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Control of Salmonellosis Pacifarin Biosynthesis by Iron

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Enterobacter cloacae strain SS_4-56 produced salmonellosis pacifarins when grown in an iron-depleted synthetic medium to which 3.16×10^{-7} g-atom of iron had been added per liter, but did not do so when grown in iron-depleted medium supplemented with 3.16×10^{-4} g-atom of iron per liter. The addition of 3.16×10^{-4} g-atom of ferric iron per liter to a pacifarin-containing culture supernatant fluid had no significant effect upon the ability of the active pacifarins present, when administered per os, to protect mice from an otherwise fatal infection produced by a sequential injection of avirulent and virulent strains of *Salmonella typhimurium*.

Mice obtained by a planned system of random breeding (4), when doubly infected with an avirulent and a virulent strain of *Salmonella typhimurium* and fed a semisynthetic basal diet supplemented with certain natural foodstuffs or with supernatant fluids from certain bacterial cultures, survive in significantly greater numbers than do comparable, similarly infected control mice fed only the basal diet, a diet demonstrably adequate for the growth and maintenance of uninfected animals (6). The agents responsible for the increase in survivorship seen in the infected mouse populations fed the protective diets appear to act neither as antibiotics with respect to the salmonellae nor as vitamins with respect to the mice. They have been referred to collectively as "the *Salmonella* resistance factor," or "SRF," and individually as "salmonellosis pacifarins" (5). The chemical identity of one of these "pacifarins" has been reported (7). Enterobactin, the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine, has been shown to have pacifarin activity at concentrations in the test diets as low as 2 mg per kg of diet. Certain bacterial products other than enterobactin are also now known to possess pacifarin activity, and two of them have recently been crystallized from *Enterobacter cloacae* culture supernatant fluids (unpublished data). Although the structures of these latter compounds are not yet fully established, all the pacifarins thus far isolated are catechol derivatives and all are very avid iron-sequestering agents.

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This report documents an experiment designed specifically to test the hypothesis that the biosynthesis of the pacifarins, but not their biological activity, is controlled by the concentration of iron in the media used to grow the bacteria which produce them. Pacifarin biosynthesis proceeds in "low-iron" environments and diminishes to nought in "high-iron" environments.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. A strain of *E. cloacae* (SS_4-56) known to generate pacifarin activity when grown on egg white or on simple, lactate-salts medium (H. A. Schneider and H. N. Wood, Fed. Proc. 18:545, 1959) was used. In the double inoculation test for pacifarin activity, the strains of *S. typhimurium* previously indicated (7) were used, and the test was carried out as previously described, with the following modification: the source of the avirulent cells used was a 13-h culture of *S. typhimurium* RIA-Tr in iron-depleted lactate-salts medium to which had been added, per liter, 3.16×10^{-7} g-atom of $FeSO_4$. This culture was appropriately diluted with sterile, physiological saline solution, for injection into the mice, at the time the bacterial cells had just begun to excrete catechol derivatives into the medium. As source of the virulent challenge organisms used in the test, an 18-h culture of *S. typhimurium* SR-11-GR-3 in Penassay broth (Difco) was utilized. The Penassay broth had been rehydrated and sterilized according to the directions given by the manufacturer. In this medium, strain SR-11-GR-3 does not excrete catechol derivatives during growth. The liquid cultures of all the bacteria used were grown in 250-ml Erlenmeyer flasks. Each flask contained either 50 or 100 ml of medium, depending on the experiment, and was covered with an inverted 50-ml beaker. The cultures were incubated at 37 C on a rotary shaker operating at 160 rpm. Their content of viable cells was estimated from the absorbance of

culture samples at 660 nm, measured by use of a Coleman Junior spectrophotometer model 6 D with 1-cm diameter tubes, and was confirmed by plate counts.

Iron-depleted lactate-salts medium. For each liter of medium, 10 ml of NH_4Cl stock solution (100 g/liter), 10 ml of KH_2PO_4 stock solution (150 g/liter), 40 ml of Na_2HPO_4 stock solution (87.5 g/liter), and 9.8 ml of reagent-grade 85% lactic acid were mixed with distilled water to make a solution about 300 ml in volume. The pH of this solution was adjusted to neutrality with 50% NaOH. The solution was transferred to a liter separatory funnel and to it was added 1 ml of a solution of 8-hydroxyquinoline in chloroform (5 mg/ml). After the mixture had been shaken vigorously for 5 min, 3 ml of chloroform was added, and the vigorous extraction was then repeated. When the phases had equilibrated, the dark, chloroform-8-hydroxyquinoline-metal-chelate layer was removed and the aqueous layer was twice extracted with 3-ml quantities of chloroform. A 5-mg amount of dry 8-hydroxyquinoline was then added to the aqueous solution, now saturated with chloroform. The mixture was shaken vigorously and then extracted three times with 3-ml portions of chloroform. This operation (addition of dry 8-hydroxyquinoline followed by repeated chloroform extractions) was repeated until the chloroform layer obtained during the first extraction subsequent to the addition of dry chelator was colorless. Finally (after the last three 3-ml chloroform extractions), the mixture was re-extracted with an additional 15 ml of chloroform and, after the layers had separated and had been drawn off into separate containers, the aqueous phase was boiled for several minutes to remove residual organic solvent. When the liquid cooled, to it were added, in the order given and with vigorous stirring after each addition, 1 ml each of the following aqueous solutions: $CuSO_4 \cdot 5H_2O$, 400 mg/liter; $MnSO_4 \cdot H_2O$, 310 mg/liter; $ZnCl_2$, 208 mg/liter; KI, 420 mg/liter; and $MgSO_4 \cdot 7H_2O$, 100 g/liter. The volume of the resulting solution was then adjusted to 990 ml with distilled water, and the medium was dispensed in 49- or 99-ml quantities into 250-ml flasks and sterilized by autoclaving at 2 atm for 15 min. To supplement the medium with iron, 1-ml quantities of filter-sterilized (0.22-μm pore size, Millipore Corp., Bedford, Mass.) freshly prepared, aqueous $FeSO_4$ solutions of the concentrations appropriate to the experiment (see Results) were added to each of the flasks. All glassware coming into contact with the medium, subsequent to the 8-hydroxyquinoline-chloroform extractions involved in its preparation, was soaked in 1% oxalic acid solution and rinsed well with distilled water before use.

Catechol derivative determinations. The catechol derivative content of bacterial culture supernatant fluids was estimated by the method of Arnow (1). We define a catechol derivative absorbancy unit as equivalent to that amount of Hoepfner-reacting material which, when present in 1 ml of an aqueous solution and combined with the quantities of reagents specified in the Arnow test, gives an absorbance of 1.0 at 510 nm in a cuvette of 1-cm light path. In these experiments, a Bausch & Lomb Spectronic-20 colorimeter was used to measure the absorbance of the test mixtures.

RESULTS

Effect of medium iron concentration on growth and on catechol derivative production. Brot and Goodwin (2) reported that the biosynthesis of 2,3-dihydroxy-*N*-benzoyl-L-serine by an auxotrophic mutant of *Escherichia coli* is repressed by the presence in the growth medium of 2×10^{-6} g-atom of iron per liter. To test the effect of medium iron concentration on the growth of and catechol derivative accumulation by cultures of *E. cloacae* SS_4-56, 0.2-ml inocula, containing 2×10^8 twice-washed cells and taken from an 18-h culture of the organism in Penassay broth, were introduced into flasks that each contained 50 ml of iron-depleted lactate-salts medium supplemented with sufficient sterile 3.16×10^{-4} M $FeSO_4$ solution, or dilutions thereof, to give media whose added iron content ranged from 10^{-8} to 3.16×10^{-6} g-atom per liter. Growth and catechol derivative accumulation were determined every 6 h.

After the cultures had been incubated for 24 h, the cultures were directly comparable (Fig. 1); the cells had entered the stationary phase and the catechol derivative accumulation was still proceeding linearly. The results plotted in Fig. 1 were obtained by averaging the data collected from triplicate cultures. If the lactate-salts medium originally used to grow the pacifarin-producing strain of *E. cloacae* is depleted of iron, as described in Materials and Methods, and inoculated with carefully washed cells, it

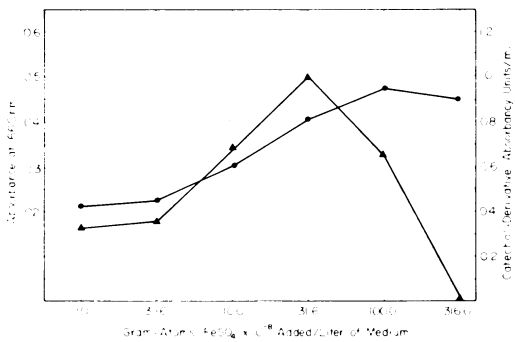

FIG. 1. *Effect of added iron concentration on growth of and catechol derivative accumulation by cultures of E. cloacae SS_4-56. Growth (●) was estimated from the absorbance at 660 nm of samples of 24-h cultures placed in tubes with a 1-cm light path. Catechol-derivative accumulation (▲) was measured by the Arnow method applied to supernatant fluids of culture samples.*

will not support the growth of the bacteria. As can be seen in Fig. 1, however, growth was sparse but optically measurable if this iron-depleted medium was enriched with at least 10^{-8} g-atom of iron per liter. Growth improved and catechol derivative production became maximal if the medium was supplemented with 3.16×10^{-7} g-atom of iron per liter. Luxuriant growth occurred if the medium was enriched with 3.16×10^{-6} g-atom of iron per liter. However, at this level of iron supplementation, the cultures no longer showed detectable production of catechol derivatives.

Effect of iron on pacifarin biosynthesis and activity. To test the hypothesis that iron concentration of the medium controls the biosynthesis of the pacifarins but does not affect pacifarin activity once the pacifarins have been produced, we performed the following experiment. Forty-five flasks, each containing 99 ml of iron-depleted lactate-salts medium, were each inoculated with 0.2 ml of a suspension in distilled water of 2×10^8 twice-washed cells of *C. cloacae* taken from an 18-h culture of the organism in Penassay broth. To each of 30 of these flasks, labeled "optimal iron," 1 ml of a filter-sterilized solution of 4.39 mg of $FeSO_4 \cdot 7H_2O$ in 500 ml of water was added to bring the iron concentration of the medium in them to 3.16×10^{-7} g-atom of added iron per liter. To each of 15 other flasks, labeled "repressive iron," 1 ml of a filter-sterilized solution of 439 mg of $FeSO_4 \cdot 7H_2O$ in 50 ml of water was added to bring the iron concentration of the medium in them to 3.16×10^{-4} g-atom of added iron per liter.

After 36 h, by which time good growth (A_{660}, 0.450) had occurred in the "optimal iron" cultures and a total of 3,200 absorbancy units of catechol derivatives had accumulated in them, whereas luxuriant growth (A_{660}, 0.600) without measurable catechol derivative accumulation had occurred in the "repressive iron" cultures, the cultures of each kind were pooled and the bacteria in them were removed by centrifugation. The pH of the resulting supernatant fluids was adjusted to 4.0 with 1 N HCl; the supernatant fluids themselves were passed through filters of 0.44 μm pore size (Millipore Corp.) and then concentrated, under reduced pressure at 40 C, to about one-fifteenth their original volume. One-half of the "optimal iron" pooled culture supernatant concentrate (containing 1,600 catechol derivative absorbancy (units) was mixed with 5 kg of basal diet 4045 (7). To the other half 128 mg of $FeCl_3 \cdot 6H_2O$, an amount of iron equivalent to that in the pooled "repressive iron" culture supernatant concentrate, was added. The resulting dark-red liquid was placed in a cooling bath at 0 C, and its pH was carefully adjusted to 7.5 with 50% NaOH. At this point, the formation of iron chelates gave it a dark purple color. The solution was then mixed with 5 kg of basal diet. The pooled "repressive iron" culture supernatant concentrate was also mixed with 5 kg of basal diet. Finally, the three supplemented diets were tested for pacifarin activity in the double inoculation mouse salmonellosis model routinely used in these investigations (intraperitoneal injection of avirulent *S. typhimurium*, followed, after a 48-hr interval, with intraperitoneal injection of virulent *S. typhimurium*).

Table 1 shows the survivorship results summarized on the 30th day of the experiment. On basal diet, 26 of 30 mice doubly infected with *S. typhimurium* had died of salmonellosis. On the basal diet supplemented with "optimal iron" *E. cloacae* culture supernatant concentrate (320 catechol derivative absorbancy units/kg of diet), only 11 of 30 doubly infected mice had died, and, on basal diet supplemented with "optimal iron" culture supernatant concentrate (320 catechol derivative absorbancy units/kg of diet) to which an excess of iron had been added prior to its incorporation into the feed, only 15 of 30 doubly infected mice had died. Thus, both diets supplemented with "optimal iron" culture

TABLE 1. *Survivorship of samples of 30 mice doubly infected with S. typhimurium, 30 days after challenge with virulent cells*

Group	Diet	Mice surviving	Net survivorship (%)	Statistical significance of net survivorship
1	Basal (diet 4045)	4	—	—
2	Basal + "optimal iron" culture supernatant	19	50	Highly significant, $P < 0.001$
3	Basal + "optimal iron" culture supernatant + Fe^{3+}	15	36.7	Highly significant, $P < 0.002$
4	Basal + "repressive iron" culture supernatant	7	10	Not significant, $P > 0.1$

supernatant concentrate mediated statistically highly significant ($P < 0.01$) increases in survivorship of the doubly infected mouse populations (an example of the classical pacifarin effect), and, because the survivorship results obtained with these two supplemented diets are themselves not statistically significantly different from each other ($P > 0.1$), both diets can be said to have possessed equivalent pacifarin activity. In contrast, on the diet supplemented with *E. cloacae* culture supernatant concentrate from a "repressive iron" culture, 23 of 30 doubly infected mice had died of salmonellosis. The result yields a survivorship response not statistically different ($P > 0.1$) from that which had been given by the unsupplemented basal diet and thus leads to the conclusion that the diet supplemented with "repressive iron" culture supernatant concentrate was devoid of pacifarin activity.

DISCUSSION

The results of these experiments suggest that, like the biosynthesis of various other iron-chelating agents by microorganisms (3), the biosynthesis of the pacifarins is controlled by the concentration of iron in the medium provided for the growth of the bacteria which produce them. Since in "optimal iron" medium a predictable amount of iron-chelating capacity is generated by our *E. cloacae* cultures, and since the amount of ferric iron needed to saturate that capacity was estimated to be about 10^{-4} g-atom/liter, we chose to investigate in this study the effects on pacifarin production and activity of a medium iron concentration of 3.16×10^{-4} g-atom/liter, even though we had determined that the biosynthesis of all catechol derivatives (and thus, presumably, of all pacifarins) by the organisms is repressed by medium iron concentrations as low as 3.16×10^{-6} g-atom/liter. The choice of the higher medium iron concentration for this investigation was prompted by our desire to make a direct comparison of the effect on pacifarin production and on pacifarin activity of an amount of iron which, when present in the medium, would be sufficient to prevent catechol derivative production by growing cultures and, when added after growth has taken place, would be sufficient completely to saturate whatever iron-chelating agents might be produced in "optimal iron" medium cultures which generate pacifarin activity. The results reported here lend strong support to the conclusion that, at the "repressive" concentration actually used in this study, iron exerts its inhibitory effect on the biosynthesis of the pacifarins but not on their biological activity once they have been formed.

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