

Role of Microbial Iron Transport Compounds in the Bacterial Spoilage of Eggs

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Microbial iron transport compounds, belonging either to the hydroxamate family excreted by pseudomonads, or to the phenolate family excreted by salmonellae, reverse the bacteriostatic effect of conalbumin on the growth of these bacteria in egg white. The presence of microgram quantities of these compounds permits both salmonellae and pseudomonads to reach dense populations in egg white. The role of these iron transport compounds in bacterial egg spoilage is discussed.

It was shown some years ago that the iron-binding protein, conalbumin, was the most important factor controlling the growth of gram-negative bacteria in egg white (5). The results of that study indicated that the bacteria under test had varying capabilities of availing themselves of iron bound by conalbumin. Such ability was postulated to correlate with the biosynthetic ability of the microorganism to excrete iron-binding compounds when grown in media containing sub-optimal amounts of iron. The role of such microbial iron transport compounds (ITC) in reversing the bacteriostatic action of conalbumin, thereby leading to the bacterial spoilage of eggs, is the subject of this report.

MATERIALS AND METHODS

Egg white. Egg white was prepared aseptically as previously described (5).

Bacteria. Bacteria used were: (i) *Salmonella typhimurium* Tm-1, the strain used in our laboratory as a reference in the study of heat resistance of *Salmonella* in egg products (9); (ii) *Pseudomonas ovalis* P-1 from our culture collection, but unclassified and designated ATCC 17399 by Stanier et al. (13).

Supplement. (i) FeSO_4 aqueous solutions containing 0.10 mg of Fe per ml were sterilized by filtration through ultrafine sintered glass funnels.

(ii) *Salmonella* ITC, phenolates [parent compound 2,3-dihydroxy benzoyl serine (12, 14)], were prepared by ether extraction of acidified cell-free supernatants from cultures grown on a medium containing limiting concentrations of iron. This preparation contained less than 0.1% iron.

(iii) *Pseudomonas* ITC, fluorescent hydroxamate preparations, were obtained by phenol extraction of acidified cell-free supernatants from cultures grown on media with limiting concentrations of iron. This preparation contained less than 0.04% iron.

Inocula. The bacteria were grown on a glucose salts medium with limiting iron for 15 hr and centrifuged;

the supernatant was discarded. The cell pellet was washed 1× with an equal amount of sterile demineralized water and again suspended in sterile demineralized water.

Growth Experiments. The amounts of iron added were approximately 5 and 20% of those required to saturate the conalbumin found in egg white. The egg white was inoculated with such suspensions to give the desired number of bacteria. After inoculation, the egg white was divided into four equal samples. The first was not supplemented, the second was supplemented with sufficient iron to give a final concentration of 1 or 4 μg of Fe per ml of egg white, the third was supplemented with microgram quantities of the respective ITC, and the fourth was supplemented with identical quantities of both iron and ITC as in the second and third samples.

The samples inoculated with *P. ovalis* P-1 were incubated at 28 C, whereas those with *S. typhimurium* Tm-1 were incubated at 35 C.

In vivo studies. Nest-clean eggs on day of lay were inoculated with *P. ovalis* P-1 by immersing the warm egg in a cold suspension of bacteria containing 10 μg of iron per ml as previously described (6). The inoculated eggs were incubated at 15 C and were examined periodically for the appearance of fluorescent pigments in the white. When these pigments, which have the iron transport capacity associated with them, were detected, the egg white was separated from the yolk aseptically on a commercial tray and separator. Examination of the separated white under "black light" (360 nm) revealed that the fluorescence was localized and concentrated in one small spot. The entire white was homogenized and placed in sterile screw-top test tubes. The number of bacteria present in the white at breakout was determined as described below. The remaining white was incubated at 15 C, and changes in bacterial numbers were determined at intervals.

Plate counts. Changes in bacterial numbers were determined by spread-plating 0.1 ml of the appropriate dilutions on Trypticase Soy Agar plates. Colonies were counted after incubating the plates at 28 C for 2 days.

RESULTS

The results obtained with *P. ovalis* P-1 are shown in Fig. 1. It is evident that the organism is not able to reproduce in either unsupplemented egg white or in egg white supplemented to give 4 μg of iron per ml. In fact, *P. ovalis* P-1 actually dies off in unsupplemented white upon incubation for periods in excess of 24 hr at 28 C.

However, in the presence of microgram quantities of the fluorescent ITC, the organism now has a mechanism which allows it to metabolize the iron present in the egg white as the iron-conalbumin complex and grow to populations approaching billions per milliliter.

Results of a similar study with *S. typhimurium* Tm-1 and the ITC it synthesizes are given in Table 1. Again there is little or no growth of bacteria in either egg white or egg white supplemented to give 1.0 μg of iron per ml. In the pres-

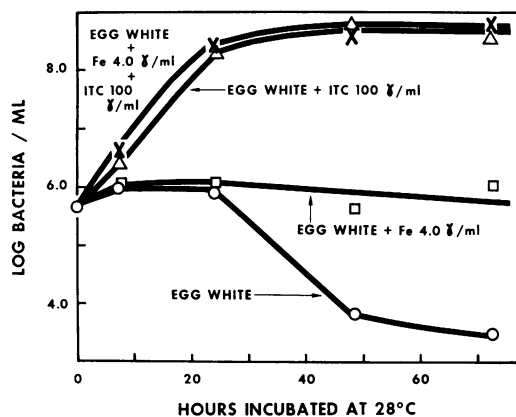


FIG. 1. Effect of fluorescent hydroxamate iron transport compounds on the growth of *P. ovalis* P-1 in egg white. This ITC preparation contained less than 0.04% iron.

TABLE 1. Effect of iron transport compounds (ITC) on growth of *Salmonella* in egg white

Medium	Population (bacteria/ml) at		
	0 hr	17 hr	25 hr
Egg white.....	10^5	7.4×10^5	7.8×10^5
Egg white + Fe (1.0 $\mu\text{g}/\text{ml}$).....	10^5	5.2×10^5	1.1×10^5
Egg white + ITC ^a (20 $\mu\text{g}/\text{ml}$).....	10^5	1.1×10^8	1.6×10^8
Egg white + Fe (1.0 $\mu\text{g}/\text{ml}$) + ITC (20 $\mu\text{g}/\text{ml}$).....	10^5	1.3×10^8	1.3×10^8

^a These ITC preparations contain <0.1% Fe.

ence of microgram quantities of a preparation of *Salmonella* ITC, the organism grows rapidly and reaches populations of a hundred million per milliliter.

Results of the experiment with the egg white from inoculated shell eggs are shown in Table 2. At time of breakout, the number of bacteria per ml of white is very low: 705/ml for egg no. 1, 45/ml for egg no. 2, and less than 10/ml for egg no. 3. However, upon further incubation at 15 C, the numbers increased quite rapidly and reached tens of millions per milliliter.

DISCUSSION

As postulated in our previous reports (5-8), microbial ITC play a significant role in reversing the bacteriostatic action of conalbumin on the growth of egg spoilage bacteria in egg white. As suggested in our earlier work and recently shown by Board et al. (1), the effect of contaminating iron introduced during washing on the course of bacterial infection of hen eggs is to promote multiplication of the bacteria at the shell membrane system. Concurrent with this multiplication, we again propose (6, 7) that the bacteria excrete ITC. These growth factors then diffuse into the white and facilitate the multiplication of the bacteria therein.

Our in vitro experiments with preparations of such compounds synthesized by salmonellae and pseudomonads certainly define the role of these compounds. It is, of course, well known that egg spoilage by fluorescent pseudomonads is always accompanied, and in fact may be preceded, by the presence of fluorescent substances in the albumin (4, 10). Such fluorescent compounds have associated with them the properties of iron hydroxamate transport compounds.

It is not unlikely that all primary bacterial egg spoilage organisms have the ability to synthesize these compounds which aid them in reversing the bacteriostatic action of conalbumin. The review by Neilands (11) names the many microorganisms which synthesize the ITC belonging to the hydrox-

TABLE 2. Growth of *Pseudomonas* in egg white from shell eggs with fluorescent albumens

Egg white from	Bacteria per ml of egg white		
	At breakout	After 96 hr at 15 C	After 144 hr at 15 C
Egg no. 1	705	2.39×10^7	1.9×10^8
Egg no. 2	45	1.72×10^7	6.56×10^7
Egg no. 3	10	2.23×10^5	2.43×10^7

amate family. Many other organisms are able to biosynthesize ITC belonging to the phenolate family. Among these are members of *Escherichia*, *Aerobacter*, *Salmonella* and *Azotobacter* (2, 3, 12, 14). *Salmonellae* are also able to overcome the bacteriostatic action of transferrin (the related blood iron-binding protein) in the presence of these phenolate compounds (14).

It appears from our *in vitro* experiments and certainly from our data with "broken out" whites that the conclusion of Board et al. (1) that "the initial contaminants of the white do not grow" is untenable. They certainly grow more rapidly and extensively when they reach the nutritious yolk, but with the aid of their own ITC they are also able to reach dense populations in egg white.

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