Induction of Pigmentation in Nonproliferating Cells of *Serratia marcescens* by Addition of Single Amino Acids

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Addition of casein hydrolysate to suspensions of washed, nonpigmented, nonproliferating *Serratia marcescens* incubating at 27°C induced biosynthesis of prodigiosin. Four amino acids of casein hydrolysate, DL-aspartic acid, L-glutamic acid, L-proline, and L-alanine caused formation of pigment when added individually. DL-Ornithine also was effective. Optimal concentrations for maximal pigmentation were 5 to 10 mg/ml; at these high concentrations, D-serine also induced biosynthesis of some prodigiosin. DL-Alanine and -ornithine were as effective as the L-isomers, but L-glutamic acid and L-proline gave better responses than their racemic mixtures. Kinetics of prodigiosin biosynthesis after addition of DL-alanine (20 mg/ml) were similar to those of cells suspended in 0.2% casein hydrolysate. The other amino acids were less effective. Addition of 5 mg of DL-alanine or casein hydrolysate per ml to minimal medium increased by 30% the amount of prodigiosin formed by growing cells after incubation for 7 days at 27°C. Cultures grown for 7 days at 27°C in 0.2% casein hydrolysate formed more prodigiosin than did suspensions of nonproliferating cells containing individual amino acids or casein hydrolysate. However, more pigment was produced by cells suspended in L-alanine (5 mg/ml) or L-proline (10 mg/ml) than when suspended in 0.4% natural or synthetic casein hydrolysate. Filtrates from suspensions of nonproliferating cells forming pigment in L-proline induced more rapid formation of prodigiosin, but filtrates from suspensions in DL-alanine did not. The data supported the hypothesis that pyrrole groups of prodigiosin may be synthesized from 5-carbon amino acids such as proline, ornithine, aspartic, and glutamic acids, but the role of alanine is unknown.

The preceding report (14) describes conditions in which multiplication of bacteria can be separated from biosynthesis of prodigiosin by *Serratia marcescens*. After growth to the stationary phase at 38°C, the nonpigmented cells are washed, suspended in saline, and incubated at 27°C. Addition of casein hydrolysate to the suspensions incubating at the lower temperature induces pigment formation without concurrent growth.

This paper establishes that the single addition of only some of the amino acids present in casein hydrolysate induces pigment formation after the shift down in temperature. By using this system, biosynthesis of prodigiosin can be investigated separately from cellular multiplication.

**MATERIALS AND METHODS**

**Organism and growth medium.** Inocula were prepared from strain Nima of *S. marcescens* as described previously (14). Cultures were grown without shaking at 38°C for 72 hr in 10 ml of minimal medium (14) contained in 50-ml Erlenmeyer flasks. Since a drop in temperature below 37°C will permit pigmentation, cultures were immersed in a water bath, and the temperature of incubation was continuously monitored with a recording thermometer. Only nonpigmented bacteria obtained from cultures continuously incubated within a range of 37.5 to 38.5°C for 72 hr were harvested for the experiments. In some experiments, cultures were grown in complete medium (14) rather than in minimal. When biosynthesis of prodigiosin was being examined, cultures were incubated at 27°C.

**Preparation of suspensions of nonproliferating cells.** After growth into the stationary phase at 38°C, nonpigmented cells were harvested from the cultures and washed three times in 0.85% saline buffered at pH 7.2 with 0.01 M phosphate buffer. The packed cells were then suspended to a density of 1.7 to 1.8 mg of protein per ml in 10 ml of 0.85% saline containing the appropriate supplement of amino acid in a 50-ml Erlenmeyer flask. Suspensions of nonproliferating cells were incubated without shaking in a water bath at 27°C for the appropriate times indicated in the experiments, before measuring prodigiosin.
Amino acid solutions. Synthetic casein hydrolysate equivalent to a concentration of 0.4% of N-Z Case peptone (a trypic digest of casein) was formulated according to data provided by Sheffield Chemical, Norwich, N.Y. The mixture contained the following concentrations of amino acids (mg/ml): L-alanine, 0.13; L-arginine, 0.14; DL-aspartic acid, 0.24; L-cystine, 0.02; L-glutamic acid, 0.91; glycine, 0.07; L-histidine monohydrochloride, 0.12; DL-isoleucine, 0.18; L-leucine, 0.41; L-lysine, 0.26; DL-methionine, 0.09; DL-phenylalanine, 0.18; L-proline, 0.4; DL-serine, 0.27; DL-threonine, 0.15; DL-tryptophan, 0.06; L-tyrosine, 0.14; and DL-valine, 0.22. The final solution was adjusted to pH 7.6 to 7.8 and sterilized by filtration. The following amino acids not contained in N-Z Case were used in some experiments: DL-α-aminobutyric acid, L-cystine hydrochloride, hydroxy-L-proline, DL-norleucine, DL-ornithine monohydrochloride, and D-serine. Amino acids were of the purest grade obtainable from either Nutritional Biochemical Corp., Cleveland, Ohio, or from Calbiochem, Los Angeles, Calif.

Preparation of filtrates. DL-Alanine (20 mg/ml) or L-proline (10 mg/ml) were added to suspensions of nonproliferating bacteria incubating at 27 C. After 1, 3, 5, or 7 days of incubation, cells were sedimented from the suspensions by centrifugation at 4 C. The supernatant fluid was decanted and sterilized by filtration through a Seitz filter. Freshly prepared suspensions of nonproliferating cells were then added to these filtrates for the experiments. No growth was evident when samples from sterilized filtrates were inoculated into complete broth (14), and the tubes were incubated at 27 and 38 C for 48 hr.

Analytical procedures. Extraction and quantitative measurement of prodigiosin was carried out by the procedures outlined previously (14). The procedure of Lowry et al. (7) was used to determine protein, by using bovine serum albumin as a standard. Viable counts were made by standard plate count procedures in complete medium (14).

RESULTS

The amino acids of casein hydrolysate were separated into six groups designed to facilitate characterization of bacterial mutants (6). Pigmentation occurred in three of the mixtures. When each amino acid of these three groups was evaluated individually to determine whether they could induce pigmentation, only 5 of the 13 tested had the ability. They were DL-aspartic acid, L-glutamic acid, L-proline, L-alanine, and DL-ornithine monohydrochloride. The optimal concentration of each of the five effective amino acids ranged from about 5 times the amount of the amino acid in 0.4% casein hydrolysate in the case of L-glutamic acid to 50 times in the case of DL-ornithine monohydrochloride (Table 1).

In some cases, the relative efficiency of L-, D-, or DL-forms of the amino acids was determined. No response occurred with D-glutamic acid, in

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Prodigiosin extracted at a concn of amino acid of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>4.0</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>2.2</td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>2.0</td>
</tr>
<tr>
<td>DL-Aspartic acid</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.8</td>
</tr>
<tr>
<td>D-Glutamic acid</td>
<td>0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.2</td>
</tr>
<tr>
<td>DL-Proline</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Ornithine monohydrochloride</td>
<td>Trace²</td>
</tr>
<tr>
<td>DL-Ornithine monohydrochloride</td>
<td>Trace³</td>
</tr>
<tr>
<td>D-Serine</td>
<td>0</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>0</td>
</tr>
</tbody>
</table>

* Suspensions of cells were incubated at 27 C in a water bath for 7 days without shaking. Protein concentration of suspensions was 1.8 mg/ml.

² At optimal concentration as determined from data in columns 2–5. L-Alanine, DL-alanine, DL-ornithine, and L-proline were used, but not D-alanine, L-ornithine, or DL-proline.

³ Although these cultures were visually pigmented, not enough prodigiosin was extracted for accurate measurement.
contrast to the L-form, and the L-form of proline was three times as effective as the Dl-form. In contrast, the Dl-forms of ornithine and of alanine were equally as efficient as the L-forms, although D-alanine alone was less effective.

Table 1 also shows the approximate time at which pigmentation first appeared when the optimal concentration of the most effective form of the amino acids was used. The appearance of pigment can be roughly categorized as early in the case of alanine, intermediate for aspartic and glutamic acids, and late for proline and ornithine. The time at which pigmentation is first detected with alanine is similar to the time pigmentation first appears in cultures of nonproliferating cells suspended in 0.2% casein hydrolysate (14).

Since the data in Table 1 indicated that high concentrations of amino acids produced more pigment in nonproliferating cells, we reexamined individually the amino acids of casein hydrolysate at concentrations of 5 and 10 mg/ml. Dl-serine was the only additional amino acid that produced visual pigmentation, and 5 and 10 mg of D-serine per ml permitted formation of a slight amount of measurable pigment (Table 1).

The much greater concentration of amino acids required for maximal production of prodigiosin by suspensions of nonproliferating cells suggested that perhaps addition of greater amounts of these amino acids to minimal medium might enhance pigment formation in growing cultures. Only Dl-alanine at a concentration of 5 mg/ml was effective (Table 2). A concentration of 20 mg of Dl-alanine per ml inhibited growth. Greater quantities of prodigiosin were also produced by growing cultures when casein hydrolysate was added to minimal medium. When 5 mg of either Dl-alanine or casein hydrolysate was added to minimal medium, about one-third more prodigiosin was formed (Table 2). However, more prodigiosin was synthesized per unit of cellular protein by cultures growing in casein hydrolysate than by those growing in alanine. Although more pigment was synthesized by cultures containing 10 mg of casein hydrolysate per ml, the efficiency of formation was less than in 5 mg/ml because of the increase in protein of the latter cultures. Greater concentrations of casein hydrolysate increased the amount of cellular protein in growing cultures, but the actual amount of prodigiosin formed decreased until, at a concentration of 50 mg of casein hydrolysate per ml, the ratio of prodigiosin to protein was only 1.2.

Prodigiosin formation by nonproliferating cells after addition of various supplements and by a culture growing in complete medium are shown in Fig. 1. At the end of incubation for 7 days, the most pigment is formed by the growing culture. The response of nonproliferating cells is almost the same for synthetic as for natural casein hydrolysate. When added at their most effective concentration, L-alanine and L-proline produced more prodigiosin in suspensions of nonproliferating cells than did natural casein hydrolysate, but combinations of the two were no more effective than each amino acid individually. Dl-Ornithine induced about 65% as much pigment as L-alanine or L-proline; L-glutamic acid, about 25%; and Dl-aspartic acid, only about 10%.

The kinetics of pigment formation by cultures of nonproliferating cells containing either DL-alanine or L-proline was similar to that of cells

### Table 2. Increased pigment formation by cultures growing in minimal medium after addition of alanine or casein hydrolysate

<table>
<thead>
<tr>
<th>Medium</th>
<th>Prodigiosin (µg/ml)</th>
<th>Protein (mg/ml)</th>
<th>Ratio of prodigiosin to protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal</td>
<td>12.7</td>
<td>2.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Minimal plus DL-alanine (5 mg/ml)</td>
<td>19.2</td>
<td>2.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Minimal plus casein hydrolysate (5 mg/ml)</td>
<td>20.0</td>
<td>1.8</td>
<td>11.1</td>
</tr>
<tr>
<td>Minimal plus casein hydrolysate (10 mg/ml)</td>
<td>22.1</td>
<td>2.1</td>
<td>10.6</td>
</tr>
</tbody>
</table>

* Cultures incubated without shaking for 7 days at 27°C before measuring prodigiosin and protein.

![Fig. 1. Prodigiosin formation by nonproliferating cells in the presence of various supplements and by a culture growing in complete medium. Prodigiosin was measured after incubation at 27°C for 7 days. Protein concentration in suspensions of nonproliferating cells was 1.7 to 1.8 mg/ml after 7 days; concentration in growing culture, 1.8 mg/ml.](http://jb.asm.org/Downloaded from http://jb.asm.org)
suspended in 0.2% casein hydrolysate (14). However, the response to proline was slower than to alanine. The lag period that occurred in cultures incubating in proline suggested that the amino acid might be converted to another substance that in turn induced formation of pigment. A series of experiments did establish that filtrates from cultures incubated for 1, 3, or 5 days in L-proline began synthesizing pigment at earlier times, although the amount of pigment formed after incubation for 7 days was not appreciably greater than in the amino acid alone.

Earlier or more rapid formation of prodigiosin did not occur in filtrates obtained from cultures of nonproliferating cells forming prodigiosin in DL-alanine. Similar experiments were not done with ornithine, although the longer lag period occurring with this amino acid before pigment formation began suggests that an intermediate may also be present. The bacteria removed from suspensions in DL-alanine contained various amounts of prodigiosin depending on the length of incubation before preparation of filtrates, but these cells were unable to produce more pigment when resuspended again in saline alone. Cells harvested while preparing filtrates from cultures containing L-proline also were unable to form additional prodigiosin when suspended in saline.

**DISCUSSION**

Several investigations indicated that amino acids are involved in the biosynthesis of prodigiosin, although the experimental results sometimes differed (1, 9, 10, 11, 12). Because these studies all used procedures in which growth of bacteria was not separated from pigment formation, the specific contribution of possible intermediates for pigment biosynthesis is difficult to determine. As Ingledew and Campbell point out (4, 5), when pigmentation is not separated from growth, the metabolic demands for intermediates during cellular multiplication can obscure the utilization of the same intermediates for pigment formation.

Marks and Bogorad (9) hypothesized that 5-carbon amino acids, such as proline, ornithine, and glutamic acid, are used for biosynthesis of prodigiosin, and that the pyrrole groups of the pigment are not synthesized through the succinate-glycine cycle, as are the pyrroles of porphyrins. Our results showed that in addition to proline, ornithine, and glutamic acid, the amino acids, alanine, aspartic acid, and serine, also induced formation of prodigiosin. Additional evidence suggesting that the pyrrole groups of prodigiosin are synthesized by a different pathway was the discovery that δ-aminolevulinic-5-14C acid was not incorporated into pigment formed by growing cells (9, 11). We found in experiments not reported here that δ-aminolevulinic acid also did not induce pigmentation in nonproliferating cells.

Preliminary experiments with L-proline suggest that an intermediate of the amino acid may be involved in biosynthesis of pigment, but more critical experiments are required before this implication can be established. The roles of other amino acids must be determined by further studies. The contribution of L-alanine is of interest because our data show that this amino acid is one of the most effective inducers of prodigiosin biosynthesis in nonproliferating cells.

In addition to a direct contribution for biosynthesis of pyrroles in prodigiosin, induction of pigmentation in nonproliferating cells by single amino acids offers other problems for investigation. Presumably, synthesis of prodigiosin involves enzymes. Does addition of the amino acid induce the required enzymes, or are the enzymes already present and merely activated? Inhibition of pigmentation in nonproliferating cells by chloramphenicol (14) suggests that protein synthesis is required. Since the nonproliferating cells are essentially starved, does addition of a single nutrient such as an amino acid enable these cells to initiate metabolic processes, including synthesis of macromolecules and intermediates, that result in biosynthesis of prodigiosin? Addition of an exogenous substrate to starved *Pseudomonas aeruginosa* enables the bacteria to reestablish their system for synthesizing protein and to form an induced enzyme (8). Perhaps addition of single amino acids to starved, nonproliferating cells of *S. marcescens* can induce metabolic processes that result in biosynthesis of prodigiosin.

If the suggestion that prodigiosin is a secondary metabolite is accepted (13), the utilization of amino acids for biosynthesis of the pigment may be less perplexing. Synthesis of secondary metabolites involves different enzymes, not common for biosynthesis of primary metabolites (2), and unknown pathways may be utilized (3). Biosynthesis of the pyrroles in prodigiosin from amino acids may reflect a form of secondary metabolism in *S. marcescens*. Suspensions of nonproliferating cells provide a system in which the biosynthesis of pigment can be separated from cellular multiplication, and investigations of pigmentation in *S. marcescens* may reveal new pathways, different from those involved in growth, for metabolism of amino acids. The principal deficiencies to be overcome are producing greater amounts of pigment more rapidly.

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This manuscript is dedicated to Stewart A. Koser on the occasion of his 75th birthday and in recognition of his distinguished career in teaching and research.

LITERATURE CITED
