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Mycobacteriocins Produced by Rapidly Growing Mycobacteria Are Tween-Hydrolyzing Esterases

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Smegmatocin, a protein produced by *Mycobacterium smegmatis* ATCC 14468, was found to have an esterase activity, hydrolyzing Tween 80, polyoxyethylene sorbitan monooleate, added to the assay medium for various "bacteriocins" from mycobacteria. Because *M. diernhoferi* ATCC 19340 (indicator strain for smegmatocin) is highly susceptible to oleic acid and smegmatocin requires Tween 80 for manifestation of its anti-*M. diernhoferi* activity, it is likely that smegmatocin-mediated antimicrobial action is caused by oleic acid generated by hydrolysis of Tween 80 by the inherent esterase action of smegmatocin. Other mycobacteriocins from rapidly growing mycobacteria also have inherent esterase activity against Tween 80 and require Tween 80 for expression of antimycobacterial action. Smegmatocin was found to hydrolyze various polyoxyethylene (sorbitan) fatty acyl esters but not sorbitan monooleate and glyceryl esters.

Some physicochemical and biological properties of bacteriocins produced by mycobacteria (mycobacteriocins) have been reported by us (4, 21) and others (2, 25–29). Biological assays for the bactericidal activity of mycobacteriocins have been performed on media supplemented with Tween 80, polyoxyethylene sorbitan monooleate (2, 4, 21, 26–29), which is a growth stimulant (9), a nontoxic reservoir for oleic acid (20), and a carbon or energy source (8, 24, 31) of mycobacteria. When attempting to elucidate mechanisms involved in the bactericidal activity of smegmatocin 14468 (mycobacteriocin produced by *Mycobacterium smegmatis* ATCC 14468), we found that Tween 80 is required for the smegmatocin-mediated killing of *M. diernhoferi* ATCC 19340 (indicator strain). This effect was also exhibited by other ester compounds having polyoxyethylene and long-chain acyl moieties, regardless of the presence of sorbitan residue. These findings suggest that smegmatocin is an esterase acting on Tween 80 molecules and generating free oleic acid, a compound which kills indicator cells. Indeed, nonesterified fatty acids such as oleic acid are known to be toxic to a number of gram-positive bacteria such as *Staphylococcus aureus* (5, 10), *Streptococcus faecalis* (6), *Bacillus subtilis* (22, 23), *Clostridium welchii* (11), and various mycobacterial species (7, 12, 17, 20).

The present paper describes the enzymatic properties of so-called mycobacteriocins, especially of smegmatocin, as Tween-hydrolyzing esterases, and the killing mechanism of indicator cells mediated by an interaction of mycobacteriocins with Tween 80 molecules.

MATERIALS AND METHODS

Bacteria. The following mycobacterial strains were used: *M. smegmatis* ATCC 14468 and ATCC 19979, *M. diernhoferi* ATCC 19340 and SN 1412, *M. chitae* ATCC 19627 and ATCC 19629, *M. phlei* ATCC 19249 and WA-289, *M. fortuitum* ATCC 6841 and ATCC 23012, *M. parafortuitum* ATCC 19686 and ATCC 19688, and *M. chelonei* supsp. *abscessus* Yamamoto and ATCC 14472. These bacteria were purified on heart infusion agar plates containing 4% glycerol and maintained on 1% Ogawa egg medium (18) or in heart infusion medium supplemented with 4% glycerol and 0.1% Tween 80.

Bacteriocins. Smegmatocin 14468 was partially purified from sonically disrupted cells of *M. smegmatis* ATCC 14468 by DEAE-cellulose, Sephadex G-150, phenyl Sepharose, and diethyl-(2-hydroxypropyl) aminoethyl Sephadex column chromatographies. By these procedures, approximately 320-fold purification was achieved. In some experiments, partially purified bacteriocins from sonically disrupted cell lysates of other rapidly growing mycobacteria by DEAE-cellulose and Sephadex G-150 column chromatographies were employed.

Assay for bactericidal activity of mycobacteriocins. (i) Method A. About 5 μl of twofold serially diluted bacteriocin solution was spotted on a heart infusion-glycerol (4%) agar plate with or without Tween 80. After drying, the plate was exposed to chloroform vapor. The basal layer of agar plate was then overlaid with 3 ml of 0.5% heart infusion-glycerol agar with or without Tween 80 containing 10^6 organisms of *M. diernhoferi* ATCC 19340 (indicator cells) per ml, unless otherwise specified. After incubation at 37°C for 3

days, the maximum dilution of the bacteriocin solution giving a clear zone of growth inhibition was recorded and referred to as arbitrary units (AU) per ml.

(ii) Method B. A paper disk (8 mm in diameter) dipped into the bacteriocin solution was placed on 3×10^6 indicator cells overlaid on the basal heart infusion-glycerol agar with or without detergent such as Tween 80. Alternatively, wells (7-mm diameter) in the same agar plate were filled with bacteriocin solution. After incubation at 37°C for 3 days, the diameter of the growth inhibitory zone was measured.

(iii) Method C. Three-day-old cultures of *M. diernhoferi* ATCC 19340 grown on Ogawa egg medium were harvested, rinsed three times with saline, and suspended in heart infusion-glycerol medium. A 0.5-ml volume of the bacterial suspension (4×10^6) was mixed with an equal volume of smegmatocin solution in phosphate-buffered saline containing 0.01% gelatin and 0.5 ml of the same buffer with or without 0.6% Tween 80 and was incubated for up to 72 h with reciprocal shaking. At various intervals, CFU of the incubation mixture were counted by seeding on the heart infusion-glycerol agar plate. In some cases, *M. diernhoferi* cells were incubated with smegmatocin in the presence of detergents other than Tween 80.

Assay for esterase activity of mycobacteriocin preparations. The hydrolyzing activity of various mycobacteriocin preparations against detergents having long-chain acyl esters was measured as follows. A 0.2-ml reaction mixture containing 1 or 1.25% substrate (such as Tween 80) and 10 μl of bacteriocin solution as the enzyme source (200 AU/ml) in 50 mM Tris-hydrochloride buffer (pH 7.5) was incubated at 37°C for 10 to 15 min. Free fatty acid liberated in the reaction mixture was measured by means of the Nonesterified Fatty Acid Test (Wako Pure Chemical, Osaka, Japan).

Antimycobacterial activity of fatty acids. The minimum inhibitory concentrations of some fatty acids against rapidly growing mycobacteria were studied by using the agar dilution method with heart infusion-glycerol agar. The test strains grown on 1% Ogawa egg medium were suspended in saline containing 0.1% Tween 80 at a concentration of 10^6 organisms per ml and were streaked 2 cm on the agar plate. Growth of bacteria was observed after incubation at 37°C for 5 days.

Special agents. Special chemicals used in this study were: polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80), and polyoxyethylene capryl phenol ether (Triton X-100) purchased from Wako Pure Chemical and polyoxyethylene monooleate, polyoxyethylene dioleate, glyceryl monooleate (monoolein), and polyoxyethylene oleoyl ether donated by Nihon Yushi Co., Tokyo.

RESULTS

Requirement of Tween 80 for expression of anti-*M. diernhoferi* activity of smegmatocin. Not only smegmatocin but also six other mycobacteriocins exhibited antimicrobial activity against *M. diernhoferi* ATCC 19340, but only when the assay agar plate was supplemented with 0.1% Tween 80 (Fig. 1). Figure 2 shows the dependency of smegmatocin activity on the concentration of Tween 80 added to the agar plate. Toxic effects of Tween 80 were observed at concentrations over 0.5%, presumably due to free oleic acid and its peroxides contained in the Tween 80 preparation. Tween 80 was required for the expression of the bactericidal action of smegmatocin 14468 (Fig. 3A). Tween 80 was also required for the manifestation of bactericidal activity of bacteriocins produced by the other six strains of group IV mycobacteria (data not shown). Some polyoxyethylene sorbitan long-chain acyl esters (Tweens 20, 40, and 60) were also effective in the manifestation of smegmatocin activity, but the degree was lower than that of Tween 80 (Fig. 3B). Figure 3C shows a comparison of various nonionic detergents with long-chain acyl esters with polyoxyethylene or sorbitan moieties to Tween 80, with regard to their activities to collaborate with smegmatocin. Polyoxyethylene mono- or dioleate esters showed almost equal activity to Tween 80. Sorbitan monooleate (Span 80) and glyceryl monooleate (monoolein) exhibited no such effect. These results indicate that both polyoxyethylene and long-chain acyl moieties (without sorbitan residue) are essential for collaboration of these detergents with smegmatocin for killing of *M. diernhoferi*.

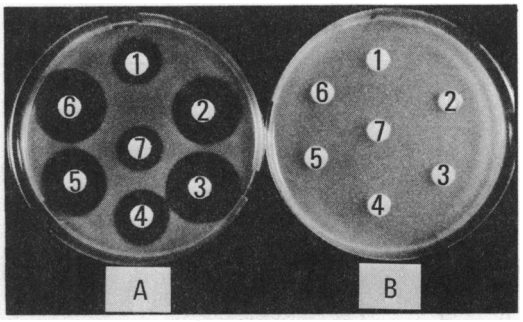

FIG. 1. Essential role of Tween 80 in the anti-*M. diernhoferi* activity of mycobacteriocin preparations from rapidly growing mycobacteria. Mycobacteriocins prepared from *M. smegmatis* ATCC 14468 (1), *M. fortuitum* ATCC 14472 (4), *M. phlei* ATCC 19249 (5), *M. chitae* ATCC 19627 (6), and *M. diernhoferi* ATCC 19340 (7) were assayed by "method B" (see the text) on heart infusion-glycerol agar plates with (A) or without (B) 0.1% Tween 80. Specific activities of these mycobacteriocin preparations are shown in Table 4.

Evidence that mycobacteriocins are esterases. Smegmatocin 14468 hydrolyzed Tween 80 molecules and liberated free oleic acid (Table 1). Moreover, the smegmatocin preparation also hydrolyzed Tweens 20, 40, and 60 and polyoxyethylene monostearate, monooleate, and dioleate esters. In contrast, the fatty acyl esters

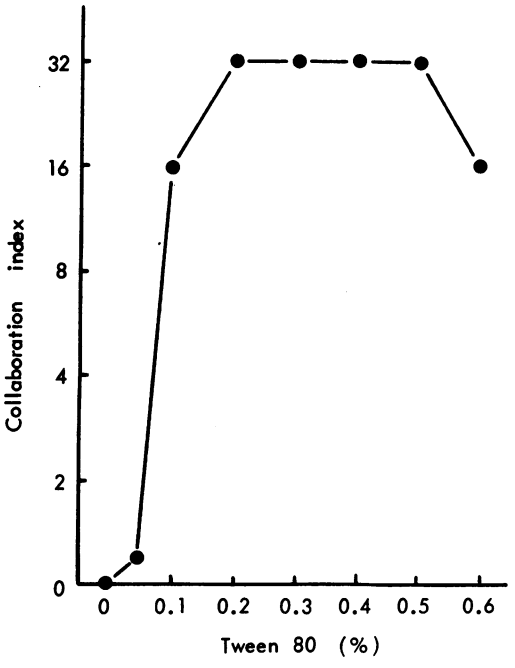

FIG. 2. Effect of Tween 80 on the expression of anti-*M. diernhoferi* ATCC 19340 action by smegmatocin 14468. Smegmatocin activity was assayed by "method A" (see the text) on the heart infusion-glycerol agar plates containing 0 to 0.6% Tween 80. The collaboration index was the maximum dilution of smegmatocin solution (16 AU/ml) to yield a clear growth inhibitory zone of the indicator strain.

lacking the polyoxyethylene moiety such as Span 80 and monoolein did not efficiently serve as the substrates for smegmatocin-mediated hydrolysis. The nearly pure (90%) smegmatocin preparation, obtained by further purification steps such as phenyl Sepharose column chromatography and subsequent preparative polyacrylamide gel electrophoresis, exhibited a similar hydrolyzing action against polyoxyethylene fatty acyl esters (data not shown).

Evidence that the antimycobacterial actions of mycobacteriocins are mediated by oleic acid. Except for *M. diernhoferi* strains ATCC 19340 and SN 1412 and *M. chitae* strains ATCC 19627 and ATCC 19629, all of the group IV mycobacteria were resistant to oleic acid (Table 2). The sensitivity of these organisms to oleic acid paralleled the smegmatocin susceptibility. Either 1% bovine serum albumin or 10% fetal bovine serum added to the agar medium for smegmatocin assay, as a reservoir for oleic acid, almost completely inhibited the action of smegmatocin (data not shown). The lauric acid (generated from Tween 20 by hydrolysis) was considerably toxic to *M. diernhoferi* strain ATCC 19340 and SN 1412 (Table 2); palmitic acid and stearic acid were substantially nontoxic (data not shown). The reaction mixture of smegmatocin and Tween 80 was subjected to chymotrypsin digestion, heat treatment, or extraction with ethyl-ether, and the resultant samples were measured for their antibacterial activities and for the amount of oleic acid (Table 3). The antibacterial substance generated in the reaction mixture of smegmatocin and Tween 80 was highly resistant to chymotrypsin and heating and transferable to the ether layer. Mycobacteriocins produced by some other rapidly growing mycobacteria were incubated with Tween 80, and the reaction mixtures were digested with chymotrypsin, extracted with ether, and assayed for anti-*M. diernhoferi* activity and oleic acid content. The antibacterial substances were also resistant to chymotrypsin and were extractable by ethyl ether (data not shown). The reaction mixtures with or without heating and the ether extracts contained sufficient free oleic acid for growth inhibition against *M. diernhoferi* (see also Table 2). The anti-*M. diernhoferi* activities of 12 mycobacteriocins, partially purified by DEAE-cellulose and Sephadex G-150 column chromatography, were compared with regard to Tween 80-hydrolyzing activities (Table 4). A significant correlation was found between these two activities of mycobacteriocins ($r = 0.908$; $P < 0.05$).

DISCUSSION

Tween 80 is added to various culture media for mycobacteria, as a wetting and growth-stimulating agent and also as a carbon source (8, 9, 20, 24, 31). This detergent also serves as a carbon source in *Pseudomonas aeruginosa* (13) and *Lactobacillus salivarius* (14). It is also known that Tween 80 specifically suppresses toxin production by *Corynebacterium ulcerans*, presumably due to its promoting action of bacterial growth (1).

We found that Tween 80 was required for the expression of mycobacteriocins produced by group IV mycobacteria. Nonionic detergents other than Tween 80 with polyoxyethylene and fatty acyl ester bonds also had a similar action, whereas fatty acyl ester derivatives lacking a polyoxyethylene moiety did not. These findings suggested that smegmatocin is an esterase having a specificity to polyoxyethylene and fatty acyl moieties. The long-chain acyl coenzyme A-hydrolyzing esterase of *P. aeruginosa* also acts on Tween 80 (19). Many of the mycobacterial species also have a Tween 80-hydrolyzing ability (3, 30); Hawkins and Steenken (12) reported some properties of crude esterases hydrolyzing Tween 80 prepared from *M. phlei* and *M. smegmatis* (*butyricum*). We examined here Tween 80-hydrolyzing activities of various mycobacterio-

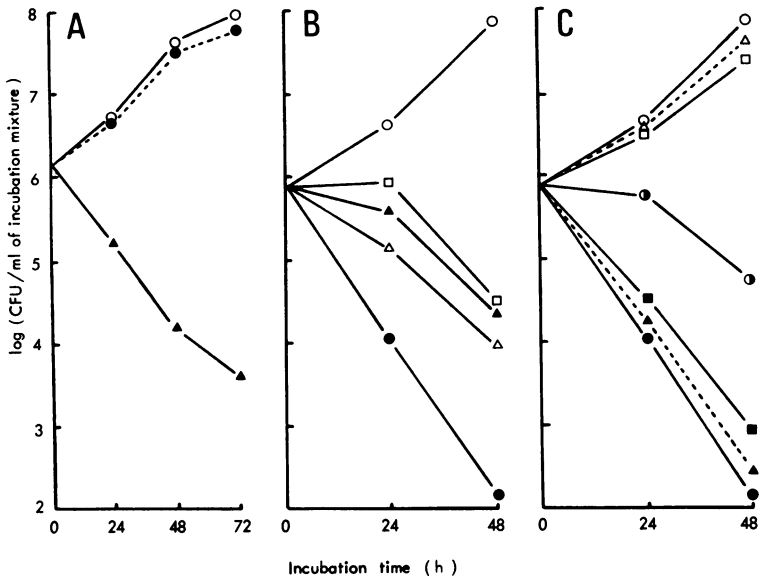

FIG. 3. Effects of various polyoxyethylene long-chain acyl esters on the manifestation of bactericidal action by smegmatocin 14468 against *M. diernhoferi* ATCC 19340. The assay for killing action of smegmatocin on *M. diernhoferi* was as described in the text. (A) Bacteria alone (○); bacteria plus smegmatocin (32 AU/ml) (●); bacteria plus smegmatocin (32 AU/ml) plus Tween 80 (0.2%) (▲). (B) Smegmatocin (128 AU/ml) in the absence (○) or the presence of either Tween 20 (△), Tween 40 (▲), Tween 60 (□), or Tween 80 (●) at 0.2%. (C) Smegmatocin (128 AU/ml) in the absence (○) or the presence of either Tween 80 (●), polyoxyethylene monooleate (▲), polyoxyethylene dioleate (■), polyoxyethylene monostearate (◑), glyceryl monooleate (□), or sorbitan monooleate (△) at 0.2%.

cins from rapidly growing mycobacteria. The specific activities of mycobacteriocin preparations for Tween hydrolysis (esterase activity) correlated fairly well with those for anti-*M.*

TABLE 1. Hydrolyzing activity of smegmatocin 14468 on polyoxyethylene long-chain acyl ester derivatives

Substrate	Liberation of free fatty acid (μmol/min per mg of protein)[a]
Tween 80	23.8
Tween 20	65.7
Tween 40	31.2
Tween 60	23.6
Polyoxyethylene monostearate	24.5
Polyoxyethylene monooleate	33.1
Polyoxyethylene dioleate	17.4
Monoolein	5.5
Span 80	0.0

[a] The reaction mixture, containing 1.25% indicated substrate, 50 mM Tris-hydrochloride (pH 7.5), and 7.5 μg of protein per ml of smegmatocin 14468 preparation (obtained by QAE-Sephadex column chromatography; see the text), was incubated at 37°C for 10 min, and the amount of free fatty acid was measured. The specific activity of this smegmatocin preparation for anti-*M. diernhoferi* action was 8,530 AU per mg of protein.

diernhoferi activity (Table 4). Moreover, the nearly purified smegmatocin 14468 had a high specific activity of Tween 80 hydrolysis (2.5×10^4 U/mg of protein). These results indicate that the antimycobacterial action of mycobacteriocins produced by group IV mycobacteria against their indicator bacteria is dependent on their esterase activity.

The question arises whether the mycobacteriocins directly act on the indicator bacterial cells or whether the antimicrobial action is merely mediated by substances generated in the reaction between mycobacteriocin and Tween 80. If the mechanism of mycobacteriocin action is the latter, "mycobacteriocins" cannot be regarded as true bacteriocins. The finding that anti-*M. diernhoferi* activity of smegmatocin was completely inhibited by the addition of 1% bovine serum albumin or 10% fetal bovine serum to the assay medium containing 0.1% Tween 80 strongly suggests the latter possibility, because serum albumin or serum proteins act as a reservoir for oleic acid (7), which is toxic to *M. diernhoferi* and *M. chitae* (Table 2) and also to *M. tuberculosis* (12). The smegmatocin sensitivities of the mycobacterial strains tested here coincided fairly well with their susceptibilities to oleic acid (Table 2). Moreover, we found that a lipophilic, heat-stable, and chymotrypsin-resis-

TABLE 2. Susceptibilities of various rapidly growing mycobacteria to smegmatocin and to some free fatty acids

Bacterial strain	Sensitivity to smegmatocin[a]	Sensitivity to fatty acid (MIC)[b]	
		Lauric acid	Oleic acid
M. diernhoferi ATCC 19340	64	25	3.2
M. diernhoferi SN 1412	32	50	3.2
M. chitae ATCC 19627	ND[c]	400	1.6
M. chitae ATCC 19629	128	50	1.6
M. smegmatis ATCC 14468	0	>400	400
M. smegmatis ATCC 19979	0	>400	200
M. fortuitum ATCC 6841	0	>400	>400
M. fortuitum ATCC 23012	0[d]	>400	>400
M. parafortuitum ATCC 19686	ND[c]	400	6.3–25[e]
M. parafortuitum ATCC 19688	0	>400	400
M. chelonei Yamamoto	0[d]	>400	>400
M. phlei ATCC 19249	0[d]	>400	12.5–25[e]
M. phlei WA-289	0[d]	>400	25–400[e]

[a] Susceptibility of indicated strains to smegmatocin was assayed by "method A" (see the text) on heart infusion-glycerol (4%) agar plates containing 0.1% Tween 80.

[b] The minimum growth inhibitory concentration (MIC) of the indicated fatty acid against each bacterial strain was measured on heart infusion-glycerol agar plates. MICs are expressed in micrograms per milliliter.

[c] ND, Not determined. In separate experiments, *M. chitae* ATCC 19267 was found to show sensitivity to smegmatocin similar to that of *M. diernhoferi* ATCC 19340.

[d] As a turbid growth inhibitory zone was observed, these strains seemed to be weakly sensitive to smegmatocin.

[e] The MIC varied among four separate experiments.

tant substance generated by Tween 80 hydrolysis by various mycobacteriocins actually produced the same clear growth inhibitory zone of *M. diernhoferi* on the same agar plate as that used for the mycobacteriocin assay (Table 3). This anti-*M. diernhoferi* substance is likely to be oleic acid liberated from Tween 80, as discussed above. Indeed, the main component in the ethyl ether extract of the reaction mixture of Tween 80 and mycobacteriocin was determined to be oleic acid by thin-layer chromatography (data not shown). However, we cannot exclude the possibility that another reaction product, polyoxyethylene sorbitan ether, participates more or less in the expression of mycobacteriocin activity as a toxic mediator, because Tweens 40 and 60 act as the collaborating agents with smegmatocin (Fig. 3), despite the finding that their fatty acid moieties, palmitic acid and stearic acid, showed no toxicity to *M. diernhoferi* and *M. chitae* (Table 2).

The mycobacteriocins produced by group IV mycobacteria are not true bacteriocins. They seem to exhibit anti-*M. diernhoferi* and anti-*M. chitae* activity as the result of oleic acid, a toxic mediator generated from Tween 80 added to the assay medium. A similar observation was reported by Hawkins and Steenken (12) that cell extracts of *M. phlei* and *M. smegmatis* (*butyricum*) exhibited antimicrobial effects against *M. tuberculosis* by liberating oleic acid through hydrolysis of Tween 80 added to the medium. In light of our findings, mycobacteriocins reported by other investigators have to be reconsidered since all were assayed for their antimicrobial activity in media containing Tween 80 (2, 4, 21, 26–29). In fact, morphological changes such as thickening of the cell envelope in *M. diernhoferi* after exposure to mycobacteriocin produced by

TABLE 3. Some properties of antimicrobial substance generated in the reaction mixture of Tween 80 and smegmatocin 14468

Treatment of reaction mixture	Concn of oleic acid[a] (μg/ml)	Diam of the growth inhibitory zone[b] (mm)
None	ND[c]	22
Chymotrypsin[d]	ND	12
Ether extraction[e]	ND	12
None	744	24
121°C[f]	750	19
Tween 80 alone[g]	52	10

[a] The reaction mixture, consisting of 1% Tween 80 and smegmatocin 14468 (160 AU/ml) in 0.05 M Tris-hydrochloride (pH 7.5), was incubated at 37°C for 2 h. After various treatments, the remaining amounts of oleic acid were determined.

[b] The reaction mixture, after various treatments, was assayed for its antimicrobial activity by "method B" (see the text), and the diameter of the growth inhibitory zone of the indicator strain (*M. diernhoferi* ATCC 19340) is shown. Smegmatocin was completely (*d* and *e*) or nearly completely (*f*) inactivated by the indicated treatments.

[c] ND, Not determined.

[d] Digested with 500 μg of chymotrypsin per ml at 37°C for 1 h.

[e] The mixture was extracted with ethyl ether, and the extract was evaporated and redissolved in 50 mM $NaHCO_3$ (0.6 volume of original reaction mixture).

[f] Heated at 121°C for 20 min.

[g] The data for the reaction mixture without smegmatocin are shown.

TABLE 4. Anti-*M. diernhoferi* activity and esterase activity of various mycobacteriocin preparations

Bacteriocin[a]	Anti-*M. diernhoferi* activity (AU/mg of protein)[b]	Esterase activity (U/mg of protein)[c]
M. smegmatis ATCC 14468	267	643
M. smegmatis ATCC 19979	267	786
M. fortuitum ATCC 6841	41	170
M. fortuitum ATCC 23012	98	333
M. parafortuitum ATCC 19688	121	618
M. chelonei ATCC 14472	16	40
M. phlei ATCC 19249	258	910
M. phlei WA-289	71	324
M. chitae ATCC 19627	67	191
M. chitae ATCC 19629	29	66
M. diernhoferi ATCC 19340	91	101
M. diernhoferi SN 1412	57	388

[a] Bacteriocin preparation partially purified by DEAE-cellulose and Sephadex G-150 column chromatography.

[b] The specific activity of anti-*M. diernhoferi* action of each preparation was assayed by "method A" on a heart infusion-glycerol agar plate containing 0.1% Tween 80.

[c] One unit of esterase activity was defined as the amount of enzyme generating 1 nmol of fatty acid per min from Tween 80.

M. smegmatis ATCC 25855 (26) resemble those caused by certain fatty acids (increase in plasma membrane permeability and swelling of cells) (6, 10, 15, 16, 24).

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