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Polyamine Distribution in Actinomycetes with Group B Peptidoglycan and Species of the Genera *Brevibacterium*, *Corynebacterium*, and *Tsukamurella*

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Polyamine patterns of 75 strains of actinobacteria belonging to the genera *Agrococcus*, *Agromyces*, *Aureobacterium*, *Brevibacterium*, *Clavibacter*, *Corynebacterium*, *Curtobacterium*, *Microbacterium*, *Rathayibacter*, and *Tsukamurella* were analyzed in order to investigate the suitability of this approach for differentiation within this group. The results revealed that the overall polyamine contents differ significantly among genera and that various patterns are present in actinobacteria. One characteristic pattern found in the genera *Clavibacter*, *Rathayibacter*, and *Curtobacterium* included a high polyamine concentration, and the polyamines were mainly spermidine and spermine. This feature distinguished the 2,4-diaminobutyric acid-containing genera *Rathayibacter*, *Clavibacter*, and *Agromyces*, which contained low concentrations of polyamines. Strains of the genus *Brevibacterium* were characterized by the presence of high concentrations of cadavarine and usually high concentrations of putrescine. Members of the genus *Corynebacterium* had relatively low polyamine contents, and usually spermidine was the major polyamine. A similar polyamine pattern was detected in the species of the genus *Tsukamurella*. No homogeneous polyamine patterns were detected in representatives of the genera *Microbacterium* and *Aureobacterium*, which are phylogenetically intermixed (M. Takeuchi and A. Yokota, FEMS Microbiol. Lett. 124:11–16, 1994). The results of polyamine analyses are in good agreement with the genetic heterogeneity within the actinobacteria and demonstrate that polyamine patterns are suitable for use in classification of actinobacterial taxa.

In recent years analysis of polyamine patterns has become well-established in the systematics of gram-negative bacteria, and its suitability for classification has been demonstrated for the class *Proteobacteria* (2–4, 6–8, 20, 22, 25, 26, 28, 30, 49, 50) and the *Flavobacterium-Cytophaga-Sphingobacterium* branch (23, 24). Attempts to introduce polyamine patterns as chemotaxonomic markers for gram-positive bacteria, such as bacilli and lactic acid bacteria (18), thermophilic bacilli (19), actinomycetes (21), and aerobic (18) and anaerobic cocci (27), did not demonstrate the suitability of this approach for the classification of these groups. However, studies on the polyamine contents of the heterogeneous group that includes the actinobacteria have not been performed previously.

In the past, actinobacteria, which are also designated the coryneform bacteria, have often been misclassified, which has led to reclassifications or descriptions of new taxa. In the search for a reliable classification and identification system, the actinobacteria have been the subject of numerous investigations, including numerical taxonomic studies (31, 42) and analyses of cell wall sugars (47), peptidoglycan types and diamino acids (41), fatty acids (5, 11, 29, 35), mycolic acids (1, 34), polar lipids (12, 35), quinones (10), and 16S rRNA sequences (9, 32, 33, 37–40, 43, 48). The combination of these different approaches has led to an improved taxonomy of actinobacteria, although there are many actinobacterial genera which exhibit similar chemotaxonomic features.

This study was initiated to evaluate the suitability of polyamine patterns for differentiating actinobacterial taxa. The results of our analysis of the polyamine patterns obtained for different actinobacterial genera are presented below; this analysis included the genera *Agrococcus*, *Agromyces*, *Aureobacterium*, *Brevibacterium*, *Clavibacter*, *Curtobacterium*, *Corynebacterium*, *Microbacterium*, *Rathayibacter*, and *Tsukamurella.*

MATERIALS AND METHODS

Strains used and culture conditions. The designations of the strains analyzed are listed in Tables 1 through 3 The cells were cultivated at 28°C on PYE medium (0.3% peptone from casein, 0.3% yeast extract) and were harvested at a density corresponding to approximately 70% of the maximum optical density determined for each strain. Since *Microbacterium laevaniformans* DSM 20140^T (T = type strain), *Tsukamurella inchoensis* DSM 44067^T, and *Tsukamurella wratislaviensis* DSM 44107^T did not grow in homogeneous suspensions, the point of harvest was estimated. To grow *Clavibacter michiganensis* subsp. *michiganensis* ICMP 1808 in mineral medium, 0.3 g of $(NH_4)_2SO_4$, 1.04 g of K_2HPO_4, 0.75 g of KH_2PO_4, 0.025 g of $CaCl_2$, and 0.2 g of $MgSO_4$ were dissolved in 1 liter of glass-distilled water, and the medium was supplemented with 3 ml of a trace metal mixture (36) and 10 mM NH_4 succinate. The mineral medium used to grow *Corynebacterium glutamicum* contained 8 g of K_2HPO_4 per liter, 3.2 g of NaH_2PO_4 per liter, 0.2 g of Na_3 citrate · $2H_2O$ per lilter, 2 mg of $FeSO_4$ per liter, 1.1 g of $(NH_4)_2SO_4$ per liter, 0.1 g of $MgSO_4 \cdot 7H_2O$ per liter, and 10 mM glucose.

Polyamine analysis. Polyamines were extracted as described previously (6). Approximately 40 mg of lyophilized cells was hydrolyzed in 1 ml of 0.2 N perchloric acid for 30 min at 100°C with occasional shaking. Each mixture contained the internal standard 1,8-diaminooctane (0.5 μmol/40 mg of cells). After extraction the samples were centrifuged, and 0.2 ml of the supernatant was transferred to a tube containing 0.3 ml of a Na_2CO_3 solution (100 mg/ml). Then 0.8 ml of a dansyl chloride solution (7.5 μg/ml in acetone) was added. The tube was tightly closed, and dansylation was performed for 20 min at 60°C. Subsequently, 0.1 ml of a proline solution (50 mg/ml) was added to bind excessive dansyl chloride during incubation for 10 min at 60°C. After cooling to 5°C, the polyamines were extracted with 0.1 ml of toluene with shaking. The upper phase was used for high-performance liquid chromatography (HPLC) analysis. The

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TABLE 1. Polyamine patterns of peptidoglycan type B and 2,4-diaminobutyric acid-containing actinobacteria[a]

Organism	Polyamine content (μmol/g [dry wt])[b]								
	DAP	PUT	CAD	NSPD	SPD	HSPD	SPM	TYR	Total
Agrococcus jenensis DSM 9580^{T}	0.03 (3.19)				0.04 (4.25)	Tr	**0.87** (92.6)	Tr	0.94
Agromyces cerinus subsp. *cerinus* VKM Ac-1340^{T} (= DSM 8595^{T})	**0.10** (38.5)	**0.11** (42.3)	0.02 (7.7)		0.02 (7.7)		0.01 (3.8)		0.26
Agromyces cerinus subsp. *nitratus* VKM Ac-1351^{T} (= DSM 8596^{T})		**0.14** (63.6)	0.01 (4.5)		0.03 (13.6)		0.04 (18.2)		0.22
Agromyces fucosus subsp. *fucosus* VKM Ac-1345^{T} (= DSM 8597^{T})	**0.05** (23.8)	**0.12** (57.1)	0.01 (4.8)		0.02 (9.5)		0.01 (4.8)		0.21
Agromyces fucosus subsp. *hippuratus* VKM Ac-1352^{T} (= DSM 8598^{T})	Tr	**0.14** (58.3)		Tr	**0.07** (29.2)	Tr	0.03 (12.5)		0.24
Agromyces mediolanus DSM 20152^{T} (= ATCC 14004^{T} = NCIB 7206^{T}) (formerly "*Corynebacterium mediolanum*")	**0.07** (25.0)	**0.21** (75.0)	Tr	Tr	Tr		Tr		0.28
Agromyces sp. strain DL-89		**0.33** (51.6)	0.02 (3.1)		0.07 (10.9)		0.12 (18.6)	0.08 (12.5)	0.62
Aureobacterium barkeri DSM 20145^{T} (= ATCC 15954^{T} = NCIB 9658^{T})		0.15 (4.8)			**2.92** (95.2)				3.07
Aureobacterium liquefaciens DSM 20638^{T} (= NCIB 11509^{T})		**0.19** (33.3)	0.02 (3.5)		**0.30** (52.6)		0.06 (10.5)		0.57
Aureobacterium testaceum DSM 20166^{T} (= ATCC 15829^{T})	**0.01** (20.0)	**0.01** (20.0)			**0.02** (40.0)		**0.01** (20.0)		0.05
"*Brevibacterium helvolum*" WS 1753 (= ATCC 13715 = DSM 20419)	**1.29** (23.4)		**4.08** (73.8)		0.02 (0.4)		0.13 (2.4)		5.52
Clavibacter michiganensis subsp. *michiganensis* ICMP 2550^{T} (= NCPPB 2979^{T})	0.04 (1.6)	0.05 (2.1)	**0.50** (20.6)		**0.87** (35.8)		**0.94** (38.7)	0.03 (1.2)	2.43
Clavibacter michiganensis subsp. *michiganensis* ICMP 2539 (= NCPPB 2034)	0.09 (4.5)	0.05 (2.5)	**0.57** (28.4)		**0.60** (29.8)	Tr	**0.70** (34.8)		2.01
Clavibacter michiganensis subsp. *michiganensis* ICMP 1808		0.21 (5.3)	0.19 (4.8)		**1.65** (41.8)	Tr	**1.90** (48.1)		3.95
Clavibacter michiganensis subsp. *michiganensis* ICMP 1808[c]		0.04 (0.3)			**14.60** (99.6)		0.02 (0.1)		14.66
Clavibacter michiganensis subsp. *michiganensis* ICMP 5026		0.17 (4.7)	0.37 (10.2)		**1.55** (42.8)		**1.36** (37.6)	0.17 (4.7)	3.62
Clavibacter michiganensis subsp. *michiganensis* ATCC 4450		0.11 (10.0)	0.19 (17.3)		**0.55** (50.0)		**0.25** (22.7)		1.10
Clavibacter michiganensis subsp. *insidiosus* ICMP 2621^{T} (= NCPPB 1109^{T})	0.08 (1.8)	0.14 (3.2)	0.07 (1.6)	0.04 (0.9)	**1.85** (42.3)		**2.19** (50.1)		4.37
Clavibacter michiganensis subsp. *insidiosus* ICMP 4192	0.06 (2.2)	0.24 (9.0)	0.20 (7.5)		**1.67** (62.3)		**0.44** (16.4)	0.07 (2.6)	2.68
Clavibacter michiganensis subsp. *insidiosus* ICMP 2615 (= NCPPB 1020)	0.03 (0.5)	0.09 (1.6)	0.22 (3.8)	Tr	**2.10** (36.2)		**3.36** (57.9)	Tr	5.80
Clavibacter michiganensis subsp. *insidiosus* ICMP 3568	Tr	0.10 (1.4)	0.13 (1.8)	Tr	**2.34** (32.3)	Tr	**4.64** (64.0)	0.04 (0.6)	7.25
Clavibacter michiganensis subsp. *nebraskensis* ICMP 3298^{T} (= NCPPB 2581^{T} = ATCC 27794^{T})	0.02 (0.5)	0.10 (2.4)	0.09 (2.2)		**1.90** (45.6)		**2.06** (49.4)		4.17
Clavibacter michiganensis subsp. *nebraskensis* ICMP 3294	0.03 (0.8)	0.17 (4.5)	0.12 (3.2)		**1.68** (44.2)	0.01 (0.3)	**1.79** (47.1)		3.80
Clavibacter michiganensis subsp. *sepedonicus* ICMP 2535^{T} (= NCPPB 2137^{T} = ATCC 33113^{T})	0.02 (0.4)	0.11 (2.1)	0.09 (1.7)		**1.84** (34.6)	Tr	**3.26** (61.3)		5.32
Clavibacter michiganensis subsp. *sepedonicus* ICMP 2531	0.04 (0.6)	0.13 (2.0)	0.14 (2.2)		**2.36** (36.3)		**3.84** (58.9)		6.51

Continued on following page

TABLE 1—*Continued*

Organism	Polyamine content (μmol/g [dry wt])[b]								
	DAP	PUT	CAD	NSPD	SPD	HSPD	SPM	TYR	Total
Clavibacter michiganensis subsp. *tesselarius* ICMP 7221^T (= ATCC 33566^T)	Tr	0.05 (1.9)	0.08 (3.0)		**1.14** (42.4)		**1.42** (52.8)		2.69
Clavibacter michiganensis subsp. *tesselarius* ICMP 7219	0.02 (0.7)	0.08 (2.8)	0.20 (7.0)		**1.26** (43.9)		**1.31** (45.6)		2.87
Clavibacter toxicus ICMP 6309^T	Tr	0.10 (4.2)	0.13 (5.5)		**0.91** (38.6)		**1.22** (51.7)		2.36
Clavibacter sp. strain VKM Ac-1371	0.02 (0.3)	0.04 (0.6)	0.05 (0.8)	Tr	**2.12** (31.6)		**4.48** (66.8)	Tr	6.71
Curtobacterium flaccumfaciens pv. *flaccumfaciens* NCPPB 178	0.05 (8.8)				**0.20** (35.1)		**0.32** (56.1)		0.57
Curtobacterium pusillum DSM 25207^T (= ATCC 19096^T = NCIB 10354^T)					**0.49** (30.4)		**1.12** (69.6)		1.61
Microbacterium arborescens DSM 20754^T (= ATCC 4358^T)	**0.21** (22.8)	**0.27** (29.3)	0.01 (1.1)		**0.36** (39.1)		0.07 (7.6)	0.92	
Microbacterium aurum DSM 8600^T (= ATCC 51345^T)	0.05 (3.5)	0.06 (4.3)			**0.70** (49.6)		**0.60** (42.6)		1.41
Microbacterium imperiale WS 1771^T (= DSM 20530^T)	0.01 (0.4)	**2.07** (88.8)		0.01 (0.4)	0.18 (7.7)		0.06 (2.6)		2.33
Microbacterium lacticum WS 2129^T (= DSM 20427^T = ATCC 8180^T)	0.04 (4.5)	**0.49** (55.0)	0.01 (1.1)	0.02 (2.2)	**0.19** (21.3)		0.14 (15.7)	Tr	0.89
Microbacterium lacticum WS 1914 (= NCPPB 2980)	0.01 (0.3)	**3.20** (91.2)	0.02 (0.6)	Tr	0.07 (1.9)		0.21 (6.0)		3.51
Microbacterium laevaniformans DSM 20140^T (= ATCC 15953^T)		**0.09** (75.0)			0.01 (8.3)		0.02 (16.7)		0.12
Rathayibacter rathayi ICMP 2574^T	0.03 (0.5)	0.10 (1.6)	0.09 (1.5)		**2.49** (40.2)		**3.44** (55.5)	0.05 (0.8)	6.20
Rathayibacter rathayi ICMP 2576	0.04 (0.7)	0.02 (0.3)	0.04 (0.7)	Tr	**2.00** (33.4)	Tr	**3.86** (64.4)	0.03 (0.5)	5.99
Rathayibacter rathayi ICMP 2579 (= NCPPB 797)	0.14 (2.3)	0.05 (0.8)	0.08 (1.3)	Tr	**1.82** (29.9)	Tr	**3.93** (64.6)	0.06 (1.0)	6.08
Rathayibacter iranicus ICMP 3496^T (= NCPPB 2253^T)	0.07 (0.4)	1.45 (8.0)	0.24 (1.3)		3.48 (19.2)		**12.91** (71.1)		18.15
Rathayibacter tritici ICMP 2626^T (= ATCC 11403^T = NCPPB 1857^T)	0.27 (2.3)	0.05 (0.4)	0.04 (0.3)		1.59 (13.2)	0.03 (0.3)	**8.08** (67.3)		10.06
Rathayibacter tritici ICMP 2623 (= NCPPB 471)	0.31 (6.4)	0.04 (0.8)	0.03 (0.6)		**1.34** (27.8)		**3.09** (64.2)		4.80
Rathayibacter sp. strain VKM Ac-1789	**0.37** (25.9)	Tr	0.03 (2.1)		0.05 (3.5)	Tr	**0.87** (60.8)	0.11 (7.7)	1.43
Rathayibacter sp. strain VKM Ac-1787	Tr	0.03 (2.3)			0.06 (4.6)	Tr	**1.13** (86.9)	0.08 (6.2)	1.30

[a] Abbreviations: DAP, 1,3-diaminopropane; PUT, putrescine; CAD, cadaverine, NSPD, *sym*-norspermidine; SPD, spermidine; HSPD, *sym*-homospermidine; SPM, spermine; TYR, tyramine; Tr, trace (less than 0.01 μmol/g [dry weight]; ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; NCIB, National Collection of Industrial Bacteria, Aberdeen, United Kingdom; NCPPB, National Collection of Plant Pathogenic Bacteria, Bertfordshire, United Kingdom; VKM, All-Russian Collection of Microorganisms, Pushchino, Russia; WS, Culture Collection, Dairy Research Institute of Weihenstephan, Freising, Germany.

[b] The values in boldface type are the values for major polyamines (polyamines which accounted for more than 20% of the total polyamines). The values in parentheses are percentages.

[c] Cells were grown in mineral medium as described in Materials and Methods.

HPLC apparatus was equipped with two Waters model 510 pumps, a model U6K injector, a column oven, a spectrofluorometric detector (Jasco model 821-FP), and a reversed-phase column (250 by 4 mm; Hypersil octyldecyl silane; 5-μm particles). To separate the polyamines, a linear 40 to 85% acetonitrile-water gradient was applied for 35 min, and this was followed by a linear 85 to 100% acetonitrile-water gradient applied for 15 min at 40°C. To detect dansylated polyamines, the excitation wavelength used was 360 nm and the emitted light was measured at 520 nm. Polyamine concentrations were calculated by internal standardization. Repeated analyses of selected samples demonstrated that the deviation of the values for major compounds was less than 10%.

RESULTS AND DISCUSSION

The representatives of the actinobacteria selected for analysis of their polyamine contents are phylogenetically located on three different branches (9, 37, 38, 40, 48), the peptidoglycan type B group comprising the genera *Agrococcus*, *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium*, *Microbacterium*, and *Rathayibacter*, the mycolic acid-containing genera *Corynebacterium* and *Tsukamurella*, and the deeply branching genus

TABLE 2. Polyamine patterns of *Brevibacterium* species[a]

Organism	Polyamine content (μmol/g [dry wt])[b]							
	DAP	PUT	CAD	NSPD	SPD	HSPD	SPM	Total
Brevibacterium casei WS 2128^{T} (= DSM 20657^{T} = ATCC 35513^{T})	0.12 (2.4)	**2.41** (47.7)	**2.41** (47.7)	0.02 (0.4)	0.03 (0.6)	0.05 (1.0)	0.01 (0.2)	5.05
Brevibacterium casei WS 2124 (= NCDO 2050)	0.08 (6.5)	**0.31** (25.0)	**0.80** (64.5)		0.03 (2.4)		0.02 (1.6)	1.24
Brevibacterium casei WS 2125 (= NCDO 2049)	0.21 (10.1)	**0.60** (29.0)	**1.21** (58.5)		0.03 (1.4)		0.02 (1.0)	2.07
Brevibacterium casei DSM 20658 (= NCDO 2051)	0.20 (13.2)	**0.40** (26.3)	**0.79** (52.0)		0.06 (3.9)		0.07 (4.6)	1.52
Brevibacterium epidermidis DSM 20660^{T} (= NCDO 2286^{T})	0.12 (8.6)	**0.35** (25.2)	**0.86** (61.9)		0.04 (2.9)		0.02 (1.4)	1.39
Brevibacterium iodinum DSM 20626^{T} (= NCDO 613^{T})	0.05 (2.7)	**0.55** (30.2)	**1.18** (64.8)		0.03 (1.6)		0.01 (0.6)	1.82
Brevibacterium linens WS 1782^{T} (= ATCC 9172^{T} = DSM 20425^{T})	0.47 (6.5)	**2.93** (40.5)	**3.68** (50.8)	0.04 (0.5)	0.08 (1.1)	0.03 (0.4)	0.01 (0.1)	7.24
Brevibacterium linens WS 1939	0.16 (6.2)	**1.08** (41.5)	**1.05** (40.4)		0.29 (11.2)		0.02 (0.8)	2.60
Brevibacterium linens WS 1954	0.03 (2.9)	0.10 (9.5)	**0.51** (48.6)		**0.26** (24.8)		0.15 (14.3)	1.05

[a] NCDO, National Collection of Dairy Organisms, Reading, United Kingdom. For an explanation of other abbreviations, see Table 1, footnote *a*.

[b] The values in boldface type are the values for major polyamines (polyamines which accounted for more than 20% of the total polyamines). The values in parentheses are percentages.

Brevibacterium. All of the actinobacteria investigated were found to contain polyamines, and characteristic polyamine patterns were detected for certain taxa or closely related groups of actinobacteria.

Polyamines in peptidoglycan type B bacteria. Species of the genera *Microbacterium* and *Aureobacterium* are intermixed and are located on a common branch that is distant from the other peptidoglycan type B bacteria. Three variations of the peptidoglycan type, B1α, B1β, and B2β, have been found in the phylogenetically analyzed species of these two genera (48, 52, 53), which demonstrates the heterogeneity in this group. The type strains of the type species of the two genera, *Microbacterium lacticum* WS 2129 and *Aureobacterium liquefaciens* DSM 20638, are phylogenetically more closely related to each other than to other species in their own genera (38, 48). This relatedness may also be indicated by the similar polyamine patterns of these organisms. Both species contained putrescine and spermidine as the predominant compounds (Table 1). A high polyamine content with putrescine as the major compound was the common feature of the second strain of *M. lactium* examined, WS 1914, and *Microbacterium imperiale* WS 1771^{T}. Physiologically, each of the two strains of *M. lacticum* (WS 2129^{T} and WS 1914) and *M. imperiale* WS 1771^{T} clustered in a different group (31). This indicates that *M. lacticum* WS 1914 may represent a species separate from *M. lacticum* WS 2129^{T} and *M. imperiale* WS 1771^{T}. *Microbacterium arborescens* DSM 20754^{T}, *Microbacterium aurum* DSM 8600^{T}, and *Microbacterium laevaniformans* DSM 20140^{T} each exhibited a specific polyamine pattern. *M. arboresceus* DSM 20754^{T} contained the major polyamines 1,3-diaminopropane, putrescine, and spermidine, *M. aurum* DSM 8600^{T} was characterized by the presence of spermidine and spermine as the predominant polyamines, and *M. laevaniformans* DSM 20140^{T} contained extremely low concentrations of polyamines (Table 1). Although putrescine was found to be the predominant polyamine, this observation is not considered significant since the amount of this polyamine was at the lower limit of the analysis system used. No common polyamine patterns were detected in *Aureobacterium barkeri* DSM 20145^{T} and *Aureobacterium testaceum* DSM 20166^{T}. *Aureobacterium barkeri* DSM 20145^{T} was characterized by the presence of the predominant compound spermidine in relatively high amounts. In *Aureobacterium testaceum* DSM 20166^{T}, only traces of polyamines were detected, and there was no predominant polyamine; thus, this organism was similar to *M. laevaniformans* DSM 20140^{T} in this respect. The different polyamine patterns obtained for the species of the genera *Microbacterium* and *Aureobacterium* indicate that the approach which we used is not suitable for clustering species belonging to this group. On the other hand, the differences in the polyamine patterns may be distinctive features for species belonging to the *Aureobacterium-Microbacterium* group which have the same peptidoglycan type (Table 4). Only a separate study that includes all species of the two genera as well as additional strains of each species may clarify whether polyamine patterns are species or strain specific within this group.

Recently, a new genus with only one species, *Agrococcus jenensis* (type strain, DSM 9580), was proposed, and this organism is genetically most closely related to the *Microbacterium-Aureobacterium* branch (16). *Agrococcus jenensis* DSM 9580^{T}, which contains 2,4-diaminobutyric acid in its cell wall, was characterized by the presence of spermine, which distinguishes it from all other members of the *Microbacterium-Aureobacterium* branch. The detection of spermine as the major polyamine can also be used to distinguish the genus *Agrococcus* from other 2,4-diaminobutyric acid-containing genera (the genera *Rathayibacter*, *Clavibacter*, and *Agromyces*). However, this pattern was similar to the patterns obtained for two *Rathayibacter* strains, *Rathayibacter* sp. strains VKM Ac-1787 and VKM Ac-1789. These two *Rathayibacter* strains can be easily distinguished from the genus *Agrococcus* by the presence of menaquinone MK-10 (12a, 13), a significant feature of the genus *Rathayibacter* (54). *Agrococcus jenensis* contains mainly menaquinones MK-11 and MK-12 (16). On the other hand, the polyamine patterns of *Rathayibacter* sp. strains VKM Ac-1787 and VKM Ac-1789 did not display the characteristic features of the genus *Rathayibacter* as they exhibited only low levels of spermidine. The observed differences indicate that the tentative identification of these two strains as members of the genus *Rathayibacter* needs to be confirmed by further studies. The peptidoglycan type-B2γ-containing genera *Rathayibacter* and *Clavibacter* are closely related phylogenetically (38). This relationship is also demonstrated by the presence of similar polyamine patterns (Table 1). Compared to other genera investigated in this study, members of recognized species of these two genera contained relatively high concentrations of polyamines, and generally spermidine and spermine were the major compounds. Two strains of *Clavibacter michiganensis* subsp. *michi-*

TABLE 3. Polyamine patterns of the mycolic acid-containing species of the genera *Corynebacterium* and *Tsukamurella*[a]

Organism	Polyamine content (μmol/g [dry wt])[b]							
	Unknown	DAP	PUT	CAD	SPD	HSPD	SPM	Total[c]
Corynebacterium ammoniagenes WS 1571^{T} (= DSM 20306^{T} = ATCC 6871^{T}			0.01 (4.3)	0.01 (4.3)	**0.12** (52.2)	Tr	**0.09** (39.1)	0.23
Corynebacterium ammoniagenes WS 1572 (= ATCC 13746)			0.01 (4.5)	0.02 (9.1)	**0.13** (59.1)	Tr	**0.06** (27.3)	0.22
Corynebacterium callunae DSM 20147^{T} (= ATCC 15991^{T} = NCIB 10338^{T})	**0.27**	0.01 (6.7)	0.01 (6.7)	0.01 (6.7)	**0.09** (60.0)		**0.03** (20.0)	0.15
Corynebacterium cystitidis WS 1701^{T} (= ATCC 29593^{T} = DSM 20524^{T})				0.01 (1.0)	**0.35** (30.0)		**0.82** (69.5)	1.18
Corynebacterium flavescens DSM 20296^{T} (= ATCC 10340^{T} = NCIB 8707^{T})		0.05 (6.2)	0.03 (3.8)	**0.28** (35.0)	**0.28** (35.0)		**0.16** (20.0)	0.80
Corynebacterium glutamicum DSM 20300^{T} (= ATCC 13032^{T} = NCIB 10025^{T})	**0.41**	0.11 (14.5)	0.05 (6.6)	0.04 (5.3)	0.13 (17.1)		**0.43** (56.6)	0.76
Corynebacterium glutamicum DSM 20300^{T}[d]	**0.08**		**0.05** (41.6)		**0.05** (41.6)		0.02 (16.7)	0.12
Corynebacterium kutscheri NCTC 949		0.07 (4.5)	0.24 (15.6)	0.01 (0.7)	**0.78** (50.6)		**0.44** (28.6)	1.54
Corynebacterium lilium DSM 20137 (= ATCC 15990 = NCIB 10337)	**0.50**	0.06 (16.2)			**0.09** (24.3)		**0.22** (59.5)	0.37
Corynebacterium minutissimum NCTC 10288^{T} (= ATCC 23348^{T} = DSM 20651^{T})		0.07 (14.9)		**0.10** (21.3)	**0.18** (38.3)		**0.12** (25.5)	0.47
Corynebacterium minutissimum NCTC 10284		0.04 (9.5)	0.05 (11.9)	0.03 (7.1)	**0.20** (47.6)		**0.10** (23.8)	0.42
Corynebacterium mycetoides WS 2140 (= CCM 576)	**1.76**		0.01 (3.1)	0.05 (15.6)	**0.19** (59.4)		**0.07** (21.9)	0.32
Corynebacterium pilosum WS 1700^{T} (= DSM 20521^{T} = ATCC 29592^{T})	**0.85**		0.05 (18.5)		**0.18** (66.7)		0.04 (14.8)	0.27
Corynebacterium pseudodiphtheriticum NCTC 11136^{T} (= ATCC 10700^{T})		**0.12** (27.3)	0.05 (11.4)	0.01 (2.3)	**0.11** (25.0)	0.08 (18.2)	0.07 (15.9)	0.44
Corynebacterium pseudodiphtheriticum NCTC 8633			**0.16** (43.2)	0.02 (5.4)	**0.12** (32.4)	Tr	0.07 (18.9)	0.37
Corynebacterium renale WS 1826^{T} (= DSM 20298^{T} = ATCC 10848^{T})		0.01 (2.0)	**0.30** (60.0)	0.01 (2.0)	**0.12** (24.0)	Tr	0.06 (12.0)	0.50
Corynebacterium renale NCTC 11141		0.01 (2.0)	0.03 (6.1)	0.01 (2.0)	**0.31** (63.3)		**0.13** (26.5)	0.49
Corynebacterium striatum WS 11129^{T} (= DSM 20668^{T} = ATCC 6940^{T})	**0.35**		**0.19** (38.0)	0.04 (8.0)	**0.16** (32.0)	0.03 (6.0)	0.08 (16.0)	0.50
Corynebacterium striatum A54/86	**0.19**		**0.03** (23.1)	0.02 (15.4)	**0.06** (46.2)		0.02 (15.4)	0.13
Corynebacterium vitarumen DSM 20294^{T} (= ATCC 10234^{T})	**0.56**	**0.18** (40.9)			**0.20** (45.5)		0.06 (13.6)	0.44
Tsukamurella inchonensis DSM 44067^{T}			0.21 (18.1)	0.09 (7.8)	**0.74** (63.8)	Tr	0.12 (10.3)	1.16
Tsukamurella paurometabola DSM 20162^{T} (= ATCC 8368^{T})	0.06			0.02 (1.6)	**1.13** (88.3)		0.13 (10.2)	1.28
Tsukamurella wratislaviensis DSM 44107^{T}		0.01 (11.1)	**0.03** (33.3)		**0.03** (33.3)	0.01 (11.1)	0.01 (11.1)	0.09

[a] NCTC, National Collection of Type Cultures, London, England; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic. For an explanation of other abbreviations, see Table 1, footnote [a].

[b] The values in boldface type are the values for the major polyamines (polyamines which accounted for more than 20% of the total amines). The values in parentheses are percentages.

[c] The values for the unknown compound are not included.

ganensis, ICMP 2250^{T} and ICMP 2539, also contained remarkable amounts of putrescine, which may be a strain-specific feature. A remarkably high concentration of polyamines in *Rathayibacter iranicus* ICMP 3496^{T} differentiated this strain from the other *Rathayibacter* strains analyzed, and this might be a species-specific characteristic. This hypothesis could not be confirmed as no more strains of this species are available. The allocation of strain *Clavibacter* sp. strain VKM Ac-1371 to the genus *Clavibacter* was supported by the detection of spermidine and spermine as the predominant polyamines in this organism. The *Clavibacter-Rathayibacter*-specific polyamine pattern distinguished these two genera from the genus *Agromyces*, which is characterized by peptidoglycan type B2γ as well (55). *Agromyces* species had rather low concentrations of polyamines, and putrescine was the predominant compound. The recently proposed species *Agromyces mediolanus* (formerly "*Corynebacterium mediolanum*") (45) displayed the characteristic polyamine pattern observed for the genus *Agromyces*, thus supporting allocation to this genus.

When *Clavibacter michiganensis* subsp. *michiganensis* ICMP 1808 was grown on mineral medium with succinate as the only carbon source, only traces of spermine were detected (Table 1), whereas the amount of spermidine increased ninefold compared to cells of the same strain grown in complex medium. This observation indicates that, as in *Escherichia coli* (46), spermine is taken up from the growth medium and that this compound substitute for spermidine. The fact that spermine is not synthesized in significant amounts by the cell itself does not reduce the importance of the presence of this polyamine for classification purposes. Species of other genera analyzed in this study, such as *Aureobacterium* species, *Agromyces* species, some *Microbacterium* species, and *Brevibacterium* species, did not contain comparable concentrations of spermine when they were grown on the same medium. Thus, the presence or absence of significant concentrations of spermine in bacterial cells can be used as a distinguishing characteristic.

Closely related to the *Clavibacter-Rathayibacter* group is the genus *Curtobacterium* (38, 48), which contains D-ornithine in

TABLE 4. Chemotaxonomic features of the taxa included in this study

Taxon	Polyamine pattern[a]	Peptidoglycan type[b]	Amino acid(s) in cell wall[c]	Major menaquinone(s)
Aureobacterium barkeri	SPD	B2β	L-Hsr, D-Orn	MK-11, MK-12
Aureobacterium liquefaciens	PUT, SPD	B2β	L-Hsr, D-Orn	MK-11, MK-12
Aureobacterium testaceum	Unspecific	B2β	L-Hsr, D-Orn	MK-11
Microbacterium imperiale	PUT	B1β	L-Hsr, L-Lys	MK-11, MK-12
Microbacterium aurum	SPD, SPM	B1β	L-Hsr, L-Lys	MK-11, MK-12
Microbacterium arborescens	DAP, PUT, SPD	B1β	L-Hsr, L-Lys	MK-11, MK-12
Microbacterium lacticum	PUT, SPD	B1α	L-Lys	MK-11, MK-12
Microbacterium laevaniformans	Unspecific	B1α	L-Lys	MK-11, MK12
Curtobacterium	SPD, SPM	B1α	L-Lys, D-Orn	MK-9
Agrococcus	SPM	Unknown	L-Dab	MK-11, MK-12
Rathayibacter	SPD, SPM	B2γ	L-Dab	MK-10
Clavibacter	SPD, SPM	B2γ	L-Dab	MK-9
Agromyces	PUT	B2γ	L-Dab	MK-11, MK-12
"*Brevibacterium helvolum*"	DAP, CAD	B2γ	L-Dab	MK-9
Brevibacterium	PUT, CAD	A1γ	*m*-Dap	MK-8(H$_2$)
Corynebacterium	SPD	A1γ	*m*-Dap	MK-8(H$_2$), MK-9(H$_2$)
Tsukamurella	SPD	A1γ	*m*-Dap	MK-9

[a] For abbreviations see Table 1, footnote [a].

[b] Peptidoglycan types as described by Schleifer and Kandler (38).

[c] *m*-Dap, *meso*-diaminopimelic acid; L-Lys, L-lysine; L-Dab, L-diaminobutyric acid; L-Hsr, L-homoserine; D-Orn, D-ornithine. See Table 1 for the strains investigated in this study.

the peptidoglycan layer. The close relationship is indicated by the presence of a similar polyamine pattern (Table 1). Despite the predominance of spermidine and spermine, the curtobacteria may be distinguished from the genera *Clavibacter* and *Rathayibacter* by their lower spermidine content.

The misnamed strain "*Brevibacterium helvolum*" WS 1753 is another representative of the peptidoglycan type B2γ bacteria located on a separate branch within this group (38). This strain contained cadaverine as the predominant compound, and there was a significant concentration of 1,3-diaminopropane in its polyamine pattern. These features were not detected in any of the other strains analyzed in this study and thus support the position of "*B. helvolum*" WS 1753 separate from other peptidoglycan type B2γ bacteria.

Polyamines in *Brevibacterium* species. The genus *Brevibacterium* is only distantly related to the other actinobacteria (9, 38). Members of this genus form a clear generic cluster and are characterized by peptidoglycan type A1γ with *meso*-diaminopimelic acid. The uniform polyamine pattern detected in the group consisting of strains obtained as *Brevibacterium linens*, *Brevibacterium casei*, *Brevibacterium epidermidis*, and *Brevibacterium iodinum* (Table 2) supports the close relatedness of these species. The polyamine patterns showed that cadaverine is the major compound but that high levels of putrescine are also present. *B. linens* WS 1954 lacked significant amounts of putrescine. This strain belongs to a group of *Brevibacterium* strains that can be distinguished from the type strain of *B. linens* by differences in the polysaccharide moiety of the cell wall (14). Based on DNA-DNA similarity studies, this group is assumed to represent a species separate from *B. linens*. The variation in the polyamine pattern might also indicate some distant relatedness of *B. linens* WS 1954 to the type strain of the species.

Polyamines in mycolic acid-containing actinobacteria. In *Corynebacterium* species the overall content of well-known polyamines such as 1,3-diaminopropane, putrescine, cadaverine, spermidine, and spermine was relatively low. One of these polyamines, spermidine, was always a predominant compound, and often high concentrations of spermine were also detected. In some species an as-yet-unidentified compound was detected. This compound eluted 0.6 min before 1,3-diaminopropane during the HPLC analysis. We do not know whether this compound can be considered a polyamine. Based on the presence of this unknown compound, the *Corynebacterium* species could be subdivided into two groups. One group consisting of *Corynebacterium callunae*, *Corynebacterium glutamicum*, *Corynebacterium mycetoides*, *Corynebacterium lilium*, *Corynebacterium pilosum*, *Corynebacterium striatum*, and *Corynebacterium vitarumen* contained the unknown compound, which was not present in *Corynebacterium ammoniagenes*, *Corynebacterium cystitidis*, *Corynebacterium flavescens*, *Corynebacterium kutscheri*, *Corynebacterium minutissimum*, *Corynebacterium pseudodiphteriticum*, and *Corynebacterium renale*. Recent analyses of the 16S ribosomal DNAs of species of the genus *Corynebacterium* have demonstrated that this taxon is rather homogeneous (37, 40). In some cases the reliability of the resulting phylogenetic trees is questionable. This is indicated by the relatively low bootstrap values and different orders of branching in the phylogenetic trees of Ruimy et al. (40) and Pascual et al. (37). All *Corynebacterium* species analyzed in this study belong to cluster I of Ruimy et al. (40). Clustering according to the presence or absence of the unknown compound did not reflect the subgroups within cluster I. So far, a closer relationship among the species containing the unknown compound cannot be assumed since it is not supported by other data. However, detection of the unknown compound in an actinobacterial strain might be of diagnostic value, since the presence of this compound may be characteristic for certain species. It was detected in *Corynebacterium glutamicum*, *Corynebacterium lilium* (which is considered synonymous with *Corynebacterium glutamicum* [44]), and two strains of *Corynebacterium striatum* (Table 3). The observation that significant amounts of this compound were found only in certain *Corynebacterium* species (Table 3) may be a chemotaxonomic indication for these species. In order to evaluate the importance of the unknown compound in classification of *Corynebacterium* species, more species of this genus must be analyzed and the branching in cluster I needs to be determined more reliably.

As in *Clavibacter michiganensis* subsp. *michiganensis* ICMP 1808, the concentration of spermine decreased significantly in

Corynebacterium glutamicum DSM 20300$^{\mathrm{T}}$ when this strain was grown in mineral medium. It can be supposed that *Corynebacterium glutamicum* DSM 20300$^{\mathrm{T}}$ is able to take up spermine from the medium as well as other *Corynebacterium* species.

Tsukamurella paurometabola DSM 20162$^{\mathrm{T}}$ and *T. inchonensis* DSM 44067$^{\mathrm{T}}$ are phylogenetic neighbors of the genus *Corynebacterium* (37, 40, 51). Both *Tsukamurella* species contained the predominant compound spermidine in their polyamine patterns. The spermidine concentrations in these species were appreciably higher than the concentrations in most *Corynebacterium* species but differed only slightly from the concentration found in *Corynebacterium kutscheri* (Table 3). In *T. wratislaviensis* DSM 44107$^{\mathrm{T}}$ (15) only traces of polyamines were detected. This might be related to the fact that the growth of the cells could not be measured spectrophotometrically since the cells did not grow in homogeneous suspensions. Thus, the point of harvest had to be estimated to obtain cells from the exponential growth phase for polyamine analysis. However, a clear distinction between species of the genera *Tsukamurella* and *Corynebacterium* does not seem to be possible due to the similarity in the polyamine patterns.

In conclusion, it is obvious from the results presented in this study that actinobacteria possess various polyamine patterns. In some cases, preliminary identification at the genus level based on the polyamine patterns seems to be possible. The closely related peptidoglycan type B2γ genera *Clavibacter* and *Rathayibacter*, which contain high concentrations of spermidine and spermine, cannot be distinguished by an analysis of their polyamine patterns. The peptidoglycan type B2γ organism "*B. helvolum*" WS 1753 and the genus *Agromyces* clearly differ in their polyamine patterns from the genera *Clavibacter* and *Rathayibacter*. "*B. helvolum*" WS 1753 is characterized by the presence of the predominant compound cadaverine, and *Agromyces* strains contain low levels of polyamines and the predominant compound is putrescine. The mycolic acid-containing genera *Tsukamurella* and *Corynebacterium*, which have peptidoglycan type A1γ cell walls, also have similar polyamine patterns, and spermidine is the predominant compound. The genus *Brevibacterium* also has peptidoglycan type A1γ cell walls but clearly differs from the genera mentioned above in its polyamine pattern, which is characterized by the predominant compound cadaverine and generally high concentrations of putrescine. However, the variety in polyamine patterns can be used as an important tool for polyphasic taxonomy within this heterogeneous group of gram-positive bacteria (Table 4).

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