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Role of L-Proline in the Biosynthesis of Prodigiosin

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Nonproliferating cells of *Serratia marcescens*, wild-type strain Nima, synthesized the pigment, prodigiosin, when saline suspensions were incubated with aeration at 27°C in the presence of proline or alanine. Mutants PutS1 and PutS2 derived from strain Nima formed prodigiosin from alanine, but not from proline, unless alanine also was added. Strain Nima utilized proline as a sole source of carbon and of nitrogen for growth, whereas Put mutants did not. Investigation of enzymes degrading proline showed that the wild-type strain contained proline oxidase, which was absent in Put mutants. The wild type, as well as the mutants, utilized alanine as the sole source of carbon and nitrogen for growth. Although nonproliferating cells of Put mutants failed to synthesize prodigiosin from proline, addition of L-[U-^{14}C]proline to suspensions metabolizing and synthesizing the pigment because of addition of alanine resulted in the incorporation of radioactive label into prodigiosin, as well as into cellular protein. Since Put mutants could not catabolize proline, the incorporation of [^{14}C]proline into the prodigiosin molecule indicated that proline was incorporated directly into the pigment.

Serratia marcescens strain Nima synthesizes the red pigment, prodigiosin, when incubated under specific environmental conditions (13, 22). However, when grown at 38°C, the pigment is not formed. When harvested and suspended in saline, these nonpigmented bacteria synthesize prodigiosin during incubation at 27°C after addition of certain amino acids (12, 13, 23, 24).

Of the amino acids that caused pigmentation in suspensions of nonproliferating cells, L-proline was of particular interest because of general similarities in chemical structure of this amino acid and the pyrrole rings of prodigiosin (Fig. 1). L-Proline also caused formation of large quantities of prodigiosin in nonproliferating cells (13) and was incorporated into the prodigiosin molecule (18, 19). Proline and other amino acids that caused pigmentation in nonproliferating cells of Nima also served as sources of carbon and nitrogen for growth (25).

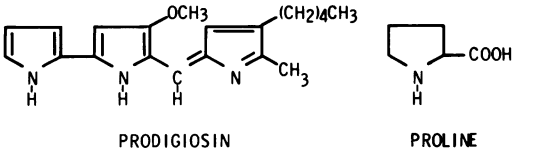
This paper presents data concerning the biochemical degradation of L-proline as a requirement for prodigiosin biosynthesis. We also confirm that proline is a direct precursor of prodigiosin.

MATERIALS AND METHODS

Bacterial strains. The prototroph, *S. marcescens* strain Nima, utilized proline as a carbon and nitrogen source for growth. PutS1 and PutS2 were mutants of Nima that were incapable of utilizing proline as either carbon or nitrogen sources for growth. These mutants were phenotypically Put mutants according to the nomenclature of Demerec et al. (4), and they behaved identically in our experiments.

Growth media. Stock cultures were carried on Trypticase soy agar (BBL) slants. Bacteria were cultivated in the minimal medium of Bunting (3). In nutritional studies, a carbon-deficient medium (C^- medium) was used that differed from the minimal medium by the omission of glycerol and ammonium citrate, and by the addition of 0.118% (wt/vol) ammonium chloride to obtain an equivalent concentration of ammonium ion. Medium lacking nitrogen (N^- medium) contained 0.65% (wt/vol) trisodium citrate instead of ammonium citrate. All three media had an initial pH of 7.1. Solid minimal medium, C^- medium, and N^- medium contained 2.0% (wt/vol) agar.

Mutagenesis, mutant selection, and growth characteristics. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) was used to induce mutations. After overnight growth in Trypticase soy broth, strain Nima was inoculated into Trypticase soy broth and incubated at 37°C for 2 h on a rotary water-bath shaker (model G76, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) at 200 rpm. The bacteria were harvested by centrifugation and resuspended in



PRODIGIOSIN PROLINE

FIG. 1. *Structures of prodigiosin and proline.*

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minimal salts medium (8). Nitrosoguanidine (0.1 ml of a 10% [wt/vol] solution in acetone) was added to 10 ml of the suspension, which then was incubated for 30 min at 37°C with shaking. The bacteria were harvested by centrifugation and then were washed once with minimal medium. The cells were resuspended in minimal medium, diluted in 0.85% (wt/vol) saline, and spread on solid minimal medium. Isolated colonies that grew on plates of minimal medium were picked with sterile toothpicks and transferred to plates of C^- and N^- media, each containing 0.1 M L-proline. Those mutants failing to utilize proline as a carbon or a nitrogen source for growth were streaked on minimal medium. Isolated colonies were purified by repeated evaluation of growth on media with or without proline as the sole source of carbon or nitrogen.

Growth of the mutants and Nima was compared by inoculation of each strain into C^- and N^- media, each containing 0.1 M L-proline. Growth was measured turbidimetrically on a Klett-Summerson colorimeter with the red filter (no. 64) to avoid interference by prodigiosin.

Mutants failing to utilize proline as a carbon or nitrogen source for growth were isolated at a frequency of less than 0.1%. As far as could be determined, the only metabolic defect in the mutants was the failure to catabolize proline.

Preparation of nonpigmented, nonproliferating cell suspensions. Nonpigmented bacteria were obtained by growth in minimal medium at 38°C on the rotary shaker for 18 to 24 h, at which time the cells were in late log or early stationary phases of growth. The cells were centrifuged at 10,000 × *g* (Sorvall model RC-2B, Ivan Sorvall, Inc., Newtown, Conn.) for 10 min at 4°C, washed three times with 0.85% saline, stored either as packed cells at −22°C or as suspensions in 0.85% saline, and then diluted with saline to give an absorbance of 3.0 at 600 nm in the spectrophotometer (Gilford model 300-N, Gilford Instruments Laboratories, Inc., Oberlin, Ohio). This value corresponded to 1.5 mg of protein/ml.

Either L-alanine or L-proline (0.1 M final concentration) was used to induce prodigiosin biosynthesis in the nonproliferating cells. L-Methionine (0.2 mM final concentration) was added to increase the amount of prodigiosin formed and to decrease the time required for appearance of pigment (11). Methionine alone did not initiate pigmentation in nonproliferating cells (11).

Quantitation of prodigiosin and protein. The acidic methanol extraction procedure of Goldschmidt and Williams (7) was used to measure prodigiosin. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

Proline transport. While in logarithmic growth in minimal medium, cells of Nima and PutS2 were measured for proline transport into the cells. Cultures were incubated at 27°C on the rotary shaker. Transport of labeled proline was measured after transfer of cultures to minimal medium containing 0.1 μCi of L-[U-^{14}C]proline (Amersham/Searle Corp., Arlington Heights, Ill.) and 10 μg of unlabeled L-proline per ml. At the times indicated (see Fig. 3), cells were placed on 0.45-nm membrane filters (Schleicher and Schuell) and washed with 10 ml of saline. The filters were dried in scintillation counting vials containing 10 ml of Omnifluor (New England Nuclear, Boston, Mass.). The amount of radioisotope transported into the cells was expressed as the percentage of total counts in the labeling medium (percent input) and was calculated by the equation: percent input = $[(\text{cpm}t_x - \text{cpm}t_0)/\text{cpm}_i] \times 100$, where cpm equals the observed counts, t_x equals various time intervals, t_0 equals zero time, and i equals total counts.

Protein synthesis in PutS2. The rate of protein synthesis was determined according to the procedure described previously (25). Rate of protein synthesis was measured in nonproliferating cells of PutS2 in 0.2 mM L-methionine and 0.1 M L-proline or L-alanine. Prodigiosin was formed in the presence of alanine but not of proline.

Proline oxidase assay. The proline oxidase activity was measured by a modification of the procedure of Dendinger and Brill (5). Growing or nonproliferating bacteria were suspended in 0.1 M sodium cacodylate buffer, pH 6.6, to which 5 μl of toluene per ml of suspension had been added. After shaking the cells for 10 min at 37°C, 1.0 ml of 1.0 M L-proline and 0.2 ml of 0.05 M *o*-aminobenzaldehyde in 20% aqueous ethanol were added. This mixture was incubated at 37°C with shaking for 45 min. The reaction was stopped with 0.2 ml of 20% (wt/vol) trichloroacetic acid, and after 5 min the precipitate was removed by centrifugation at 11,100 × *g* for 10 min. The absorbance of the supernatant at 443 nm was measured against water in a Gilford model 300-N microsample spectrophotometer. The absorbance was corrected against a blank containing the same reaction mixture to which 0.2 ml of 20% trichloroacetic acid was added before incubation. The enzyme activity was determined using 2.71 as the millimolar extinction coefficient of the Δ′-pyrroline 5-carboxylate-*o*-aminobenzaldehyde complex (17). The activity of proline oxidase in *Serratia marcescens* was linear with respect to time of reaction and enzyme concentration.

Proline incorporation into prodigiosin. Alanine and methionine were added to suspensions of nonproliferating cells of Nima, PutS1, and PutS2, and the cultures were incubated at 27°C on the rotary shaker. Labeled proline was added at the 10th h of incubation, after pigmentation had begun. One-milliliter samples of the nonproliferating cells were removed periodically and added to 1.0 ml of acidic methanol. Another 1.0-ml sample was removed at the same time for quantitation of prodigiosin.

Prodigiosin was extracted and purified according to the method described previously (14). After the pigment was dried in a rotary evaporator, prodigiosin was solubilized in 10 ml of toluene fluor containing TLA-mix (Fluoralloy Formula TLA; Beckman Instruments, Inc., Fullerton, Calif.), transferred to a vial, and counted for 10 min.

The residual cell material then was treated with 5.0 ml of 5% trichloroacetic acid and boiled for 20 min to solubilize nucleic acids. The material insoluble in trichloroacetic acid was collected by centrifu-

gation, washed with 5.0 ml of 5% trichloroacetic acid, solubilized in 1 ml of Soluene 100 (Packard Instrument Co., Inc., Downers Grove, Ill.), and neutralized with 0.04 ml of glacial acetic acid per ml of Soluene. The TLA-mix toluene fluor was added, and the mixture was transferred to a scintillation vial.

The total amount of radiotracer per milliliter of nonproliferating cells was determined in duplicate in 0.2-ml samples dissolved in 1 ml of Soluene. After the sample was dissolved, fluor was added, and the amount of [^{14}C]proline incorporated was determined and expressed as percentage of the total amount of isotope added.

RESULTS

Growth characterization of Nima and mutants. The growth curves in Fig. 2 illustrate the similarity of growth of Nima and PutS2 in minimal medium and the dissimilarity of growth when proline is used as a carbon or nitrogen source. As the cells entered stationary phase, prodigiosin was synthesized by both Nima and the mutants in any medium in which growth occurred. Nima used proline as a carbon or nitrogen source for growth, whereas the mutant did not. These results indicated that the mutant was defective either in proline transport or degradation, or both. The possibility of defective proline transport was eliminated by the observations that [^{14}C]proline was taken up by the mutant (Fig. 3) and was incorporated into cellular protein (Table 1).

Proline transport in Nima and PutS2. Figure 3 presents a comparison of proline transport by Nima and PutS2. In both exponentially growing and nonproliferating cells, the mutant consistently retained larger amounts of proline from the medium than Nima. Nima accumulated 21% of the total counts, whereas PutS2 took up 34% after 15 min of incubation.

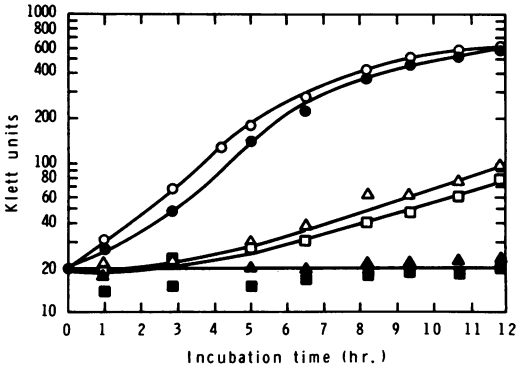

FIG. 2. *Growth curves of Nima and PutS2 at 27°C. Growth of Nima (open symbols) and PutS2 (closed symbols) is compared in minimal medium (circles), C$^-$ medium + 0.1 M L-proline (squares), and N$^-$ medium + 0.1 M L-proline (triangles). Inoculation was from an overnight culture in minimal medium.*

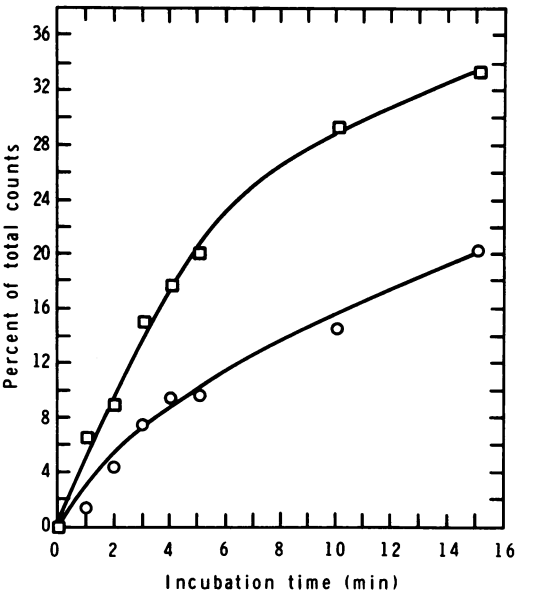

FIG. 3. *Proline transport in Nima* (○) *and PutS2* (□). *Equivalent concentrations of cells in exponential growth in minimal medium were measured for proline transport in minimal medium containing 0.1 μCi of [^{14}C]proline and 10 μg of proline/ml. At various times during incubation at 27°C on a rotary shaker water bath, cells were placed on 0.45-nm membrane filters and washed with 10 ml of 0.85% saline. The ^{14}C content of cells was counted in a scintillation counter and expressed as percentage of total counts.*

Protein synthesis in PutS2. At 27°C, the increase in the rate of protein synthesis paralleled the biosynthesis of prodigiosin (25). This metabolic activity was present in a suspension of Nima after addition of either proline or alanine. No increase in the rate of protein synthesis occurred in mutant PutS2 after addition of proline to nonproliferating cells, whereas alanine stimulated a marked increase (Fig. 4). Incubation of nonproliferating cells of mutant PutS2 with alanine resulted in prodigiosin synthesis within 8 h, whereas no pigmentation occurred in the presence of proline.

Proline oxidase activity. To determine the involvement of proline degradation in prodigiosin biosynthesis, proline oxidase was measured during a typical growth cycle and also in suspensions of nonproliferating bacteria. In bacteria cultured at 38 or 27°C in minimal medium without added proline, an increase in specific activity of proline oxidase coincided with the end of exponential growth (Fig. 5). In cells incubated at 27°C, this higher specific activity oc-

TABLE 1. *Incorporation of [^{14}C]proline into prodigiosin and bacterial protein*[a]

Component	Bacterial strain								
	Nima			PutS1			PutS2		
	Total counts (cpm/ml)	Utilization (% input)[b]	Sp act (cpm/ μg)	Total counts (cpm/ml)	Utilization (% input)	Sp act (cpm/ μg)	Total counts (cpm/ml)	Utilization (% input)	Sp act (cpm/ μg)
Prodigiosin	28,885	6.94	250.9	71,984	15.31	297.4	85,314	18.17	308.8
Protein	46,976	11.29	23.5	34,621	7.37	17.8	25,550	5.44	13.5

[a] Each suspension was allowed to synthesize prodigiosin by addition of 0.1 M alanine plus 0.2 mM methionine. After 10 h of incubation, [^{14}C]proline was added (4.15 × 10^5 cpm/ml, final concentration). At 17 h of incubation, samples were removed for purification of prodigiosin and protein. The total amount of prodigiosin formed between 10 and 17 h of incubation, expressed as micrograms per milliliter of bacterial suspensions, was for Nima, 115.0, PutS1, 242.0, and PutS2, 276.3. Specific activity of the [^{14}C]proline in the suspensions was 4.15 × 10^5 cpm per 0.209 μg of proline/ml.

[b] Definition of percentage of input is given in Materials and Methods.

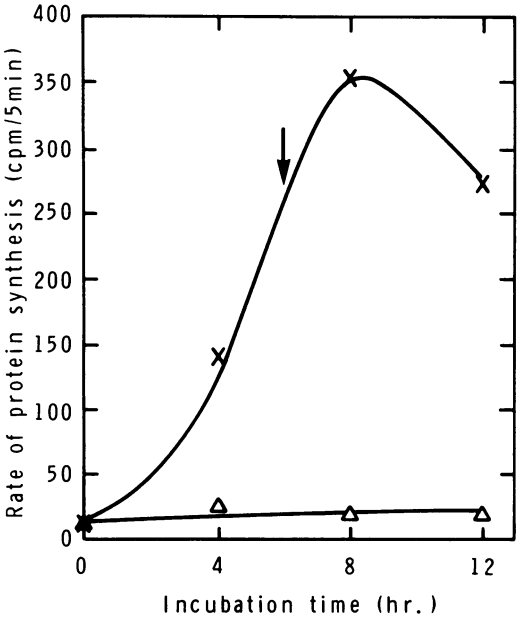

FIG. 4. *Rate of protein synthesis in nonproliferating cells of PutS2 incubated at 27°C in a rotary shaker water bath. Rate of* L-[^{14}C]*leucine incorporation into protein was determined after addition of 0.2 mM* L*-methionine and 0.1 M* L*-proline* (△) *or* L*-alanine* (×). *The arrow indicates the time at which prodigiosin was first observed in suspensions containing alanine.*

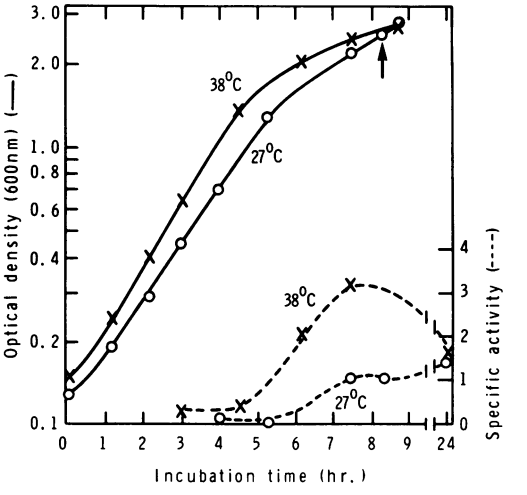

FIG. 5. *Growth (solid lines) and proline-oxidase activity (dashed line) measured in wild-type strain Nima grown on minimal medium at 38°C* (×) *or 27°C* (○) *in a rotary shaker water bath. Enzyme activity was determined at various times during growth. The arrow indicates the time at which prodigiosin was first observed in cultures grown at 27°C. No prodigiosin was synthesized at 38°C.*

curred just before the onset of prodigiosin biosynthesis. Minimal medium contained no proline, a fact that probably accounted for the lack of proline oxidase in the early phases of bacterial growth.

When a suspension of nonproliferating cells was prepared from *S. marcescens* strain Nima grown at 38°C, the initial level of proline oxidase reflected enzyme in cells harvested from minimal medium during stationary phase (Fig. 6). Incubation of these nonproliferating cells with proline caused a significant increase in specific activity by 6 h when prodigiosin synthesis commenced (Fig. 6). During incubation of nonproliferating cells of Nima with L-alanine, a continual decline in the specific activity of proline oxidase was observed, although pigmentation started at the same time as when incubated with proline.

Neither PutS1 nor PutS2 formed prodigiosin in suspensions of nonproliferating cells during

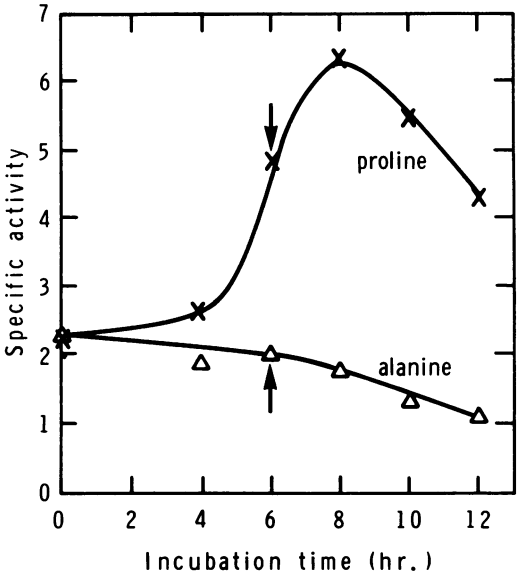

FIG. 6. *Proline oxidase activity in nonproliferating cells of Nima. The specific activity is expressed as pyrroline-5-carboxylate-o-aminobenzaldehyde complex formed in the presence of 0.2 mM* L*-methionine and either 0.1 M* L*-proline* (×) *or 0.1 M* L*-alanine* (△). *The arrows indicate the time at which prodigiosin was first observed.*

incubation with 0.1 M L-proline plus 0.2 mM L-methionine, but both synthesized prodigiosin when incubated with 0.1 M L-alanine plus 0.2 mM methionine. When grown on minimal medium or minimal medium supplemented with 0.1 M proline, proline oxidase activity was not detected in either mutant.

Incorporation of proline into prodigiosin and protein. To determine if the two mutants deficient in proline catabolism could incorporate carbon from proline into prodigiosin, nonproliferating cells of Nima, PutS1, and PutS2 were induced to synthesize prodigiosin by addition of alanine plus methionine. During incubation at 27°C in the rotary shaker water bath, prodigiosin was observed after 6 h of incubation and continued to accumulate to the 10th h of incubation, when [^{14}C]proline was added. After additional incubation for 7 h, all three strains incorporated the [^{14}C]proline into cellular protein and into prodigiosin (Table 1). The incorporation of ^{14}C from proline into protein indicated the existence of proline transport in the Put mutants. Although proline was not catabolized by the mutants and did not induce prodigiosin biosynthesis in nonproliferating cells of these mutants, [^{14}C]proline was incorporated into prodigiosin, when metabolism of the bacteria was effected by addition of alanine. These observations indicated that proline oxidase was not required for biosynthesis of prodigiosin and that proline was incorporated intact into the pigment.

DISCUSSION

Several amino acids caused biosynthesis of prodigiosin when added to suspensions of nonproliferating *S. marcescens* incubated at 27°C (13, 23). All of these amino acids also were used as sole sources of carbon and nitrogen for growth of strain Nima (25), and carbon from some of them was incorporated into prodigiosin synthesized by growing bacteria (10, 15, 18, 19). An example of the incorporation was proline, whose carbon structure was found intact in the pigment (18, 19). Our present study was initiated to answer two questions about the role of proline in the biosynthesis of prodigiosin by nonproliferating bacteria. First, must proline be degraded and utilized for metabolism before it could cause prodigiosin formation? And second, if mutants that could not catabolize proline were caused to synthesize prodigiosin by addition of another amino acid, would added [^{14}C]proline be incorporated into the pigment? A positive answer to the latter question would suggest a novel biosynthetic role for proline and also might provide a system uncomplicated by metabolic degradation and subsequent utilization to investigate the enzymatic reactions for incorporation of intact proline into prodigiosin. As presented in our results, the answer to both questions was positive. When added by itself to suspensions of nonproliferating bacteria, proline did not cause biosynthesis of prodigiosin unless it was catabolized. However, if addition of another amino acid such as alanine promoted metabolism and biosynthesis of the pigment in Put mutants, proline was incorporated, although not catabolized.

Since the prototroph, Nima, used proline as a carbon and nitrogen source for growth (25; Fig. 2), it must degrade the amino acid. Degradation of proline in *Salmonella typhimurium* (5) and *Escherichia coli* (6) involved two enzymes: proline oxidase that converted L-proline to Δ'-pyrroline 5-carboxylic acid (P5C) and P5C dehydrogenase that converted P5C to glutamic acid. The first enzyme in proline degradation, proline oxidase, is functional in *S. marcescens*. However, proline oxidase did not participate directly in prodigiosin biosynthesis since Put mutants synthesized prodigiosin in the stationary phase of growing cultures and as suspensions of nonproliferating bacteria when incubated with alanine or histidine (25), when no

proline oxidase was detected.

Since the Put mutants were incapable of degrading proline, the presence of ^{14}C from proline in the prodigiosin extracted from these mutants, after pigment biosynthesis was effected by addition of alanine, indicated that proline was a precursor to prodigiosin. In an examination of the products formed from chemical degradation of radiolabeled prodigiosin isolated from growing cells (18), all of the carbon atoms of proline were accounted for in the prodigiosin molecule and in a configuration corresponding to proline. This conclusion was substantiated by studies using ^{13}C labeling of prodigiosin and nuclear magnetic resonance analysis (19). The inability of proline alone to cause pigmentation in nonproliferating cells of the Put mutants arose from the lack of degradation of proline and the resulting loss of both the energetics for cellular metabolism and metabolites necessary for prodigiosin biosynthesis.

Amino acids like alanine that cause pigmentation during incubation of nonproliferating bacteria also may serve the dual role of precursor and primary metabolite. Some of the other amino acids like histidine may serve only as primary metabolites and not direct precursors to prodigiosin.

Synthesis of secondary metabolites may provide a mechanism to dispose of potentially toxic amounts of primary metabolites (20, 21) and to establish intracellular conditions least harmful to the cell. As shown in Fig. 5, increases in proline oxidase and prodigiosin biosynthesis occur sequentially at the onset of the stationary phase of growth at 27°C in growing cultures of *S. marcescens* strain Nima. Juxtaposition of proline catabolism with the end of active bacterial growth suggests degradation of amino acids as one mechanism for the bacteria to maintain metabolism during the stationary phase. However, this suggestion does not explain the role of proline as a direct precursor for prodigiosin.

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