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Phylogenetic Diversity of Acidophilic Sporoactinobacteria Isolated from Various Soils

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Spore forming actinobacteria (sporoactinobacteria) isolated from soils with an acidic pH in *Pinus thunbergii* forests and coal mine waste were subjected to taxonomic characterization. For the isolation of acidophilic actinobacteria, acidified starch casein agar (pH adjusted to 4-5) was used. The numbers of actinobacteria growing in acidic media were between 3.2 × 10^4 and 8.0 × 10^6 CFU/g soil. Forty three acidophilic actinobacterial strains were isolated and their 16S rDNA sequences were determined. The isolates were divided into eight distinctive phylogenetic clusters within the variation encompassed by the family *Streptomycetaceae*. Four clusters among them were assigned to the genus *Streptacidiphilus*, whereas the remaining four were assigned to *Streptomyces*. The clusters belonging to either *Streptomyces* or *Streptacidiphilus* did not form a monophyletic clade. The growth pH profiles indicated that the representative isolates grew best between pH 5 and 6. It is evident from this study that acidity has played a critical role in the differentiation of the family *Streptomycetaceae*, and also that different mechanisms might have resulted in the evolution of two groups, *Streptacidiphilus* (strict acidophiles) and neutrotolerant acidophilic *Streptomyces*. The effect of geographic separation was clearly seen among the *Streptacidiphilus* isolates, which may be a key factor in speciation of the genus.

Keywords: acidophilic sporoactinobacteria, neutrotolerant acidophile, *Streptomycetaceae*, *Streptomyces*, *Streptacidiphilus*

Acidophilic actinobacteria are filamentous, Gram positive, aerobic and chemoorganotrophic organisms belonging to *Actinomycetales* that grow in acidic environments (Lonsdale, 1985; Seong, 1992; Kim *et al.*, 2003). Their main morphological features are quite similar to those of the genus *Streptomyces*, including extensive branching of mycelia, formation of arthrospores with straight or flexuous spore chains, and smooth spore surfaces. Their optimal growth occurs at a pH of approximately 4.5-5 in mesophilic temperature ranges. The main chemotaxonomic properties are the presence of *LL*-diaminopimelic acid as the major diamino acid in the cell wall, galactose as the main diagnostic sugar in the whole cell hydrolysates, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides as the main polar lipids, and octahydrated menaquinones (MK-9[H]$_8$) with nine isoprene units as the major respiratory quinones. Earlier studies indicated that there are two main groups of acidophilic actinobacteria according to growth pH profiles, namely strict acidophilic groups and neutrotolerant acidophilic groups (Lonsdale, 1985; Seong, 1992). Probabilistic identification matrices have been constructed for acidophilic actinobacteria using the phenotypic properties (Seong *et al.*, 1995). Strict acidophiles were found to form a new genus, designated *Streptacidiphilus*, within the family *Streptomycetaceae* (Kim *et al.*, 2003), and three new species, namely *Streptacidiphilus albus*, *S. carbonis* and *S. neutrinimicus* were initially described. Two more species, *S. jianxiensis* (Huang *et al.*, 2004) and *S. oryzae* (Wang *et al.*, 2006), were described afterwards. In contrast, neutrotolerant acidophiles described to date (*S. yeochonensis*, *S. guanduensis*, *S. paucisporeus*, *S. rubidus*, and *S. yanglinensis*) were classified into *Streptomyces*. (Kim *et al.*, 2004; Xu *et al.*, 2006).

Seong (1992) reported that the number of acidophilic sporoactinobacteria may exceed 10^6 CFU/ml in envi-

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ronments such as soils inhabited by several species of *Pinus* and coal waste sites. However, there are only a handful of reports on the isolation and taxonomic characterization of these acidophilic actinobacteria, and their true diversity has yet to be explored. In this study, acidophilic actinobacteria were isolated from *P. thunbergii* soils and taxonomically characterized. Their phylogenetic positions using 16S rDNA sequences and pH profiles for growth were analyzed. The possible driving forces for the differentiation and evolution of these acidophiles are discussed.

Materials and Methods

Selective isolation and cultivation of acidophilic actinobacteria

Soil samples were collected from terrestrial environments in Korea (Table 1). Samples were heat treated following the procedure previously described by Seong (1992). For bacterial isolation, 10 ml of sterile 1/4 strength Ringer solution (Seong, 1992) was added to 1 g soil sample. These diluted samples were then shaken on a reciprocal shaker for 20 min followed by incubation at 50°C for 15 min. Aliquots (0.2 ml) of each dilution were spread onto acidified (pH 4-5) starch casein agar (SCA) supplemented with cycloheximide (final concentration of 50 μg/ml) and nystatin (50 μg/ml). Inoculated plates were then incubated at 30°C for at least one week, colonies characteristic of actinobacteria were then selected and subcultured using the same media.

DNA extraction, PCR amplification and sequencing of 16S rDNA

Total DNA was extracted from one week-old cultures as described by Park *et al.* (2005). The size and amount of extracted DNA were determined by agarose gel (1%) electrophoresis. Extracted DNAs were stored at −20°C until needed. PCR amplification of 16S rDNA was carried out using two universal primers, 27f; 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492r; 5'-GGY TAC CTT GTT ACG ACT T-3', which were also used for sequencing. The PCR consisted of an initial denaturation step at 95°C for 3 min, which was followed by 30 cycles of 95°C for 1 min, 55°C for 40 sec and 72°C for 1 min. Sequencing was performed using the service of COSMO Co. (Korea) and Solgent Co. (Korea).

Phylogenetic analysis

The sequences were proofread, edited and merged into full length sequences using the PHYDIT program version 3.0 (available at http://plaza.sun.ac.kr/~jchun/phydit). The sequences were aligned with those of reference taxa retrieved from public databases. Distance-based phylogenetic trees were generated using the model of Jukes and Cantor (1969) and neighbor-joining algorithm (Saitou and Nei, 1987). The topology

Table 1. Viable counts of acidophilic actinobacteria in soil samples

Location	Sampling site	Viable count (CFU/ml)
P. thunbergii soil, Sambong, Chungnam	0-3 cm depth	8.0×10^6
	3-6 cm depth	2.3×10^6
	12-15 cm depth	3.8×10^5
P. thunbergii soil, Anmyeon, Chungnam	0-3 cm depth	6.4×10^6
P. thunbergii soil, Yeochon, Chonnam†	L horizon	9.4×10^5
	F horizon	1.4×10^6
	H horizon	1.1×10^6
	A1 horizon	2.7×10^5
	A2 horizon	2.7×10^5
	C horizon	3.2×10^4
Coal waste, Hwasun, Chonnam†	Site 1	2.2×10^5
	Site 2	9.8×10^5
	Site 3	6.3×10^5
	Site 4	1.1×10^6

† Data from Seong (1992)

of phylogenetic trees was evaluated using bootstrap analysis with 1,000 resampled dataset. PHYLIP program ver. 3.5c (Felsenstein, 1993) was used for the analyses.

Results and Discussion

Isolation and viable counts of acidophilic sporoactinobacteria

Non-spore-forming microorganisms were eliminated from samples by drying and heat treatment. Growth of fungi, potential competitors of acidophilic actinobacteria, was prevented by using the fungal antibiotics nystatin and cycloheximide. Colonies that showed typical characteristics of actinobacteria were counted from the acidified SCA plates (Table 1). Viable counts of samples from *P. thunbergii* forests were 6.4×10^6 CFU/g soil in Anmyeon, Chungnam, $2.3 - 8 \times 10^6$ CFU/g soil in Sambong, Chungnam, and $3.2 \times 10^4 - 1.4 \times 10^6$ CFU/g soil in Yeochon, Chonnam, respectively. The counts of acidophilic actinobacteria from coal waste sites ranged between 2.2×10^5 and 1.1×10^6 CFU/g soil. The viable count data indicate that acidophilic actinobacteria are commonly occurring in soil microbial flora, as seen in previous observations (Lonsdale, 1985; Seong, 1992).

Forty three pure cultures obtained from this study as well as strains CN 668 and CN 671 isolated by Seong (1992) were subjected to further taxonomic characterization. The strains used in this study are listed in Table 2.

Phylogenetic analysis using 16S rDNA

The 16S rDNA sequences of 43 acidophilic actinobacteria were determined and the initial identification using a BLAST search indicated that the isolates could be assigned to either *Streptomyces* or *Streptacidiphilus* (Table 2). From the phylogenetic analysis using 1,358 nucleotide positions (*Streptacidiphilus*) and 1,368 nucleotide positions (*Streptomyces*) of 16S rDNA sequences, the isolates fell into eight distinctive phylogenetic clusters within the taxonomic variation encompassed by the two genera (Fig. 1). Four clusters (designated Clusters 1, 2, 3, and 4) among them were assigned to the genus *Streptacidiphilus*, whereas the remaining four were assigned to *Streptomyces*. (designated Clusters 5, 6, 7 and 8). Clusters 4 and 8 were single-membered clusters.

Amplified 16S ribosomal DNA restriction analysis (ARDRA) was performed for the rapid typing of isolates using procedures described elsewhere (Lim *et al.*, 2005; Park *et al.*, 2005; Yeon *et al.*, 2005). The typing based on ARDRA patterns, however, was not consistent with results of the phylogenetic analysis (data not shown).

Composition of clusters

Cluster 1 consisted of 10 strains assigned to *Streptacidiphilus*, all obtained from Anmyeon soil (Table 2). The closest neighbors to the cluster were the strains of *Streptacidiphilus jiangxiensis*, indicated by similarity values of 16S rDNA sequences between strains of Cluster 1 and *S. jiangxiensis* of 99.3 - 99.6%. All members of Cluster 1 shared identical sequences.

Cluster 2 also consisted of 10 *Streptacidiphilus* strains, including 8 isolates from Anmyeon soil and 2 isolates from a coal waste site, CN 668 and CN 671 (Seong, 1992). The latter two strains formed an independent subcluster (Fig. 1A). The closest neighbors in the phylogenetic tree were strains of *S. jiangxiensis*, which had 16S rDNA sequence similarities of 98.0 - 99.4%.

Cluster 3 contained three *Streptacidiphilus* strains from Sambong soil (Table 2). The strains were most closely related to strains of Cluster 1 and Cluster 4 with 99.3% and 99.5% 16S rDNA sequence similarities, respectively. The strains also shared 98.8 - 99.0% sequence similarities with strains of *S. jiangxiensis*. However, the three strains clearly formed an independent phylogenetic line (Fig. 1A).

Cluster 4 was a single-membered cluster containing strain AM-23. The strain exhibited high levels of similarity with Cluster 1 (99.9%), Cluster 2 (98.7 - 99.9%), Cluster 3 (99.5%) and strains of S. *jiangxiensis* (99.1 - 99.4%). It is not clear from the phylogenetic analysis alone if Clusters 1, 3, 4 and strains of *S. jiangxiensis* can be recognized as a single species. The four clusters shared greater than 99% 16S rDNA sequence similarities with one another, and further study is necessary to clarify the relationship among them.

Cluster 5 consisted of 13 *Streptomyces* strains from Sambong soils (Table 2). All of the strains shared identical 16S rDNA sequences. *S. lucensis* (98.9%) and *S. diastatochromogenes* subsp. *luteus* (98.9%) were found to be distant neighbors to the cluster.

Cluster 6 consisted of 5 *Streptomyces* strains from Sambong soils (Table 2). The strains of the cluster shared identical 16S rDNA sequences. *S. psammoticus* and *S. miharaensis* were the distant neighbors, sharing only 98.2% 16S rDNA sequence similarities with members of this cluster. It is likely that strains of Cluster 6 represent a novel species of *Streptomyces*.

Cluster 7 contained two *Streptomyces* strains from Anmyeon soil. This cluster was closely related with *Streptomyces rubidus* 13c15^T, a validly described neutrotolerant acidophilic *Streptomyces* species (Xu *et al.*, 2006), with a 16S rDNA similarity of 99.7%. The cluster was also related with *S. yeochonensis* CN 732^T (Kim *et al.*, 2004), sharing 98.5% 16S rDNA se-

Table 2. Actinobacterial strains used in this study

Cluster	Strain	Source	Accession no.
1	AM-06	*P. thunbergii* soil, A layer, Anmyeon	DQ904536
	AM-08	〃	DQ904538
	AM-17	〃	DQ904541
	AM-10	〃	DQ904545
	AM-11	〃	DQ904546
	AM-18	〃	DQ904548
	AM-24	〃	DQ904550
	AM-02	〃	DQ904535
	AM-30	〃	DQ994691
	AM-25	〃	DQ994695
2	AM-16	*P. thunbergii* soil, A layer, Anmyeon	DQ904547
	AM-20	〃	DQ904549
	AM-27	〃	DQ904551
	AM-29	〃	DQ994692
	AM-28	〃	DQ994693
	AM-26	〃	DQ994694
	AM-22	〃	DQ994697
	AM-21	〃	DQ994698
	CN 668†	Coal waste, Hwasun	AF074414
	CN 671†	〃	AF074411
3	SB-B35	*P. thunbergii* soil, C layer, Sambong	DQ904528
	SB-B34	〃	DQ994689
	SB-B33	*P. thunbergii* soil, B layer, Sambong	DQ994690
4	AM-23	*P. thunbergii* soil, A layer, Anmyeon	DQ904542
5	SB-B48	*P. thunbergii* soil, A layer, Sambong	DQ904532
	SB-B27	〃	DQ904539
	SB-B47	〃	DQ904555
	SB-12	〃	DQ904558
	SB-22	〃	DQ904559
	SB-B38	〃	DQ904552
	SB-B50	*P. thunbergii* soil, B layer, Sambong	DQ904533
	SB-B51	〃	DQ904534
	SB-B28	〃	DQ904540
	SB-B24	〃	DQ904543
	SB-B41	〃	DQ904544
	SB-B43	〃	DQ904553
	SB-B40	〃	DQ904529
6	SB-B44	*P. thunbergii* soil, A layer, Sambong	DQ904530
	SB-B45		DQ904531
	SB-B46		DQ904554
	SB-S31		DQ904556
	SB-25		DQ994688
7	AM-07	*P. thunbergii* soil, A layer, Anmyeon	DQ904537
	AM-04	〃	DQ994696
8	SB-04	*P. thunbergii* soil, A layer, Sambong	DQ904557

† Isolates obtained in the study by Seong (1992)

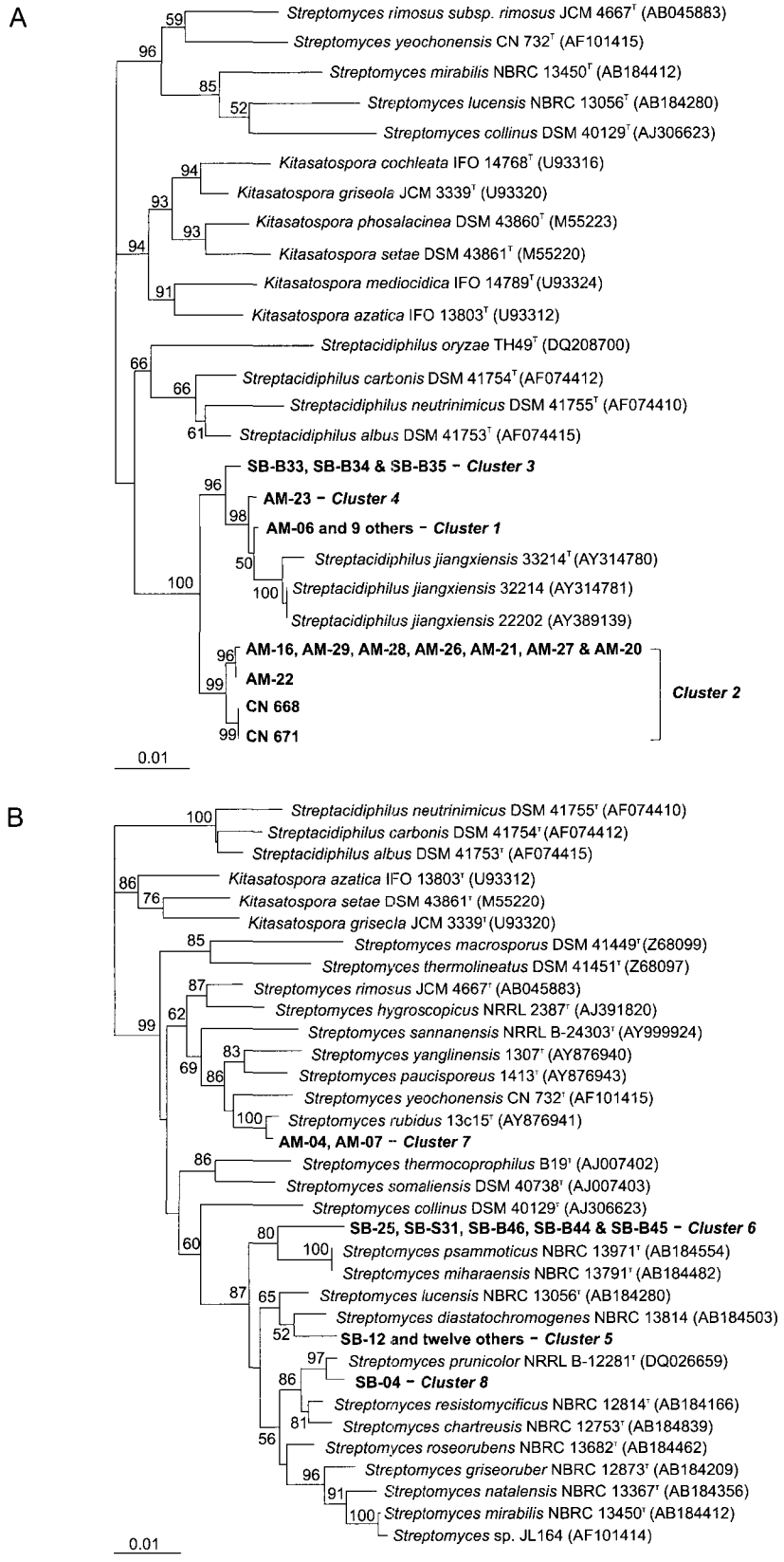

Fig. 1. Phylogenetic trees based on the 16S rDNA sequences of acidophilic actinobacterial isolates. A, isolates belonging to *Streptacidiphilus*. B, isolates belonging to *Streptomyces*. Numbers at nodes indicate levels of bootstrap support (%) determined from 1,000 resampled data. Scale bar corresponds to 0.01 substitutions per nucleotide position.

quence similarity. *Streptomyces yeochonensis* CN 732^T shared 98.5% nucleotide similarity with *S. rubidus*.

Cluster 8 was a single-membered cluster, containing strain SB-04. The strain was close to *S. prunicolor* with 99.5% 16S rDNA sequence similarity.

These phylogenetic data suggest that each of Clusters 2 and 3 belongs to a well circumscribed taxon within the genus *Streptacidiphilus* that can be equated to a novel species (Fig. 1A). Cluster 6 forms an independent phylogenetic lineage within *Streptomyces*, and thus can also be recognized as a new species of the genus (Fig. 1B).

Growth pH profile

The optimal pH for growth of strains *Streptacidiphilus* sp. CN 668 and CN 671 belonging to Cluster 2, and *Streptomyces yeochonensis* CN 732^T has been determined in a previous study (Seong, 1992). The optimal pH values were 5.0 for CN 671, 5.5 for CN 668 and 6.0 for *S. yeochonensis* CN 732. Seong (1992) reported that the optimal pH values ranged between 4.5 and 5.5 for the strict acidophilic group, whereas those for the neutrotolerant group were between 5.5 and 6.5. These findings are consistent with other observations that strict acidophilic sporactinobacteria comprise the genus *Streptacidiphilus*, whereas neutrotolerant counterparts belong to *Streptomyces* (Kim *et al.*, 2003, 2004; Huang *et al.*, 2004; Wang *et al.*, 2006; Xu *et al.*, 2006).

Acidity and evolution

Members of the family *Streptomycetaceae* are common constituents of microbiota in terrestrial environments. Soils are generally acidic due to the microbial decomposition of plant materials, therefore it would not be surprising to find high numbers of acidophilic actinobacteria belonging to *Streptomycetaceae* in soils. Previously acidophilic spore forming actinobacteria have been considered to be members of *Streptomyces* because of the similarities in morphological and chemotaxonomic properties. However, Kim *et al.* (2003) found that the strict acidophilic group is closer to *Kitasatospora* than to *Streptomyces*. Species of *Kitasatospora* can be differentiated from the strict acidophilic group and *Streptomyces* by the presence of *meso*-diaminopimelic acid in vegetative mycelia (Kim *et al.*, 2003).

Acidic environments are apparently the major selective pressure on members of *Streptacidiphilus*. The differentiation of streptacidiphili seems to be a relatively recent event, as the average 16S rDNA sequence similarities with members of *Kitasatospora* and *Streptomyces* are as high as 96.6% and 95.1%, respectively (Kim *et al.*, 2003). In contrast, members of the neutrotolerant acidophilic group are scattered within *Streptomyces*, indicating that neutrotolerant acidophily is not a character shared by an independent phylogenetic lineage (Fig. 1B).

In this study, the effect of geographic separation was demonstrated (Fig. 1, Table 2) by the observation that isolates from Anmyeon soil formed three distinct clusters within *Streptacidiphilus* and one within *Streptomyces*. The isolates from Sambong soils also formed one distinct *Streptacidiphilus* cluster and three distinct *Streptomyces* clusters. Mixed presence of *Streptacidiphilus* and *Streptomyces* strains was also notable, implying that two different phylogenetic lineages may have been exposed and adapted to the same environment.

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