FACTORS INFLUENCING PIGMENT PRODUCTION IN A MUTANT STRAIN OF SERRATIA MARCESCENS

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A high frequency of mutations affecting color production is characteristic of the HY strain of Serratia marcescens. After ultraviolet irradiation, mutants ranging from white, through pink and orange, to deep red shades darker than the wild type can be obtained (Labrum and Bunting, 1953). Many of the white and pink mutants emit diffusible substances which can be utilized by other color mutants growing in the same medium to produce red pigment (Rizki, 1954a, b). Some of these substances may be precursors of prodigiosin, the red pigment of the parent strain, as shown by the isolation and chemical analysis of one of these diffusible products from a mutant strain (Santer and Vogel, 1956). It is hoped that this approach to the biosynthesis of red pigment in S. marcescens will help to elucidate the pathways of its production. With this ultimate aim, numerous mutants of S. marcescens have been isolated and tested for their interaction with other mutants, i.e., whether they produce any substance which can be utilized by other mutants to form red pigment, or whether a particular mutant will respond to the presence of another mutant by developing red pigment. During this survey of mutant types, an unusual mutant was isolated and this report presents the conditions under which this mutant of S. marcescens will produce red pigment.

METHODS AND RESULTS

Mutant 254 was obtained after ultraviolet irradiation of the HY strain. This mutant had been maintained on a defined medium on which it produced no pigment and had been classified as one of the white mutants. Cultures of mutant 254 were noted to have a strong odor reminiscent of indole. Such a feature had never been noticed in any of the other mutants of Serratia which had been isolated in our laboratory even though hundreds of mutants affecting color were examined. When a drop of Kovac's reagent was placed on a growing culture of 254 in a petri dish, a strong positive test was obtained. This reaction was further confirmed by the standard procedure of the Kovac test. A section of agar culture medium on which 254 was growing was transferred to a test tube with a cotton plug containing a drop of Kovac medium. After heating the test tube by placing it in boiling water, a cherry red color developed on the cotton plug.

Defined broth medium was inoculated with mutant 254 and the culture flasks were placed on a mechanical shaker at room temperature. After a growth period of 75 hr, the bacteria were removed by centrifugation and the supernant was steam distilled. The distillate, which gave a strong positive test with Ehrlich's reagent, was extracted with ether. After evaporation of the ether extract, a crystalline residue was obtained.

The nature of this residue was tested by paper chromatography using a solvent of 10 parts isopropanol, 1 part ammonia, and 1 part water with Whatman no. 1 paper. The Rf values corresponded with those of a known sample of indole (figure 1). Some of the crystalline residue was dissolved in cyclohexane and an absorption spectrum obtained on a DK1 Beckman spectrophotometer. A comparison of this curve with that of a sample of indole (Eastman Kodak) in cyclohexane is given in figure 2 and illustrates the degree of similarity between the two samples. Skatole with an Rf value close to that of indole had a very different absorption spectrum from indole or the isolated sample.

Mutant 254 was grown on defined medium to which individual amino acids were added in the first step of a survey to determine whether color production could be influenced by known chemical substances. The amino acids which were tested included arginine, alanine, aspartic acid, cysteine, cystine, glutamine, glycine, histidine,
hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Each substance was placed on the agar surface on which mutant 254 was plated. When tryptophan was added to the medium, mutant 254 grew as intensely red as the wild-type HY strain. Pigment was also produced when glycine was added to the medium. Tryptophan and glycine were the only two amino acids tested which showed any effect on pigmentation of 254. There was a delay in the development of pigment in the medium supplemented with glycine, but cultures plated on medium supplemented with tryptophan showed intense coloration as soon as growth on the petri dishes became apparent.

Cultures of mutant 254 were grown on a defined medium supplemented with tryptophan. The bacteria were harvested by scraping from the medium in the petri dishes and the combined sample from three petri dishes was lysed with KOH, precipitated with ethanol, and extracted with petroleum ether. The petroleum ether fraction was repeatedly washed with distilled water. A pigment sample was obtained by evaporation of the petroleum ether. An absorption spectrum of this pigment in cyclohexane was identical with that of the pigment extracted from the HY strain in a similar manner. This pigment production in 254 occurred only with the addition of L-tryptophan, since no effect was produced by the presence of the isomer, D-tryptophan.

Compounds known to be involved in the metabolism of tryptophan were also tested for possible influences on pigment production in mutant 254. The list of substances used included: niacin, anthranilic acid, kynurenine, tryptamine, 3-hydroxyanthranilic acid, peridoxal phosphate, serine + peridoxal phosphate, and serotinin. In addition, isatin, dihydroxyphenylalanine, and catechol were tested. No effect on color production was noted in any of these experiments.

The indole odor was not apparent when mutant 254 was producing red pigment on medium supplemented with tryptophan. Cultures of 254 were plated on small sections of defined medium and defined medium supplemented with tryptophan. These small agar blocks were then transferred to a unidirectional set-up (Rizki, 1958) so that defined medium and tryptophan medium began diffusing down the filter paper strips containing the corresponding blocks of medium. When red pigment had developed in the culture on the tryptophan medium, the metabolites diffusing with the medium along the paper strip were tested for the presence of indole by placing a drop of p-dimethylaminobenzaldehyde solution (1 per cent in 1 N HCl) on the paper strip. A positive test for indole was obtained on the paper strip with the culture growing on defined medium, but not with the pigmented culture growing on medium supplemented with tryptophan.

To determine whether any of the other mutants of S. marcescens produce substances which will influence color production in mutant 254, or whether metabolites from mutant 254 will supplement the pigment synthesis of other color mutants of S. marcescens, mutant 254 was tested with a series of color mutants which had been previously studied and selected for their color interactions. Each of these mutants was plated adjacent to a streak of mutant 254 and the plates were examined for color production after

Figure 1. Chromatogram of a known sample of indole and the accumulated metabolite from mutant 254. (Whatman no. 1 paper; isopropanol:ammonia:water solvent; sprayed with p-dimethylaminobenzaldehyde in 1 N HCl)
a growth period of 3 or 4 days. The results of this survey are presented in figure 3. In this schema the normal red pigment has been assumed as the final goal of a pattern of metabolic pathways. Each arrow leads from a mutant which is an "auxochromotroph" to another mutant which produces the chromogenic metabolite utilized by the former auxochromotroph for color production. Some of the mutants behave as chromogenic inductors for several mutants, but it is not necessary to assume that the inductor activity from such a mutant is due to a single metabolic product. That is, a mutant may be accumulating several chromogenic metabolites. This point can be illustrated by mutant P_{18}. Mutant P_{18} accumulates a substance which causes red pigment formation by mutant P_{254}. This induced pigment has the same absorption spectrum as the red pigment of the normal HY strain. The presence of glycine will also induce the same red pigment in P_{254}. On the other hand, P_{18} is also a strong inductor of color for W_{11} and all the mutants preceding it by arrows except P_{3}. Glycine does not cause pigment to develop in any of these mutants. Similarly, P_{18} will induce pigment formation in mutant 254, and 254 will also pro-
duce pigment when grown with tryptophan or glycine. The addition of tryptophan causes no pigmentary response from any of the other mutants in this schema. Thus each inductor mutant may accumulate a complex of metabolites and one of the substances of this complex used by a mutant to produce pigment may not necessarily be the same substance utilized by another mutant responding to the presence of the inductor mutant. C\(\alpha_1\) and C\(\alpha_2\) are points of metabolic convergence and are hypothetical additions to the schema. The mutant P\(18\) is an inductor for W\(11\) and P\(5\), but not for P\(1\), whereas W\(11\) will act as an inductor for both P\(2\) and P\(4\). The mutants P\(2\) and P\(3\) can serve as pigment inducers for mutants W\(5\), O\(3\), W\(1\), W\(6\), and W\(w\). Some of the mutants in this schema can mutually supplement each other when grown together. In all of these cases of double interaction in the chart, one mutant reacts strongly by producing red pigment whereas the other mutant shows only a slight pigment production. The direction of the strong reaction is shown by the long arrows and the weak reaction is indicated in the chart by the arrow with the short line.

**DISCUSSION**

The red pigment prodigiosin of Serratia has a tripyrrole structure (Wrede and Rothhaas, 1934). The use of glycine by Serratia mutants to produce pigment suggests that the formation of the pyrrole rings for pigment production may be related to the succinate-glycine cycle as postulated by Shemin (1956) for the biosynthesis of porphyrins. The metabolism of glycine in this cycle involves the condensation of succinate on the \(\alpha\)-carbon atom of glycine as a step in the process leading to the formation of \(\delta\)-aminolevulinic acid. The \(\delta\)-aminolevulinic acid may then be utilized in a variety of pathways, notably for porphyrin synthesis, or further metabolism may render the \(\delta\)-carbon atom useful in the synthesis of purines, the \(\beta\)-carbon atom of serine, and for methyl groups. Hubbard and Rimington (1950) have demonstrated the incorporation of the nitrogen and \(\alpha\)-carbon atoms of glycine in the pigment molecule of Serratia. Santer and Vogel (1956) also utilized glycine-2-C\(^14\) to label one of the precursors accumulated by a mutant strain of *S. marcescens*. This labeled precursor, suggested to contain two pyrrole rings, was then utilized by another mutant strain (W\(1\) given in the present schema) in the formation of red pigment. In addition, a random sampling of ultraviolet-induced mutations of Serratia showed that many of the white and light pink mutants will form red pigment when glycine is supplemented in the medium (Rizki, *unpublished data*). Therefore a relationship between glycine metabolism and pigment synthesis in Serratia has been demonstrated, and this relationship may explain the role of glycine in the proposed schema of color mutants in figure 3, particularly with regard to mutant 254.

The use of tryptophan to produce color by mutant 254, as well as the accumulation of indole...
when the mutant is growing white, suggests that the metabolism of these aromatic compounds may also be involved in the synthesis of the pigment of Serratia. Tryptophan biosynthesis has been extensively studied utilizing mutants of Neurospora and Escherichia coli, and a review of these biosynthetic pathways revolves around the reactions catalyzed by the enzyme, tryptophan synthetase (Bonner, 1959). The biosynthesis of tryptophan in these microorganisms normally proceeds via a pathway involving anthranilic acid with the elaboration of indole-3-glycerol-phosphate as an intermediate compound. An exchange reaction between serine and indole-glycerol-phosphate produces the molecule of tryptophan (Yanofsky, 1956, 1957; Yanofsky and Rachmeler, 1958). Two other biochemical reactions are catalyzed by this same enzyme, tryptophan synthetase: the production of indole and triose phosphate from indole-glycerol-phosphate; and the condensation of serine with indole to form tryptophan. Crawford and Yanofsky (1958) have separated tryptophan synthetase into two protein fractions and have examined the properties and reactions of these two components. They have shown that the combination of both components of the enzyme is necessary for the progress of the three reactions catalyzed by tryptophan synthetase. These studies on Neurospora and E. coli have all utilized mutants which require tryptophan for growth. Mutant 254 in S. marcescens is not an auxotroph, and the sole detectable difference produced by exogenous tryptophan is in the coloration of the bacterial culture. When mutant 254 is growing in the presence of tryptophan, indole accumulation is suppressed, at least below the point of detection in the present experiments. Speculation as to the role of tryptophan in prodigiosin synthesis must await further examination of tryptophan metabolism in Serratia. Since mutant 254 does not require exogenous tryptophan for growth, it appears that the required quantity of tryptophan needed for growth of the organism is synthesized by this mutant, but tryptophan synthesis is not sufficient in quantity to enable pigment production. Of particular interest to the present investigation is a consideration of the molecular structure of tryptophan, and pending further experimentation, it is satisfying to suggest the possibility that cleavage of the aromatic ring of tryptophan could result in a source of pyrrole fragments for prodigiosin synthesis.

SUMMARY

Mutant 254 of Serratia marcescens is white when grown on a defined medium, but will develop the characteristic red pigment of Serratia if tryptophan is added to the medium. On a defined medium, indole is accumulated by this mutant, but no detectable amounts of indole are present when red color is produced by the mutant growing on medium supplemented with tryptophan. Glycine will also initiate color production in mutant 254, although in this case, the pigmentation is less intense than with tryptophan. A schema of pigment synthesis in S. marcescens is developed basing the steps on the color interaction of mutants when they are grown adjacent to one another on the same medium.

REFERENCES


RIZKI


