Biochemical Characterization of Unidentified Microaerophilic Cocci Isolated from Heifer and Dry-Cow Mastitis

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Thirty-nine strains of gram-positive microaerophilic cocci isolated from cases of heifer and dry-cow mastitis were biochemically characterized with the API 50E and API-ZYM test kit systems, gas-liquid chromatography for analysis of end products of glucose metabolism, and anaerobic biochemical tests (L. V. Holdeman, E. P. Cato, and W. E. C. Moore, *Anaerobe Laboratory Manual*, Virginia Polytechnic Institute, Blacksburg, 1977). Strains were screened for production of a variety of extracellular enzymes on substrate-containing agar plates and for hemolysin and coagulase production. Antibiotic susceptibility and sensitivity tests were also performed. The microaerophilic cocci displayed homogeneity with respect to the majority of the biochemical tests used; i.e., $\geq 90\%$ of the strains were consistently positive or negative in any one test and probably represent one species. All produced deoxyribonuclease, ribonuclease, and hyaluronidase, and 92% were positive for chondroitin sulfatase. Catalase and coagulase tests were negative. Greening was observed on bovine blood agar. Acetic and succinic acids were produced by all strains as the only detectable products of glucose metabolism. The strains were susceptible to penicillin G, cefoxitin, doxycycline, and chloramphenicol and were resistant to clindamycin, novobiocin, and metronidazole. Their taxonomic position remains unclear.

Heifer mastitis and dry-cow mastitis are types of mastitis in which several bacterial species have been incriminated (12, 13, 20, 21, 23, 24, 29; G. G. Steiner, D.V.M. inaugural dissertation, Justus Liebig University, Giessen, West Germany, 1975). A bacterial combination of *Corynebacterium pyogenes*, *Peptococcus indolicus*, and unclassified microaerophilic cocci is thought to be of etiological importance (12, 13, 21, 24).

Reports of biochemical characteristics of *C. pyogenes* are numerous (for references, see Høj Sørensen [11]), and a few reports concerning the identification of *P. indolicus* are available (3, 8, 10, 20). In contrast, the microaerophilic cocci have not been characterized apart from limited observations of Stuart et al. (24).

Microaerophilic cocci have been used in experimental mixed infections to study the pathogenesis of heifer mastitis (2, 9; Schwan, unpublished data), but it is not possible to gauge whether all investigators using "an unidentified microaerophilic coccus" have used the same bacterium.

The lack of information on these microaerophilic cocci and the uncertainties with respect to species singularity and taxonomic position

prompted this investigation. Biochemical and enzymatic test kit systems as well as substrate-containing agar plate assays were used to obtain as broad a picture as possible of the biochemical activity to these bacteria. Moreover, as the distinction between microaerophilic and anaerobic cocci has been questioned (7, 28), techniques such as analysis of end products of glucose metabolism by gas-liquid chromatography, which are routinely used to identify anaerobic bacteria, were used in attempts to classify the microaerophilic cocci.

MATERIALS AND METHODS

Chemicals. Heart infusion agar (used as blood agar base), Noble agar, peptone, yeast extract, heart infusion broth, and brain heart infusion broth were obtained from Difco Laboratories, Detroit, Mich. API 50 *Enterobacteriaceae* (API 50E) and API-ZYM test kits were from API System S.A., La Balme les Grottes, France. Horse serum was purchased from the National Bacteriological Laboratory, Stockholm, Sweden. Bovine and sheep blood was purchased from the National Veterinary Institute, Stockholm, and the horse blood was collected by venipuncture at the College of Veterinary Medicine, Uppsala, Sweden. Novobiocin disks were obtained from Biodisk AB, Stockholm. Soluble

starch was from Merck AG, Darmstadt, West Germany. Yeast ribonucleic acid, elastin powder, sodium hyaluronidate, chondroitin sulfate, and bovine serum albumin fraction V were from Sigma Chemical Co., St. Louis, Mo. Skimmed-milk powder and fresh eggs were obtained from a retail supplier. Antibiotics used for susceptibility tests were kindly donated as follows: benzylpenicillin by Kabi AB, Stockholm; cefoxitin by Merck, Sharpe and Dohme, Rahway, N.J.; clindamycin by The Upjohn Co., Kalamazoo, Mich.; chloramphenicol by Park-Davis, Detroit, Mich.; doxycycline by Pfizer, New York, N.Y.; and metronidazole by Leo AB, Helsingborg, Sweden.

Strains. Thirty-nine strains of microaerophilic gram-positive cocci originating from heifer and dry-cow mastitis (21) were examined in the present investigation. The bacteria had been isolated from blood agar plates on which they occurred in mixed culture mostly with *C. pyogenes*, *P. indolicus*, and *Streptococcus dysgalactiae*. Primary identification of the microaerophilic cocci was performed on the basis of growth characteristics and bacterial morphology (24) and some additional biochemical tests, namely, indole, lactose, salicin, maltose, mannite, Voges-Proskauer, and nitrate tests (Holmberg, unpublished data). Strains were subsequently stored in heart infusion broth containing 1% (wt/vol) glucose at -70°C .

Growth characteristics, colony morphology, and bacterial morphology. Comparative studies of growth were done using aerobic incubation, anaerobic incubation in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.), and incubation in $\sim 2.5\%$ CO_2 (candle jar system) on 5% (vol/vol) horse blood agar plates. Bacterial morphology was determined using cells from blood agar plates and cells from peptone-yeast extract-glucose broth (8) and serum broth (heart infusion broth containing 10% [vol/vol] horse serum, pH 7.3) at 37°C for 48 h.

Capsule staining. The strains were grown in peptone-yeast extract-glucose broth (8) for 24 h at 37°C and were investigated for the presence or absence of capsules by staining with safranin followed by contrasting with India ink according to Duguid (5).

Biochemical testing according to VPI. Tests for the fermentation of cellobiose, fructose, glucose, lactose, maltose, sorbitol, and sucrose as well as tests for gelatinase and indole production were performed anaerobically according to procedures described in the Virginia Polytechnic Institute (VPI) laboratory manual (8).

Catalase. Tests were performed according to Cowan (4). The bacteria were grown on 10% (vol/vol) serum-heart infusion agar for 48 h, and 3% H_2O_2 was used as reagent.

Coagulase. Coagulase tests were performed according to the Subcommittee on Taxonomy of Staphylococci and Micrococci, using rabbit plasma (25).

API 50E. Biotyping of isolates was performed using the API 50E test kit system. All strains were grown in serum broth (see above) at 37°C for 24 h. The bacteria were sedimented by centrifugation ($3,250 \times g$, 10 min) and resuspended in serum broth. The absorbance of the bacterial suspensions was adjusted to 0.2 at 600 nm in a 10-mm light path. Test kits were inoculated according to the instructions of the manufacturer. A

test kit was also inoculated with sterile serum broth as a control to aid in reading of the test strips. The API 50E were incubated anaerobically in GasPak jars (BBL) for 48 h at 37°C . The color reactions were divided into four groups (0 through 3), where 2 and 3 were regarded as positive and 0 and 1 were regarded as negative. All strains were tested twice.

API-ZYM tests. Bacterial growth from five horse blood agar plates (37°C , 48 h) was suspended in 2 ml of distilled water to provide a heavy bacterial suspension (26). Two drops of the bacterial suspension were added to each cupule of the API-ZYM strips. The strips were incubated in moist chambers for 4 h at 37°C . API-ZYM developing reagents were added to the cupules, and the color reactions which developed within 5 min were read according to a scaled color chart supplied by the manufacturer. Tests giving grade 0 reactions twice or grade 0 once and grade 1 once were regarded as negative (–). Reproducible reactions of grades 1 through 3 were considered weak to moderate positive reactions (+), whereas reactions of grades 4 and 5 were considered strongly positive (++)

Detection of extracellular enzymes. The ability to produce various extracellular enzymes was determined by growth anaerobically on substrate-containing agar plates. Observation of zones of hydrolysis was performed routinely after 48 to 72 h at 37°C with the exception of the hyaluronidase and chondroitin sulfatase tests, which were incubated for up to 5 days. Heart infusion agar was used as the basal medium in all tests except those for hyaluronidase and chondroitin sulfatase production (see below). Production of amylase was studied on 0.9% (wt/vol) starch agar (27), production of protease on 15% (vol/vol) skimmed-milk agar (1), production of ribonuclease on 0.4% (wt/vol) ribonucleic acid agar (17), production of egg yolk-reactive factors on 5% (vol/vol) egg yolk agar (16), and production of elastase on 0.9% (wt/vol) elastin agar (19).

Production of hyaluronidase and chondroitin sulfatase were studied according to Smith and Willett (22). The basic medium consisted of brain heart infusion broth to which 1% (wt/vol) Noble agar was added. Aqueous solutions of 2 mg of sodium hyaluronidate per ml, 4 mg of chondroitin sulfate per ml, and 5% (wt/vol) bovine serum albumin fraction V were sterilized by filtration through Seitz filters ($0.45 \mu\text{m}$). Each substrate was added to cooled medium to give a final concentration of 400 $\mu\text{g/ml}$. Bovine serum albumin fraction V was then added to give a final concentration of 1% (wt/vol). After incubation at 37°C the plates were flooded with 2 N acetic acid for 10 min.

Hemolysis. Hemolysis on 5% (vol/vol) horse, bovine, and sheep blood agar plates was checked after incubation anaerobically at 37°C for 48 h. The plates were then allowed to stand at room temperature for 1 week to detect color changes in the blood agar (24).

Gas chromatography. Samples of cultures in peptone-yeast extract-glucose medium were treated and analyzed for volatile and nonvolatile fatty acids by gas chromatographic analysis according to Holdeman et al. (8) in a gas chromatograph model Varian 920 (Varian, Palo Alto, Calif.).

Antibiotic susceptibility tests. The tests of sus-

ceptibility were carried out on 29 of the 39 isolates, using Schaedler agar medium (BBL), by the agar dilution method according to Ericsson and Sherris (6). The strains to be tested were grown anaerobically in peptone-yeast extract-glucose broth for 24 h. Samples (50 μ l, ca. 10^6 bacteria) of these broth cultures were transferred to cups arranged complementary to the 32 prongs in a Steers replicator. Each plate was spot inoculated with the replicating device. All plates were incubated for 48 h at 37°C in anaerobic jars. Minimum inhibitory concentration (MIC) denoted the lowest concentration of an antibiotic which inhibited growth completely.

Sensitivity to novobiocin. A bacterial suspension was prepared in physiological saline from a 48-h horse blood agar plate. The suspension was spread over the surface of a P-agar plate (14) containing 5% horse blood. The plate was then allowed to dry, and a novobiocin disk containing 5 μ g of novobiocin was applied on the surface. The plates were allowed to stand at room temperature for 30 min, after which they were incubated anaerobically in the GasPak system (BBL) for 48 h at 37°C. A novobiocin-sensitive *Staphylococcus aureus* strain was used as a control.

RESULTS

Growth characteristics. Of 39 strains, 36 grew both aerobically and anaerobically after 2 days of incubation, although the aerobic growth was weak. The three remaining strains failed to grow aerobically even after 7 days of incubation. Two days of incubation anaerobically or in a candle jar gave small, translucent colonies with entire edges. In Gram smears from broth cultures the bacteria were coccoid (0.5 to 1.0 μ m) and were arranged in clumps, pairs, or short chains. None of the strains produced capsules detectable in the India ink preparations or stained, dried films. Addition of horse serum to nutrient broth such as heart infusion broth enhances growth.

API 50E. All strains were reproducibly positive in the *d*-(-)-levulose, *N*-acetylglucosamine, dextrin, starch, glycogen, and deoxyribonuclease tests, and all were reproducibly negative in the erythritol, *d*-(-)-arabinose, *d*-(+)-xylose, *l*-(-)-xylose, methyl xyloside, *d*-(+)-melibiose, mucate, gluconate, lipase, tetrathionate reductase, pectate, Christensen's citrate, malonate, acetate, and methyl red tests. The results for the rest of the substrates are listed in Table 1. The reactivity patterns on the different substrates of API 50E were consistent for 39 of the 49 tests, where a typical reaction is defined by a positive or negative reaction with $\geq 90\%$ of the strains.

Biotyping according to VPI. The results of biochemical tests are shown in Table 2. Most strains, i.e., $\geq 90\%$, could be considered weak acid producers (pH 5.6 to 6.1) with the substrates tested. None of the strains produced indole or was positive in the gelatinase test.

TABLE 1. Variable characters for the 39 microaerophilic cocci tested on API 50E

Microtube test	% of strains yielding indicated reactions ^a		
	+	-	v
Galactose	97	3	
<i>d</i> -(+)-Glucose	97	3	
<i>d</i> -(+)-Mannose	97	3	
Maltose	97	3	
Lactose	97	3	
Esculin	87	5	8
Salicin	87	5	8
<i>d</i> -(-)-Trehalose	87	3	10
Arbutin	85	5	10
<i>d</i> -(+)-Cellobiose	85	8	7
<i>l</i> -(+)-Arabinose		97	3
<i>l</i> -(-)-Sorbitol	3	97	
Rhamnose	3	97	
Dulcitol	3	97	
<i>meso</i> -Inositol	3	97	
Methyl-D-mannoside	3	97	
Methyl-D-glucoside	3	97	
Mannitol	3	97	
Adonitol		92	8
Inulin		92	8
<i>d</i> -(+)-Melezitose		92	8
Amylose	5	92	3
<i>d</i> -(+)-Raffinose		90	10
Sorbitol	3	85	12
Ribose	3	85	12
Glycerol	3	80	17
Saccharose	3	74	23
Amygdalin	18	59	23

^a +, Positive test, acid production; -, negative test; v, variable reactions, interpretation of test as positive and negative on different occasions.

API-ZYM. Strains were tested on two or more occasions. All were negative for lipase, valine aminopeptidase, trypsin- and chymotrypsin-like proteases, α -galactosidase, β -glucosidase, α -mannosidase, and α -fucosidase. The variations in reactions with the other substrates are given in Table 3.

Production of extracellular enzymes. None of the strains produced amylase, protease, egg yolk factor, or elastase, whereas all strains were weakly positive for ribonuclease and hyaluronidase and 92% produced chondroitin sulfatase.

Hemolysis. None of the strains produced hemolysis on bovine, horse, or sheep blood agar plates. However, on subsequent standing at room temperature for 7 days, all strains produced zones of greening around the colonies on bovine blood agar plates. Greening of bovine blood agar was also detected after incubation at 37°C for 48 h in candle jars.

Coagulase and catalase. None of the strains

TABLE 2. Production of acid by microaerophilic cocci according to standard anaerobic procedures^a

Substrate ^b	No. of strains achieving final pH:										
	5.2	5.3	5.5	5.6	5.7	5.8	5.9	6.0	6.1	6.2	6.3
Cellobiose	1	1		1	2	4	8	7	10	4	1
Fructose				1	2	5	11	8	11	1	
Glucose						2	16	6	15		
Lactose			1	1	2		7	14	11	3	
Maltose					1		12	10	10	6	
Sorbitol							2	22	12	3	
Sucrose		1	2				1	25	8	1	1

^a Holdeman et al. (8).^b Initial pH of all substrates was pH 6.9.

TABLE 3. Variable characters for the 39 strains of microaerophilic cocci tested on API-ZYM

Enzyme	% of strains giving indicated interpretation ^a		
	-	+	++
Alkaline phosphatase	13	74	13
Esterase		87	13
Esterase lipase		64	36
Leucine aminopeptidase	64	36	
Cystine aminopeptidase	67	33	
Acid phosphatase	5		95
Phosphoamidase	85	15	
β -Galactosidase	85	15	
β -Glucuronidase	46	36	18
α -Glucosidase	97	3	
β -Glucosaminidase	26	67	7

^a API-ZYM color grade 0 or 0 and 1 on separate occasions, negative (-); grades 1 through 3, positive (+); grades 4 and 5, positive (++)

produced coagulase or catalase.

Gas chromatography. Analysis for volatile and nonvolatile fatty acids as end products of glucose metabolism revealed production of acetic and succinic acids by all strains.

Antibiotic susceptibility and sensitivity tests. The cumulative susceptibilities of the 29 strains for four antibiotics are shown in Fig. 1. According to the recommendations of the Swedish Medical Association, all strains were susceptible to penicillin G (MIC, ≤ 1 μ g/ml), cefoxitin (MIC, ≤ 2 μ g/ml), and chloramphenicol (MIC, ≤ 8 μ g/ml) and all but one were susceptible to doxycycline (MIC, ≤ 1 μ g/ml). All strains were resistant to clindamycin (MIC, ≥ 8 μ g/ml; data not shown in Fig. 1). In addition, all strains were resistant to novobiocin and metronidazole by the disk sensitivity method.

DISCUSSION

By and large, the microaerophilic cocci studied herein displayed homogeneity with respect to their reactions in many of the biochemical tests used. It thus seems probable that the strains examined represent one species. Watt

and Jack (28) have discussed the problems of differentiating microaerophilic and anaerobic cocci and have suggested the use of metronidazole resistance and sensitivity, respectively, as a distinguishing feature. Using this criterion, and the fact that they grow aerobically, albeit poorly, the investigated cocci would be considered microaerophilic.

Conclusions regarding identification of these microaerophilic cocci to the genus and species levels are somewhat difficult on the basis of the present findings. Novobiocin resistance appears to be useful for primary differentiation of peptococci and peptostreptococci (30) and of micrococci and staphylococci (18) with some exceptions (14). Taking this test together with the catalase and coagulase results, identification of the microaerophilic cocci as micrococci, staphylococci, or peptostreptococci is highly unlikely. The end products of glucose fermentation of streptococci are mainly lactic acid, with small

FIG. 1. Cumulative susceptibility of strains of microaerophilic cocci to four antibiotics. MIC, Minimal inhibitory concentration. Symbols: ■, penicillin G; ●, doxycycline; ▲, cefoxitin; ○, chloramphenicol. Cumulative susceptibility calculated as percentage of the 29 strains tested.

amounts of acetic and formic acids. By comparison, the microaerophilic cocci produced acetic and succinic acids as the only detectable end products. The end products of glucose metabolism are not consistent with those of *Gemminger*, *Coprococcus*, and *Ruminococcus* (8).

On the whole, our studies confirm the limited description of these microaerophilic cocci by Stuart et al. (24). Their sugar fermentation tests are not in full agreement with the API 50E findings, but the minor differences are probably due to methodological variations which affect interpretation of results, e.g., indicator, sugar purity, incubation time, and inoculum of bacteria. Such problems are even illustrated within this study. The major indicator used in API 50E (with some exceptions) is phenol red, which yields a positive test for acid production at pH values <6.8. According to the VPI system, final pH values > 6.1 are considered to indicate negative tests. Although most of tests with sucrose and sorbitol according to the VPI system would be regarded as giving weak positive reactions, only 3% of the strains gave positive reactions in the API 50E system with these sugars.

In certain species of bacteria, e.g., staphylococci, streptococci, and clostridia, extracellular enzymes and factors such as coagulase have been regarded as potential virulence factors. Our findings on production of hyaluronidase by the microaerophilic cocci confirm Høi Sørensen's observations (9). In addition, deoxyribonuclease, ribonuclease, and chondroitin sulfatase were demonstrated herein. However, the role of such factors in mixed infections with *C. pyogenes* and *P. indolicus* is unclear.

The present uncertainties with respect to the taxonomic position of these bacteria may be resolved when their cell wall composition and the guanine-plus-cytosine content of their deoxyribonucleic acid have been determined.

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