A Family of Novel Macrocyclic Lactones, the Saccharocarcins Produced by *Saccharothrix aerocolonigenes* subsp. *antibiotica*

I. Taxonomy, Fermentation, Isolation and Biological Properties

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A nocardioform actinomycete, SCC 1886, isolated from a soil sample collected in Ohio was found to produce, in fermentation, six novel macrocyclic lactones, the saccharocarcins. The producing culture was identified as *Saccharothrix aerocolonigenes* subsp. *antibiotica* based on the formation of fragmenting substrate mycelia, aerial mycelia that coalesce to form aerial colonies, whole-cell hydrolysates that contain *meso*-diaminopimelic acid, galactose and rhamnose and physiological comparisons to type species of the genus. Peak production of the saccharocarcins occurred after 95 hours of fermentation in a starch rich medium. The compounds were isolated from the fermentation broth by solvent extraction and purified by HPLC. Isolated compounds were active against *Micrococcus luteus*, *Staphylococcus aureus* and *Chlamydia trachomatis*; none were cytotoxic at concentrations up to 1.0 μg/ml.

Actinomycetes continue to be a major source of fermentation derived samples for screening. In our screening program we focus on actinomycete genera other than *Streptomyces*, with particular emphasis on organisms with cell walls of type III and IV[1]. Organisms classified as *Saccharothrix*[2~5], nocardioform actinomycetes with a cell wall of type III, have been shown to be producers of novel secondary metabolites[6~9], with individual strains often producing numerous analogs in a single fermentation[10]. In this paper we describe a novel subspecies of *Saccharothrix aerocolonigenes* that produces, in fermentation, six related macrocyclic lactones, designated saccharocarcins. Fermentation parameters, the methods used to isolate the individual compounds and their biological activities are also presented.

Materials and Methods

The Microorganisms

Strain SCC 1886 was isolated from a soil sample collected from a grassland in Ohio which was air dried and plated on the following medium; molasses 1.0 ml, $NaNO_3$ 0.1 g, agar 15 g, distilled water 1 liter. Plates were incubated at 30°C, 50% humidity for 14 to 21 days. SCC 1886 appeared as a soft filamentous beige colony which was isolated to purity.

The culture was compared directly to *Saccharothrix aerocolonigenes* ATCC 23870[3], *S. aerocolonigenes* ATCC 39243[6], *S. waywayandensis* NRRL B-16159[5], and *S. texasensis* NRRL B-16134[5]. Source materials used for the described studies were prepared as described by HORAN and BRODSKY[11].

Microscopic and Macroscopic Observations

Washed cells were inoculated onto the surface of petri dishes containing either tap water agar, inorganic salts-starch agar (ISP 4), or Czapek-sucrose agar, incubated at 28°C for 28 days and examined weekly under a microscope (400X). Strain SCC1886 was cultivated on standard actinomycete media as described by SHIRLING and GOTTLIEB[12] and WAKSMAN[13] Each color designation assigned to the vegetative mycelial pigments consisted of a color name and a color number[14].

Biochemical and Physiological Tests

Acid production from carbohydrates and utilization of organic acids were tested using the method of GORDON *et al.*[15] and utilization of carbohydrates by the method of SHIRLING and GOTTLIEB[12]. The procedures described by GOODFELLOW[16] and GORDON *et al.*[15] for decomposition of organic compounds were followed except that glucose-yeast extract agar (glucose 10 g, yeast extract 10 g, agar 15 g; pH 7.2) was substituted for nutrient agar. Determination of growth temperature and ability to survive at 50°C for 8 hours were conducted according to the procedures of GORDON *et al.*[15] using ATCC Medium 172[17]. Growth in the presence of antibiotics was performed according to the procedure of HORAN and BRODSKY[11].

Chemotaxonomy

Whole-cell and cell wall preparations were analyzed for diaminopimelic acid[18,19], sugars[20,21], phospholipids[22], and mycolic acids[23].

Pulsed Field Gel Electrophoresis

Isolation of the DNA, restriction with Ase I and Dra I and subsequent gel analysis followed the procedures of BEYAZOVA *et al.*[24].

Fermentation

The strain was grown in a seed medium consisting of glucose 1%, trehalose 1%, enzymatically hydrolyzed casein 0.5%, soy flour 0.5%, yeast extract 0.5% and calcium carbonate 0.2%. A 48 hour culture in 250-ml Erlenmeyer flasks containing 70 ml of seed medium was inoculated (10%) into 500 ml of the same medium in 2-liter Erlenmeyer flasks. A 48 hour culture was then inoculated (10%) into 10 liters of production medium consisting of PD 650 dextrin 3%, molasses 1%, soluble starch 0.5%, pH 6.5 prior to sterilization. Fermentations were carried out at 30°C for 120 hours in a 14-liter New Brunswick Microgen fermenter, batched to 10 liters, aerated at 3.5 lpm and agitated at 350 rpm. Production was monitored by agar diffusion using *M. luteus* ATCC 9341 grown in Difco Nutrient Agar.

Isolation

The isolation of the saccharocarcins is outlined in Fig. 1. Separation was monitored by activity against *M. luteus* ATCC 9341 and by HPLC using a UV detector at 265 nm.

Determination of Antibacterial and Antichlamydial Activity

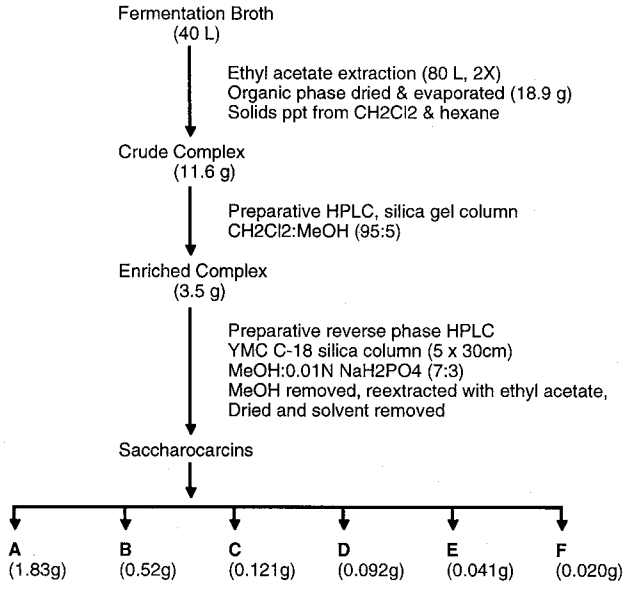
Paper discs (6.5 mm) were saturated with a solution of each saccharocarcin resulting in a concentration of 300 μg of compound per disc. Duplicate discs were tested for activity (zones of inhibition) against *Staphylococcus aureus*, *M. luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Activity against *Chlamydia trachomatis* was measured in McCoy cells using a fluorescein conjugated antichlamydia monoclonal antibody to measure inclusion bodies. Cytotoxicity was measured visually. (Activity against *Chlamydia* and cytotoxicity tests were performed by ViroMed Laboratories, Inc., 5500 Feltl Road, Minneapolis, MN 55343).

Results and Discussion

Isolation from Soil

SCC 1886 was isolated from soil under conditions shown to favor nocardioform actinomycetes and reduce the population of streptomycetes, including air drying of the soil and the use of inorganic nitrogen in the medium[25].

Fig. 1. Isolation and purification scheme for the saccharocarcins.



Growth Characteristics and Morphological Observations

Table 1 details the macroscopic appearance of SCC 1886 on various descriptive media. The substrate mycelia is plicate and varies from pale yellow to brownish orange to light brown. The culture tends to produce little, if any, visible aerial mycelia. If present, as on CZAPEK's sucrose agar, it is observed as scattered white patches. Soluble pigments vary from pale yellow to pinkish white to brownish orange. Colorless crystals are formed on Bennett agar, glucose-yeast extract agar, yeast extract-malt extract agar and inorganic salts-starch agar. SCC 1886 is a gram positive, filamentous actinomycete that forms a well developed, moderately branching substrate mycelia with hyphae approximately 0.4 to 0.8 μm in diameter. The substrate mycelia has a tendency to fragment into coccobacillary elements as the culture ages. The aerial mycelia, when present, is frequently sterile; however, occasional fragmentation of the hyphae into spores can be observed. The spore chains are straight to flexuous and vary in length from less than 10 spores to frequently more than 50 spores per chain. The spores are cylindrical and somewhat irregular in size (approximately 0.6 to 0.8 μm wide by 0.8 to 4.3 μm long). On inorganic salts-starch agar, when the aerial mycelia becomes sufficiently dense, characteristic clumps of interwoven aerial hyphae or 'aerial colonies' are sometimes observed. No motile elements were observed in either the substrate or aerial mycelia.

Table 1. Growth characteristics of strain SCC 1886 on various media.

Medium	Growth and color[14]	Aerial mycelia	Diffusible pigment
Bennett agar	+, Off-white to grayish yellow	Absent	Absent
Glycerol-asparagine agar (ISP 5)	+/−, Off-white	Absent	Absent
Glucose-yeast extract agar	+++, Greyish yellow (4B3) to amber (4B6)	Absent	Pale yellow brown
Yeast extract-malt extract agar (ISP 2)	+++, Grayish yellow (4B4) to brownish orange (5C6)	Absent	Absent
Oatmeal Agar (ISP 3)	+++, Yellowish white (3A2) to grayish yellow (4B4)	Absent	Pale yellow to yellow brown
Inorganic salts-starch agar (ISP 4)	+++, Yellowish white (4A2) to grayish yellow (4B4)	Absent	Pale yellow brown
Starch agar (Waksman #21)	+++, Pale yellow (4A3) to grayish orange (5B4)	Absent	Pinkish white (7A2) to grayish orange (6B4)
Tap water agar	+/−, Off-white	Absent	Absent
Peptone-yeast extract-iron agar (ISP 6)	+, Grayish yellow (4B4)	Absent	Pale yellow brown
Czapek-sucrose agar	+++, Light brown (6D6) to brownish orange (5C5)	Sparse, white	Brownish orange (6C6 to 6C8)
ATCC Medium 172	+++, Brownish orange (5C5 to 5C6)	Absent	Yellow brown to brownish orange (6C7)

+/−: Poor; +: fair; ++: good; +++: excellent.

Table 2. Physiological and biochemical characteristics of SCC 1886.

Test	Result	Test	Result
Decomposition of:		Growth at:	
Adenine	−	10°C	+ to ++
Amylopectin	+	28°C	+++
Casein	+	37°C	+++
Elastin	+	40°C	++
Guanine	−	42°C	+/−
Gelatin	+	45°C	−
Hypoxanthine	+	50°C	−
Starch	+	Survival at:	
L-Tyrosine	+	50°C for 8 hours	−
Xanthine	−	Growth on NaCl (%):	
Xylan	+	3.0	+++
Hydrolysis of:		4.0	++
Urea	+	5.0	++
Arbutin	+	6.0	+/− to +
Allantoin	+	7.0	−
Esculin	+	Growth in the presence	
Hippurate	+	of 50 μg/ml of:	
Hydantoin	−	Gentamicin	−
Production of:		Sisomicin	−
Nitrate Reductase	+	Neomycin	+
Catalase	+	Kanamycin	v
Phosphatase	+	Streptomycin	−
Sulfatase	+	Paromomycin	−
Melanin	−	Nalidixic acid	+
Utilization of:		Novobiocin	−
Acetate	+	Rifamycin	−
Benzoate	−	Everninomicin	−
Citrate	+	Rosaramicin	−
Oleate	−	Erythromycin	+
Oxalate	+	Tetracycline	−
Propionate	+	Cycloserine	+
Succinate	+	Penicillin G	+
Tartrate	−	Cephalothin	+
Lysozyme resistance:	+	Lincomycin	+
		Clindamycin	−
		10 μg/ml:	
		Chloramphenicol	+
		Bacitracin	+

−: Negative; +: positive; +/−: weak; v: variable.

Physiological and Biochemical Characteristics

The physiological and biochemical characteristics of SCC 1886 are presented in Table 2. Acid production from carbohydrates and carbohydrate utilization are shown in Table 3. The culture is very active metabolically utilizing a wide variety of carbon sources and hydrolyzing the majority of compounds tested.

Chemotaxonomic Analysis

Purified cell wall preparations of SCC 1886 contain *meso*-diaminopimelic acid, glutamic acid, alanine, galactosamine, glucosamine, muramic acid, galactose, ribose and rhamnose (cell wall Type III). Whole-cell hydrolysates contain galactose, glucose, mannose, ribose and rhamnose. Phospholipids present are phosphatidylinositol mannosides, phosphatidylinositol, diphosphatidylglycerol and phosphatidylethanolamine acylated with hydroxy fatty acids and normal fatty acids (Type PII). No mycolates are present.

Identity of Strain SCC1886

The formation of fragmenting substrate and aerial mycelia, cell walls containing *meso*-diaminopimelic acid, the presence of rhamnose and galactose as whole-cell diagnostic sugars and phosphatidylethanolamine as the diagnostic phospholipid places SCC 1886 into the genus *Saccharothrix*[2]. Strains belonging to the genus *Saccharothrix* are morphologically similar forming plicate, yellow to yellow brown substrate mycelia and sparse white to yellowish white aerial mycelia that eventually fragment into coccobacillary forms. Of the described species of *Saccharothrix*, SCC 1886 was easily differentiated from *S. mutabilis*[4,26] and *S. espanaensis*[4] on the basis of phospholipid patterns; both species have a type PIV pattern. *S. australiensis*[2] differs from SCC 1886 in failing to hydrolyze urea, hippurate or hypoxanthine; producing acid from erythritol but not inositol, lactose, melibiose, raffinose, rhamnose, sucrose or xylose; failing to utilize citrate or oxalate; and, producing brown melanoid pigments.

Based on literature data SCC 1886 was found to closely resemble *S. texasensis*[5], *S. waywayandensis*[5] and *S. aerocolonigenes*[3,27] and was, therefore, compared

Table 3. Acid production from and utilization of carbohydrates by strain SCC 1886.

Test	Results	Test	Results
Acid production from:		Utilization of:	
Adonitol	+	Adonitol	−
D-Arabinose	+	D-Arabinose	+
L-Arabinose	+	L-Arabinose	+++[a]
D-Cellobiose	+	D-Cellobiose	+++
Dextrin	+	Dextrin	+++
Dulcitol	−	Dulcitol	−
i-Erythritol	−	*i*-Erythritol	−
D-Fructose	+	D-Fructose	+++
L-Fucose	+	L-Fucose	+++
D-Galactose	+	D-Galactose	+++
Glucose	+	Glucose	+++
Glycerol	+	Glycerol	++
i-Inositol	+	*i*-Inositol	+++
Inulin	−	Inulin	−
Lactose	+	Lactose	+++
Maltose	+	Maltose	+++
D-Mannitol	+	D-Mannitol	+++
D-Mannose	+	D-Mannose	+++
D-Melezitose	−	D-Melezitose	−
D-Melibiose	+	D-Melibiose	+++
α-Methyl-D-glucoside	−	α-Methyl-D-glucoside	−
D-Raffinose	+	D-Raffinose	+/−
L-Rhamnose	+	L-Rhamnose	+ to ++
D-Ribose	+	D-Ribose	+++
Salicin	−	Salicin	+
D-Sorbitol	−	D-Sorbitol	−
L-Sorbose	−	L-Sorbose	−
Sucrose	+	Sucrose	+++
D-Trehalose	+	D-Trehalose	+++
D-Xylose	+	D-Xylose	+++

[a] All +++ utilization, yellow diffusible pigment was formed.
Acid production: +: positive; −: negative.
Utilization: −: no utilization; +/−: poor; +: fair; ++: good; +++: excellent.

directly with these cultures. Differentiating characteristics are presented in Table 4. SCC 1886 is most closely related to *S. aerocolonigenes* ATCC 23870. Both strains form 'aerial colonies', a key characteristic of organisms identified as *S. aerocolonogenes*, and differ physiologically in very few characteristics including acid production from adonitol and raffinose, the ability to hydrolyze arbutin, growth in the presence of 10 μg/ml novobiocin and the ability to produce macrocyclic lactone antibiotics.

The Ase I (AT'TAAT) and Dra I (TTT'AAA) DNA restriction patterns of SCC 1886 were resolved by pulsed-field gel electrophoresis and compared to the DNA patterns obtained with *S. aerocolonigenes* ATCC 23870 and *S. aerocolonigenes* ATCC 39243, an antibiotic producing strain[6]. The number and distribution of the restriction bands with both rarely cutting enzymes indicates that these three strains are not clones of each other[28] (Fig. 2).

We consider SCC 1886 to be a new subspecies of *S. aerocolonigenes* designated *S. aerocolonigenes* subsp. *antibiotica* subsp. nov. in reference to the novel antibiotics produced by this strain. A subculture of SCC 1886 has been depositied in the American Type Culture Collection (ATCC), Rockville, MD, U.S.A., where it has been assigned the number ATCC 55003.

Table 4. Characteristics differentiating SCC 1886 from *S. aerocolonigenes* ATCC 23870, *S. waywayandensis* NRRL B-16159 and *S. texasensis* NRRL B-16134.

Test	SCC 1886	ATCC 23870	NRRL B16159	NRRL B16134
Acid production from:				
Adonitol	+	−	+	−
Raffinose	+	−	+	−
Ribose	+	+	+	−
Utilization of:				
Citrate	+	+	+	−
Malonate	+	+	+	−
Oxalate	+	+	+	−
Hydrolysis of:				
Allantoin	+	v	+	−
Arbutin	+	−	−	+
Hippurate	+	+	−	w
Growth at:				
10°C	+	−	+	+
37°C	+	+	−	+
40°C	+	+	−	+
Growth in the presence of:				
50 μg/ml				
Novobiocin	−	−	−	+
Cephalothin	+	+	+	−
Penicillin G	+	+	+	−
10 μg/ml				
Erythromycin	+	+	+	−
Kanamycin	+	+	+	−
Neomycin	+	+	+	−
Novobiocin	+	−	−	+
Tetracycline	+	+	−	+
Nitrate reductase:	+	+	−	+

−: Negative; +: positive; w: weak; v: variable.

Fermentation

The fermentation profile is shown in Fig. 3. Peak saccharocarcin production occurred after 95 hours of fermentation. The pH remained around 7.5 during the course of the fermentation and therefore did not require adjusting. Cell growth, measured by packed cell volume (PCV), reached a maximum at approximately 18 hours and coincided with glucose depletion.

Fig. 2. PFGE of Ase I and Dra I restriction digests of *S. aerocolonigenes* strains.

Lanes 1, 2 and 3, are Ase I digests: Lanes 4, 5 and 6 are Dra I digests. Lanes 1 and 4, ATCC 23870: Lanes 2 and 5, SCC 1886: Lanes 3 and 6, ATCC 39243: Lane 7, lambda concatamer.

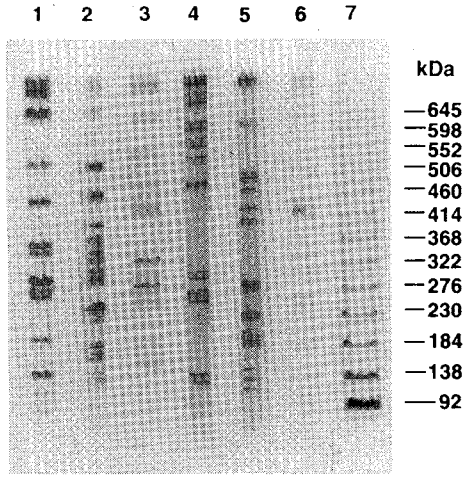


Fig. 3. Fermentation profile of *S. aerocolonigenes* subsp. *antibiotica* SCC 1886.

● Activity, ▼ glucose %, ■ PCV, ◆ pH.

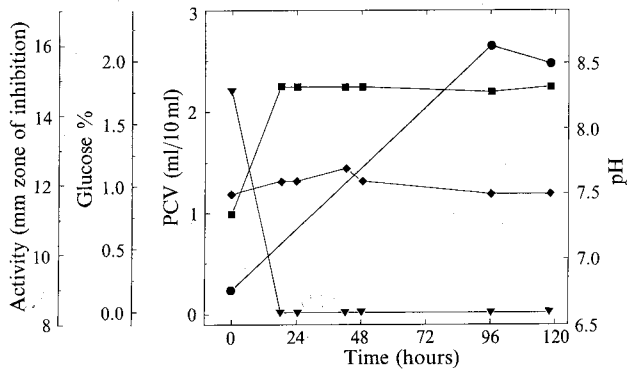


Table 5. Antimicrobial activity of the saccharocarcins.

Test organisms	Zone size (mm) at 300 μg						Kijanimicin (300 μg)	Gentamicin (10 μg)
	A	B	C	D	E	F		
Micrococcus luteus ATCC 9341	14	15	15	8	7	7	14	17
Staphylococcus aureus ATCC 209P	8	8	9	8	8	8	9	16
Escherichia coli ATCC 10536	7	7	7	7	7	7	7	—
Pseudomonas aeruginosa ATCC 25619	7	7	7	7	7	7	7	15
Candida albicans Wisconsin	7	7	7	7	7	7	7	7
	% Inhibition							
Chlamydia trachomatis serotype H (0.5 μg/ml)[a]	88	100	14	26	0	0	NT	NT
Cytotoxicity vrs McCoy cells (1.0 μg/ml)[b]	0	0	0	0	0	0	NT	NT

[a] Lowest concentration exhibiting activity.
[b] Highest concentration tested.
NT = Not tested.

Isolation

From 80 liters of fermentation, six compounds, saccharocarcins A through F, ranging in weight from 1.83 g to 0.20 g, were isolated and purified. Based on physical and spectroscopic data (presented in the accompanying paper) the compounds belong to the actinomycete produced macrocyclic tetronic acid class of secondary metabolites which includes kijanimicin[29], antlermicins[30,31], tetrocarcins[32], MM46115[33], chlorothricin[34] and PA-46101[35]. This is the first report of macrocyclic tetronic acids produced by a species of *Saccharothrix*.

Biological Activity

The antimicrobial activity of the saccharocarcins are presented in Table 5. The compounds exhibited Gram-positive activity similar to the activity exhibited by kijanimicin[29]. In addition, saccharocarcins A and B were active against *C. trachomatis* serotype H at concentrations as low as 0.5 μg/ml. All of the components were non-toxic against McCoy cells at the highest concentration tested, 1.0 μg/ml.

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