Staphylococcus aureus subsp. *anaerobius* subsp. nov., the Causal Agent of Abscess Disease of Sheep

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A new subspecies, *Staphylococcus aureus* subsp. *anaerobius*, is described on the basis of a study of 84 strains isolated from young sheep affected by the so-called "abscess disease." The strains of this new subspecies grow well under anaerobic conditions, but not at all or only very weakly under aerobic conditions. They are catalase and benzidine negative and form small unpigmented colonies. Anaerobically they produce L-lactic acid from glucose. The chemical composition of the cell wall, the results of deoxyribonucleic acid-deoxyribonucleic acid hybridization experiments, and the immunological relationships among L-lactate dehydrogenases demonstrated that these strains are closely related to *Staphylococcus aureus*. The type strain is strain MVF-7(= ATCC 35844).

In the 1920s Aynaud (2-5) and Carré (9-11) described a gram-positive coccus that was isolated from young sheep affected by the "abscess disease," an ailment symptomatically similar to caseous lymphadenitis and diagnosed principally by French workers. This organism did not grow on usual solid media aerobically (2-5, 7-11, 37; De la Fuente and Suárez, Zentralbl. Veterinärmed. Reihe B, in press). Carré (9-11) separated this coccus from staphylococci on the basis of cultural characteristics. Aynaud (2-5), however, found many biochemical characters in common with staphylococci and considered this organism to be a staphylococcus adapted to sheep, although modified by symbiosis.

In 1957, Benito and Borrel (7) reported that the causal agent of the abscess disease produced hemolysins, coagulase, and proteases. This description contradicted the results of Joubert (25), who found that this coccus did not produce hemolysins, coagulase, or proteases. Benito and Borrel (7) and Joubert (25) considered the organism to be an autonomous species that had not yet been described, for which they proposed the names "*Micrococcus pyogenes ovis*" and "*Micrococcus abscedens ovis*," respectively.

On the basis of the anaerobic growth of this organism, Bergey's Manual of Determinative Bacteriology, 8th ed. (32), lists "*M. abscedens ovis*" as belonging to the genus *Peptococcus*. It could be similar to group VIIb in the classification scheme of anaerobic cocci of Hare (22).

Blanco Loizelier (8) diagnosed the abscess disease in Spain and identified the etiologic agent as the organism described by Benito and Borrel. In Kenya, Shirlaw and Ashford (37) isolated microaerophilic, catalase-negative cocci from abscesses of sheep; these organisms were very similar to the organism studied by the French workers, but the former did not produce coagulase.

The abscess disease and its etiology have been mentioned more recently in Iran and Hungary (1, 6).

In a recent study, De la Fuente and Suárez demonstrated that the etiologic agent of the abscess disease is a catalase- and benzidine-negative *Staphylococcus*; they considered it to be a respiratory-deficient *Staphylococcus aureus* (De la Fuente and Suárez, in press).

A few catalase-negative *S. aureus* strains have been isolated (17, 28, 36, 38, 39); the anaerobic organism "*Pepto-*

coccus saccharolyticus" has been shown to belong to the genus *Staphylococcus* (27).

On the basis of phenotypic properties and etiological aspects (mainly specific pathogenicity), in this paper we describe the etiologic agent of the abscess disease as a new subspecies of *Staphylococcus aureus*, *Staphylococcus aureus* subsp. *anaerobius*.

MATERIALS AND METHODS

Bacterial strains. In this study 84 strains were investigated. These organisms were isolated from abscesses of young sheep diagnosed as suffering from the abscess disease. The strains were isolated on sheep blood agar and in Baird-Parker medium incubated under microaerophilic conditions (candle jar system) or under anaerobic conditions in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.). The 84 strains were catalase and benzidine negative, and 80 were previously identified as respiratory-deficient strains of *S. aureus* (De la Fuente and Suárez, in press).

Methods. The cultural characteristics and many biochemical and physiological properties of the strains, together with their levels of pathogenicity for mice, guinea pigs, rabbits, and sheep, have been described elsewhere (De la Fuente and Suárez, in press).

All tests on agar plates were incubated under microaerophilic conditions.

The strains were also characterized by API-ZYM tests (API System S.A., La Balme les Grottes, France) done according to the instructions of the manufacturer.

Samples of cultures in peptone-yeast extract-glucose medium incubated anaerobically were treated and analyzed for volatile and nonvolatile fatty acids by gas chromatography, using the method of Holdeman et al. (23) and a Hewlett Packard model 5830A gas chromatograph. The configuration of the lactic acid produced was determined as described by Schleifer and Kocur (34).

Preparation of cell walls and determination of peptidoglycan type were carried out as described by Schleifer and Kandler (33). Cell wall teichoic acids were extracted with 60% hydrofluoric acid and were characterized by hydrolysis with 2 N HCl at 100°C for 3 h, followed by gas chromatography of the *N,O*-trifluoroacetylated products (16).

The class of fructose-1,6-bisphosphate aldolase was determined as described by Götz et al. (18).

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The quantitative relationship between the L-lactate dehydrogenase of *S. aureus* ATCC 12600^T (T = type strain) and that of the causal agent of the abscess disease was determined by microcomplement fixation (35), using antiserum against purified L-lactate dehydrogenase of *S. aureus* ATCC 12600^T (A. Hartinger and K. H. Schleifer, unpublished data). The results are expressed in immunological distance units. A gel electrophoretic analysis of superoxide dismutase was carried out as described by Zitzelsberger et al. (41).

The guanine-plus-cytosine content of the DNA was determined by the thermal denaturation method of Marmur and Doty (29). Isolation of DNA and DNA-DNA hybridization experiments were accomplished as described previously (26, 30).

Enterotoxin production. The cellophane-over-agar method of Hallander (21) as modified by Jarvis and Lawrence (24) and the sac culture method of Donnelly et al. (15) were used to produce enterotoxins. In both methods the inoculated medium (brain heart infusion agar and brain heart infusion broth) was incubated under microaerophilic conditions for 72 h at 37°C. Enterotoxins were detected with the microslide test of Casman et al. (12) and with the optimal sensitivity plate method of Robbins et al. (31). Highly purified enterotoxins (serotypes A,B,C,D, and E) and their corresponding antisera were kindly provided by M. S. Bergdoll.

RESULTS AND DISCUSSION

The organisms used in this study were originally identified as respiratory-deficient strains of *S. aureus*. In agreement with a report by De la Fuente and Suárez (in press), all of the strains reacted uniformly in all tests. Additional chemical and biochemical properties of five strains (strains MVF-7^T, MVF-25, MVF-39, MVF-41, and MVF-43) that were originally isolated from abscesses of young sheep were also determined. All of these strains exhibited a cell wall composition typical of *S. aureus* (namely, peptidoglycan type Lys-Gly_{5,6} and ribitol teichoic acid substituted with *N*-acetylglucosamine). They possessed class I fructose-1,6-bisphosphate aldolase, as do most staphylococci, and showed the same superoxide dismutase pattern after separation on polyacrylamide gel electrophoresis as the type strain of *S. aureus*. The five respiratory-deficient strains produced only L-lactate from glucose, whereas most *S. aureus* strains produce D- and L-lactate (34). However, the L-lactate dehydrogenase of *S. aureus* ATCC 12600^T is immunologically very closely related to that of the respiratory-deficient strains, as indicated by the low immunological distance values of 4 to 6. Finally, DNA-DNA hybridization studies between three strains (strains MVF-7^T, MVF-25, and MVF-41) and *S. aureus* ATCC 12600^T were carried out. Homology values of more than 80% indicated that these strains are closely related to *S. aureus* at the species level. However, on the basis of biochemical distinctiveness (catalase negative, benzidine negative, no or only weak growth under aerobic conditions) and the etiological importance of these respiratory-deficient strains, we propose to classify them as a subspecies of *S. aureus*.

Description of *Staphylococcus aureus* subsp. *anaerobius* subsp. nov. *Staphylococcus aureus* subsp. *anaerobius* (an.ae.ro'bi. us. Gr. pref. *an* not; Gr. n. *aer* air; Gr. n. *bios* life; M.L. adj. *anaerobius* not living in air) cells are cocci that are 0.8 to 1.0 µm in diameter and occur singly, in pairs, and predominantly in irregular clusters. Gram positive. Nonmotile. Nonsporeforming.

On the primary isolation medium, growth is obtained only in media that are supplemented with blood, serum, or egg

yolk and incubated microaerobically or anaerobically. Colonies on blood agar (Oxoid Ltd., London, England) after 2 days of incubation are very small (1 to 3 mm in diameter), low convex, circular, entire, smooth, glistening, and opaque. Pigment is not produced. Luxuriant growth is obtained on Dorset egg medium, with colony diameters of 4 to 6 mm. The strains produce unevenly disseminated growth on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) after 3 days of microaerophilic incubation. They grow as dwarf colonies, among which a few colonies of normal size are observed.

In broth cultures (3 days), a white granular deposit without turbidity is formed. When broth media are supplemented with 5% serum, the typical deposit appears after 24 h.

After several subcultures, the strains are able to grow aerobically on solid media, but aerobic growth is much worse than anaerobic growth. Growth is weak to moderate in the anaerobic portion of a semisolid thioglycolate medium after 4 to 5 days.

Analysis for volatile and nonvolatile fatty acids as end products of anaerobic glucose metabolism indicates production of acetic, lactic, and succinic acids by all strains.

The five strains studied in detail produce L-lactic acid from glucose under anaerobic conditions, but not D-lactic acid. Most *S. aureus* strains produce D- and L-lactic acids, and only rare strains (about 5%) produce solely L-lactic acid.

All strains grow well at NaCl concentrations up to 10%; 70% of the strains grow at a NaCl concentration of 12.5%, and 85% do not grow in the presence of 15% NaCl. The optimal growth temperature range is 30 to 40°C. No strain grows at 20 or 45°C.

All strains are catalase, benzidine, and oxidase negative. Catalase and cytochrome synthesis cannot be induced by adding small amounts of H₂O₂ or hemin to the culture media. Catalase-negative staphylococci have been described occasionally (17, 28, 36, 38, 39), but the strains on which the benzidine test was performed (36, 38, 39) contained the cytochrome system; since catalase is ubiquitously present in aerobic bacteria containing a cytochrome system (13), the absence of catalase activity in these strains could be interpreted as a mutation affecting the synthesis or function of this enzyme. On the other hand, Kilpper-Bälz and Schleifer (27) reported that the amount of cytochromes is markedly reduced in the anaerobic species *Staphylococcus saccharolyticus*.

Like most staphylococci, the *S. aureus* subsp. *anaerobius* strains contain class I fructose-1,6-bisphosphate aldolase.

The strains produce coagulase (tube test positive between 3 and 8 h) and heat-stable and heat-labile nucleases and are clumping-factor negative. They produce hemolysis in sheep, bovine, rabbit, and human blood agar media. On sheep or bovine blood agar, a wide zone of β-hemolysis (sphingomyelinase) is observed. This hemolytic effect is a characteristic of many coagulase-positive staphylococci of animal origin (20). They do not ferment mannitol, reduce nitrates, or produce acetylmethylcarbinol. Phosphatase activity is positive.

They produce acid from glucose, fructose, maltose, and sucrose. No acid is produced from galactose, mannose, rhamnose, xylose, arabinose, ribose, lactose, trehalose, cellobiose, melibiose, gentiobiose, melezitose, raffinose, glycerol, inositol, xylitol, sorbitol, salicin, galactitol, inulin, or amygdalin.

The strains show strong caseinolytic and gelatinase activities and produce egg yolk factor, but lipolytic activity on

TABLE 1. Differentiation of coagulase-positive species and subspecies of the genus *Staphylococcus*^a

Characteristic	<i>S. aureus</i>	<i>S. aureus</i> subsp. <i>anaerobius</i>	<i>Staphylococcus</i> <i>intermedius</i>	<i>Staphylococcus</i> <i>hyicus</i> subsp. <i>hyicus</i>
Aerobic growth	+ ^b	w or -	+	+
Coagulase (rabbit plasma)	+	+	+	D
Clumping factor	+	-	D	-
Heat-stable nuclease	+	+	+	+
Hemolysins	+	+	+	-
Fermentation of mannitol (anaerobically)	+	-	-	-
Hyaluronidase production	+	+	-	+
Acetoin production (from glucose)	+	-	-	-
Nitrate reduction	+	-	+	-
Hydrolysis of Tween 80	D	-	+	+
Pigment production	+	-	-	-
Phosphatase activity	+	+	+	+
Acid from maltose	+	+	-	-
Acid from galactose	+	-	+	+
Acid from mannose	+	-	+	+
Acid from trehalose	+	-	+	+

^a Data from references 12, 13, 17, 18, and 27.^b +, 90% of the strains are positive; -, 90% of the strains are negative; D, 11 to 89% of the strains are positive; w, weak.

Tween 80 is negative. They are hyaluronidase positive and staphylokinase negative. They reduce tellurite.

The strains of *S. aureus* subsp. *anaerobius* are pathogenic for sheep, in which they produce the abscess disease, and experimentally for goats (3-5), but not for mice, guinea pigs, or rabbits (2-5, 7-11, 37; De la Fuente and Suárez, in press).

The cultural characteristics, the pathogenicity, and some biochemical properties of *S. aureus* subsp. *anaerobius* are similar to those of the organism studied by Aynaud (2-5) and Carré (9-11) and subsequently named "*M. pyogenes ovis*" (7) and "*M. abscedens ovis*" (25). Contrary to the description of "*M. pyogenes ovis*" by Benito and Borrel and the results of the present study, Joubert found that "*M. abscedens ovis*" did not produce hemolysins, coagulase, or proteases. The organism studied by Shirlaw and Ashford (37) closely resembles *S. aureus* subsp. *anaerobius*, but it did not produce coagulase.

None of the strains studied showed production of enterotoxins A through E by the methods used.

When we used the API-ZYM tests, all *S. aureus* subsp. *anaerobius* strains exhibited strong esterase-lipase and acid phosphatase activities and moderate phosphamidase and alkaline phosphatase activities. All strains were negative for lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin-like protease, chymotrypsin-like protease, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase activities.

The strains were very susceptible to lysostaphin (minimal inhibitory concentration, 12.5 μ g/ml) and resistant to lysozyme (minimal inhibitory concentration, 1,000 μ g/ml). Agar disk diffusion tests indicated that all of the strains were susceptible to novobiocin, penicillin G, methicillin, cephalothin, gentamicin, tetracycline, chloramphenicol, erythromycin, and vancomycin. Growth was inhibited in a medium supplemented with 25 μ g of furazolidone per ml.

All strains studied exhibited the same cell wall composition as *S. aureus* (i.e., peptidoglycan type L-LysGly_{5,6} and ribitol teichoic acid substituted with *N*-acetylglucosamine).

Protein A was not detected by a passive hemagglutination method (40), using the commercial reagent AUREA-KIT (bio-Merieux).

None of the 20 selected strains tested was susceptible to phages of the human *S. aureus* typing set.

The guanine-plus-cytosine contents of the DNAs, as determined by melting temperature in five strains (strains MVF-7^T, MVF-25, MVF-39, MVF-41, and MVF-43) ranged from 31.5 to 32.7 mol%.

S. aureus subsp. *anaerobius* can be distinguished from all other staphylococci primarily by its weak or negative aerobic growth, negative catalase activity, and negative benzidine test. *S. aureus* subsp. *anaerobius* is easily differentiated from the anaerobic species *S. saccharolyticus* by colonial morphology, catalase activity, the benzidine test, coagulase and thermonuclease production, and hemolytic activity.

Simple characters that are useful in identification of *S. aureus* subsp. *anaerobius* and separation of this taxon from other coagulase-positive staphylococcal species and subspecies are listed in Table 1.

The characteristics of type strain MVF 7 are the same as those described above for the subspecies; the guanine-plus-cytosine content of its DNA is 31.7 mol%.

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