JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 124-130
Vol. 129, No. 1

Printed in U.S.A.

Biosynthesis of Prodigiosin by Non-Proliferating Wild-Type *Serratia marcescens* and Mutants Deficient in Catabolism of Alanine, Histidine, and Proline

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Received for publication 19 August 1976

Mutants of *Serratia marcescens* Nima, designated as Aut, Hut, or Put, did not utilize L-alanine, L-histidine, or L-proline, respectively, as a sole carbon source but did utilize other amino acids or glycerol as carbon sources. The bacteria were permeable to alanine, histidine, and proline but lacked the enzymes responsible for degradation of these amino acids. The Aut mutant contained no L-alanine dehydrogenase activity, whereas the Hut and Put mutants contained only 7 and 4% of the histidase and proline oxidase activities, respectively, found in the wild-type strain. Rates of oxygen uptake and protein synthesis were significantly lower when the mutants were incubated in the presence of amino acids they could not degrade. Studies of L-[^{14}C]alanine, L-[^{14}C]histidine, and L-[^{14}C]proline incorporation into prodigiosin synthesized by these mutants and the wild-type strain revealed that proline was incorporated intact, whereas all of alanine except the carboxyl group was incorporated into the pigment molecule. Histidine did not enter prodigiosin directly. These data suggested that the presence of unique biosynthetic pathways, independent of primary metabolism, leads to formation of prodigiosin from specific amino acids.

Certain amino acids contribute to the formation and structure of prodigiosin, a secondary metabolite of *Serratia marcescens*. Wasserman et al. (14), by using ^{13}C Fourier transform nuclear magnetic resonance, established that proline was incorporated intact into prodigiosin, whereas all of alanine, except the carboxyl group, contributed to the pigment molecule. They also showed that portions of acetate, glycine, methionine, and serine entered the molecule. Qadri and Williams (7, 8, 16) developed a system in which addition of single amino acids such as L-alanine, L-histidine, or L-proline to non-proliferating bacteria caused formation of prodigiosin. Williams et al. (18) established that these three amino acids served as sources of carbon and nitrogen for the non-proliferating bacteria to increase macromolecular synthesis, as well as prodigiosin biosynthesis.

This non-proliferating cell system, in which pigment synthesis and not bacterial multiplication is a major metabolic activity (15), can be used to investigate prodigiosin synthesis. However, metabolic degradation of amino acids used to induce pigmentation in such a system interferes with studies of specific biosynthetic pathways that incorporate these amino acids into prodigiosin. This problem can be resolved by the use of mutants that are unable to catabolize specific amino acids, but are still able to synthesize prodigiosin and to incorporate these amino acids into the pigment.

In this paper we describe mutants that are unable to catabolize L-alanine, L-histidine, or L-proline. These mutants, designated as Aut, Hut, or Put, lack the enzymes responsible for the catabolism of alanine, histidine, or proline, respectively, but still can synthesize prodigiosin in the presence of other amino acids.

MATERIALS AND METHODS

Organism and growth media. *S. marcescens* Nima was carried as a stock culture on Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.). Bacteria were grown either in liquid minimal medium containing (wt/vol) 1.0% glycerol, 0.5% ammonium citrate, 0.05% $MgSO_4 \cdot 7H_2O$, 1.0% K_2HPO_4, 0.5% NaCl, and 0.005% ferric ammonium citrate, or in liquid complete medium containing the same ingredients plus 0.1% yeast extract (Difco Laboratories, Detroit, Mich.) and 0.2% casein hydrolysate (Sigma Chemical Co., St. Louis, Mo.). Utilization of amino acids as a carbon source for growth was tested in a modified minimal medium containing a 0.1 M concentration of the appropriate amino acid in place of glycerol, and with the substitution of 0.118% ammonium chloride and 1.0% KH_2PO_4 for ammonium citrate and K_2HPO_4, respectively. All

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media were adjusted to pH 7.2. Agar (Difco; 1.5% [wt/vol]) was added to solidify the minimal medium.

Preparation of non-proliferating bacteria was described previously (8). These bacteria contained no prodigiosin. A saline suspension of the bacteria at an optical density of 2.5 at 600 nm represented 1.8 mg of protein per ml. Prodigiosin biosynthesis in the non-proliferating cells was effected by addition of 0.1 M L-alanine, L-histidine, or L-proline to suspensions incubated at 27°C on a rotary shaker water bath (model G76, New Brunswick Scientific Co., New Brunswick, N.J.) set at 200 rpm (8).

Mutagenesis. Mutants deficient in catabolism of an amino acid were isolated by treatment of wild-type strain Nima with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by the procedure of Adelberg et al. (1). Bacteria were grown in liquid minimal medium at 39°C on a rotary shaker water bath to a density of 1.4×10^9 bacteria per ml, as determined by viable cell count on Trypticase soy agar plates. After centrifugation of cultures at 14,000 × *g* in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall Inc., Nowalk, Conn.), the pellet of bacteria was washed in a buffer that contained (per 100 ml): tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 6.0), 0.6 g; $(NH_4)_2SO_4$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; $CaNO_3$, 0.0005 g; $FeSO_4 \cdot 7H_2O$, 0.00025 g; and maleic acid, 0.58 g. The pellet was resuspended in this buffer to 1/5 of the original volume of culture, and nitrosoguanidine was added to the suspension to a final concentration of 1 mg/ml. After 30 min of incubation at 39°C, the suspension was added to an equal volume of cold 0.05 M potassium phosphate buffer (pH 7.0). The treated bacteria were centrifuged, washed with potassium phosphate buffer, suspended in liquid minimal medium, and then incubated at 39°C with shaking for 15 h prior to being streaked on plates. Mutants deficient in catabolism of the amino acid were isolated by using replica plates of modified minimal medium that contained L-alanine, L-histidine, or L-proline as the sole source of carbon. Bacteria unable to catabolize these amino acids were designated as Aut, Hut, and Put (Table 1) for deficiencies in the *autA*, *hutH*, and *putA* genes, respectively (2).

Enzyme assays. Activities of catabolic enzymes were measured in non-proliferating bacteria incubated in 0.1 M L-histidine or L-proline for 7 or 6 h, respectively, and in cell extracts prepared from non-proliferating bacteria incubated in 0.1 M L-alanine for 4 h at 27°C in a rotary shaker water bath. For measurement of L-alanine dehydrogenase (L-alanine:nicotinamide adenine dinucleotide [NAD] oxidoreductase, deaminating; EC 1.4.1.1.) activity, bacteria incubated in alanine were centrifuged, washed with an equal volume of 0.05 M Tris-hydrochloride (pH 8.0), and suspended in the Tris buffer to 1/6 of the original volume. Glass beads (5 μm in diameter; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) were added in a ratio of 1:3 to the total volume of suspension, with 0.01 M $MgCl_2$ and 0.01 M 2-mercaptoethanol. The bacteria were treated in a Bronwill Biosonik Sonicator (Bronwill Scientific, Rochester, N.Y.) for 4 min at intervals of 1 min, with intermittent cooling to 0°C, and then were centrifuged for 30 min at 30,000 × *g*. The supernatant fluid was collected and used for assay of the oxidative deamination of L-alanine by the procedure of Yoshida and Freese (19). The reaction mixture for this assay contained 0.1 M sodium carbonate (pH 10.0), 0.3 mM NAD, 5 mM L-alanine, and enzyme from the disrupted bacteria. L-Alanine was replaced by water in controls. One unit of L-alanine dehydrogenase activity caused NAD to be reduced at an initial rate of 1 pmol/min at 27°C, as determined by the increase in absorbance at a wavelength of 340 nm on a Gilford model 300-N Micro-sample spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The rates of this reaction and the reactions for histidase activity and proline oxidase activity were linear and directly proportional to the amount of protein in the assay mixtures. Specific activity was expressed as the number of enzyme units per milligram of protein.

Histidase (L-histidine ammonia-lyase, EC 4.3.1.3) activity was measured by the procedure of Prival and Magasanik (11) in non-proliferating bacteria incubated in histidine. The bacteria were centrifuged, washed in an equal volume of 0.1 M diethanolamine · HCl buffer (pH 9.4) that contained 20 μg of hexadecyltrimethyl-ammonium bromide per ml, and were resuspended in the buffer to 18 times the original volume of the culture. After addition of 0.005 M reduced glutathione the suspension was incubated for 10 min at 27°C. L-Histidine (or water, for controls) was added, and histidase activity was determined by measuring the appearance of urocanate at 277 nm on a spectrophotometer. Specific activity was expressed as picomoles of urocanate formed per minute per milligram of protein.

The procedure of Dendinger and Brill (3) was followed to measure proline oxidase (L-proline:O_2 oxidoreductase, EC 1.4.3.2) activity in non-proliferating bacteria incubated in proline. Bacteria were centrifuged, washed in an equal volume of 0.1 M sodium cacodylate buffer (pH 6.6), and suspended, to the original volume of culture, in the buffer containing 1% (vol/vol) toluene. The reaction mixture to measure enzyme activity contained 0.5 M L-proline (or water, for controls), 0.005 M *o*-aminobenzaldehyde, and bacteria. After 45 min of shaking at 27°C, the reaction was stopped by addition of trichloroacetic acid, at a final concentration of 2%, to the mix-

TABLE 1. *Bacterial strains used*[a]

Strain	Phenotypic designation	Genotype	Characteristics
Nima		Wild type	
BCMM-1	Aut	*autA2*	Deficient in L-alanine dehydrogenase activity
BCMM-11	Hut	*hutH6*	Deficient in histidase activity
BCMM-21	Put	*putA1*	Deficient in proline oxidase activity

[a] All mutant strains were derived from the wild-type strain Nima after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

ture. The bacteria were centrifuged, and the absorbance of the supernatant fluid was read at 443 nm on a spectrophotometer for the quantity of pyrroline-5-carboxylate formed from proline. Specific activity was expressed as picomoles of pyrroline-5-carboxylate produced per minute per milligram of protein.

Labeling procedures. Permeability of the bacteria to amino acids was determined by the incorporation of L-[U-^{14}C]alanine, L-[U-^{14}C]histidine, or L-[U-^{14}C]proline into protein. These labeled amino acids (specific activity, 0.5 μCi/μmol) were added to 5 ml of non-proliferating bacteria in a 50-ml flask, and the suspensions were incubated at 27°C on a rotary shaker water bath. After 30 min of incubation, the cultures were divided into two 2-ml portions. Unlabeled amino acids (final concentration, 0.05 M) was added to one sample, and incubation of both parts was continued. Samples of 0.1 ml were removed from the cultures at different times and mixed with 1 ml of cold 5% trichloroacetic acid. After 1 h at 0°C, the samples were filtered onto Whatman GF/C glass-fiber filters (W&R Balston, Ltd., England), and the acid-precipitable material on the filter was washed with 10 ml of cold 5% trichloroacetic acid and 3 ml of 95% (vol/vol) ethanol. The filters were dried for 15 h in an oven at 60°C, cooled to 25°C, and dissolved in 5 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole and 0.06 g of *p*-bis-[2]-(5-phenyloxazolyl)benzene (New England Nuclear Corp., Boston, Mass.) per liter of Scintanalyzed toluene (Fisher Scientific Co., Fairlawn, N.J.). The radioactivity in the samples was determined in a Beckman model LS 250 liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.). The scintillation counter had a counting efficiency of 65% for ^{3}H and 95% for ^{14}C.

Rates of protein synthesis were determined by the procedure of Williams et al. (18). Non-proliferating bacteria (6 ml) were incubated at 27°C in a rotary shaker water bath in the presence of 0.1 M L-alanine, L-histidine, or L-proline. After 12 h of incubation, the rates of protein synthesis were determined in a labeling medium that contained L-[^{14}C]leucine (specific activity, 11 μCi/μmol). Incorporation of labeled amino acids into prodigiosin was measured by the procedure of Lim et al. (5). Activity was expressed as picomoles of amino acid incorporated per microgram of purified prodigiosin.

Measurement of oxygen uptake. The rate of oxygen uptake by suspensions of non-proliferating cells was measured on a GME-Lardy Warburg apparatus (Gilson Medical Electronics, Middleton, Wis.) by the procedure of Umbreit et al. (13). The Warburg flasks contained 0.2 ml of 20% (wt/vol) KOH in the center well, 0.25 ml of amino acid or saline in each of two side arms, and 2.4 ml of non-proliferating cells. Total volume in each flask was 3.1 ml. The final concentration of amino acid present in each flask was 0.1 M. The flasks were incubated with shaking at 30°C in the Warburg apparatus, and the rate of oxygen uptake by the bacteria was measured at 1-h intervals for 8 h. The oxygen quotient [$Q(O_2)$] was expressed as microliters of oxygen taken up per milligram of cellular protein per hour.

Quantitative measurements. Prodigiosin was extracted from bacteria with acidic methanol, and the quantity of pigment was measured spectrophotometrically at a wavelength of 534 nm by the procedure of Goldschmidt and Williams (4). Protein was determined by the method of Lowry et al. (6), with bovine serum albumin as a standard for the assay.

Materials. All chemicals used were of reagent or analytical grade, unless otherwise noted. Amino acids were purchased from Sigma Chemical Co., St. Louis, Mo. DL-[COOH-^{14}C]proline was purchased from Research Products International Corp., Elk Grove Village, Ill., and all other isotopes were obtained from Amersham/Searle Corp., Arlington Heights, Ill.

RESULTS

Growth of strain Nima and mutants. There was no difference in the growth of wild-type strain Nima or the Aut (BCMM-1), Hut (BCMM-11), or Put (BCMM-21) mutants in a liquid minimal medium that contained glycerol as the only source of carbon (Fig. 1). All four strains grew well in the medium, an indication that the mutants had no requirements for specific amino acids.

The minimal medium did not support growth of the Aut, Hut, and Put mutants when glycerol was replaced as the sole source of carbon by

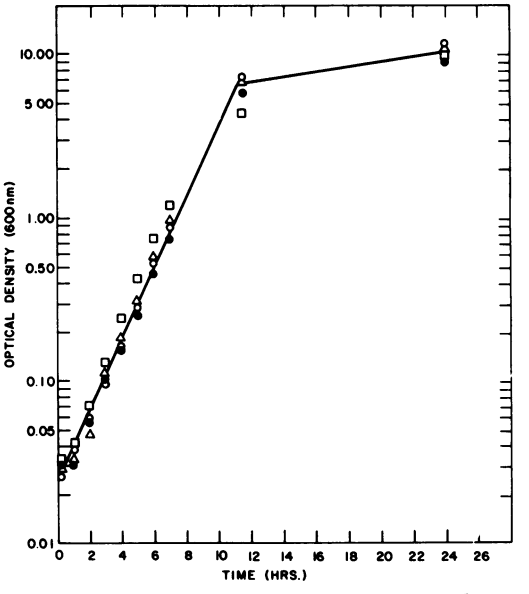

FIG. 1. *Growth of wild-type strain Nima and mutants in liquid minimal medium. Cultures of Nima, BCMM-1, BCMM-11, BCMM-21, grown in liquid minimal medium at 27°C in a rotary shaker water bath for 16 h, were used to inoculate into 50 ml of minimal medium in 250-ml Erlenmeyer flasks. These flasks were incubated at 27°C with shaking, and growth of the bacteria was measured by the increase in absorbance at 600 nm on a spectrophotometer. Symbols:* ●, *Nima;* ○, *BCMM-1;* □, *BCMM-11;* △, *BCMM-21.*

L-alanine, L-histidine, or L-proline, respectively (Fig. 2), although each mutant grew as well as strain Nima in the presence of either of the other two amino acids. These data suggested that the mutants were deficient in the catabolism of only one specific amino acid. Strain Nima grew in the presence of any of the amino acids but did not grow when the medium contained no carbon source. In all cases, prodigiosin was synthesized by strain Nima and its mutants when growth occurred.

Permeability of mutants to amino acids. The inability of the Aut, Hut, and Put mutants to catabolize certain amino acids for growth and metabolism was not a result of decreased cellular permeability to the amino acids. The mutants incorporated labeled L-alanine, L-histidine, and L-proline into protein without difficulty (Fig. 3). The incorporation of label represented active protein synthesis by the bacteria, since addition of unlabeled amino acids to the bacterial suspensions resulted in a decrease in radiolabel found in acid-precipitable protein. Strain Nima incorporated all three labeled amino acids into protein in exactly the same pattern as shown in Fig. 3.

Activity of catabolic enzymes in strain Nima and the mutants. L-Alanine dehydrogenase (19), histidase (11), and proline oxidase (3) are the primary enzymes responsible for the catabolism of L-alanine, L-histidine, and L-proline, respectively, by bacteria. These enzymes were measured in preparations of non-proliferating cells of strain Nima and the mutants were incubated in the presence of different amino acids (Table 2). The Aut mutant contained no L-alanine dehydrogenase activity

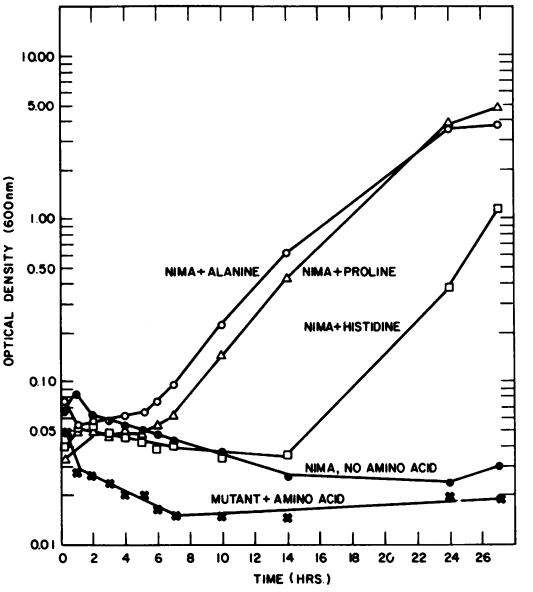

FIG. 2. *Growth of wild-type strain Nima and mutants in modified liquid minimal medium in which the indicated amino acid was the sole source of carbon. Cultures, grown as described in the legend of Fig. 1, were used to inoculate 50 ml of modified minimal medium containing a 0.1 M concentration of L-alanine, L-histidine, or L-proline. The cultures were incubated in 250-ml Erlenmeyer flasks, and growth of the bacteria was measured as indicated in the legend of Fig. 1. Symbols: ●, Nima, with no amino acid present in the medium; ○, Nima, with L-alanine; □, Nima, with L-histidine; △, Nima with L-proline; X, BCMM-1, BCMM-11, and BCMM-21, with L-alanine, L-histidine, and L-proline, respectively, as the sole carbon source present in the medium. The curve is representative of the data obtained with each of the three mutant strains.*

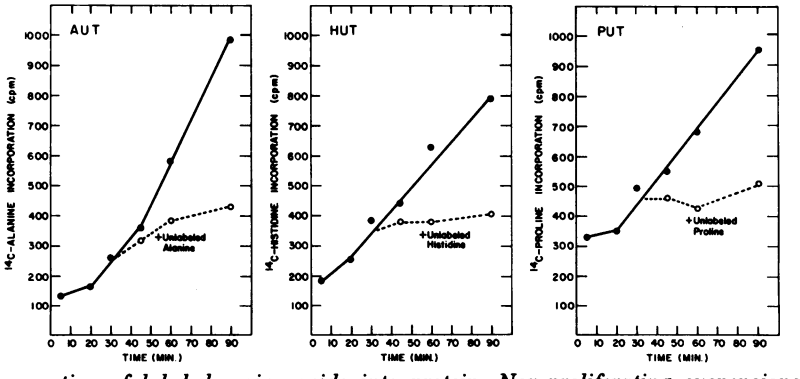

FIG. 3. *Incorporation of labeled amino acids into protein. Non-proliferating suspensions of the Aut (BCMM-1), Hut (BCMM-11), and Put (BCMM-21) mutants were incubated at 27°C in a rotary shaker water bath in the presence of L-[^{14}C]alanine, L-[^{14}C]histidine, and L-[^{14}C]proline, respectively, to measure incorporation of these amino acids into protein. Samples (0.1 ml) were removed from the cultures at different times for determination of acid-precipitable material. After 30 min of incubation, the cultures were divided into two 2-ml portions. Unlabeled amino acid was added to one part as a chase, and incubation of both parts was continued. Symbols: ●, incorporation of label into acid-precipitable material; ○, incorporation of label into acid-precipitable material after chase with unlabeled amino acid.*

TABLE 2. *Catabolic enzyme activities in strain Nima and its mutants*

Bacterial strain	Sp act[a] after addition of:		
	L-Alanine (L-alanine dehydrogenase)	L-Histidine (histidase)	L-Proline (proline oxidase)
Nima	10.3	22,076.1	160.6
BCMM-1 (Aut)	0.0	12,238.4	145.7
BCMM-11 (Hut)	10.6	1,611.2	110.9
BCMM-21 (Put)	12.6	10,589.1	12.7

[a] Enzyme activities were measured in non-proliferating bacteria incubated in the presence of L-alanine, L-histidine, or L-proline. Specific activities are expressed as the picomoles of product formed per minute per milligram of cellular protein.

when incubated in the presence of alanine, although the mutant contained high levels of histidase and proline oxidase activities when incubated in the presence of histidine and proline, respectively. The Hut and Put mutants contained only 7 and 4% of the histidase and proline oxidase activities, respectively, that were found in the wild-type strain Nima, although both mutants contained normal levels of catabolic enzyme activities in the presence of amino acids that they could catabolize. In the presence of alanine, histidine, and proline, strain Nima contained high levels of activity of each of the three catabolic enzymes.

Metabolism. Metabolism in strain Nima and in the mutants was determined by measurement of the rates of protein synthesis and oxygen uptake in non-proliferating bacteria. The rate of protein synthesis was lowest when the Aut, Hut, and Put mutants were incubated in the presence of amino acids they could not catabolize (Table 3). The decrease in rates of synthesis suggested that under these conditions the mutants could not degrade the amino acids for use in primary metabolic pathways, and therefore they could not synthesize protein.

Although the wild-type strain Nima took up oxygen in the presence of different amino acids, as measured by the Warburg respirometer, the Aut, Hut, and Put mutants had no oxygen uptake in the presence of L-alanine, L-histidine, and L-proline, respectively (Table 4). This absence of oxygen uptake, measured between 5 and 6 h of incubation, suggested that the mutants were not metabolizing in the presence of these amino acids, although they could take up oxygen and metabolize in the presence of utilizable amino acids (10).

In addition, paper chromatography of cell fractions of the Aut, Hut, and Put mutants incubated in the presence of radioactive alanine, histidine, and proline, respectively, showed label appearing only in free amino acids, protein, and prodigiosin (unpublished data).

Incorporation of amino acids into prodigiosin. Since the Aut, Hut, and Put mutants were permeable to L-alanine, L-histidine, and L-proline, respectively, but did not catabolize these specific amino acids, the strains might be used to follow direct incorporation of the amino acids into prodigiosin. Such a study was made possible by addition of proline to non-proliferating suspensions of strain Nima, the Aut mutant, and the Hut mutant, and of alanine to non-proliferating suspensions of the Put mutant, to induce metabolism and pigmentation (Table 5). L-Methionine was added to these suspensions to increase the quantity of pigment formed (9). Once pigment was formed, labeled alanine, histidine, or proline was added to the cell suspensions, and the incorporation of these amino acids into the pigment was followed. The results of these studies showed that proline, in-

TABLE 3. *Rates of protein synthesis by strain Nima and its mutants*

Bacterial strain	Rate of synthesis[a] (cpm/10 min per 0.1 ml of cells) in presence of:			
	No amino acid	L-Alanine	L-Histidine	L-Proline
Nima	796	1,015	1,571	3,263
BCMM-1 (Aut)	687	311	1,645	2,850
BCMM-11 (Hut)	808	1,450	600	3,756
BCMM-21 (Put)	848	1,996	2,575	558

[a] Rates of protein synthesis were measured after incubation of non-proliferating bacteria for 12 h at 27°C in a rotary shaker water bath.

TABLE 4. *Rates of oxygen uptake by strain Nima and its mutants*

Bacterial strain	$Q(O_2)$[a] in presence of:			
	No amino acid	L-Alanine	L-Histidine	L-Proline
Nima	6	106	34	84
BCMM-1 (Aut)	20	19	62	112
BCMM-11 (Hut)	10	79	17	79
BCMM-21 (Put)	10	90	58	10

[a] Oxygen uptake is expressed as microliters of oxygen taken up by the bacteria per milligram of cellular protein per hour. Non-proliferating bacteria (1.8 mg of protein per ml) were incubated in Warburg flasks as described in the text. After 5 h of incubation at 30°C, the rate of oxygen uptake was measured for a 1-h period. The readings obtained from each flask were corrected against the reading obtained from a thermobarometer that contained saline in place of the bacteria and amino acids in the flask.

TABLE 5. *Incorporation of labeled amino acids into prodigiosin*

Bacterial strain	Amino acid added at 0 h[a]	Labeled amino acid added at 12 h	Sp act of label (mCi/mmol)	Quantity of label added (μCi/10-ml reaction mix)	Activity[b]
Nima	L-Proline	L-[U-^{14}C]Alanine	0.0625	0.16	42
	L-Proline	L-[U-^{14}C]Histidine	0.0625	0.16	34
	L-Alanine	L-[U-^{14}C]Proline	0.0625	0.16	243
BCMM-1 (Aut)	L-Proline	L-[U-^{14}C]Alanine	0.0625	0.16	58
	L-Proline	L-[^{14}COOH]Alanine	0.0625	0.16	2
BCMM-11 (Hut)	L-Proline	L-[U-^{14}C]Histidine	0.0625	0.16	0
	L-Proline	L-[^{14}COOH]Histidine	0.0625	0.16	0
BCMM-21 (Put)	L-Alanine	L-[U-^{14}C]Proline	0.0625	0.16	624
	L-Alanine	DL-[^{14}COOH]Proline	0.0625	0.32	615

[a] Suspensions of non-proliferating cells containing a 0.1 M concentration of the added amino acid and a 0.27 mM concentration of L-methionine were incubated at 27°C in a rotary shaker water bath for 12 h before addition of the labeled amino acid. After an additional 4 h of incubation, prodigiosin was extracted from the cells, purified, and counted for radioactivity.

[b] Activity is expressed as picomoles of amino acid incorporated per microgram of prodigiosin.

cluding the carboxyl group, was incorporated intact into prodigiosin, while the carboxyl group of alanine did not enter the pigment. Histidine was not incorporated into prodigiosin synthesized by the Hut mutant, although label from the amino acid did appear in prodigiosin synthesized by strain Nima.

DISCUSSION

Synthesis of prodigiosin by non-proliferating cells of *S. marcescens* depends upon the presence of carbon and nitrogen sources (18). The pigment is synthesized only after an increase in the rates of ribonucleic acid and protein synthesis (18), and its synthesis may involve intermediates unique in the formation of the secondary metabolite (5, 15). Proline and alanine are known to be incorporated, either intact or in part, into prodigiosin (12, 14). However, the role of histidine in prodigiosin biosynthesis is not known. Although addition of histidine to non-proliferating bacteria causes pigmentation after a lag period (7, 8), our data show that the amino acid does not enter prodigiosin directly. All three amino acids are used as carbon and nitrogen sources by the wild-type strain Nima (18) and, therefore, can be degraded into metabolic intermediates by the bacteria.

Catabolism of alanine, histidine, and proline by wild-type *S. marcescens* interferes in studies of their role in prodigiosin biosynthesis. The use of Aut, Hut, and Put mutants that are unable to catabolize these amino acids (Table 2) eliminates such interference. Our experiments with Aut (BCMM-1), Hut (BCMM-11), and Put (BCMM-21) mutants show that, if other amino acids are utilized as carbon and nitrogen sources for synthesis of pigment, degradation of alanine, histidine, and proline is not necessary for prodigiosin synthesis (Fig. 4). L-Proline, although not catabolized by the Put mutant, enters intact into prodigiosin synthesized by non-proliferating suspensions of bacteria after the addition of alanine as a carbon and nitrogen source to effect pigmentation. This incorporation of proline is directed enzymatically by a biochemical pathway unique to prodigiosin synthesis (5). The carbon chain of L-alanine is decarboxylated by the Aut mutant prior to insertion into the pigment. Since the Aut mutant does not contain L-alanine dehydrogenase to catabolize alanine by normal degradative pathways, this decarboxylation of the alanine carbon chain may be caused by an enzyme unique in prodigiosin biosynthesis. L-Histidine does not enter prodigiosin synthesized in the presence of proline. Although radiolabel from histidine appears in prodigiosin purified from non-proliferating suspensions of strain Nima (Table 5), this pattern of incorporation can be misleading since the label may come from degradative products of histidine rather than directly from the amino acid during prodigiosin synthesis. Use of the Hut mutant that does not catabolize histidine shows that the amino acid does not enter directly into prodigiosin.

Since proline enters intact into prodigiosin, it contributes the greatest number of carbon atoms to the pigment (Table 5). Tanaka et al. (12) and Wasserman et al. (14) proposed that carbon atoms from the first pyrrole group of prodigiosin (A, Fig. 4) originated from proline. Our experiments with incorporation of labeled pro-

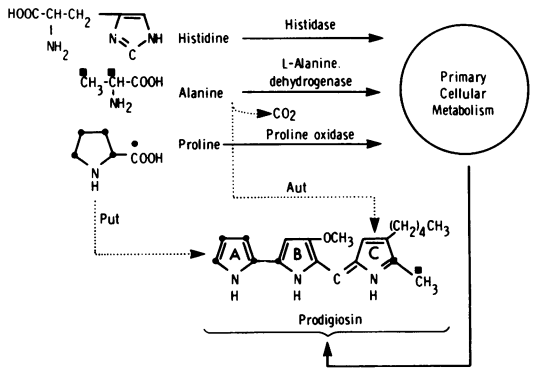

FIG. 4. *Scheme for biosynthesis of prodigiosin from L-alanine, L-histidine, and L-proline. All three amino acids can be degraded by catabolic enzymes to enter primary cellular metabolism that can result in synthesis of prodigiosin. The Aut and Put mutants, unable to catabolize alanine and proline, insert these amino acids directly into prodigiosin by unique biosynthetic pathways.*

line into prodigiosin synthesized by the Put mutant support their conclusions. In addition, the quantity of radiolabel from proline found in prodigiosin (measured as activity) is greater in pigment synthesized by the Put mutant than by the wild-type strain Nima. Since proline is not catabolized by the Put mutant, one explanation for this higher activity may be a shift of the amino acid to the biosynthetic pathway leading to formation of prodigiosin. A similar, although smaller, increase in activity of alanine is found in prodigiosin synthesized by the Aut mutant as compared to pigment synthesized by strain Nima. Although alanine contributes fewer carbon atoms than proline to the structure of prodigiosin, its contribution is significant in comparison to that of histidine.

These data suggest that Aut and Put mutants can be valuable when the direct incorporation of alanine and proline, respectively, into prodigiosin is studied, since these amino acids are not catabolized into other metabolic intermediates by the mutants. In addition, the data indicate that alanine and proline serve dual roles in prodigiosin biosynthesis (Fig. 4). Not only do the two amino acids contribute to the structure of prodigiosin, but they also are effective in inducing pigmentation in non-proliferating bacteria. The use of Aut and Put mutants may provide information by which alanine and proline participate in unique pathways involved in the biosynthesis of prodigiosin. Since histidine is not incorporated directly into prodigiosin, but can cause delayed pigmentation, it would also be of interest to investigate the role of this amino acid in prodigiosin synthesis.

ACKNOWLEDGMENTS

These studies were supported by grant Q-359 from the Robert A. Welch Foundation, Houston, Tex. Daniel V. Lim is a postdoctoral fellow supported by the Foundation.

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