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Purification and Partial Characterization of a Bacteriocin from *Serratia marcescens*

JOHN FOULDS

University of Connecticut Health Center, Farmington, Connecticut 06032

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Bacteriocin JF246 has been purified from mitomycin C-induced *Serratia marcescens* cells by salt extraction, ammonium sulfate fractionation, and chromatography on QAE-Sephadex and SP-Sephadex. The purified material is homogeneous on polyacrylamide gel electrophoresis in the presence of 2% sodium dodecyl sulfate or 6 M urea. In the absence of these agents, the bacteriocin associates into aggregates which can be dissociated with 0.4 M NaCl. The bacteriocin is probably composed of a single subunit with a molecular weight of 64,000 daltons. Analytical studies show the bacteriocin to be essentially protein in nature containing less than one residue of glucose or phosphorus per 64,000 daltons.

Bacteriocins are high-molecular-weight antibiotics synthesized by a variety of gram-positive and gram-negative bacterial strains. Bacteriocins are extracellular substances which often remain bound to the cell surface of the producer strain (26). Bacteriocin synthesis can be induced by agents which interfere with deoxyribonucleic acid (DNA) synthesis, such as ultraviolet (UV) light (14) or mitomycin C (13).

Bacteriocins exhibit a remarkable degree of specificity. Susceptibility to a given bacteriocin is usually restricted to bacterial species which carry a specific receptor on the cell envelope. Different bacteriocins may attach to the same receptor but differ in their mode of action. Similarly, bacteriocins with similar modes of action may attach to different receptors. Thus, each bacteriocin has a specificity of attachment and a specific mode of action, and these specificities are independent (23).

A number of bacteriocins have been purified and characterized. Most appear to be essentially, if not entirely, protein in nature. Certain bacteriocins, such as pyocin R (14) and colicin 15 (27), have very high molecular weights and when observed by electron microscopy resemble bacteriophage parts such as tails. Other bacteriocins, including colicins E1 (28), E2, E3 (11), Ia, Ib (17), and K (3, 6, 18), have been shown to be simple proteins with molecular weights in the range of 55,000 to 80,000.

Bacteriocin production in the genus *Serratia* was first described by Hamon and Peron in 1961 who found that 86% of the *Serratia* strains tested produced at least one bacteriocin (10). This report describes the purification and partial characterization of a bacteriocin produced in *S. marcescens* strain JF246.

Treatment of sensitive cells with bacteriocin JF246 results in the cessation of all macromolecular synthesis (5), a mode of action similar to that described for colicin A (21), E1 (15), I (20), and K (22). However, bacteriocin JF246 is not identical to any of these colicins, for mutants resistant to colicins A, E1, I, or K remain sensitive to bacteriocin JF246 (6).

MATERIALS AND METHODS

Bacterial strains and media. *S. marcescens* JF246 and *Escherichia coli* JF135 have already been described (5). For production of bacteriocin, *S. marcescens* JF246 was grown in CY medium. This medium contained 80 mM potassium phosphate (*p*H 7.0), 1 mM $MgSO_4$, 1% Casamino Acids (Difco), 0.5% yeast extract (Difco), and 1% glucose. The bacteriocin-sensitive indicator strain, *E. coli* JF135, was grown in M9 (5) medium containing 0.4% glucose and 20 μg/ml each of leucine, isoleucine, valine, and tryptophan. Other media have already been described (5).

Reagents and biochemicals. Bovine serum albumin (fraction V), Coomassie Brilliant Blue, mitomycin C, pyronin Y, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co., St. Louis, Mo. Reagents for the preparation of polyacrylamide gels and guanidine thiocyanate were purchased from Eastman Kodak, Rochester, N.Y. QAE-Sephadex (diethyl-2-hydroxypropyl-aminoethyl dextran), SP-Sephadex (sulfopropyl dextran) and G-50 Sephadex were obtained from Pharmacia, Piscataway, N.J. Bio-Gel A1.5 was from Bio-Rad, Rich-

mond, Calif. Ammonium sulfate (special enzyme grade), urea, and proteins used as molecular-weight markers (see Fig. 5) were purchased from Mann Research Laboratories.

Bacteriocin assay. Bacteriocin activity during purification was determined by serially diluting the solution to be tested with PPBE broth (5) and spotting a small drop (10–20 μliters) on PPBE soft agar containing approximately 10^7 freshly grown JF135 cells. After incubation at 37 C for 6 hr, the plates were scored. The number of bacteriocin units per milliliter was defined as the reciprocal of the highest dilution which gave a clear zone of inhibition of growth of the indicator strain.

Lacunae counts. Lacunae counts were performed by the method of Ozeki et al. (24) with *E. coli* JF135 as the indicator strain. Lacunae were visible as tiny clear areas (0.5–1.0 mm) in a lawn of cells after incubation of the plates for 6 to 8 hr.

Analytical methods. Protein concentration was determined by the method of Lowry et al. (19) with bovine serum albumin as the standard. Specific activity of bacteriocin solutions is defined as the number of bacteriocin units per milligram of protein.

Phosphorus was determined by the method of Ames and Dubin (1) after exhaustive dialysis of the sample. Before analysis for reducing sugars, samples of bacteriocin JF246 were hydrolyzed for 4 hr in vacuo at 100 C in 2 N HCl, and the *p*H was adjusted to approximately 7.0 with NaOH. Reducing sugars were then estimated by the method of Park and Johnson with glucose as a standard (25).

Identification of sugars present in purified bacteriocin preparations was made by gas chromatography after hydrolysis and reduction (4).

Amino acid analysis. Samples of purified bacteriocin JF246 in a volume of 10 ml were dialyzed for 4 days against 2 liters of deionized water. The water was changed daily. A portion of the dialyzed material containing 0.2 mg of protein was lyophilized in a hydrolysis tube. The lyophilized material was then taken up in 1 ml of 6 N HCl, and the tube was sealed under vacuum. The protein was hydrolyzed at 110 C for 22 to 24 hr, and the amino acid composition of the hydrolysate was determined on an automatic amino acid analyzer.

Electrophoresis. Polyacrylamide disc gel electrophoresis at *p*H 9.5 was carried out with 7.5% gels as described in the Canalco manual, supplied by the Canal Industrial Corporation, Bethesda, Md., except that the stacking gel was omitted. Sample application was accomplished after the individual gel columns were positioned in the electrophoresis apparatus and the electrophoresis buffers were in place. The density of the sample was increased by the addition of a few crystals of sucrose (8), and with a micropipet the sample was carefully applied in a layer at the top of the gel. The gels (0.5 by 8 cm) were run at room temperature with a current of 3 ma per gel. The gels were stained for 4 hr with a solution of 0.25% Coomassie Brilliant Blue in 10% acetic acid and 25% isopropanol. Destaining was accomplished by leaching the gels in 10% acetic acid. Polyacrylamide (6.5%) gel electrophoresis in the presence of 6 M urea was carried out at *p*H 8.5 with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-glycine buffer (alkaline urea). The samples were prepared by dissolving crystalline urea in 200 μliters of sample in 0.1 M Tris-glycine buffer at *p*H 8.5, such that the final concentration of urea was 6 M. After the addition of a few crystals of sucrose and 5 μliters of a 1% solution of pyronin Y, 100-μliter samples were layered on the gels and subjected to electrophoresis as described above. Staining and destaining of the urea gels were accomplished as described above. SDS polyacrylamide gel electrophoresis was carried out as described by Fairbanks (G. Fairbanks, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1969). Samples containing 30 to 150 μg of protein in 50 to 150 μliters were mixed with SDS and dithiothreitol (DTT) such that the final concentration was 2% and 10^{-3} M, respectively. After a 1-hr incubation period at 37 C, 0.1 volume of a 0.5% solution of pyronin Y (as a marker) in 0.4 M Tris-hydrochloride, *p*H 7.5, containing 0.2 M sodium acetate and 20 mM ethylenediaminetetraacetic acid (EDTA) was added, and a few crystals of sucrose were dissolved in the sample. This mixture was layered over an SDS polyacrylamide gel (0.5 by 10 cm), and electrophoresis was begun immediately. The gels were run at room temperature with a current of 3 ma per gel. When the pyronin Y had migrated to the bottom of the gel, the gels were removed and fixed overnight at 4 C in a solution of 15% trichloroacetic acid and 25% isopropanol (G. Fairbanks, Ph.D. thesis). The gels were then soaked at room temperature for 4 to 6 hr in a solution containing 10% acetic acid and 25% isopropanol. After this, the gels were stained and destained as described above. Where the protein bands were found to be only weakly stained, the staining and destaining steps were repeated. This invariably resulted in more distinct bands, probably as a result of the leaching of residual SDS which interferes with staining during the first staining and destaining.

For molecular-weight determinations, the migration of the stained band from the origin was measured and compared with the migration of standard proteins of known molecular weight.

Bacteriocin activity could be leached from slices of polyacrylamide gels by soaking the slice overnight with 0.5 ml of 0.05 M KPO_4, *p*H 7.0.

Protease assay. The presence of protease activity in bacteriocin extracts was monitored by a procedure described by Broussard (E. A. Broussard, M.S. thesis, Louisiana State Univ., 1968) which measures the hydrolysis of azocasein. One unit of activity was equal to the solubilization of 0.1 absorbancy unit in a 15-min assay.

RESULTS

Growth and induction of bacteriocingenic strain. The cells from a 200-ml overnight culture of *S. marcescens* JF246 in CY medium were collected by centrifugation and, after resuspension, inoculated into 12 liters of sterile CY medium. The culture was grown in a New Brunswick microferm at 39 C with vigorous aeration (6 liters air/min) and the fermentor

agitation control at 400 rev/min. Antifoam A (Dow-Corning, food grade) was added to control foaming as needed. (No foaming occurred until the optical density at 650 nm reached 2–3.) The *p*H of the culture was maintained at 7.0 with a radiometer model TTT-1 automatic titrator equipped with a magnetic valve which regulated the addition of 10% Na_2CO_3. Typically, 250 ml of Na_2CO_3 solution was required.

When the optical density (650 nm) of the culture reached 0.5, mitomycin C was added at a final concentration of 0.5 mg/liter. Growth was continued for 2.5 hr. The cells were then collected at a flow rate of 400 ml/min by connecting the exit port of the fermentor to a continuous-flow refrigerated centrifuge. The cell pellet, 150 to 200 g (wet weight), was chilled in an ice bath, and from this point all operations were performed at 0 to 4 C. Figure 1 shows the appearance of bacteriocin JF246 activity in the culture after addition of mitomycin C. When the culture was allowed to incubate longer than 2.5 hr in the presence of mitomycin C, a dramatic decrease in bacteriocin titer occurred. This was presumably due to destruction of the bacteriocin by an extracellular protease produced by *Serratia* (2).

Extraction of cell-bound bacteriocin. Under these growth conditions, greater than 95% of the bacteriocin activity in the culture was found in the cell pellet. The extracellular nature of the bacteriocin permitted the extraction of activity without disruption of the cells by simply suspending the cell pellet in 1 liter of 0.05 M KPO_4, *p*H 7.0, containing 1 M NaCl. The cells were mixed with the extractant for 1 hr, and the suspension was centrifuged at 10,000 × *g* for 20 min. The milky supernatant fluid was decanted and sterilized by shaking briefly with 5 ml of chloroform. Usually, over 90% of the activity in the cell pellet was solubilized by this procedure. Residual bacteriocin could be solubilized by simply repeating the NaCl extraction.

Ammonium sulfate fractionation. After excess chloroform was removed by decantation, solid ammonium sulfate (200 g/liter of solution) was slowly added, and the solution was stirred for 1 hr. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 20 min and discarded. Additional ammonium sulfate (134 g/liter of original solution) was slowly added to the supernatant solution, and, after 1 hr of stirring, the resulting precipitate, containing 80 to 95% of the bacteriocin activity, was collected by centrifugation at 10,000 × *g* for 20 min. The precipitate was dissolved in 200 ml of 0.02 M Tris-hydrochloride, *p*H 8.0, containing 10^{-3} M EDTA and 10^{-4} M DTT and dialyzed overnight against 6 liters of the same buffer. A precipitate which usually formed on dialysis was removed by centrifugation at 100,000 × *g* for 60 min. The resulting clear supernatant solution was distinctly yellow and contained all of the bacteriocin activity.

QAE-Sephadex column chromatography. The material was applied to a QAE-Sephadex column previously equilibrated with 0.02 M Tris-hydrochloride, *p*H 8.0, containing 10^{-3} M EDTA and 10^{-4} M DTT. Chromatographic separation was accomplished under starting conditions by washing the column with equilibration buffer. A distinct yellowish band remained at the top of the column, and the bacteriocin activity was found in the earliest fractions containing material which absorbed light at 280 nm. No additional bacteriocin activity could be removed from the column by adding NaCl at a final concentration of 1 M to the elution buffer or by washing the column with 0.02 M sodium acetate buffer, *p*H 4.0.

Figure 2 shows a typical elution profile from QAE-Sephadex. Peak fractions (fractions 25–33) were pooled and dialyzed overnight against 0.05 M KPO_4, *p*H 6.5, containing 10^{-3} M EDTA and 10^{-4} M DTT. Examination of this material by SDS-polyacrylamide gel electrophoresis (Fig. 4A) showed eight bands, one of which contained bacteriocin activity.

SP-Sephadex chromatography. The

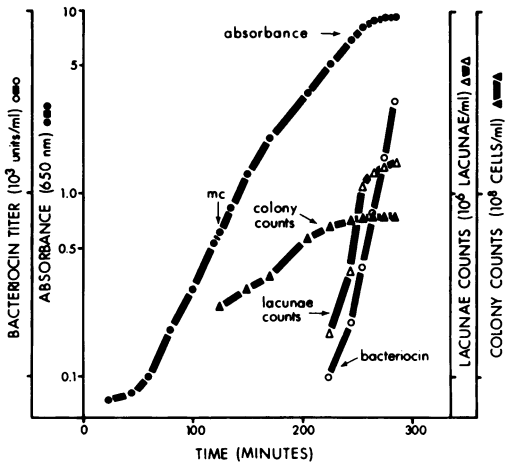

FIG. 1. *Induction of bacteriocin synthesis in Serratia marcescens JF246. Cells were grown as described in Materials and Methods. At 125 min (indicated by the arrow), 0.5 mg of mitomycin C was added per liter.*

pooled fractions from the QAE-Sephadex column were applied to an SP-Sephadex column (5.0 by 75 cm) previously equilibrated with 0.02 M Tris-hydrochloride, *p*H 8.0, containing 10^{-3} M EDTA and 10^{-4} M DTT. The column was washed with 1,500 ml of equilibration buffer, and a linear NaCl gradient was begun (1,000 ml of equilibration buffer in the mixing chamber and 1,000 ml of equilibration buffer containing 1.0 M NaCl in the reservoir). Figure 3 shows that the bacteriocin activity eluted as a single peak at an NaCl concentration of approximately 0.15 M.

The most active fractions were pooled. The biological activity of this material was stable for at least 3 months when frozen. For most of the studies described here, the low-molecular-weight salts were removed by gel filtration of 15-ml samples on a Sephadex G-50 column (2.5 by 40 cm) which was equilibrated with deionized water. Fractions containing bacteriocin activity were lyophilized.

Comments on the purification. The results of the purification of bacteriocin JF246 are summarized in Table 1. Usually, the purified bacteriocin had a specific activity 500 to 1,000-fold higher than found in the NaCl supernatant solution. Bacteriocin production was much greater in PPBE broth or a minimal medium supplemented with Casamino Acids and yeast extract than in an unsupplemented minimal medium. *p*H also had a marked effect on the yield and a sharp increase in maximal bacteriocin titer occurred at *p*H 7.0. Of the other variables tested, temperature (25, 30, and 39 C), aeration (no aeration or 6 liters/min), carbon source (glucose, glycerol, lactate, or succinate) and inducing agent (mitomycin C, thymine starvation, UV irradiation, or thiouracil), none had a marked effect on the yield.

Estimation of the fraction of *S. marcescens* cells which produced bacteriocin was made by the lacunae assay to detect bacteriocin production by individual cells (Fig. 1). When the number of lacunae was compared to the total number of viable cells, bacteriocin production could be detected in only 5 to 10% of the cells. In an attempt to increase the number of lacunae, cultures were treated with various concentrations of mitomycin C (0.1–2.0 mg/liter) and under conditions where mitomycin C was

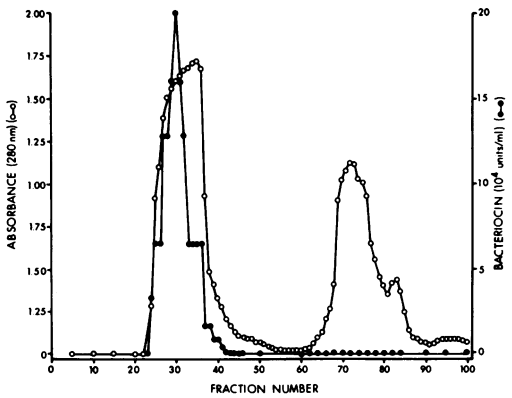

FIG. 2. *QAE-Sephadex chromatography of bacteriocin JF246. Conditions are described in the text. The column (5.0 by 79 cm) was loaded with 1.8 g of protein collected after ammonium sulfate treatment and dialysis against the equilibration buffer. Fractions 25 to 33 were pooled and dialyzed against the equilibration buffer for the SP-Sephadex column.*

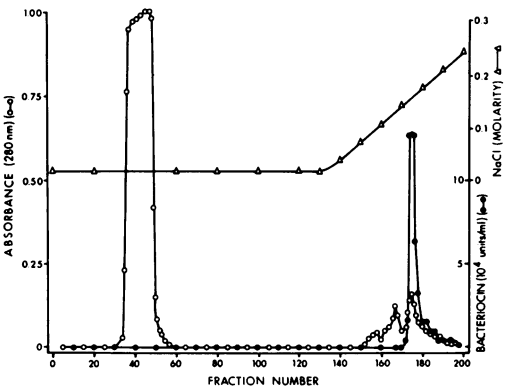

FIG. 3. *SP-Sephadex chromatography of bacteriocin JF246. Conditions are described in the text. The column (5.0 by 75 cm) was loaded with material containing 0.315 g of protein. Fractions 173 to 180 were pooled, dialyzed, and lyophilized.*

TABLE 1. *Purification of bacteriocin JF246 from Serratia marcescens*

Step	Volume (ml)	Bacteriocin activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)
NaCl extract	2,000	2×10^4	15.5	1.3×10^3	
0–33% Ammonium sulfate supernatant fluid	1,850	2×10^4	4.9	4.0×10^3	92
33–53% Ammonium sulfate pellet	245	1.6×10^5	10.6	1.5×10^4	97
QAE-Sephadex (peak fractions)	294	6.4×10^4	2.4	2.7×10^4	45
SP-Sephadex (peak fractions)	99	1.3×10^5	0.12	9.9×10^5	32

added in samples over a period of time. These procedures did not improve the ratio of lacunae to total cells.

Protease. *S. marcescens* strains excrete an inducible extracellular protease (2) which is capable of inactivating bacteriocin JF246 (see Table 3). Protease activity was assayed at various stages in the purification of the bacteriocin. Although strain JF246 produces only about 10% of the wild-type level of protease (7), this activity was found in the culture supernatant fluid (3 units/ml) and the ammonium sulfate fractions, but it was not present in detectable (<0.1 units/ml) amounts after the QAE-Sephadex step.

Electrophoretic homogeneity of purified bacteriocin. Electrophoresis of the lyophilized bacteriocin on polyacrylamide gels at *p*H 9.5 showed four bands staining with Coomassie Brilliant Blue (Fig. 4B). When examined by electrophoresis in polyacrylamide gels containing 2% SDS, the same preparation of bacteriocin migrated in a single band (Fig. 4C and 4D). Where the sample of purified bacteriocin was incubated at 100 C before electrophoresis, an identical single band was observed (gel not shown). Electrophoresis in gels containing 6 M urea at *p*H 8.5 (Fig. 4E, alkaline urea) showed a single band in the gel plus some material not entering the gel. The material not entering the gel is assumed to be bacteriocin not denatured by urea because when the sample containing urea was heated at 60 C for 60 min prior to application to the gel no band at the top of the gel was seen. This treatment completely destroyed bacteriocin activity. When bacteriocin activity was measured in eluates of gel slices prepared from duplicate gels in each instance (standard gel at *p*H 9.5, SDS gels, and alkaline urea gels), the position of the gel slices containing activity coincided with the position of the bands which stained with Coomassie Brilliant Blue. In the case of the multiple bands observed in the standard *p*H 9.5 gels, each band was found to contain bacteriocin activity.

Molecular-weight determination. The molecular weight of bacteriocin JF246 was estimated by SDS-gel electrophoresis (29) and an apparent molecular weight of approximately 64,000 was obtained. The data for an

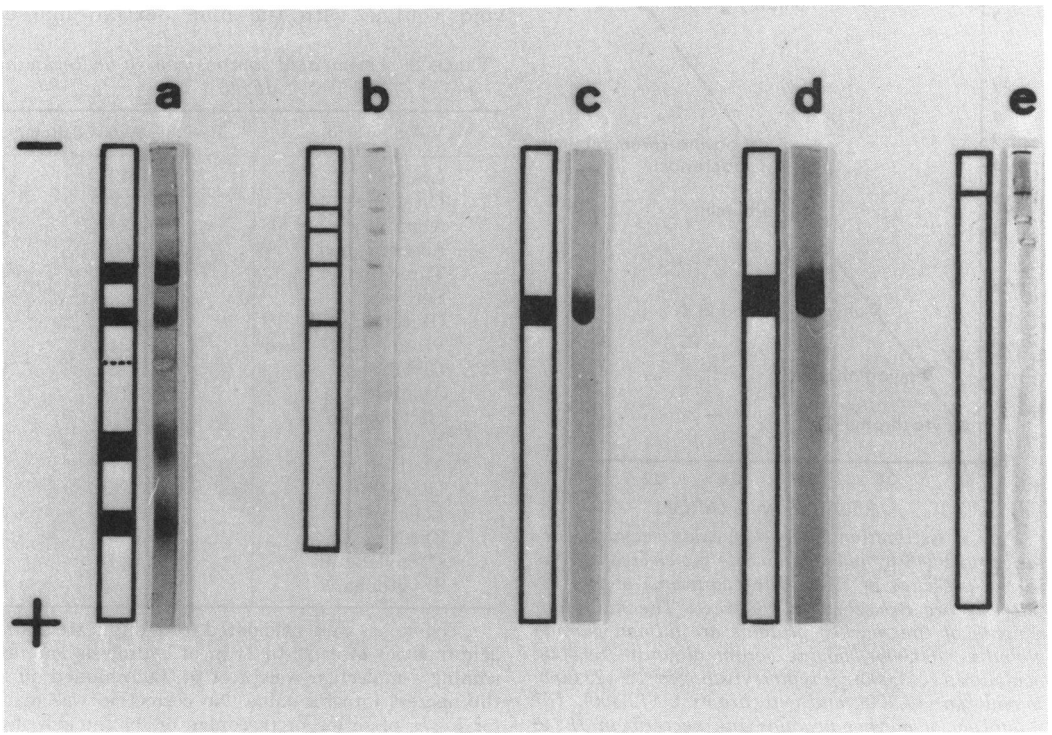

FIG. 4. *Polyacrylamide gel electrophoresis. (a) SDS gel of pooled fractions from QAE-Sephadex. (b) Standard 7.5% gel at pH 9.5 with 30 μg of purified bacteriocin. (c) SDS gel of 30 μg of purified bacteriocin. (d) SDS gel of 120 μg of purified bacteriocin. (e) Alkaline urea gel at pH 8.5 of 30 μg of purified bacteriocin. Note that this gel separated during staining. A diagrammatic representation of each gel is included for clarity.*

experiment in which bacteriocin JF246 was run in gels together with one or more standard proteins is summarized in Fig. 5.

Analytical results. Analysis for phosphate using 0.8 mg of bacteriocin showed that less than 1 nmole was present in the sample. Assuming a molecular weight of 64,000, this indicates less than 0.1 mole of phosphate per mole of bacteriocin.

Analysis for reducing sugars showed the presence of 6 to 48 μg of reducing sugar, expressed as glucose, per mg of bacteriocin. The sugar was identified as glucose after reduction and gas chromatography.

The sugar could be removed by treatment of the purified bacteriocin with 10^{-2} M DTT in 2% SDS for 1 hr at 37 C, followed by chromatography on a Sephadex G-200 column which had been equilibrated with 0.02 M Tris-hydrochloride buffer, *p*H 7.8, containing 2% SDS and 10^{-4} M DTT. The column fractions containing bacteriocin activity were pooled, and the SDS was removed by precipitation as the potassium salt after addition of KPO_4, *p*H 7.5, to a final concentration of 0.05 M. After lyophilization, less than 0.4 μg of glucose per mg of bacteriocin was found by gas chromatography. The removal of glucose by this procedure indicates that glucose was not covalently linked to bacteriocin. There was a 50 to 75% decline in bacteriocin activity after the chromatography in SDS. The activity lost was not restored by the addition of 10^{-2} M glucose. It should be noted that these data may be subject to error for they are based on protein concentrations with bovine serum albumin as a standard. Amino acid analysis of purified bacteriocin yielded the data in Table 2.

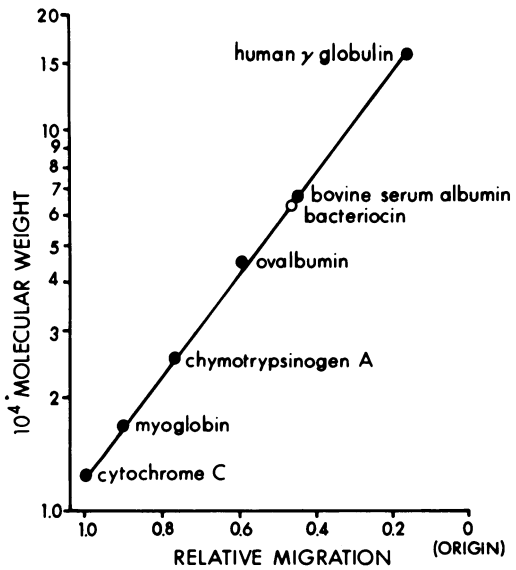

FIG. 5. *Estimation of the molecular weight of bacteriocin JF246 by polyacrylamide gel electrophoresis in the presence of SDS. The conditions of electrophoresis are described in the text. The molecular weights of the marker proteins are human gamma globulin (160,000), bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (25,000), myoglobin (17,200), and cytochrome C (12,400). The migration of marker proteins and bacteriocin JF246 is expressed relative to the migration of cytochrome C. A molecular weight of 64,000 can be extrapolated from the relative migration of bacteriocin JF246 (0.46).*

Aggregation of bacteriocin JF246. The presence of a single band in SDS gels indicated that the bacteriocin was made up of at least one polypeptide with a molecular weight of approximately 64,000. The behavior of the bacteriocin on agarose columns suggested that in buffers of low ionic strength the polypeptide interacts to form large aggregates. Figure 6 shows the chromatography of purified bacteriocin on a Bio-Gel A1.5 column equilibrated with 0.02 M Tris-hydrochloride, *p*H 7.8. The majority of the bacteriocin activity was excluded from the column and appeared at the void volume with the blue dextran marker

TABLE 2. *Amino acid composition of bacteriocin JF246*

Amino acid	Residues/64,000 daltons[a]
Lysine	49
Histidine	9
Arginine	24
Aspartic acid	60
Threonine	27
Serine	29
Glutamic acid	50
Proline	24
Glycine	73
Alanine	72
Half-cystine	ND[b]
Valine	33
Methionine	ND
Isoleucine	25
Leucine	36
Tyrosine	10
Phenylalanine	4
Tryptophan[c]	4

[a] The values were calculated from duplicate amino acid analysis after 22 to 24 hr of hydrolysis and, assuming a molecular weight of 64,000, rounded off to the nearest integral value. No correction was made for losses of serine or threonine or for the slow hydrolysis of valine and isoleucine.

[b] ND, not determined.

[c] Tryptophan was determined spectrophotometrically (11).

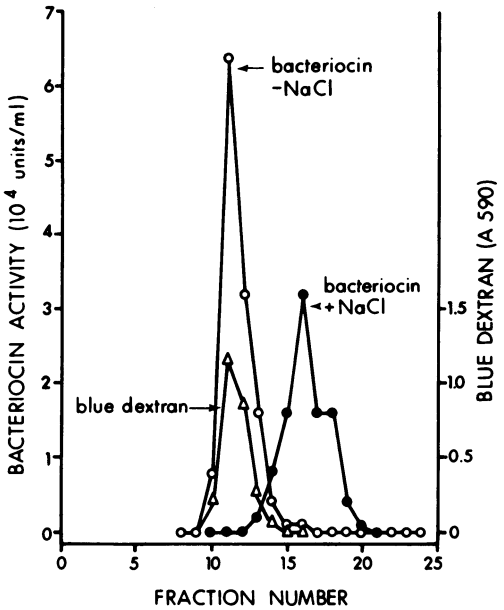

FIG. 6. *Dissociation of an aggregate of bacteriocin JF246 with NaCl. Purified bacteriocin, 0.8 ml, was mixed with 0.2 ml of blue dextran in 0.02 M Tris-hydrochloride buffer, pH 7.5, in the presence or absence of 0.4 M NaCl and separately applied to a Bio-Gel A1.5 column (0.9 by 56 cm) equilibrated with the appropriate buffer. Fractions of 2.4 ml were collected.*

(molecular weight ca. 1.5×10^6). When the bacteriocin was applied to the same column equilibrated with a 0.02 M Tris-hydrochloride buffer, pH 7.8, containing 0.4 M NaCl, the bacteriocin activity was eluted in later fractions.

Stability of bacteriocin JF246. Solutions of purified bacteriocin JF246 in 0.02 M KPO_4, pH 7.0, were stable at 4 C for at least 6 weeks. The bacteriocin was remarkably stable (or reversibly denatured) in the presence of certain agents which denature some proteins. For example, bacteriocidal activity was not diminished by a 60-minute incubation period at 25 C of bacteriocin in the presence of 2% SDS and 1% mercaptoethanol. In addition, 4 M guanidine thiocyanate or 6 M urea did not irreversibly denature the bacteriocin, for after dialysis to remove the denaturing agent most of the bacteriocin activity was recovered. On the other hand, the bacteriocin was completely inactivated by heating at 60 C in 0.02 M KPO_4 buffer, pH 7.0, for 1 hr or by brief exposure to proteolytic enzymes. A sulfhydral group appeared to be important, for activity was lost after incubation of bacteriocin JF246 with iodoacetate. These results are summarized in Table 3.

DISCUSSION

Bacteriocin synthesis can be induced in *S. marcescens* JF246 by a variety of techniques which interfere with DNA metabolism and typically induce phage synthesis in lysogenic strains. In contrast to what has been observed for phage induction, efficient oxygenation of the induced culture was not required for high yields of bacteriocin JF246. Unexpectedly, bacteriocin synthesis could be detected by the lacunae assay in only 5 to 10% of the cells after the addition of the inducing agent (mitomycin C). This was true even when the level of mitomycin was increased 10-fold or when mitomycin was added in several portions over a period of time. No explanation can presently be offered for the apparent low probability of induction. It may simply represent an inability to detect all cells which are producing bacteriocin by the lacunae assay. A number of workers have noted that the synthesis of bacteriocin results in the death of the producing cell. For example, Herschman and Helinski have demonstrated a decline in colony-forming ability with a concomitant increase in lacunae counts after induction of colicin E2 with mitomycin C (12). A marked decrease in viability of the *S. marcescens* culture after addition of mitomycin C would be expected if bacteriocin synthesis were a lethal biosynthesis and a large fraction of the population were involved in

TABLE 3. *Effect of various agents on JF256 bacteriocin*

Treatment[a]	Final titer of bacteriocin
None	10,000
2% SDS, 1% βME	5,000
4 M Guanidine thiocyanate	10,000
6 M Urea	10,000
Heat:	
60 min at 60 C	<10
30 min at 60 C	1,000
60 min at 50 C	10,000
Iodoacetate (5 mM)	<100
Trypsin (0.2 mg/ml)	0
Pronase (0.2 mg/ml)	0
Extracellular protease from *Serratia* (0.1 mg/ml)	0

[a] A solution of purified JF246 bacteriocin in 0.02 M potassium phosphate, pH 7.0, was used for all procedures. Sodium dodecyl sulfate (SDS), β-mercaptoethanol (βME), guanidine thiocyanate, or urea was added, and the mixture was incubated for 60 min at 37 C. The incubation in the presence of iodoacetate was for 15 min at 37 C. The incubation with the proteolytic enzyme was for 5 min at 25 C.

bacteriocin synthesis. This was not observed. Mitomycin C stopped cell division (the number of cells, enumerated electronically with a Coulter counter, did not increase), but it did not stop the increase in cell mass, as filamentous forms were seen.

The purification of bacteriocin JF246 was facilitated by the observation that under the conditions of growth and induction described over 95% of the bacteriocin activity could be sedimented with the bacteria. The bacteriocin, which may be bound to the cell surface electrostatically, was solubilized by 1 M NaCl. Although bacteriocins have been described as extracellular products released into the culture fluid, other bacteriocins have also been found complexed to the surface of induced cells (11).

Bacteriocin JF246 appears to be simple protein. The biological activity of purified bacteriocin was rapidly destroyed by several proteases tested and by a number of other treatments which are known to affect the activity of proteins. Amino acid analysis showed that the bacteriocin was rich in charged amino acids.

The glucose found in preparations of purified bacteriocin was not covalently linked to the bacteriocin for it was separated by chromatography in the presence of 2% SDS. The question of whether glucose was required for full activity of the bacteriocin cannot be answered at present for the chromatography in SDS resulted in a 50 to 75% decrease in bacteriocin activity which was not restored by the addition of glucose.

The sensitivity of the bacteriocin to an extracellular protease purified from *S. marcescens* demonstrates an interesting point. Synthesis of extracellular protease could mask the phenotypic expression of concommitant bacteriocin synthesis. Such an effect has, in fact, been postulated to explain the conditional phenotypic expression of concomitant bactein in the strain of *S. marcescens* from which JF246 was isolated (7). In addition, the protease activity found in crude preparations will decrease the yield of bacteriocin. Therefore, the purification should be carried through the QAE-Sephadex step without delay.

Bacteriocin JF246 readily aggregated to give forms with apparent molecular weights of over 10^6. These aggregates could be dissociated with 0.4 M NaCl. The monomeric form may not be active, for no bacteriocin activity could be demonstrated on plates containing 0.4 M NaCl. The electrophoresis of purified bacteriocin on polyacrylamide gels at *p*H 8.6 gave a series of protein-containing bands, each of which contained bacteriocin activity. When the same material was subjected to electrophoresis on polyacrylamide gels containing SDS or on alkaline urea gels, only a single band could be seen, indicating that the bacteriocin was composed of a single subunit species or of subunits of similar molecular weights and electrophoretic mobilities in these systems.

The mode of action of bacteriocin JF246 is similar to that described for several colicins (5). However, comparison of the amino acid analysis of bacteriocin JF246 with, for example, that of colicin E1 (11), does not reveal any marked similarities. Of course, this does not preclude significant areas of sequence homology.

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