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Influence of Temperature of Incubation and Type of Growth Medium on Pigmentation in *Serratia marcescens*

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Maximal amounts of prodigiosin were synthesized in either minimal or complete medium after incubation of cultures at 27 C for 7 days. Biosynthesis of prodigiosin began earlier and the range of temperature for formation was greater in complete medium. No prodigiosin was formed in either medium when cultures were incubated at 38 C; however, after a shift to 27 C, pigmentation ensued, provided the period of incubation at 38 C was not longer than 36 hr for minimal medium or 48 hr for complete medium. Washed, nonpigmented cells grown in either medium at 38 C for 72 hr could synthesize prodigiosin when suspended in saline at 27 C when casein hydrolysate was added. These suspensions produced less prodigiosin at a slower rate than did cultures growing in casein hydrolysate at 27 C without prior incubation at 38 C. Optimal concentration of casein hydrolysate for pigment formation by suspensions was 0.4%; optimal temperature was 27 C. Anaerobic incubation, shift back to 38 C, killing cells by heating, or chloramphenicol (25 μg/ml) inhibited pigmentation. Suspensions of washed cells forming pigment reached *p*H 8.0 to 8.3 rapidly and maintained this *p*H throughout incubation for 7 days. Measurements of viable count and of protein, plus other data, indicated that cellular multiplication did not occur in suspensions of washed cells during pigment formation. By this procedure utilizing a shift down in temperature, biosynthesis of prodigiosin by washed cells could be separated from multiplication of bacteria.

The salient characteristic of typical strains of *Serratia marcescens* is production of the red pigment, prodigiosin, at room temperature. Environmental conditions such as temperature of incubation and type of growth medium influence pigment formation, although the effects of these conditions were not thoroughly examined in quantitative terms. This shortcoming is unfortunate because a better understanding of conditions for pigmentation might provide the experimental knowledge for developing a system to divorce growth of *S. marcescens* from synthesis of prodigiosin. Such a system is needed for definitive investigations on biosynthesis of the pigment. This paper describes the influence of incubation temperature and the effect of growth in minimal or in complete medium upon pigment production by a strain of *S. marcescens*. By manipulation of the two conditions, biosynthesis of prodigiosin can be separated from multiplication of the bacteria.

MATERIALS AND METHODS

Organism and growth media. The Nima strain of *S. marcescens* was carried as a stock culture on Trypticase Soy Agar (Difco). Periodic examination of isolated colonies growing on the surface of the agar established that the strain contained only bacteria capable of growing at room temperature as phenotypically red colonies of *S. marcescens*. Inocula were prepared from stock cultures by growing bacteria in liquid minimal medium in test tubes at 27 C for 24 hr without shaking; 0.1 ml of the culture was inoculated into a second test tube containing 10 ml of minimal medium. After growth without shaking at 27 C for 24 hr, the bacteria were harvested by centrifugation and washed three times with 0.85% saline buffered at *p*H 7.2 with 0.01 M phosphate buffer. The washed bacteria were slightly pink. For the experiments, 0.1 ml of a suspension containing 10^6 washed bacteria/ml of buffered saline was inoculated into 10 ml of medium contained in 50-ml Erlenmeyer flasks. The flasks were incubated without shaking for the desired period of time in a water bath that maintained the proper temperature within $\pm$0.5 C, as continuously monitored during the experiments by a recording thermometer.

Liquid minimal medium (2) contained: glycerol, 1.0%; ammonium citrate, 0.5%; magnesium sulfate, 0.05%; potassium secondary phosphate, 1.0%; sodium chloride, 0.5%; and ferric ammonium citrate, 0.005%.

Addition of 0.1% yeast extract (Difco) and 0.2% N-Z Case peptone (Sheffield Chemical, Norwich, N. Y.) to minimal medium made complete medium. Media were adjusted to *p*H 7.2 before autoclaving.

Preparation of cell suspensions. Some experiments were made with nonpigmented cultures grown at 38 C, followed by suspension of cells at 27 C for determination of their ability to form pigment. The suspensions were prepared by growing cultures without shaking for 72 hr at 38 C in complete or in minimal medium. The nonpigmented cells were harvested by centrifugation, washed three times in buffered saline, and suspended to one-half the original volume of the culture in 0.85% saline; the supplements were then added. Ten milliliters of this suspension was incubated at 27 C without shaking in 10-ml Erlenmeyer flasks. Pigment formation was determined after incubation for 7 days.

Analytical procedures. Protein was determined by the method of Lowry et al. (7) with bovine serum albumin as a standard. Prodigiosin was measured by a modification of the method of Hubbard and Rimington (4) in which the bacteria were digested by boiling with 1 N NaOH for 1 hr in a water bath. Pigment was extracted from the digest with absolute ethanol and from the ethanolic solution with petroleum ether. The latter extract was dried in a boiling-water bath; the residue was dissolved in acidified ethanol (10 ml of ethanol plus 1 ml of 1 N HCl); and the absorbancy of the solution was measured in a Beckman spectrophotometer (model DU) at 535 nm. Concentrations of pigment were then calculated by using 51.5×10^3 liter per g per cm as the specific absorbancy of prodigiosin (8).

Viable counts were determined by standard plate count procedures by using complete medium solidified with agar. After incubation at 27 C for 48 hr, plates were counted. In counting, no distinction was made between surface or subsurface, or pigmented or nonpigmented, colonies. A Petroff-Hausser counting chamber was used to determine total counts.

RESULTS

Figure 1 shows the effect of temperature upon the variables measured after 7 days of incubation. The values are similar for both complete and minimal media. Although growth, as measured by either viable count or protein, occurs over a wide range of temperatures, cells form pigment only within a relatively narrow range. The data confirm the general assumption that formation of prodigiosin is maximal near room temperature, the actual peak occurring near 27 C after incubation for 7 days. Complete medium supports pigment formation better at the extremes of temperature, but both media yield about the same amount of prodigiosin at temperatures close to 27 C. However, maximal growth, whether measured by viable count, protein or nitrogen, is at a lower temperature, near 16 C, after this length of incubation. Measurements of bacterial nitrogen are included as a control to determine whether protein determinations were a valid measure of cell substance. Protein and nitrogen values for growth in minimal medium closely parallel one another (Fig. 1).

The kinetics of growth of strain Nima at 27 C in complete or in minimal medium are shown in Fig. 2. When incubated at 27 C, pigment is produced in both media, and the concentration at the end of 7 days is almost the same. Growth and pigment production begin earlier in complete medium. But, although the stationary phase of growth is reached sooner in complete medium, maximal values for prodigiosin occur at about the same time in both media. The greatest amount of pigment is synthesized during the stationary phase of growth. The maximal values for viable count and protein are similar in the two media.

When incubated at 38 C, the cultures do not form pigment in either medium. The curves for viable count and protein are flatter than those seen at 27 C, and less protein is formed throughout the incubation period. The latter observation is most apparent in minimal medium.

During the course of the above investigations, a chance observation indicated that, after incubation at 38 C, nonpigmented cultures could form pigment if the flasks were shifted to 27 C, provided the length of incubation time at 38 C was not too long. Since some of these cultures had already reached the stationary phase of growth at 38 C, we speculated that an investigation of

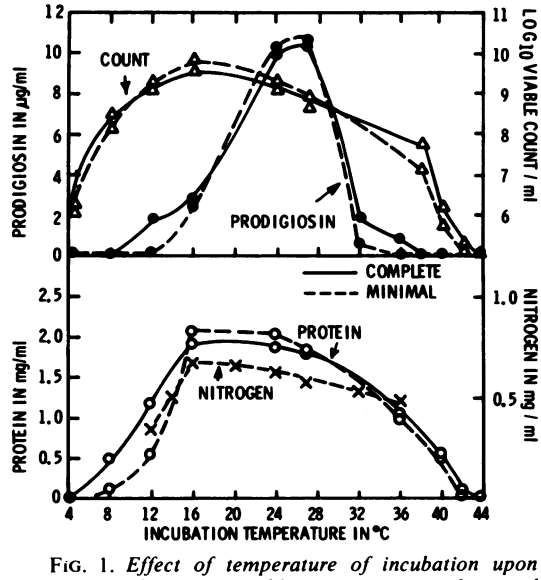

FIG. 1. *Effect of temperature of incubation upon prodigiosin formation, viable count, protein values, and nitrogen values of Serratia marcescens strain Nima grown in complete or in minimal medium. Values determined after incubation for 7 days at the indicated temperatures.*

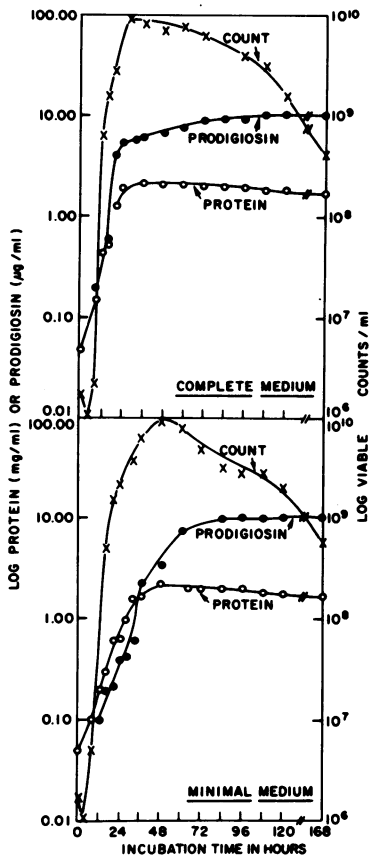

FIG. 2. *Kinetics of growth, as determined by protein and viable count, and of prodigiosin formation of Serratia marcescens strain Nima incubated at 27 C in complete or in minimal medium.*

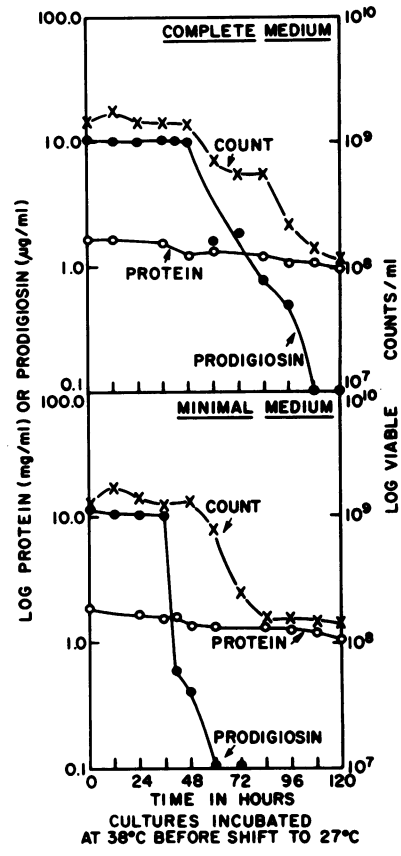

FIG. 3. *Effect of growth in complete or in minimal medium at 38 C upon prodigiosin formation after cultures were shifted to 27 C at times indicated. Prodigiosin, protein, and viable count were determined after incubation for 7 days at 27 C.*

the shift down in incubation temperature might permit pigmentation in a suspension of nonproliferating cells. Pigmentation at 27 C could occur after a longer period of incubation at 38 C in complete medium than in minimal, suggesting that casein hydrolysate or yeast extract supplied nutrients permitting formation of prodigiosin. Thus, a study of the phenomenon might also provide information relative to the nutritional requirements for biosynthesis of prodigiosin.

Cultures were grown in complete or in minimal medium at 38 C, and then, after incubation for the times indicated on the abscissa of Fig. 3, the flasks were shifted to 27 C. All cultures were incubated at the lower temperature for an additional 7 days, at which time prodigiosin, protein, and viable count were determined. Ability to form pigment at 27 C after shift down of nonpigmented cultures grown in minimal medium dropped precipitously after incubation for 36 hr at 38 C and ceased completely after 48 to 60 hr. A similar sharp decline occurred after a period of 48 hr at 38 C for cultures incubated in complete medium, but the ability to form small amounts of pigment after the shift down continued for a period of 96 hr of prior incubation at 38 C. Although values for viable count and protein gradually declined as the period of incubation at 38 C increased to 120 hr, followed by an additional 7 days at 27 C, little variation occurred between the two media.

The data from Fig. 3 suggest that complete medium contains substances that permit pigmentation after the shift down either because of their presence in the growth medium at 38 C or because of their presence in the medium after the shift to 27 C. Table 1 shows that casein hydrolysate will permit pigmentation to occur in suspensions at 27 C. Bacteria can be grown in minimal medium at 38 C for as long at 72 hr and still form pigment when shifted to 27 C, provided casein hydrolysate is added to the cultures. Washed cells obtained from cultures grown for

TABLE 1. *Induction of pigment at 27 C after growth of bacteria at 38 C*

Growth medium at 38 C[a]	Induction at 27 C		Pigment after 7 days at 27 C
	Suspension liquid[c]	Supplement added	
Complete or minimal[b]	Complete medium	None	+
	Minimal medium	None	−
	Saline	Casein hydrolysate (0.1%)	+
	Saline	Casein hydrolysate (0.2%)	+
	Saline	Yeast extract (0.1%)	−
	Saline	Yeast extract (0.2%)	+
Minimal plus casein hydrolysate (0.2%) or yeast extract (0.1%)	Saline	None	−
	Saline	Casein hydrolysate (0.1%)	+
	Saline	Yeast extract (0.1%)	−
	Saline	Yeast extract (0.2%)	+

[a] All cultures were incubated for 72 hr; no pigment was formed at this temperature.

[b] Identical results were obtained with complete or minimal media, or with minimal medium containing either casein hydrolysate or yeast extract.

[c] Washed suspensions of bacteria were resuspended in fresh medium or in 0.85% saline plus supplement.

72 hr at 38 C in various media will form pigment when suspended in 0.85% saline at 27 C, provided casein hydrolysate is present. This fact not only affirms the requirement for casein hydrolysate but also indicates that no substances which are needed for pigmentation after the shift to 27 C accumulate in the growth medium at 38 C. Although yeast extract at a concentration of 0.1% in minimal medium will support growth at 38 C, this concentration will not permit pigmentation after a shift down to 27 C. However, if the concentration of yeast extract is 0.2%, pigment will form after the shift down, apparently because this higher concentration provides enough of the required substances that are present in casein hydrolysate. The data establish that casein hydrolysate provides essential substance(s) for pigment formation after a shift in temperature from 38 to 27 C.

Figure 4 compares the kinetics of pigment formation at 27 C in a culture growing in 0.85% saline plus 0.2% casein hydrolysate to a suspension of cells grown at 38 C and suspended in the same medium. The shift down suspension contained cells that were harvested from cultures grown for 72 hr in minimal medium. Pigment formation was slower after shift down; after incubation for 7 days, the amount of pigment formed is not quite two-thirds of that produced by the growing culture. Shift-down cultures incubated for as long as 14 days showed no further increase in pigment beyond the amount present at 7 days. The amount of protein in the shift-down culture increases slightly for the first 24 hr of incubation but then slowly declines along with the viable count. Prodigiosin continues to be synthesized although protein and viable count are declining.

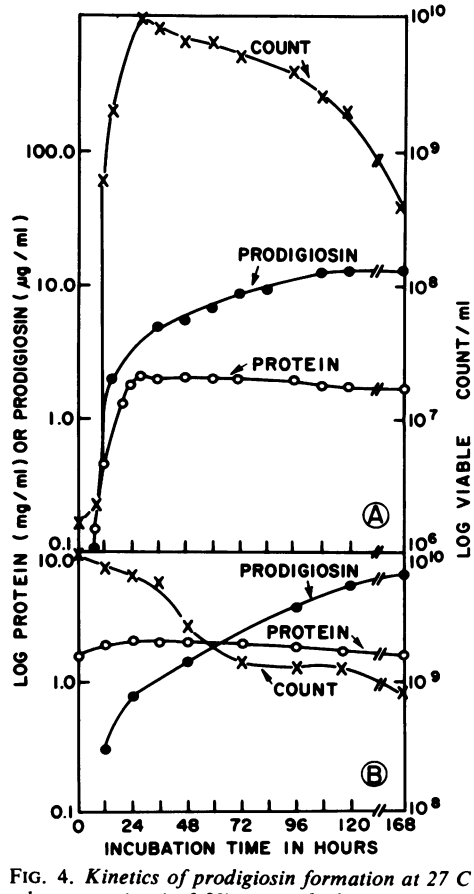

FIG. 4. *Kinetics of prodigiosin formation at 27 C in a culture growing in 0.2% casein hydrolysate (A) and in a culture of nonpigmented, washed cells resuspended in 0.85% saline plus 0.2% casein hydrolysate (B). Measurements of protein and viable count are also indicated.*

The data for viable count shown in Fig. 4 suggested that the bacteria did not proliferate in the shift-down cultures forming pigment from casein hydrolysate. Additional experiments confirmed this conclusion. When total counts were made, the values paralleled those of the viable count. Microscopically, individual rods first elongated and then, at about 48 hr, degenerated into bizarre forms of many shapes. In addition, the data in Table 1 showed that a concentration of 0.1% yeast extract, which supported multiplication (growth) from small inocula equivalent to 0.2% casein hydrolysate, did not support pigmentation in shift-down cultures. These results indicated that multiplication of cells and ability to form pigment are not necessarily coupled. DL-Alanine at a concentration of 20 mg/ml inhibited growth of strain Nima but supported pigmentation in shift-down cultures (9). Data from Fig. 2 and Table 1 demonstrated that pigmentation in shift-down cultures occurred in suspensions of cells harvested from the stationary phase of growth after incubation for 72 hr at 38 C. The cells were suspended in saline at 27 C to one-half their concentration reached while growing in medium at 38 C. These conditions probably precluded further multiplication of the bacteria. The nonpigmented cells could also be suspended in 2% agar at 27 C and could form pigment when 0.2% casein hydrolysate was added to hollow glass cylinders placed on the agar surface. Only the area surrounding the cylinders showed pigment. No macroscopic or microscopic colonies formed either in or on the agar, again indicating that the suspensions were not multiplying.

The maximal amount of pigment was synthesized by the nonproliferating cells after incubation for 7 days in a concentration of 0.4% casein hydrolysate. Comparative amounts of prodigiosin formed for different amounts of the added supplement were: 1.8, 2.4, 2.6, 2.1, and 1.5 μg/mg of protein for concentrations of 0.1, 0.2, 0.4, 0.8, and 2.0% of casein hydrolysate, respectively.

The temperature for pigment formation in shift-down cultures was similar to the range for growing cells. The maximal amount of pigment was synthesized at 27 C. Narrower limits of temperature were evident for the shift-down system since no pigment formed at 16 or 32 C, whereas growing cultures still produced some pigment at these points (Fig. 1).

Incubation under anaerobic conditions, killing cells by heating to 56 C for 1 hr before incubation, or a shift back to 38 C before pigment formation began at 27 C completely inhibited pigmentation in the shift-down system. A concentration 5 μg of chloramphenicol per ml added at the start of incubation inhibited pigmentation in shift-down cultures by 50%; a concentration of 25 μg/ml completely prevented pigment formation. Other experiments demonstrated that complete inhibition of pigment formation by bacteria growing in complete medium required a concentration of 250 μg of chloramphenicol per ml. These data again emphasized the difference between biosynthesis of prodigiosin in shift down and in growing cultures. Interestingly, although chloramphenicol inhibits pigment formation by growing cells, we were unable to inhibit growth of strain Nima, as measured by either viable count or protein, with concentrations of the antibiotic as high as 1,000 μg/ml.

Efforts to investigate the effect of *p*H upon pigment formation in the nonproliferating cells failed because the suspensions themselves provided such great buffering. Extremes of *p*H either in the acid range below 3.0 or in the alkaline range above 10.0 prevented pigmentation. Between these ranges no matter what buffer was tried, pigmentation always occurred, and the cultures rapidly approached a *p*H of 8.0 to 8.3, maintaining this range throughout incubation for 7 days.

DISCUSSION

Our quantitative data confirm the common belief that maximal pigmentation in *S. marcescens* occurs near room temperature. Other investigators report that some strains of *S. marcescens* can form pigment when incubated at 37 C (3). However, we have not seen a strain that will pigment in any medium at incubation temperatures of 37 C or higher, if the temperature remains at that point throughout incubation. Perhaps the discrepancy between our results and those of others is explained by the careful control we provided for temperature by using a water bath.

When cells are growing in casein hydrolysate alone, prodigiosin biosynthesis appears to begin earlier (Fig. 4) than in cultures growing in complete or minimal media (Fig. 2). This observation suggests that some ingredients of the latter media, perhaps citrate or glycerol, may repress or inhibit enzymes required for pigment formation. The kinetics for prodigiosin biosynthesis by all growing cultures indicate that pigmentation begins in the exponential phase, and that maximal production occurs in the stationary phase. These data corroborate the opinion that prodigiosin can be regarded as a secondary metabolite (10).

Pigment formation after shift down in temperature may involve several processes. In addition to furnishing precursors for biosynthesis, casein

hydrolysate may also be required for derepression of the enzymes synthesizing prodigiosin. Whether the initial rise in protein reflects increased protein synthesis needed for pigment biosynthesis must await further investigations.

Ingledew and Campbell (5, 6) suggest that investigations on the biosynthesis of pyocyanine were complicated and hampered by failure to discover conditions that separate pigment biosynthesis from growth of *Pseudomonas aeruginosa*. The shift-down system we describe separates growth and pigment formation in *S. marcescens*. Although pigment biosynthesis by nonproliferating cells is slow, and the amount of pigment formed is less than in growing cultures, an experiment of Blizzard and Peterson (1) suggests that shaking the suspensions might achieve more rapid formation of prodigiosin. Detailed quantitative investigations are needed to define the optimal conditions for maximal pigment formation by the nonproliferating cells.

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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.

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