Factors Affecting the Growth of *Pseudomonas fluorescens* in Liquid Egg White

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*Pseudomonas fluorescens* grew rapidly in fresh egg albumen diluted with water. Growth of the bacteria in egg albumen was stimulated by the addition of carbohydrate and ovomucoid-rich egg exudate. Polyacrylamide-gel electrophoresis for residual egg albumen revealed extensive proteolysis of albumen inoculated with the organism. A fluorescent compound with absorption maximum at 408 nm was isolated from a defined salt medium inoculated with *P. fluorescens*. It shortened the lag phase and increased the final cell yield of the organism when added to the salt medium (100 μg/ml).

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Growth of bacteria in egg white is limited due to several inhibitory factors: lysozyme (7), conalbumin (6), ovomucoid (1), avidin (4), high pH (16), and the intact nature of its protein (15). Conalbumin forms a stable complex with iron which renders the medium deficient in available iron and the protein resistant to proteolysis (2). Garibaldi (9) showed reversal of inhibition of the gram-negative bacteria in egg white by saturating conalbumin with Fe⁺⁺. However, *Pseudomonas ovalis* did not grow in albumen supplemented with Fe⁺⁺ (4 μg/ml) (10). Gardner and Nikooppour (8) demonstrated growth of *P. fluorescens* in purified 1% conalbumin, 1% lysozyme, and on ovalbumin substrates (14). Recently, Garibaldi (10) isolated an iron transport compound which reversed the bacteriostatic action of conalbumin. There is lack of agreement on the factors affecting the growth of *Pseudomonas* sp. in egg white, namely, the role of conalbumin, lysozyme, and ovomucoid, the antitrypsin factor (12).

Egg white contains about 88% water (15) held mostly as water of hydration of proteins. Lack of this solvent may be responsible for the poor overall growth of bacteria in albumen. This study was undertaken to study the effect of addition of water and egg exudate on the growth of *P. fluorescens* in egg albumen.

**MATERIALS AND METHODS**

**Egg white.** Eggs laid within 1 to 2 h were washed with egg washing compound and sanitizer (Agway), and egg albumen was prepared aseptically as described by Garibaldi (9).

**Bacteria.** *P. fluorescens* was isolated in our laboratory. The stock culture was incubated at 25°C on Trypticase soy agar (BBL) slants and held at 4°C. Before using, the culture was activated by two serial transfers in Trypticase soy broth (BBL), incubated in a shaker at 21°C. The cells were removed by centrifugation, washed once with sterile distilled water, and appropriate dilutions were made to give about 40 × 10⁶ organisms per ml of egg white.

**Egg exudate.** Cooked egg exudate was prepared and freeze-dried as described by Nath et al. (13). Eggs that are cooked, peeled, and packaged in Cryovac bags give out a greemish-yellow liquid. The liquid is called the exudate.

**Fluorescent compound production.** The medium used by Shimane and Neiland (17) was modified by replacing sucrose with dextrose. One liter of the medium contained ammonium citrate, 14.0 g; dextrose, 6.0 g; NaH₂PO₄·H₂O, 1.38 g; K₂HPO₄, 2.28 g; NH₄Cl, 1.8 g; MgSO₄·6H₂O, 0.5 g; and CaCl₂·2H₂O, 0.01 g. Dextrose was filter sterilized by passing through a Millipore filter (0.45 μm) and added to the sterile salt medium (121°C for 15 min). The medium was tempered to 18°C, inoculated, and incubated in a shaker at 18°C for 4 to 5 days. Cells were removed by centrifugation and the fluorescent compound was isolated by adsorption onto Dowex 50 W (H⁺, 200 mesh) and eluted with 6 N HCl as described by Shimane and Neiland (17).

**Growth experiment.** The organism was grown in tubes containing (i) 8 ml of egg albumen plus 4 ml of distilled water; (ii) 11 ml of egg albumen plus 1 ml of distilled water; (iii) 12 ml of egg albumen; (iv) 8 ml of egg albumen plus 4 ml of 3% egg exudate; (v) 11 ml of egg albumen plus 1 ml of pigment (2 mg/ml); and (vi) 8 ml of egg albumen plus 3 ml of distilled water plus 0.5 ml of lysozyme (1%) plus 0.5 ml of conalbumin (1%). Lysozyme and conalbumin were added to the diluted albumen to avoid the dilution effect of these inhibitory fractions in egg white. Lysozyme (Nutritional Biochemicals Corp.), conalbumin (Nutritional...
Biochemicals Corp.) each 1%, exudate (3%), and pigment (0.2%) solutions were filter sterilized by using a Millipore filter (0.45 μm). Inoculated tubes were incubated at 25°C and samples for plating were drawn every 24 h.

Plate count. After mixing, the tube contents were plated with standard plate count agar (Difco) and counts were made after incubation for 48 h at 30°C.

Effect of pigment on growth. Sterile modified salt medium (250 ml) was inoculated with 0.25 ml of washed and resuspended cells. After thorough mixing, 50 ml of the medium was transferred to each of the three sterile flasks. Flasks 1 and 2 were supplemented with 2 and 5 mg, respectively, of sterile fluorescent compound, whereas the third contained water equal in amount to that added with the pigment and served as a control. The flasks were incubated in a shaker at 18°C. The growth in each flask was monitored by reading absorbancy at 660 nm.

Chemical analysis. At the end of the incubation period of 15 days, 2 ml of inoculated or control alburnen from the growth experiment was diluted with 10 ml of distilled water. The contents were thoroughly mixed, heated in a boiling water bath for 10 min, and immediately cooled in ice water. The tubes were covered with marbles during heating. The tube contents were filtered through Whatman no. 1 filter paper. The filtrate was centrifuged at 12,000 x g. The clear supernatant fluid obtained was used for chemical analysis. Total carbohydrate and protein in the supernatant fluid were determined by the method of Tillmans and Philippi (18) and Gornall et al. (11), respectively. The corresponding control for each treatment was treated in a similar manner.

Electrophoresis. Albumen (0.2 ml) was diluted with 0.2 ml of tris(hydroxymethyl)aminomethane-borate buffer, pH 8.9, and two drops of saturated sucrose solution were added to the mixture. Samples (35 or 50 μlitters) were applied on a 7.5% polyacrylamide-gel, and electrophoresis was performed at 250 V for 5 h. The gel was stained with amido black and destained with 7% acetic acid. Densitometer tracings of the destained gels were made at 588 nm by using a Beckman spectrophotometer model Acta III.

RESULTS

Growth of P. fluorescens in egg alburnen is shown in Fig. 1. The organism grew readily in the tube supplemented with egg exudate and the tube containing 4 ml of water. There was little growth in the other tubes, indicating the lack of some important factor other than energy source. At 96 h the counts in these two tubes declined slightly and then rose again. At this time, we are unable to explain this phenomenon fully. Growth in the culture tubes can be assessed by examining the tubes for fluorescence development in ultraviolet light (320–400 nm). Albumen containing pigment fluoresced strongly at zero hour and gradually declined over the period indicated in Table 1. It is clear that the organism grew only in the tube containing exudate and the tube with added water.

The inoculated albumen containing exudate was heat coagulated and then centrifuged. The supernatant liquid absorbed maximally at 408 nm. The fluorescent compound from the salt medium also had an absorption maximum at 408 nm. Since fluorescence in the tube containing pigment diminished over 72 h with no noticeable growth, it was thought that the organism might utilize this compound. It is clear from Fig. 2 that 100 μg/ml of this compound shortened the lag-phase period and increased the overall growth yield of this organism in the salt medium.

At the end of the 15-day incubation, water extracts of the heat-coagulated albumen were analyzed for total carbohydrate and protein. The data in Table 2 indicate that the organism proteolyzed egg albumen. A fresh or intact albumen is readily coagulated by the heat treatment employed, but as the albumen is proteolyzed, the small peptides or the low-molecular-weight fractions produced are not coagulated and appear in the supernatant fluid. The data on total carbohydrate suggest that some glycoproteins in the egg albumen may also be proteolyzed.

To further study proteolysis, the samples inoculated and incubated with P. fluorescens were examined for residual albumen components by polyacrylamide-gel electrophoresis. Figure 3 shows extensive proteolysis of the samples containing egg exudate and added water. Numbers 1 and 2 in Fig. 3 refer to ovalbumin and no. 13 refers to ovotransferrin (conalbumin).
TABLE 1. Development of fluorescence in egg albumen inoculated with Pseudomonas fluorescens

| Sample no. | Treatment (ml) | Fluorescence |
|------------|----------------|--------------|
| Sample no. | Albumen  | Water  | Exudate* | 0  | 24  | 48  | 72  | 96  | 168 |
| 1          | 8        | 4      | 0       | -   | -   | +   | +   | +   | +   |
| 2          | 11       | 1      | 0       | -   | -   | -   | -   | -   | -   |
| 3          | 12       | 0      | 0       | -   | -   | -   | -   | -   | -   |
| 4          | 8        | 0      | 4       | +   | ++  | ++  | ++  | ++  | ++  |
| 5          | 11       | 1c     | 0       | +   | ++  | ++  | ++  | +   | +   |
| 6          | 12d      | 0      | 0       | -   | -   | -   | -   | -   | -   |

*Three percent solution of freeze-dried exudate.
*Numbers across represent number of hours.
*One milliliter contained 2 mg of fluorescent compound.
*Incubated control.

Table 2. Carbohydrate and protein content in the extract of inoculated egg albumen after 15 days incubation at 25 C

| Sample no.* | Carbohydrate (mg/ml) | Protein (mg/ml) |
|--------------|----------------------|-----------------|
|              | Incubated Control    | Incubated Control |
| 1            | 1.3                  | 0.75            |
|              |                       |                 |
| 2            | 1.4                  | 1.1             |
|              |                       |                 |
| 3            | 1.75                 | 1.7             |
|              |                       |                 |

*1. Egg albumen with water added; 2. egg albumen with exudate added; 3. uninoculated control.
*Δ, Incubated minus control.

DISCUSSION

Garibaldi (9) postulated the reversal of the bacteriostatic action of conalbumin by iron and by iron transport compound (10). In the present report, it is clear that P. fluorescens grew readily only in the albumen supplemented with egg exudate and in the albumen diluted with water. It is expected that the dilution of egg white with water or egg exudate would lower conalbumin and lysozyme concentrations in the medium. Addition of 5 mg of conalbumin and 5 mg of lysozyme to 12 ml of diluted albumen did not inhibit growth of the organism in the present study; on the contrary, the
numbers increased from $16 \times 10^4$ at zero hour to $17 \times 10^7$ at 72 h. Gardner and Nikoopour (8) observed no inhibition of \textit{P. fluorescens} in 1% conalbumin and 1% lysozyme substrates. Briggs and Millin (3) have shown that hydroxamates (the iron transport compounds) may be produced during proteolysis of proteins. In our experiments, there is a considerable proteolysis (Fig. 3), but we could not detect hydroxamates in the albumen or its extracts as determined by the procedure of Emery and Neilands (5). The growth of the organism inoculated in the salt medium was stimulated by the fluorescent compound (100 $\mu$g/ml). This may also explain the disappearance of fluorescence from the tube containing pigment (Table 1). At higher concentrations (1 mg/ml) this compound suppressed the growth of the organism in the salt medium.

It appears that the addition of water to albumen helps the organism grow better, probably by making more nutrients available. This observation is also supported by the growth curve obtained with albumen with added egg exudate. The exudate is proteinaceous and rich in carbohydrate and also contains some riboflavin (13).

It may be suggested that along with other bacteriostatic factors present in the egg white, lack of free available water for dissolution of assimilable nutrients is an important factor in controlling growth of the bacterium in egg albumen.

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\begin{figure}
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\caption{Densitometer tracings of polyacrylamide gel electrophoretic patterns of inoculated egg albumen after 15 days of incubation at 25 C. Symbols: ---, control; ----, egg albumen with water added (4 ml); -----, egg albumen with exudate added (4 ml).}
\end{figure}
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