NOTE**Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis**

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The nucleotide sequences of the 16S rRNA gene (rDNA) in 38 taxa of the genus *Staphylococcus* were compared phylogenetically. Based on phylogenetic tree analysis, staphylococcal species were divided into 12 cluster groups. These cluster groups were in very good agreement with species groups determined by DNA–DNA reassociation studies. These genealogical classifications were consistent with the results of the production of coagulase or oxidase and with resistance to novobiocin. These suggest that the phylogenetic relationship of the genus *Staphylococcus* is accurately represented by the results obtained from the sequence analysis of 16S rDNA.

Keywords: *Staphylococcus*, 16S rDNA, phylogeny

The genus *Staphylococcus* comprised more than 30 taxa (species and subspecies) (Kloos *et al.*, 1991). Five species and four subspecies of *Staphylococcus* have been added to the list of bacteria as newly identified organisms from studies conducted in the 1990s (Bannerman & Kloos, 1991; Chesneau *et al.*, 1993; Hájek *et al.*, 1992; Igimi *et al.*, 1990; Kloos & Wolfshohl, 1991; Tanasupawat *et al.*, 1992; Webster *et al.*, 1994; Zakrzewska-Czerwínska *et al.*, 1995). More recently, Gram-positive bacteria isolated from European otters have been proposed as a new species, *Staphylococcus lutrae* (Foster *et al.*, 1997). Some of these studies employed sequence analysis of the 16S rRNA or its gene (Hájek *et al.*, 1992; Zakrzewska-Czerwínska *et al.*, 1995). This analysis has been established as a method available for determining phylogenetic relationships (Olsen & Woese, 1993). The intermittent appearance of a highly conserved sequence and integration of sequence data are useful for correcting sequence alignment.

Ribotyping is a probe-based fragment analyses targeting rRNA operons and the pattern of DNA fragments hybridized with an rDNA probe can be highly discriminatory, and enable strain typing (Jordens, 1998). This method has been applied to the taxonomy at the species and subspecies levels in the genus *Staphylococcus* (Chesneau *et al.*, 1993; Webster *et al.*, 1994; De Buyser *et al.*, 1992; Thomson-Carter *et al.*, 1989). Probably, ribotyping is advantageous to 16S rRNA sequence analysis in terms of detecting variations within and flanking the probe sequence.

However, phylogenetic relationships can be inferred more precisely by the sequencing method because of its reliability in the calculation of DNA similarity (Olsen & Woese, 1993).

Earlier studies on the taxonomy of species of the genus *Staphylococcus*, based on DNA–DNA reassociation, have indicated that genealogically distinct groups exist within the genus as species groups represented by the species of *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus simulans*, *Staphylococcus intermedius*, *Staphylococcus hyicus*, *Staphylococcus sciuri*, *Staphylococcus aureus* and *Staphylococcus caseolyticus* (Kloos & Schleifer, 1986; Kloos *et al.*, 1991). Our previous study on 23 taxa of the genus *Staphylococcus* using 16S rDNA sequence analyses has shown the existence of 10 cluster groups (Takahashi *et al.*, 1997). These classifications based on the whole genome and 16S rDNA analysis were nearly identical to each other; however, the branching pattern in the phylogenetic tree may change at the nodes because of the limited number of species used for the analyses. Therefore, it is necessary to complete the 16S rDNA sequence database for all of the type strains of the undetermined taxa of the genus *Staphylococcus*.

In order to have an overview of intrageneric relationships of *Staphylococcus*, we examined the 16S rDNA sequences of the type strain of each of 38 taxa by phylogenetic analysis and compared the results from DNA–DNA reassociation studies.

The *Staphylococcus* strains determined in this study

Fig. 1. Unrooted neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships among the species of the genus *Staphylococcus*. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis (Felsenstein, 1985). The scale bar indicates the evolutionary distance value (K_{nuc}) between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms. 'Cluster group' was designated based on the branching pattern and was compared with the 'species group' described by Kloos et al. (1991). The accession numbers of the sequences of the organisms used in the analyses are as follows: *S. caseolyticus*, D83359; *S. sciuri*, S83569; *S. lentus*, D83370; *S. pulvereri*, AB009942; *S. vitulinus*, AB009946; *S. simulans*, D83373; *S. piscifermentans*, AB009943; *S. carnosus*, AB009934; *S. muscae*, S83566; *S. chromogenes*, D83360; *S. hyicus*, D83360; *S. lutrae*, X84731; *S. felis*, D83364; *S. schleiferi* subsp. *coagulans*, AB009945; *S. schleiferi* subsp. *schleiferi*, S83568; *S. intermedius*, D83369; *S. delphini*, AB009938; *S. auricularis*, D83358; *S. kloosii*, AB009940; *S. gallinarum*, D83366; *S. arlettae*, AB009933; *S. saprophyticus*, D83371; *S. xylosum*, D83374; *S. equorum*, AB009939; *S. cohnii* subsp. *cohnii*, D83361; *S. cohnii* subsp. *urealyticus*, AB009936; *S. lugdunensis*, AB009941; *S. haemolyticus*, D83367; *S. hominis*, X66101; *S. pasteurii*, AB009944; *S. warneri*, L37603; *S. aureus* subsp. *aureus*, D83357; *S. aureus* subsp. *anaerobius*, D83355; *S. epidermidis*, D83363; *S. saccharolyticus*, L37602; *S. capitis* subsp. *urealyticus*, AB009937; *S. caprae*, AB009935; and *S. capitis* subsp. *capitis*, L37599. The origin of strain codes, CCM, DSM and MAFF are the Czechoslovak Collection of Microorganisms, Deutsche Sammlung von Mikroorganismen und Zellkulturen and the Ministry of Agriculture, Forestry and Fisheries of Japan, respectively.

were obtained from the American Type Culture Collection (ATCC). The nucleotide sequence data reported in this paper appear in the GSDB, DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers shown in the legend for Fig. 1. The bacterial culture, DNA extraction, gene amplification and sequencing techniques were described previously (Takahashi et al., 1997). Sequence alignment was performed from nucleotide residues 28–1490, based on the *Escherichia coli* numbering system (Brosius et al., 1978). Evolutionary distance values (K_{nuc}) were calculated by Kimura's two-parameter method (Kimura, 1980) with the BioResearch SINCA program package. The neighbour-joining method of Saitou & Nei (1987) was employed to

construct a phylogenetic tree. The topology of the tree was evaluated by a bootstrapping method (Felsenstein, 1985).

The relatively close relationships among species of the genus *Staphylococcus* was confirmed by phylogenetic analyses based on the gene sequencing employed in this study. However, the phylogenetic tree showed that staphylococcal strains were divided into distinct subgroups that were recovered in high percentages of the bootstrap trees. Based on criteria of the branching patterns and the bootstrap values (80% or more), 12 cluster groups were designated, as shown in Fig. 1. They are *S. caseolyticus*, *S. sciuri*, *S. carnosus*, *S. simulans*, *S. hyicus-intermedius*, *S. auricularis*, *S.*

saprophyticus, *S. lugdunensis*, *S. haemolyticus*, *S. warneri*, *S. epidermidis* and *S. aureus*.

The phylogenetic position of *S. caseolyticus* is the most distant from any other taxa of the genus *Staphylococcus*. A root for *Bacillus subtilis* as an outgroup was branched off from a line between *S. caseolyticus* and the others (data not shown). The staphylococcal species groups determined by DNA–DNA reassociation studies (Kloos & Schleifer, 1986; Kloos *et al.*, 1991) have been supported by phenotypic character analysis. In many cases, the classifications were consistent with our results. The correspondence between the species groups and the cluster groups can be seen in Fig. 1.

The close relationship between *S. sciuri* and *Staphylococcus lentus* of the *S. sciuri* species group was reproduced in the results from our treeing analysis based on 16S rDNA sequences. In the tree, the newly established species *Staphylococcus vitulinus* and *Staphylococcus pulvereri* formed a specific cluster within the clade of *S. sciuri* and *S. lentus*. These four species of the *S. sciuri* cluster group were novobiocin-resistant and oxidase-positive, as was *S. caseolyticus*.

A *S. simulans* species group consisting of *S. simulans* and *Staphylococcus carnosus* may not be related monophyletically with *Staphylococcus piscifermentans* in Fig. 1 (59% of the bootstrap value). Since *S. carnosus* and *S. piscifermentans* were specifically related to each other and *S. simulans* appeared to form a deep subline within the group, we divided this group into cluster groups of *S. simulans* and *S. carnosus*. *Staphylococcus felis* is thought to be related to members of the *S. simulans* group; however, the phylogenetic position of the organism was distinctive in a clade of *S. hyicus* or *S. intermedius*.

The *S. hyicus* species group consisting of *S. hyicus* and *Staphylococcus chromogenes*, and a *S. intermedius* species group consisting of *S. intermedius* and *Staphylococcus delphini*, were closely related in our phylogenetic tree, and they formed a distinct subgroup with *S. felis*, '*Staphylococcus lutrea*', *Staphylococcus muscae* and two subspecies of *Staphylococcus schleiferi*. It is notable that all coagulase-positive taxa, except for *S. aureus*, belonged to a monophyletic clade of this cluster group, which was recovered in 93% by the bootstrapping tree.

The *S. saprophyticus* species group consisting of *S. saprophyticus*, *Staphylococcus arlettae*, *Staphylococcus cohnii*, *Staphylococcus equorum*, *Staphylococcus gallinarum*, *Staphylococcus kloosii* and *Staphylococcus xylosus*, which were characterized as being novobiocin-resistant and oxidase-negative, appeared to form a monophyletic clade in our phylogenetic tree with 91% of the bootstrap value. *S. kloosii* has a relatively deep subline within a cluster group of *S. saprophyticus* and may be distinct from others in this group.

The *S. epidermidis* species group of *S. epidermidis*, *Staphylococcus capitis*, *Staphylococcus caprae*, *Staphyl-*

ococcus haemolyticus, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saccharolyticus* and *Staphylococcus warneri* were somewhat closely related with each other and with the recently added species *Staphylococcus pasteurii*. However, specific association among these species was not exhibited in our treeing analysis. Based on the above-described criteria, we divided these species into five cluster groups: *S. lugdunensis*, *S. haemolyticus*, *S. warneri*, *S. epidermidis* and *S. aureus* (Fig. 1). The branching order of *S. lugdunensis*, *S. haemolyticus* and *S. hominis* was uncertain in our phylogenetic tree; however, it is in agreement with the results from DNA–DNA reassociation studies (Kloos & Schleifer, 1986; Kloos *et al.*, 1991). In the *S. epidermidis* cluster group, 16S rDNA sequences of *S. caprae* and two subspecies of *S. capitis* were nearly indistinguishable. This was also consistent with the results obtained from DNA–DNA hybridization study (Bannerman & Kloos *et al.*, 1991).

From the results of the sequence analysis of 23 staphylococcal taxa in our previous study (Takahashi *et al.*, 1997), the nucleotide residues 72–97, 183–203, 999–1022 and 1290–1293 in the variable regions of V1, V3, V7 and V9 designated by Raué *et al.* (1990), respectively, possibly discriminate the staphylococcal species except for two subspecies of *S. aureus*. In the present study, some of the 38 taxa of the genus exhibited identical or similar sequence stretches in the above regions. Such identical or similar sequence stretches were found between *S. pulvereri* and *S. vitulinus*; among *S. saccharolyticus*, *S. capitis* subsp. *urealyticus* and *S. caprae*, between two subspecies of *S. aureus*, and between two subspecies of *S. cohnii*. These were probably due to the limitation in the discriminating power of 16S rDNA sequence analysis, as described by Palys *et al.* (1997). In other taxa having longer sublines, however, these regions may be useful for the assignment of staphylococcal strains. For example, nucleotide stretches of ATTA at the residues 1290–1293 were found in the cluster group *S. sciuri*, while those of ACCA, and GTTG or GCCT were found in *S. caseolyticus* and in the other groups, respectively. Such a possibly specific sequence was also found in the above-mentioned regions for the group of *S. aureus*, *S. epidermidis* and *S. haemolyticus*.

A few disagreements, such as the phylogenetic position of *S. felis*, were found between our cluster groups and species groups based on DNA–DNA reassociation studies. These confusions were quantified as probability by means of bootstrapping analysis. In other words, branching patterns in a phylogenetic tree based on the 16S rDNA sequence are reliable if the bootstrap value is sufficiently high. In the present study, the degree of nucleotide change among species of the genus *Staphylococcus* was relatively small, but the bootstrapping tree of the species was recovered in high probabilities. Considering the stability of the 16S rDNA sequence, nucleotide stretches uniquely found in a certain cluster group may be group-specific.

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