Physicochemical and Biological Properties of Mycobacteriocin M12 Produced by *Mycobacterium smegmatis* ATCC 25855

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A mycobacteriocin (M12) produced by *Mycobacterium smegmatis* ATCC 25855 was partially purified by ammonium sulphate precipitation followed by DEAE-cellulose chromatography and Sephadex G100 chromatography. Production of M12 was maximal when bacteria were harvested after 3 d cultivation in liquid medium and disrupted by sonication. The molecular weight of M12, estimated by Sephadex G100 chromatography, was about 85000. M12 was sensitive to proteolytic enzymes but resistant to DNAase and RNAase, and was relatively stable to heat treatment, sonication, ultraviolet irradiation and pH over the range 4 to 8. When sensitive bacteria were exposed to the mycobacteriocin, the number of viable cells began to decrease after about 6 h incubation. The killing curve of M12 thus appeared to be a multiple-hit curve. Electron microscopic observation revealed that the mycobacteriocin induced morphological changes in the cells: these were partial loss of ribosomes, enlargement of lipoidal inclusion bodies and thickening of the cell envelope. The activity spectrum of M12 was restricted to the genus *Mycobacterium*.

INTRODUCTION

The presence of bacteriocins of mycobacteria was suggested by Adámek *et al.* (1968), and independently, by Imaeda & Rieber (1968). Later, we confirmed that when appropriate indicator strains were used for the detection of bacteriocin or bacteriocin-like substances, many strains belonging to the genus *Mycobacterium* produced these substances; hence, classification and typing of mycobacteria were possible by their activity spectra (Takeya & Tokiwa, 1972; Takeya & Tokiwa, 1974).

Very little is known of the characteristics of the mycobacteriocins. We started, therefore, to investigate the nature and function of a mycobacteriocin. This paper describes some physicochemical and biological properties of mycobacteriocin M12 which is produced by *Mycobacterium smegmatis* ATCC 25855.

METHODS

Bacterial strains. *Mycobacterium smegmatis* ATCC 25855, *M. vaccae* ATCC 25952, *M. aurum* strains ATCC 25790, ATCC 25792, ATCC 25794, ATCC 25800 and *M. fortuitum* ATCC 1701 were tested for the production of mycobacteriocins. *Mycobacterium diernhoferi* ATCC 19340 was used as an indicator strain.

For measurement of the activity spectrum of mycobacteriocin M12, the strains listed in Table 4 were used.

Media. Heart Infusion Broth (HIB; Eiken, Tokyo, Japan) supplemented with 0.1% (v/v) Tween 80 and 4% (v/v) glycerol was used as a liquid medium. To solidify the medium, 1.5 or 0.6% (w/v) agar was added (HIA or soft agar, respectively). Ogawa's egg medium was also used as a solid medium.

Assay of bacteriocin activity. The indicator strain was subcultured on Ogawa's egg medium for 48 h at 37 °C. A suspension containing 1×10^8 bacteria ml⁻¹ was prepared using the cells from the egg medium. A portion of this suspension (0.5 ml) was mixed with soft agar (5 ml) and poured on to an HIA plate. Drops of

twofold serial dilutions of a bacteriocin suspension, sterilized by either ultraviolet (u.v.) irradiation or chloroform treatment, were spotted on to the lawn of the indicator strain. The reciprocal of the maximum dilution which produced a growth inhibition zone on the indicator strain was taken as the concentration of bacteriocin in arbitrary units (U).

Purification procedure. An exponential phase culture of *M. smegmatis* (40 ml) was inoculated into 8 l HIB and incubated at 37 °C for 72 h in a jar fermenter (Microferm; New Brunswick Scientific Co.). After incubation, bacteria were harvested by centrifugation and washed three times in Tris/NaCl/MgCl₂ buffer (Tris buffer; 10 mM-Tris adjusted to pH 7.5 with HCl, 100 mM-NaCl and 10 mM-MgCl₂). About 20 g wet wt bacteria were obtained from 8 l HIB. Bacteria (50 g wet wt) were suspended in 300 ml of the same buffer and disrupted ultrasonically (Branson Sonifier Cell Disruptor 200, Branson Sonic Power Co., Danbury, Conn., U.S.A.). Cell debris was removed by centrifuging twice (13000 g for 20 min and 80000 g for 240 min), and the supernatant fluid was used as the starting material for purification.

Solid ammonium sulphate was added slowly to the supernatant fluid until the solution was 40 % saturated. After 2 h at 4 °C, the precipitate was collected by centrifuging at 7000 g for 30 min and discarded. Ammonium sulphate was added to the supernatant until the solution was 60 % saturated. After 2 h at 4 °C, the precipitate was collected by centrifugation, re-dissolved in 16 ml Tris buffer and dialysed against the same buffer for 24 h.

A portion (10 ml) of the ammonium sulphate fraction was applied to a DEAE-cellulose column (1.5 × 25 cm) pre-equilibrated with Tris buffer. Fractions of 5 ml were collected; after elution of 40 fractions, the concentration of NaCl was raised to 0.7 M with a linear gradient over 81 fractions. Fractions 48 to 57 were pooled and concentrated to 2 ml using an ultrafiltration membrane (Amicon UM10). After centrifugation at 13000 g for 20 min, 1.5 ml of the fraction was applied to a Sephadex G100 column (1.5 × 80 cm) equilibrated with Tris buffer. The void volume of the column was 52 ml and the flow rate was 3 ml h⁻¹. Fractions of 1 ml were collected at 4 °C, and their absorbances at 280 nm and mycobacteriocin activities were assayed.

Molecular weight estimation. The molecular weight of M12 was estimated by Sephadex G100 column chromatography. The elution position of M12 in relation to other proteins of known molecular weight (Combitek calibration protein kit size 1; Boehringer-Mannheim) was determined.

Physicochemical properties. The heat stability of the ammonium sulphate precipitated fraction of M12 was tested by heating at 40, 50, 56, 60, 70, 80 and 100 °C for 30 min. Residual activities were assayed by the method described above.

The ammonium sulphate fraction of M12 was treated for 30 min at 37 °C with the following enzymes (each at 200 µg ml⁻¹): trypsin (Difco), pronase (Kaken Co., Tokyo, Japan), α-chymotrypsin (Sigma), deoxyribonuclease I (DNAase; Sigma), ribonuclease (RNAase; Sigma). After incubation, residual activities were assayed. In the case of trypsin, trypsin inhibitor (Sigma) was added at the end of the incubation period.

The ammonium sulphate fraction of M12 in Tris buffer was mixed with equal volumes of either 0.01 M-acetate buffer (pH 4.0 and 5.0), 0.01 M-phosphate buffer (pH 6.0) or 0.01 M-Tris buffer (pH 7.0 and 8.0). Preparations were dialysed against the same buffers for 24 h and then assayed.

M12 was also subjected to u.v. irradiation (1 × 10⁻² J mm⁻²; Toshiba germicidal lamp 25 W; Toshiba Electric Co., Tokyo, Japan), ultrasonic oscillation (200 W, 20 min; Kubota insonator 200M; Kubota Seisakusho, Tokyo, Japan), and freezing and thawing (20 times).

Assay of protein. Protein was assayed by the method of Lowry *et al.* (1951).

Kinetics of killing. A suspension of the indicator strain containing about 10⁷ bacteria ml⁻¹ was prepared after growth on Ogawa's egg medium or in HIB. To 1.6 ml of the suspension, 0.4 ml M12 (256 U ml⁻¹) was added and the mixture was incubated at 37 °C. At intervals, samples were withdrawn, diluted appropriately, and plated. Colonies were counted after about 1 week incubation at 37 °C.

Electron microscopy. *Mycobacterium diernhoferi* (10⁸ bacteria ml⁻¹) exposed to M12 (final concentration 56 U ml⁻¹) for 24 or 48 h were prefixed in HIB by adding OsO₄ to a final concentration of 0.1 % (w/v) and keeping the suspension at room temperature for about 20 min. They were postfixed with 1 % (w/v) OsO₄ and processed further according to the method of Ryter *et al.* (1958). Observations were made using a JEM 100C electron microscope.

Assay of active spectrum. Mycobacterial test strains were subcultured on Ogawa's medium for 2 d, and then suspensions of these bacteria were plated on HIA with 5 ml soft agar. A drop of M12 suspension containing 128 U ml⁻¹ was then spotted and the results were scored after 2 to 4 d incubation at 37 °C. Strains other than mycobacteria were subcultured on HIA without Tween 80 or glycerol and the same method was used to determine the susceptibility of these strains.

Table 1. *Adsorption of mycobacteriocin M12 to M. diernhoferi* ATCC 19340 grown on different media

Suspensions containing 1×10^8 bacteria ml^{-1} were mixed with M12 preparation (final concentration 32 U ml^{-1}). After 2 or 6 h incubation at 37 °C, bacteria were removed by centrifugation and the activities in the supernatant were assayed.

Bacteria obtained from:	Unadsorbed mycobacteriocin (U ml^{-1})		Adsorption (%)	
	2 h	6 h	2 h	6 h
Ogawa's slant	4	2	87.5	93.7
Heart Infusion Broth	16	8	50	75

Table 2. *Production of mycobacteriocins active against M. diernhoferi* ATCC 19340 from various mycobacterial strains

Bacteria (5 g wet wt) were suspended in 20 ml Tris buffer and disrupted ultrasonically. Activity in the supernatant was assayed after removing cell debris by centrifugation.

Strain	Activity (U ml^{-1})
<i>M. vaccae</i> ATCC 25952	8
<i>M. aurum</i> ATCC 25790	8
<i>M. aurum</i> ATCC 25792	8
<i>M. aurum</i> ATCC 25794	8
<i>M. aurum</i> ATCC 25800	8
<i>M. smegmatis</i> ATCC 25855	32
<i>M. fortuitum</i> ATCC 1701	16

RESULTS

Sensitivity of indicator strain

The sensitivity of *M. diernhoferi* to a suspension of M12 was greatly influenced by the physiological condition of the bacteria. A suspension of M12 apparently contained 256 U when tested against cells cultured on solid media (Ogawa's egg or HIA) for 48 h, but 64 U when tested against cells grown overnight in liquid medium (exponential phase culture). A suspension containing 10^8 bacteria ml^{-1} obtained from Ogawa's egg medium subcultured for 48 h was employed routinely.

The variations in sensitivity of the indicator strain might be due to differences in adsorption of M12 on to the cell surface. To test this possibility, adsorption experiments were carried out. Suspensions (10^8 bacteria ml^{-1}) prepared from each of the different media were mixed with M12 preparation (final concentration 32 U ml^{-1}), and the mixtures were incubated for 6 h at 37 °C with gentle shaking. At 2 and 6 h, samples were withdrawn, centrifuged to remove the cells, and the residual activities in the supernatant were assayed using bacteria from Ogawa's medium. Adsorption of M12 to the bacteria cultured on Ogawa's slant for 48 h was faster than to those in exponential phase obtained from liquid HIB medium (Table 1).

Production of mycobacteriocins

Seven strains of rapidly growing mycobacteria were tested for their ability to produce mycobacteriocins active against *M. diernhoferi*. None of them released bacteriocins into the medium even after 4 to 5 d incubation in HIB. Attempts to induce release of bacteriocin by u.v.-irradiation or mitomycin C treatment were unsuccessful. It was necessary to disrupt the bacteria by sonication to release mycobacteriocins. From 5 g (wet wt) bacteria suspended in 20 ml Tris buffer, activities of 8 to 32 U ml^{-1} were obtained (Table 2). The activity was highest in *M. smegmatis*.

Table 3. *Purification of mycobacteriocin M12*

Material	Activity (U ml ⁻¹)	Total activity (U)	Protein (mg ml ⁻¹)	Specific activity (U mg ⁻¹)	Recovery (%)	Purifi- cation factor
Ultracentrifugation supernatant	16	3840	11.0	1.4	100	1
Ammonium sulphate fraction	128	2048	15.4	8	53	6
DEAE-cellulose	256	512	6.2	41	13	30
Sephadex G100	64	96	0.52	123	3	88

Fig. 1. Elution of mycobacteriocin M12 from DEAE-cellulose. M12 ammonium sulphate fraction (10 ml) was applied to a DEAE-cellulose column. Fractions of 5 ml were collected. After elution of 40 fractions, the concentration of NaCl was raised from 0.1 to 0.7 M over 81 fractions with a linear gradient. —, Absorbance at 280 nm; ---, NaCl concentration; dotted histogram, activity of M12.

Partial purification of M12

The partial purification of M12, described in Methods, is summarized in Table 3. M12 activity was eluted from DEAE-cellulose as a single component at about 0.25 M-NaCl (Fig. 1). When the combined active fractions were applied to a Sephadex G100 column, the activity was eluted as a single component (Fig. 2). Only 3% of the initial activity could be recovered after the Sephadex G100 chromatography. Purification was calculated to be 88-fold, but was not complete since polyacrylamide gel electrophoresis showed at least four to five different components. The elution position of M12 in relation to cytochrome *c*, chymotrypsin, hen egg albumin, bovine serum albumin, aldolase and catalase showed that the molecular weight of M12 was about 85000.

Physicochemical properties of M12

M12 (ammonium sulphate fraction) was sensitive to treatment with trypsin, α -chymotrypsin and pronase but resistant to DNAase and RNAase.

No decrease in activity was observed after 30 min incubation at temperatures up to 56 °C, but after 30 min at 60 °C, about 75% of the activity was lost. Surprisingly, slight activity was still detectable in the undiluted suspension after 30 min heating at 100 °C.

M12 was stable to u.v. irradiation, freezing and thawing, ultrasonic oscillation and changes in pH from 4 to 8.

Killing curve

When *M. diernhoferi* grown on Ogawa's medium for 48 h was used for the killing experiments, the number of viable cells did not decrease until after 6 h incubation. The proportion of viable cells then decreased gradually to about 8% at 24 h and 0.03% after 48 h (Fig. 3*a*).

Fig. 2. Partial purification of mycobacteriocin M12 by chromatography on Sephadex G100. The concentrated preparation of M12 from DEAE-cellulose chromatography was applied to a column of Sephadex G100. The void volume, determined from the elution volume of Blue dextran, was 52 ml. —, Absorbance at 280 nm; dotted histogram, activity of M12.

Fig. 3. Mycobacteriocin sensitivity of *M. diernhoferi* grown on solid medium (a) or in liquid medium (b). Suspensions of *M. diernhoferi* were mixed with 52 U of mycobacteriocin. Samples were removed at intervals and assayed for viable colony count: ○, without mycobacteriocin; ●, with mycobacteriocin.

The results indicated that killing of indicator bacteria by M12 was very slow. The curve had a shoulder, suggesting a multiple-hit process. The control culture (without M12) had a long lag time and the number of bacteria doubled after 18 to 20 h.

Killing of indicator strain by M12 was much slower when exponential phase bacteria grown in liquid medium were employed. The number of viable cells increased during the first 6 h even when M12 was present in the mixture (Fig. 3b). After that the number of viable cells decreased rather rapidly; at 24 h, about 31% of the initial cells were viable and at 48 h, less than 0.1%. Since most of the cells in the exponential phase (liquid) culture were growing, it seems unlikely that adsorption or killing requires growing cells. The generation time of *M. diernhoferi* in the untreated exponential culture was between 5 and 6 h.

Morphological changes in *M. diernhoferi* after exposure to M12

A section of an unexposed control cell of *M. diernhoferi* is shown in Fig. 4 (a); nucleoid, ribosomes and vacuole-like lipoidal inclusion bodies (Barksdale & Kim, 1977) can be seen in the cells. Morphological changes were induced by M12. After 24 h (Fig. 4b), when about

Fig. 4. Thin sections of *M. diernhoferi*, before exposure to mycobacteriocin M12 (a) and after exposure to M12 for 24 h (b) or 40 h (c). Bar markers represent 0.5 μm . CE, Cell envelope; CM, cytoplasmic membrane; N, nucleoid; R, ribosomes; LIB, lipoidal inclusion body.

Fig. 5. Cell envelopes of *M. diernhoferi* before (a) and after (b) exposure to mycobacteriocin M12. Bar marker represents 0.1 μm . CE, Cell envelope; CM, cytoplasmic membrane.

Table 4. Activity spectrum of mycobacteriocin M12

One drop of M12 suspension containing 128 U ml⁻¹ was spotted on to the lawn of test strain.

Strain tested	Sensitivity
<i>Mycobacterium chelonae</i> ATCC 19977	+
<i>M. aurum</i> ATCC 25790	+
<i>M. chitae</i> ATCC 19627	+
<i>M. flavescens</i> ATCC 14474	+
<i>M. fortuitum</i> ATCC 19542	+
<i>M. parafortuitum</i> ATCC 19686	+
<i>M. phlei</i> ATCC 11758	+
<i>M. smegmatis</i> ATCC 14468	—
<i>M. thermoresistibile</i> ATCC 19527	+
<i>M. vaccae</i> ATCC 15483	+
<i>Nocardia asteroides</i>	—
<i>Corynebacterium diphtheriae</i>	—
<i>Listeria monocytogenes</i>	—
<i>Bacillus subtilis</i>	—
<i>Klebsiella pneumoniae</i>	—
<i>Pseudomonas aeruginosa</i>	—
<i>Staphylococcus aureus</i>	—

+, Inhibition of growth; —, no growth inhibition.

10% of the cells were still viable, there was partial loss of ribosomes and thickening of the cell envelope. After 40 h (Fig. 4c), the changes were much more striking; there was loss of ribosomes and enlargement of lipoidal inclusion bodies but cell lysis was not observed.

The appearance of the normal envelope (Fig. 5a) was different from that after 40 h treatment with M12 (Fig. 5b). The increased thickness brought about by M12 treatment is clearly visible.

Spectrum of activity

All the rapidly growing strains of mycobacteria used in this experiment, except *M. smegmatis*, were sensitive to M12 (Table 4). Bacteria other than mycobacteria were resistant.

DISCUSSION

The sensitivity of *M. diernhoferi* to mycobacteriocin M12 was affected by the physiological condition of the cells. Such differences in sensitivity appear to be a more general

phenomenon in the titration of bacteriocins; the titre of several types of bacteriocin must be determined under carefully standardized conditions (Tagg *et al.*, 1976). Exponentially growing organisms are usually the most sensitive to bacteriocins and are normally used in assays. However, exponential phase cultures of *M. diernhoferi* (obtained from liquid medium) were relatively insensitive to M12. This might be due to a difference in adsorption of M12 on to the bacterial surface, since the decrease in bacteriocin titre after mixing was less than that obtained using bacteria grown on Ogawa's solid medium. Presumably, the latter have more exposed receptor sites than those from liquid medium.

Since the yield from *M. smegmatis* was very poor, we tried several culture media to increase the production of M12. However, none of them appeared to be effective (unpublished data), and we failed to demonstrate M12 activity in culture filtrates, even after induction. Production of bacteriocins by many Gram-positive bacteria is poor and is affected by culture conditions (Tagg *et al.*, 1976). Mycobacteriocin M12 is thus similar to bacteriocins produced by Gram-positive bacteria. Like staphylococcin 414 (Gagliano & Hinsdill, 1970), M12 was released from *M. smegmatis* by mechanical disruption of the cells.

Attempts to purify M12 were not satisfactory because of the low initial activity and extensive loss of activity during the purification procedure, a common problem in purifying bacteriocins of Gram-positive bacteria (Tagg *et al.*, 1976). Stability of bacteriocins produced by *Clostridium botulinum*, *C. perfringens* and group A streptococci decreases dramatically with increased purification (Ellison & Kautler, 1970; Mahony, 1974; Tagg *et al.*, 1973*a*).

Bacteriocin M12 is small, with a molecular weight of about 85000. Its active moiety seems to be a protein, since it is sensitive to proteolytic enzymes. A mycobacteriocin produced by *M. tuberculosis* was also sensitive to proteolytic enzymes (Takeya & Tokiwa, 1974).

The very slow progress of killing suggested either that adsorption of M12 on to the cell surface was very slow or that there might be a reversible period during which binding does not necessarily result in death. Adsorption experiments (Table 1) indicated the latter possibility, since 87.5% of the bacteriocin was adsorbed to the cell surface after 2 h incubation. However, cell concentrations used in the adsorption experiments were at least 50 times higher than those in the killing experiments, so the amount of adsorbed bacteriocin in the killing experiments must be less than those observed in the adsorption experiments.

The killing curve of M12 resembled a multiple-hit curve, suggesting that cumulative action by more than one bacteriocin molecule may be necessary to kill the cells. Most bacteriocins kill sensitive cells by single-hit kinetics. One possible explanation of multiple-hit killing is that the cells used in the experiments were clumped. However, this seems unlikely, because 70 to 90% of the population were seen by microscopy to be single cells, whilst by extrapolation from Fig. 3(*b*), the number of hits is estimated to be between 5 and 10. Whatever the mechanism of the multiple-hit killing, this character of M12 may contribute to the slow progress of killing.

There have been relatively few reports on the morphological changes induced by bacteriocins. Ohnishi (1969) observed degradation of the nucleoid of *Pseudomonas aeruginosa* by pyocin 28. After exposure of group A streptococcus to staphylococcin C55, condensation of nuclear material, partial loss of ribosomes, modification of mesosomes and eventual dissolution of cell contents occurred (Clawson & Dajani, 1970). Similar changes were observed with streptococcin A-FF22 (Tagg *et al.*, 1973*b*) and boticin E-S5₁ (Ellison *et al.*, 1971). Changes induced by mycobacteriocin M12 seem to be similar to those induced by streptococcin C55, except for the changes in cell envelope. M12 did not lyse cells; indeed it induced a thickening of the cell envelope. It is not known what metabolic disorder induced such morphological changes. Attempts to detect the specific biochemical lesions within affected cells are in progress.

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