Anticapsin, a New Biologically Active Metabolite: Screening and Assay Procedures

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In addition to its implication in the virulence of *Streptococcus pyogenes*, the hyaluronic acid capsule produced by this bacterium renders it resistant to infection by bacteriophage. A method employing *S. pyogenes* and a bacteriophage incorporated into an agar plate was devised as a screen to detect compounds that inhibit the formation of the hyaluronic acid capsule. Filter-paper discs saturated with experimental compounds were applied to the surface of test plates containing host plus phage and control plates of host only. After incubation, inhibition of capsule synthesis was indicated by the presence of clear zones where phage infection and lysis had occurred. Zones of growth inhibition on control plates represented classical antibacterial activity. During the testing of over 6,000 fermentation samples, anticapsin, a unique metabolite, was discovered. Modification of incubation temperature, thickness of agar layers, and host-phage input ratios resulted in a quantitative assay method having a dose-response range of 4 to 160 μg of anticapsin.

Although many infectious diseases can now be controlled, the precise biochemical mechanisms whereby microorganisms cause disease generally remain obscure. Considerable investigation of the parameters for pathogenicity has implicated a number of specific cellular structures and products in microbial virulence. These cellular entities, which may be termed virulence factors, include capsules, slime layers, a variety of enzymes and toxins, antigens, cell wall mucopolypeptides, and lipopolysaccharides. Five of these factors (M-antigen, capsule, slime layer, mucopolypeptides, and lipopolysaccharides), occurring as structural components in or at the periphery of the cell, may contribute to the virulence of the organism by inhibiting phagocytosis (2, 4, 6, 8, 10–12).

The capsule of the group A hemolytic streptococci has been identified as hyaluronic acid by Kendall et al. (3). In addition to the implication of the capsule in the virulence of the organism, Maxted (5) observed that treatment of encapsulated, phage-resistant streptococci with the enzyme hyaluronidase rendered the culture phage susceptible, thus demonstrating that the capsule is also a barrier to phage infection.

This report describes a screening system in which *Streptococcus pyogenes* and a bacteriophage that infects only the nonencapsulated host were used to select compounds inhibiting synthesis of the hyaluronic acid capsule of the bacterium. A modification of the method for use in the quantitative assay of one such compound, anticapsin (7, 9), having the structure shown in Fig. 1, will also be presented.

MATERIALS AND METHODS

**Bacterial culture.** The strain of *S. pyogenes* used throughout this study was Lilly culture X240.1, a single-colony isolate of ATCC 12384.

**Media and growth conditions.** Stock cultures were prepared by incubating cells overnight under static conditions at 37 C in either Difco brain heart infusion (BHI) broth or on BHI agar. All media were supplemented with 5% (v/v) sterile normal horse serum. These cultures were stored at 4 C and used for 7 days as stock cultures. Cultures were prepared each day by inoculating 10 ml of broth with 0.5 ml of liquid stock cultures and incubating overnight at 37 C. Cultures used as inocula for assay and screening plates were prepared by inoculating 50 ml of BHI broth with 0.6 ml of overnight culture. After incubation at 37 C for 3 hr, the optical density was adjusted to 0.25 as measured at 540 nm on a Bausch & Lomb Spectronic 20.

**Bacteriophage.** Plates for the isolation, assay,
and propagation of the bacteriophage were double-layered BHI agar plates prepared as described by Adams (1), except that the soft agar layers were supplemented with 125 international units (IU) of hyaluronidase per ml (obtained from Nutritional Biochemicals Corp.). The bacteriophage was isolated from Selas-filtered raw sewage, having a titer of $1 \times 10^6$ plaque-forming units (PFU)/ml, were routinely stored in the vapor phase of liquid nitrogen.

**Screen and assay plates.** Plates for the screening of experimental compounds were prepared by using 100 by 100 by 15 mm, square petri dishes. Test plates contained 12 ml of BHI 0.8% agar at 46 C, 0.6 ml of sterile horse serum, 0.2 ml of phage stock ($1 \times 10^4$ PFU/ml), and 1.0 ml of host inoculum. Control plates were prepared in the same manner except that no phage was added. Assay plates were prepared by adding the following to 100 ml of BHI 0.8% agar held at 46 C: 5 ml of sterile horse serum, 2 ml of host inoculum, and 1.6 ml of phage stock. An 8-ml amount of the inoculated medium was dispensed into non-sterile plastic petri dishes (100 by 20 mm), and the plates were chilled until used, normally 1 to 2 hr after preparation. Screen and assay plates were incubated overnight at 30 C.

**RESULTS AND DISCUSSION**

The anticapsular screen involved the use of test and control plates as indicated in Fig. 2. After solidification of the agar, 6.35-mm filter-paper discs were dipped into each sample being screened and were applied to the surface of both plates. Samples for screening consisted of filtered actinomycete and mold fermentation broths and solutions of organic compounds, all having little or no antibiotic activity. Paper discs saturated with solutions of organic compounds were air-dried before application to the plates to minimize the effects of solvents. Controls consisting of discs saturated with hyaluronidase solution at 150 IU/ml were included in each test. Those samples producing zones on both test and control plates were considered to be antibiotics and were consequently discarded. Samples producing zones of phage-induced lysis on test plates only were assumed to contain anticapsular compounds. The hyaluronidase control, allowing phage infection and lysis on the test plate, was a measure of the viability of the host-phage system. After screening of more than 6,000 samples, one showing reproducible activity was selected for chemical isolation and provided the compound anticapsin.

To facilitate chemical isolation of anticapsin, modifications of the screening method were made to devise a quantitative paper-disc-agar diffusion assay for anticapsular activity. The original assay standard was a lyophilized fer-

**Fig. 1. Structure of anticapsin.**

**Fig. 2. Plates for screen: control (A) and test (B). Reading clockwise from upper left, discs represent antibiotic activity, hyaluronidase control, capsule inhibition, and inactive sample.**
mentation broth having anticapsular activity as detected in the screen. Isolation and purification of anticapsin, the active factor in the broth, provided preparations for use as standards in later assays.

The standard dose-response curve was run with 160, 80, 40, and 20 μg of anticapsin activity per ml. A typical assay plate for the dose-response curve is presented in Fig. 3. Ten such plates were used for the standard curve, each containing four filter-paper assay discs (12.7 mm in diameter) saturated with the four concentrations of the standard. Zones of lysis were measured on a modified Fisher-Lilly antibiotic zone reader, and the average zone diameters were plotted versus the standard concentrations on semi-log paper. The resulting dose-response curve is shown in Fig. 4.

Samples to be assayed were diluted in sterile, deionized water to give activity levels within the range of the standard curve. The appropriate dilutions were applied to the assay plates via 12.7-mm paper discs. Four plates, each containing two discs saturated with the sample dilution being assayed and two discs saturated with a standard reference solution of 40 μg of anticapsin per ml, were used to assay each sample. The reference solution was routinely employed to allow correction of sample zone diameters for plate-to-plate variations. The corrected sample zone diameters were used to read the anticapsular activity in micrograms per milliliter from the dose-response curve. All assay values were adjusted for sample dilution.

The sensitivity of the assay was found to be influenced by several factors. Reducing the amount of agar medium to the minimum necessary for good growth increased sensitivity. The optimum temperature for incubation of the assay plates was 30°C; temperatures below 30°C resulted in reduced zone sizes, whereas higher temperatures produced zones diminished in clarity. Maximum zone sizes were obtained with phage stocks having titers of $1 \times 10^9$ PFU/ml; lower titers gave sharply reduced zone sizes.

These data outline screening and assay procedures for compounds that inhibit the synthesis of the hyaluronate capsule of S. pyogenes X240.1. The zones of phage-induced lysis upon which the screen and assay are based require either the removal of preformed capsule or inhibition of synthesis of capsular material. Although hyaluronidase was used in the isolation and propagation of the bacteriophage and as a positive control for the screen, anticapsular activities found to date are nonenzymatic.

![Fig. 3. Anticapsular assay plate. Reading counterclockwise, discs represent 160, 80, 40, and 20 μg of anticapsin activity per ml.](image)

![Fig. 4. Dose-response curve for anticapsin.](image)

The development of the anticapsular screen and assay represents a more specialized approach to the search for biologically active compounds. Considering the diverse occurrence of macromolecules such as hyaluronic acid, the study of anticapsin and similar compounds in chemotherapy and research is currently being investigated.

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