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A Proposal To Revive the Genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982)

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We determined almost complete 16S ribosomal DNA sequences for 12 actinomycete strains which were either previously classified as *Kitasatospora* strains or defined as *Streptomyces* strains but shown to contain major amounts of *meso*-diaminopimelic acid in their whole-cell hydrolysates. These sequences were subjected to phylogenetic analyses together with the sequences of 34 *Streptomyces* species. Phylogenetic trees were reconstructed by using both neighbor-joining and maximum-parsimony methods. The *Kitasatospora* species always formed a stable monophyletic clade. However, the genus *Kitasatospora* appeared to be either a sister taxon of the genus *Streptomyces* or a lineage that originated from within *Streptomyces* species, depending on the outgroup used. Phylogenetic trees were also constructed by using the sequences of the 16S-23S rRNA gene spacers. *Streptomyces* and *Kitasatospora* species were consistently recovered as two distinct clades independent of the outgroup used. On the basis of phylogenetic, chemotaxonomic, and phenotypic evidence, we propose that the genus *Kitasatospora* Omura et al. 1982 should be revived.

The genus *Kitasatospora* was proposed by Omura et al. in 1982 for actinomycete strains which were phenotypically similar to *Streptomyces* strains but contained major amounts of the *meso* isomers of diaminopimelic acid (DAP) and galactose in whole-cell hydrolysates (15, 16). Takahashi et al. (24) studied the distribution of the two isomers of DAP in cells at different stages of differentiation and growth and found that *Streptomyces* species contained only LL-DAP in both aerial and vegetative mycelia, while *Kitasatospora* species contained LL-DAP in the aerial mycelium and *meso*-DAP in the vegetative mycelium. Although the relative amounts of the two isomers seemed to vary in different experiments and some *Streptomyces* species were also found to contain various amounts of *meso*-DAP, *Kitasatospora* species consistently had a much higher ratio of *meso*-DAP to LL-DAP than *Streptomyces* species (29). The *Kitasatospora* species are also characterized by their resistance to polyvalent *Streptomyces* phages (29) and the formation of submerged spores in liquid culture (15, 16, 24), which are features rarely observed in *Streptomyces* species.

Wellington et al. (29) reported that the 16S rRNA sequence of *Kitasatospora setae* showed 91.6% similarity to the 16S rRNA sequence of *Streptomyces baldaccii* and that a *Streptomyces*-specific oligonucleotide probe could recognize all four valid *Kitasatospora* species. On the basis of these observations and phenotypic properties shared by *Kitasatospora* and *Streptomyces* species, these authors proposed that the name *Kitasatospora* should be reduced to synonymy with the name *Streptomyces*. Ochi and Hiranuma (14) later supported this proposal on the basis of the results of an analysis of the N-terminal sequences of ribosomal protein AT-L30. However, the unification of these two genera is not unequivocal. For instance, Nakagaito et al. (12), using the results of DNA-DNA reassociation and phenetic studies, found that the *Kitasatospora* species and the original *Streptomyces* species formed two distinct clusters. Recently, Kim et al. (8) conducted a 16S rRNA sequence-based phylogenetic analysis of a large number of *Streptomyces* species and observed that three *Kitasatospora* species formed a distant and stable clade outside the clade comprising *Streptomyces* species.

In order to further clarify the phylogenetic relationship between the genera *Kitasatospora* and *Streptomyces*, we determined the nucleotide sequences of 16S rRNA genes and 16S-23S rRNA gene spacers of 12 actinomycete strains which were either previously classified as *Kitasatospora* strains or defined as *Streptomyces* strains but shown to contain major amounts of *meso*-DAP in their whole-cell hydrolysates. Here, we report the results of phylogenetic analyses in which we used sequences of both 16S ribosomal DNAs (rDNAs) and 16S-23S rRNA gene spacers, and we propose that the genus *Kitasatospora* Omura et al. 1982 (15) should be revived.

MATERIALS AND METHODS

Organisms and culture conditions. The actinomycete strains used in this study were purchased from the Japan Collection of Microorganisms (Wako, Japan) and the Institute for Fermentation (Osaka, Japan). Strain designations are listed in Table 1. Cells were cultured in media as described by the suppliers.

Preparation of genomic DNAs. The genomic DNAs were prepared as previously described (27, 28).

PCR amplifications. The pair of oligonucleotides and PCR conditions used for amplification of the nearly complete 16S rRNA genes have been described previously (27, 28). For amplification of the 16S-23S rRNA gene spacers, one primer was designed to target the conserved sequence at the end of the 16S rRNA gene, and the second primer was specific for a conserved block at the beginning of the 23S rRNA gene. The sequences of the two oligonucleotides are as follows: 5′ CCG GGA TCC GGT TGG ATC CAC CTC CTT3′ (nucleotides 1525 to 1542; *Escherichia coli* numbering [1]) and 5′ AAG GGA TCC TGC CAA GGC ATC CAC C3′ (nucleotides 33 to 48; *E. coli* numbering). The restriction site for *Bam*HI (underlined nucleotides) was added to each primer for convenient cloning of the PCR-amplified fragments. The PCR conditions were basically the same as those used for amplifying 16S rRNA genes (27, 28), except that the elongation time was 20 s.

Cloning and sequence analysis. Cloning and sequencing of the PCR-amplified rDNA and spacer fragments were carried out as described previously (27, 28).

Sequence alignment and phylogenetic analysis. Multiple alignment of sequences and computation of sequence similarities were carried out by using the Clustal method of the DNASTAR program (DNASTAR, Inc., Madison, Wis.). Evolutionary distance matrices were generated by the method of Jukes and Cantor (6). Phylogenetic trees were constructed by using both the maximum-parsimony method of the PAUP program (23) and the neighbor-joining method described by Saitou and Nei (18). The confidence level of phylogenetic tree topology was evaluated by performing 1,000 bootstrap replications and using the bootstrap program contained in the ClustalW package (4).

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TABLE 1. Actinomycete strains used

Taxon	Strain	GenBank accession no.	
		16S rDNA	Spacer region
Kitasatospora kifunense	JCM9081^T	U93322	U93323
"*Kitasatospora brunnea*"	IFO14627	U93314	U93315
"*Kitasatospora cystarginea*"	JCM7356^T	U93318	U93319
Kitasatospora griseola	JCM3339^T	U93320	U93321
Kitasatospora mediocidica	IFO14879	U93324	U93325
"*Kitasatospora melanogena*"	JCM3337^T	U93326	U93327
Kitasatospora phosalacinea	JCM3340^T	U93330	U93331
Kitasatospora setae	JCM3304^T	U93332	U93333
"*Nocardiopsis streptosporus*"	IFO14362	U93334	U93335
"*Streptomyces azaticus*"	IFO13803$_T$	U93312	U93313
"*Streptomyces cochleatus*"	IFO14768$_T$	U93316	U93317
"*Streptomyces paracochleatus*"	IFO14769$_T$	U93328	U93329
"*Kitasatospora grisea*"	JCM7249	U93336	U93337
"*Kitasatospora papulosa*"	JCM7250	U93338	U93339

RESULTS AND DISCUSSION

Organisms used in this study. The organisms used in this study are listed in Table 1. The original names of these organisms are used below. There are four valid *Kitasatospora* species (*Kitasatospora setae* [15], *Kitasatospora griseola* [25], *Kitasatospora phosalacinea* [25], and *Kitasatospora mediocidica* [10]) and four invalid *Kitasatospora* species ("*Kitasatospora melanogena*" [20], "*Kitasatospora brunnea*" [21], "*Kitasatospora cystarginea*" [9], and "*Kitasatospora kifunense*" [5]). "*Streptomyces cochleatus*" and "*Streptomyces paracochleatus*" (12) were defined after the proposed unification of the genera *Kitasatospora* with *Streptomyces*. These two species and the previously defined taxon "*Streptomyces azaticus*" (3, 12) were shown to contain major amounts of *meso*-DAP in their cell walls and to exhibit closer DNA-DNA relatedness with *Kitasatospora* species than with members of the original genus *Streptomyces* (12). "*Nocardiopsis streptosporus*" (11) was combined, by Nakagaito et al. (12), with *K. phosalacinea* and "*K. brunnea*" in one species. For convenience, we refer to the species mentioned above as members of the genus *Kitasatospora* throughout this paper. We also included "*Kitasatospora papulosa*" (13) and "*Kitasatospora grisea*" (13), which were originally classified as *Kitasatospora* species but later were found to belong to the original genus *Streptomyces* because no *meso*-DAP was detected in their cell walls (12).

Analysis of 16S rDNA sequences. We obtained almost complete 16S rDNA sequences (nucleotides 7 to 1507; *E. coli* numbering [1]) from all of the organisms described above and subjected them to phylogenetic analyses. The initial analysis, which included sequences from representative species of most actinomycete groups, confirmed that the kitasatosporae and streptomycetes were most closely related to each other in the order *Actinomycetales* (data not shown) (8, 29). We then carried out more detailed analyses in which we focused on sequences from members of the two groups of organisms. Figure 1 presents the phylogenetic trees inferred when the neighbor-joining method (18) was used. When the alignment gaps were included as a fifth base and *Nocardia asteroides* and *Bacillus subtilis* were used as outgroups (Fig. 1A), all of the *Kitasatospora* species aggregated, with a bootstrap value of 100%, in one clade separate from the clade containing *Streptomyces* species. This clade contained "*S. cochleatus*," "*S. paracochleatus*," "*S. azaticus*," and "*Nocardiopsis streptosporus*," which confirmed the close relationships of these species with members of the genus *Kitasatospora* determined by Nakagaito et al. (12). The misclassified taxa "*K. papulosa*" and "*K. grisea*" aggregated closely with six *Streptomyces* species. When the nucleotide positions corresponding to the alignment gaps were excluded from the analysis (Fig. 1B), the *Kitasatospora* clade was shown to originate from within the radiation of the genus *Streptomyces* and to form a sister group of the group containing several *Streptomyces* species, represented by *Streptomyces bikiniensis*. Similar trees were also obtained when *Streptosporangium* and *Micromonospora* species were used as outgroups with or without inclusion of the alignment gaps in the analysis and when the maximum-parsimony method (23) was used (data not shown). Kim et al. (8) also noticed the effect of different outgroups on the relationship between kitasatosporae and streptomycetes. However, in no case were *Kitasatospora* species intermixed with *Streptomyces* species, and the bootstrap value for the *Kitasatospora* clade was always more than 95%. The integrity of the *Kitasatospora* group was also demonstrated by the results of a pairwise analysis of the levels of nucleotide sequence similarity (data not shown). The average level of sequence similarity between members of the genus *Kitasatospora* was 97.65%, and the levels of sequence similarity ranged from 95.9% (between "*S. azaticus*" and "*K. brunnea*") to 99.5% (between "*S. cochleatus*" and "*S. paracochleatus*"), whereas the average level of sequence similarity between *Kitasatospora* and *Streptomyces* species was much lower, 93% (range, 90.7 to 95.6%).

Analysis of 16S-23S rRNA gene spacers. The 16S rRNA sequence analysis described above could not distinguish between the following two possible relationships of kitasatosporae to streptomycetes: (i) both groups are monophyletic, and the two taxa are sister taxa; and (ii) the kitasatosporae represent a lineage that originated from within the radiation of the genus *Streptomyces*, in which case the genus *Streptomyces* would be paraphyletic. An example of the second type of relationship is the evolutionary origin of the genus *Nocardia* from within the radiation of *Rhodococcus* species (17).

16S rRNA sequences are highly conserved in evolution (31), and closely related species are often found to have identical or nearly identical sequences (22). Thus, 16S rRNA sequence-based phylogenetic analysis may not be powerful enough to reliably resolve close phylogenetic relationships, such as the relationship between the kitasatosporae and the streptomycetes (22). Nucleotide sequences of genes that are evolutionarily more variable than the rRNA genes may provide useful information for the resolution of close relationships. To further investigate the phylogenetic relationship between the two groups of organisms, we analyzed the internally transcribed spacers between the 16S and 23S rRNA genes, a region known to be more variable than 16S rRNA genes (2). We cloned and sequenced the 16S-23S rRNA gene spacers of all of the strains listed in Table 1 and of species belonging to the genera *Streptosporangium*, *Micromonospora*, *Microtetraspora*, and *Microbispora* for use as outgroups in phylogenetic analyses. Figure 2 shows the phylogenetic trees inferred when the spacer sequences were used. Although considerable gaps had to be introduced in some regions in the alignment (Fig. 3), the kitasatosporae and streptomycetes were consistently separated into two distinct clades irrespective of the inclusion (Fig. 2A) or exclusion (Fig. 2B) of the gaps. The separation of the two groups was substantiated by high bootstrap values and was not affected by the different outgroups used. Spacers from more distant groups of actinomycetes could not be used as outgroups, because they exhibit very low levels of homology with the spacers of streptomycetes and kitasatosporae. These results strongly suggest that the genera *Kitasatospora* and *Streptomyces* are sister taxa.

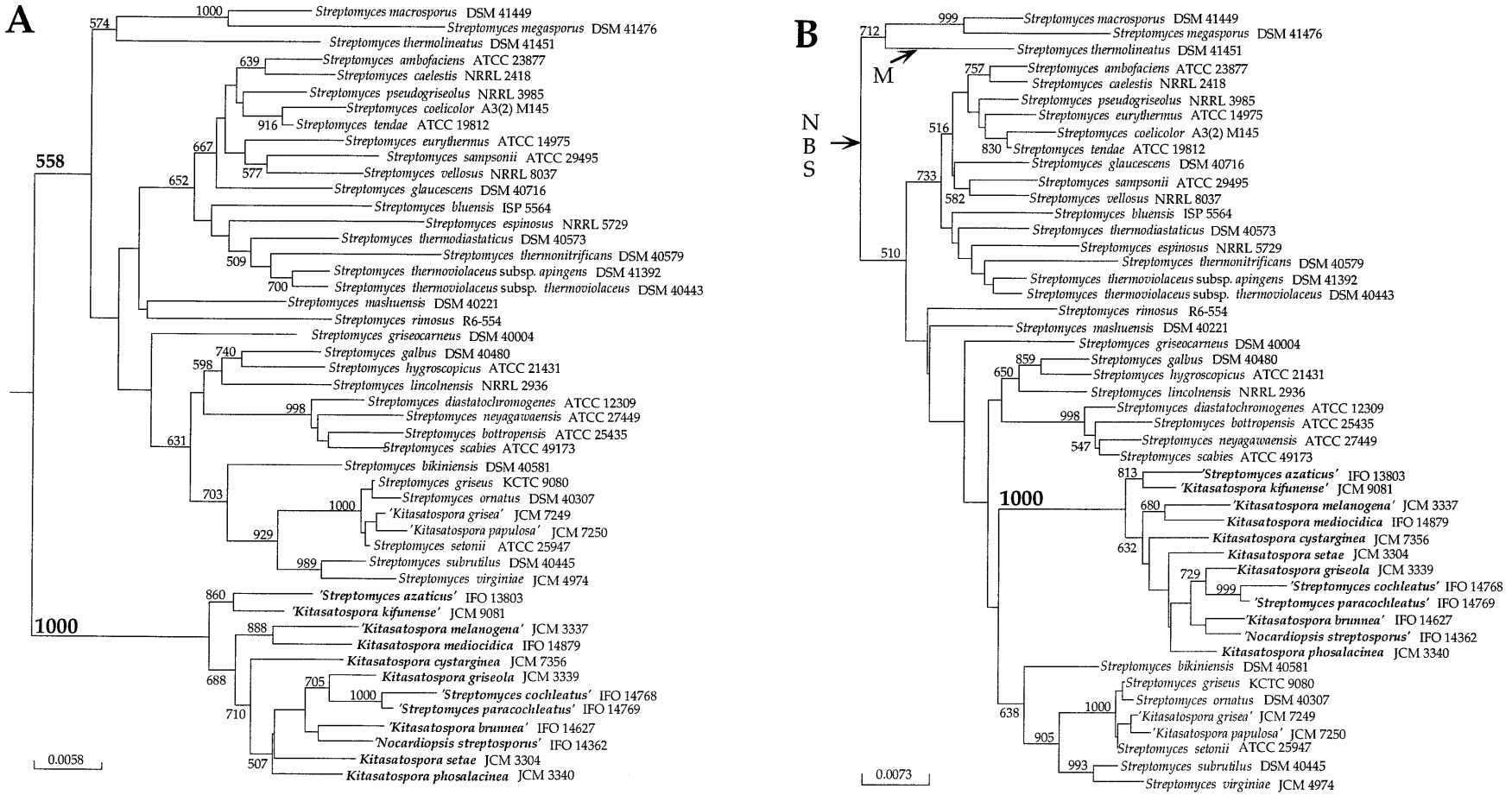

FIG. 1. Neighbor-joining trees generated by using 16S rDNA sequences from members of the genera *Kitasatospora* and *Streptomyces*. The numbers at the nodes indicate the levels of bootstrap support based on 1,000 resamplings. Bootstrap values lower than 500 are not shown. The bars indicate the numbers of inferred substitutions per nucleotide. The sequences for *Streptomyces* species were retrieved from the GenBank database. (A) The gaps in the multiple-sequence alignment were treated as a fifth base. The position of the root was determined by using *Nocardia asteroides* and *Bacillus subtilis* as outgroups. (B) The nucleotide positions corresponding to the alignment gaps were excluded from the analysis. The arrows indicate the positions of the root when *Nocardia asteroides* (N), *Bacillus subtilis* (B), *Micromonospora* species (M), and *Streptosporangium* species (S) were used as outgroups.

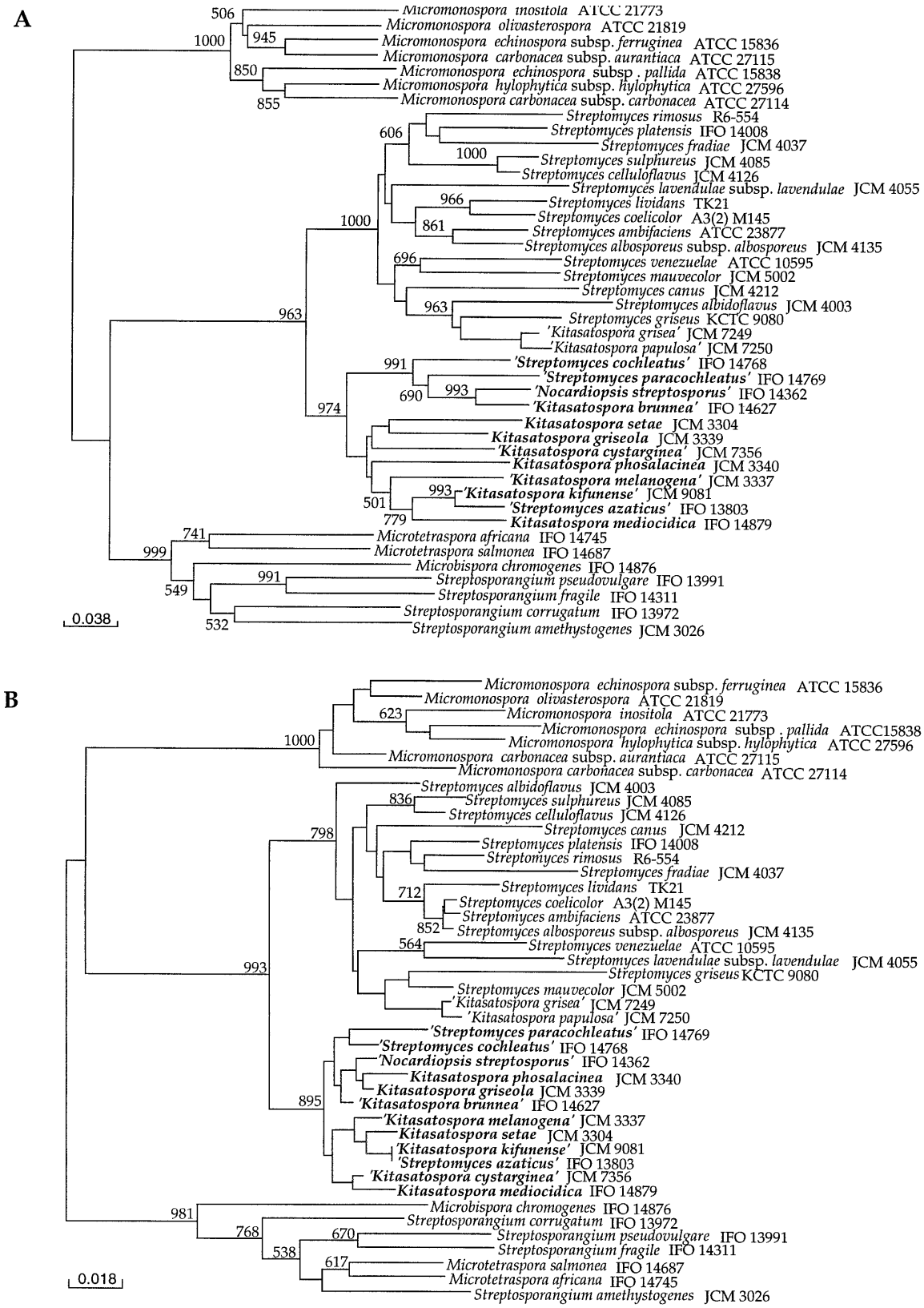

FIG. 2. Neighbor-joining trees generated by using the sequences of 16S-23S rRNA gene spacers. For details, see the legend to Fig. 1. The GenBank accession numbers for the spacer sequences of non-*Kitasatospora* species and subspecies are as follows: *Micromonospora carbonacea* subsp. *carbonacea*, AF004000; *Micromonospora carbonacea* subsp. *aurantiaca*, AF004001; *Micromonospora hylophytica* subsp. *hylophytica*, AF004002; *Micromonospora inositola*, AF004003; *Micromonospora olivasterospora*, AF004004; *Micromonospora echinospora* subsp. *ferruginea*, AF004005; *Micromonospora echinospora* subsp. *pallida*, AF004006; *Microbispora chromogenes*, AF004007; *Microtetraspora africana*, AF004008; *Microtetraspora salmonea*, AF004009; *Streptosporangium amethystogenes*, AF004010; *Streptosporangium corrugatum*, AF004011; *Streptosporangium fragile*, AF004012; and *Streptosporangium pseudovulgare*, AF004013.

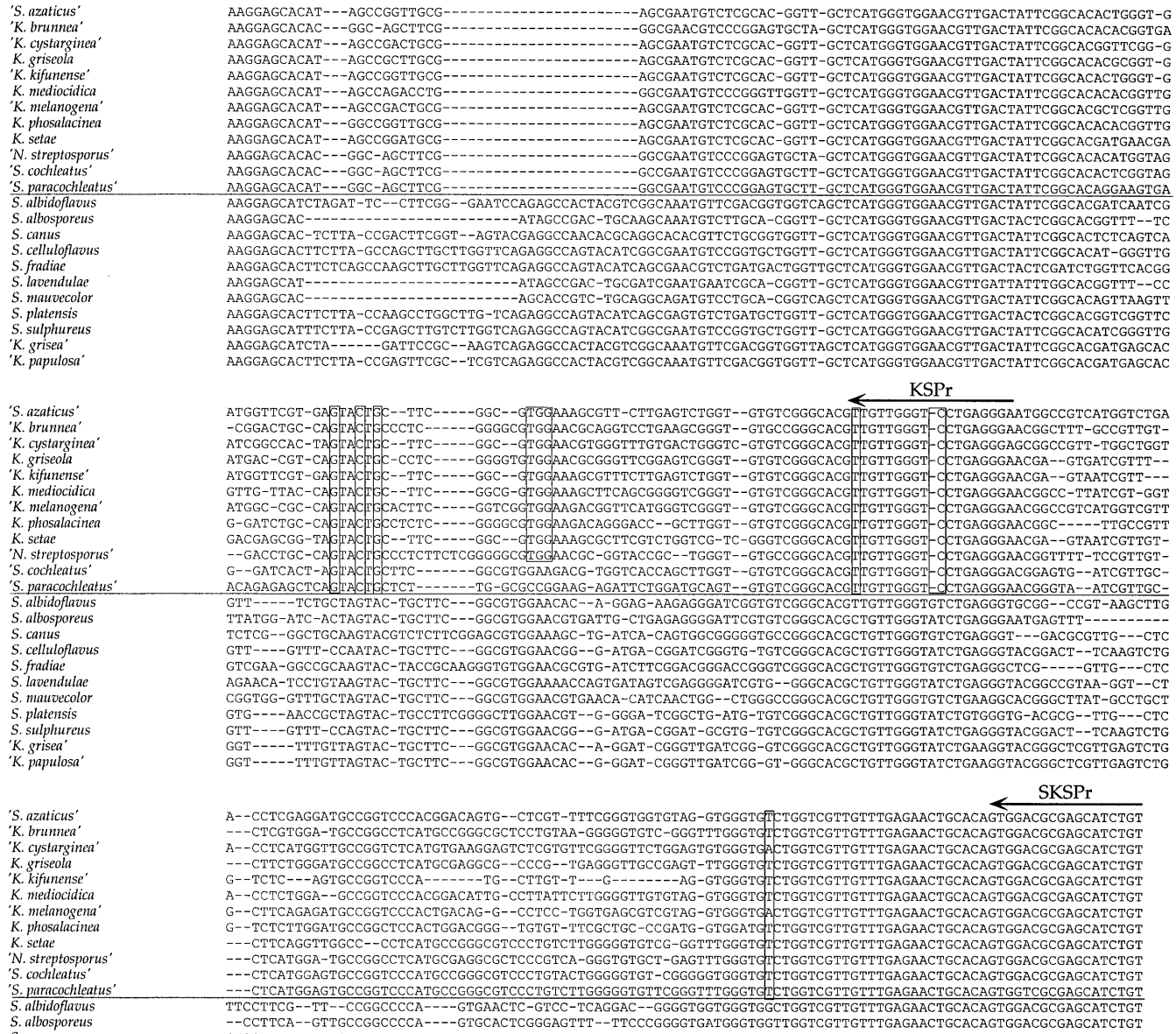

FIG. 3. Multiple-sequence alignment of the sequences of 16S-23S rRNA gene spacers determined by using the Clustal method of the DNASTAR program. The sequences of members of the genus *Kitasatospora* and the sequences of members of the genus *Streptomyces* are divided by a line. The nucleotides specific for the members of the genus *Kitasatospora* are enclosed in a box. The arrows indicate the positions of two oligonucleotide primers which we used for identification of *Kitasatospora* species by PCR (see Fig. 4).

Specific nucleotide signatures in the spacer region. The spacer sequence alignment revealed nucleotide signatures specific for each group of organisms. For example, there are nine nucleotides (Fig. 3) unique to members of the genus *Kitasatospora*. Such signatures can be targeted by oligonucleotides for convenient discrimination between kitasatosporae and streptomycetes by hybridization or PCR. Figure 4 shows that a pair of oligonucleotide primers designed to be specific for the *Kitasatospora* species amplified an expected fragment only from members of the genus *Kitasatospora.*

Conclusion. The results of our phylogenetic analyses, based on the sequences of both 16S rRNA genes and 16S-23S gene spacers, strongly suggest that the genus *Kitasatospora* is a taxon that is separate from the genus *Streptomyces*. These results do not totally disagree with the observations of Wellington et al. (29) and Ochi and Hiranuma (14) in that the two groups of

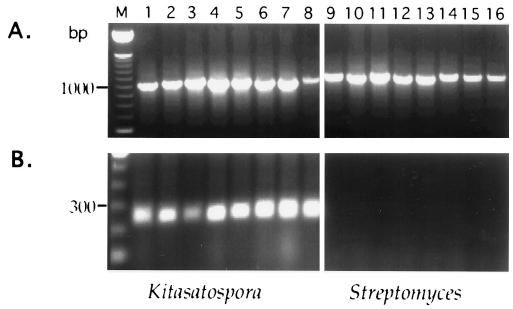

FIG. 4. Differentiation of *Kitasatospora* species from *Streptomyces* species by PCR by using *Kitasatospora*-specific oligonucleotides. Genomic DNA was prepared from each organism and was used as a PCR template. Lanes 1 to 8, amplification from members of the genus *Kitasatospora*, (*K. griseola*, "*S. azaticus*," "*Nocardiopsis streptosporus*," *K. phosalacinea*, *K. brunnea*, "*S. cochleatus*," "*S. paracochleatus*," and *K. setae*, respectively); lanes 9 to 16, amplification from some randomly picked *Streptomyces* species (*Streptomyces* sp. strain MA6858 = [ATCC 55098], *Streptomyces griseus* JCM4047, *Streptomyces fradiae* ATCC 19609, *Streptomyces nodosus* ATCC 14899, *Streptomyces hygroscopicus* JCM4772, *Streptomyces rimosus* JCM4073, *Streptomyces noursei* ATCC 11455, and *Streptomyces* sp. strain ATCC 43620, respectively). (A) Positive control showing the successful amplification of an approximately 1-kb fragment from all of the templates. The forward primer (5′ GAA CAG GAT TAG ATA CCC 3′) targets nucleotides 781 to 796 (*E. coli* numbering [1]) of the 16S rRNAs of all of the organisms, and the reverse primer is SKSPr (5′ ACA GAT GCT CGC GTC CAC 3′), which targets the end of the 16S-23S rRNA gene spacer (see Fig. 3). (B) Specific amplification from members of the genus *Kitasatospora*. The forward primer (5′ CCG TCG AAG GTG GGA CCA 3′) corresponds to nucleotides 1463 to 1480 of the 16S rRNA (*E. coli* numbering [1]), and the reverse primer is KSPr (5′ TCC CTC AGG ACC CAA CAA 3′), as shown in Fig. 3.

organisms are indeed closely related. However, in the study of Wellington et al. (29), which was based on partial 16S rRNA sequences from only one *Kitasatospora* species, *K. setae*, and four *Streptomyces* species, the authors could not possibly discover the phylogenetic separation of the two groups of actinomycetes. In addition to the phylogenetic evidence, the chemotaxonomic differences between the members of the genus *Kitasatospora* and the members of the genus *Streptomyces* are also substantial. It is well-accepted that both DAP and galactose have great discriminatory value in classification and identification of actinomycetes at the generic and suprageneric levels (7, 19). The presence of major amounts of *meso*-DAP and galactose in the members of the genus *Kitasatospora* (12, 15, 25) but not in the members of the genus *Streptomyces* reflects the phylogenetic integrity of each group and the distance between the two groups of organisms. The formation of submerged spores in liquid culture and resistance to polyvalent *Streptomyces* phages are other characteristics that may distinguish the members of the genus *Kitasatospora* from at least the majority of *Streptomyces* species.

On the basis of the phylogenetic, chemotaxonomic, and phenotypic evidence described above, we propose that the genus *Kitasatospora* Omura, Takahashi, Iwai, and Tanaka 1982 (15) should be revived.

Nomenclature considerations. As a result of the revival of the genus *Kitasatospora*, the description of the genus *Streptomyces* Waksman and Henrici 1948 (26) should follow the description given by Witt and Stackebrandt (30). On the basis of chemotaxonomic and phenotypic properties determined by Nakagaito et al. (12) and the result of this study, the *Streptomyces* species "*S. azaticus*," "*S. cochleatus*," and "*S. paracochleatus*" should be transferred to the genus *Kitasatospora.*

Emended description of the genus *Kitasatospora* Omura, Takahashi, Iwai, and Tanaka 1982. *Kitasatospora* (Ki.ta.sa.to.spo′ra. Jpn. n. Kitasato, a Japanese microbiologist; M.L. fem. n. *spora* spore; M.L. fem. n. *Kitasatospora*, Kitasato spore). The description below is based on data from this study and previous studies (12, 15, 29).

The substrate mycelium is as well-developed as *Streptomyces* substrate mycelium, and the aerial mycelium bears long spore chains containing more than 20 spores. No fragmentation of substrate mycelium occurs. No sporangia are formed. The major constituents of the cell wall are glycine, galactose, and *meso*-DAP or LL-DAP, depending on the type of cells analyzed. When cells are grown on agar media, the aerial spores contain LL-DAP, whereas the substrate mycelium contains *meso*-DAP. When cells are grown in liquid media, submerged spores are formed, and the spores contain LL-DAP and the filamentous mycelia contain *meso*-DAP. Whole-cell hydrolysates contain galactose, but lack arabinose, madurose, and xylose. The phopholipid type is type II. The glycolate test is negative. The organism is gram positive, aerobic, and chemoorganotrophic. The growth temperature range is 15 to 42°C, and the pH range is 5.5 to 9.0. The G+C content is 66 to 73 mol%.

The genus *Kitasatospora* can be distinguished from the genus *Streptomyces* by the ratio of *meso*-DAP to LL-DAP in whole-cell hydrolysates. The *meso*-DAP content is 49 to 89% in *Kitasatospora* strains and 1 to 16% in *Streptomyces* strains. Galactose is present in the whole-cell hydrolysates of *Kitasatospora* strains but not in the whole-cell hydrolysates of *Streptomyces* strains. In the dendrogram constructed by using 16S rRNA sequences of actinomycete species, *Kitasatospora* species form a tight clade which excludes all *Streptomyces* species; and in the dendrogram based on the 16S-23S rRNA gene spacers, the genera *Kitasatospora* and *Streptomyces* form distinct clades. The genus *Kitasatospora* can be readily distinguished from the genus *Streptomyces* by specific nucleotide signatures in the sequences of both the 16S rRNA and the 16S-23S rRNA gene spacers.

The type species of the genus is *Kitasatospora setae* Omura, Takahashi, Iwai, and Tanaka 1982 (15).

Descriptions of new combinations. The description of *Kitasatospora azatica* comb. nov. (a.za′ti.ca. L. adj. *azatica*, referring to the product aza amino acid, an antitumor agent) is the same as that given by Nakagaito et al. (12).

The description of *Kitasatospora cochleata* comb. nov. (coch′le-.a.ta. L. adj. *cochleata*, spiral, referring to the formation of spiral aerial mycelium) is the same as that given by Nakagaito et al. (12).

The description of *Kitasatospora paracochleata* comb. nov. (pa.ra.coch′le.a.ta. L. pron. *para*, along side of, resembling; L. adj. *paracochleata*, a species like *K. cochleata*) is the same as that given by Nakagaito et al. (12).

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