new approach to the problem of control of pigment synthesis in *Serratia*.

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LITERATURE CITED

1. BUNTING, M. I. 1940. A description of some color variants produced by *Serratia marcescens*, strain 274. *J. Bacteriol.* **40**:57-68.
2. HEARN, W. R., R. E. WORTHINGTON, R. C. BURGUS, AND R. P. WILLIAMS. 1964. Norprodigiosin: occurrence in a mutant of *Serratia marcescens*. *Biochem. Biophys. Res. Commun.* **17**:517-520.
3. RAPOPORT, H., AND K. G. HOLDEN. 1960. The synthesis of prodigiosin. *J. Am. Chem. Soc.* **82**:5510-5511.
4. RAPOPORT, H., AND K. G. HOLDEN. 1962. The synthesis of prodigiosin. *J. Am. Chem. Soc.* **84**:635-642.
5. RIZKI, M. T. M. 1954. Diffusion of chromogenic inductors of *Serratia marcescens*. *Proc. Natl. Acad. Sci. U.S.A.* **40**:1057-1060.
6. SANTER, U. V., AND H. J. VOGEL. 1956. Prodigiosin synthesis in *Serratia marcescens*: isolation of a pyrrole-containing precursor. *Biochim. Biophys. Acta* **19**:578-579.
7. WASSERMAN, H. H., J. E. McKEON, AND U. V. SANTER. 1960. Studies related to the biosynthesis

- of prodigiosin in *Serratia marcescens*. Biochem. Biophys. Res. Commun. 3:146-149.
8. WASSERMAN, H. H., J. E. McKEON, L. S. SMITH, AND P. F. FORGIONE. 1960. Prodigiosin: structure and partial synthesis. J. Am. Chem. Soc. 82:506-507.
9. WILLIAMS, R. P., J. A. GREEN, AND D. A. RAPPO-PORT. 1956. Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. J. Bacteriol. 71:115-120.