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Suprageneric classification of peptidoglycan group B actinomycetes by nucleotide sequencing of 5S ribosomal RNA

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Abstract

5S ribosomal RNA sequences were determined for thirteen actinomycetes mainly representatives with the rare group B type peptidoglycan. The primary and secondary structure of the resultant sequences were of the type characteristic of Gram-positive bacteria with DNA rich in guanine plus cytosine. The sequencing and associated chemotaxonomic data provide compelling grounds for classifying actinomycetes with a group B type peptidoglycan in a single family. The family *Microbacteriaceae* fam. nov. is proposed to accommodate actinomycetes classified in the genera *Agromyces, Aureobacterium, Clavibacter, Curtobacterium* and *Microbacterium.*

Introduction

The determination of peptidoglycan types provide valuable data for the classification of bacteria and for the detection of heterogeneous taxa and misclassified strains (Schleifer & Kandler, 1972; Suzuki et al. 1993). Studies on the chemical structure of the peptidoglycan of Gram-positive bacteria, notably actinomycetes correlate well with whole-organism lipid and ribosomal (r) RNA sequence data (Goodfellow 1989). Most bacteria have group A type peptidoglycan which is characterised by a cross-linkage extending from the ω-amino group of the diamino acid in position 3 of the peptide subunit to the carboxyl group of the C-terminal D-alanine in position 4 of the adjacent peptide subunit. In contrast, some Gram-positive taxa, notably actinomycetes belonging to the genera *Agromyces, Aureobacterium, Clavibacter, Curtobacterium* and *Microbacterium*, have the unusual group B type peptidoglycan where the cross-linkage is between the α-carboxyl group of D-glutamic acid in position 2 of the peptide subunit and the C-terminal D-alanine of the adjacent peptide subunit (Schleifer & Kandler 1972; Yokota et al. 1993a,b).

The genera that encompass actinomycetes with a group B type peptidoglycan can be distinguished using a combination of chemical and morphological features (Collins et al. 1983; Davis et al. 1984; Zgursckaya et al. 1992). Döpfer et al. (1982) consid-

ered that actinomycetes with a group B type peptidoglycan should be assigned to a single genus whereas Collins and Bradbury (1986) believed that the genera currently recognised should be classified in a single family. Most actinomycete genera have been assigned to families based on chemical, morphological and molecular systematic data (Stackebrandt et al. 1983; Embley et al. 1988; Goodfellow et al. 1990).

Small rRNA sequencing studies have been used to establish fine evolutionary relationships between prokaryotes (Hori & Osawa 1986; Specht et al. 1990; Van den Eynde et al. 1990), including pleomorphic actinomycetes (Dekio et al. 1984; Park et al. 1987a), rhodococci (Park et al. 1987b), streptomycetes (Park et al. 1991) and thermoactinomycetes (Park et al. 1993). In the present study, 5S rRNA sequence data generated mainly from representative actinomycetes with a group B type peptidoglycan were compared with corresponding results from earlier studies.

Materials and methods

Strains and culture conditions

The source and taxonomic histories of the test strains are given in Table 1. The *Agromyces, Aureobacterium, Clavibacter, Microbacterium* and *Oerskovia* strains were grown at 30° C in shake flasks containing medium R (Yamada & Komagata 1972) and the *Mycobacterium phlei* and *Mycobacterium smegmatis* cultures similarly but at 37° C in Trypticase soy medium (BBL, Cockeysville, MD; USA). The strains were checked for purity after 7 days and harvested by centrifugation.

Isolation and sequencing of 5S rRNA

Wet biomass (5 g) was homogenised with 10ml of RNAzol B (Biotecx Laboratories, Houston, Texas, USA), mixed with 1.5 ml chloroform and the homogenate centrifuged (12,000 Xg) for 15 minutes at 4° C. The aqueous phase was transferred to an equal volume of isopropanol and the preparation

Table 1. Source and taxonomic histories of test strains.

Laboratory number (KCTC)	Name	Source
9232^T	*Agromyces ramosus*	JCM 3108; KCC A-0108; L.E. Casida, PSU 38L
9227^T	*Aureobacterium liquefaciens*	JCM 3879; DSM 20638; K. Robinson, Mbm 15
9231	*Clavibacter michiganense*	JCM 1371; K-I.Suzuki,CNF 097; AJ 1391; ATCC 4450; R.E. Buchanan; M. Patel; N. Brown
9229^T	*Microbacterium imperiale*	JCM 1378; ATCC 8365; E.A. Steinhaus, 72
9230^T	*Microbacterium lacticum*	JCM 1379; IAM 1640; Shionogi & Co., Ltd., ATCC 8180; C.S. Pederson, 5; Orla-Jensen
9084^T	*Oerskovia turbata*	JCM 3160; KCC A-0160; IMRU 689; Statens Seruminstitut, Copenhagen, strain 891 (J. Oerskov, strain 27)
9085^T	*Oerskovia xanthineolytica*	JCM 3164; KCC A-0164; M.P. Lechevalier, LL G62 (IMRU 3959)
9087^T	*Mycobacterium phlei*	JCM 5865; NCTC 8151; G. Penso, strain Timoteo
9108^T	*Mycobacterium smegmatis*	JCM 5866; NCTC 8159; R.E. Gordon; Hagan, Alvarez and Tavel, Cornell 3

T Type strain

AJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; ATCC, American Type Culture Collection, Rockville, M.D., U.S.A., DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; IMRU, Waksman Institute of Microbiology, Rutgers University, Piscataway, N.J., JCM, Japan Collection of Microorganisms, RIKEN, Wako-shi, Saitama, Japan; KCC, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan; KCTC, Korean Collection for Type Cultures, Genetic Engineering Research Centre, Taejeon, Republic of Korea; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K., NCTC, National Collection of Type Cultures, Colindale, London, England, UK.

	KCTC	A	aLb	B	bLc	C	cLc'
		1 10		20		30	40
*Corynebacterium aquaticum	9233	GUUUCGGCGGC	CAUA	GCGAGAGG	GAAAC	GCCCGG	UUAUAUUCCGAAC
*Aureobacterium testaceum	9228	GUUUCGGCGGC	CAUA	GCGAGAGG	GAAAC	GCCCGG	UUAUAUUCCGAAC
Clavibacter michiganense	9231	GUUUCGGCGGC	CAUA	GCGAGAGG	GAAAC	GCCCGG	UCACAUUCCGAAC
Agromyces ramosus	9232	GUUUCGGCGGC	CAUA	GCGCGAGG	GAAAC	GCCCGG	ACACAUUCCGAAC
Aureobacterium liquefaciens	9227	GUUUCGGCGGC	CAUA	GCGUGAGG	GAAAC	GCCCGG	UUACAUUCCGAAC
Microbacterium lacticum	9230	GUUUCGGCGGC	CAUA	GCGUGAGG	GAAAC	GCCCGG	UUACAUUCCGAAC
Microbacterium imperiale	9229	GUUUCGGCGGC	CAUA	GCGUGAGG	GAAAC	GCCCGG	UUACAUUCCGAAC
*Curtobacterium citreum	9100	GUUCCGGUGGU	GAUA	GCGAGAGG	GAAAC	GCCCGG	UGAGAUUCCGAAC
*Corynebacterium glutamicum	9234	UGUGUCGGUGGU	GAUA	GUAGCAGG	GAAAC	GCCCGG	UCCCUUUCCGAAC
Oerskovia turbata	9084	GUUACGGCGGU	CAUA	GCGAGAGG	GAAAC	GCCCGG	UCCCAUUCCGAAC
Oerskovia xanthineolytica	9085	GUUACGGCGGU	CACA	GCGAAGGG	GAAAC	GCCCGG	UCCCAUACCGAAC
Mycobacterium phlei	9087	GUUACGGCGGU	CAAUAGCGGCAGG		GAAAC	GCCCGG	UCCCAUCCCGAAC
Mycobacterium smegmatis	9108	GUUACGGCGGU	CCAUAGCGGCAGG		GAAAC	GCCCGG	UCCCAUCCCGAAC

C'	c'Lb'	B'	b'Ld	D	dLd'	D'	d'La'	A'
50		60	70	80	90		100	110 120
CCGGAAGC	UAAG	ACUCUCUGC	GCCGAUGGUA	CUGCAGGG	GGGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACU
CCGGAAGC	UAAG	CCUCUCAGC	GCCGAUGGUA	CUGCAGGG	GGGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACU
CCGGAAGC	UAAG	ACUCUCAGC	GCCGAUGGUA	CUGCAGGG	GGGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACU
CCGGAAGC	UAAG	ACUCGCAGC	GCCGAUGGUA	CUGCAGGG	GCGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACA
CCGGAAGC	UAAG	CCUCACAGC	GCCGAUGGUA	CUGCAGGG	GGGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACU
CCGGAAGC	UAAG	CCUCACAGC	GCCGAUGGUA	CUGCAGGG	-GGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACUU
CCGGAAGC	UAAG	CCUCACAGC	GCCGAUGGUA	CUGCAGGG	-GGA	CCCUGUGG	GAGAGUAGGAC	ACCGCCGGACUU
CCGGAAGC	UAAG	CCUCUCAGC	GCCGAUGGUA	CUGCAAGG	GGGA	CCUUGUGG	GAGAGUAGGAC	GCCGCCGGACUCA
CCGGAAGC	UAAG	CCUGGUUAC	GCUGAUGGUA	CUGCACUC	GGGA	GGGUGUGG	GAGAGUAGGUU	ACCGCCGACCA
CCGGAAGC	UAAG	CCUCUCAGC	GCCGAUGGUA	CUGCCCUG	GAGA	CGGGGUGG	GAGAGUAGGUC	GCCGCCGGACA
CCGGAAGC	UAAG	CCCUUCAGC	GCCGAUGGUA	CUGCCCUG	GAGA	CGGGGUGG	GAGAGUAGGAC	GCCGCCGGACA
CCGGAAGC	UAAG	CCUGCCAGC	GCCAAUGAUA	CUGCCC--UCACC		--GGGUGG	AAAAGUAGGAC	ACCGCCGAACAC
CCGGAAGC	UAAG	CCUGCCAGC	GCCGAUGAUA	CUACCC--	AUCC	--GGGUGG	AAAAGUAGGAC	ACCGCCGAACAC

Fig. 1. Sequence alignment of 5S rRNAs from selected actinomycetes including eight strains with a group B peptidoglycan type. Base-paired regions: A,A′, B,B′, C,C′, and D,D′; loop regions: aLb, bLc, cLc′, c′Lb′, b′Ld, dLd′, and d′La′;. *·⁺ Sequence data from Park et al. (1987a).

stored at 4° C for 15 minutes. 5S rRNA was obtained by polyacrylamide gel electrophoresis of total rRNA, as described previously (Park et al. 1987a). The nucleotide sequences of the 5S rRNA preparations were determined using both chemical and enzymatic methods (Peattie 1979; Donis-Keller 1980). Ambiguous parts of the nucleotide sequences were confirmed by electrophoresis on a hot plate at 70° C. The identity of 5′-labeled termini was done by thin layer chromatography with RNase T_2 digest of 3′-^{32}P-labeled RNA on cellulose plates. Models of 5S rRNA secondary structure were constructed using the method of Tinoco et al. (1971), as adapted by Hori and Osawa (1986).

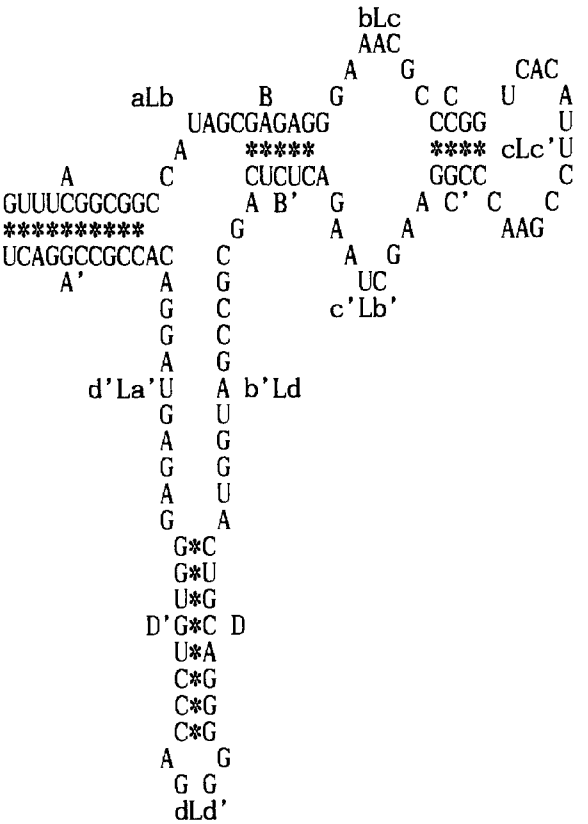

Fig. 2. Secondary structure of 5S rRNA from *Clavibacter michiganense* KCTC 9231. Asterisks and dotted lines indicate base pairing.

Phylogenetic analysis

The evolutionary distances, Knuc, between nucleotide sequences were calculated after Kimura (1980). Knuc corresponds to the number of base substitutions per nucleotide site that occurred in the course of evolution:

$$Knuc = (1/2) \log_e [(1-2P-Q)(1-2Q)^{1/2}]$$

where P and Q are fractions of nucleotide sites showing transition – and transversion – type differences, respectively. An evolutionary tree was generated by applying the unweighted pair group method with mean averages (UPGMA) algorithm to data that included information derived from earlier studies on representative Gram-positive bacteria (Park et al. 1987a).

Results and discussion

Bacterial 5S rRNA can be assigned to three groups based on differences in their primary and secondary structures (Hori & Osawa 1986; Park et al. 1987a, b, 1991, 1993). Those from Gram-positive bacteria with a low genomic G+C content (< 55 mole %) usually have 116 nucleotides, five base-pairs and a U-U mismatch in the D-D′ helix. Ribosomal RNAs from Gram-negative bacteria and actinomycetes have around 120 nucleotides and eight basepairs in the D-D′ region. In addition, 5S rRNAs from pleomorphic actinomycetes have a bulge in the A-A′ helix, apart from *Brevibacterium* and *Corynebacterium* (*Caseobacter*) strains (Park et al. 1987a, b). The

Table 2. Homology percentage matrix of 5S rRNA sequences.

		KCTC	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]
1.	*Corynebacterium aquaticum*	9233	100												
2.	*Aureobacterium testaceum*	9228	98	100											
3.	*Clavibacter michiganense*	9231	97	97	100										
4.	*Agroryces ramosus*	9232	93	93	95	100									
5.	*Aureobacterium liquefaciens*	9227	95	97	96	94	100								
6.	*Microbacterium lacticum*	9230	94	95	95	92	98	100							
7.	*Microbacterium imperiale*	9229	94	95	95	92	98	100	100						
8.	*Curtobacterium citreum*	9100	89	90	90	86	89	89	89	100					
9.	*Corynebacterium glutamicum*	9234	75	75	76	76	76	75	75	74	100				
10.	*Oerskovia turbata*	9084	85	87	88	86	86	85	85	86	79	100			
11.	*Oerskovia xanthineolytica*	9085	81	83	84	82	82	81	81	81	75	94	100		
12.	*Mycobacterium phlei*	9087	76	77	78	78	78	78	78	76	74	84	82	100	
13.	*Mycobacterium smegmatis*	9108	75	77	77	77	77	77	77	75	73	82	81	94	100

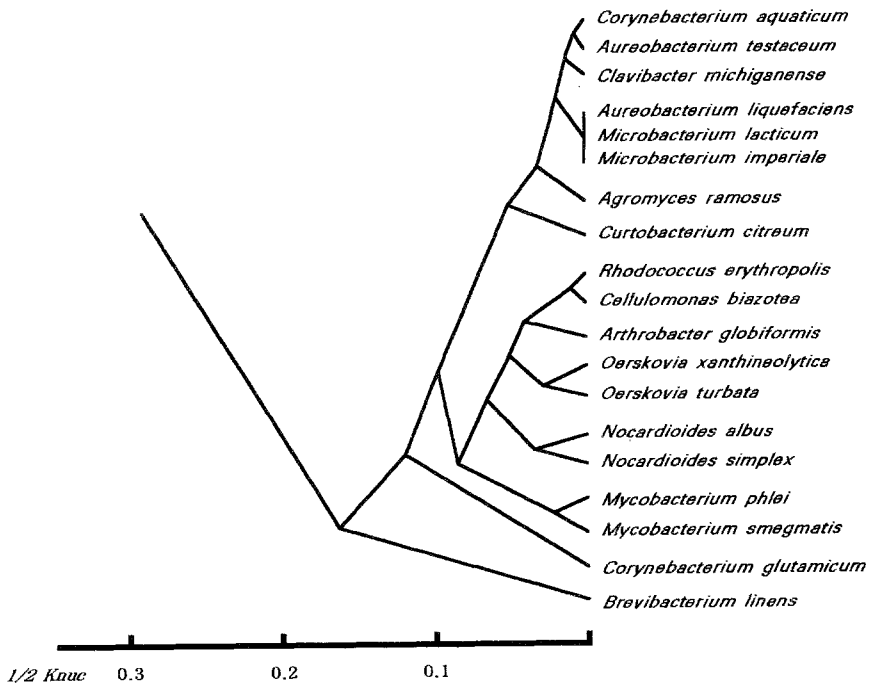

Fig. 3. Phylogenic tree showing suprageneric relationships amongst actinomycetes with a group B peptidoglycan type and selected Gram-positive and Gram-negative bacteria based on 5S rRNA sequence data.

bulge has also been detected in strains of *Micrococcus (Dekio et al. 1984), Mycobacterium* (Dams et al. 1987) and *Streptomyces* (Simoncsits 1980).

The length of the 5S rRNA was 120 nucleotides for *Agromyces ramosus* KCTC 9232, *Aureobacterium liquefaciens* KCTC 9227, *Clavibacter michiganense* KCTC 9231, *Microbacterium imperiale* KCTC 9229, *Microbacterium lacticum* KCTC 9230, *Oerskovia turbata* KCTC 9084 and *Oerskovia xanthineolytica* KCTC 9085, and 119 nucleotides for *Mycobacterium phlei* KCTC 9087, and *Mycobacterium smegmatis* KCTC 9108. The sequences were aligned by juxtaposing the secondary structures prior to their division into 15 regions (Fig. 1); A and A′, B and B′, C and C′ and D and D′ are, respectively, the sequences that can base-pair with one another. The loop region aLb connects the base-pair regions A and B, the remaining base-pair regions are connected by bLc, cLc′, c′Lb′, b′Ld, dLd′ and d′La′ as shown. The secondary structure of the 5S rRNA of *Clavibacter michiganense* KCTC 9231 is given in Fig. 2. The percentage sequence homology values of the 5S rRNAs are given in Table 2.

It is clear from the primary and secondary structure of the 5S rRNAs that actinomycetes with a group B peptidoglycan belong to the 120 nucleotide type (Hori & Osawa, 1986; Park et al. 1987a,b, 1991, 1993), as do the remaining test strains. The representatives of the genera *Agromyces, Aureobacterium, Clavibacter, Microbacterium* and *Oerskovia* have a bulge in the A-A′ helix, and unique sequences, such as 5′-CUGCA-3′ and 5′-UGUGG-3′ in the D-D′ region. The *Mycobacterium* strains also have a bulge in this region. The nucleotide sequence found in *Mycobacterium phlei* KCTC 9087 differed at positions 82, 87 and 119, and by one base insertion in the dLd′ region, compared with the sequence reported by Dams et al. (1987) for the same organism albeit with a different strain history. Similarly, the nucleotide sequence for *Mycobacterium smegmatis* KCTC 9108 (ATCC 19420) differed by substitutions at positions 86 and 118 compared with the sequence of *Mycobacterium smegmatis* strain Rabinowitsch.

It is clear from the phylogenetic tree generated from 5S rRNA sequencing data that the genera *Agromyces, Aureobacterium, Clavibacter, Curtobacterium* and *Microbacterium* form a phyletic line that can be readily separated from actinomycetes

with a group A type peptidoglycan (Fig. 3). '*Corynebacterium aquaticum*' KCTC 9233, originally described by Leifson in 1962, is also a member of this group. The results of the 5S rRNA sequencing studies taken together with associated chemotaxonomic data (Collins & Bradbury, 1986, 1991) provide compelling evidence that the genera *Agromyces, Aureobacterium, Clavibacter, Curtobacterium* and *Microbacterium* should be assigned to a single family as suggested by Collins and Bradbury (1986).

Description of Microbacteriaceae fam. nov.

Mic.ro.bac.te'ri.um. M.L. neut.n. *Microbacterium*, type genus of the family; suff.-aceae, denoting family; M.L. neut. pl. n. *Microbacteriaceae, the Microbacterium* family. The description is taken from Collins and Bradbury (1991). Obligately aerobic to facultatively anaerobic, Gram-positive, acid-fast negative, nonsporeforming bacteria that show a range of morphological features which extend from small irregular rods to branched filamentous elements (1 μm or less in diameter) that undergo septation and fragmentation to yield coccoid and irregular forms. All strains have a group B type peptidoglycan. The diamino acids in the wall peptidoglycan include diaminobutyric acid (B2γ peptidoglycan; *Agromyces, Clavibacter*), lysine (B1α, B1β peptidoglycan; *Microbacterium*) and ornithine (B2β peptidoglycan; *Aureobacterium, Curtobacterium*). Other common chemical features include the unsaturated menaquinones with 9 to 12 isoprene units, and diphosphatidylglycerol, phosphatidylglycerol and a variety of glycolipids. The G+C content of the DNA lies within the range of 66 to 78 mole%. The family can be distinguished from other suprageneric groups of actinomycetes by rRNA sequence data. Isolated from diverse habitats including dairy, sewage and insect sources. Some strains are pathogenic for plants. The type genus is *Microbacterium* Orla-Jensen 1919.

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