JOURNAL OF BACTERIOLOGY, June 1984, p. 1128–1132
0021-9193/84/061128-05$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 158, No. 3

Cloning and Expression in *Escherichia coli* of *Serratia marcescens* Genes Encoding Prodigiosin Biosynthesis

STEVEN A. DAUENHAUER,† RICHARD A. HULL, AND ROBERT P. WILLIAMS*
Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Received 27 October 1983/Accepted 15 March 1984

Prodigiosin, the bright red pigment produced by many strains of *Serratia marcescens*, is synthesized by a bifurcated pathway that terminates in the enzymatic condensation of the two final products, a monopyrrole and a bipyrrole. *Sau*3A fragments of *S. marcescens* (Nima) DNA were introduced into a strain of *Escherichia coli* K-12 by use of the cosmid vector pHC79, and transformed clones were selected based on resistance to ampicillin. Among 879 transformants screened, 2 could be induced to synthesize prodigiosin when supplied with either one or both terminal products of the bifurcated pathway. Data are presented to support the idea that production of prodigiosin is not usually mediated by a plasmid.

Downloaded from jb.asm.org at CAMBRIDGE UNIVERSITY LIBRARY on July 24, 2009

Many strains of *Serratia marcescens* produce pigment via a bifurcated pathway in which 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2′-bipyrrole-5-carboxyaldehyde (MBC) are enzymatically condensed into 2-methyl-3-amyl-6-methoxypyrodigiosene, or prodigiosin (Fig. 1). Several mutants of *S. marcescens* have been identified as being blocked in either the MAP or MBC pathway. However, little is known about the precursors accumulated by these mutants, and nothing is known about the enzymes or gene products involved (8).

Prodigiosin is an easily assayed secondary metabolite of *S. marcescens* and may be useful as a model system to study the mechanism of expression of secondary metabolites in bacteria. Isolation of recombinant molecules encoding the prodigiosin biosynthetic pathway would provide an approach to identifying gene products and understanding the enzymology and the genetics of prodigiosin biosynthesis.

In this paper, we describe the isolation of DNA sequences encoding part of the prodigiosin biosynthetic pathway by use of a cosmid vector-*Escherichia coli* cloning system. In addition, we present a novel method for the screening of bacterial clones for prodigiosin genes, using mutant strains of *S. marcescens* that are defective in pigment production.

MATERIALS AND METHODS

Bacteria and conditions of growth. Wild-type strains of *S. marcescens*, Nima and Hy, were grown routinely at 37°C on peptone-glycerol agar (PGA; 0.5% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 1% glycerol, and 1.5% agar) and were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Mutant strains of *S. marcescens*, 933 and WF, were cultured and maintained under the conditions indicated above.

E. coli HB101 (*pro leu thi lacY hsdR endA recA rpsL20 ara-4 galK2 xyl-5 mtl-1 supE44*) and PK243 (HB101[pHC79]) (2) were cultivated at 37°C. *E. coli* NS428 (N205[λ *A*am11 *b2 red*3 *c*Its857 *S*am7]) and NS433 (N205[λ *E*am4 *b2 red*3 *c*Its857 *S*am7]) were grown at 30°C on L agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) unless otherwise indicated.

* Corresponding author.
† Present address: Louisiana Reference Laboratories, International Clinical Laboratories, Baton Rouge, LA 70806.

Purification of DNA from *S. marcescens*. Two milliliters of an overnight broth culture of *S. marcescens* (Nima) was transferred to 100 ml of fresh tryptic soy broth (Difco) and incubated with shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.3. The bacteria were centrifuged for 10 min at 5,500 × *g* and 4°C, suspended in TEN buffer (50 mM Tris-hydrochloride [pH 8.0], 10 mM EDTA, 50 mM NaCl), centrifuged again, and then suspended in 4 ml of a solution of 25% sucrose in 50 mM Tris-hydrochloride (pH 8.0) and 1 mM EDTA. This suspension was equally divided into two VTi-50 Quick-seal (Beckman Instruments, Inc., Palo Alto, Calif.) centrifuge tubes, and to each tube was added 100 μl of aqueous lysozyme (20 mg/ml; Sigma Chemical Co., St. Louis, Mo.). After gentle swirling in ice for 5 min, 10 μl of proteinase K (20 mg/ml; Sigma) was mixed into the suspension. To each tube 0.4 ml of 0.5 M EDTA (pH 8.0) was added with gentle mixing, and finally, 0.25 ml of 10% N-lauroyl sarcosine (Sigma) was added. The suspension was mixed, and the tubes were allowed to remain in ice until the mixtures became clear. The tubes were covered with aluminum foil and incubated at 50°C in a water bath overnight.

A 10-mg/ml stock solution of phenylmethylsulfonyl fluoride (Calbiochem-Behring, La Jolla, Calif.) was prepared in 95% ethanol and was mixed with TEN buffer to a final concentration of 50 μg/ml. Cesium chloride was dissolved in the phenylmethylsulfonyl fluoride-TEN buffer and mixed with the DNA to achieve a starting density of 1.717 g/cm^3. The tubes were centrifuged in a Beckman type 60 Ti rotor for 48 h at 35,000 rpm and 20°C. The purified DNA was dialyzed (six changes, 2 liters per change) against a solution of 6 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, and 0.5 mM EDTA and stored at 4°C (6).

Partial digestion of intact DNA. Into each of 25 microcentrifuge tubes in ice was added 20 μl of purified *S. marcescens* (Nima) DNA, 1.2 μl of 0.1 M $MgCl_2$, 1.0 μl of bovine serum albumin (2.8 μg/μl; Sigma), and 2 to 4 U of the restriction endonuclease *Sau*3A (New England Biolabs, Beverly, Mass.). The tubes were transferred to room temperature, and at various time intervals (5, 8, 10, 12, 15 min, etc.), some of the tubes were returned to the ice bath. Enzyme digestion in each tube was terminated by the addition of a 0.1 volume of a 1:100 dilution of diethylpyrocarbinate (Sigma) in 95% ethanol followed by incubation at 37°C for 15 min.

The contents of all tubes were pooled and centrifuged in 5 to 20% NaCl gradients for 4.5 h in a Beckman SW41 Ti rotor

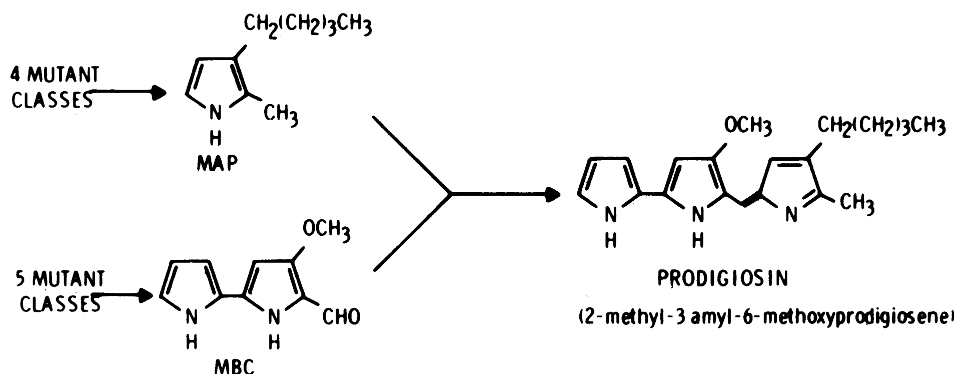

FIG. 1. End products of the bifurcated prodigiosin biosynthetic pathway in *S. marcescens*. MAP and MBC are enzymatically condensed into prodigiosin. Several mutants of *S. marcescens* have been characterized that possess genetic lesions in the biosynthesis of one or the other precursor compounds.

Downloaded from jb.asm.org at CAMBRIDGE UNIVERSITY LIBRARY on July 24, 2009

at 150,000 × *g* and 4°C. Gradients were collected dropwise in 0.5-ml fractions by puncturing the bottom of the tubes, and a 7-μl sample from alternating fractions was electrophoresed in 0.35% agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) slab gels. Those fractions containing *Sau*3A fragments in the size range of 25 megadaltons (md) were pooled and precipitated in 95% ethanol at −20°C overnight. After centrifugation, the DNA was dissolved in TE buffer (50 mM Tris-hydrochloride [pH 8.0], 10 mM EDTA), and the OD_{260} was measured.

Ligation of the 25-md DNA fragments with the cosmid vector. The amount of pHC79 vector DNA employed was ca. one-fifth the number of micrograms of DNA fragments ligated to assure nearly equal numbers of vector and *Serratia* DNA molecules. A predetermined amount of cosmid DNA was digested to completion with *Bam*HI (New England Biolabs). Endonuclease activity was terminated by the addition of a 1:1,000 volume of diethylpyrocarbinate. The reaction mixture was subjected to vacuum desiccation for 20 min and incubated at 65°C for 5 min. The volume was adjusted to 200 μl with TE buffer, and the DNA was precipitated with a 0.1125 volume of 3 M sodium acetate and a 0.625 volume of ice-cold isopropanol at −20°C for 1 h. After centrifugation, the DNA was dissolved in TE buffer at a concentration of 50 μg/ml.

DNA from the cosmid and from *S. marcescens* was combined in a ratio of 1:5 (measured in micrograms) and coprecipitated. The DNA mixture was suspended in 10 μl of ligation buffer (20 mM Tris-hydrochloride [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 10 mM dithiothreitol [Sigma], 40 μM ATP [Sigma]) to which was added 1 U of T4 DNA ligase (New England Biolabs). Ligation proceeded overnight on ice.

Preparation of bacteriophage lambda packaging mix. The procedure was a modification of that described by Sternberg et al. (7). Briefly, cultures of *E. coli* NS428 and NS433 were each grown overnight at 32°C in 10 ml of Davis-Mingioli minimal salts media (3) containing 0.4% glucose and 2.5% Casamino Acids. Each culture was diluted 100-fold (total volume: NS428 in 150 ml; NS433 in 600 ml) in fresh media and grown at 32°C to an OD_{600} of 0.3. The bacteriophage then was induced by swirling the cultures in a 90°C water bath until the fluid temperature rose to 42°C. Incubation was continued with vigorous aeration at 42°C for 20 min. The cultures were shifted to 38°C for an additional 70 min and then chilled to 4°C.

Extract A. The NS428 culture was mixed with an equal volume of the NS433 culture and centrifuged for 10 min at 11,300 × *g* and 4°C. The pellet was suspended in a solution containing 0.6 ml of buffer A (20 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol [Fisher Scientific Co., Fair Lawn, N.J.]) and transferred to a chilled plastic tube (Falcon no. 2054; Becton Dickinson Labware, Oxnard, Calif.). The bacteria were disrupted with sonic vibration at full power by use of a fine-tip probe (Biosonik III; Bronwill Scientific Inc., Rochester, N.Y.) and three 5-s bursts.

Extract B. The remainder of the NS433 culture was centrifuged as described above and suspended in 1.2 ml of 10% sucrose in 50 mM Tris-hydrochloride (pH 7.4). The bacteria were transferred to a 25-ml Erlenmeyer flask and frozen and thawed twice in liquid nitrogen, followed by immersion in ice water. For each 100 μl of cell suspension, 5 μl of lysozyme (1 mg/ml in 0.25 M Tris-hydrochloride [pH 7.4]) was added. This mixture was incubated for 30 min at 4°C. Lastly, 10 μl of buffer B (6 mM Tris-hydrochloride [pH 7.4], 15 mM ATP, 16 mM $MgCl_2$, 30 mM spermidine [Sigma], 60 mM putrescine [Sigma], 30 mM 2-mercaptoethanol) was added and gently mixed into the cell lysate.

Glycerol was added to each extract to a final concentration of 20%. The extracts were distributed separately into microcentrifuge tubes in a liquid nitrogen bath. Extract A was stored in 25-μl volumes, and extract B was stored in 125-μl volumes, both at −70°C.

Packaging reaction and infection of the bacterial host. To a 1.5-ml microcentrifuge tube was added 30 μl of buffer A, 5 μl of the ligated DNA preparation (concentration of ca. 5 μg/ml), 2 μl of buffer B, and 20 μl of extract A. After incubation at room temperature for 10 min, 100 μl of extract B was added to the mixture, and incubation was continued for 1 h at 35°C. The reaction was terminated by the addition of 150 μl of TMG (10 mM Tris-hydrochloride [pH 7.4], 10 mM $MgSO_4$, 0.1% gelatin) and incubation for 10 min at 35°C.

A 1-ml overnight broth culture of *E. coli* K-12 HB101 was transferred to 9 ml of fresh medium (1% tryptone [Difco], 0.5% NaCl, 0.2% maltose, 10 mM $MgCl_2$), and the suspension was grown on a shaker at 37°C to an OD_{600} of 0.5.

Bacteria (0.5 ml) were combined with 0.1 ml of the preparation of packaged lambda phage, and the mixture was incubated at 37°C for 15 min. Samples (0.1 ml) were then spread evenly on the surface of six plates of L agar that contained 100 μg of ampicillin per ml (Sigma). The plates were

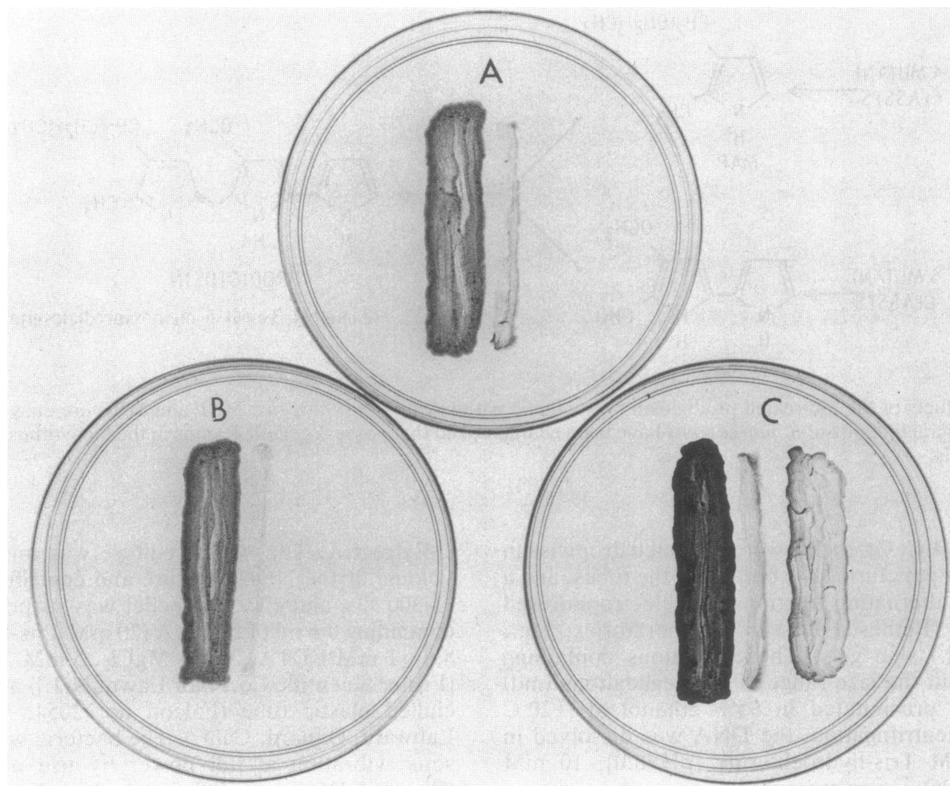

FIG. 2. Feeding experiments demonstrating the potential of *E. coli* K-12 transformants to produce prodigiosin. In each experiment, *S. marcescens* 933 was inoculated 24 h before inoculation of the *E. coli* transformant to allow the diffusible MBC compound to permeate the agar. (A) Strain SAD400 (right) manifests pigment when provided only MBC from *S. marcescens* 933 (left). (B) Strain SAD757 (right) does not produce pigment in the presence of *S. marcescens* 933 alone (left). (C) Strain SAD757 (center) exhibits pigment production when supplied with both MBC and *S. marcescens* 933 (left) and MAP from strain WF (right).

incubated at 37°C overnight. Colonies that arose were subcultured, cataloged, and screened immediately for phenotypes associated with prodigiosin biosynthesis.

Plasmid amplification and DNA purification. The methods employed have been previously described (1, 4).

RESULTS

Screening of *E. coli* isolates for recombinant molecules containing prodigiosin gene sequences. Four separate experiments were done in which cosmid DNA containing 25-md *Serratia* fragments was packaged into lambda phage that was used to infect *E. coli*. A total of 879 transformants were isolated and examined for prodigiosin production. As none of the isolated colonies were found to express prodigiosin autonomously, a procedure was developed to clarify whether some of the pathway sequences of DNA might be present. The method involved supplying MAP, MBC, or both to complement precursors of prodigiosin that might be produced by a transformant and result in formation of pigment.

In the first phase of the screening procedure, *S. marcescens* 933 was inoculated as a 3-cm spot onto PGA and grown for 24 to 48 h at 30°C. Colonies of transformed *E. coli* growing on L agar containing ampicillin were picked and then inoculated as spots on the plates of PGA within 1 to 3 mm of the circle of strain 933 growth. The plates were incubated for an additional 24 h at 30°C. *S. marcescens* 933 is defective in production of the volatile MAP precursor but synthesizes the diffusible MBC precursor (Fig. 1). Formation of prodigiosin by recombinant bacteria supplied only with MBC by diffusion from strain 933 would suggest that sequences were present in the *E. coli* isolates that encoded for the production of enzymes necessary for synthesis of MAP. One *E. coli* isolate, designated SAD400, examined by this method produced red pigment (Fig. 2).

Transformants not producing red pigment in the presence of MBC were further incubated in the presence of MAP supplied by *S. marcescens* mutant WF that lacks the ability to synthesize MBC. Bacteria that can synthesize prodigiosin only when furnished with both final precursors, MAP and MBC, must have the genetic information for an enzyme or enzymes that condense these molecules into prodigiosin. In this second step of the screening procedure, strain WF was inoculated onto a PGA plate and incubated at 30°C to obtain a lawn of growth. This agar plate was substituted for the cover of the plate containing the colonies of *E. coli* transformants surrounding a circle of growth of strain 933 so that the volatile MAP produced by mutant WF could saturate the atmosphere of the culture during incubation at 30°C for 24 h. A single isolate, designated SAD757, was found to produce red pigment only in the presence of both MBC and MAP (Fig. 2). No isolate examined synthesized prodigiosin when furnished only with MAP.

Verification of prodigiosin production by absorption spectrophotometry. In addition to the observable presence of red color that developed in both clones SAD400 and SAD757, a further test was made to confirm that the pigment produced

Downloaded from jb.asm.org at CAMBRIDGE UNIVERSITY LIBRARY on July 24, 2009

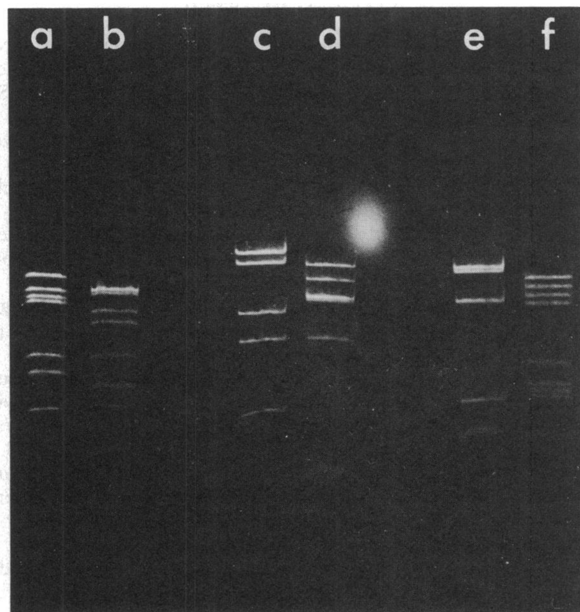

FIG. 3. Comparison of pSAD400 DNA (lanes a, c, and e) restriction endonuclease digestion profiles (fragment size range, 13 to 1 md) with those of purified *S. marcescens* Hy plasmid DNA (lanes b, d, and f). Enzymes used to digest the DNA in each pair were *Cla*I (lanes a and b), *Eco*RI (lanes c and d), and *Sal*I (lanes e and f).

was, in fact, prodigiosin. Prodigiosin extracted with ethanol from pigment-producing strains of *S. marcescens* shows characteristic absorption maxima of 535 and 470 nm in acid and alkaline solutions, respectively (5). Pigment extracted from the Nima strain was measured in the range of 300 to 700 nm, and the readings obtained served as a control.

Both clones were propagated on PGA at room temperature in the presence of the *Serratia* mutant (or mutants) which induced pigment formation. The *E. coli* were collected and treated as the *Serratia* control, and the pigments were analyzed spectrophotometrically. Absorption maxima at 535 and 470 nm in acid and alkaline solutions, respectively, from both clones confirmed that the pigment produced was prodigiosin.

Comparison of cloned *Serratia* DNA fragments with *S. marcescens* Hy plasmid DNA. One question addressed in this investigation was whether genetic information encoding prodigiosin biosynthesis resided totally or partially on extrachromosomal DNA in *S. marcescens*. The question could be answered by comparison, after digestion with various restriction endonucleases and electrophoresis of fragments in agarose gels, of fragment profiles of recombinant plasmid DNA from *E. coli* that produced prodigiosin and plasmid DNA from a strain of *S. marcescens* that synthesized prodigiosin. The consistent finding of one or more fragments of the same size when the two DNA molecules were cut with several different enzymes would provide evidence that the comigrating fragments were identical sequences. *S. marcescens* Hy that contains a single plasmid species (ca. 25 md) was used in these experiments.

Cosmid DNA extracted from *E. coli* SAD400 and SAD757 and plasmid DNA from *S. marcescens* Hy were purified by dye-buoyant density centrifugation in cesium chloride with ethidium bromide. Samples of both cosmid DNA and strain Hy plasmid DNA were digested with *Cla*I, *Eco*RI, and *Sal*I. The digestion products of cosmid DNA obtained with each enzyme were separated by electrophoresis in agarose slab gels. Corresponding digests of strain Hy plasmid DNA were run in adjacent lanes for comparison. No consistent fragments of identical size were found from the comparison of cosmid (Fig. 3 and 4, lanes a, c, and e) and plasmid DNA (lanes b, d, and f). These data suggest that the cloned sequences from the prodigiosin biosynthetic pathway were not located on Hy plasmid DNA.

DISCUSSION

Of 879 *E. coli* recombinant clones examined, none contained the sequences encoding the entire prodigiosin pathway. However, two clones responded to formation of syntrophic pigment with mutant strains of *S. marcescens*. It is possible that the cloning frequency of MBC is somewhat less than 1 in 879 or that these genes contained an abundance of *Sau*3A sites that were cleaved during partial digestion. Alternatively, the reason for failure to isolate these genes may be that they are widely distributed on the *Serratia* chromosome such that all individual genes would not be contained on a single 25-md fragment. Another reason could be that the MBC pathway is more complex than that for production of MAP and may depend upon the occurrence of other metabolic events. For example, a proline molecule is incorporated intact into one of the pyrrole groups of MBC, suggesting that biosynthesis might be linked to utilization of proline (8).

An important question addressed in this study was the location of pathway genes. Some strains of *S. marcescens*, such as Hy, demonstrate a high rate of spontaneous mutation to nonpigmented variants. Although a number of these variants promptly revert to pigmented bacteria (8), the high rate of spontaneous mutation suggests that extrachromosomal elements could be responsible for prodigiosin biosynthesis, at least in some strains. Our data provide two lines of

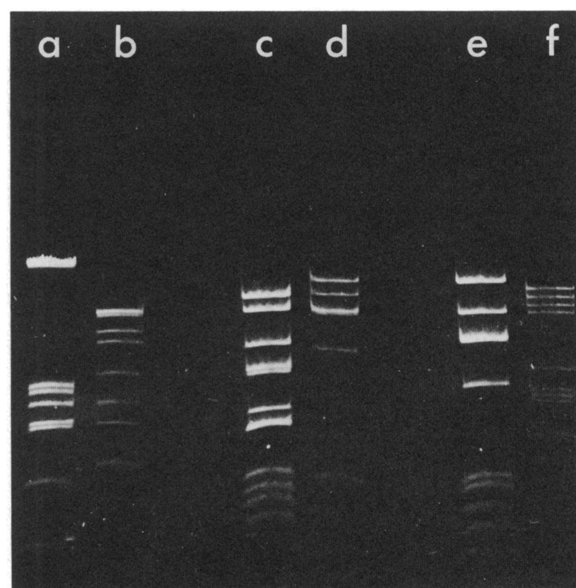

FIG. 4. Comparison of pSAD757 DNA (lanes a, c, and e) restriction endonuclease digestion profiles (fragment size range, 14.5 to 0.5 md) with those of purified *S. marcescens* Hy plasmid DNA (lanes b, d, and f). Enzymes used to digest the DNA in each pair were *Cla*I (lanes a and b), *Eco*RI (lanes c and d), and *Sal*I (lanes e and f).

Downloaded from jb.asm.org at CAMBRIDGE UNIVERSITY LIBRARY on July 24, 2009

evidence that genes for prodigiosin may not be entirely contained on plasmids. First, no detectable plasmids could be found in strain Nima, from which the sequences we characterized originated. Second, comparison of restriction endonuclease digests of cloned DNA with those of plasmid DNA isolated from the prodigiosin-producing Hy strains did not reveal sets of fragments of similar size when several different enzymes were used. Experiments in which cloned *Serratia* DNA fragments are radiolabeled and used as probes for hybridization to chromosomal DNA of strains Nima and Hy, as well as Hy plasmid DNA, should establish whether any of the prodigiosin genes are carried on plasmid DNA.

ACKNOWLEDGMENTS

These investigations were supported by U.S. Public Health Service training grant AI 07145 and research grant AI 18462 from the National Institute of Allergy and Infectious Diseases.

We thank Richard L. Ziprin, Veterinary Toxicology and Entomology Research Laboratory, U.S. Department of Agriculture Agricultural Research Service, College Station, Tex., for running the spectra.

LITERATURE CITED

1. **Clewell, D., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
2. **Collins, J.** 1979. *Escherichia coli* plasmids packageable *in vitro* in λ bacteriophage particles. Methods Enzymol. **68**:309–326.
3. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
4. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 116–119. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Ding, M.-J., and R. P. Williams.** 1983. Biosynthesis of prodigiosin by white strains of *Serratia marcescens* isolated from patients. J. Clin. Microbiol. **17**:476–480.
6. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
7. **Sternberg, N., D. Tiemeier, and L. Enquist.** 1977. *In vitro* packaging of a λ *Dam* vector containing EcoRI DNA fragments of *Escherichia coli* and phage P1. Gene **1**:255–280.
8. **Williams, R. P., and S. M. H. Qadri.** 1980. The pigment of *Serratia*, p. 31–75. *In* A. von Graevenitz and S. J. Rubin (ed.), The genus *Serratia*. CRC Press, Inc., Boca Raton, Fla.

Downloaded from jb.asm.org at CAMBRIDGE UNIVERSITY LIBRARY on July 24, 2009