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Evaluation of Methods for Identification and Determination of the Taxonomic Status of Strains Belonging to the *Streptococcus porcinus-Streptococcus pseudoporcinus* Complex Isolated from Animal, Human, and Dairy Sources

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Ninety-seven animal, human, and dairy *Streptococcus porcinus* or *Streptococcus pseudoporcinus* isolates in the CDC *Streptococcus* strain collection were evaluated on the basis of DNA-DNA reassociation, 16S rRNA and *rpoB* gene sequencing, conventional biochemical and Rapid ID 32 Strep identification methods, and antimicrobial susceptibility testing to determine their taxonomic status, characteristics for species differentiation, antimicrobial susceptibility, and relevance of clinical source. Nineteen of the 97 isolates (1 human, 18 swine) were identified as *S. porcinus*. The remaining 72 human isolates and 6 dairy isolates were identified as *S. pseudoporcinus*. The use of 16S rRNA or *rpoB* gene sequencing was required to differentiate *S. porcinus* from *S. pseudoporcinus*. The human and dairy *S. pseudoporcinus* isolates were biochemically distinct from each other as well as distinct by 16S rRNA and *rpoB* gene sequencing. Therefore, we propose the subspecies denominations *S. pseudoporcinus* subsp. *hominis* subsp. nov. for the human isolates and *S. pseudoporcinus* subsp. *lactis* subsp. nov. for the dairy isolates. Most strains were susceptible to the antimicrobials tested, with the exception of tetracycline. Two strains of each species were also resistant to clindamycin and erythromycin and carried the *erm*(A) (*S. pseudoporcinus*) or the *erm*(B) (*S. porcinus*) gene. *S. porcinus* was identified from a single human isolate recovered from a wound in an abattoir worker. *S. pseudoporcinus* was primarily isolated from the genitourinary tract of women but was also associated with blood, placental, and wound infections. Isolates reacting with group B antiserum and demonstrating wide beta-hemolysis should be suspected of being *S. pseudoporcinus* and not *S. agalactiae*.

Due to the advancement and use of genetic sequence markers, the taxonomic standing of many microorganisms characterized in the past has been reevaluated. A recent example involves isolates that were previously identified as *Streptococcus porcinus*. This species was originally described in 1984 (8) as a pathogen associated with swine. The isolation from human sources of strains with nearly identical phenotypic characteristics was later described (10, 14, 22). A more recent publication describing a study that used 16S rRNA gene sequencing revealed that isolates primarily from the genital tract of female patients were significantly different (greater than 2.1% dissimilarity) and constituted a separate species, named *Streptococcus pseudoporcinus* (3). Consequently, we decided to reevaluate the identification of *S. porcinus* isolates from our previous studies (10, 14, 32) and more recent isolates included in the CDC *Streptococcus* Laboratory collection. The goal of this study was to determine the association of *S. porcinus* and *S. pseudoporcinus* isolates with different sources, analyze genetic and phenotype characteristics useful for their differentiation and precise identification, and examine potential differences in antimicrobial susceptibility patterns. Accordingly, isolates were evaluated by 16S rRNA and *rpoB* gene sequencing, DNA-DNA reassociation experiments, conventional biochemical testing, Rapid ID 32 Strep panels, and antimicrobial susceptibility patterns.

MATERIALS AND METHODS

Bacterial strains. We included in the present study a total of 97 strains belonging to the *S. porcinus*/*S. pseudoporcinus* complex, comprising most ($n = 42$) of the strains described in previous publications of our group (10, 14, 32), 2 additional group E reference strains, and 53 additional isolates obtained between 1984 and 2010. From the last group, 22 strains were received from the collection of the Wadsworth Center, New York State Department of Health, and 3 strains were recovered from dairy products that were cultured during the investigation to elucidate a nephritis outbreak in a Brazilian city (2). The type strains of several other streptococcal species were also included for comparative purposes.

Phenotypic characteristics. The isolates were tested for their phenotypic characteristics by using a conventional biochemical identification schema (13, 12) and the Rapid ID 32 Strep system (18) (bioMérieux, Inc.). Serogroups were determined by using the Lancefield method and antisera for groups B, E, P, U, V, NG1, NG2, and NG3, as previously described (10, 14, 16). Reactivity with group B antiserum was also evaluated using a PathoDX Strep grouping latex agglutination test kit (Remel, Lenexa, KS), in accordance with the manufacturer's package insert.

Antimicrobial susceptibility testing. MICs were determined by using a broth microdilution panel method according to the manufacturer's instructions (PML Microbiologicals, Wilsonville, Oregon). The following antibiotics were tested: ampicillin, cefotaxime, clindamycin, erythromy-

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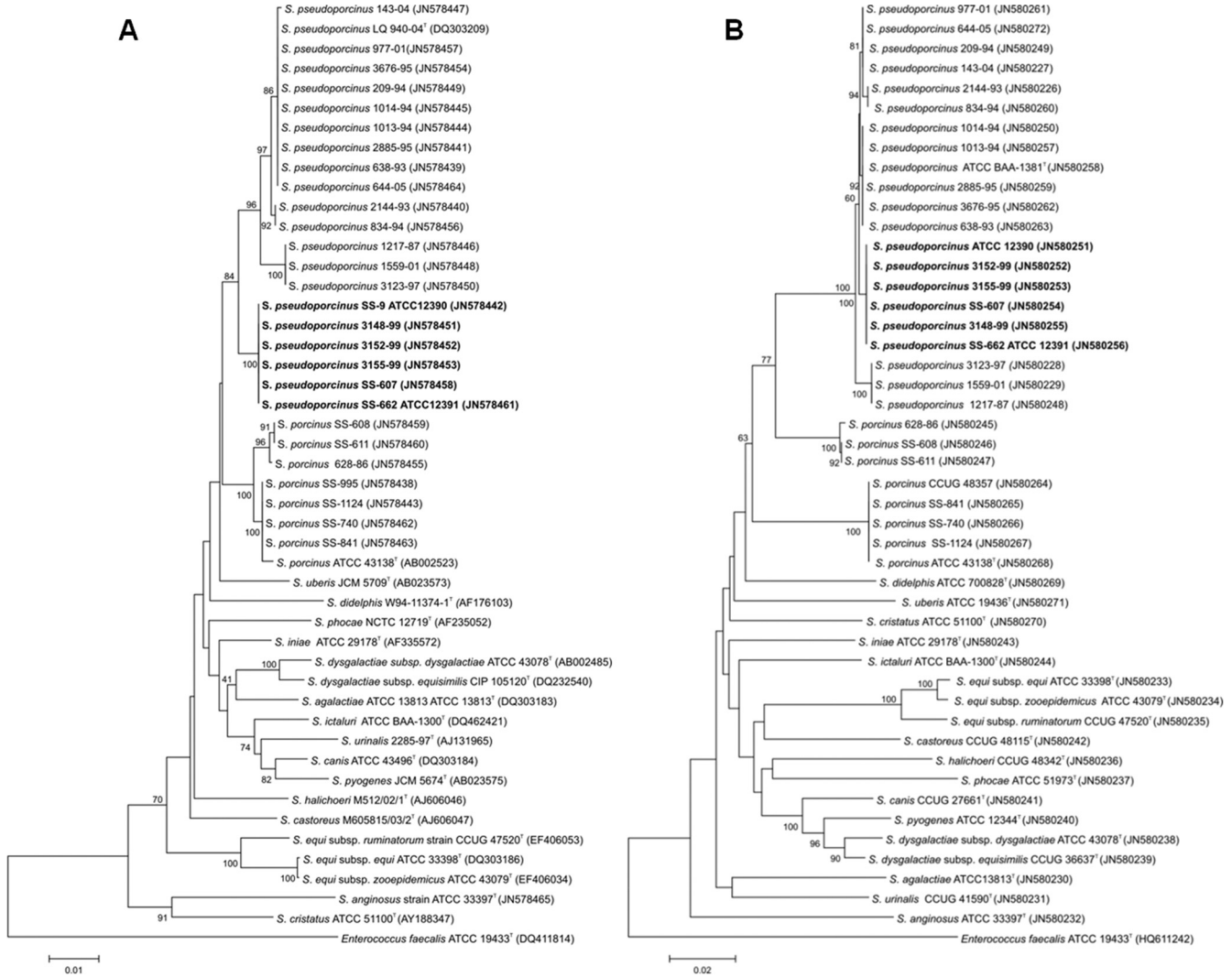

FIG 1 Phylogenetic trees from 16S rRNA gene (A) and *rpoB* gene (B) sequence data of representative strains belonging to the *Streptococcus porcinus*/*Streptococcus pseudoporcinus* complex and other select species of *Streptococcus*. GenBank accession numbers are given in parentheses. The evolutionary history was inferred by using the neighbor-joining method. The associated taxa clustered together in 60% or more of the 1,000 replicate trees. *Enterococcus faecalis* was used as the outgroup sequence. The scale bar indicates the units of the number of base substitutions per site.

cin, levofloxacin, penicillin, tetracycline, and vancomycin. The MIC interpretive standards for beta-hemolytic streptococci were used (7).

Determination of macrolide resistance phenotypes and genotypes. Macrolide resistance phenotypes were determined by using a modification of the double-disk test described by Seppälä et al. (24). Disks (Oxoid Ltd., Basingstoke, United Kingdom) of erythromycin (15 mg) and clindamycin (2 mg) were placed 12 mm apart on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, MD) supplemented with 5% sheep blood which had been inoculated with a swab dipped into a bacterial suspension with a turbidity equivalent to a 0.5 McFarland standard. Resistant isolates were evaluated for the presence of macrolide resistance genes by PCR on the basis of previous recommendations (11, 29). Briefly, one loopful from an overnight culture on 5% sheep blood agar was suspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1 mg of lysozyme and 5 U of mutanolysin. After 30 min incubation at 37°C, the suspensions were heated at 100°C for 5 min and the lysates were stored at −20°C. PCR conditions were as described by Sutcliffe et al. (29). The PCRs were done separately for each erythromycin-resistant determinant, with 4 mM magnesium used for both the *erm*(A) and *mef* primer sets and 2 mM used for the *erm*(B) primer set.

16S rRNA gene sequencing. Amplification and sequencing of the 16S rRNA gene were performed as previously described (25, 26). Sequencing reaction products were purified with Centri-Sep plates (Princeton Separations, Princeton, NJ). Reaction mixtures were electrophoresed on an ABI 3130 or 3730 apparatus using POP-7 polymer (Applied Biosystems). Chromatograms were assembled and analyzed in Seqmerge (Wisconsin Package, version 10.3; Accelrys Inc., San Diego, CA). The consensus sequences were aligned by using the CLUSTAL W program (33) and trimmed to 1,419 bp to create a phylogenetic tree (Fig. 1A). Evolutionary analyses were conducted in the MEGA5 program (31). The evolutionary history was inferred using the neighbor-joining method (23). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was determined (17). The evolutionary distances were computed using the maximum composite likelihood method (30).

***rpoB* gene sequencing.** Approximately 1,200 bp of the RNA polymerase beta subunit (*rpoB*) gene from each isolate was amplified with HotStarTaq (Qiagen, Valencia, CA) using primers UnivrpoB3F (5′-ATGGGNDCGNAAYATGCA) and UnivrpoB23R (5′-GAYATGGAYGTNTGYGC) in a 50-μl PCR mixture. The thermal cycling conditions were 95°C

for 5 min; 15 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a decrease of the annealing temperature by 1°C at each cycle; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; 72°C for 10 min; and a final hold at 4°C. The PCR amplicons were purified through a Nucleospin column (Clontech) and eluted in 120 μl PCR-grade water. Cycle sequencing was performed as described above for the 16S rRNA gene but instead using primers UnivrpoB3F, UnivrpoB23R, UnivrpoBseq1 (5′-GGNGAYAARNT NKSNRR), and UnivrpoBseq2 (5′-YYNSMNANYTTRTCNCC). Raw sequence data were assembled in Geneious (version 5.4) software (9), and a neighbor-joining tree was created in MEGA5 using an alignment of 1,149 bp (Fig. 1B).

DNA-DNA reassociation experiments. The cultures were grown in 2-liter flasks containing 1 liter Todd-Hewitt broth and incubated at 35°C for 18 h to 24 h on a rotary shaker. The cells were centrifuged to obtain a pellet, and total DNA was isolated with phenol-chloroform extraction and purified. DNA-DNA reassociation experiments were performed as previously described (4, 27). The temperatures used for DNA reassociation were 55°C (optimal conditions) and 70°C (stringent conditions).

Nucleotide sequence accession numbers. The 16S rRNA and *rpoB* gene sequences for strains belonging to the *S. porcinus*/*S. pseudoporcinus* complex generated during this study have been deposited in GenBank. The accession numbers are shown in Fig. 1. The sequences of the 16S rRNA genes determined in this study have GenBank accession numbers JN578438 to JN578465. The partial sequences of the *rpoB* genes determined in this study have GenBank accession numbers JN580226 to JN580272.

RESULTS AND DISCUSSION

16S rRNA gene and *rpoB* gene sequence analyses. The results of comparative 16S rRNA and *rpoB* gene sequence analysis for the isolates were confirmed with DNA-DNA reassociation experiments and are shown in Fig. 1A and B. Of the 97 isolates, 19 showed greater than 99% 16S rRNA gene sequence identity to the type strain of *S. porcinus* (GenBank accession number AB002523), and 78 isolates had greater than 98% sequence identity to *S. pseudoporcinus* (GenBank accession number DQ303209). However, six of these *S. pseudoporcinus* isolates, including SS-662 (ATCC 12391, a dairy isolate), formed a separate clade on the dendrogram (Fig. 1A) and differed significantly from other *S. pseudoporcinus* isolates on the basis of source and biochemical data. The sequence from SS-662 was 98.8% identical to the 16S rRNA gene sequence of the *S. pseudoporcinus* type strain and 98.3% identical to that of the *S. porcinus* type strain, values which exceed the criteria recommended for species identification (6). The identification of the dairy isolates as *S. pseudoporcinus* was possible on the basis of a signature sequence in the first 100 nucleotides of the 16S rRNA gene. All *S. pseudoporcinus* 16S rRNA gene sequences were identical and differed by 7 nucleotides from the *S. porcinus* sequence (region from positions 61 to 91 of the *Escherichia coli* 16S rRNA gene sequence, GenBank accession number X80725). The sequences are as follows: TGAGGTCTGGTGCTTGCACTAGAC CAAG for *S. pseudoporcinus* and AGAGGACAGGTGCTTGCAC CAGTCTAAT for *S. porcinus*. The *S. pseudoporcinus* dairy isolates could also be distinguished from the *S. pseudoporcinus* human isolates by a signature sequence in the region from positions 175 to 218 of the *E. coli* 16S rRNA gene sequence, GenBank accession number X80725. The sequences are as follows: CAATAGAGTAC ACATGTACTTAATTTAAAAGGGGCAACTGCTC for dairy isolates and GACTGGGGTTCACATGAACCCGAGTTAAAAGGA GCAAAAGCTT for human isolates. DNA-DNA hybridization or sequencing of an additional genetic target, the *rpoB* gene, was required for unambiguous identification of the dairy isolates as *S. pseudoporcinus*.

Overall, the *rpoB* gene sequencing results corroborated those obtained by 16S rRNA gene sequencing. Comparative analysis of the *rpoB* gene sequencing results is shown in Fig. 1B. The *rpoB* gene, however, is a more powerful tool to distinguish these two difficult-to-separate species. The *rpoB* sequence of *S. pseudoporcinus* strain SS-662 was 99.2% identical to that of *S. pseudoporcinus* and 89.6% identical to that of *S. porcinus*. In both the 16S rRNA and *rpoB* trees, the six *S. pseudoporcinus* dairy isolates, including SS-662, were strongly grouped together in a clade (100% bootstrap values). To the best of our knowledge, this is the first report on the application of *rpoB* gene sequencing for the study of microorganisms included in the *S. porcinus*/*S. pseudoporcinus* complex.

In a publication prior to the species description of *S. pseudoporcinus*, restriction fragment length polymorphism (RFLP) of the 16S rRNA gene with the enzyme BpiI was used to distinguish *S. porcinus* (group E, P, U, V, NG1, NG2, and NG3) strains from other *Streptococcus* species (1). *In silico* analysis of the 16S rRNA gene sequences for all *S. pseudoporcinus* strains in our study revealed that none of these isolates has the restriction enzyme site BpiI. Analysis of the 16S rRNA gene sequence of all 19 *S. porcinus* isolates in our study confirmed the presence of the BpiI restriction site. Thus, the BpiI RFLP method may be useful to distinguish between *S. porcinus* and *S. pseudoporcinus* and should be evaluated.

DNA-DNA reassociation experiments. The type strains for *S. porcinus* (ATCC 43138) and *S. pseudoporcinus* (ATCC BAA-1381), as well as SS-662 (ATCC 12391, a select strain representative of the dairy isolates), were labeled with ^{32}P for the DNA reassociation studies and hybridized with representative strains, as shown in Table 1. The representative group of strains was selected for inclusion in DNA-DNA reassociation studies on the basis of differences in 16S rRNA gene sequences, differences in source, and/or slight differences in biochemical patterns. Two strains are considered the same species if their DNA-DNA relatedness is 70% or higher at the optimal reassociation temperature and 55% or more at the stringent temperature with 5% divergence or less (34). The 6 *S. porcinus* isolates and human clinical isolate 628-86 were confirmed to be *S. porcinus*. *S. porcinus* ATCC 43183^T was less than 58% related at the optimal temperature and less than 33% related at the stringent temperature and showed 5.0% or greater divergence from *S. pseudoporcinus* type strain ATCC BAA-1381 and reference dairy strain SS-662 (ATCC 12391), indicating that these were a different species. *S. pseudoporcinus* ATCC BAA-1381^T showed 24% or less relatedness when hybridized against the type strains of other phylogenetically related species of *Streptococcus*. In reciprocal experiments, DNA studies with labeled *S. pseudoporcinus* ATCC BAA-1381^T showed greater than 70% relatedness at the optimal and stringent temperatures to all 11 *S. pseudoporcinus* clinical isolates and the SS-662 dairy isolate and showed 24% or less relatedness to the other *Streptococcus* species tested. In addition, in experiments with labeled strain SS-662, all 5 additional dairy isolates and *S. pseudoporcinus* ATCC BAA-1381^T were greater than 79% related at the optimal and stringent temperatures with less that 2% divergence. Strain SS-662 (ATCC 12391) was 48% related to *S. porcinus* ATCC 43183^T with a divergence of 9.0%, indicating that it is not *S. porcinus*. Results of DNA-DNA reassociation with SS-662 against other *Streptococcus* species revealed 20% or less relatedness.

TABLE 1 Levels of DNA relatedness between *Streptococcus pseudoporcinus* human isolates, *S. pseudoporcinus* dairy isolates, *Streptococcus porcinus*, and other selected species of *Streptococcus*

Strain source of unlabeled DNA	Result with labeled DNA from[a]:								
	S. pseudoporcinus ATCC BAA-1381^T (human isolate)			*S. pseudoporcinus* ATCC 12391 (SS-662) (dairy isolate)			*S. porcinus* ATCC 43138^T		
	RBR at 55°C	% D	RBR at 70°C	RBR at 55°C	% D	RBR at 70°C	RBR at 55°C	% D	RBR at 70°C
S. porcinus/*S. pseudoporcinus* reference strains									
S. pseudoporcinus ATCC BAA-1381^T (SS-1796)	100	0.0	100	79	2.0	79	60	5.0	33
S. pseudoporcinus dairy ATCC 12391^T (SS-662)	90	2.0	70	100	0.0	100	58	6.0	18
S. porcinus ATCC 43138^T (SS-1029)	58	7.0	20	48	9.0	25	100	0.0	100
S. porcinus/*S. pseudoporcinus* test strains[b]									
S. pseudoporcinus human isolates (11 strains)	83–100	0.0–1.5	71–100	NP	NP	NP	NP	NP	NP
S. pseudoporcinus dairy isolates (5 strains)	NP	NP	NP	90–100	0.0–0.5	85–100	NP	NP	NP
S. porcinus animal isolates (6 strains)	NP	NP	NP	NP	NP	NP	83–100	0.5–4.5	83–92
S. porcinus human isolate (1 strain)	NP	NP	NP	NP	NP	NP	97	1.5	92
Reference strains of other species									
S. iniae ATCC 29178^T	20	NP	NP	19	NP	NP	19	NP	NP
S. uberis ATCC 19436^T	17	NP	NP	17	NP	NP	16	NP	NP
S. cristatus ATCC 51100^T	24	NP	NP	24	NP	NP	23	NP	NP
S. halichoeri CCUG 48342^T	3	NP	NP	4	NP	NP	6	NP	NP
S. didelphis ATCC 700828^T	19	NP	NP	20	NP	NP	20	NP	NP
S. agalactiae ATCC 13813^T	17	NP	NP	18	NP	NP	19	NP	NP
S. pyogenes ATCC 12344^T	18	NP	NP	18	NP	NP	19	NP	NP
S. dysgalactiae subsp. *dysgalactiae* ATCC 43078^T	19	NP	NP	20	NP	NP	20	NP	NP
S. dysgalactiae subsp. *equisimilis* CCUG 36637	21	NP	NP	21	NP	NP	20	NP	NP
S. equi subsp. *equi* ATCC 33398^T	17	NP	NP	18	NP	NP	20	NP	NP
S. equi subsp. *zooepidemicus* ATCC 43079^T	18	NP	NP	18	NP	NP	19	NP	NP
S. equi subsp. *ruminatorum* CCUG 47520^T	10	NP	NP	11	NP	NP	11	NP	NP
S. canis ATCC 43496^T	21	NP	NP	23	NP	NP	21	NP	NP
S. anginosus ATCC 33397^T	11	NP	NP	10	NP	NP	11	NP	NP
S. phocae ATCC 51973^T	8	NP	NP	9	NP	NP	8	NP	NP

[a] RBR, relative binding ratio; % D, percent divergence, calculated to the nearest 0.5%; NP, not performed.
[b] Numbers represent average values of the results obtained for the number of strains indicated in parentheses for each species or category.

Phenotypic characteristics. Very few biochemical differences were observed between *S. porcinus* and *S. pseudoporcinus*, as shown in Table 2. Using conventional biochemical testing, hippurate hydrolysis was the key test in differentiating *S. porcinus* from *S. pseudoporcinus*. The six dairy source isolates were distinguished from both *S. porcinus* and *S. pseudoporcinus* by their inability to grow in 6.5% NaCl and to acidify pyruvate broth. The 100% positive results for Vogues-Proskauer and lactose tests observed for the dairy isolates are more consistent with *S. porcinus* (84% positive compared to 18% positive for *S. pseudoporcinus*). It is noteworthy that the different methodologies used gave different results for the hippurate hydrolysis test. This test is based on the bacterium's ability to hydrolyze hippurate to glycine and benzoic acid. The conventional tube method uses ferric chloride to form a precipitate with benzoic acid (15). The method on the Rapid ID 32 Strep panel uses ninhydrin to detect glycine (20). The ninhydrin method appears to be more sensitive, as the majority of *S. porcinus* and *S. pseudoporcinus* isolates were positive when tested by the Rapid ID 32 Strep system. This finding is consistent with that described in a previous publication describing a study that used several of the same strains (10). The Rapid ID 32 Strep system was not very useful in separating these species, as even fewer differences than the number obtained with the traditional biochemical method were observed. Again, it is noteworthy that with this method, 68% of *S. porcinus* isolates were hippurate positive with ninhydrin; however, all tested negative using the conventional method with ferric chloride. Using the Rapid ID 32 Strep system, all 19 *S. porcinus* isolates were identified as *S. porcinus*, with 18 strains having a confidence level of greater than 90%. All 72 *S. pseudoporcinus* strains were identified as *S. porcinus* with a confidence level of greater than 90%. Three *S. pseudoporcinus* dairy isolates were identified as *S. porcinus*, but with a confidence level of 81.7%, and the other three were identified as *S. uberis* with a confidence level of 74.1%.

Information available in the literature was reviewed (10, 13, 32), and more recent isolates were tested to analyze the serogroup data for *S. porcinus* and *S. pseudoporcinus.* The animal *S. porcinus* strains SS-995 and SS-996 cross-reacted with CDC group B antisera as well as with several commercial kits. In addition, the clinical isolate 1256-95, described as reacting with all commercial group B antisera, has subsequently been identified as *S. pseudoporcinus*. A noteworthy finding was that only two of the *S. porcinus* strains tested reacted with NG1 antisera, whereas almost all *S. pseudoporcinus* strains reacted. Interestingly, NG1 group antiserum was derived from *S. porcinus* SS-995, an animal isolate. Five of the six *S. pseudoporcinus* dairy isolates reacted with group E antisera, one isolate was nongroupable, and none reacted with group B or NG1 antisera. This would indicate that antigenic entities are

TABLE 2 Phenotypic characteristics of *Streptococcus pseudoporcinus* and *Streptococcus porcinus* isolates[a]

Test and phenotypic characteristic	*S. pseudoporcinus* human isolates (total $n = 72$)			*S. pseudoporcinus* dairy strains (total $n = 6$)			*S. porcinus* (total $n = 19$)		
	No.	%	RXN	No.	%	RXN	No.	%	RXN
Conventional tests									
Pyrase	59	83	V	6 (1)[b]	100	+	17 (6)	89	V
Bile esculin	30	41	V	0	0	−	1	5	−
6.5% NaCl	72	100	+	0	0	−	19	100	+
Esculin	72	100	+	6	100	+	19	100	+
Arginine	72	100	+	6	100	+	19	100	+
Hippurate	67	93	+	0	0	−	0	0	−
Pyruvate	60	83	V	0	0	−	14	74	V
Voges-Proskauer	13	18	V	6	100	+	16 (5)	84	V
CAMP	70	97	+	6	100	+	19	100	+
Acid from:									
Glycerol	52	72	V	0	0	−	1	5	−
Lactose	2	3	−	6	100	+	6	32	V
Maltose	72	100	+	6	100	+	19	100	+
Mannitol	72	100	+	6	100	+	19	100	+
Ribose	72	100	+	6	100	+	19	100	+
Sorbitol	72	100	+	6	100	+	19	100	+
Sucrose	72	100	+	6	100	+	19	100	+
Trehalose	72	100	+	6	100	+	19	100	+
MGP[c]	69	97	+	6	100	+	14	74	V
Rapid ID 32 Strep tests[d]									
α-Galactosidase	40	56	V	0	0	−	16	84	V
Lactose	0	0	−	6	100	+	9	43	V
Hippurate	68	94	+	6	100	+	13	68	V
Pullulan	70	97	+	0	0	−	17	89	V
M-β-Glu[e]	5	7	−	0	0	−	12	63	V

[a] No., number of strains with positive results; RXN, expected reaction (+, 90% or more of the strains were positive; −, 90% or more of the strains were negative; V, variable [11 to 89% of the strains were positive]).
[b] Numbers in parentheses indicate the number of strains with weak reactions.
[c] MGP, methyl-α-D-glucopyranoside.
[d] Only differential tests are listed.
[e] M-β-Glu, methyl-β-D-glucopyranoside.

shared between *S. porcinus*, *S. pseudoporcinus*, and group B *Streptococcus agalactiae*. While serogroup may be helpful with identification, it would not be conclusive in identifying *S. pseudoporcinus*.

Current testing with the Remel PathoDx Strep group B latex reagent revealed that one *S. porcinus* isolate (5%) reacted strongly (greater than 2+ agglutination), 74% ($n = 14$) showed a weak reaction (grainy agglutination), and 21% ($n = 4$) did not react. Ten (14%) of the *S. pseudoporcinus* strains reacted strongly, 69% ($n = 50$) reacted weakly, and 17% ($n = 12$) gave no reaction. None of the *S. pseudoporcinus* dairy isolates reacted with the PathoDx group B reagent. In addition, two *S. porcinus* isolates and all the human *S. pseudoporcinus* isolates reacted with NG1 antisera. None of the six *S. pseudoporcinus* dairy isolates reacted with NG1 antisera. These six dairy isolates demonstrated serogroup E in previous studies (10). The two additional serogroup E reference strains isolated from pigs, SS-608 and SS-611 (which were not included in the earlier studies), were retested with the current methodology and identified as *S. porcinus* by 16S rRNA and *rpoB* gene sequencing and DNA-DNA reassociation. This finding is somewhat expected, since serogroup is often not species specific, and other species of *Streptococcus* (e.g., *S. uberis*) also react with serogroup E antisera (35). On the basis of results of this study and previous studies, *S. porcinus* may possess Lancefield group B, E, P, U, V, NG1, NG2, or NG3 or no group antigen; human *S. pseudoporcinus* may possess B, P, or NG1 or no group antigen; and dairy *S. pseudoporcinus* isolates may possess E or no group antigen. Isolates reacting with group B antiserum and demonstrating wide zones of beta hemolysis should be suspected of being *S. pseudoporcinus* and not *S. agalactiae*.

Antimicrobial susceptibility. There was little difference in the susceptibility of *S. porcinus* and *S. pseudoporcinus* strains to the antimicrobials tested, as shown in Table 3. A few strains of both species showed resistance to both clindamycin and erythromycin, and more than half of all the isolates were resistant to tetracycline. The finding of high percentages of resistance to tetracycline is in agreement with previously published studies (10, 19). Two *S. porcinus* isolates showed multiresistance to clindamycin, erythromycin, and tetracycline. The constitutive macrolide, lincosamide, streptogramin B (cMLS) phenotype and the *erm*(B) gene were detected in these two erythromycin-resistant *S. porcinus* isolates. Over 72% of the *S. pseudoporcinus* strains were resistant to tetracycline. Two *S. pseudoporcinus* strains also showed resistance to both clindamycin and erythromycin, indicative of the cMLS phenotype. The *erm*(A) gene was detected in these two *S. pseudoporcinus* isolates. This is the first report of the presence of the *erm* genes among strains of *S. porcinus* and *S. pseudoporcinus*. The six *S.*

TABLE 3 Antimicrobial susceptibility of 72 *Streptococcus pseudoporcinus* human isolates, 6 *S. pseudoporcinus* dairy isolates, and 19 *Streptococcus porcinus* isolates

	S. pseudoporcinus human isolates ($n = 72$)				*S. pseudoporcinus* dairy isolates ($n = 6$)				*S. porcinus* ($n = 19$)			
	MIC (μg/ml)			No. of isolates susceptible (resistant)[a]	MIC (μg/ml)			No. of isolates susceptible	MIC (μg/ml)			No. of isolates susceptible (resistant)
Antimicrobial agent	Range	50%	90%		Range	50%	90%		Range	50%	90%	
Ampicillin	0.03–0.06	0.06	0.06	72	0.015–.03	0.03	0.03	6	0.03–0.06	0.06	0.06	19
Cefotaxime	0.03–0.06	0.03	0.06	72	0.015–0.015	0.015	0.015	6	0.03–0.06	0.03	0.06	19
Clindamycin	0.03–>32	0.06	0.12	70 (2[b])	0.03–0.03	0.03	0.03	6	0.06–>32	0.06	0.06	17 (2[c])
Erythromycin	0.008–>32	0.06	0.12	70 (2[b])	0.03–0.03	0.03	0.03	6	0.03–>32	0.06	>32	17 (2[c])
Levofloxacin	0.5–1	1	1	72	0.05–0.5	0.5	0.5	6	0.5–1	1	1	19
Penicillin	0.008–0.03	0.015	0.015	72	0.008–0.008	0.008	0.008	6	0.008–0.03	0.03	0.03	19
Tetracycline	1–>16	>16	>16	20 (52)	1–1	1	1	6	1–>16	1	>16	10 (9)
Vancomycin	0.25–1	0.5	0.5	72	0.5–1.0	0.5	1	6	0.25–1	1	1	

[a] CLSI interpretive standards for large-colony beta-hemolytic *Streptococcus* were used.
[b] These two *S. pseudoporcinus* isolates carried the *erm*(A) gene.
[c] These two *S. porcinus* isolates carried the *erm*(B) gene.

TABLE 4 Sources and patient characteristics of 78 *Streptococcus pseudoporcinus* and 19 *Streptococcus porcinus* isolates included in the present study

Source or characteristic[a]	*S. pseudoporcinus*[b] ($n = 78$)	*S. porcinus*[c] ($n = 19$)
No. of isolates from:		
Animals	0	18
Porcines	0	18
Dairy products	6	0
Milk	5	0
Cheese	1	0
Humans	73	1
No. of human subjects with characteristic/total no. (%)		
Gender ($n = 53/59$, 90%)		
Male	3/53 (5.7)	1/19 (5.3)
Female	50/53 (94.3)	
Range (mean) age (yr) by gender ($n = 28/59$, 47%)		
Male (3/28)	41–47 (43)	54
Female (25/28)	Newborn–56 (30)	
Clinical source of isolates ($n = 55/59$, 93%)		
Blood	5/55 (9.1)	
Wound	7/55 (12.7)	1
Placenta/umbilicus	8/55 (14.5)	
Urine	4/55 (7.4)	
Cervix	4/55 (7.3)	
Genital	25/55 (45.4)	

[a] No information (sex, age, or source) was provided for 13/72 (18%) of *S. pseudoporcinus* human isolates; for the remaining 59/72, at least one piece of information was provided.
[b] All three *S. pseudoporcinus* isolates from males were from wounds (sinus, scrotum, and no specific anatomic location provided).
[c] The one human *S. porcinus* isolate was from the wound of a male abattoir worker.

pseudoporcinus dairy isolates, isolated from milk and cheese, were very susceptible to all antibiotics.

Sources and clinical significance. Analysis of historical records of the *S. porcinus* isolates confirmed only one *S. porcinus* strain that was associated with human clinical disease. This strain was isolated from a skin lesion of a 54-year-old male abattoir worker, and zoonotic transmission is presumed. The remaining 18 *S. porcinus* isolates were from porcine sources. *S. pseudoporcinus* was not identified among the swine isolates in our collection.

Seventy-eight isolates were identified as *S. pseudoporcinus* on the basis of 16S rRNA and *rpoB* gene sequencing and DNA-DNA hybridization. The majority ($n = 72$) of the *S. pseudoporcinus* isolates were obtained from clinical human sources and 6 were from dairy sources. The demographic data for the patients and the sources of isolation for 59 of the 72 clinical *S. pseudoporcinus* isolates are shown in Table 4. Of the 56 isolates of which the source was provided, 78% (44/56) were recovered from the reproductive tract or urine of females aged from newborn to 56 years. We were not provided with patient nationality and cannot comment on the findings of a previous study (19) linking the isolation of *S. pseudoporcinus* isolates to patients originating from the Caribbean and sub-Saharan Africa. The gender was not provided for the source of 21 of the 72 isolates, so the percentage of isolates from males could be slightly higher. A recent report describes the isolation of *S. pseudoporcinus* from a thumb infection of a 33-year-old male (21), which is in agreement with our finding that *S. pseudoporcinus* infection in males is typically associated with wound infections (Table 4). Blood was listed as the source for 5 isolates (4 from females and 1 from an individual whose gender was not provided). However, in a recent population-based study of invasive disease due to beta-hemolytic streptococci other than groups A and B (5), the CDC *Streptococcus* Laboratory did not identify any *S. porcinus* or *S. pseudoporcinus* isolates. Either the incidence of *S. pseudoporcinus* associated with invasive disease is very low in the general population, or these isolates were misidentified as *S. agalactiae* on the basis of their occasional cross-reactivity with group B antiserum (28). We have not retested group B isolates in our population-based study collection to answer this question.

We have applied a polyphasic approach for the characterization of strains belonging to the *S. porcinus*/*S. pseudoporcinus* complex. Historical and recent isolates were examined to determine methods for the identification and determination of the antimicrobial susceptibility and occurrence of *S. porcinus* and *S. pseudoporcinus* isolates from human, animal, and dairy sources. Sequencing of the *rpoB* gene was used for the first time to identify these difficult-to-differentiate microorganisms. In spite of nearly identical phenotypic characteristics, DNA-DNA reassociation studies as well 16S rRNA and *rpoB* gene sequencing confirm that *S. porcinus* and *S. pseudoporcinus* are clearly two distinct species. Isolates from human sources were predominantly identified as *S. pseudoporcinus*, while isolates from animals were identified as *S. porcinus*. Only one human isolate was identified as *S. porcinus*. In addition, dairy isolates from bovine milk and cheese were identified as *S. pseudoporcinus*, and biochemical reactions and source differences in conjunction with molecular data showed that they form distinct subclusters, suggesting that these strains may represent a *S. pseudoporcinus* subspecies.

On the basis of these findings, we propose to subdivide the species *S. pseudoporcinus* into two subspecies. For the human isolates that usually grow in the presence of 6.5% NaCl, are positive for hippurate hydrolysis and pyruvate tests, and do not produce acids from lactose, we propose the denomination *S. pseudoporcinus* subsp. *hominis* subsp. nov. The second subspecies, for which we propose the denomination *S. pseudoporcinus* subsp. *lactis* subsp. nov., accommodates the dairy isolates that do not grow in the presence of 6.5% NaCl, are negative for hippurate hydrolysis and pyruvate tests, and produce acids from lactose.

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