Phylogenetic diversity in the genus *Bacillus* and comparative ribosomal protein AT-L30 analyses of the genus *Thermoactinomyces* and relatives

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The ribosomal L30 proteins from strains of 27 species belonging to the genera *Bacillus*, *Escherichia*, *Staphylococcus*, *Lactobacillus*, *Leuconostoc* and *Thermoactinomyces* were analysed, together with AT-L30 proteins from selected actinomycetes. The results of partial amino acid sequencing of L30 preparations revealed that the members of the genera *Escherichia*, *Staphylococcus* and *Thermoactinomyces* were homogeneous within each genus. In contrast, phylogenetic diversity existed in the genus *Bacillus*, which contained at least four clusters. One cluster that contained *Bacillus subtilis* and *Bacillus stearothermophilus* was more closely related to the genus *Staphylococcus* than to members of the other three *Bacillus* clusters. Members of the genus *Thermoactinomyces* were most closely related to the '*Bacillus subtilis* cluster', but less related to the other three *Bacillus* clusters. A distant phylogenetic relationship was detected between the genus *Thermoactinomyces* and its morphological relative, *Thermomonospora*.

Keywords: *Bacillus*, *Thermoactinomyces*, ribosomal protein AT-L30, amino acid sequencing

INTRODUCTION

It has been recognized that *Bacillus* is a very diverse genus, as indicated by the enormous range of genomic guanine-plus-cytosine (G+C) contents (32–69 mol%: Priest, 1981; Claus & Berkeley, 1986) and by the variety of phenotypes (Gordon, 1981; Logan & Berkeley, 1981). Moreover, recent phylogenetic investigations by 16S rRNA sequencing have shown the heterogeneity of *Bacillus* at the genus level. Based on the data set of 16S rRNA sequences Rössler *et al.* (1991) divided nine *Bacillus* species into four clusters. Similarly, Ash *et al.* (1991) and Farrow *et al.* (1992) recognized that over 50 species of *Bacillus* examined form five phylogenetically distinct clusters on the basis of 16S rRNA sequence data. The phylogenetic incoherency of *Bacillus* has also been shown by Stackebrandt *et al.* (1987) and Wisotzkey *et al.* (1992). At present 40 *Bacillus* species are listed in *Bergey's Manual of Systematic Bacteriology* (Claus & Berkeley, 1986). It is evident that the *Bacillus* species should be subjected to further phylogenetic investigation for the systematic reclassification of this genus.

The genus *Thermoactinomyces* contains thermophilic bacteria which form endospores like those of the genus *Bacillus*. It contains seven species in *Bergey's Manual of Systematic Bacteriology* (Lacey & Cross, 1989) and occupies a unique position in bacterial taxonomy. Until recently, it was considered to be an actinomycete, mainly because of its ability to form aerial mycelium when cultured on solid media. However, chemotaxonomic studies have cast doubts on the status of the members of the genus *Thermoactinomyces* as true actinomycetes: their wall peptidoglycan contains meso-diaminopimelic acid but no diagnostic sugars (wall chemotype III) (Becker *et al.*, 1965); their menaquinones are unsaturated with seven or nine isoprene units (Collins *et al.*, 1982); and spores are formed as endospores and contain dipicolinic acid (Cross *et al.*, 1971; Lacey, 1971). These characters are all shared with the genera *Bacillus* and *Clostridium* but not *Streptomyces*, a representative actinomycete genus (reviewed by Lacey & Cross, 1989). Moreover, the DNA base composition of *Thermoactinomyces* species has a G+C content (52–55 mol%) lower than that found in other actinomycete genera (about 70 mol%) but similar to that of *Bacillus* species (32–69 mol%) (Craveri & Manachini, 1966; Fritzsche, 1967). Recent phylogenetic investigations have supported the above concept and 16S rRNA data (Stackebrandt & Woese, 1981; Stackebrandt *et al.*,

Abbreviations: REM, relative electrophoretic mobility; SAS, similarity of amino acid sequences.

Table 1. Strains used in this study

Species	Strain designation*	Species abbreviation used in this paper
<i>Bacillus alvei</i>	ATCC 6344 ^T	Bal
<i>Bacillus brevis</i>	JCM 2503 ^T (= ATCC 8246 ^T)	Bbr
<i>Bacillus cereus</i>	JCM 2152 ^T (= ATCC 14579 ^T)	Bce
<i>Bacillus cycloheptanicus</i>	ATCC 49028 ^T	Bcy
<i>Bacillus laterosporus</i>	JCM 2496 ^T (= ATCC 8248 ^T)	Bla
<i>Bacillus macerans</i>	JCM 2500 ^T (= ATCC 8244 ^T)	Bma
<i>Bacillus macquariensis</i>	ATCC 23464 ^T	Bmq
<i>Bacillus megaterium</i>	JCM 2506 ^T (= ATCC 14581 ^T)	Bme
<i>Bacillus polymyxa</i>	JCM 2507 ^T (= ATCC 842 ^T)	Bpo
<i>Bacillus stearothermophilus</i>	NCA 1503	Bst
<i>Bacillus subtilis</i>	JCM 1465 ^T (= ATCC 6051 ^T)	Bsu
<i>Bacillus subtilis</i>	Marburg	Bsu
<i>Escherichia coli</i>	K12	Eco
<i>Escherichia fergusonii</i>	JCM 5897 (= ATCC 35471)	Efe
<i>Escherichia hermannii</i>	JCM 1473 ^T (= ATCC 33650 ^T)	Ehe
<i>Escherichia vulneris</i>	JCM 1688 ^T (= ATCC 33821 ^T)	Evu
<i>Lactobacillus plantarum</i>	JCM 1149 ^T (= ATCC 14917 ^T)	Lpl
<i>Leuconostoc mesenteroides</i>	JCM 6124 ^T (= ATCC 8293 ^T)	Lme
subsp. <i>mesenteroides</i>		
<i>Nocardia asteroides</i>	JCM 3384 ^T (= ATCC 19247 ^T)	Nas
<i>Staphylococcus aureus</i>	JCM 2151 (= ATCC 6538P)	Sau
<i>Staphylococcus aureus</i>	209P	Sau
<i>Staphylococcus epidermidis</i>	JCM 2414 ^T (= ATCC 14990 ^T)	Sep
<i>Staphylococcus hyicus</i> subsp. <i>hyicus</i>	JCM 2423 ^T (= ATCC 11249 ^T)	Shy
<i>Staphylococcus xylosus</i>	JCM 2418 ^T (= ATCC 29971 ^T)	Sxy
<i>Streptomyces parvulus</i>	ATCC 12434 ^T	Spa
<i>Thermoactinomyces intermedius</i>	JCM 3312 ^T (= ATCC 33205 ^T)	Tin
<i>Thermoactinomyces sacchari</i>	JCM 3137 ^T (= ATCC 27375 ^T)	Tsa
<i>Thermoactinomyces thalpopophilus</i>	JCM 3217 ^T	Tth
<i>Thermoactinomyces vulgaris</i>	JCM 3162 ^T	Tvu
<i>Thermomonospora chromogena</i>	JCM 6244 ^T	Tch
<i>Thermomonospora curvata</i>	JCM 3096 ^T (= ATCC 19995 ^T)	Tcu
<i>Thermomonospora mesophila</i>	JCM 3151 ^T (= ATCC 27303 ^T)	Tme

* T, type strain.

1987) have established that the genus *Thermoactinomyces* is within the bacillus group and not the actinomycete group.

Ribosomes and their components have provided an excellent means of studying the evolution of organisms, since they are essential components for all cells. In addition to rRNA sequencing, ribosomal protein sequencing has also proved a useful approach, as demonstrated for members of the archaeobacteria (Wittmann-Liebold *et al.*, 1990). During the last few years I have developed a novel method for the identification and classification of actinomycetes which depends on the heterogeneity of the ribosomal AT-L30 proteins among actinomycete genera (Ochi, 1989, 1992a, b). Analysis of the AT-L30 proteins by two-dimensional PAGE or by N-terminal amino acid sequencing has been used successfully to review the taxonomic status of various actinomycete genera (Ochi & Yoshida, 1992; Ochi & Miyadoh, 1992; Ochi *et al.*, 1991, 1993a, b; Ochi & Hiranuma, 1994). The

extent of amino acid sequence homology among AT-L30 proteins has been a useful standard for classification at the genus level. The aim of the present study was to review the taxonomic status of the genera *Bacillus* and *Thermoactinomyces* by analysing the amino acid sequence of their L30 proteins.

METHODS

Bacterial strains. Almost all the strains used in this study were type strains (Table 1) that were obtained from the Japan Collection of Micro-organisms, Wako-shi, Saitama, Japan, and the American Type Culture Collection, Rockville, MD, USA. Although *Bacillus cycloheptanicus* is currently assigned to the genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992), the earlier name has been used for convenience in this study. The strains were grown to mid-exponential phase in soluble starch/polypeptone/yeast extract medium at 30 °C (for actinomycetes) or 20–55 °C (for other eubacteria).

Two-dimensional PAGE. Two-dimensional PAGE by the

method of Kaltschmidt & Wittmann (1970) has been described in detail previously (Ochi, 1989). The L30 or AT-L30 protein spot was identified after each slab gel had been stained with Coomassie blue. The electrophoretic mobilities (in the first dimension) of the L30 or AT-L30 proteins were expressed as relative electrophoretic mobilities (REMs) compared with the electrophoretic mobility of *Saccharomonospora viridis* AT-L30 protein, which exhibited the greatest mobility of all of the actinomycete AT-L30 proteins examined (Ochi, 1992a).

Determination of amino acid sequence. Amino acid sequence analysis of each L30 or AT-L30 protein was carried out as described in detail by Ochi & Miyadoh (1992), using a model 470A protein sequencer (Applied Biosystems). The method is based upon the Edman degradation, and determination of a maximum of 27 N-terminal amino acids was possible when about 100 pmol of L30 preparation was sampled. In *Escherichia coli* the L30 protein that corresponds to the actinomycete AT-L30 proteins consists of 58 amino acid residues. The 27 N-terminal amino acids, therefore, represent nearly half of the AT-L30 protein, assuming that the L30 protein from *E. coli* and the AT-L30 protein from actinomycetes are of the same size.

RESULTS

Two-dimensional PAGE analysis of ribosomal proteins

The ribosomal proteins of 27 species of the Gram-positive or -negative bacteria (Table 1) were extracted with acetic acid and separated by two-dimensional PAGE. The electrophoretic mobilities of the proteins assigned as L30 proteins in the first dimension of gel electrophoresis were similar for the species of the genera *Escherichia*, *Bacillus*, *Staphylococcus*, *Lactobacillus* and *Leuconostoc* (REMs 68–76). Similar REM values (65–73) were detected for the four *Thermoactinomyces* species examined. Thus, eubacteria, including the genus *Thermoactinomyces*, appear to have a constant REM value of about 70. These results contrast with a previous observation that among the genera of actinomycetes there exists a considerable electrophoretic heterogeneity of ribosomal AT-L30 proteins (Ochi, 1992a, b). The experimental error for the REMs was $\leq 5\%$, as determined from several gel runs of the same sample. To obtain further insight into the phylogenetic relationship we next conducted an amino acid sequence analysis of the L30 or AT-L30 proteins.

Amino acid sequences of L30 or AT-L30 proteins (1) Genera *Escherichia* and *Staphylococcus*

We first analysed L30 proteins from four species each of the genera *Staphylococcus* and *Escherichia*, which were taken as representatives of Gram-positive and -negative bacteria, respectively (Fig. 1). To express quantitatively the levels of similarity of the amino acid sequences (SAS values), we determined the frequency of appearance of the same amino acid in 20 N-terminal amino acids of each L30 protein for every combination of strains tested (Table 2).

Staphylococcus epidermidis and *Staphylococcus xylosus*, isolated from human skin, are validly described by Schleifer & Kloos (1975) as new species in the genus *Staphylococcus*. The former strain, together with *S. aureus*, has been

subjected to 5S rRNA sequence analysis (Dekio *et al.*, 1984). *Staphylococcus hyicus* was validly published by Devriese *et al.* (1978). As shown in Fig. 1 and Table 2, these four species revealed a high level of amino acid sequence homology (SAS $\geq 94\%$) for every combination of strains. Complete homology was detected between *S. aureus*, *S. epidermidis* and *S. xylosus*.

	1	5	10	15	20	25
Eco	A--KT	IKIT	QTRSA	IGRL	PKHKATLLGLGL	
Efe	A--KT	IKIT	QTRSA	IGRL	PK?KATLLG	
Ehe	A--KT	IKIT	QTRSA	IGRL	P??KA	
Evu	A--KT	IKVT	QTRSA	IGRL	PKHKATLLG	
Sau	A--K--	LQITL	TRSV	IGRP	PETQRKTVEAL	
Sau	A--K--	LQITL	TRSV	IGRP	PET	
Sep	A--K--	LQITL	TRSV	IGRP	PET	
Shy	A--K--	IQITL	TRSV	IGRP	PETQ?KTV?AL	
Sxy	A--K--	LQITL	TRSV	IGRP	PETQ?K?V?AL	
Bsu	A--K--	LEITL	KRSV	IGRP	EDQRVTV?TLGL	
Bsu	A--K--	LEITL	KRSV	IGRP	EDQRVTV?TLGL	
Bme	ANK--	LAITL	TRSV	IGRP	EDQR	
Bce	A--KKLE	ITL	TRSV	IGRP	EDQR	
Bst	A--KKLA	ITL	TRSV	IGRP	EDQRITVRTLGL	
Bmq	T--KQLE	ITL	VRSL	IGRP	GDQRATVN	
Bma	A--K--	LQITL	VRSL	IGRP	EP?QRT	
Bal	A--K--	LQITL	VRSL	IGRP	ET--RT	
Bpo	A--K--	LEITL	VRSL	IGRP	GTQRT	
Bla	A--KSL	QITL	KRSV	IGRP	ADQK	
Bbr	A--K--	LQITL	KRSV	IGRP	TVDR	
Bcy	ASK--	LAITL	KKSP	IGRP	ENQRKTA	
Tin	A--KKL	AITL	KRSV	IGRP	ESQR	
Tsa	A--KKL	AVTL	KRSV	IGRP	EPVQR	
Tth	A--KKL	AVTL	KRSV	IGRP	EPVQR	
Tvu	A--KKL	AITL	KRSV	IGRP	ESQVRTL	
Lpl	A----	QLKIT	TLVRSAA	?RL	LPKQK	
Lme	A----	DLKIT	TLIKSAVHR	LPKQRAIVKSL		
Spa	A----	QLKIT	QVKS	YIGSKQ	N??DTL?SLGL	
Nas	A----	DLKIT	QVKS	YIGSKQ	NQKDSL	
Tch	A----	RLKIT	QVRS	KIGGKQ	NQ?DSL??L	
Tcu	S----	RLKIT	QVRS	KIGGKQ	NQ?N	
Tme	A----	RLKIT	QLRS	KIGGKQ	NQ?D?L	

Fig. 1. Primary structures of N-termini of the L30 or AT-L30 proteins from eubacteria or actinomycetes. The abbreviation used for each organism is presented in Table 1. The amino acid numbering is given above the sequence of *E. coli*. Question marks indicate amino acids that were not determined. The dotted lines indicate deletions. The common sequence found in all strains or in a specified genus is indicated by an arrow or box, respectively. Sau (upper line), *Staphylococcus aureus* JCM 2151; Sau (lower line), *Staphylococcus aureus* 209P; Bsu (upper line), *Bacillus subtilis* JCM 1465^T; Bsu (lower line), *Bacillus subtilis* Marburg. The data for *Escherichia coli* K12 and *Bacillus stearothermophilus* NCA 1503 are from Ritter & Wittmann-Liebold (1975) and Kimura (1984), respectively. JP accession numbers are as follows: Efe (JP0042), Ehe (JP0043), Evu (JP0044), Sau (JP0045), Sep (JP0046), Shy (JP0047), Sxy (JP0048), Bsu (JP0049), Bme (JP0050), Bce (JP0051), Bmq (JP0052), Bma (JP0053), Bal (JP0054), Bpo (JP0055), Bla (JP0056), Bbr (JP0057), Bcy (JP0058), Tin (JP0059), Tsa (JP0060), Tth (JP0061), Tvu (JP0062), Lpl (JP0063), Lme (JP0064), Spa (JP0065), Nas (JP0066), Tch (JP0067), Tcu (JP0068), Tme (JP0069).

Table 2. SAS values for the amino acid sequences of L30 or AT-L30 proteins

The SAS values for each pair of organisms for 20 N-terminal amino acids of the L30 or AT-L30 proteins was determined on the basis of the data shown in Fig. 1. Non-determined positions were not weighted for the calculation of SAS values. The abbreviations used for each organism are presented in Table 1.

Species	SAS values (%)																			
	Eco	Evu	Sau	Shy	Bsu	Bme	Bce	Bpo	Bmq	Bma	Bal	Bst	Bla	Bbr	Bcy	Tsa	Tvu	Lpl	Lme	Spa
Eco	95																			
Evu	50	45																		
Sau	58	53	94																	
Shy	45	40	84	78																
Bsu	45	40	80	74	80															
Bme	50	45	85	79	90	90														
Bce	45	40	79	72	79	65	75													
Bpo	40	35	65	58	75	65	75	85												
Bmq	47	42	89	82	83	74	79	89	79											
Bma	45	40	84	78	74	65	70	84	70	94										
Bal	50	45	85	79	85	95	95	70	70	79	70									
Bst	50	45	70	68	75	65	70	70	70	79	70	70								
Bla	45	40	74	67	79	65	70	74	70	83	74	70	80							
Bbr	35	30	65	58	70	75	70	60	55	68	60	75	60	60						
Bcy	40	45	70	63	75	75	75	65	60	74	65	80	65	65	75					
Tsa	45	40	75	68	80	80	80	70	65	79	70	85	70	70	80	90				
Tvu	63	58	47	44	47	47	47	53	53	56	47	47	53	47	42	42	47			
Lpl	50	45	45	37	45	45	45	45	40	47	40	45	40	45	50	40	45	72		
Lme	44	39	39	33	39	39	39	44	44	47	44	39	39	39	50	33	39	50	41	
Spa	40	45	35	32	35	35	35	35	30	37	30	35	45	35	45	40	35	39	47	65
Nas	47	42	47	42	47	47	47	53	47	56	47	47	47	47	47	42	47	53	39	76
Tch	47	42	47	42	47	47	47	47	42	50	42	47	47	47	47	42	47	47	39	61
Tme																				94

	1	5	10	15	20																
1	M	K	A	K	E	L	R	E	K	S	V	E	E	L	N	T	E	L	L	N	L
2	M	K	A	K	E	L	R	E	K	S	V	E	E	L	N	T	E	L	L	N	
3	M	K	A	K	E	L	R	E	K	S	V	E	E	L	N	T	E	L	L	N	L
4	A	V	Q	Q	N	K	P	T	R	S	K	R	G	M	R						
5	A	V	P	A	K	P	T	S	?	V											
6	A	T	P	S	N	K	N	S	K												

Fig. 2. Primary structures of N-termini of the L29 proteins from *Escherichia coli* K12 (line 1), *Escherichia fergusonii* JCM 5897 (line 2) and *Escherichia vulneris* JCM 1688^T (line 3), or the L32 proteins from *Escherichia coli* K12 (line 4), *Lactobacillus plantarum* JCM 1149^T (line 5) and *Leuconostoc mesenteroides* JCM 6124^T (line 6). The data for the L29 or L32 protein from *E. coli* are from Bitar (1974) and Wittmann-Liebold *et al.* (1975), respectively. JP accession numbers are as follows: Efe (JP0070), Evu (JP0071), Lpl (JP0072), Lme (JP0073).

Escherichia hermannii, *E. vulneris* and *E. fergusonii* were reported to be new species in the genus *Escherichia*, mainly on the basis of DNA relatedness or biochemical aspects (Brenner *et al.*, 1982a, b; Farmer *et al.*, 1985). Amino acid sequence data of L30 proteins of these species, together with that of *Escherichia coli*, are shown in Fig. 1. These

species revealed a high level of homology (SAS $\geq 95\%$) for every combination of strains (Table 1). Complete homology was detected between *E. coli*, *E. hermannii* and *E. fergusonii*. In contrast, a low level of homology (SAS 50%) was detected between *E. coli* and *S. aureus* (Table 2). Amino acid sequence analysis of another ribosomal protein L29, from three *Escherichia* species demonstrated a close relatedness (SAS 100%) among the species examined (Fig. 2), leading to a conclusion similar to that obtained from the L30 analysis. Thus, within a single genus of eubacteria, the homology level of amino acid sequences in L30 proteins is very high. This has previously been reported for actinomycetes, in which a homology level higher than 85% was usually detected within a single genus (Ochi & Miyadoh, 1992; Ochi *et al.*, 1993a, b).

(2) Genus *Bacillus*

The eleven species of the genus *Bacillus* which we examined revealed considerable diversity in amino acid sequences of their L30 proteins (Fig. 1). The homology level was as low as 55% between *B. cycloheptanicus* and *B. macquariensis*, while it was as high as 95% between *B. cereus* and *B. stearothermophilus* (Table 2). On the basis of these SAS values we compiled a dendrogram (Fig. 3). The tree

Fig. 3. Clustering of L30 or AT-L30 protein SAS values. The dendrogram was constructed using the data in Fig. 1. The abbreviation used for each organism is presented in Table 1.

was constructed by using a phylogenetic algorithm to minimize the fluctuation. In the tree, eleven *Bacillus* species were divided into four major clusters; the first cluster included *B. subtilis*, *B. stearothermophilus*, *B. cereus* and *B. megaterium*; the second cluster included *B. alvei*, *B. macerans*, *B. polymyxa* and *B. macquariensis*; the third cluster included *B. brevis* and *B. laterosporus*; and the fourth branch had a single representative *B. cycloheptanicus*. Strikingly, this classification was entirely consistent with the results from 16S rRNA sequence analysis conducted by Rössler *et al.* (1991). Within each of three multi-species clusters, greater than 80% sequence homology existed between all combinations of members, whereas between the four clusters less than 70% sequence homology was found. Surprisingly, members of the first cluster including *B. subtilis* shared more sequence homology with the genus *Staphylococcus* than any of the other *Bacillus* clusters (Fig. 3). The result would support the need for reclassification of *Bacillus* at the genus level. *Bacillus cycloheptanicus* was most distantly related to other members of *Bacillus*.

(3) Genus *Thermoactinomyces*

The *Approved List of Bacterial Names* (Skerman *et al.*, 1980) includes five species of the genus *Thermoactinomyces*, and *Bergey's Manual of Systematic Bacteriology* (Lacey & Cross, 1989) recognizes seven, including *Thermo-*

actinomyces vulgaris, *T. intermedius*, *T. sacchari* and *T. thalpopophilus*. These four species are all thermophilic, growing at 30–60 °C. Endospores formed singly on both aerial and substrate mycelium are either sessile (*T. thalpopophilus*) or on unbranched sporophores (*T. vulgaris*, *T. sacchari*). The endospores of *T. intermedius* are sessile or on short sporophores. Highly homologous sequences (Fig. 1) were found among the four species examined (SAS 90–100%, Table 2), indicating that the genus *Thermoactinomyces* is phylogenetically homogeneous. AT-L30 proteins (homologous to the *E. coli* L30 protein) from *Streptomyces parvulus*, *Nocardia asteroides* and members of the genus *Thermomonospora* were also analysed as representatives of other actinomycete genera. The sequences for *Thermoactinomyces* species exhibited a higher level of homology to those of eubacteria than to those of actinomycetes (Fig. 1). It is especially noteworthy that, as shown in the dendrogram, the genus *Thermoactinomyces* is closely related to the members of the *Bacillus subtilis* cluster, the highest homology being with *Bacillus stearothermophilus* (SAS 85%, Fig. 3). *Thermoactinomyces* strains can resemble members of the actinomycete genus *Thermomonospora* in their morphology but with these latter organisms a distant phylogenetic relatedness was revealed (Fig. 3).

(4) Genera *Lactobacillus* and *Leuconostoc*

Lactobacillus plantarum and *Leuconostoc mesenteroides*, members of the lactic bacteria, were placed at a distant phylogenetic position not only from *Escherichia coli* but also from *Bacillus* species (Fig. 3). A distant relatedness between *E. coli* and *L. plantarum* or *L. mesenteroides* was also supported by the low level of homology in another ribosomal protein, L32 (Fig. 2).

Amino acid sequences characteristic for each genus

In addition to a simple comparison of the homology of amino acid sequences, it was possible to pinpoint amino acids, located at specific positions, which characterize each genus. Such amino acids are boxed in Fig. 1. The results can be summarized as follows. (1) In eubacteria, including the genus *Thermoactinomyces*, position 16 or 17 is characterized by proline, while in actinomycetes it is lysine. (2) Position 15 is characterized by arginine in eubacteria. (3) In actinomycetes, positions 5, 18 and 20 are characterized by lysine, asparagine and aspartic acid, respectively. Also, a deletion at position 2 appears to be a characteristic for actinomycetes. (4) Positions 7 (threonine) and 11 (serine) were invariable throughout the bacteria examined. (5) It is especially noteworthy that position 12 appears to reflect the classification at the genus level, as seen from alanine for the genus *Escherichia*, valine for the genus *Staphylococcus*, methionine for the genus *Thermoactinomyces*, and lysine for the genus *Thermomonospora*. More than 30 members of the genus *Streptomyces* examined were all characterized by tyrosine at this position (Ochi & Hiranuma, 1994). Similarly, the observed distinction among the *Bacillus* clusters can be further emphasized. For example, the members of the

genus *Bacillus* examined could be divided into three groups from the amino acid at position 12: a 'valine group' including *B. subtilis*, *B. megaterium*, *B. cereus* and *B. stearothermophilus*; a 'leucine group' including *B. alvei*, *B. polymyxa*, *B. macquariensis*, *B. macerans*, *B. brevis* and *B. laterosporus*; and a 'proline group' represented by *B. cycloheptanicus* as a single species (Fig. 1). It is striking that this grouping is consistent with the grouping based on the protein L30 sequence homology (Fig. 3), with the exception that the *B. subtilis* cluster and the *B. brevis* cluster were not discriminated.

Thus, together with comparison by sequence homology, position 12 can be used as a good standard for differentiation of bacteria at the genus level. For example, since methionine has not been found in any L30 or AT-L30 proteins examined so far, its presence at position 12 should be a characteristic typical for the genus *Thermoactinomyces*.

It was also noteworthy that in members of the *B. alvei* cluster including *B. macerans*, *B. macquariensis* and *B. polymyxa* a large fraction (15–70%) of the L30 proteins retained methionine residues in their N-termini.

DISCUSSION

This paper is a further expansion of our new approach to the classification of micro-organisms and raises an intriguing possibility that L30 protein analysis may be effective for the taxonomy of not only actinomycetes but also other eubacteria. Despite the similar electrophoretic properties of L30 proteins, eubacteria still exhibited extensive variability in amino acid sequence of the proteins. In the present study a close phylogenetic relationship between the genus *Thermoactinomyces* and the *B. subtilis* cluster within the genus *Bacillus* was demonstrated. Thus, despite the entirely different taxonomic approaches used, we reached a similar conclusion to that of Stackebrandt *et al.* (1987): that the genus *Thermoactinomyces* should be classified in the family *Bacillaceae* and not with the actinomycetes.

In the phylogenetic tree (Fig. 3) we can see three main stems, representing Gram-positive and Gram-negative bacteria, and actinomycetes. Although actinomycetes exhibit a Gram-positive staining reaction they form a deep branch from the main stem for Gram-positive eubacteria. This view is consistent with the work of Stackebrandt & Woese (1981) using 16S rRNA oligonucleotide cataloguing. Their work also demonstrated that the genus *Nocardia*, although it exhibits several eubacteria-like characters (Goodfellow & Lechevalier, 1989), belongs to the actinomycetes (Stackebrandt & Woese, 1981). Our results support the latter conclusion (Fig. 3).

The taxonomic status of *Thermoactinomyces vulgaris* and *T. thalophilus* has been confused at the species level: the epithet '*vulgaris*' is often used in the literature to describe *T. thalophilus*. These two organisms, however, could be clearly discriminated by differences in the L30 amino acid sequence at positions 6 and 18 (Fig. 1). Since 100% homology in AT-L30 amino acid sequence has been

detected in various *Streptomyces* species (Ochi & Hiranuma, 1994), the homology level (100% or not) might be used to demonstrate differentiation at the species level. Likewise, *Escherichia vulneris* and *S. hyicus* could be discriminated from *E. coli* and *S. aureus*, respectively, confirming that these are separable at the species level (Fig. 1).

It is generally believed that many kinds of ribosomal proteins, including L30 proteins, are assembled into ribosomal RNA after undergoing processing of their native forms (Srivastava & Schlessinger, 1990). Ribosomes containing pre-16S rRNA are not biologically active: processing to mature 16S rRNA molecules is obligate for competence in protein synthesis (Wireman & Sypherd, 1974). Therefore, it is curious that there are ribosomal L30 proteins with methionine in their N-termini. These unprocessed (or incompletely processed) L30 proteins in the ribosome fraction have been found only in the members of the *B. alvei* cluster of the genus *Bacillus* among the bacteria examined so far. This phenomenon may thus be a characteristic typical for this cluster.

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