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Induction of Prodigiosin Biosynthesis after Shift-Down in Temperature of Nonproliferating Cells of *Serratia marcescens*

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Nonpigmented bacteria obtained by growth of *Serratia marcescens* at 38 C synthesized prodigiosin at 25 C if certain individual amino acids were added to cultures of nonproliferating cells. In order of effectiveness, the amino acids were: DL-histidine, L-proline, L-hydroxyproline, DL-alanine, L-alanine, DL-aspartic acid, D-alanine, DL-proline, L-serine, L-ornithine, L-glutamic acid, and D-proline. DL-Histidine at its optimal concentration (20 mg/ml) induced formation of prodigiosin (198 μg of prodigiosin per mg of bacterial protein) after incubation of cultures for 54 hr. Lower concentrations (10 mg/ml) of the other amino acids usually were optimum but less prodigiosin was synthesized, and the maximal amount of pigment occurred between 36 and 48 hr. DL-Methionine was not effective alone but at a low concentration (40 μg/ml) enhanced and accelerated biosynthesis of prodigiosin in the presence of other suitable amino acids. Addition of 2 mg of L-proline per ml at 0 hr induced formation of only 30 μg of prodigiosin after incubation for 42 hr, but addition at 36 hr of 5 mg more of L-proline per ml increased synthesis to 120 μg at 42 hr. Again, DL-methionine markedly augmented prodigiosin biosynthesis in these cultures. Synthesis of prodigiosin ceased if cultures were shifted from 25 to 38 C. Prodigiosin biosynthesis by the nonproliferating cells was maximum when cultures were aerated, the amount of bacterial protein was about 2.0 mg/ml, and amino acids were added at 0 hr. Bacteria synthesized prodigiosin most efficiently when they were harvested from aerated cultures grown at 38 C for 24 hr in a complete medium in a fermentor.

Serratia marcescens does not synthesize prodigiosin when cultures are grown at 38 C (5). However, if these nonpigmented bacteria are suspended in saline at 27 C, formation of prodigiosin can be induced in the nonproliferating cells by individual addition of certain amino acids (4). Although in this system biosynthesis of prodigiosin can be investigated separately from cellular multiplication, the slow production of small amounts of pigment will hamper such studies. In this paper we detail investigations to determine what factors influencing growth at 38 C and prodigiosin biosynthesis at 25 C can be manipulated to enable nonproliferating cells to synthesize greater amounts of pigment more rapidly. The results establish conditions whereby nonproliferating cells not only synthesize as much prodigiosin as growing cultures but whereby maximal synthesis occurs earlier as well.

MATERIALS AND METHODS

Organism, growth media, and growth conditions. *S. marcescens* strain Nima was carried in stock as previously described (5). For inoculation of cultures, bacteria were grown in liquid minimal medium at 38 C for 24 hr on a rotary shaker operating at 200 rev/min (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). The nonpigmented bacteria were harvested from the cultures by centrifugation, were washed three times with distilled water, and then were suspended to a concentration of 0.8 mg (dry weight) of cells per ml of distilled water. One milliliter of this suspension was inoculated per liter of the various growth media.

Cultures were grown in either a minimal or a complete liquid medium (5). Small quantities of bacteria for experiments were obtained by growing cultures in 250- or 1,000-ml Erlenmeyer flasks containing 25 or 100 ml of medium, respectively. Flasks were incubated at 38 C either on the rotary shaker operating at 200 rev/min or as stationary cultures. Larger quantities of nonpigmented bacteria were

produced by growth in a 14-liter fermentor (The Virtis Co., Inc., Gardiner, N.Y.) containing 8 liters of the appropriate medium. Cultures were incubated in the fermentor at 38 C while being aerated with air at a pressure of 15% and while being stirred at 400 rev/min. No prodigiosin was synthesized by cultures grown at 38 C.

Preparation of suspensions of nonpigmented, nonproliferating cells. After growth at 38 C, nonpigmented bacteria were harvested from the culture vessels by centrifugation at 10,000 rev/min for 20 min at 5 C in a refrigerated centrifuge (Sorvall RC2-B, Ivan Sorvall, Inc., Norwalk, Conn.). The cells were washed three times with distilled water by similar centrifugation and then were suspended in 0.85% (w/v) saline to give an optical density of 2.5 to 2.6 at 600 nm in a spectrophotometer. This density was equivalent to about 1.8 to 2.0 mg of protein per ml, or about 10^9 viable cells per ml. Suspensions of cells for experiments were distributed in 30-ml quantities into 250-ml Erlenmeyer flasks stoppered with cotton. Except for studies to determine the response of nonproliferating cells to various amino acids, all suspensions contained 10 mg of L-proline per ml to induce prodigiosin biosynthesis (4). After addition of amino acids to the flasks, the cultures were incubated at 25 C for the appropriate length of time on the rotary shaker set at 200 rev/min.

Analytical procedures. Prodigiosin was extracted from cells with acidic methanol (4.0 ml of 1 N HCl-96.0 ml of methanol) and then was measured by the procedure of Goldschmidt and Williams (1) in which a difference in absorbancy of 1.0 between 534 and 655 nm is equivalent to 19.3 μg of prodigiosin. Protein was measured by the method of Lowry et al. (2) by using bovine serum albumin as a standard. All spectrophotometric determinations were measured in an Hitachi-Perkin-Elmer, model 139, spectrophotometer.

Viable counts were determined by standard plate count procedures by using complete medium solidified with agar. After incubation at 27 C for 48 hr, plates were counted. In counting, no distinction was made between surface or subsurface or between pigmented or nonpigmented colonies.

Chemicals. Amino acids were the purest grade obtainable from either Nutritional Biochemicals Corp., Cleveland, Ohio, or from Calbiochem, Los Angeles, Calif. The compounds were used as purchased without additional purification.

RESULTS

Influence of growth conditions at 38 C on induction of pigmentation in nonproliferating cells at 25 C. The data in Fig. 1 and Table 1 show that prodigiosin biosynthesis by nonproliferating cells incubated at 25 C following growth at 38 C was affected by a number of factors such as the medium in which the bacteria were grown, the extent of aeration of the growing cultures, and the time at which the nonpigmented cells were harvested. Bacteria harvested after 24 hr of growth in complete medium in a fermentor produced the maximal amount of prodigiosin at 25 C. These growth conditions were utilized for the rest of the experiment.

Bacteria harvested after growth at 38 C can be stored frozen at −10 C as packed cells for a

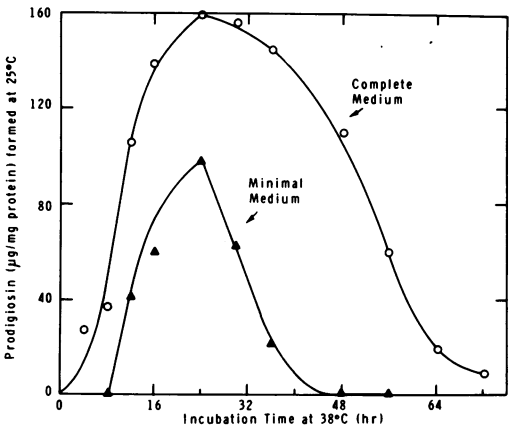

FIG. 1. *Biosynthesis of prodigiosin at 25 C by nonproliferating cells of Serratia marcescens harvested after aerated growth at 38 C in a fermentor for various lengths of time in minimal or complete medium. All cultures incubated at 25 C contained 1.9 mg of bacterial protein and 10 mg of L-proline per ml. Prodigiosin was measured after incubation for 48 hr on a rotary shaker operating at 200 rev/min.*

TABLE 1. *Effect of growth conditions at 38 C on subsequent pigmentation at 25 C by nonproliferating bacteria*[a]

Cultural condition for growth at 38 C	Medium	Time[b] bacteria were harvested (hr)	Maximal amount of prodigiosin (μg/mg of protein) formed at 25 C	Time[c] prodigiosin was measured (hr)
Stationary	Minimal	38	56	120
	Complete	38	86	120
Shaken	Minimal	24	84	48
	Complete	24	121	48
Fermentor	Minimal	24	98	40
	Complete	24	159	40

[a] Cultures of nonproliferating cells at 25 C contained 1.9 mg of bacterial protein and 10 mg of L-proline per ml and were incubated on a rotary shaker operating at 200 rev/min.

[b] Optimal time of growth at 38 C that yielded maximal amount of prodigiosin at 25 C.

[c] Time at which maximal synthesis of prodigiosin occurred at 25 C. This time varied little from cells grown in minimal or complete medium at 38 C.

limited period of time. The maximal amount of prodigiosin synthesized at 25 C dropped about 5% after storage for 1 week and about 25% after 2 weeks. Only about 40% of the original activity remained after storage for 5 weeks. In contrast, suspensions stored at 4 C lost 70% of their activity within 1 week and at the end of 5 weeks retained less than 10% of their activity. Suspensions incubated at 25 C lost all activity by 48 hr (Fig. 3).

Factors affecting prodigiosin biosynthesis by nonproliferating cells incubated at 25 C. The amount of prodigiosin synthesized by nonproliferating cells increased almost linearly between protein concentrations of 1 to 2 mg/ml (Fig. 2). Above a concentration of 2 mg/ml the linear relationship dwindled, and the maximal amounts of pigment synthesized varied only slightly betwee 2.6 and 3.0 mg of bacterial protein per ml.

Saline suspensions of nonproliferating bacteria incubated in L-proline (10 mg/ml) on a rotary shaker synthesized about four times as much prodigiosin as similar preparations incubated as stationary cultures (Table 2). Pigment also is detected earlier and reaches a maximal concentration sooner. As recently reported by us (3), addition of a low concentration of DL-methionine (40 μg/ml) markedly enhances and accelerates biosynthesis of prodigiosin by nonproliferating bacteria incubated with L-proline (Table 2; Fig. 4). Cysteine effects a similar, but lesser, response. Table 2 shows that the amount of prodigiosin synthesized by nonproliferating cells suspended in methionine and proline was as great as the amount formed by bacteria growing in complete medium. Prodigiosin biosynthesis always proceeds more quickly in cultures of nonproliferating cells

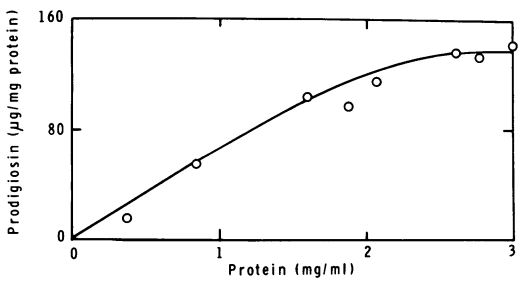

FIG. 2. *Effect of concentration of bacterial protein upon biosynthesis of prodigiosin by nonproliferating cells. Nonpigmented bacteria were harvested after growth at 38 C in complete medium for 24 hr in a fermentor. Saline suspensions of nonproliferating bacteria were incubated in 10 mg of L-proline per ml at 25 C for 48 hr on a rotary shaker operating at 200 rev/min.*

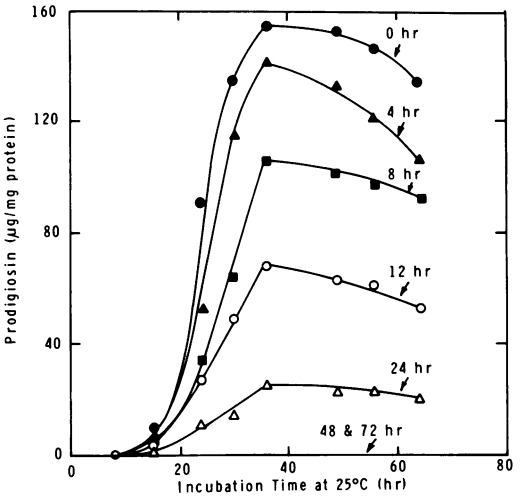

FIG. 3. *Kinetics of prodigiosin biosynthesis when L-proline (10 mg/ml) is added to nonproliferating bacteria (2.0 mg of protein per ml) at different periods of time after initiating incubation at 25 C on a shaker. Times of addition are indicated on the curves. Nonpigmented cells were prepared as indicated in Fig. 2.*

that are shaken than in growing bacteria incubated under similar conditions.

Individual addition of 8 amino acids, out of 24 examined, induced biosynthesis of prodigiosin in shaking cultures of nonproliferating cells (Table 3). Histidine and hydroxyproline were effective in addition to the other amino acids that induced pigmentation in stationary cultures of nonproliferating cells (4). Although methionine and cysteine markedly enhanced biosynthesis of prodigiosin (Table 2; Fig. 4), neither of these amino acids alone at any concentration could induce pigmentation. Except for histidine and alanine, the maximal amount of pigment was synthesized at an amino acid concentration of 10 mg/ml. At a concentration of 20 mg/ml, histidine induced formation of the greatest amount of prodigiosin. Whereas in stationary cultures of nonproliferating cells alanine (5 mg/ml) and proline (10 mg/ml) were about equally effective (4), in shaking cultures more than twice as much prodigiosin was synthesized after the addition of proline (Table 3). The D- and DL-forms of proline were inefficient when compared with the L-form, and D-alanine was about half as efficient as the L- or DL-forms. In suspensions of cells containing 5 mg each of D- and L-proline per ml, biosynthesis of prodigiosin was not inhibited when compared with a control containing only 5 mg of L-proline per ml.

As shown in Fig. 3, if addition of L-proline

TABLE 2. *Biosynthesis of prodigiosin by growing culture and by nonproliferating bacteria suspended in L-proline*[a]

Culture	Medium	Biosynthesis of prodigiosin		
		Time first detected (hr)	Time of maximal content (hr)	Maximal amount (μg/mg of protein)
Growing on shaker	Complete	18	48	302
Nonproliferating stationary	L-Proline (10 mg/ml)	24	120	42
Nonproliferating on shaker	L-Proline (10 mg/ml)	10	40	160
	L-Proline (10 mg/ml) plus DL-methionine (40 μg/ml)	6	16	360

[a] Cultures were incubated at 25 C, and, when indicated, on a rotary shaker operating at 200 rev/min. Protein concentration of a growing culture was 2.0 mg/ml at 48 hr and of all nonproliferating cultures, 1.9 mg/ml. Amino acids were added to saline suspensions of nonproliferating cells at 0 hr. Nonproliferating cells were prepared from bacteria grown at 38 C for 24 hr in a fermentor in complete medium.

was delayed, biosynthesis of prodigiosin by nonproliferating cells decreased until no pigment was produced after a delay of between 24 and 48 hr. A similar set of curves was obtained for the other amino acids capable of inducing pigmentation. Thus, once suspensions of nonproliferating cells have begun incubating at 25 C, amino acids must be added almost immediately to induce maximal synthesis of prodigiosin.

A rapid increase in biosynthesis of prodigiosin by nonproliferating cells could be achieved by the manipulation shown in Fig. 4. Nonpigmented cells were induced to pigment by addition of a small amount of L-proline (2 mg/ml), with or without DL-methionine (40 μg/ml), that permitted synthesis of a small amount of prodigiosin. When the amount of prodigiosin reached a maximum at 36 hr, more proline (5 mg/ml) was added to the culture. Biosynthesis of prodigiosin rose sharply to a maximum 8 hr later. The initiating and stimulatory amino acids need not be identical, i.e., pigment formation could be initiated by addition of a small concentration of proline and then stimulated by addition of a larger concentration of proline, alanine, or histidine. Similarly, the initiating amino acid could be alanine or histidine, and the stimulatory addition proline, alanine, or histidine. The initial presence of methionine always markedly increased the amount of pigmentation but had no effect if added along with the stimulatory amino acid at 36 hr.

Growing cultures of *S. marcescens* strain Nima do not synthesize prodigiosin when incubated at 38 C. Data in Table 4 show that, if nonpigmented, nonproliferating bacteria induced to synthesize prodigiosin at 25 C by addition of L-proline are shifted to 38 C, not only does formation of pigment cease, but the amount of prodigiosin decreases after incubation for 48 hr at the higher temperature. We have not examined the kinetics of decrease in amount of prodigiosin after shift to 38 C, but the results establish that nonproliferating cells do not continue to synthesize prodigiosin when shifted to the higher temperature.

DISCUSSION

Formation of prodigiosin by nonproliferating cells is influenced not only by conditions of incubation at 25 C but also by the antecedents of the nonpigmented bacteria. Growth at 38 C, under highly aerobic conditions in a rich medium, may provide actively metabolizing cells that contain intermediates needed for biosynthesis of pigment. During storage of nonprolif-

TABLE 3. *Induction by individual amino acids of prodigiosin biosynthesis in nonproliferating bacteria*[a]

Amino acid	Values for maximal pigmentation			Time pigmentation first detected (hr)
	Concentration of amino acid (mg/ml)	Amount of prodigiosin (μg/mg of protein)	Age of culture (hr)	
L-Alanine	5	68	36	8
D-Alanine	5	32	42	12
DL-Alanine	10	70	42	8
DL-Aspartic acid	10	37	45	18
L-Glutamic acid	10	7	48	24
DL-Histidine	20	198	54	18
L-Hydroxyproline	10	75	48	10
L-Ornithine	10	10	48	24
L-Proline	10	165	36	10
D-Proline	10	4	48	24
DL-Proline	10	28	42	10
L-Serine	10	25	48	18

[a] Cultures were incubated at 25 C on a rotary shaker operating at 200 rev/min. Protein concentration of all suspensions was 2.0 mg/ml; amino acids were added at 0 hr. Nonproliferating cells were prepared from bacteria grown at 38 C for 24 hr in a fermentor in complete medium.

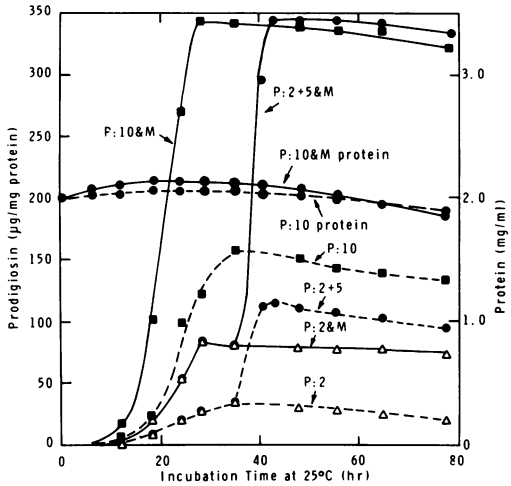

FIG. 4. *Effect of sequential addition of* L-*proline, or* L-*proline plus* DL-*methionine, upon kinetics of prodigiosin biosynthesis by nonproliferating cells. Initial addition of proline (2 mg/ml) without (curve P:2) or with 40 µg of* DL-*methionine per ml (curve P:2 & M) was at 0 hr. At 36 hr the cultures were divided, 5 mg of* L-*proline per ml was added to half of each (curves P:2 + 5, and P:2 + 5 & M), and incubation of all cultures continued. Control cultures contained* L-*proline (10 mg/ml) without (curve P:10) or with 40 µg of* L-*methionine per ml (curve P:10 & M) added at 0 time. Protein concentrations measured after extraction of prodigiosin from the control cultures are shown by curves P:10 protein and P:10 & M protein. Preparation of bacteria and conditions of incubation were as indicated in Table 3.*

erating cells, these intermediates may be depleted so that pigmentation cannot be initiated. The requisite early addition of amino acids at 25 C corroborates the hypothesis that certain cellular metabolites must be preserved in nonproliferating cells. More aerobic cultural conditions also influence the metabolism of nonproliferating cells because the amino acid requirements and concentrations for induction of prodigiosin biosynthesis (Table 3) vary from those found for stationary cultures (4).

The puzzling requirement for high concentrations of amino acids for most effective induction of pigmentation may also be related to the need for intermediates. In addition to utilization for formation of prodigiosin, the amino acids may be utilized as carbon and nitrogen sources for cellular energy and other biosyntheses. Lower concentrations may be ineffective for prodigiosin biosynthesis because pigmentation is a secondary process that can utilize excess concentrations of amino acids only after primary biosyntheses have occurred. Contrasted to these needs is the marked stimulation provided by a small amount of methionine. This amino acid may stabilize nonproliferating bacteria in some way to provide enhanced biosynthesis of prodigiosin, as well as contribute methyl groups to the pigment (3).

TABLE 4. *Inhibition of prodigiosin biosynthesis by nonproliferating bacteria by a shift from 25 to 38 C*[a]

Age of culture at 25 C (hr)	Prodigiosin concentration (µg/mg of protein)	
	Immediately before shift	After 48 hr at 38 C
0	0	0
6	0	0
12	6	0
18	24	8
24	98	29
28	133	41
35	157	56
48	156	64
Control (48)	152	

[a] Preparation and incubation of nonproliferating cells was as described in Table 3. L-Proline (10 mg/ml) was added at 0 hr to all cultures. At the times indicated, prodigiosin concentration of a sample was measured, and the culture was moved to 38 C. After incubation for an additional 48 hr at 38 C on a shaker, prodigiosin was measured again.

Rapid formation by nonproliferating cells of an ample quantity of prodigiosin, particularly by the procedure shown in Fig. 4, will permit isotopic studies. Combination of this procedure with a shift to higher temperature to inhibit pigment formation may permit detection of intermediates. Such investigations may answer the questions about why high concentrations of amino acids are required, whether the amino acids are needed for primary biosyntheses before they can be utilized for pigment formation, and the extent to which various amino acids are involved in biosynthesis of the two moieties of prodigiosin, 2-methyl-3-amylpyrrole and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (6).

ACKNOWLEDGMENTS

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