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Rapid Plate Method for Screening Hyaluronidase and Chondroitin Sulfatase-producing Microorganisms

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Microbial hyaluronidase and chondroitin sulfatase have been studied because of the possible associations between these enzymes and microbial mechanisms of pathogenicity. One of the most sensitive biological assays for mucopolysaccharide-degrading enzymes is the turbidity reducing unit (TRU) method (3). In most assays for these two enzymes, measurement of activity involves the conjugation of bovine serum fractions with nondepolymerized substrate in acetic acid. The turbidity produced by the conjugate or the lack of it can be directly related to the amount of substrate depolymerized in solution. Recently, a direct localization and visualization technique to study hyaluronate lyase by agar-gel electrophoresis was reported (1). This technique was modified as a cultivable screening plate method for bacteria.

The basic medium consisted of Brain Heart Infusion broth (BBL) prepared to make 100 ml, to which was added 1 g of Noble agar (Difco). The medium was autoclaved at 121 C for 15 min and cooled to 46 C. Aqueous solutions of 2 mg of umbilical sodium hyaluronidate (Sigma Chemical Co., St. Louis, Mo.) per ml, 4 mg of bovine nasal chondroitin sulfate (Pentex, Inc., Kankakee, Ill.) per ml, and 5% bovine albumin fraction V (Sigma) were sterilized by filtration with 0.20-μm Nalgene filter units (Nalge Co., Inc., Rochester, N.Y.). Each substrate was added to the cooled media to give final concentrations of 400 μg/ml. The bovine albumin fraction-V was then added with constant stirring to give a final concentration of 1%. The agar was poured to a depth of 3 to 4 mm. The final *p*H of each medium was 6.8 ± 0.1. After solidification, plates were tempered at 4 C to provide a firm surface for streaking or swabbing. Pure or mixed cultures can be examined for enzyme activity. After incubation at 37 C, the plates were flooded with 2 *N* acetic acid for 10 min.

The nondegraded substrate precipitates as a conjugate with the albumin, leaving a clear zone around those colonies which produce soluble enzymes that attack the hyaluronate or chondroitin sulfate (Table 1; Fig. 1A–E). Hyaluronidase activity is well known among coagulase-positive staphylococci and group-A hemolytic streptococci. Both groups were represented among the test cultures. The undesignated strains of *Corynebacterium acnes* were from a collection recently characterized by Smith and Bodily (2) who also found that, of 27 strains of *C. acnes*

TABLE 1. *Hyaluronidase and chondroitin sulfatase activity of selected bacterial strains tested by the plate method*

Strains[a]	No. tested	Hyaluronidase	Chondroitin sulfatase	Days incubated
Staphylococcus aureus	5	+	−	1
S. epidermidis	7	−	−	1
Streptococcus pyogenes	1	+	−	1
S. agalactiae	5	+	−	1
Corynebacterium xerosis ATCC 373 and Midwest	2	−	−	3
C. stratium ATCC 6940	1	−	−	3
C. pseudodiphtheriticum Midwest	1	−	−	2
C. acnes	2	+	+	5
Propionibacterium acnes ATCC 11828	1	+	+	5
Clostridium perfringens	1	+	−	5
Odontomyces viscosus ATCC 15987	1	−	−	6
Escherichia coli	1	−	−	2
Aerobacter aerogenes	1	−	−	2
Pseudomonas aeruginosa	1	−	−	1

[a] Undesignated strains were part of the departmental stock collection. Reference strains were obtained directly from reference services shown. Facultative organisms were streaked on plates from BHI broth. Anaerobic cultures were grown in thioglycollate broth (BBL). Anaerobic strains were incubated in Brewer jars with Gaspaks (BBL).

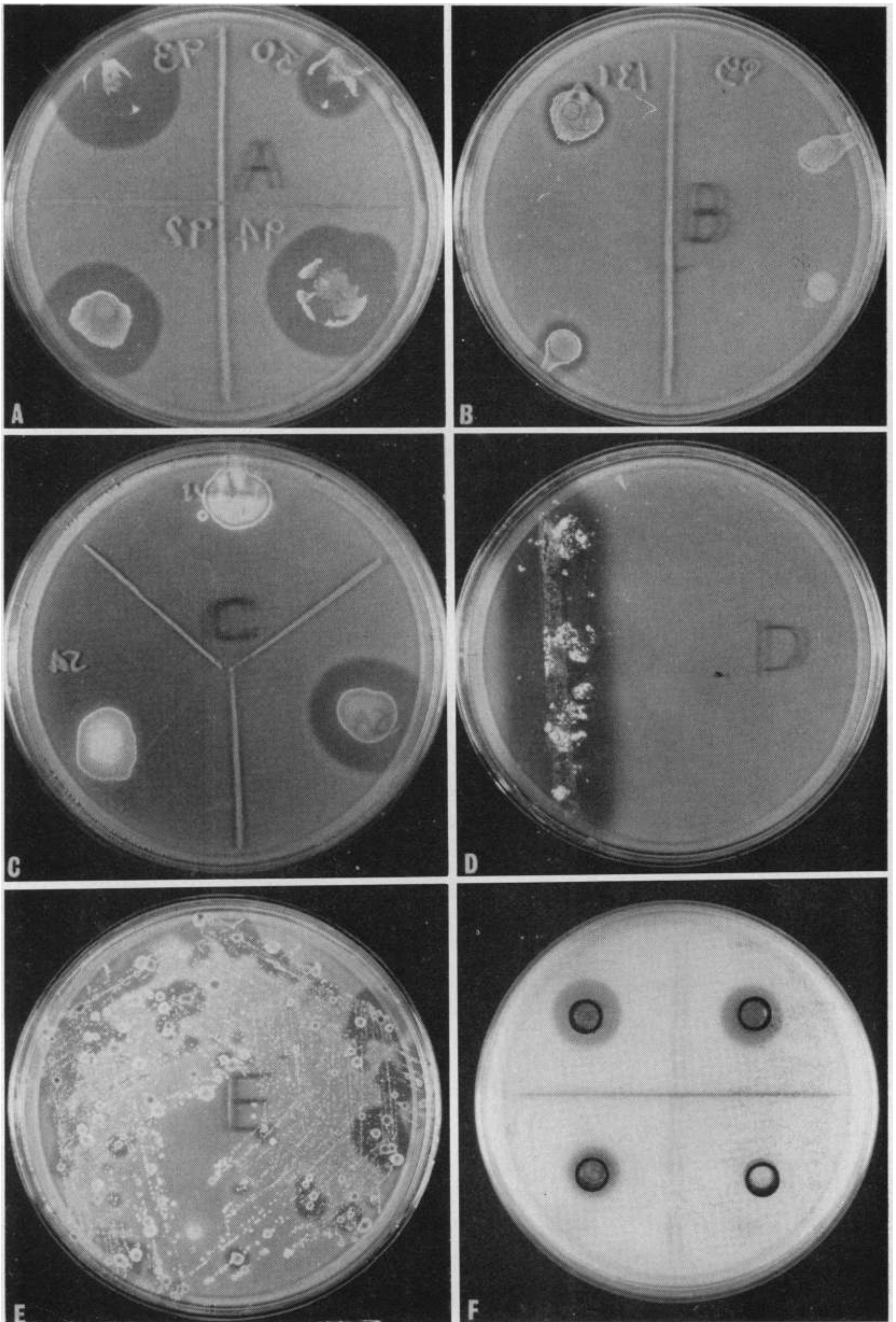

FIG. 1. *Illustrations of the plate method.* (A) *Hyaluronidase-positive strains of Streptococcus agalactiae inoculated by the drop method. Part of colonies washed off from acid treatment.* (B) *Hyaluronidase-positive and -negative streptococci.* (C) *Staphylococcus aureus-positive and S. epidemidis-negative strains on hyaluronidase test plates.* (D) *Propionibacterium acnes ATCC 11828 showing chondroitin sulfatase action.* (E) *Mixed culture of salivary bacteria showing areas of hyaluronidase-positive bacteria among a mixed population of negative organisms. Plate was incubated 48 hr anaerobically. Chondroitin sulfate plate streaked from same inoculum had no positive colonies.* (F) *Testis hyaluronidase. Lower left, upper right, and upper left, 29, 58, and 116 USP units per cylinder, respectively. Upper left, saline control.*

tested by the TRU method, 20 strains produced hyaluronidase and 11 produced chondroitin sulfatase (Smith and Bodily, *unpublished data*). This study was not intended as a survey but rather to show the effectiveness of a screening test method by using a small but diverse group of bacteria. Acidification of the media inoculated with positive or negative cultures containing each substrate or the albumin reagent alone produced only a faint opacity which does not interfere with the interpretation of a positive test. Sensitivity of the method was shown by placing aqueous testis hyaluronidase (Worthington Biochemical Corp., Freehold, N.J.) into penicylinders and incubating the plates 12 to 18 hr at 37 C; this produced clear zones (Fig. 1F).

Although this method does not distinguish constitutive from inducibly produced enzymes, a large number of isolates can be rapidly screened and positive strains identified from among those without activity. The final medium can be supplemented with 5 μg of hemin per ml, 0.25 μg of menadione per ml, and 0.1% KNO_3 to enhance the cultivation of fastidious anaerobic bacteria. A 100-mg amount of substrate provides enough plates (about 25) to directly screen as many as 100 bacterial strains for enzyme activity. Replica plating or other micromethods could probably screen more. Individually filtered substrates and the conjugating albumin reagent can be stored at −20 C and the autoclaved basal medium stored at 4 C indefinitely so plates can be prepared for use in less than 1 hr. After the incubated plates have been acidified and examined, the areas of precipitated, nondegraded substrate remain opaque and the clear zones remain stable. Therefore, plates can be refrigerated for making comparisons to other strains tested at different times.

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