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Hydrolytic Enzymes of "*Streptococcus milleri*"

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Seventy-two isolates classified as "*Streptococcus milleri*" were examined for the presence of various hydrolytic enzymes. While no protein or lipid-degrading activities were demonstrated, some isolates showed DNase and mucopolysaccharide-degrading activities. Beta-hemolytic isolates were more likely to produce these enzymes than were nonhemolytic strains. Isolates of one "*S. milleri*" biotype (mannitol fermentation positive) were uniformly devoid of all enzyme activities tested.

The designation "*Streptococcus milleri*" has been applied to a group of clinically important streptococci displaying various hemolytic, serological, and physiological traits (3). Whereas Facklam (5) advocates the separation of these organisms into distinct species (*Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus*), others have suggested that these organisms are closely enough related to be considered as a single species (3, 9, 22). In spite of the uncertain taxonomic status of these streptococci, they have been repeatedly isolated from a variety of purulent infections (1, 10, 11, 13, 17, 20).

Although "*S. milleri*" is a well-recognized agent of infection, little is known about its pathogenic mechanisms. Extracellular hydrolytic enzymes which may contribute to pathogenicity have been assayed in isolates classified as "*S. milleri*." Hyaluronidase (3, 14), DNase (15), and lipase (2) have been observed in some strains, whereas no isolates examined have been able to hydrolyze gelatin (2, 9). Three isolates examined by Bridge and Sneath (2) were reported to hydrolyze casein, but this activity was not detected in 86 isolates examined by Lutticken and associates (9). In the study reported here, we examined a collection of "*S. milleri*" strains with diverse characteristics for the presence of the aforementioned, as well as other, enzymes in an effort to learn more about the possible pathogenic mechanisms of these organisms.

MATERIALS AND METHODS

Bacteria. All of the strains examined were isolated from human clinical specimens and stored as frozen suspensions in horse blood at −70°C. Thawed suspensions were propagated on brucella agar plates containing 5% horse blood (GIBCO Diagnostics, Madison, Wis.) incubated at 35°C in an atmosphere containing 5% CO_2. Growth from these plates served as inocula for all tests on the isolates.

Identification methods. All isolates were tested for the presence of Lancefield groups A through D and F and G. Some isolates were examined by using the lysozyme-albus enzyme extraction method of Watson et al. (21), followed by capillary precipitin testing with antisera obtained from Burroughs Wellcome (Wellcome Reagents Ltd., Beckenham, England). Other isolates were grouped by using the Streptex method (Wellcome Diagnostics, Temple Hill Dartford, England). All strains were identified as one of three biotypes of "*S. milleri*" detected by the API Rapid Strep system (Analytab Products, Plainview, N.Y.).

Determination of enzymatic activities. Hyaluronidase and chondroitin sulfatase were assayed with the plate method described by Smith and Willet (18). The method of Lutticken and co-workers (9) was used for testing the ability of isolates to hydrolyze casein. DNase agar (Scott, Fiskeville, R.I.) was used for DNase tests. The method of Janda (6) was used to test for elastolytic activity, with brain heart infusion agar serving as the basal medium. Lipase and lecithinase production was tested on egg yolk agar composed of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing 10% egg yolk enrichment 50% (Difco Laboratories, Detroit, Mich.). Unless otherwise noted, media were obtained from Difco, and other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. All of the plated media for the above tests, except elastase, were incubated at 35°C in the presence of 5% CO_2 for 6 days before final readings were made. Elastin plates were incubated for 10 days. Tubed nutrient gelatin medium (GIBCO) was inoculated and incubated at 35°C for 2 weeks before gelatinase activity was determined.

The following organisms were used as positive control strains for the various enzymatic assays: DNase, *Staphylococcus aureus*; lipase, *Clostridium sporogenes*; lecithinase, *Clostridium perfringens*; gelatinase, *Serratia marcescens*; caseinase, *Bacillus subtilis*; hyaluronidase, *Streptococcus pyogenes*; chondroitin sulfatase, *Bacteroides fragilis*; elastase, *Pseudomonas aeruginosa*. The *B. fragilis* and *P. aeruginosa* strains were clinical isolates, the *B. subtilis* strain was obtained from Difco, and all other control organisms were obtained from the Massachusetts General Hospital Bacteriology Laboratory quality control collection.

RESULTS

All isolates examined gave negative reactions in tests for gelatinase, lipase, lecithinase, elastase, and casein hydrolysis. Table 1 displays the frequency with which DNase, hyaluronidase, and chondroitin sulfatase activities were observed among the various biotypes of "*S. milleri*" examined. The non-beta-hemolytic mannitol nonfermenters capable of acidifying lactose correspond to *S. intermedius* in Facklam's system of nomenclature (5). Some of these strains, along with their lactose-negative counterparts (*S. constellatus* in Facklam's scheme), elaborated DNase or hyaluronidase, but only a few strains possessed both enzyme

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TABLE 1. Enzymatic activities of biotypes of "*S. milleri*"

"*S. milleri*" biotype			No. of isolates with the following serological reaction(s)[a]	No. of strains examined	No. (%) of strains positive for:		
Beta-hemolytic	Mannitol fermentating	Lactose fermentating			DNase	Hyaluronidase	Chondroitin sulfatase
−	−	+	1 A, 1 C, 5 F	15	9 (60)	3 (20)	0 (0)
−	−	−	5 F	14	5 (36)	9 (64)	0 (0)
−	+	+	9 F	16	0 (0)	0 (0)	0 (0)
+	−	−	3 A	3	0 (0)	3 (100)	0 (0)
+	−	3 +, 4 −	7 C	7	6 (86)	7 (100)	4 (57)
+	−	2 +, 4 −	6 F	6	4 (67)	5 (83)	0 (0)
+	−	5 +, 1 −	6 G	6	5 (83)	2 (33)	0 (0)
+	−	1 +, 4 −		5	3 (60)	5 (100)	0 (0)

[a] The remaining isolates in each group and all isolates in the last line of the table were unreactive with group A, B, C, D, F, and G reagents.

activities. Hyaluronidase was produced by 11 of 17 nongroupable mannitol-negative nonhemolytic isolates, but only 1 of 12 strains with demonstrable Lancefield antigens showed this activity. If all of the isolates are considered solely on the basis of serological reactions, there seem to be no striking differences in DNase and hyaluronidase production between nongroupable strains and strains with Lancefield antigens. Of the 29 nongroupable isolates, 35% produced DNase and 55% elaborated hyaluronidase, while 52% of the 43 groupable strains showed evidence of DNase production, and hyaluronidase was detected in 42% of these isolates. The non-beta-hemolytic mannitol-fermenting isolates examined (which correspond to Facklam's mannitol-positive *S. intermedius* strains) were uniformly devoid of all of the enzyme activities assayed.

The beta-hemolytic strains examined (*S. anginosus* in Facklam's nomenclature) are divided in Table 1 according to serological reactions. DNase and hyaluronidase activities were found among these isolates. Of the 27 strains examined, 16 produced both of these enzymes. Four of the seven beta-hemolytic group C isolates hydrolyzed chondroitin sulfate. Three of these chondroitin sulfatase-positive strains produced both DNase and hyaluronidase, while one produced only hyaluronidase.

DISCUSSION

Our observation of the absence of gelatin- and casein-hydrolyzing activities among the "*S. milleri*" strains examined is in agreement with the report of Lutticken and co-workers (9), who were unable to detect these activities in 86 "*S. milleri*" strains. Bridge and Sneath (2), who included only three "*S. milleri*" isolates in their study, found no gelatinase activity but reported that all isolates hydrolyzed casein. Bridge and Sneath also found that two of the three strains they examined produced lipase, but in our study this enzyme, along with lecithinase, was undetected in all strains. Although elastase activity has been described in fungi and gram-positive and gram-negative bacteria, to our knowledge there have been no reports of assays of this activity in "*S. milleri*." None of the isolates we examined showed evidence of elastase production.

In general, the beta-hemolytic isolates we examined were more active enzymatically than the non-beta-hemolytic strains. This observation is in accord with those of previous studies in the case of hyaluronidase (3, 14). Kilpper-Balz and co-workers (7) found that the ability to produce hyaluronidase was usually found in "*S. milleri*" strains that were unable to ferment lactose. Our results agree with their observations since only 9 of 34 hyaluronidase-producing strains we examined were lactose fermenters. DNase activity was absent in all three isolates of "*S. milleri*" examined by Bridge and Sneath (2) but present in 23 of 69 "*S. milleri*" strains examined by Pulliam and associates (15). Although approximately one-fourth of these strains were beta-hemolytic, presentation of the data did not permit determination of the incidence of DNase production in nonhemolytic versus beta-hemolytic strains. In our study, this enzyme was more prevalent in the beta-hemolytic isolates examined.

Chondroitin sulfatase activity was noted only among the beta-hemolytic group C isolates. To our knowledge, this enzyme has not been previously described in streptococci, although it has been detected in both gram-positive and gram-negative anaerobes (18, 19).

Absence of extracellular enzymes was observed in all 10 of the mannitol-fermenting "*S. milleri*" strains we examined. These organisms rarely play significant roles in infection. No mannitol-fermenting isolates were found among the 81 "*S. milleri*" strains from serious infections studied by Parker and Ball (12). In a study of 346 clinically significant "*S. milleri*" isolates, Ball and Parker (1) reported that only 4% of the strains displayed the mannitol-positive biotype. In our own laboratory during 1986, we identified only one mannitol-fermenting "*S. milleri*" strain among 225 non-beta-hemolytic "*S. milleri*" isolates judged to be of clinical importance. Another study suggested that these strains play no pathogenic role in urinary tract infections, even when present in pure culture in urine (16). Although a recent report (4) describes two cases of neonatal sepsis due to this "*S. milleri*" biotype, it appears to be much less common than other biotypes among clinically significant isolates.

Although DNase and mucopolysaccharide-degrading enzymes were commonly found among the beta-hemolytic isolates we examined, only 22% of Parker and Ball's (12) "*S. milleri*" strains and 25% of the isolates in the study of Ball and Parker (1) were beta-hemolytic. Our beta-hemolytic group C "*S. milleri*" strains were the most enzymatically active of all the isolates tested. Lebrun and co-workers (8) recently demonstrated the absence of receptors for the Fc fragment of human immunoglobulin G, a possible virulence factor, in these organisms.

Although we were unable to arrive at any clear-cut conclusions about the relationship between enzymatic activities and pathogenic potential of "*S. milleri*" from the data presented here, it appears that different "*S. milleri*" biotypes display various capacities for production of hydrolytic enzymes. Further studies of these organisms and their interaction with their hosts are necessary to elucidate the pathogenic mechanisms of these streptococci.

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