DISTRIBUTION OF β-LACTAM AND β-LACTONE PRODUCING BACTERIA IN NATURE

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Over one million bacteria were isolated from a large variety of soil, plant and water samples collected from different environments and examined in an extremely sensitive and highly specific screen for β-lactam production.

A group of seven related monocyclic β-lactams (monobactams) were isolated from strains representing four genera–*Agrobacterium*, *Chromobacterium*, *Gluconobacter* and *Pseudomonas*. Monobactam-producing strains of *Agrobacterium* and *Pseudomonas* were isolated only rarely. Producing strains of *Chromobacterium* were isolated from a relatively limited number of habitats while the *Gluconobacter* strains appeared to be widespread in nature. In addition, three closely related β-lactone-containing molecules were isolated from strains representing three genera–*Arthrobacter*, *Bacillus* and *Pseudomonas*. The *Bacillus* and *Pseudomonas* strains were isolated infrequently but from a variety of samples. The producing strain of *Arthrobacter* was isolated only once.

Screening of microorganisms for the production of antibiotics has provided the cornerstone of antibiotic research programs for the past thirty years. The great majority of such studies have been carried out with fungi and the actinomycetes which are capable of producing natural products with widely divergent chemical structures.

Until 1970, only two classes of naturally occurring β-lactam antibiotics, the penicillins and cephalosporins, were known. However, with the advent of new screening and isolation techniques a variety of β-lactam-containing molecules were identified, as evidenced by the discovery of the cephamycins[1], clavulanic acid[2], nocardicins[3], and the carbapenems[4~7]. Moreover, all the novel β-lactam antibiotics identified over the last ten years are products of the actinomycetes.

In an attempt to discover novel β-lactam-containing molecules from nature, we developed a highly sensitive screen, capable of handling large numbers of organisms and highly specific for β-lactam-containing molecules. Employing this technology we screened large numbers of fungi and actinomycetes only to find many of the known β-lactams. It was at this stage, we turned our attention to the bacteria. This paper reports on the isolation from nature of β-lactam- and β-lactone-producing bacteria[8]. With the exception of tabtoxin produced by species of *Pseudomonas*[9], bacteria have only recently been reported to produce β-lactam-containing molecules[10,11].

Methods

Isolation of Bacteria

Soil and plant material (Table 1) were collected in sterile plastic bags (Whirl-pak, American Scientific Products) and brought to the laboratory for immediate examination or stored at 5°C and examined within 24~48 hours. Water samples (Table 1), collected in sterile plastic bottles, were treated similarly.

Table 1. Collection areas for soil, plant and water samples.

Collection area	Types of samples
Agricultural crop lands	Soils & plants
Agricultural pasture lands	Soils, plants, mushrooms, water from ponds & streams
Gardens	Soils, plants, mushrooms & composts
Forests	Soils, plants, mushrooms, leaf litter & tree bark
Mountains	Soils, plants & waters
Pine barren	Soils, plants & waters
Desert	Soils
Swamps	Muds & plants
Salt marshes	Soils, muds, waters & plants
Beaches	Sand & waters
Garbage dumps	Soils, plants & decaying matter
Sewage plants	Waters
Industrial wastes	Waters
Rivers	Waters, muds & plants
Lakes	Waters, muds & plants
Canals	Waters, muds & plants

Suspensions of bacteria present in soil and plant samples were prepared by adding five grams of soil or one gram of cut up plant material to 99 ml of diluent, *e.g.* phosphate-buffered water or saline. The samples were mixed by shaking on a rotary shaker for 20 minutes at 150 rpm. Bacteria present in water samples were concentrated on a Millipore filter (0.45 μ) and washed off the filter pad with distilled water. Serial dilutions were prepared of all the processed samples and 0.1-ml aliquots of three dilutions were surface-plated onto at least four isolation agar plates. A standard dehydrated culture medium, *e.g.* Brilliant green bile, MACCONKEY, ENDO, Eosin methylene blue (BBL), selective for Gram-negative bacteria was always included as one of the four isolation media. The composition of the other media was dependent on the type of samples being examined; pH and salinity were often adjusted to simulate the environmental conditions from which the samples were collected. In various media, soil and plant extracts were substituted for water. All the isolation media contained actidione (50 μg/ml) to retard fungal growth. Plates were incubated at room temperature for 2~3 days. Water samples were plated onto six isolation media and incubated overnight at room temperature and then at 20°C for an additional four days.

In addition to direct examinations, many soil and plant samples were enriched by the addition of colloidal chitin, cellulose, pectin or various amino acids to enhance the numbers of specific types of bacteria. After a 7~10-day incubation period at room temperature, dilutions were prepared and the samples were plated onto the isolation plates as previously described.

Antibiotic Characterization

Preliminary characterization of antibiotics was based on thin-layer chromatography (F1440 cellulose Schleicher and Schuell, Keine, NJ, and polysilicic acid gel impregnated glass fiber sheets, Gelman Instrument Co., Ann Arbor, MI) employing acetonitrile - water (7: 3, 4: 1 or 5: 1) and high voltage electrophoresis at 2,000 volts for 30 minutes at pH 2, 7 and 9. Antibiotics were visualized by bioassay using *B. licheniformis* SC 9262. In addition, all compounds were tested for stability to a range of β-lactamases as described by SYKES, *et al.*[11].

Characterization of Producing Organisms

All producing cultures were examined initially for their Gram reaction and by phase contrast microscopy for morphology and motility. Additional standard taxonomic characterization was carried out based on BERGEY's Manual of Determinative Bacteriology (8th edition). Antibiograms were performed against a battery of 24 antibiotics using a disc diffusion assay (Table 2).

Screening

The screen developed to identify novel β-lactam-containing molecules employed *Bacillus licheniformis* SC 9262 as the assay organism[11]. Over a million bacteria were tested leading to the discovery of seven related β-lactam and three related β-lactone-containing molecules.

Results

β-Lactam-Producing Bacteria

Monobactams (monocyclic derivatives of 3-amino-2-oxoazetidine-1-sulfonic acid)[11] have been

Table 2. Antibiograms of bacteria producing β-lactams and β-lactones.

Compound	Sensi-Disc (μg/disc)	*Chromobacterium violaceum*	*Agrobacterium radiobacter*	*Gluconobacter* sp.	*Pseudomonas* sp.	*Bacillus* sp.	*Arthrobacter* sp.
Amikacin	10	18.4[a]	20.3	14.2	18.9	26.5	19.7
Ampicillin	10	—[b]	—	—	—	13.6	16.2
Bacitracin	10	9.5*	—	—	—	18.7	34.4
Carbenicillin	50	—	22.2	—	—	12.4	13.8
Cephalothin	30	—	—	—	—	12.7	27.6
Chloramphenicol	30	17.2	9.3*	—	—	30.6	32.9
Colistin	10	—	15.4	—	13.5	—	14.8
Erythromycin	15	21.7	12.9	—	—	31.2	35.2
Gentamicin	10	16.2	19.6	13.2	17.0	27.6	19.2
Kanamycin	30	23.7	20.5	18.8	23.5	23.8	21.4
Nafcillin	1	—	—	—	—	—	10.8
Nalidixic acid	30	57.4	—	21.9	15.2	26.9	16.8
Neomycin	30	17.6	19.4	13.2	16.9	24.8	19.6
Nitrofurantoin	300	36.0	11.7*	—	—	21.9	—
Novobiocin	30	43.6	27.7	18.5	—	25.9	36.5
Penicillin G	10 units	—	—	—	—	10.2	11.9
Polymyxin B	300 units	—	17.9	—	15.5	10.9	17.6
Lincomycin	2	—	—	—	—	13.3	—
Rifampin	5	47.5	26.4	15.7	16.4	21.0	41.8
Streptomycin	10	23.0	16.9	9.5*	18.0	23.9	21.8
Sulfachloropyrazine	250	15.4*	—	—	—	28.3	28.9
Tetracycline	30	39.0	39.0	23.8	29.4	25.2	36.6
Tobramycin	10	18.2	17.1	12.2	17.0	19.9	14.6
Vancomycin	30	19.6*	14.6	—	—	19.9	31.0

[a] Diameter (mm) of zone of inhibition. [b] No zone of inhibition.
* Indicates variation among strains tested.

detected from strains of *Chromobacterium violaceum, Gluconobacter* sp., *Pseudomonas* sp., and *Agrobacterium radiobacter*. The simplest molecule SQ 26,180 (Fig. 1) is produced by strains of *Chromobacterium violaceum*[12]. Although these organisms were isolated frequently from nature, monobactam-producing strains were isolated from a relatively limited number of habitats[13] (Table 3). In addition, a large number of pigmentless *C. violaceum* strains producing SQ 26,180 were isolated from samples collected in the New Jersey pine barrens. These isolates possessed the same key biochemical characters (Table 4) and antibiotic susceptibility pattern (Table 5) as *C. violaceum* SC 11,378 and represent natural variants of this species. *C. violaceum* SC 11,378, producing SQ 26,180, has been deposited with the American Type Culture Collection (ATCC) under the accession number of ATCC 31,532. This organism was isolated from a soil sample collected in a cedar forest in the New Jersey pine barrens.

The most commonly encountered monobactam was SQ 26,445 (Fig. 2). This compound was produced by strains of bacteria identified as *Gluconobacter* sp.[11] and strains of a fluorescent *Pseudomonas* sp. SQ 26,445[14] is identical with sulfazecin, a monocyclic β-lactam produced by strains of *Pseudomonas acidophila*[10,15]. Samples of *Gluconobacter* sp. producing SQ 26,445 were deposited with the ATCC under the accession number of ATCC 31,581. *Gluconobacter* sp. ATCC 31,581 was initially isolated from a sample of ground moss collected at New Hope, Pennsylvania. Additional SQ 26,445-producing strains

Fig. 1. Structure of SQ 26,180.

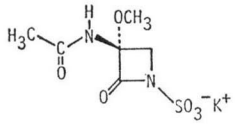


Fig. 2. Structure of SQ 26,445.

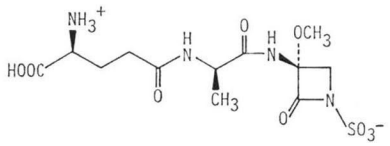
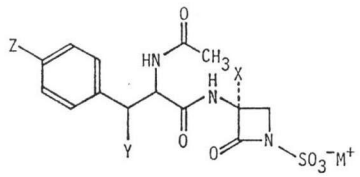


Fig. 3. Structures of monobactams isolated from *Agrobacterium radiobacter* fermentations.



SQ No.	X	Y	Z	M
26,823	OCH_3	H	H	Na
26,875	OCH_3	H	OH	K
26,700	H	H	OH	K
26,970	OCH_3	OH	$OSO_3^-Na^+$	Na
26,812	OCH_3	$OSO_3^-Na^+$	$OSO_3^-Na^+$	Na

Table 3. Collection sites of *Chromobacterium violaceum* strains producing SQ 26,180.

Sample	Collection site
Cedar forest soil	New Jersey pine barrens, New Jersey
Swamp water	New Jersey pine barrens, New Jersey
Lake water	New Jersey pine barrens, New Jersey
Creek water	New Jersey pine barrens, New Jersey
Water lily (leaves and stem)	New Jersey pine barrens, New Jersey
Cranberry bog	New Jersey pine barrens, New Jersey
Blue Spruce needle compost pile	Waterbury, Connecticut
Small unidentified plant	Barnegat Bay, New Jersey
Sulfur-iron bog sediment	Great Swamp Refuge, New Jersey
Decaying root mass of swamp plant	Mercer County Park, New Jersey
Forest soil	West Windsor, New Jersey
Oak-leaf litter	Hacklebarney State Park, New Jersey

Table 4. Key taxonomic characters demonstrating similarity of pigmentless bacteria with *C. violaceum* SC 11,378.

Key characters	SQ 26,180 producing strains				
	Pigmentless				Pigmented
	F-3600	F-3615	F-3616	F-3617	*C. violaceum* SC 11,378
Gram reaction	−	−	−	−	−
Motility	+	+	+	+	+
Oxid./Ferm.	F	F	F	F	F
Casein hydrolysis	+	+	+	+	+
Acid from trehalose	+	+	+	+	+
Aesculin hydrolysis	−	−	−	−	−
Production of HCN	+	+	+	+	+
Arginine decarboxylase	+	+	+	+	+
Violacein production	−	−	−	−	+

of *Gluconobacter* sp. were isolated from a variety of environments as shown in Table 6. The fluorescent *Pseudomonas* that produces SQ 26,445 was isolated on only two occasions from water samples collected at the same site in Regensburg, Germany. Ten strains of *Gluconobacter* and *Acetobacter* (Table 7) obtained from the ATCC produced SQ 26,445.

A mixture of monobactams was found to be produced by bacterial isolates identified as strains of

Table 5. Susceptibility of *C. violaceum* SC 11,378 (ATCC 31,532) and four pigmentless strains of *C. violaceum* to 24 antibiotics.

Compound	Sensi-disc (μg/disc)	*C. violaceum* SC 11,378	F-3600	F-3615	F-3616	F-3617
Amikacin	10	14.7[a]	15.0	13.7	14.5	14.5
Ampicillin	10	—[b]	—	—	—	—
Bacitracin	10	—	—	—	—	—
Carbenicillin	50	—	—	—	—	—
Cephalothin	30	—	—	—	—	—
Chloramphenicol	30	16.5	17.8	15.7	13.3	15.0
Colistin	10	—	—	—	—	—
Erythromycin	15	21.1	26.4	12.9	19.4	23.9
Gentamicin	10	15.6	15.4	15.0	15.5	14.7
Kanamycin	30	21.5	21.5	21.5	20.2	20.9
Nafcillin	1	—	—	—	—	—
Nalidixic Acid	30	43.4	45.5	40.6	43.8	45.3
Neomycin	30	15.5	15.1	9.9	14.7	14.7
Nitrofurantoin	300	29.1	28.7	22.1	29.0	28.7
Novobiocin	30	37.3	41.0	39.6	39.7	37.6
Penicillin G	10 units	—	—	—	—	—
Polymyxin B	300 units	—	—	—	—	—
Lincomycin	1	—	—	—	—	—
Rifampin	5	27.2	28.7	32.0	27.8	30.9
Streptomycin	10	18.9	17.7	17.2	18.0	19.0
Sulfachloropyrazine	250	18.7	24.0	21.4	17.8	17.9
Tetracycline	30	32.0	34.6	34.0	34.3	34.3
Tobramycin	10	15.4	14.5	14.5	14.3	14.8
Vancomycin	30	9.5	10.7	11.6	12.4	10.0

[a] Diameter (mm) of zones of inhibition.
[b] No zone of inhibition.

Table 6. Types and numbers of samples containing SQ 26,445-producing *Gluconobacter* and *Pseudomonas* strains.

Sample	No. Locations
Ground mosses (Pennsylvania and New Jersey):	4
Fungi (New Jersey): *Polyporus* sp., *Amanita* sp. and unidentified species of bird's-nest fungi, white bracket fungi and a brown gilled fungus	11
Soils (New Jersey, California, Iowa and Germany):	13
Plants (New Jersey):	4
Decaying plants (Pennsylvania, New Jersey, New York, Maine and Germany):	13
Water (Germany):	1

Table 7. SQ 26,445-producing bacteria obtained from the American Type Culture Collection (ATCC).

Culture	ATCC No.
Acetobacter aceti subsp. *aceti*	15973
Acetobacter aceti subsp. *liquefaciens*	23751
Acetobacter pasteurianus subsp. *pasteurianus*	6033
Acetobacter peroxydans	12874
Acetobacter sp.	21760
Gluconobacter oxydans subsp. *oxydans*	15178 and 19357
Gluconobacter oxydans subsp. *suboxydans*	19441 and 23773
Gluconobacter oxydans subsp. *industrius*	11894

Agrobacterium radiobacter[11,16]. The structures of five of these compounds are shown in Fig. 3[17]. SQ 26,700 was the first non-methoxylated monobactam derivative isolated in quantity. Samples of *A. radiobacter* producing monobactams were deposited at the ATCC under the accession number of ATCC 31,700. Monobactam-producing *A. radiobacter* strains were isolated on only three occasions, twice from plant samples collected in Germany and once from mud and leaf litter collected in New Jersey.

β-Lactone-Producing Bacteria

During our search for β-lactam-producing bacteria we isolated a number of β-lactone containing molecules[8,18]. These compounds contain a monocyclic ring system in which oxygen replaces nitrogen of the β-lactam.

The first β-lactone encountered, SQ 26,517 (Fig. 4), contains the same acyl side chain as the monobactam, SQ 26,180, but has a β-methyl group at position-4. The bacterium producing SQ 26,517 is an aerobic, Gram-positive, spore-forming rod and was therefore assigned to the genus *Bacillus*. Strains of SQ 26,517-producing bacilli were isolated relatively infrequently but from samples collected worldwide (Table 8). An antimicrobial evaluation of racemic SQ 26,517, synthesized from DL-allothreonine,

Fig. 4. Structure of SQ 26,517.

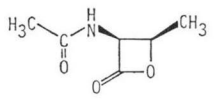


Fig. 5. Structure of SQ 27,012.

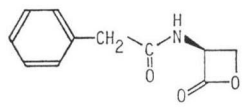


Fig. 6. Basic structure of EM5357 and EM5395.

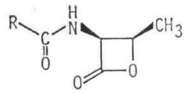


Table 8. Types and number of samples containing SQ 26,517-producing *Bacillus* strains.

Sample	No. Locations
Soils (Hawaii, Iowa, Louisiana, New Jersey, Argentina, Brazil, Chile and Germany):	10
Moss (Germany):	1
Sand (Germany):	1
Plant debris (Pennsylvania):	1
Mud (New Jersey):	1
Water (Pennsylvania):	1

Table 9. Antibacterial activity of racemic SQ 26,517.

Organism			MIC (μg/ml)
Staph. aureus	SC	1276	>100
Staph. aureus	SC	2399	>100
Staph. aureus	SC	2400	>100
Staph. aureus	SC	10165	>100
Strep. faecalis	SC	9011	>100
Strep. agalactiae	SC	9287	50
Micro. luteus	SC	2495	100
E. coli	SC	8294	>100
E. coli	SC	10857	>100
E. coli	SC	10896	>100
E. coli	SC	10909	>100
K. pneumoniae	SC	10440	>100
K. pneumoniae	SC	9527	>100
Prot. mirabilis	SC	3855	>100
Prot. rettgeri	SC	8479	>100
Prot. vulgaris	SC	9416	100
Sal. typhosa	SC	1195	>100
Shig. sonnei	SC	8449	>100
Ent. cloacae	SC	8236	>100
Ent. aerogenes	SC	10078	>100
Citro. freundii	SC	9518	>100
Ser. marcescens	SC	9783	>100
Ps. aeruginosa	SC	9545	>100
Ps. aeruginosa	SC	8329	>100
Acineto. calcoaceticus	SC	8333	>100

Minimum inhibitory concentrations were determined by a two fold agar dilution method on DST agar (Oxoid). Final inoculum level was 10^4 colony-forming units.

showed weak activity (Table 9). SQ 27,012, a synthetic analog of SQ 26,517 (Fig. 5), produced no zone of inhibition when tested against a limited number of microorganisms. Interaction of SQ 27,012 with β-lactamases is shown in Table 10. With the exception of the K-1 enzyme from *Klebsiella pneumoniae*, the compound was stable to β-lactamase attack.

β-Lactones EM5395 and EM5357 shown in Fig. 6 were produced by strains of bacteria identified as *Pseudomonas* sp. and a soil coryneform, respectively. The bacterium producing EM5395 is a Gram-negative polar flagellated rod. Growth did not occur at pH 4.5 nor at temperatures of 41°C. The culture was cytochrome-oxidase and arginine-dihydrolase positive. Fluorescent pigment was produced on KING's medium B[19]. On the basis of these characteristics, the organism was assigned to the genus *Pseudomonas*. EM5395-producing strains of *Pseudomonas* were isolated most frequently from a variety of mushrooms (Table 11). The bacterium producing EM5357 is a Gram-positive, pleomorphic non-motile rod. Based on morphology and analysis of cell wall hydrolysates, the culture was assigned to the soil coryneform group and tentatively placed in the genus *Arthrobacter*. The culture was isolated only once from a sample of decaying leaf litter collected at a site in New Jersey.

Table 10. Action of β-lactamases on SQ 27,012.*

Compound	Relative rate of hydrolysis with β-lactamase type		
	TEM-2	K-1	P-99
Cephaloridine	100	100	100
SQ 27,012	<0.01	0.6	0.04

* Studies were performed using spectrophotometric assays at 25°C in 0.1 M phosphate buffer, pH 7.0.

Table 11. Collection sites of *Pseudomonas* strains producing the β-lactone EM5395.

Sample	Site
Hygrophorus cantharellus mushroom	Washington Crossing State Park, New Jersey
Yellow *Hygrophorus* sp.	Washington Crossing State Park, New Jersey
Tan-grey, small, umbonated mushroom on dead tree stump	Washington Crossing State Park, New Jersey
Decaying *Armillariella* sp. mushroom	Princeton, New Jersey
Amanita chlorinosma mushroom	Princeton, New Jersey
Tan gilled mushroom from dead tree	Princeton, New Jersey
Decaying unidentified mushroom	Princeton, New Jersey
Unidentified mushroom on rotting log	Hacklebarney State Park, New Jersey
Leaf litter and soil	Hacklebarney State Park, New Jersey
Water and leaves from small spring	Jamesburg, New Jersey
Algae and beach sand	Bahamas

Discussion

The ability to produce monocyclic β-lactams and β-lactones is possessed by strains of bacteria representing a number of genera. Although some of the strains appeared in a limited number of samples, others were isolated from a wide variety of natural environments.

The discovery of these compounds has opened up a new era of antibiotic research. One exciting aspect of this research has been the total chemical synthesis of these compounds. Furthermore, as with the penicillins and cephalosporins, chemical modification of the monobactams has led to compounds with superior properties over the naturally occurring molecules[20]. One of these compounds, SQ 26,776 (Azthreonam) a highly active β-lactamase-stable compound has been developed for clinical evaluation[21].

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