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Enhancement by Sodium Dodecyl Sulfate of Pigment Formation in *Serratia marcescens* O8

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Three methods were used to determine the enhancement by sodium dodecyl sulfate (SDS) of prodigiosin formation in *Serratia marcescens* O8. The results of the agar disk diffusion method indicated that pigment formation was dependent upon the concentration of SDS. Diameters of the pigment zones were proportional to the logarithm of SDS concentrations of 300 to 1,500 μg/ml. When bacteria were grown in broth containing SDS from 0 to 800 μg/ml and the pigment extracts were analyzed spectrophotometrically, a similar enhancement of pigment formation was observed. Finally, these results were confirmed by high-performance liquid chromatographic analysis of the extracts. Prodigiosin appeared to be the sole component with increased synthesis. The possible mechanism of the SDS enhancement effect could be explained by an increase in negative binding sites by the association of SDS with a cell envelope component(s). These binding sites may be required for prodigiosin synthesis.

Serratia marcescens, which was generally considered to be a harmless saprophyte, is now recognized as a cause of serious infections, especially nosocomial infections (20). Consequently, more and more attention is being focused on its biochemical and physiological properties. Some strains of *S. marcescens* synthesize a linear tripyrrole pigment, prodigiosin, which has some antimicrobial activity (18). Although the complete biosynthetic pathway of prodigiosin in *S. marcescens* remains unclear, its biosynthesis is influenced by various environmental and nutritional factors (18). Recently, Tsang and Sheung (12) observed that the pigmentation of *S. marcescens* O8 was inhibited, without cellular growth being disturbed, by low concentrations of polymyxin B, a cationic antibiotic detergent. Since polymyxin B is capable of binding to several outer membrane components, they suggested that the binding of polymyxin B to these components may competitively bind to one or more of the macromolecular sites required for pigment synthesis. Thus, it may be possible that some of the anionic detergents capable of binding to a bacterial envelope component(s) may exert a positive effect on the pigment formation. This paper reports the enhancement effect of sodium dodecyl sulfate (SDS), an anionic detergent, on prodigiosin synthesis in *S. marcescens* O8.

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MATERIALS AND METHODS

Bacteria. *S. marcescens* O8 (a pigmented wild type) was used. All plates and cultures were incubated at room temperature (24 to 25°C) under continuous fluorescent lights to allow light exposure at all times during growth.

Agar diffusion method. Tryptic soy agar (Difco Laboratories) was used to prepare plates. Each petri dish (100 by 15 mm; Fisher Scientific Co.) contained 20 ml of autoclaved rehydrated tryptic soy agar. A single colony of *S. marcescens* O8 was transferred into 10 ml of tryptic soy broth (Difco); then the latter was incubated for 6 h. A sample of 0.2 ml of this bacterial suspension was spread on each tryptic soy agar plate. After 20 to 30 min, the sterilized disk blanks (6.5 mm in diameter; Difco) impregnated with different concentrations of SDS (Matheson Coleman & Bell), ranging from 0 to 1,500 μg/ml, were placed on the surface of the seeded agar plates. Each concentration of SDS was repeated in four to six disks. Plates were then incubated for 24 h. After this time, the bacteria grew well and the lawn was an even orange-red color. When there was an enhancement of pigmentation, the red color around the disk appeared deeper than the adjacent area. The diameter of the pigment zone, if any, was measured directly with a ruler to 0.1 mm.

Growth of bacteria in broth and extraction of pigment. Bacteria were grown in a 125-ml Erlenmeyer flask containing 30 ml of tryptic soy broth and shaken on a shaker bath (model 50, Precision Scientific Co.) at 100 rpm. The concentrations of SDS in broth were 0, 100, 200, 300, 400, 500, 600, and 800 μg/ml. After incubation for 48 h (pH of the cultures, about 8.0 with or without addition of SDS), 0.25 ml of culture was pipetted and diluted appropriately to determine the turbidity at 650 nm (Bausch & Lomb Spectronic 20 photometer), and 1 ml of culture was used for viable

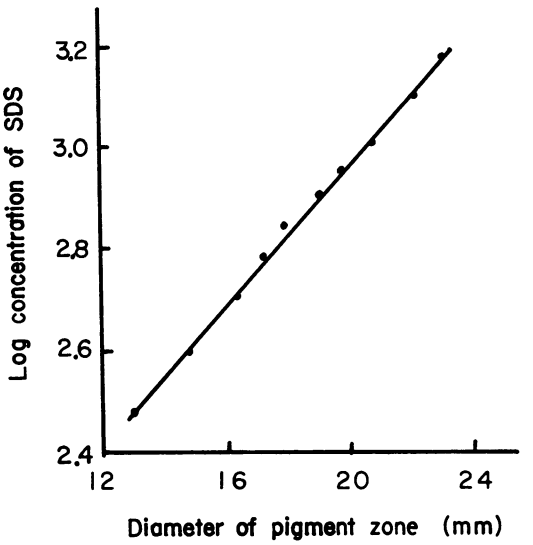

FIG. 1. Effect of SDS concentration on zone of pigmentation in *S. marcescens* O8.

counts by the standard technique. A 5-ml portion of the same culture was used for cell protein analysis (6). The rest of the culture was centrifuged at 10,000 rpm (Beckman model J2-21 centrifuge) for 30 min. The cells were washed with 5 ml of deionized water, suspended in 4 ml of deionized water, and extracted with acetone according to the method of Williams et al. (17). The petroleum ether extract was evaporated to dryness and reconstituted in 2 ml of dichloroethylene for analysis.

Spectrophotometric analysis of pigment. The reconstituted samples were diluted appropriately (2 to 30 times) for spectrophotometric analysis (Beckman model 35 spectrophotometer). The absorbance at 537 nm (17) multiplied by the dilution factor was used to express the amount of prodigiosin in the sample.

High-performance liquid chromatography (HPLC) analysis of extracts. A Beckman model 330 isocratic high-performance liquid chromatograph with a Rheodyne injector valve containing a 10-μl sample loop and a Perkin-Elmer LC-55 variable-wavelength UV visible detector was used. The column was a 25-cm by 4.6-mm-inside diameter, stainless-steel column packed with Lichrosorb RP-18 of 10-μm particle diameter (Alltech Associates). The mobile phase was 25% dichloroethylene in methanol containing 10 ppm (10 μl/liter) of concentrated HCl (3). Samples were analyzed at a flow rate of 2.00 ml/min and monitored at 537 or 272 nm (3, 17). The 537-nm absorption is one of the characteristics of prodigiosin, whereas 272 nm has been used to monitor the precursors of prodigiosin.

RESULTS

Agar disk diffusion test. Pigment zones of the agar plates at various concentrations of SDS are shown in Fig. 1. The minimal concentration that enhanced pigment formation was estimated to be 300 μg/ml. At 200 μg/ml, no enhancement of pigment was observed. It appeared that pigment enhancement by SDS was dependent upon the concentration of SDS. A plot of the logarithm of SDS concentrations versus the diameters of the pigment zones exhibited a linear relationship (Fig. 1).

Growth of bacteria and spectrophotometric analysis of extracted pigment. The culture became turbid after about 4 h of incubation, and pigmentation generally began after 18 to 24 h of incubation. As growth of the bacteria continued, differences in color between cultures were more and more obvious. Pigmentation of the cells reached the highest level at about 48 h. After that time the color intensity decreased slowly. Pigment formation in cultures containing different SDS concentrations after 48 h of incubation is shown in Fig. 2. Enhancement by SDS increased as SDS concentration increased and reached a maximum at a concentration of 500 μg/ml (Fig. 2a). The relationship between pigment enhancement and SDS concentrations of 100 to 500 μg/ml is almost linear; above 500 μg/ml the enhancement effect declined significantly. At the same time, the growth of the bacteria in tryptic soy broth was inhibited by SDS, as shown by the turbidity of the cultures and viable counts of the cells (Fig. 2b). This observation is consistent with the results reported in an earlier study (G. G. Patel, M.S. thesis, Illinois State University, Normal, 1980). However, it was reported on other occasions in *Escherichia coli* that SDS at a concentration of 100 μg/ml neither inhibited the growth nor injured cell structures (9).

HPLC analysis of pigment extracts. When samples were analyzed by HPLC monitored at 537 nm, a single symmetric peak at a retention time of 6.7 min was obtained. This result was indicative of the presence of prodigiosin (3; Fig. 3a). As expected, the peak areas (measured by a planimeter and expressed as square centimeters per milligram of protein) of the prodigiosin component in the pigment extract was a function of SDS concentrations in the cultures (Fig. 2a). From these results the relationship between SDS concentration and its enhancement of prodigiosin formation was firmly established. When monitored at 272 nm for possible monopyrrole and bipyrrole precursors, three well-resolved peaks with retention times of 1.6, 4.1, and 5.6 min, respectively, were obtained (Fig. 3b). At 272 nm no difference in the HPLC profiles was observed as the concentration of SDS was varied in cultures.

DISCUSSION

Other than the antibiotic property, the physiological function of prodigiosin in *S. marcescens* is unknown. It is generally considered to be a

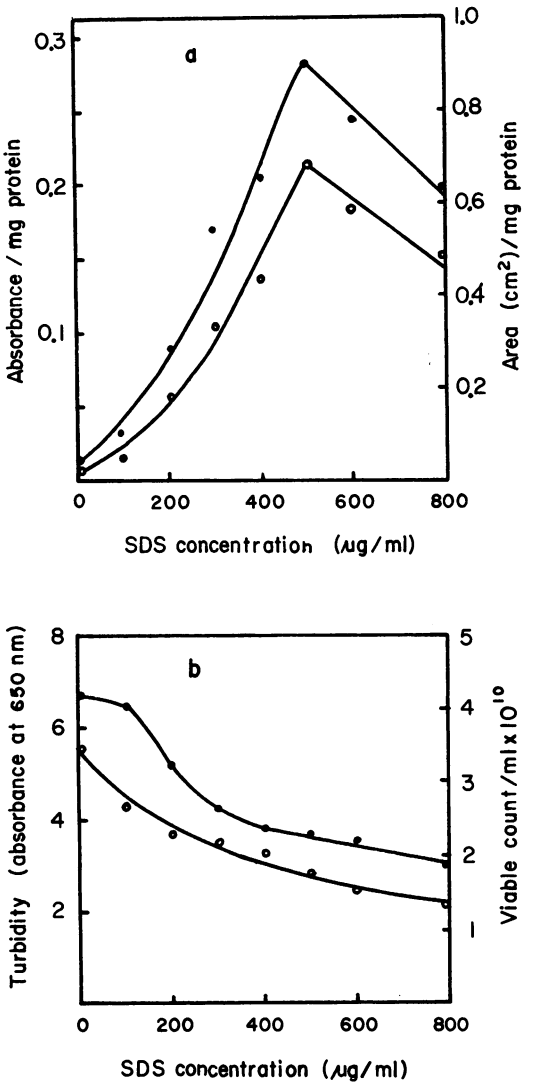

FIG. 2. Prodigiosin formation in cells grown at different SDS concentrations. (a) Symbols: (●) amount of prodigiosin expressed as absorbance at 537 nm per milligram of protein; (○) amount of prodigiosin in pigment extract after HPLC separation, expressed as peak area. (b) Bacterial growth after 48 h at different SDS concentrations. Symbols: (●) turbidity of culture expressed as absorbance at 650 nm; (○) viable counts.

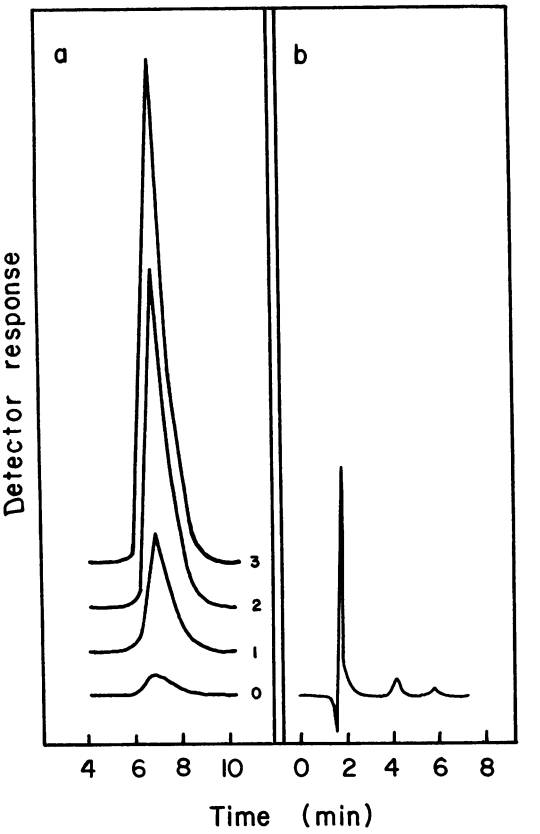

FIG. 3. HPLC profiles of extracts from *S. marcescens* O8. (a) Detected at 537 nm of pigment fractions extracted from cells grown in the presence of various concentrations of SDS: 0 = no SDS; 1 = 200 µg/ml; 2 = 800 µg/ml; 3 = 500 µg/ml. (b) Detected at 272 nm of pigment fraction extracted from cells grown in the absence of SDS.

secondary metabolite (15). Some nutrient factors, such as thiamine (2) and ferric ion (11), are needed for optimal synthesis of prodigiosin, but others, such as inorganic phosphate (19), ATP, and ribose (5), as well as sodium chloride (11), inhibit its synthesis. Several antimicrobial compounds also inhibit the pigment synthesis (12, 16, 18). However, no detailed studies on the enhancement have been reported. Our experiments clearly show that SDS, an anionic detergent, substantially enhances the production of prodigiosin in *S. marcescens* O8. Its enhancement increased with increasing SDS concentration within a definite range and reached a maximum at 500 µg/ml.

The color of *S. marcescens* cells capable of prodigiosin synthesis changes under different cultural conditions (1). The quantity of prodigiosin determined visually should be confirmed by other techniques. It may be more reliable if the pigment is extracted and then determined spectrophotometrically (17). HPLC, a fast analytical technique developed in the past 10 years, is an excellent tool for various areas of analyses, especially those in life sciences (4). Because of its high separation ability, analysis by this method could be used as a definition of purity. Using this approach, Kalanik et al. developed a method to analyze the prodigiosin component in

pigment extracts of *S. marcescens* O8 (3). In light of the results of HPLC analysis obtained in this study, we conclude that SDS enhances the pigmentation of *S. marcescens* O8 under the experimental conditions described, and the pigment component that was actually enhanced was prodigiosin.

Tsang and Sheung suggested that the binding of cationic polymyxin B to several outer membrane components and competitively displacing prodigiosin or its precursors from one or more of the macromolecular sites required for pigment synthesis may be the mechanisms through which polymyxin B exerts its inhibitory effect (12). If this is the case, SDS, an anionic detergent, may be expected to enhance prodigiosin synthesis. When a gram-negative bacterial cell is in direct contact with SDS, the detergent may interact with the cell wall lipoprotein and lipopolysaccharides (10). Indeed, cell envelope glycoproteins have been successfully extracted by SDS from two strains of *S. marcescens* by Tsang et al. (13). Recently, Rosenbusch identified the main envelope protein capable of binding SDS in *E. coli* (7). Even after SDS treatment of *E. coli* cells, some envelope proteins were able to retain their biochemical functions, such as phospholipase A_1 (8), NADH oxidase, and ATPase activities (14). Therefore, we suggest that SDS may bind certain components in the living cell envelope of *S. marcescens* O8. This binding may increase an extra negative macromolecular site(s) needed for the binding or synthesis or both of the positively charged prodigiosin or for its precursors to condense.

Based on the above assumption, we intend to confirm our suggestion by analyzing the effects on pigment formation in *S. marcescens* O8 of other anionic detergents, such as sodium deoxycholate, and other cationic detergents, such as cetyltrimethyl ammonium bromide, as well as that of the neutral detergent Triton X-100. These future studies will provide further evidence for the charge effect of the detergents on prodigiosin synthesis in *S. marcescens*.

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