Bacteriocin from *Bacillus megaterium* ATCC 19213: Comparative Studies with Megacin A-216

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A bacteriocin produced by *Bacillus megaterium* ATCC 19213 was identified, purified, and compared with megacin A from *B. megaterium* 216. The ATCC 19213 bacteriocin was inducible with mitomycin C and showed phospholipase A activity. Both megacin A-216 and megacin A-19213 contained two dissimilar polypeptide subunits. Megacin A-216 contains a 30,000-dalton α subunit and a 15,000-dalton β subunit. Megacin A-19213 is composed of an α subunit 18,000 daltons in mass and a β subunit about 7,500 daltons in mass. No sequence similarities between α and β subunits of either megacin were detected. The two megacins were further distinguished by quantitative differences in activity spectra and by immunodiffusion analyses.

Strains of the gram-positive bacterium *Bacillus megaterium* produce several bacteriocins active against other strains of *B. megaterium* (9-11). These megacins have been classified into three categories by Holland and Roberts (9) on the basis of inducibility, mode of action, and spectrum of activity. Megacin A is inducible by treatment of logarithmically growing cultures with low levels of mitomycin C or UV irradiation. Megacin A shows phospholipase activity which can be assayed by the conversion of phospholipids to the corresponding lysophospholipids (19). Most strains of *B. megaterium* are sensitive to megacin A. Megacin C is noninducible, has a more limited activity spectrum than megacin A, and apparently causes the activation of DNase activities in sensitive bacteria (8, 9). A third megacin, exhibiting an intermediate activity spectrum, is megacin B. This megacin is poorly characterized and is demonstrable only on solid media (9).

B. megaterium strain 216, the type producer of megacin A, produces extremely high levels of megacin A, often as much as 400 times more than most other megacin A producers (13). Routine screening of other strains of *B. megaterium* for bacteriocin production revealed that strain ATCC 19213 produces an inducible megacin at a level comparable to that of strain 216. This paper describes the purification and properties of this megacin, which show that it conforms most closely to a type A megacin. We also report comparative studies with megacin A-216.

MATERIALS AND METHODS

Reagents. Bacterial growth media were obtained from Difco Laboratories, Detroit, Mich.; papain, mito-

mycin C, lysozyme, Sephadex G-100, DEAE A-50, L- α -phosphatidylcholine (dipalmitoyl) (PC), L- α -lysophosphatidylcholine (palmitoyl), and Tris were obtained from Sigma Chemical Co., St. Louis, Mo. Acrylamide, bis-acrylamide, and Cellex D-DEAE were from Bio-Rad Laboratories, Richmond, Calif. *Staphylococcus aureus* V8 proteinase and immunological adjuvants were from Miles Laboratories, Elkhart, Ind. Sodium phosphate was obtained from Mallinckrodt Chemical Co., St. Louis, Mo. Ultrogel A5A was from LKB Instruments Inc., Rockville, Md. Sodium dodecyl sulfate (SDS) was purchased from BDH Chemicals Ltd., Poole, England.

Bacterial strains. *B. megaterium* 216 MegA⁺ MegC⁺ was obtained from G. Ivanovics. *B. megaterium* ATCC 19213 was obtained from A. D. Hitchins. Strains QMB1551, PV5, and JV78 were obtained from P. S. Vary. The properties of these wild-type strains as well as of a number of indicator strains are summarized in the accompanying paper (23).

Induction and titration of megacins. The yeast extract-tryptone broth medium of Tikhonenko et al. (22) was used to grow bacteria for megacin production. Cultures were grown at 37°C to a Klett turbidity reading of 15 to 30 (red filter) and then induced by addition of 1 μ g of mitomycin C per ml. Incubation was continued until the cultures had lysed. Cell debris was removed by centrifugation at 12,000 $\times$ g for 10 min before titration of megacin activity in culture supernatants. The activity of megacin preparations was determined as the reciprocal of the greatest dilution that inhibited the growth of 50% of the indicator bacterial strain 2165 (11). Endpoint titers are expressed without regard to the volume of megacin assayed. Protein concentrations were determined by the method of Lowry et al. (16).

Protoplast lysis by megacins. Indicator strain *B. megaterium* 2165 was grown to mid-logarithmic phase in yeast extract-tryptone broth at 37°C. Cells were pelleted by centrifugation and then resuspended in 20% sucrose-50 mM NaPO₄, pH 7.0 (PS buffer),

washed with PS buffer at room temperature, and treated with 100 μ g of lysozyme per ml. After 15 min at room temperature the resulting protoplasts were centrifuged, resuspended, and washed as before. Preparations were diluted in PS buffer to an optical density of 0.28 at 600 nm before the addition of megacins. Purified megacins were added so that protoplast preparations contained 500 endpoint units of activity. Lysis of protoplasts was detected by the change in absorbance at 600 nm.

Phospholipase assay. The enzymatic conversion of PC to L- α -lysophosphatidylcholine by megacin A (19) was assayed qualitatively by thin-layer chromatography. Synthetic PC was solubilized at 1.0 mg/ml in 50 mM Tris-hydrochloride buffer-1% Triton X-100-6 mM CaCl_2 , pH 7.1. A 50- μ l portion of PC solution was mixed with an equal volume containing 5×10^4 U of megacin A in 50 mM sodium borate, pH 8.0, to initiate the phospholipase reaction. The reaction mixtures were incubated for 2 h at 37°C and then applied to Silica Gel G (Merck) thin-layer chromatography plates. The plates were developed in chloroform-methanol-5 M ammonium hydroxide (65:30:5, vol/vol/vol), and the reaction products were visualized with the lipid phosphate spray reagent of Dittmer and Lester (5).

Antisera to megacin A-216. Antisera to megacin A-216 were prepared by injecting young New Zealand white rabbits subcutaneously with 200 μ g of purified megacin A mixed with Freund complete adjuvant. Two subsequent injections of megacin (200 μ g) with incomplete adjuvant were given at 2-week intervals. Antisera were collected 6 weeks after the initial injections. Crude immunoglobulin G was precipitated three times at 30% saturation with ammonium sulfate.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) in gels of 12.5 or 15% acrylamide was conducted as described by Laemmli (15). Stacking gels 6.0 cm or more in height were generally used to ensure complete stacking of low-molecular-weight proteins. Nondenaturing disc polyacrylamide gel electrophoresis was conducted as described by Davis (3).

Peptide mapping by limited proteolysis. Peptide mapping was conducted as described by Cleveland et al. (2). α and β subunits of megacins A-216 and A-19213 were resolved preparatively on SDS-15% polyacrylamide gels 1.5 mm thick. Gel pieces containing subunit proteins were cut into thin slices and placed directly in the wells of a second SDS-15% polyacrylamide gel, 0.7 mm thick. The gel pieces were overlaid with *S. aureus* V8 proteinase. Electrophoresis was conducted at 20 mA (constant current) such that electrophoresis through the stacking gel (8.0 cm) generally required 2.5 h. Under these conditions proteolysis occurs during migration through the stacking gel.

RESULTS

Megacin activity of *B. megaterium* ATCC 19213. Early logarithmic-phase cultures of *B. megaterium* ATCC 19213 undergo lysis after low levels of mitomycin C are added to the cultures (Fig. 1). Concomitant with the loss of turbidity, bacteriocin activity against other strains of *B. megaterium* was detected in culture filtrates. Fully induced cultures of strain ATCC 19213

FIG. 1. Growth of *B. megaterium* ATCC 19213 after megacin induction. Megacin production was induced with mitomycin C. After complete clearing of the induced culture, both cultures were harvested and the megacin titers were determined. Symbols: ●, mitomycin C added at 1 μ g/ml at 0 min; ○, uninduced control culture.

produced between 2,500 and 3,500 U of megacin activity when tested against strain 2165, a sensitive megacin indicator strain. This level of megacin activity was somewhat higher than the amount of megacin A produced by induced cultures of strain 216, the type producer of megacin A. Uninduced cultures of both Meg⁺ strains showed no antibacterial activity against strain 2165.

Proteins present in induced lysates from strain 216 showed a fast-migrating band (indicated by the arrow, Fig. 2) that is not present in uninduced strain 216 lysates. This protein is megacin A-216 and accounts for a substantial fraction of the total cellular protein. Induced strain ATCC 19213 also produced a fast-migrating band (arrow) not present in uninduced lysates. This protein contains the megacin activity. Both proteins identified in crude lysates as megacin A-216 and megacin from ATCC 19213 comigrate in nondenaturing disc gels with purified megacins (data not shown).

Purification of megacin A-216 and megacin from ATCC 19213. The purification of megacins, modified from the procedure of Holland (7) for

FIG. 2. PAGE (7.5%) profiles of proteins present in induced and uninduced cultures of *B. megaterium*. Crude megacin lysates from 5.0 ml of yeast extract-tryptone broth cultures were prepared as described in the text. After lysis of induced cultures, the debris was removed by centrifugation. The supernatant solutions were adjusted to 80% of saturation with solid ammonium sulfate and stirred for 2 h at 4°C. The resulting precipitates were collected by low-speed centrifugation and resuspended in 0.25 ml of 10 mM Tris-hydrochloride buffer, pH 7.4. The concentrated lysates were dialyzed against the same buffer, and 50 μ l of each preparation was applied to a 7.5% polyacrylamide gel. The arrows indicate the known positions of megacins A-216 and A-19213. 1, Uninduced control culture of strain 216; 2 and 3, mitomycin C-induced megacin lysate of strain 216; 4, uninduced control lysate of ATCC 19213; 5 and 6, induced megacin lysate of ATCC 19213.

megacin A-216, is summarized in Table 1. The purification scheme for megacin A-216 included several column steps which appeared necessary to remove contaminating peptides still evident by SDS-PAGE, after using the purification procedures of Holland (7). At least one additional peptide of an apparent molecular mass of 40,000 daltons copurified with megacin A-216. This peptide may be associated with megacin A-216

in solution, since SDS-PAGE of megacin A-216, electroeluted from preparative disc acrylamide gels, demonstrated the 40,000-dalton peptide as well as both subunits of megacin A-216. After the purification steps listed in Table 1, megacin A-19213 was homogeneous, and megacin A-216 was at least 95% homogeneous, as judged by electrophoresis in SDS-polyacrylamide gels.

Classification of megacin from *B. megaterium* ATCC 19213. Megacin from ATCC 19213 was classified to determine whether this megacin conforms most closely to an A, B, or C type megacin in the classification scheme proposed by Holland and Roberts (9). Megacin from ATCC 19213 was inducible by mild treatment with UV or mitomycin C. Like megacin A-216, induction of megacin from ATCC 19213 resulted in lysis of the producer bacteria; this effect was observed in liquid and on solid media.

Megacin from ATCC 19213 was active against all strains of *B. megaterium* tested (Table 2), unlike megacin B- and megacin C-producing strains, which are inactive against strains that produce megacin A (9).

Addition of megacin from ATCC 19213 or megacin A-216 to protoplast suspensions of strain 2165 led to the rapid lysis of protoplasts (Fig. 3). The phospholipase A activity of megacin A (19) was also demonstrated directly (Fig. 4). Both megacin A-216 and megacin from ATCC 19213 catalyzed the conversion of PC to L- α -lysophosphatidylcholine. The results of these experiments justify the classification of megacin from ATCC 19213 in the A group. Following the nomenclature of Reeves (20), we propose to name this megacin, megacin A-19213.

Comparative studies of megacins A-216 and A-19213. Preparations of megacins A-216 and A-19213, adjusted to the same activity against strain 2165, showed significantly different levels of activity when tested against a number of other indicator strains (Table 2). Both producer strains 216 and ATCC 19213 exhibited low sensitivity to

TABLE 1. Purification of megacins

Step	Megacin A-216 ^a	Step	Megacin A-19213
1	Acid precipitation of crude supernatant at pH 4.0 ^b	1	50–70% ammonium sulfate fractionation of crude culture supernatant ^b
2	50–60% ammonium sulfate fractionation	2	Sephadex A-50–DEAE ion-exchange chromatography, pH 7.4
3	Streptomycin sulfate precipitation	3	Sephadex G-100 chromatography
4	Cellex D-DEAE ion-exchange chromatography, pH 7.0		
5	First Sephadex G-150 chromatography		
6	Second Sephadex G-150 chromatography		
7	Cellex D-DEAE ion-exchange chromatography, pH 6.3		

^a Modified from Holland (7).

^b Crude supernatants were prepared as described in the text.

TABLE 2. Endpoint sensitivity of indicator strains of *B. megaterium* to megacin A-216 and megacin A-19213

Strain	Sensitivity ^a to:	
	Megacin A-216	Megacin A-19213
216	5.0×10^2	3.8×10^3
ATCC 19213	1.3×10^5	3.8×10^2
2165	1.5×10^5	1.5×10^5
VT131	5.0×10^4	3.8×10^3
VT145	1.0×10^4	1.9×10^3
VT1206	1.0×10^5	3.8×10^3
JV78	1.5×10^5	1.5×10^5
QMB1551	1.5×10^4	3.8×10^4
PV5	7.0×10^4	2.6×10^4
PV5-T7	7.0×10^4	2.6×10^4

^a A series of dilutions of purified megacin in 50 mM NaPO₄ (pH 7.1) was prepared and applied to the surface of nutrient agar plates previously seeded with indicator bacteria. Endpoint sensitivity was determined as the reciprocal of the highest dilution that inhibited the growth of the indicator bacteria by at least 50%. The indicator strains are described in the accompanying paper (23).

the megacin they produce, but were significantly more sensitive to the other megacin. ATCC 19213 was almost as sensitive to megacin A-216 as either 2165 or JV78, both very sensitive indicator strains. Type A megacins have previously been shown to be active against strains producing other type A megacins (9).

Strains that have been cured of megacin A-216 or A-19213 production showed increased sensi-

FIG. 3. Protoplast lysis by megacins. Megacin (500 U) was added to protoplast suspensions at 0 min. Symbols: ○, megacin A-216; ●, megacin A-19213; ×, no added megacin. A₆₀₀, Absorbance at 600 nm.

FIG. 4. Phospholipase A activity on purified PC of megacin A assayed by thin-layer chromatography. 1, PC standard; 2, PC treated with megacin A-216; 3, PC treated with megacin from ATCC 19213; 4, L-α-lysophosphatidylcholine standard.

tivity to their parental megacin but not to the other megacin. VT131 and VT145 are derivatives of strain 216 cured for megacin A-216 production (23). These strains were, respectively, 100- and 20-fold more sensitive to megacin A-216 than strain 216, yet showed approximately the same sensitivity to megacin A-19213 as strain 216. Strain VT1206 (a cured derivative of ATCC 19213 [23]) was 10 times more sensitive to megacin A-19213 than its parent strain, yet somewhat less sensitive to megacin A-216 than its parent strain.

Strain QMB1551 and its derivative PV5 produce a noninducible megacin (M. J. Weickert and P. S. Vary, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H21, p. 116) which is probably a type C megacin. Strain PV5-T7 has been cured of the ability to produce this megacin (M. Von Tersch, unpublished data). This cured derivative showed approximately the same sensitivity to both type A megacins as its parental strain.

The molecular weights of purified native megacins A-216 and A-19213 were estimated by gel filtration chromatography on Ultrogel A5A. The results indicated apparent molecular masses of 66,000 daltons for active megacin A-216 and 39,000 daltons for active megacin A-19213 (data not shown).

The subunit compositions and subunit molecular weights of both megacins were determined by SDS-PAGE (Fig. 5). Both megacins are composed of two dissimilar size subunits. Megacin A-216 is composed of an α subunit of 30,000

FIG. 5. SDS-PAGE of purified megacins A-216 and A-19213. 1, Megacin A-19213; 2, standard protein mix, containing (from top to bottom; molecular weights in parentheses) bovine serum albumin (68,000), ovalbumin (44,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,400); 3, megacin A-216.

daltons and a β subunit of 15,000 daltons. Megacin A-19213 contains an α subunit of 18,500 daltons and a β subunit of approximately 7,500 daltons. This conclusion was confirmed in the following manner: each megacin was preparatively electroeluted from single bands on non-denaturing 7.5 or 12% disc acrylamide gels (3) and then electrophoresed on SDS-15% polyacrylamide gels. The gels revealed two subunits for each megacin (see Fig. 5).

Peptide analysis by limited proteolysis. Since the α subunits are approximately two times greater in mass than the β subunits, we considered the possibility that a precursor-product relationship exists between the two subunits. Peptide mapping by limited proteolysis (Fig. 6)

FIG. 6. Limited proteolysis of megacins with *S. aureus* V8 proteinase. Before the second SDS-PAGE, the gel pieces containing resolved subunits were overlaid with either 2.5 (lanes 2 to 5) or 0.5 (lanes 6 to 9) μ g of V8 proteinase. The positions of marker proteins are indicated on the right. 1, *S. aureus* V8 proteinase; 2 and 6, megacin A-216 α subunit; 3 and 7, megacin A-216 β subunit; 4 and 8, megacin A-19213 α subunit; 5 and 9, megacin A-19213 β subunit.

showed that the α subunit of each megacin was cleaved by *S. aureus* V8 proteinase to a series of small peptides, whereas the β subunit of each megacin remained undigested.

Immunological analysis. Megacin A-19213 was tested for cross-reaction with antiserum to megacin A-216 in a double-immunodiffusion assay (Fig. 7). Megacin A-19213 failed to cross-react with antiserum directed against megacin A-216.

DISCUSSION

An inducible bacteriocin from *B. megaterium* ATCC 19213 has been identified, purified, and characterized. Because this megacin can be induced with mitomycin C, shows a wide activity spectrum, and possesses phospholipase A activity, it conforms most closely to the A group of megacins. Comparative analysis of this purified megacin (A-19213) with megacin A-216 revealed that these proteins are not identical. Although both proteins are composed of two dissimilar subunits, none of the subunits are common to both megacins. Limited proteolysis does not support a precursor-product relationship between subunits. These megacins can be distinguished immunologically and by quantitative differences in activity spectra. In this regard, it has previously been recognized that the A group of megacins probably represents a heterogeneous array of bacteriocins (9, 17).

Our results indicated a molecular mass of 66,000 daltons for native megacin A-216 and subunit molecular masses of 30,000 and 15,000 daltons, which suggests the possibility that the active bacteriocin may be a trimer with an $\alpha\beta_2$ configuration. Holland (7) has reported the mo-

FIG. 7. Double-diffusion immunoprecipitation analysis of megacins A-216 and A-19213. Center well, 2 μ l of antimegacin A-216 antiserum; outer wells: 1, 1.3 μ g of native megacin A-216; 2, 10 μ g of megacin A-19213; 3, 1.0 μ g of denatured megacin A-216, eluted from a 7.5% disc polyacrylamide gel into SDS-PAGE sample buffer (15); 4, 1.0 μ g of denatured megacin A-19213 eluted from a 7.5% disc polyacrylamide gel into SDS-PAGE sample buffer.

molecular weight of megacin A-216 to be 51,000, which is more suggestive of an $\alpha\beta$ stoichiometry. Subunit molecular masses of 18,500 and 7,500 daltons for the α and β subunits of megacin A-19213 correlate poorly with a molecular mass of 39,000 daltons for the native megacin. Thus, our results do not allow an unambiguous estimation of subunit stoichiometry for either megacin.

Quantitative sensitivity testing of a number of indicator strains of *B. megaterium* has helped to distinguish megacin A-216 from megacin A-19213. Strains cured of the ability to produce either megacin were more sensitive than their parent strains to their parental megacin. These findings suggest that there may exist specific immunity substances for both megacins A-216 and A-19213. Proteins that confer immunity to individual bacteriocins, including colicin E2 (21), colicin E3 (14), colicin Ia (24), colicin V (6), and cloacin DF13 (4), have been identified. An inhibitor of megacin A-216 has been identified in mutants of strain 216 that show decreased sensitivity to megacin A-216 compared with wild-type 216 (18). This inhibitor remains largely uncharacterized. It has not been shown to be a protein, although this is suspected to be the case. In the accompanying paper (23), we show that megacin immunity can be used as a positive selection for transformation for megacin production for both megacins A-216 and A-19213. This observation further argues that immunity functions exist for both megacins and that the genes encoding the immunity functions are probably closely linked to the structural genes for their respective megacins.

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