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Hydrolytic Enzymes of *Moraxella bovis*

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Certain strains of *Moraxella bovis* produce tissue-damaging enzymes which may initiate or potentiate infectious bovine keratoconjunctivitis. Thirteen reference strains of this species were characterized physiologically and screened for production of various enzymes by some conventional biochemical tests and the API ZYM system (Analytab Products, Plainview, N.Y.). All 13 strains were hemolytic. All hydrolyzed Tween 80 and Tween 85 and displayed C4 esterase, C8 esterase-lipase, and C14 lipase activities. All produced phosphoamidase and phosphatase. All were able to hydrolyze casein and gelatin. All produced leucine and valine aminopeptidases and fibrinolysin. Twelve produced hyaluronidase or were agarolytic. Three hydrolyzed chondroitin sulfate. Nine strains autoagglutinated. Five produced catalase, and two produced cystine aminopeptidase.

Moraxella bovis is isolated most commonly from field cases of infectious bovine keratoconjunctivitis (IBK; bovine pinkeye). The incidence, predisposing factors, clinical signs, etiology, treatment, and control of IBK have been reviewed most recently and extensively by Baptista (1). The disease is becoming more prevalent, and current control measures are unsatisfactory. The role of *M. bovis* in IBK is somewhat uncertain because of variations in strain virulence. Additionally, laboratory identification of the nonfermenter *M. bovis* presents some difficulty. Since there is a need to develop more biochemical tests for use in the identification of nonfermenters (6) and because of the obscure nature of the pathogenesis of IBK, we performed a physiological characterization and metabolic study of 13 reference strains. The conventional procedures and the API ZYM system (Analytab Products, Plainview, N.Y.) (which has not been used extensively in clinical bacteriology and can detect activity of 19 hydrolytic enzymes) were chosen for this investigation because many of the substrates could become available to *M. bovis* in diseased ocular tissue. Of 31 tested substrates, 19 were attacked (13 were attacked by all strains).

MATERIALS AND METHODS

Reference strains. Eleven reference strains designated WSE64(13R), WSE64(2L), KGD63(1L), IVI64(58L), RM69(7R), IBH63(383R), IBH64(400R), IBH65(424L), IBH66(643R), IBH67(741L), and Fla64 were received from the National Animal Disease Center, Ames, Iowa. The neotype strain 10900 was obtained from the American Type Culture Collection. An additional Norden Laboratories field strain designated SKF1 was also examined. Lyophilized cultures were reconstituted and subcultured to Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing 5% sheep blood (GIBCO Laboratories, Grand Island, N.Y.). The identification of each strain was confirmed by the criteria of Tatum et al. (18) before characterization. Test inocula were obtained from blood agar plates incubated for 24 h at 37°C and for 6 h at 22°C.

Conventional tests. Hemolysin production was determined on sheep blood agar plates incubated for 24 h at 37°C and for 24 h at 22°C. Autoagglutinating ability was determined by suspending growth in normal saline. Areas of plates from which growth had been removed were examined for agarolysis (circular depressions or craters in the medium beneath colonies). Growth was suspended in 3% H_2O_2 to determine catalase production.

The methods for preparation of chondroitin sulfate and hyaluronic acid agars (17) were modified slightly. Both substrates were obtained from Sigma Chemical Co., St. Louis, Mo. A total of 10 ml of filter-sterilized 0.4% chondroitin sulfate was added to an autoclaved and cooled basal medium that contained 4 g of Trypticase soy agar in 70 ml of distilled water. The medium was stirred while 20 ml of filter-sterilized 5% bovine albumin (Sigma) was added, and the completed medium was poured into petri dishes. A total of 20 ml of filter-sterilized 0.2% hyaluronic acid was added to an autoclaved and cooled basal medium composed of 4 g of Trypticase soy agar in 60 ml of distilled water. The medium was stirred while 20 ml of bovine albumin was added, and plates were poured. Chondroitin sulfate and hyaluronic acid agar plates were spot inoculated with growth and incubated at 37°C for 5 days and at 22°C for an additional 2 days. Plates were flooded with 2 N acetic acid for 10 min and observed for clear zones surrounding growth (positive test).

Preparation of solid media containing lecithin (phospholipase substrate), Tween 80 (esterase substrate), Tween 85 (lipase substrate), casein (nonspecific protease substrate), and gelatin was described previously (20); Frazier reagent was used to flood gelatin plates after 4 days of incubation at 37°C,

enabling observation of clear zones (positive test).

Fibrinogen plates were prepared as described previously (11). Test strains were spot inoculated onto the plates and incubated at 37°C for 24 h. Collagen agar contained 0.5 ml of Vitrogen 100 (Collagen Corp., Palo Alto, Calif.) in 100 ml of previously autoclaved and cooled Trypticase soy agar. The medium was stirred very briefly, immediately poured into plates, and allowed to solidify upright in a 37°C incubator for collagen gelation. Collagen plates were spot inoculated and incubated at 37°C for 5 days. Both fibrinogen and collagen plates were examined for clearing (positive test).

API ZYM. Constitutive enzymes were assayed colorimetrically with the API ZYM system. The instructions of the manufacturer were followed. Briefly, 2 drops of a heavy bacterial suspension (McFarland no. 5 standard) were added to each microcupule. Strips were incubated in the humidified chamber at 37°C for 4 h. The provided reagents were added, and the color was allowed to develop for 5 min. Each strip was placed 4 in. (ca. 10.2 cm) beneath a sunlamp (Westinghouse Electric Corp., Bloomfield, N.J.) for 4 min to remove residual reagent color in negative cupules. The intensity of each reaction was estimated with the reading scale of the manufacturer.

RESULTS

Conventional tests. All 13 strains of *M. bovis* produced hemolysin, were fibrinolytic, and hydrolyzed Tween 80, Tween 85, casein, and gelatin. Of these, 12 strains were agarolytic, and 12 produced hyaluronidase. Nine strains autoagglutinated. Five produced catalase. Three strains attacked chondroitin sulfate. No strain produced lecithinase or collagenase.

API ZYM. All 13 strains displayed constitutive acid and alkaline phosphatase, phosphoamidase, C4 esterase, C8 esterase-lipase, C14 lipase, and leucine and valine aminopeptidase activities. Leucine aminopeptidase and C8 esterase-lipase activities were quite intense. Only two strains produced cystine aminopeptidase. No strain produced trypsin- or chymotrypsin-like proteases, α or β-galactosidase, β-glucuronidase, α- or β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, or α-fucosidase.

DISCUSSION

The strains of *M. bovis* studied had relatively homogeneous cultural and biochemical characteristics. Determinations of esterase and lipase activities with the API ZYM system were in agreement with esterase and lipase tests in which Tween 80 and Tween 85 were used as substrates. The API ZYM system has been used to identify staphylococci, *Erwinia*, nonhemolytic streptococci, and gram-negative anaerobes (19) and to characterize gram-positive microaerophilic cocci (16). The system also might be used to characterize *M. bovis*. Such characterization may give us some idea of the pathogenic mechanisms involved in IBK. The positive results obtained with two (acid and alkaline) phosphatase assays suggest that *M. bovis* produces phosphatase which is active over a wide pH range. Phosphatases (2) and hyaluronidase (2, 9) may be allergenic. An allergic response could contribute to the pathogenicity of *M. bovis*.

The hemolytic toxin, or hemolysin, of *M. bovis* has been investigated (8, 12). Because the studied strains were hemolytic but failed to produce lecithinase, we do not believe that the hemolysin is a phospholipase. Nonhemolytic *M. bovis* organisms were found to be unable to induce IBK (14).

There may be some relationship between the ability to attack the sulfated polysaccharides (agar, chondroitin sulfate, and hyaluronic acid) and the ability to adsorb to or desorb from ocular tissue. The cornea is rich in mucopolysaccharides. Chondroitin sulfate, abundant in the cornea, and hyaluronic acid, present in the aqueous humor, have somewhat similar structures. Alternating *N*-acetylgalactosamine and glucuronic acid units of chondroitin sulfate are connected by β1-4 linkages. Hyaluronic acid contains *N*-acetylglucosamine and glucuronic acid units connected by alternating β1-3 and β1-4 linkages. Agar contains complex polysaccharide chains having alternating α1-3 and β1-4 linkages. A single *M. bovis* enzyme with β1-4 linkage specificity might be capable of hydrolyzing all three polysaccharides. The release of mucopolysaccharide split products from ocular tissue could influence the development of an inflammatory reaction (7). Hydrolysis of β1-4 linkages in connective tissue could yield ocular products having terminal glucuronic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine residues. Infiltrating polymorphonuclear leukocytes and eosinophils (with lysosomal β-glucuronidase) and macrophages (which can produce inducible *N*-acetylglucosaminidase) might contribute to tissue damage. Production of hyaluronidase has been associated with virulence of *Pasteurella multocida* (4), *Staphylococcus aureus* and *Clostridium perfringens* (17), and streptococci (10, 17). Interestingly, beta-hemolytic streptococci produce both hyaluronidase and hyaluronic acid capsules, and destruction of these capsules by the streptococcal enzyme may account for capsule instability (10). Capsular material is responsible for specific adsorption of some pathogenic bacteria to host tissue sites, an initial step in pathogenesis; bacteria also adsorb to each other via capsular interaction (5). Capsular material could be responsible for both autoagglutination

and adsorption. These phenomena could also be mediated by fimbriae. Pederson et al. found that only fimbriated *M. bovis* strains were able to colonize the bovine conjunctival mucosa (13). Fimbriae have been associated with an autoagglutinating (15) and agarolytic (spreading-corroding) colony type (3, 13). Therefore, it may be important to determine whether *M. bovis* strains possess polymeric fibrils in addition to fimbriae and whether either or both structures mediate adsorption.

Although the reference strains examined produced various proteolytic enzymes, they were unable to hydrolyze collagen, a principal component of sclera, corneal, and substantia propria basement membranes. Collagenase is probably important to the progression of canine corneal ulcers and could originate from host epithelium, fibroblasts, neutrophils, or other attracted inflammatory cells. Endogenous collagenase within a corneal defect could degrade aggregated collagen fibrils to component peptides. Subsequent hydrolysis of such peptides by proteolytic *M. bovis* (perhaps via production of aminopeptidases to split intermediate degradation products and phosphoamidase to hydrolyze amino acids and acid amides) could lead to dissolution of ocular tissue matrix proteins and result in corneal ulceration.

Fibrin deposition often accompanies inflammatory tissue destruction. All of the *M. bovis* strains studied produced fibrinolysin. Casein, used as a substrate for microbial proteases and for fibrinolysin (plasmin), was also hydrolyzed by all strains. These results suggest that *M. bovis* fibrinolysin may be responsible for casein hydrolysis.

In summary, adsorption of *M. bovis* to the bovine eye followed by production of hydrolytic enzymes capable of damaging tissue lipids, mucopolysaccharides, and matrix proteins and the allergenicity of certain of these enzymes may account for the heretofore obscure pathogenesis of this species. Results of this study point to several potential pathogenic mechanisms. Examination of these mechanisms may lead us nearer to the eventual control of IBK. Additionally, many biochemical characteristics that we have observed in these reference strains should prove useful in clinical laboratory identification of the nonfermenter *M. bovis*.

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