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Biochemical, Enzymatic, and Serological Differentiation of *Peptococcus indolicus* (Christiansen) Sørensen from *Peptococcus asaccharolyticus* (Distaso) Douglas

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Peptococcus indolicus (formerly *Micrococcus indolicus*) is an asaccharolytic anaerobic coccus that is frequently isolated from udder secretions from cases of heifer and dry-cow mastitis (summer mastitis). To facilitate better identification and its differentiation from *Peptococcus asaccharolyticus*, a variety of biochemical, enzymatic, and serological properties were studied. Seventy-nine strains of *P. indolicus* of bovine origin and 10 strains of *P. asaccharolyticus* of human origin were examined using the API 20A and API-ZYM test kit systems. In addition, production of extracellular enzymes by using sensitive substrate-containing agar plate tests, production of peptocoagulase (a plasma-clotting factor), hemolytic properties, metabolic end products by gas chromatography, and serological characteristics with a set of *P. indolicus* typing antisera were investigated. *P. indolicus* and *P. asaccharolyticus* were not satisfactorily differentiated solely by the API 20A system. *P. indolicus* differed from *P. asaccharolyticus* in producing H_2S, reducing nitrate to nitrite, producing peptocoagulase, possessing alkaline phosphatase, and producing large amounts of propionate from lactate. Moreover, none of the strains of *P. asaccharolyticus* was typable with the *P. indolicus* typing antisera. The majority (88%) of *P. indolicus* strains also gave weak hydrolysis of ribonucleic acid, and 6 out of 79 produced deoxyribonuclease. All strains in this study were sensitive to metronidazole (5 μg) by disk diffusion tests.

Bacteriological investigations of summer mastitis (heifer and dry-cow mastitis) have often revealed conflicting pictures of the etiology of this disease (7, 25, 29, 30, 35; G. G. Steiner, D.V.M. inaugural dissertation, Justus Liebig University, Giessen, 1975). Although its pathogenesis remains poorly defined and its etiology complex, the possible involvement of anaerobic cocci has been evidenced in several reports (3, 4, 21, 23, 25; G. G. Steiner, D.V.M. inaugural dissertation). These anaerobic cocci have also been isolated from about 25% of various specimens from healthy heifers and cows (27).

The first taxonomic description of these anaerobic cocci was provided by Christiansen (3), who assigned the name *Micrococcus indolicus*. Physiological, biochemical, and serological properties of this species have been recorded in recent years by Sørensen (24, 26). On the basis of his studies, Sørensen (26; G. H. Sørensen, XIth Conf. Taxon. Bact., 23-25 September, 1974, Brno, Czechoslovakia, abstr. no. 49) proposed that *M. indolicus* (Christiansen) be renamed *Peptococcus indolicus*.

Differentiation of these asaccharolytic anaerobic cocci from human strains of *Peptococcus asaccharolyticus* has been based primarily upon the production of a plasma-clotting factor by *P. indolicus* and a small number of additional but variable characters for *P. asaccharolyticus* (24). However, in general the classification of obligately anaerobic cocci is a somewhat confused taxonomic field (22). Indeed, Watt and Jack (34) have questioned the very definition of anaerobic cocci.

For assessment of the potential role of *P. indolicus* in health and disease, clear guidelines for its delineation are required. Apart from the studies of Sørensen (24, 26), no reports on intensive characterization of this organism have appeared. This investigation was thus undertaken to extend the number of characteristics on which identification and classification of *P. indolicus* might be based and which might be readily used in its differentiation from *P. asaccharolyticus*. This involved the use of newly available test kit systems for biochemical and enzymatic characterization, the use of gas chromatography, which

is now considered to be of primary value in species identification of anaerobes (1, 10), and serological analysis.

MATERIALS AND METHODS

Chemicals. Trypticase soy broth and indole-nitrate medium were obtained from Baltimore Biological Laboratory (BBL), Cockeysville, Md. Heart infusion agar, yeast extract, and proteose peptone were from Difco Laboratories, Detroit, Mich. API 20A (Anaerobe) and API-ZYM test kits were from API System S.A., La Balme les Grottes, France. Horse blood (heparinized) was obtained by venipuncture from stalled animals at the College of Veterinary Medicine, Uppsala. Bovine blood (citrated) was from Statens Veterinärmedicinska Anstalt, Stockholm. Citrated rabbit plasma and prereduced chopped-meat medium were from Statens Bakteriologiska Laboratorium, Stockholm. Ethyl ether, glucose, and soluble starch were from Merck AG, Darmstadt, West Germany. Deoxyribonuclease agar was purchased from Oxoid Ltd., Basingstoke, England. Oxygen-free carbon dioxide (>99.99%) was from AGA, Stockholm. Metronidazole disks were from Biodisk AB, Stockholm. Torula yeast RNA, elastin powder, and Coomassie brilliant blue R-250 were from Sigma Chemical Co., St. Louis, Mo. Skimmed-milk powder and fresh eggs were obtained from a retail supplier.

Strains. Seventy-nine strains of anaerobic gram-positive cocci were isolated from udder secretions of individual cases of heifer and dry-cow mastitis in different herds in six regions of Sweden between July 1976 and June 1977 (21). All were identified as *P. indolicus* according to the criteria of Sørensen (24, 26). Eight and two human clinical strains of *P. asaccharolyticus* were kindly supplied by C.-E. Nord, Statens Bakteriologiska Laboratorium, Stockholm and A. C. Rydén, Akademiska sjukhuset, Uppsala, respectively. These had been identified according to Holdeman and Moore (10). Strains were inoculated into prereduced chopped-meat medium under a continuous stream of oxygen-free carbon dioxide and incubated at 37°C for 24 h prior to freezing of the entire cultures at −70°C.

Biochemical testing. Biotyping of the isolates was performed using the API 20A test system. Inocula were prepared from 48-h blood agar (5% [vol/vol] horse blood in heart infusion agar) cultures by suspension in API anaerobe basal medium. The instructions of the manufacturer for inoculation were followed. Strips were incubated anaerobically in the BBL GasPak System for 48 h at 37°C before the tests were read.

For the nitrate reduction test, indole-nitrate medium was used. The sulfanilic acid and dimethyl-α-naphthylamine test for nitrite ions was performed on 48-h anaerobic cultures. Citrate utilization tests were carried out anaerobically according to Cowan (5).

For tests of gas production, strains were cultivated in prereduced, anaerobically sterilized peptone–yeast extract–glucose medium (PYG) (11) for 48 h at 37°C in rubber-stoppered tubes (20 ml). A U-tube manometer (internal diameter 11 mm, limbs 120 mm long) containing 10 ml of water was sealed at one end with a rubber stopper. A piece of rubber tubing with an inoculation needle at both ends was used to connect the culture tube to the manometer by stabbing through the rubber corks. Gas production was indicated by a rise and a fall in the water levels in the open and closed arms, respectively, of the manometer. No tests were performed on the composition of the gases produced.

API-ZYM tests. API-ZYM comprises enzymatic tests performed on dried substrates for the following: alkaline and acid phosphatases, esterase, esterase-lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin- and chymotrypsin-like proteases, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase, and α-fucosidase.

Growth from five horse blood agar plates (37°C, 48 h) was suspended in 2 ml of distilled water to provide heavy bacterial suspensions (32). Two drops of the bacterial suspension were added to each cupule of the API-ZYM strip. Strips were incubated in moist chambers for 4 h at 37°C. API 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.

Gas chromatography. A Perkin-Elmer F11 gas chromatograph with a flame-ionizer detector was used for analysis of volatile metabolic end products of each strain. The column (stainless steel, 3.2-mm ID, 1.84 m long) consisted of 5% (wt/wt) FFAP (Supelco, Inc., Bellefonte, Pa.) on Chromosorb WHP 80–100 mesh. The column was run at 120°C and the injector and detector at 140°C. The carrier gas was nitrogen. Strains were grown in prereduced PYG medium for 48 h at 37°C. Ethyl ether extracts of cultures were prepared according to Holdeman and Moore (10). Volumes (2 μl) of extracts were chromatographed. Standard solutions of fatty acids treated as above were run as controls to permit identification of the peaks recorded for each of the samples.

Lactate utilization test. Strains were grown in lactate–peptone–yeast extract medium and PY medium (11). After acidification of cultures to pH 2, ethyl extracts of both cultures were compared by gas-liquid chromatography.

Peptocoagulase. Culture filtrates from 24-h cultures in prereduced PYG medium were assayed for their abilities to clot citrated rabbit plasma according to procedures described elsewhere (31). In addition cell-bound peptocoagulase activity was tested with saline-washed cell suspensions (31).

Detection of extracellular enzymes. The ability to produce various extracellular enzymes was determined by growth anaerobically on substrate-containing agar plates and observation of zones of hydrolysis after 48 or 72 h at 37°C. Heart infusion agar was used as the basal medium in all cases except for detection of deoxyribonuclease. Production of amylase was studied on 0.9% (wt/vol) starch agar (33), production of protease on 15% (vol/vol) skim-milk agar (2), production of ribonuclease on 0.4% (wt/vol) ribonucleic acid agar (14), production of egg yolk reactive factors on 5% (vol/vol) egg yolk agar (13), and production of elastase on 0.9% (wt/vol) elastin agar (20). Deoxyri-

bonuclease production was detected on deoxyribonucleic acid agar (Oxoid) according to DiSalvo (6). Known positive bacterial species were included as positive controls for production of enzymes.

Hemolysis. Hemolysis on 5% (vol/vol) horse and bovine blood agar plates was checked after incubation anaerobically at 37°C for 48 h.

Metronidazole disk sensitivity test. A loopful of growth (about 10 colonies) from a 48-h horse blood agar plate was spread evenly over the surface of a fresh horse blood agar plate. A 5-μg metronidazole disk was applied, and the zone of inhibition was measured after growth for 48 h at 37°C (34). Inhibition zones of <10 mm were considered to indicate metronidazole resistance, whereas zones of >22 mm indicated sensitivity (34).

Serotyping. The procedure described by Sørensen (24) was followed. Reference serotype strains of *P. indolicus* and typing antisera were kindly provided by G. H. Sørensen, State Veterinary Laboratory, Department of Jutland, Århus, Denmark. All strains were grown for 48 h at 37°C in 15 ml of chopped-meat medium, and the bacteria were harvested by centrifugation of the supernatant cultures. Bacterial pellets were suspended in 0.75 ml of distilled water and autoclaved for 20 min at 120°C. Supernatant extracts obtained by centrifugation were stored at −70°C. Each extract was tested by double immunodiffusion analysis against the seven typing antisera. Identification of serotypes was then confirmed by testing for reactions of identity and nonidentity with extracts of reference serotype strains using homologous and heterologous antisera. The presence of immunoprecipitates was read before and after staining with Coomassie brilliant blue R-250. Washing, drying, and staining of gels was performed according to the procedure described by Weeke (36).

RESULTS

API 20A. All 79 strains of *P. indolicus* and 10 strains of *P. asaccharolyticus* gave an identical seven-digit profile (1000-000). The only difference revealed by this test kit was production of H_2S by all *P. indolicus* strains, whereas *P. asaccharolyticus* strains were negative (Table 1). Ehrlich reagent, as recommended for use with the indole test, sometimes gave negative results, even when indole tests using this reagent were positive when performed in parallel with cultures grown in chopped-meat medium according to the procedure of Holdeman and Moore (10). However, the API 20A indole reaction was always positive when Kovac indole reagent was substituted (5).

API-ZYM. None of the strains possessed glycosidases (Table 1). The enzyme profile for *P. indolicus* strains was identical, whereas the patterns for leucine aminopeptidase and esterase-lipase with *P. asaccharolyticus* were variable. Reactions recorded as weak were reproducible. Only the tests for alkaline phosphatase, acid phosphatase, esterase (butyrate), and esterase-lipase gave reproducibly strong or distinct reactions. The major difference between *P. indolicus* and *P. asaccharolyticus* strains was the presence and absence, respectively, of alkaline phosphatase (Table 1).

Miscellaneous tests. *P. indolicus* but not *P. asaccharolyticus* strains reduced nitrate to nitrite. Both were unable to utilize citrate as sole carbon and energy source. Gas was produced by all strains upon cultivation in PYG medium (Table 1).

Metronidazole sensitivity. According to the criterion of Watt and Jack (34), all peptococcal isolates in this study appeared sensitive to metronidazole by the disk diffusion test, implying that they were obligately anaerobic bacteria (Table 1).

Gas chromatography. Analysis of ethyl ether extracts of PYG broth cultures of *P. indolicus* yielded highly reproducible identical profiles (Table 1). Acetic, propionic, and butyric acids were produced by all strains. The picture with the *P. asaccharolyticus* strains was variable (Table 1). All produced acetic and butyric acids, whereas some produced propionic acid in addition. Tests for lactate utilization resulted in large increases in the amounts of propionic acid in the ethyl ether extract profiles for 10 *P. indolicus* strains tested, whereas no, or only slight, production was noted for *P. asaccharolyticus* (Table 1).

Extracellular and cell-bound factors. Extracellular peptocoagulase, a plasma-clotting factor that activates prothrombin (31), was produced by 93% of the *P. indolicus* strains. Washed-cell suspensions of all strains also caused plasma clotting, indicating the presence of a cell-bound form of peptocoagulase (31). None of the *P. asaccharolyticus* strains possessed cell-bound or free peptocoagulase (Table 1). Weak hydrolysis of ribonucleic acid was observed with 89% of *P. indolicus* strains, compared with 10% of *P. asaccharolyticus* strains. A small number of *P. indolicus* strains (7%) were deoxyribonuclease positive. None of the strains of either species produced hemolysis on horse or bovine blood agar. Other tests were all negative (Table 1).

Serotyping. All the *P. indolicus* isolates could be serotyped into groups corresponding to those described by Sørensen (24). No strains belonged to the new serotype detected by Sørensen (personal communication), which corresponds to strain PcBH II. Some strains reacted with more than one typing serum. However, strains could be assigned to a serotype on the basis of the dominating immunoprecipitate. The cause(s) of cross-reactions are being further investigated. None of the *P. asaccharolyticus*

TABLE 1. *Biochemical test characteristics of P. indolicus and P. asaccharolyticus strains*

System or test kit	Biochemical test or characteristic	*P. indolicus* (79)[a]	*P. asaccharolyticus* (10)[a]
API 20A	Indole production	+	+
	Gelatin hydrolysis	−	−
	Esculin hydrolysis	−	−
	Urease production	−	−
	Acid from various saccharides[b]	−	−
	H_2S production[c]	+	−
	Catalase	−	−
API-ZYM[d]	Alkaline phosphatase	++	−
	Acid phosphatase	++	++
	Leucine aminopeptidase	w	w(6) or −(4)
	Cystine aminopeptidase	w	−
	Glycosidases[e]	−	−
	Trypsin, chymotrypsin, lipase, valine aminopeptidase	−	−
	Phosphoamidase	w	w
	Esterase (butyrate)	+	+
	Esterase-lipase (caprylate)	w	+(9) or w(1)
Miscellaneous tests	Nitrate reduction	+	−
	Citrate utilization	−	−
	Gas production	+	+
Gas-liquid chromatography[f]	Metabolites in PYG medium	A, p, B[g]	A, b or A, p, B
	Lactate utilization	A, P,B[h]	A, b or A, p, B
Hemolysis	Horse or bovine blood agar	−	−
Peptocoagulase	Cell bound	+	−
	Extracellular (free)	+ (74)	−
Agar plate tests for extracellular enzymes	Ribonuclease	w (70)[i]	−(9) or +(1)
	Deoxyribonuclease	+ (6)	−
	Amylase, elastase, protease, egg yolk factor	−	−
Serotyping[j]	A (R3)	6 [7]	0
	B (R8)	17 [21]	0
	C (R13)	16 [20]	0
	D (R14)	18 [22]	0
	E (R33)	21 [26]	0
	F (Gr31)	1 [1]	0
Metronidazole disk test	Sensitive (zone diameter >22 mm)	+	+

[a] Number of strains.

[b] Glucose, mannitol, lactose, saccharose, maltose, salicin, *d*-(+)-xylose, *d*-(+)-arabinose, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, trehalose.

[c] As detected by a smoky black color throughout the esculin cupule of the API 20A.

[d] ++, Strong reaction (grade 5 according to color chart); +, medium reaction (grade 2 or 3); w, weak reaction (grade 1); −, negative (grade 0).

[e] α-Galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase, α-fucosidase.

[f] Ethyl ether extracts only.

[g] A, Large amount of acetic acid; B, large amount of butyric acid; P, large amount of propionic acid; p, small amount of propionic acid.

[h] 10 out of 10 strains tested representing different serotypes.

[i] w, weak hydrolysis.

[j] According to Sørensen's serotype classification (24); serotype strains given in parentheses; numbers of strains of each serotype indicated in Table, with percentage of strains in brackets.

strains could be serotyped with the Sørensen set of *P. indolicus* typing sera.

DISCUSSION

The anaerobic cocci studied herein are identifiable as belonging to the genus *Peptococcus* according to the criteria set out in *Bergey's Manual of Determinative Bacteriology* (19). Indeed, by the metronidazole test criterion (34), all can be defined as obligate anaerobes rather than microaerophilic cocci. Finegold (8) and Smith (22) have recognized *P. asaccharolyticus* as being synonymous with *Micrococcus indolicus*. However, the name *Peptococcus indolicus* has been proposed by Sørensen (26; G. H. Sørensen, XIth Conf. Taxon. Bact., 23–25 September 1974, Brno, Czechoslovakia, abstr. no. 49) for these nonsaccharolytic bovine peptococci on the basis of biochemical and serological studies comparing strains of bovine, human, and other animal origin (24). Subsequently, *P. indolicus* was recognized as a species distinct from *P. asaccharolyticus* by Holdeman et al. (10) and also has been placed on the approved list of bacterial names of the International Committee on Systematic Bacteriology (12).

The present study provides new and confirmatory evidence for the validity of the classification of bovine peptococcal isolates from summer mastitis as a distinct species, namely *P. indolicus*. This species could be differentiated from *P. asaccharolyticus* by the following criteria: (i) production of H_2S; (ii) reduction of nitrate to nitrite; (iii) production of peptocoagulase; (iv) possession of alkaline phosphatase; (v) production of large amounts of propionate from lactate; and (vi) serological analysis.

Although reproducible patterns were obtained using API 20A with both *P. indolicus* and *P. asaccharolyticus*, these species should not be differentiated solely on the basis of H_2S production in the esculin cupule. Previous authors have reported that the indole test in the API 20A kit did not give a satisfactory correlation with conventional test procedures (15, 16, 28). In contrast to results reported by Moore et al. (15), Kovac reagent gave reliable results, whereas Ehrlich reagent sometimes gave negative reactions.

The taxonomic criteria for the classification of anaerobic cocci might be usefully extended by the use of the API-ZYM tests. The findings herein support the contention of Tharagonnet et al. (32) that the enzyme profile can be a useful criterion for distinguishing between closely related anaerobic species. The reproducibility of tests was good, but it must be emphasized that the color intensities of the reactions depend to some extent on the density of the bacterial suspensions used and that reproducibility requires careful preparation of such inocula. Phosphatase activities in a variety of anaerobes have been previously reported (9, 17). Interestingly, Porschen and Spaulding (17) observed that the majority of 13 strains of *P. asaccharolyticus* were phosphatase negative. However, they did not clearly differentiate between the occurrence of acid and alkaline phosphatases. More strains of asaccharolytic peptococci of various animal origin should be examined to establish defined phosphatase profiles. Differentiation of *P. indolicus* from *P. asaccharolyticus* on the basis of weak API-ZYM reactions, e.g. leucine or cystine aminopeptidase, is not to be recommended. The esterase-lipase test, however, might prove to be of use when more human *P. asaccharolyticus* strains have been examined.

Peptocoagulase, first described by Sørensen (24), has been shown to be a true staphylocoagulase-like clotting factor (31). Pulverer et al. (18) have investigated the occurrence of clotting activities in different subspecies of *Bacteroides melaninogenicus*. Although 78.3% of these strains caused clotting of rabbit plasma, this activity appeared to be due to limited proteolysis of prothrombin by proteolytic enzymes produced by *B. melaninogenicus* rather than to the existence of a specific coagulase (37).

Production of enzymes and toxins by anaerobic bacteria other than clostridia is receiving increasing attention (e.g. 9, 18, 24, 26, 31, 37). Production of ribonuclease and deoxyribonuclease by *P. indolicus* strains has not been previously reported. No hemolytic peptococci were observed in the present material as described by Sørensen (26).

Gas chromatographic analysis of metabolic end products is now a standard method for the identification of anaerobes in the clinical bacteriological laboratory. C_1 to C_4 volatile fatty acid profiles of ethyl ether extracts of PYG medium gave a highly reproducible pattern with *P. indolicus* strains. The *P. asaccharolyticus* profiles were slightly variable. The present extensive findings have been subsequently compared with profiles presented in the 4th edition of the *Anaerobe Laboratory Manual* (10) and are in agreement. Moreover, the biochemical description herein complements data in that manual. Although no attempts were made herein to analyze the gas produced by *P. indolicus* and *P. asaccharolyticus*, Holdeman et al. (10) state that both produce abundant H_2 gas. No reports on the presence or absence of other gases (e.g. CO_2) from cultures of these species have been published.

The distribution of serotypes among the *P. indolicus* strains examined by Sørensen (24) was very similar to that described in Table 1 (A,

2%; B, 18%; C, 35%; D, 23%; E, 22%). Serotype F has only been previously described in porcine isolates of *P. indolicus* (24).

In summary, it can be stated that differentiation of *P. indolicus* from *P. assacharolyticus* is valid and can be readily achieved by the use of commercially available kit systems in addition to gas chromatographic analysis and tests for extracellular or cell-bound enzymes and peptocoagulase.

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