Staphylococcus pulvereri sp. nov., Isolated from Human and Animal Specimens

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A new coagulase-negative species of the genus *Staphylococcus*, *Staphylococcus pulvereri*, was isolated from human and animal specimens. The complete 16S rRNA sequence of the type strain of *S. pulvereri*, NT215, was determined and compared with the sequences of 16S rRNAs from the other staphylococci. Strains of *S. pulvereri* were differentiated from other novobiocin-resistant *Staphylococcus* species by their biochemical activities, cell wall composition, and levels of genetic relatedness. The type strain of this species is NT215 (=PCM 2443^T).

The genus *Staphylococcus* comprises 30 species (6), including the recently described *S. delphini* (21), *S. felis* (5), *S. muscae* (4), *S. pasteurii* (1), and *S. piscifermentans* (20). The taxonomic status of these species was determined by a combination of biochemical, chemical, molecular, and physiological properties. *Staphylococcus* species have been isolated from various mammals and birds, as well as from food of animal origin. It is likely that novel species will be discovered if additional host organisms and new environments are examined.

In the course of the identification of staphylococci from various human and animal specimens, several previously undescribed coagulase-negative strains were found. In this report, we depict a new species, *S. pulvereri*.

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MATERIALS AND METHODS

Bacterial strains. Five epidemiologically unrelated coagulase-negative staphylococcal strains were examined during our taxonomic studies (Table 1). Glucose-yeast extract-peptone was used as a basal medium.

Morphological and colonial characteristics. Colony morphology and pigmentation were observed on P agar plates, as reported by Kloos et al. (6, 7). Cell morphology was observed from Gram-stained smears.

Phenotypic characteristics. The production of free coagulase was determined by the method established by Sperber and Tatini (19). In order to detect catalase activity, a small amount of cells was taken directly from a colony on a plate, added to a drop of 3% H₂O₂, and examined for release of oxygen bubbles. Anaerobic growth was tested in Evans' semisolid thioglycolate medium (2) or in Wilkins agar (23). Growth was investigated at different temperatures and NaCl concentrations (7.5, 10, 15, 17.5, and 20%), as described by Kloos et al. (7). Enzymatic reactions were detected with the ID32 Staph kit (Biomerieux, Marcy l'Etoile, France). Susceptibility to various antibiotics was determined on Mueller-Hinton agar by a standard diffusion technique with disks containing ampicillin, cephaloridine, chloramphenicol, clindamycin, erythromycin, fosfomicin, fusidic acid, gentamicin, kanamycin, lincomycin, minocycline, nitrofurantoin, novobiocin, oxacillin, penicillin, pristinamycin, rifampin, tetracycline, tobramycin, and vancomycin.

Isolation and analysis of teichoic acids. Teichoic acids were isolated according to the method of Schleifer et al. (17). Liberated sugars were analyzed as alditol acetates by gas chromatography-mass spectrometry on a Hewlett-Packard apparatus (model 5971A) with a type HP-1 glass capillary column (0.2 mm by 12 m), with temperature programming at 8°/min, from 150 to 270°C in the electron ionization (70 eV) mode.

Isolation of cell wall and amino acid analysis. From approximately 500 mg of dry cells, walls were prepared by mechanical disruption with an X-press

(Pharmacia LKB, Uppsala, Sweden). After further purification, carried out as depicted by Schleifer (16), the amino acid composition of complete cell wall was analyzed by gas chromatography-mass spectrometry with a Hewlett-Packard 5971A apparatus under the conditions described above. Standards of amino acids were hydrolyzed, derivatized, and analyzed in the same way.

Analysis of fatty acids. The fatty acid spectrum of whole-cell methanolsolates was determined according to the method of Minnikin and Goodfellow (10). Methyl esters of fatty acids were analyzed by gas chromatography-mass spectrometry (Hewlett-Packard model 5971A) under the conditions established for the analysis of the teichoic acids (described above). The relative ratio among different fatty acids was expressed as a mean value of 3 days.

DNA isolation and manipulations. Genomic DNA was prepared according to the method of Mordarski et al. (11). Lysozyme-resistant strains were treated with lysothaphin (Sigma; 5 U/g [wet biomass]) for 3 h at 37°C. Purification of DNA fragments and cloning techniques were described previously (24).

PCR. PCR was performed in a UNO-thermoblock (Biometra). The reaction mixture (100 µl) contained 1 µM primers, 1 µg of DNA template, all four deoxynucleotide triphosphates at 0.2 mM each, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, and 2.5 U of *Taq* polymerase.

DNA sequence determination. 16S genes coding for rRNA of *S. pulvereri* NT215^T were amplified in vitro by PCR (13) with oligonucleotide primers complementary to highly conserved regions of the 16S rRNA (8). The amplified DNA was sequenced either directly or after its cloning in the vector pUC18. Sequencing of both DNA strands was performed with the dideoxy-chain termination method (15) with T7 DNA polymerase (Promega Corp., Madison, Wis.) and [α -³⁵S]ATP (Amersham).

16S rRNA sequence analysis. The 16S rRNA sequences of the following organisms belonging to the genus *Staphylococcus* found in the GenBank Data Library were used for comparison (accession numbers are in parentheses): *S. aureus* (X68417), *S. epidermidis* (Z26894; Z22809), *S. haemolyticus* (X66100), *S. hominis* (X66101), and *S. saprophyticus* (L20250). The sequences of *S. muscae*, *S. schleiferi*, and *S. sciuri* were taken from reference 4.

The 16S rRNA gene sequence of *S. pulvereri* consisted of 1,009 nucleotides corresponding to 65% of the full 16S rRNA primary structure. Approximately 1,000 nucleotides of this sequence (ranging from positions 80 to 1077; *Escherichia coli* numbering system) were aligned with those of other bacteria, and sequence similarities were calculated (22). A phylogenetic tree was generated by the neighbor-joining method (14). Bootstrap values were obtained by using the programs of the PHYLIP package (3).

DNA base composition. The G+C content of DNA was extrapolated from the

TABLE 1. Designations and sources of the *S. pulvereri* strains

Strain	Other designation	Source
NT215 ^T	B92/78 Lodenkamper, Sweden	Hip infection
NT165	Marples 100 844 (phage study, British Isles)	Human
NT310	Marples 100 940 (phage study, Sweden)	Human
NT312	Marples 100 958 (phage study, Sweden)	Human
NT322	1xK7/22, Heczko, Poland	Carcass of diseased chicken

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TABLE 2. Characteristics of the *S. pulvereri* strains

Characteristic	NT215 ^T	NT165	NT310	NT312	NT322
Colony size ≥ 6 mm	+	+	+	—	ND ^a
Aerobic growth	+	+	+	+	+
Anaerobic growth	±	±	±	±	±
Catalase	+	+	+	+	+
Clumping factor	—	—	—	—	—
Staphylocoagulase	—	—	—	—	—
Acid produced aerobically from:					
D-Arabinose	—	—	—	—	—
D-Cellobiose	—	—	—	—	—
D-Fructose	+	+	+	+	+
D-Glucose	+	+	+	+	+
Lactose	—	+	—	+	+
Maltose	+	+	+	+	+
D-Mannitol	—	+	—	—	—
D-Mannose	—	—	—	+	+
N-Acetylglucosamine	—	—	—	—	+
Raffinose	—	—	—	—	—
D-Ribose	—	—	—	—	—
Sucrose	+	+	+	+	+
D-Trehalose	+	—	—	—	+
D-Turanose	—	—	—	—	—
Enzyme tests					
Acetoin production	+	+	+	+	—
Alkaline phosphatase	—	+	+	+	—
Arginine arylamidase	—	—	—	—	—
Arginine dihydrolase	—	—	+	+	+
Esculin (hydrolysis)	—	—	?	—	—
β -Galactosidase activity	—	—	—	—	+
β -Glucuronidase activity	+	—	—	—	+
Nitrate reduction	—	?	+	+	+
Ornithine decarboxylase	—	—	—	—	—
Pyrrolidonyl arylamidase	—	—	—	—	+
Urease activity	+	—	+	—	+
Resistance to:					
Lysostaphin (200 μ g/ml)	—	—	—	—	—
Lysozyme (400 μ g/ml)	+	+	+	+	+
Growth in the presence of 15% (wt/vol) NaCl	+	+	+	+	+

^a ND, not determined.

DNA melting temperature as described by Marmur and Doty (9). DNA isolated from *E. coli* K-12 (51.5 mol% G+C) was used as a control.

DNA relatedness. DNA from various strains was fixed on nitrocellulose membranes (25). After preincubation in Denhardt's solution, hybridizations were performed at 54°C for 24 h in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 20% deionized formamide. Genomic DNA was labelled in vitro by nick translation (12) with deoxy-[1',2',5'-³H]cytidine 5'-triphosphate (Amersham). All experiments were repeated three times, and average readings were recorded.

Nucleotide sequence accession number. The 16S rRNA sequence of the type strain of *S. pulvereri*, NT215, was deposited in the GenBank database under accession number U12764.

RESULTS AND DISCUSSION

Physiological and biochemical characteristics. The characteristics of five strains of *S. pulvereri* are shown in Table 2. All of the strains were coagulase negative and resistant to novobiocin. They all produced catalase, grew aerobically, were facultatively anaerobic, and were susceptible to ampicillin, cephaloridine, chloramphenicol, clindamycin, erythromycin, fosfomicin, fusidic acid, gentamicin, kanamycin, lincomycin, minocycline, nitrofurantoin, oxacillin, penicillin, pristinamycin, and vancomycin. Four strains (NT165, NT215^T, NT310, NT312) were resistant to tetracycline.

When the *S. pulvereri* strains were tested by the micro-method ID32 Staph System, the reactions of three of them (NT165, NT310, NT312) resembled those of *S. epidermidis*, whereas the reactions of the strains NT215^T and NT322 corresponded to those of *S. warneri* and *S. simulans*, respectively.

Molecular characterization. (i) The G+C content of DNA from *S. pulvereri* strains. The DNA base compositions of the five strains ranged from 27 to 30 mol% G+C; these values are slightly lower than the values (30 to 39 mol%) obtained for the previously described staphylococci (6).

(ii) DNA-DNA relatedness. The DNAs from all five strains shared more than 85% homology (Table 3) but only up to 36% homology with 30 other defined *Staphylococcus* species.

(iii) Sequence of the 16S rRNA gene. A DNA fragment from *S. pulvereri* NT215^T containing a 16S rRNA gene was amplified, cloned, and sequenced. A comparison of this sequence with the ones published from *S. haemolyticus*, *S. hominis*, *S.*

TABLE 3. Levels of DNA-DNA relatedness between *S. pulvereri* strains and other species in the genus *Staphylococcus*^a

Source of unlabeled DNA ^b	% Relatedness with <i>S. pulvereri</i> NT215 ^T	Source of unlabeled DNA ^b	% Relatedness with <i>S. pulvereri</i> NT215 ^T
<i>S. arlettae</i> DSM 20672.....	17	<i>S. kloosii</i> DSM 20676.....	9
<i>S. aureus</i> DSM 20231.....	5	<i>S. lentus</i> DSM 20352.....	36
<i>S. auricularis</i> DSM 20609.....	7	<i>S. lugdunensis</i> ATCC 43808.....	3
<i>S. capitis</i> DSM 20326.....	14	<i>S. muscae</i> CCM 4175.....	5
<i>S. caprae</i> DSM 20608.....	24	<i>S. piscifermentans</i> JCM 6057.....	4
<i>S. carnosus</i> DSM 20501.....	18	<i>S. pulvereri</i> NT165.....	86
<i>S. caseolyticus</i> DSM 20597.....	9	<i>S. pulvereri</i> NT215 ^T	100
<i>S. chromogenes</i> DSM 20454.....	15	<i>S. pulvereri</i> NT310.....	94
<i>S. cohnii</i> DSM 20260.....	22	<i>S. pulvereri</i> NT312.....	100
<i>S. delphini</i> CCM 4184.....	5	<i>S. pulvereri</i> NT320.....	85
<i>S. epidermidis</i> DSM 20044.....	13	<i>S. saccharolyticus</i> DSM 20359.....	8
<i>S. equorum</i> DSM 20674.....	12	<i>S. saprophyticus</i> DSM 20229.....	17
<i>S. felis</i> DSM 7377.....	12	<i>S. schleiferi</i> ATCC 43809.....	8
<i>S. gallinarum</i> DSM 20610.....	26	<i>S. sciuri</i> DSM 20345.....	12
<i>S. haemolyticus</i> DSM 20263.....	16	<i>S. simulans</i> DSM 20322.....	9
<i>S. hominis</i> DSM 20328.....	11	<i>S. warneri</i> DSM 20316.....	14
<i>S. hyicus</i> DSM 20459.....	6	<i>S. xylosus</i> DSM 20266.....	17
<i>S. intermedius</i> DSM 20373.....	4		

^a Hybridizations were carried out under stringent conditions.^b Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Riken, Saitama, Japan.

TABLE 4. Similarity values for sequences of genes coding for 16S rRNA of *S. pulvereri* and other staphylococcal species

Species	% Similarity to:							
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. haemolyticus</i>	<i>S. hominis</i>	<i>S. muscae</i>	<i>S. pulvereri</i>	<i>S. saprophyticus</i>	<i>S. schleiferi</i>
<i>S. aureus</i>								
<i>S. epidermidis</i>	98.2							
<i>S. haemolyticus</i>	97.5	97.2						
<i>S. hominis</i>	97.3	97.7	98.5					
<i>S. muscae</i>	96.7	97.2	96.6	97.0				
<i>S. pulvereri</i>	96.5	96.1	95.5	96.3	95.5			
<i>S. saprophyticus</i>	97.3	96.8	97.8	97.6	96.3	96.0		
<i>S. schleiferi</i>	96.3	96.9	95.8	96.2	97.9	95.8	96.0	
<i>S. sciuri</i>	97.3	96.9	96.4	96.6	96.5	98.4	96.7	96.6

muscae, *S. saprophyticus*, *S. sciuri*, and *S. schleiferi* showed that the strain NT215^T was a member of the genus *Staphylococcus* and is most closely related to *S. sciuri* (98.4% similarity [Table 4 and Fig. 1]).

Cell wall analysis. (i) Amino acids. The cell walls of all five *S. pulvereri* strains contained a glycine-rich peptidoglycan, which is typical of staphylococci. All of them had an L-Lys-Ala-Gly₄₋₅ peptidoglycan type. The presence of L-Ala in the interpeptide bridge is also a characteristic feature of *S. lentus* and *S. sciuri* (18). Serine was not detected in any of the strains analyzed.

(ii) Teichoic acid. The teichoic acids from all five strains analyzed corresponded to the poly(glycerolphosphate) type, with ribose and *N*-acetylglucosamine (GlcNAc) as the main constituents. Ribitol could not be detected.

(iii) Cellular fatty acid composition. Methanolysates of complete *S. pulvereri* cells were mainly composed of *iso*- and *anteiso*-branched fatty acids with 15, 17, and 19 carbon atoms. Long-chain-saturated C_{20:0} and *iso*-C_{21:0} were found, in addition to small amounts of unsaturated C_{17:1} acids. C_{20:0} fatty

FIG. 1. Phylogenetic tree constructed by the neighbor-joining method showing the relationships among *S. pulvereri* and other staphylococcal species. Bootstrap values are indicated at branching points.

TABLE 5. Characteristics that differentiate *S. pulvereri* from other novobiocin-resistant staphylococci^a

Characteristic	<i>S. arlettae</i>	<i>S. cohnii</i>	<i>S. equorum</i>	<i>S. gallinarum</i>	<i>S. kloosi</i>	<i>S. lentus</i>	<i>S. pulvereri</i>	<i>S. saprophyticus</i>	<i>S. sciuri</i>	<i>S. xylosus</i>
Colony size ≥ 6 mm	d	d	—	+	d	—	d	+	+	+
Colony pigment	+	—	—	d	d	d	d	d	d	d
Alkaline phosphatase	(+)	—	(+)	(+)	d	(±)	d	—	+	d
Esculin (hydrolysis)	—	—	d	+	d	+	—	—	+	d
Acetoin production	—	d	—	—	d	—	d	+	—	d
Urease	—	—	+	+	d	—	d	+	—	+
Acid produced from:										
D-Maltose	+	(d)	d	+	d	d	+	+	(d)	+
D-Mannitol	+	d	+	+	+	+	d	d	+	+
Raffinose	+	—	—	+	—	+	—	—	—	—
Sucrose	+	—	+	+	(±)	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	d	+	+	+
D-Turanose	+	—	d	+	—	(±)	—	+	(±)	d

^a +, 90% or more strains positive; ±, 90% or more strains weak positive; —, 90% or more strains negative; d, 11 to 89% of strains positive. Parentheses indicate a delayed reaction. Table 5 is adapted from work by Kloos et al. (6).

acid had recently been reported to be characteristic for the genus (18).

(iv) Description of *S. pulvereri* sp. nov. The description of the species *S. pulvereri* (pul' ver. er.i. L. gen. n. *pulvereri*, in honor of the German microbiologist Gerhard Pulverer for contributions to the study of staphylococcal infections in clinical microbiology) is based on a total of five strains isolated from different sources. The cells are nonsporulating, nonmotile, gram-positive cocci. Agar colonies are circular, entire, smooth, nonpigmented or white with yellowish tint, and 4.0 to 8.0 mm in diameter. All strains are facultative anaerobes which grow best under aerobic conditions. Anaerobic growth on Wilkins agar is evident after 24 to 48 h. The strains cannot be cultivated in semisolid thioglycolate medium. NaCl does not affect the growth up to concentrations of 15%; the strains are killed in the presence of 17.5% NaCl. Colonies develop at 25 and 37°C after 48 and 24 h, respectively. Weak growth is observed at 10°C, while at 45°C there is no growth.

The five strains produce neither extracellular nor cell-associated coagulase (clumping factor). All of them are resistant to novobiocin. The characteristics are listed in Table 2.

The DNA base composition ranges from 27.0 to 30.0 mol% G+C. The peptidoglycan structure is of the L-Lys-Ala-Gly₄₋₅ type. The cell wall teichoic acids contain glycerol, ribose, and *N*-acetylglucosamine. The dominant cellular fatty acids are *iso* and *anteiso* branched with 15, 17, and 19 carbon atoms.

Description of the type strain. The type strain, NT215 (=PCM 2443^T; Polish Collection of Microorganisms, Wrocław, Poland), was isolated from a hip infection. The characteristics of the type strain are the same as those given above for the species and in Table 2. The G+C content is 27 mol%.

The selected characteristics are sufficient to differentiate *S. pulvereri* from other novobiocin-resistant, coagulase-negative *Staphylococcus* species (Table 5). *S. pulvereri* can be distinguished from other species by its production of acids from maltose and sucrose (but not from raffinose and D-turanose), by its lack of esculin activity, its peptidoglycan type, and its comparatively low G+C content.

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