ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2000, p. 2154–2159 Vol. 44, No. 8
0066-4804/00/$04.00+0

Saccharomicins, Novel Heptadecaglycoside Antibiotics Produced by *Saccharothrix espanaensis*: Antibacterial and Mechanistic Activities

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Received 18 November 1999/Returned for modification 12 March 2000/Accepted 12 May 2000

Saccharomicins A and B, two new heptadecaglycoside antibiotics, were isolated from the fermentation broth of the rare actinomycete *Saccharothrix espanaensis*. They represent a novel class of bactericidal antibiotics that are active both in vitro and in vivo against bacteria and yeast (MICs: *Staphylococcus aureus*, <0.12 to 0.5; vancomycin-resistant enterococci, 0.25 to 16; gram-negative bacteria, 0.25 to >128; and yeast, >128 μg/ml), including multiply resistant strains. Saccharomicins protected mice from lethal challenges by staphylococci (subcutaneous 50% effective dose range of 0.06 to 2.6 mg/kg of body weight, depending on the *S. aureus* strain). The 50% lethal dose by the subcutaneous route was 16 mg/kg. Mechanistic studies with *Escherichia coli imp* and *Bacillus subtilis* suggested complete, nonspecific inhibition of DNA, RNA, and protein biosynthesis within 10 min of drug treatment. Microscopic examination of drug-treated cells also suggested cell lysis. These data are consistent with a strong membrane-disruptive activity. The antibacterial activities of the saccharomicins against gram-positive bacteria were unaffected by the presence of Ca^{2+} or Mg^{2+}, but activity against gram-negative bacteria was substantially reduced.

For the last 5 decades, antibiotics have revolutionized medicine by providing cures for life-threatening infections (2). However, the 1990s has been a period of growing anxiety about the emergence and management of antibiotic-resistant bacteria (1, 4, 10–12). The need to discover and develop more effective antibiotics with unique modes of action is greater than ever. With this goal, we have continued to screen diverse groups of organisms for antimicrobial activity, using a variety of conventional and target-based screening methods. In this paper, we report biological and mechanistic studies of two novel heptadecaglycoside antibiotics, saccharomicins A and B (Fig. 1), which were isolated and characterized from culture broths of the rare actinomycete *Saccharothrix espanaensis* LL-C19004 (5, 6). This culture (NRRL 15764) was originally isolated from a soil sample collected in Puerto Liano, Spain.

(A preliminary report of these studies was presented at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy held in San Francisco, Calif., 1999.)

MATERIALS AND METHODS

Bacterial strains. Clinical isolates were collected from various medical centers in the United States, and quality control strains were obtained from the American Type Culture Collection (ATCC), Rockville, Md. *Bacillus subtilis trpC2* BGSC1A1 and *Escherichia coli imp* BAS849 were obtained from the Bacillus Genetic Stock Center, Columbus, Ohio, and S. A. Benson (13), respectively. Identification of each culture was done by conventional methods: gram-negative rods by API 20E (Analytab Products, Plainview, N.Y.) and NF systems (Remel, Lenexa, Kans.) and staphylococci by Staph Trac (Analytab Products). All clinical isolates were stored frozen in skim milk at −70°C, and other strains were stored frozen at −80°C in 20% dimethyl sulfoxide.

Media. All media were prepared in distilled deionized (DI) water. Mueller-Hinton (MH) media was purchased from Becton Dickinson Microbiology Systems, Cockeysville, Md. Modified minimal medium (used for mechanistic studies in *E. coli imp*) contained the following (per liter): dextrose, 4 g; NH_4Cl, 1 g; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; and vitamin-free Casamino Acids, 2 g. ATCC medium 21 (K_2HPO_4, 0.5 g; ferric ammonium citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; glycerol, 20 g; citric acid, 2 g; L-glutamic acid, 4 g per liter [pH 7.4]), supplemented with 20 mg of L-tryptophan/liter, was used for studies with *B. subtilis*. All ingredients in the minimal medium were purchased from Sigma Chemical Co., St. Louis, Mo., with the exception of the Casamino Acids, which were purchased from Difco Laboratories, Detroit, Mich.

Chemicals. [^{3}H]thymidine ([^{3}H]TdR, TRK.686, 90 Ci/mmol), [^{3}H]uridine ([^{3}H]UdR, TRK.410, 49 Ci/mmol), and ^{3}H-labeled amino acids (TRK.550; a mixture of leucine, lysine, phenylalanine, proline, and tyrosine, with specific activities of 135, 83, 123, 103, and 118 Ci/mmol, respectively) were purchased from Amersham Corp., Arlington Heights, Ill. All reference antimicrobial agents and all other medium components were purchased from Sigma Chemical Co.

In vitro antibacterial activity. The in vitro antibacterial activities were determined by agar or microdilution methods as described earlier (9, 14). MH II broth or agar was used for nonfastidious aerobic bacteria, and the medium was supplemented with 5% lysed horse blood for *Streptococcus* spp. The inoculum of each culture was approximately 1×10^4 to 5×10^4 CFU applied with the Steers multiple-inocula replicator to plates containing the antibiotic in MH agar. Wilkins-Chalgren agar was used for the anaerobes. The plates were incubated at 35°C for 18 h. The agar MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism. Broth MICs were determined by adding 5 μl of an overnight broth bacterial culture (adjusted to a density of 1×10^7 to 5×10^7 CFU/ml) to 0.1 ml of broth medium in polystyrene plates containing the drug at 0.03 to 128 μg/ml. The broth MIC was defined as the lowest concentration of antibiotic which prevented visual turbidity after 18 h of incubation at 37°C. Since mechanistic studies were done with exponential-phase cultures with an absorbance at 600 nm (A_{600}) of 0.20, the growth-inhibitory concentration of each drug was also determined at this inoculum level by measuring the absorbance of drug-treated cultures after 3 h of incubation at 37°C with agitation. The minimum concentration of drug preventing any increase in the absorbance was called the MIC_{3h}. MH broth was supplemented with the desired levels of divalent cations to study their effects on the antibacterial activity. Calcium chloride, magnesium chloride, ferrous sulfate, and copper sulfate were used as the divalent cations.

Bactericidal activity (kill kinetics). The test organism was grown overnight, transferred to fresh broth, and incubated at 35°C and 200 rpm to logarithmic phase. The culture (50 ml) was then added to a 250-ml Erlenmeyer flask containing 50 ml of antibiotic solution in broth, yielding a final concentration of 10^6 CFU/ml and an antibiotic concentration of 4 or 10 times the MIC. The flasks were incubated for 24 h with shaking at 35°C. Viable cell counts were performed at various time points.

Bactericidal activity against *E. coli imp* was determined by monitoring the change in absorbance at 490 nm using the Molecular Devices Thermomax plate reader, which was set up to read in the negative kinetic mode every 5 min for 4 h at 37°C. Reversal of the saccharomicins' activity by cations was also studied this way by using an exponential-phase culture of *Pseudomonas aeruginosa* ATCC 27853 at final A_{600} of 0.0076 to 0.20. Any increase or decrease in absorbance of the culture suggested growth or inhibition and lysis, respectively.

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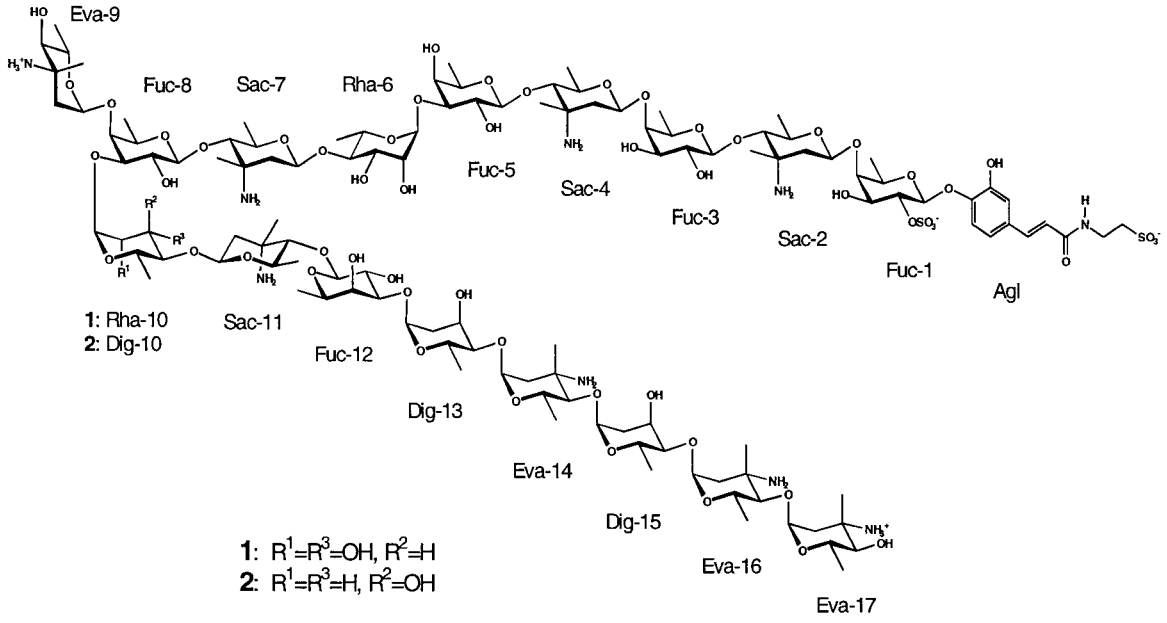

FIG. 1. Structure of saccharomicins A (1) and B (2). Abbreviations: Agl, aglycon; Fuc, fucose; Sac, saccharosamine; Rha, rhamnose; Eva, 4-epi-vancosamine; Dig, digitoxose.

In vivo activity. In vivo activity was assessed in female strain CD-1 mice (Charles River Laboratories, Kingston, N.Y.), weighing 20 ± 2 g each, which were infected intraperitoneally with sufficient bacterial cells suspended in broth or with about 5% mucin to kill 95 to 100% of the untreated mice within 48 h. Antibiotic was administered in single doses 0.5 h after infection. Seven-day survival ratios from three or four separate tests, each with five dose levels and five animals per dose level, were pooled for the determination of the median effective dose (ED_{50}) by probit analysis.

Incorporation of radiolabeled precursors. Macromolecular synthesis in *E. coli imp* and *B. subtilis* was studied by measuring the incorporation of appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material (14). Overnight cultures were diluted 1:500 in fresh modified minimal medium (50 ml of medium in a 250-ml Erlenmeyer flask) and incubated at 37°C and 200 rpm to an A_{600} of 0.20. Aliquots of 100 ml were dispensed into microtiter wells containing 5 μl of antibiotic, and plates were incubated for 2 to 14 min at 37°C with vigorous agitation. Cells were pulse-labeled for 5 min by adding one of the following radiolabeled precursors at the indicated final concentrations: for *E. coli imp*, [^{3}H]TdR (1 μCi/ml, with 0.05 μg of unlabeled thymidine/ml), [^{3}H]UdR (1 μCi/ml, with 0.12 μg of unlabeled uridine/ml), or ^{3}H-labeled amino acids (10 μCi/ml); for *B. subtilis*, [^{3}H]TdR (2 μCi/ml), [^{3}H]UdR (2 μCi/ml, with 0.35 μg of unlabeled uridine/ml), or ^{3}H-labeled amino acid mixture (2 μCi/ml, with 0.035 μg each of leucine, lysine, phenylalanine, proline, and tyrosine/ml). To determine specific incorporation into DNA, RNA, and protein, 100 μl of chilled (4°C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per ml was added to each well, and the plate was immediately refrigerated for 1 h. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205-404) using a Skatron 96-well cell harvester (model 11050) programmed for a 3-s prewet with chilled DI water, a 12-s wash with 5% chilled TCA, and a 5-s drying cycle. To assess the effects of the drugs on cellular uptake of radiolabeled precursors, the step with addition of TCA to the microtiter plate was eliminated, and the contents of each well were harvested onto a glass fiber filter by the Skatron 96-well cell harvester programmed for a 3-s prewet with chilled DI water, a 10-s wash with chilled normal saline (0.9% NaCl in DI water), and a 5-s drying cycle. Filter mats were dried for 7 min at high power in a microwave oven (Quasar, 700 W), solid scintillant (Meltilex B/HS, Wallac 1205-442) was applied, and the isotope retained on the filter was quantitated in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of [^{3}H]TdR, [^{3}H]UdR, and ^{3}H-labeled amino acids are expressed as the percentage relative to the untreated control.

Effect on intracellular potassium of *E. coli imp*. Effects on intracellular potassium in *E. coli imp* were studied in a saline buffer (10 mM HEPES buffer containing 150 mM NaCl and 0.1 mM KCl, pH 7.0). Each log-phase culture was washed twice with saline buffer, and the pellet was resuspended in the same buffer to an optical density at 600 nm of 2.00. One milliliter of the bacterial suspension was treated with the test compounds at various concentrations for 1 h, and cells were pelleted by centrifugation (at 10,000 × *g* for 2 min). The resulting supernatant was diluted 1:10 in high-pressure liquid chromatography-grade water and analyzed for potassium ion by atomic absorption spectrophotometry (Instrumentation Laboratories 551). For determination of the total potassium level, 1 ml of the culture was hydrolyzed in 2 M sulfuric acid by heat (100°C for 1 h), chilled for 1 h, and centrifuged (at 10,000 × *g* for 2 min). The supernatant was then diluted 1:10 and analyzed for potassium ion concentration.

Lysis of human RBCs. One milliliter of freshly pooled human blood was centrifuged (at 10,000 × *g* for 2 min), the pellet was washed four times with normal saline by repeated resuspension and centrifugation, and then the pellet was resuspended in 1 ml of red blood cell (RBC) buffer (10 mM Na_2HPO_4–150 mM NaCl–1 mM $MgCl_2$, pH 7.4). Twenty-five microliters of the RBC suspension was added to a microcentrifuge tube containing 1 ml of drug solution (final concentration ranging from 1 to 128 μg/ml) prepared in duplicate in RBC buffer. After 2 h of treatment, the contents of the tube was centrifuged (at 10,000 × *g* for 2 min) and absorbance (at 540 nm) of the supernatant was measured. For 100% lysis, 25 μl of the RBC suspension was added to 1 ml of water.

RESULTS AND DISCUSSION

A systematic reevaluation of our antibiotic compound library of the last 5 decades provided some extracts and complexes with very interesting bioactivity and novel chemistry. LL-C19004 was one such complex, produced by the rare actinomycete *Saccharothrix espanaensis*. Further characterization of this complex led to the discovery of two novel, high-molecular-mass (2,800 Da) heptadecaglycoside antibiotics, saccharomicins A and B (Fig. 1).

In vitro activity. The antibacterial activity of saccharomicins against a diverse group of clinical isolates is presented in Tables 1 to 3. Although saccharomicins A and B are slightly different in chemical structure, both were mostly equipotent against gram-positive and gram-negative bacteria (Table 1). They exhibited good activity against gram-positive organisms and good to moderate activity against gram-negative organisms. The MICs for gram-negative bacteria were 8 to 16 times higher in the medium supplemented with divalent cations, which suggested some sort of an antagonistic effect of cations on this antibiotic (Table 3). Saccharomicins exhibited good activity against methicillin-susceptible and methicillin-resistant staphylococci, with the majority of the MICs being between <0.12 and 0.5 μg/ml. Over 50% of the methicillin-resistant staphylococci tested were also resistant to minocycline, gentamicin, and erythromycin, and several staphylococcal isolates

TABLE 1. In vitro antibacterial activity

Organism[c]	MIC (μg/ml)[a]				
	Piperacillin	Vancomycin	Ciprofloxacin	Saccharomicin A	Saccharomicin B
S. aureus GC 4536	0.50	0.50	<0.12	0.25	0.25
S. aureus GC 1131	>128	1	32	0.25	0.25
S. aureus GC 4541	>128	0.50	32	0.25	0.50
S. aureus GC 4543	0.50	0.50	<0.12	<0.12	0.25
S. aureus GC 4543[b]	1	1	<0.12	<0.12	<0.12
S. aureus GC 2216	4	0.25	0.25	0.25	0.25
Staphylococcus haemolyticus GC 4547	>128	2	0.25	0.50	1
CoNS GC 4537	8	1	<0.12	0.50	0.50
CoNS GC 4538	32	1	<0.12	0.50	0.50
CoNS GC 4547	>128	2	<0.12	0.50	0.50
MRCoNS GC 4548	32	1	<0.12	0.25	0.25
MRCoNS GC 4549	16	0.50	<0.12	<0.12	<0.12
MRCoNS GC 6257	2	0.50	<0.12	0.25	0.25
Enterococcus faecalis GC 842	2	0.50	0.50	8	4
E. faecalis GC 4555	1	2	1	1	1
E. faecalis GC 2242	2	>128	0.25	4	2
E. faecalis GC 6189	2	>128	1	1	1
Enterococcus faecium GC 4556	2	0.50	0.25	8	4
E. faecium GC 2243	128	>128	2	2	2
E. faecium GC 4558	>128	>128	2	16	8
Streptococcus pneumoniae GC 1894[b]	2	<0.12	0.50	0.50	<0.12
S. pneumoniae GC 6242[b]	<0.12	<0.12	0.50	<0.12	<0.12
E. coli GC 2203	1	>128	<0.12	4	4
E. coli GC 4559	1	>128	<0.12	4	4
E. coli imp GC 4560	<0.12	0.25	<0.12	0.50	0.50
E. coli GC 3226	0.50	>128	<0.12	2	2
Serratia marcescens GC 4077	2	>128	<0.12	16	8
Morganella morganii GC 4531	128	>128	<0.12	8	64
Klebsiella pneumoniae GC 4534	4	>128	<0.12	8	4
Enterobacter cloacae GC 3783	32	>128	<0.12	8	4
P. aeruginosa GC 2214	2	>128	<0.12	32	32
P. aeruginosa GC 4532	4	>128	0.25	16	64
C. albicans GC 3066	>128	>128	>128	>128	>128

[a] Broth microdilution method in MH broth II.
[b] In medium supplemented with 5% lysed horse blood.
[c] CoNS, coagulase-negative staphylococcus; MRCoNS, methicillin-resistant coagulase-negative staphylococcus.

were multiresistant to antibiotics like β-lactams, tetracyclines, streptomycin, tobramycin, chloramphenicol, quinolones, and rifampin (data not shown). All of these antibiotic-resistant isolates were susceptible to saccharomicins (MIC range, <0.12 to 0.5 μg/ml). Saccharomicins were slightly less active against enterococcus species (MIC range, 0.25 to 16 μg/ml), but they were equally active against vancomycin-resistant and -susceptible isolates (Tables 1 and 2). These data on activity of saccharomicins suggest that cross-resistance to other classes of antibiotics would not be expected. The presence of 5% lysed horse blood in the medium did not increase the MICs for the staphylococcal and streptococcal strains tested. Generally, antibiotics with strong protein binding properties have much higher MICs under such conditions. Activity against gram-negative bacteria was good to moderate in cation-deficient medium, but the presence of divalent cations drastically affected the activity (Table 3). The decreased activity against gram-negative bacteria may also be due to the presence of the outer membrane, since saccharomicins exhibited two- to eight-fold-higher activity against an *E. coli imp* strain (Tables 1 and 3). The *imp* mutation increases the permeability of the cell membrane and renders the bacteria much more susceptible to many antibacterial agents, especially larger molecules (10). These antibiotics were inactive against *Candida albicans*. Although more active against gram-positive bacteria, saccharomicin A was bactericidal for both gram-positive and gram-

TABLE 2. Spectrum of activity of saccharomicin AB complex (1:1 ratio)

Organism	No. of strains	MIC (μg/ml)[a]	
		Range	90%
Gram positive			
S. aureus	61	0.12–2	0.5
S. aureus (MRSA)[b]	22	0.12–0.5	0.5
S. haemolyticus	11	1–2	2
S. pneumoniae	11	0.5–2	2
Enterococcus spp.	30	0.25–8	4
Gram negative			
Neisseria gonorrhoeae	11	0.25–1	1
Haemophilus influenzae	13	1–4	4
E. coli	15	16–32	32
Klebsiella spp.	15	16–32	32
Enterobacter spp.	15	4–64	64
Serratia spp.	15	32–>128	32
Proteus spp.	17	16–>128	>128
Acinetobacter spp.	15	4–16	16
Pseudomonas spp.	15	32–128	128
Anaerobic			
Bacteroides fragilis	15	>128	>128

[a] Agar dilution method.
[b] MRSA, methicillin-resistant *S. aureus*.

TABLE 3. Effect of divalent cations on antibacterial activity[a]

Organism[c]	MIC (MH/MH + Ca^{2+}, Mg^{2+})[b] (μg/ml)			
	Saccharomicin A	Saccharomicin B	Polymyxin B	Penicillin G
S. aureus 375 (MSSA)	0.12/0.06	0.12/0.06	16/16	0.06/0.06
S. aureus 310 (MRSA)	0.12/0.12	0.12/0.12	32/32	>128/>128
S. aureus ATCC 25923	0.12/0.12	0.12/0.12	32/32	0.06/0.06
E. faecalis ATCC 31186	0.50/0.50	0.50/0.50	>128/>128	2/1
E. faecium 379 (VREF)	1/1	1/1	NT	NT
E. coli imp 389	0.12/0.12	0.12/0.12	1/1	1/1
E. coli 442 (parent of 389)	0.25/2	0.25/2	1/1	16/16
E. coli ATCC 25922	0.50/4	0.50/4	2/2	64/64
P. aeruginosa ATCC 27853	2/32	2/32	1/4	>128/>128

[a] Broth microdilution method, with inoculum of 1×10^5 to 5×10^5 CFU/ml and incubation at 37°C for 18 h.
[b] MIC in MH broth versus MIC in MH broth plus Ca^{2+} (40 μg/ml) and Mg^{2+} (20 μg/ml). NT, not tested.
[c] MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VREF, vancomycin-resistant *E. faecium*.

negative organisms. Killing of *Staphylococcus aureus* Smith was more rapid with saccharomicin A than with vancomycin (Fig. 2). Killing of *E. coli imp* was more rapid in stationary-phase cells suspended in an isotonic saline buffer than in those in minimal medium (Fig. 3). This reduced bactericidal activity of saccharomicin A in medium containing magnesium and possible sources of calcium suggested that divalent cations may diminish the activity of the antibiotic.

Reversal of activity by divalent cations. MICs of the saccharomicins for all gram-positive bacteria and *E. coli imp* were unaffected by the presence of divalent cations (Ca^{2+} and Mg^{2+}) in the medium, but 8- and 16-fold increases in MICs were observed for *E. coli* and *P. aeruginosa*, respectively (Table 3). Growth inhibition of *P. aeruginosa* by saccharomicin A was neutralized by calcium and magnesium ions in a concentration-dependent manner (Table 4). The control antibiotic, polymyxin B, behaved similarly. Calcium ion was about twofold more effective than magnesium ion in neutralizing the activity. The concentration needed to neutralize the antibacterial activity of saccharomicin A also depended upon bacterial inoculum density and the concentration of the drug used. Higher inoculum density required a lower cation concentration for neutralization, and higher drug concentrations needed higher cation concentrations for the reversal of antibacterial activity (data not shown). Fe^{2+} and Cu^{2+} had no effect (data not shown). These data suggest that the saccharomicins do not act as general chelators like EDTA, but it is more likely that they interact with outer membrane-bound cations of gram-negative bacteria, which contribute to the maintenance of stability of this outermost layer of the cell wall. By this mechanism, the high-molecular-weight saccharomicins may be expected to self-promote their uptake into these organisms in a manner similar to polymyxin B, aminoglycosides, and quinolones (3, 7, 8). The self-promoted uptake of these antibiotics involves an interaction with the binding sites of the divalent cations on the lipopolysaccharide, and the addition of calcium and magnesium ions counteracts the displacement of the cations by these antibiotics. Binding of the saccharomicins to such sites on the lipopolysaccharide has not been established, but the observed effect of divalent cations upon antibacterial activity may involve a similar mechanism. This hypothesis is further supported by the fact that an *E. coli imp* mutant which is deficient

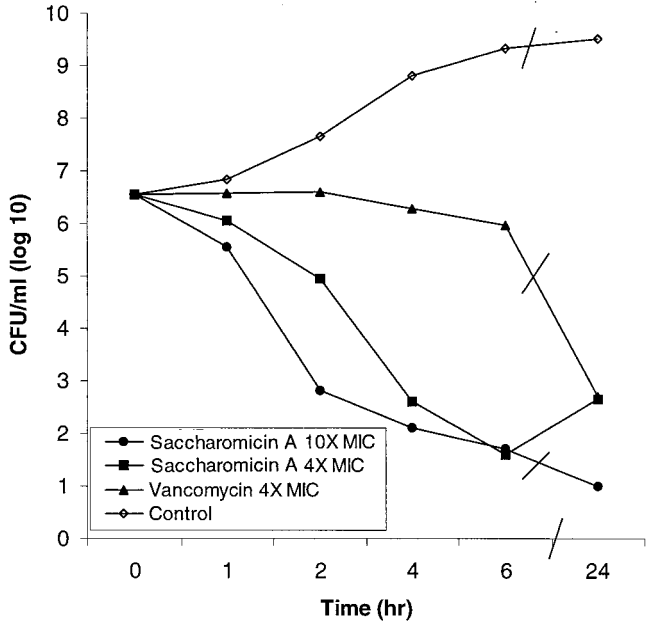

FIG. 2. Kill kinetics of *S. aureus* Smith.

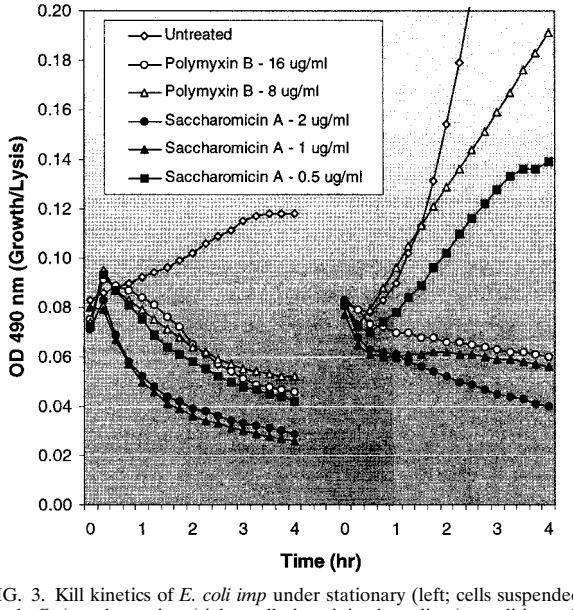

FIG. 3. Kill kinetics of *E. coli imp* under stationary (left; cells suspended in saline buffer) and growing (right; cells in minimal medium) conditions. OD, optical density.

TABLE 4. Relationship between MIC of saccharomicin A and cation concentration for *P. aeruginosa* ATCC 27853

Cation	Concn		MIC (μg/ml)	
	μg/ml	mM	Saccharomicin A	Polymyxin B
Ca^{2+}	1,000	25	>64	>16
	500	12.5	>64	8
	250	6.25	>64	4
	125	3.12	64	2
	62.5	1.56	16	1
	31.2	0.78	8	1
	15.6	0.39	4	1
	7.8	0.2	2	1
Mg^{2+}	1,000	41	>64	>16
	500	20.5	>64	>16
	250	10.2	64	8
	125	5.1	32	4
	62.5	2.5	16	2
	31.2	1.25	8	1
	15.6	0.62	4	1
	7.8	0.31	2	1
Ca^{2+} + Mg^{2+}	1,000 + 500		>64	>16
	500 + 250		>64	>16
	250 + 125		>64	8
	125 + 62.5		32	4
	62.5 + 31.2		16	2
	31.2 + 15.6		8	1
	15.6 + 7.8		4	1
	7.8 + 3.9		2	1

in this permeability barrier was two- to eightfold more sensitive to saccharomicins than the wild-type strains, and the addition of cations did not interfere with the susceptibility of this organism (Table 3).

Incorporation of radiolabeled precursors. Inhibition of DNA, RNA, and protein syntheses was determined by measuring the incorporation of [^{3}H]TdR, [^{3}H]UdR, and ^{3}H-labeled amino acids, respectively, into the TCA-precipitable material of log-phase cultures of *E. coli imp* and *B. subtilis*. The effects of drugs on the cellular uptake of radiolabeled precursors were determined by measuring radioactivity retained in saline-washed cells under the same experimental conditions. For each drug tested at one to two times the MIC_{3h}, uptake of the three radiolabeled precursors was unaffected relative to the specific inhibition of incorporation into TCA-precipitable material. Since both saccharomicins were equally active in MIC tests, saccharomicin A was used for mechanistic studies in bacteria. Within 5 min of saccharomicin A treatment at 4 μg/ml (twice the MIC_{3h}), all three macromolecular syntheses were shut down in *E. coli imp* (Table 5). At 2 μg/ml (MIC_{3h}), uptake and incorporation of amino acids appeared to be more affected than those of the other two precursors. During the same period, three control drugs, ciprofloxacin, rifampin, and chloramphenicol, predominantly inhibited DNA, RNA, and protein synthesis, respectively. Polymyxin B inhibited uptake and incorporation of all three precursors tested.

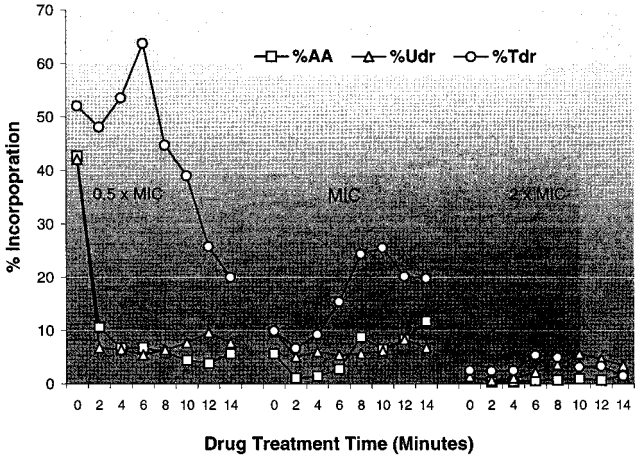

FIG. 4. Effects on macromolecular synthesis in an exponential-phase culture of *B. subtilis* treated with 0.03, 0.06 (MIC), and 0.12 μg of saccharomicin A/ml. Data are percentages of incorporation relative to untreated control for 2 to 14 min of antibiotic treatment and 5 min of pulse-labeling with [^{3}H]TdR, [^{3}H]UdR, and ^{3}H-labeled amino acids (AA).

Radiolabeling of an exponential-phase culture of *B. subtilis* pretreated with saccharomicin A for 0 to 14 min yielded similar results (Fig. 4). At 0.5 times the MIC_{3h}, incorporation of uridine and amino acids was shut down very rapidly (within 2 min), but thymidine incorporation declined slowly. At twice the MIC_{3h}, however, all three macromolecular processes were terminated almost instantly. The observed nonspecific inhibition of all three macromolecular processes (DNA, RNA, and protein synthesis) in saccharomicin A-treated bacterial cells suggested an instant disruption of membrane integrity in the bacteria.

Effects on prokaryotic and eukaryotic membranes. The membrane-damaging effects of the antibiotics on *E. coli imp* and human RBCs were studied by measuring the leakage of intracellular potassium and hemolysis, respectively. Saccha-

TABLE 5. Effects on uptake and incorporation of radiolabeled precursors for DNA, RNA, and protein in *E. coli imp*[a]

Compound	Concn (μg/ml)	% [^{3}H]TdR		% [^{3}H]UdR		% ^{3}H-labeled amino acids	
		Uptake	Incorp.	Uptake	Incorp.	Uptake	Incorp.
Saccharomicin A	8	2	1	2	0	12	8
	4	3	4	3	3	14	8
	2[b]	109	84	94	77	66	59
	1	96	104	97	100	100	93
Ciprofloxacin	0.25[b]	110	4	98	NT[c]	97	NT
Rifampin	0.25[b]	135	NT	100	2	91	26
Chloramphenicol	8[b]	110	NT	109	NT	99	20
Polymyxin B	8[b]	3	1	7	2	21	3

[a] Exponential-phase culture growing in minimal medium was pretreated with a drug for 5 min and then pulse-labeled for 5 min. Results are percentages of untreated control.
[b] MIC_{3h}.
[c] NT, not tested.

TABLE 6. Effects on prokaryotic and eukaryotic membranes

Drug	Concn (μg/ml)	K^+ leakage (%)[a]	RBC lysis (%)[b]
Saccharomicin A	2	37	0.00
	4	80	0.00
	8	94	0.00
	128	95	0.28
Polymyxin B	16	31	NT[c]
	32	43	NT
	64	54	0.84
Amphotericin B	2	0.00	12
	4	0.00	43
	8	0.00	81

[a] Percentage of total intracellular potassium that leaked from *E. coli imp* in 1 h.
[b] Percent lysis of human RBCs in 2 h.
[c] NT, not tested.

romicin A did not cause hemolysis of RBCs, but it led to extensive leakage of intracellular potassium from *E. coli* (Table 6). These data are consistent with the membrane-damaging effects of saccharomicin A in bacteria but not in eukaryotic cells.

In vivo efficacy and toxicity. The ability of saccharomicins to protect mice from lethal challenges by various pathogenic bacteria was compared to that of vancomycin (Table 7). The ED_{50}s for saccharomicin activity against gram-positive infections by subcutaneous administration were in the range of 0.06 to 2.6 mg/kg, which was comparable to the range for vancomycin. The 50% lethal dose (LD_{50}) for the saccharomicins varied from 2 to 360 mg/kg, depending upon the route of administration. Saccharomicins administered orally and subcutaneously protected the mice from a lethal challenge by gram-positive bacteria, but they were unable to protect mice from a lethal challenge of gram-negative bacteria at desirable dosages.

TABLE 7. In vivo activity (acute lethal infection in mice) and toxicity

Organism	Route	Saccharomicin AB complex		Vancomycin	
		ED_{50}	LD_{50}/ED_{50}[d]	ED_{50}	LD_{50}/ED_{50}[e]
S. aureus Smith	SOD[a]	17	21	230	ND[c]
S. aureus Smith	SSC[b]	0.06	260	1.00	>1,000
S. aureus Rose	SSC	2.60	5	5.40	>190
Streptococcus pyogenes C203	SSC	0.90	18	1.10	>930
S. pneumoniae SV1	SSC	0.85	18	0.54	>1,900
E. coli 331	SSC	>8	ND[c]	300	ND
K. pneumoniae AD	SSC	>8	ND	>256	ND

[a] SOD, single oral dose.
[b] SSC, single subcutaneous dose.
[c] ND, not determined.
[d] LD_{50}s of saccharomicin A were 360, 16, and 2 to 4 mg/kg by oral, subcutaneous, and intravenous routes, respectively.
[e] LD_{50}s of vancomycin were >1,024 mg/kg by oral and subcutaneous routes and 256 to 512 mg/kg by intravenous routes.

The ED_{50} of saccharomicins against *S. aureus* Smith by the subcutaneous route was 260 times lower than the LD_{50}. However, the ED_{50}-to-LD_{50} ratio for other gram-positive organisms ranged from 1:5 to 1:18, a narrow therapeutic window for this antibiotic compared to that of vancomycin.

The saccharomicins are novel, potent antibiotics with excellent in vivo activity against gram-positive bacteria. The in vitro antibacterial activities of the saccharomicins against gram-positive bacteria were unaffected by the presence of Ca^{2+} or Mg^{2+}, but activity against gram-negative bacteria was substantially reduced by these divalent cations. Based on mechanistic studies with gram-positive and gram-negative bacteria, the primary cellular target appeared to be the bacterial membrane. In view of the lower safety margins for the saccharomicin AB complex in mouse models, it does not warrant further development as a systemic antibiotic. However, the individual components A and B should be tested to understand their relative safety profiles in vivo. Since saccharomicins are highly water soluble and since cross-resistance with other known antibiotics does not seem to occur, they may be considered for topical applications.

ACKNOWLEDGMENTS

We thank Scott Luckman and Jeffrey Janso for providing the hemolytic data and Norman Canfield for assisting in the atomic absorption spectrophotometric determination of potassium ions.

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