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# Salmonellosis Pacifarin Activity of Enterobactin

**(mouse/2,3-dihydroxy-*N*-benzoylserine/survival from infection/bacterial iron metabolism/*aerobacter* metabolite)**

E. J. WAWSZKIEWICZ*, H. A. SCHNEIDER†, B. STARCHER‡, J. POLLACK§, AND J. B. NEILANDS

Institute for Biomedical Research, American Medical Association Education and Research Foundation, Chicago, Illinois 60610; and Department of Biochemistry, University of California, Berkeley, Calif. 94720

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**ABSTRACT Salmonellosis pacifarin activity is detected by an increased survivorship of mice, doubly infected with avirulent and virulent *Salmonella typhimurium*, when heretofore unknown agents, found in certain natural foodstuffs and in the supernatants of certain bacterial cultures, are fed to the infected animals as dietary supplements. We now announce the identity of one of these agents: it is *enterobactin*, a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine. Basal diets enriched with as little as 2 mg of crystalline trimer per kg of diet show pacifarin activity to a statistically significant degree. Diets supplemented with as much as 100 mg of the monomer per kg of diet show no such activity.**

When randomly bred mice are injected with an avirulent strain of *Salmonella typhimurium* and then, after a 24-48 hr interval, with a highly virulent strain of the same pathogen, proportionately greater numbers of them survive if they are fed certain diets containing natural foodstuffs than survive if they are fed semisynthetic diets. The agents responsible for the increase in survivors among the animals fed the "protective" diets have been called collectively "*Salmonella* resistance factor" or *salmonellosis pacifarin* (1).

The first attempts to isolate these active substances used whole wheat as starting material. Subsequently it was discovered that commercially dried egg-white was an even more potent and reliable source. However, when it was discovered that, in contrast to the active egg-white then obtainable from grocers' shelves (which was a fermented product), lyophilized, sterile egg-white has no activity, efforts were made to isolate from commercially dried material the microorganisms in it that must have been responsible for its pacifarin content. Eventually, a strain of *Aerobacter cloacae* was found which generated pacifarin activity both when grown in egg-white and in simple, synthetic medium (2). In addition to this strain of *A. cloacae*, strains of *A. aerogenes* (62-1), *Escherichia coli* (2266) and *S. typhimurium* (RIA-Tr, GR-3, and LT-2) generate pacifarin activity when grown under appropriate conditions (unpublished observations).

It was shown as early as 1963 that *Aerobacter*-derived pacifarin activity is associated with iron-chelating agents that arise in the bacterial cultures (3). In culture media whose content of iron is sufficient to repress the formation of iron-chelating agents by the bacteria, the biosynthesis of pacifarin activity cannot be detected (4). Of the several iron-chelating agents synthesized during iron-limited growth of *A. cloacae* and the other pacifarin-producing organisms listed above, the catechol derivatives 2,3-dihydroxy-*N*-benzoyl-L-serine, and di- and trimers thereof, account for about 80–90% of the total iron-chelating capacity produced. We now present evidence that the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine, enterobactin, is active in the mouse salmonellosis model, and that the monomer is not.

## MATERIALS AND METHODS

*Animals.* For these experiments, we used 6- to 9-week-old, randomly bred, Caesarean-derived and barrier-sustained, male, Swiss-Webster mice known to be free of *Salmonella*, *Pseudomonas*, and *Proteus*. Unless otherwise indicated, the mice were maintained on sterile stock diet (Wayne Sterilizable Lab-Blox, Allied Mills, Libertyville, Ill.) until 4 days before the initial *Salmonella* injection, at which time they were transferred to individual screen-bottomed cages and fed *ad libitum* a 30% casein, semisynthetic basal diet (4045), with or without supplements. Tap water was in constant supply. Each animal test group consisted of 30 mice, divided for purposes of statistical analysis into three subgroups of 10 each.

*Basal Diet.* Basal diet 4045 had the following composition per kilogram: 300 g of vitamin-free casein (Labco, Borden, Inc., New York, N.Y.), 605.5 g of glucose monohydrate (Cerelose, Corn Products Co., Argo, Ill.), 50 g of Wesson oil (Hunt-Wesson Foods, Inc., Fullerton, Calif.), 40 g of salts W-3 (salt mixture W-2 (5), modified to contain 0.55 g of $ZnCl_2$ per batch rather than 0.25 g as specified in W-2), 2 g of L-cystine, 1 g of inositol, 6.75 mg of beta-carotene, 3.9 mg of 2-methyl-naphthoquinone (menadione), 160 mg of irradiated ergosterol solution (Viosterol, Nutritional Biochemicals Corp., Cleveland, Ohio), 108.5 mg of alpha-tocopherol acetate, 2 mg of biotin, 0.4 mg of 5,6-dimethyl-benzimidazolylcyanocobamide (vitamin $B_{12}$), 10 mg of folic acid, 62.5 mg of thiamine hydrochloride, 62.5 mg of pyridoxine hydrochloride, 125 mg of *p*-aminobenzoic acid, 625 mg of nicotinic acid, 100 mg of calcium pantothenate, 125 mg of riboflavin, and 1 g of choline chloride.

* Present address: Department of Microbiology, University of Illinois at the Medical Center, P.O. Box 6998, Chicago, Ill. 60680
† Present address: Institute of Nutrition, University of North Carolina, Chapel Hill, N.C. 27514.
‡ Present address: Department of Pathology, University of Colorado School of Medicine, Denver, Colo. 80220
§ Present address: Department of Biology, Princeton University, Princeton, N.J. 08540

*Bacterial Cultures.* To infect the mice, we used *S. typhimurium* strains RIA-Tr, and SR-11–GR-3 and to prepare enterobactin, strain LT-2. RIA-Tr is a clone derived from a translucent colony of strain RIA and is relatively avirulent for Swiss-Webster mice. SR-11–GR-3 is a clone of SR-11, a strain derived from $BA_2$ of Mackenzie *et al.* (6), selected differentially for its ability to ferment xylose, a genetic marker indifferent in terms of virulence (7). SR-11–GR-3 is so virulent that the intraperitoneal injection of about 1 viable cell into a Swiss-Webster mouse constitutes a lethal dose. Stock cultures were grown in screw-capped tubes on nutrient agar at 37°C for 24 hr, then maintained at room temperature in the dark until needed. They were transferred to fresh medium at about 6-week intervals.

*Chemicals.* Enterobactin was isolated and crystallized as described (8). Biologically produced 2,3-dihydroxy-*N*-benzoyl-L-serine was a gift of Dr. Nathan Brot of the Roche Institute of Molecular Biology, Nutley, N.J. Synthetic 2,3-dihydroxy-*N*-benzoyl-DL-serine was prepared by the method of O'Brien, Cox, and Gibson (9).

*Special Media.* Previous studies (10) had shown that the pacifarin effect is most pronounced in the mouse salmonellosis model when the avirulent salmonellae injected into the mice are capable of producing catechol derivatives at the time of injection and the virulent challenge organisms are not. To obtain cells with these respective properties, we grew the two strains of *S. typhimurium* used for the mouse injections in *Chelexed penassay broth* containing different concentrations of iron. Chelexed penassay broth was prepared as follows: 17.5 g of dehydrated Penassay Broth (B243, Difco Laboratories, Detroit, Mich.) was dissolved in about 250 ml of distilled water, and the resulting solution was passed through 150 ml of Chelex-100 resin (BioRad Laboratories, Richmond, Calif.) in a 3-cm diameter column, the resin bed having previously been washed with 600 ml of 0.5 M $Na_2HPO_4$–HCl buffer (pH 7) and rinsed with 600 ml of water. After the concentrated broth had passed through the resin, 600 ml of water was allowed to drain through the the bed and to combine with the initial filtrate. 1 ml of each of following aqueous salt solutions—10 g/100 ml $MgSO_4 \cdot 7H_2O$; 40 mg/100 ml $CuSO_4 \cdot 5H_2O$; 31 mg/100 ml $MnSO_4 \cdot H_2O$; 20.8 mg/100 ml $ZnCl_2$; 42 mg/100 ml KI—was added to the resulting broth with stirring and the pH of the medium was adjusted to 7.0 with 12 N HCl. Finally, the volume of the medium was brought to 1 liter with water, and the liquid was dispensed in 50-ml portions into 250-ml beaker-covered Erlenmeyer flasks. The medium was sterilized by autoclaving at 2 atm of pressure for 20 min. All the glass and plastic containers used in the preparation and sterilization of the medium were soaked several hours in 1% aqueous oxalic-acid solution, then well rinsed with distilled water and dried before use. The requisite amount of freshly prepared and filter-sterilized (Millipore Corp., Bedford, Mass., 0.22-$\mu$m pore size) $FeCl_3$ solution was added aseptically to each flask shortly before use to give broth either *optimal* in iron concentration for catechol-derivative production ($3.16 \times 10^{-7}$ gram-atoms of Fe added per liter), or clearly *repressive* ($1 \times 10^{-4}$ gram-atoms of Fe added per liter). In the first instance, 0.5 ml of a solution of 4.27 mg of $FeCl_3$ in 500 ml of water was added to each 50 ml of Chelexed broth; in the second instance, 1.6 ml of a solution of 4.27 mg of $FeCl_3$ in 50 ml of water was added.

*Preparation of Bacterial Suspensions for Injections.* For preparation of the standard RIA-Tr mouse inoculum, the bacteria were grown at 37°C. Two successive 24-hr slant cultures on nutrient agar were prepared, and a flask of Chelexed penassay broth, adjusted to the optimal iron concentration, was inoculated from the second slant. The liquid culture was incubated for 24 hr on a rotary shaker (160 rpm), and then a second flask of the broth was inoculated lightly (surface of the medium touched with a stab inoculating needle) from it and incubated in the same manner. The catechol-derivative content of the second broth culture was measured at 24 hr by the method of Arnow (11) and, if the determination, performed on 1 ml of culture supernatant, gave the anticipated absorbance (0.100–0.150 at 510 nm in a cuvette of 1-cm light path), the bacterial population density was estimated from the absorbance of the culture at 640 nm. A sample was then appropriately diluted with sterile saline to give a suspension containing $4 \times 10^3$ viable cells/ml. 0.25 ml of the suspension was injected intraperitoneally into each mouse. The viable-cell count was verified by standard plating procedures on penassay broth solidified with 1.5% agar.

The preparation of SR-11–GR-3 for mouse inoculation proceeded in a like manner, except that the liquid medium used for the cultivation of the organisms was Chelexed penassay broth adjusted to a repressive iron concentration. In the broth that contained a repressive concentration of iron the bacteria grew to even higher cell densities than in optimal-iron medium, but the catechol-derivative content of 24-hr cultures was undetectable. Again, 0.25 ml of a suspension in saline of $4 \times 10^3$ viable cells/ml was injected intraperitoneally into each mouse.

The mice themselves were housed in a constant-temperature room maintained at 24 ± 1°C and illuminated each day for only 12 hr (from 6 a.m. to 6 p.m.). The injections took place between 10 a.m. and 12 noon. RIA-Tr was injected first, then 48 hr later, SR-11–GR-3. This regimen regularly resulted in a death rate of 27 ± 3 mice/30 mice in 30 days when the mice were fed basal diet.

*Preparation of Dietary Supplements.* 2,3-Dihydroxy-*N*-benzoylserine was added to the basal diet as a solution of the free acid in water at 60°C, with dilute NaOH added cautiously to pH 4. Enterobactin was added as a mixture of about 1 part of a solution of enterobactin crystals dissolved in an excess of ethanol, and of 1 part of 0.1 M $KH_2PO_4$. Diets supplemented with ethanolic mixtures of this kind were dried at room temperature overnight in shallow layers before they were fed to the mice.

## RESULTS

### Pacifarin activity of enterobactin

Table 1 lists the results of separate experiments in which the effects of 2,3-dihydroxy-*N*-benzoylserine, supplied at two concentrations (6.6 mg/kg of diet and 100 mg/kg), were tested, and an experiment in which enterobactin at the concentration of 5 mg/kg of diet was tested for possible activity in the mouse salmonellosis system. The raw data (numbers of mice dead/30 in 30 days) were transformed by the method of Weibull (12) and subjected to variance analysis. This com-

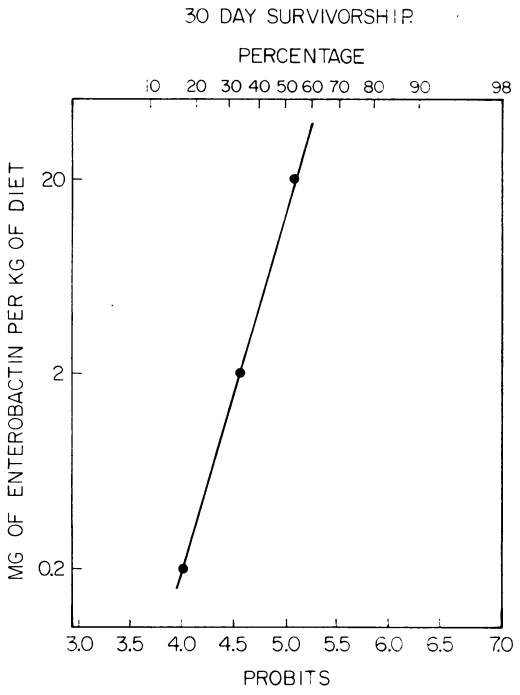


FIG. 1. Survivorship response of Swiss-Webster mouse populations, doubly infected with *S. typhimurium*, to various dietary concentrations of enterobactin.

putation showed that of the test diets listed in the table, only the one that contained enterobactin caused a significant increase in the survivorship of the infected animals.

Encouraged by this result, we ascertained the character of the dose-response curve of enterobactin. Previous studies had shown that the survivorship response of doubly-infected Swiss-Webster mouse populations was related linearly to the logarithm of the dietary "pacifarin" concentration when survivorship frequencies were transformed from percentages into population probits (13). Fig. 1 shows the result of an experiment in which diets containing, respectively, 0.2, 2, and 20 mg of enterobactin/kg were fed to the test mice 2 days prior to the initial *Salmonella* injection, and until 2 days after virulent challenge, then replaced by the unsupplemented basal diet. A previous experiment had shown that doubly-infected mice fed a pacifarin-active diet during this critical early period survived to the same extent as did mice fed active diet throughout the full course of the experiment (unpublished observations). It can be seen in Fig. 1 that, as is the case with pacifarin activity found in natural foodstuffs, enterobactin-associated protection of doubly-infected mouse populations is linearly related to the log of its dietary concentration when the survivorship is expressed in probits. Actually, statistical calculations performed on the survivorship data (transformed according to Weibull) indicate that the probability that this observed linearity of the dose-response curve could have arisen by chance is less than 0.005, and that the small deviation from linearity that is observed can readily be ascribed to random effects ($P \gg 0.25$). Furthermore, direct comparisons of the test groups show that the increase in survivorship in the group fed diet that contained 20 mg of enterobactin/kg is highly significant ($P < 0.01$), and that even in the group fed 2 mg/kg it is significant ($P < 0.05$). From these observations, we conclude that enterobactin is a pacifarin.

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

| Experiment | Diet | Mice surviving (%) | Net survivorship (%) | Statistical significance of net survivorship |
|---|---|---|---|---|
| 1 | Basal (Diet 4045) | 13.3 | 3.3 | Not significant |
| | Basal + 6.6 mg DHBS*/kg | 16.6 | | |
| 2 | Basal (Diet 4045) | 0 | 6.6 | Not significant |
| | Basal + 100 mg DHBS†/kg | 6.6 | | |
| 3 | Basal (Diet 4045) | 3.3 | 20.0 | Significant $P < 0.05$ |
| | Basal + 5 mg enterobactin/kg | 23.3 | | |

* 2,3-Dihydroxy-*N*-benzoyl-L-serine, isolated from *E. coli* cultures.

† Chemically synthesized, racemic mixture.

## DISCUSSION

Enterobactin (*enterochelin* in the terminology of Australian workers (14)) has now been shown to be biosynthesized by several strains of *Aerobacter*, *Escherichia*, and *Salmonella* grown in iron-low environments (8, 14). Salmonellosis pacifarin activity was first detected in certain samples of whole wheat, localized in the germ and outer layers of the grain (15). Later it was shown to occur in samples of fermented, dried egg-white. Egg-white contains a powerful iron-binding protein, conalbumin, whose activity removes any free iron that might be present. Although *Aerobacter* strains are widely distributed in nature, their normal habitat is the surface of plants and of grains and, to a varying degree, the intestinal canal of animals. Thus, it is quite conceivable to us that the pacifarin activity associated with whole wheat and with egg-white may have as its source enterobactin produced by *Aerobacter* growing on the small amount of fermentable carbohydrate they both contain.

During growth on glucose, *Aerobacter* produces acids, which lower the pH of its microenvironment and create a milieu in which enterobactin is relatively stable. In an alkaline environment such as that which results, for example, from growth of the bacteria on lactate, enterobactin is rapidly hydrolyzed to 2,3-dihydroxy-*N*-benzoyl-L-serine. Under these conditions, in iron-limited media, certain strains of *Aerobacter* produce pacifarins other than enterobactin. These additional pacifarins have also been isolated and crystallized, but their structures have not yet been fully established.

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**Correction.** In the article "Cytoplasmic DNA Synthesis Induced by RNA Tumor Viruses", by M. Hatanaka, T. Kakefuda, R. V. Gilden, and E. A. O. Callan, which appeared in the August 1971 issue of *Proc. Nat. Acad. Sci. USA*, **68**, 1844–1847, the following corrections should be made: see p. 1846. The last two sentences in the legend to Fig. 2 should read "The arrow (*insert*) shows a labeled mitochondrion; heavy labeling of a. . .". In Table 2 (Uninfected BALB/3T3), the 0–4 grain counts were ~0.25. The third column heading in this table should read "UV-treated MSV".