Identification of "*Staphylococcus staphylolyticus*" NRRL B-2628 as a Biovar of *Staphylococcus simulans*

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"*Staphylococcus staphylolyticus*" NRRL B-2628, the lysostaphin-producing organism, is identified as a biovar of *S. simulans*. The membership of this organism in the genus *Staphylococcus* is based on the guanine-plus-cytosine content of its deoxyribonucleic acid, its anaerobic growth in thioglycolate medium, the presence of teichoic acids in its cell wall, and the composition of its cell wall peptidoglycan. The identification of the organism as a member of *S. simulans* is based on its lack of production of coagulase and acetylmethylcarbinol, its carbohydrate reaction pattern, similarities in other biochemical characteristics, and the results of deoxyribonucleic acid-deoxyribonucleic acid hybridization studies.

Lysostaphin is a commercially available protein preparation obtained from culture filtrates of an organism (NRRL B-2628) designated "*Staphylococcus staphylolyticus*" (23). (This name is not on the Approved Lists of Bacterial Names (27), has not been validly published since 1 January 1980, and therefore has no standing in bacterial nomenclature). This preparation has been shown to be capable of specifically hydrolyzing the cell walls of other staphylococci, especially *S. aureus* (9, 22, 23). "*S. staphylolyticus*" was originally designated a *Micrococcus* species by Schindler (C. A. Schindler, Ph.D. thesis, The University of Texas, Austin, 1961) and later reassigned to the genus *Staphylococcus* by Schindler and Schuhardt (23) on the basis of its ability to tolerate high salt concentrations and to ferment glucose anaerobically.

A large number of new species of *Staphylococcus*, in addition to *S. aureus*, *S. epidermidis*, and *S. saprophyticus*, have recently been described. These include *S. cohnii*, *S. haemolyticus*, and *S. xylosus* (24); *S. warneri*, *S. capitis*, *S. hominis*, and *S. simulans* (16); *S. sciuri* (17); *S. intermedius* (13); and *S. hyicus* (11). This report deals with the characterization of "*S. staphylolyticus*" strain NRRL B-2628 to determine its relationship among these species and offers a new nomenclature for this unique organism.

MATERIALS AND METHODS

Bacterial strains. "*S. staphylolyticus*" NRRL B-2628 was kindly provided by W. C. Haynes (Northern Utilization Research and Development Division, Peoria, Ill.). Cultures were maintained at 4°C on agar slants of lysostaphin production medium (21). The type strains *S. simulans* ATCC 27848, *S. haemolyticus*

ATCC 29970, *S. aureus* ATCC 12600, and *S. cohnii* ATCC 29974, used as sources of deoxyribonucleic acid (DNA), have been described previously (7, 16, 24). Cultures of these strains were maintained at 4°C on P agar slants (18).

Culture conditions. All test media were inoculated with a 24-h-old culture grown on lysostaphin production medium agar slants and were incubated at 37°C for 1 to 5 days, unless otherwise stated.

Morphological and colonial characteristics. Colony characteristics and cell morphology were determined as described previously (18).

Physiological characters. Oxygen and temperature requirements, motility, and NaCl tolerance were determined as described previously (18).

Biochemical characters. Catalase activity was determined by inoculating a small amount of plate culture in a drop of 3% H₂O₂. The benzidine test was performed by the method of Deibel and Evans (10). The production of free coagulase was tested by the method of Fisk, as reported by Baird-Parker (1). Hemolysis was determined on sheep and human blood agar plates after 24, 48, and 72 h by using streak cultures. Acid production from carbohydrates was determined with filter-sterilized carbohydrates at a final concentration in phenol red broth base (Difco) of 0.5% (wt/vol). Tubes were observed for up to 1 month for acid production. Final pH in glucose-yeast extract broth was determined after 4 days of incubation. Acetylmethylcarbinol production was determined by Barritt's method (3). Phosphatase activity was detected by the method of Baird-Parker (1). The method of Lachica et al. (20) was used to determine deoxyribonuclease production and stability. Nitrate reduction was tested by the sulfanilic acid and α -naphthylamine method (8) in nitrate tubes (Difco) incubated for 48 h. The configuration of the lactic acid produced was determined by the method of Hohorst (14); D-lactic dehydrogenase isolated from *Leuconostoc mesenteroides* ATCC 13391 was used according to the method of Schleifer and Kocur (25). The range of bacteriolytic activity of lysostaphin has been reported previously by

Schindler and Schuhardt (23). Protein A determination was carried out as described by Hájek (13).

Lysostaphin susceptibility. For determination of lysostaphin susceptibility, washed cells were suspended in 0.05 M tris(hydroxymethyl)aminomethane-saline buffer (21), pH 7.5, to an optical density of 1.0 at 620 nm. Lysostaphin was added to give a final concentration of 40 µg/ml, and the mixture was allowed to react at 37°C. Change in optical density was read at 10-min intervals between 0 and 60 min.

Lysozyme susceptibility. Susceptibility to lysozyme (final concentration, 600 µg/ml) was determined in essentially the same manner as lysostaphin susceptibility.

Antibiotic susceptibilities. The Kirby-Bauer disk method (4) was used to determine antibiotic susceptibilities. The following antibiotic disks (Difco) were placed on a lawn of cells on Mueller-Hinton agar plates (Difco): penicillin G (2 U), novobiocin (5 µg), erythromycin (2 µg), and tetracycline (20 µg). Zones of inhibition were noted at 18 h.

Cell wall preparation and analysis. Isolation and analysis of the cell wall peptidoglycan from *S. staphylolyticus* have been described previously (21). The procedure of Wolin et al. (30) was used for qualitative examination of the cell wall teichoic acids.

DNA isolation. Staphylococci were grown in a modified CH broth medium (26) and were lysed by procedures described previously (19). DNA was isolated and purified by the procedures of Brenner and co-workers (5) as modified by Kloos and Wolfshohl (19).

DNA:DNA hybridization. DNA reassociation reactions and the separation of single-stranded, unreacted DNA from double-stranded, hybridized DNA on hydroxyapatite were performed by the procedures described by Brenner and co-workers (5). The percent relative binding was determined by normalizing the amount of labeled DNA bound to hydroxyapatite in heterologous reactions to that bound in the homologous reaction (where labeled and unlabeled DNA are from the same organism).

Chemicals. Egg white lysozyme was purchased from Sigma Chemical Co., St. Louis, Mo.; lysostaphin (237 U/mg) was purchased from Schwarz/Mann, Orangeburg, N.Y.; all other chemicals were reagent or analytical grade and were obtained from commercial sources.

RESULTS AND DISCUSSION

Identification of "*S. staphylolyticus*" NRRL B-2628 at the generic level. The major characteristics used here to differentiate the genera *Staphylococcus* and *Micrococcus* included the guanine-plus-cytosine (G+C) content of the DNA, anaerobic growth in thioglycolate medium, the presence of teichoic acids in the cell walls, lysostaphin endopeptidase susceptibility, and differences in the cell wall peptidoglycan (2). The chemical composition of the peptidoglycan and lysostaphin susceptibility are interrelated properties. Staphylococci have glycine-rich peptidoglycans in their cell walls and are susceptible or slightly resistant to the lysostaphin endopeptidase, which is specific for glycylglycine bonds

(6, 28). Micrococci have a remarkably different peptidoglycan composition and are therefore resistant to the lysostaphin endopeptidase. We previously reported that "*S. staphylolyticus*" peptidoglycan contains glycine and suggested possible reasons for the organism's resistance to its own staphylolytic glycylglycine endopeptidase (21). "*S. staphylolyticus*" also displayed good diffuse growth in the anaerobic portion of thioglycolate medium and, as is typical of staphylococci, contained cell-wall teichoic acid. The G+C contents of the DNAs of staphylococci are low (30 to 38 mol%), whereas the micrococci possess high G+C contents (66 to 73 mol%) in their DNAs. "*S. staphylolyticus*" NRRL B-2628 had a G+C content in its DNA of 35.1 mol%, definitely establishing it as not a member of the genus *Micrococcus*. On the other hand, its G+C content is consistent with that expected for a staphylococcus.

Identification of "*S. staphylolyticus*" NRRL B-2628 at the specific level. The production of coagulase is a major criterion in the classification of the staphylococci. "*S. staphylolyticus*" NRRL B-2628 was found to be coagulase negative. A large number of coagulase-negative species have been described recently, and the characteristics used in their classification have been used to identify "*S. staphylolyticus*" NRRL B-2628. A description of this strain follows:

The cells were gram-positive cocci, 0.9 to 1.2 µm in diameter, nonmotile and nonsporeforming; they occurred singly and in pairs, short chains, and clusters, occasionally in tetrads.

Colonies on P agar medium were 7.1 to 7.5 mm in diameter, raised, gray-white, and entire.

The organism was facultatively anaerobic. It demonstrated the fermentation of glucose by lowering the pH of glucose-yeast extract broth from 6.8 to 4.7 after anaerobic incubation. Both L- and D-lactic acid isomers were produced, with the L-isomer constituting 92% of the total.

The organism grew well in 10% NaCl and had weak growth at an NaCl concentration of 15%. The optimal growth range was 25 to 40°C. It grew well at 15 and 45°C.

The organism gave positive reactions for catalase and the benzidine test and demonstrated weak hemolysis on sheep blood and good hemolysis on human blood. Coagulase and acetyl-methylcarbinol were not produced.

The organism reduced nitrates, demonstrated phosphatase activity, and produced a heat-stable deoxyribonuclease.

Acid was produced aerobically from glucose, fructose, lactose, sucrose, glycerol, mannose, trehalose, ribose, and galactose. No acid was produced from mannitol, maltose, rhamnose, xylose, arabinose, turanose, gentiobiose, cellobiose, melezitose, xylitol, sorbitol, inositol, sali-

cin, adonitol, dulcitol, arabinol, erythritol, erythrose, raffinose, melibiose, fucose, tagatose, lyxose, or sorbose.

The organism possessed good bacteriolytic activity, producing the staphylolytic lysostaphin endopeptidase and an endo- β -*N*-acetylglucosaminidase (hexosaminidase) which lyses members of the genus *Micrococcus* (15, 29). It also produced an acetylmuramic acid-L-alanine amidase which appears not to be bacteriolytic per se but capable of acting only on previously solubilized peptidoglycan (29).

The organism was resistant to lysozyme and penicillin. The organism was resistant to lysostaphin under conditions in which lysostaphin was produced (21). It was susceptible to streptomycin, novobiocin, erythromycin, and tetracycline.

TABLE 1. Character differences between "*S. staphylolyticus*" NRRL B-2628 and the type strain (ATCC 27848) of *S. simulans*

Characteristic	<i>S. simulans</i> ATCC 27848	" <i>S. staphylolyticus</i> " NRRL B-2628
Hemolysis on sheep blood	— ^a	+
Phosphatase	Weak	+
DNase	Weak	+
Growth with 15% NaCl	— ^b	Weak
Acid produced aerobically from:		
Maltose	Weak ^c	—
Mannitol	+ ^d	—
Galactose	— ^e	+
Mannose	— ^f	+
Teichoic acid type	Glycerol + galactosamine	Glycerol
Peptidoglycan (mol/mol of lysine)		
Serine	0.2	1.3
Glycine	4.9	2.3
Lysostaphin susceptibility	S	R
G+C content (mol%)	34.09 ^g	35.1

^a Data in this and the following footnotes are from Kloos and Schleifer (16). Fifty-four percent of *S. simulans* strains tested showed weak hemolysis of sheep blood.

^b Seventy-seven percent of *S. simulans* strains tested showed weak growth in 15% NaCl.

^c Sixty-nine percent of *S. simulans* strains tested showed no fermentation of maltose.

^d Eight percent of *S. simulans* strains tested showed no fermentation of mannitol.

^e Eight percent of *S. simulans* strains tested showed fermentation of galactose.

^f Seventy-seven percent of *S. simulans* strains tested showed fermentation of mannose.

^g The range of the G+C contents of the DNAs of the strains of *S. simulans* tested was 34.0 to 37.2 mol%.

TABLE 2. Reassociation reactions of [*methyl*-³H]thymidine-labeled DNA from type strains of some representative *Staphylococcus* species with unlabeled DNA from "*S. staphylolyticus*" NRRL B-2628

Source of labeled DNA	Reaction temp (C)	% Relative binding of labeled DNA to unlabeled " <i>S. staphylolyticus</i> " NRRL B-2628 DNA ^a
<i>S. simulans</i>	55	100 ^b
ATCC 27848	70	82
<i>S. haemolyticus</i>	70	6
ATCC 29970		
<i>S. aureus</i>	70	4
ATCC 12600		
<i>S. cohnii</i>	70	7
ATCC 29974		

^a The actual binding of labeled DNA fragments to unlabeled DNA from the same source (homologous reactions) was 81 $\pm$ 7%.

^b Relative binding data represent the average of duplicate reassociation reactions.

The organism contained peptidoglycan of the type L-Lys-Gly_{2,3}, L-Ser_{1,3} (21). The cell wall teichoic acid contained glycerol, with no other neutral sugar or additional amino sugar detected. No protein A was found associated with the cell wall.

The G+C content of the DNA was 35.1 mol%.

As with *S. simulans*, "*S. staphylolyticus*" is distinguished from other staphylococci by its lack of acetylmethylcarbinol production, carbohydrate reaction pattern, and cell wall composition. A large number of other properties demonstrated that this organism is closely related to or identical with *S. simulans*. Differences between "*S. staphylolyticus*" and the type strain of *S. simulans* (ATCC 27848) are summarized in Table 1. As can be seen in Table 1, the only major differences between "*S. staphylolyticus*" and the various strains of *S. simulans* that have been characterized were in the composition of the cell wall. This is not surprising considering that "*S. staphylolyticus*" produces an enzyme that lyses most other staphylococcal cells, including *S. simulans* (16).

Kloos and Schleifer reported that several properties of *S. simulans* overlap with properties of human strains of *S. aureus* (16). Likewise, several characteristics of "*S. staphylolyticus*" NRRL B-2628 suggest that this strain may be phenotypically related to *S. aureus* (J. M. Robinson, C. J. Oliver, J. K. Hardman, and G. L. Sloan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K168, p. 214). The heat-stable deoxyribonuclease activity found in this strain would appear to be unusual for a coagulase-negative staphylococcus. However, Gramoli and Wilkinson (12) recently isolated some heat-

stable, deoxyribonuclease-positive strains of *S. simulans*. This property does not appear to be limited to *S. aureus*.

The results of DNA reassociation reactions performed at the stringent criterion (70°C) indicate that strain NRRL B-2628 labeled as "*S. staphylolyticus*" is a member of the species *S. simulans* (Table 2). This contention is supported further by the high relative binding of labeled *S. simulans* DNA at the optimal criterion (55°C). Considering the high binding of *S. simulans* DNA to the DNA of strain NRRL B-2628, it would not be necessary to examine DNA relatedness to other species, except perhaps to serve as suitable controls. As can be seen from the data in Table 2, strain NRRL B-2628 is not closely related to *S. haemolyticus*, *S. aureus*, or *S. cohnii*, which represent several divergent groups.

On the basis of the results presented here, we suggest that "*Staphylococcus staphylolyticus*" NRRL B-2628 be reclassified as *Staphylococcus simulans* biovar *staphylolyticus*. This properly classifies this organism with regard to the new species which have been described yet retains its identity as the lysostaphin-producing organism.

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REPRINT REQUESTS

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