1997

Characterization of group B streptococcal oligopeptidase and hyaluronate lyase.

Bo Lin
University of Alabama at Birmingham

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

CHARACTERIZATION OF GROUP B STREPTOCOCCAL OLIGOPEPTIDASE AND HYALURONATE LYASE

by

BO LIN

A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

1997

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Doctor of Philosophy Program Microbiology

Name of Candidate Bo Lin

Committee Chair Dr. David G. Pritchard

Title Characterization of Group B Streptococcal Oligopeptidase and Hyaluronate Lyase

Group B streptococcus (GBS) is the most common cause of potentially deadly infections in newborn infants. GBS pathogenesis is not fully understood, but virulence appears to be associated with the organism's ability to systemically invade a susceptible host. It is very important, therefore, to elucidate the nature of the bacterial factors that contribute to the invasive properties of GBS. Factors likely to be important include two GBS hydrolytic enzymes, PepB oligopeptidase, and hyaluronate lyase (HylB). These enzymes are the primary focus of this dissertation.

We purified and characterized GBS oligopeptidase, an enzyme that had been incorrectly identified as a collagenase based on its ability to hydrolyze the synthetic collagen-like substrate FALGPA (N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala). Our substrate specificity studies demonstrated that neither the purified enzyme nor the crude GBS cell lysate solubilized a film of reconstituted rat tail collagen, an activity regarded as the obligatory criterion of a true collagenase. The enzyme also exhibited no detectable gelatinase or other proteolytic activity. The gene for the enzyme was then cloned by screening a lambda phage library of GBS chromosomal DNA fragments. The deduced amino acid sequence of the cloned gene showed 66.4% identity to PepF oligopeptidase from *Lactococcus lactis*, a member of the M3 or thimet family of zinc metallopeptidases.

We found that the GBS enzyme also showed oligopeptidase activity and degraded a variety of bioactive peptides, including bradykinin and neurotensin, as well as peptide fragments of substance P and adrenocorticotropic hormone. Our evidence clearly demonstrated that the GBS enzyme is not a collagenase, but is actually an endo-oligopeptidase. We designated it PepB oligopeptidase.

The gene for GBS hyaluronate lyase (*hyl*B) was also cloned in this study. Degenerate oligonucleotide primers were designed, based upon the sequences of tryptic peptides prepared from purified enzyme, and used to PCR amplify a 363 base pair internal gene fragment. A λ phage library of GBS chromosomal DNA fragments was constructed and screened. Since no single clone contained the entire gene, it was necessary to reconstruct it from two plasmid clones containing suitable overlapping sequences. The full length gene (3.4 kb) contained one open reading frame capable of coding for an 111 kDa protein. When it was cloned into an expression vector and transformed into *Escerichia coli*, high level expression of hyaluronate lyase activity was obtained. Finally, a *hylB*⁻ mutant strain was constructed by insertional mutagenesis using a specially constructed plasmid vector. Southern blotting and PCR mapping experiments indicated that two copies of part of vector were specifically inserted at the target site. The availability of this *hylB*⁻ mutant GBS strain, defective in the production of hyaluronate lyase, should greatly facilitate future studies of the pathogenic roles of the enzyme.

DEDICATION

With much love, I dedicate this dissertation to my parents, Hui Fang and ChuanShou Lin. I also dedicate this work to my beloved husband, Yang Wang, and son, Jason Linn Wang. Their love, support and enormous sacrifices throughout my graduate education have made the accomplishment of this task much easier.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. David G. Pritchard for what I consider the very privileged opportunity of having worked and studied in his laboratory. His conviction to student training is based on enthusiasm, knowledge, and honor, and has continued to inspire me during my tenure in his laboratory. I also wish to thank the members of my dissertation committee, Drs. Marianne L. Egan, Janet Yother, Susan K. Hollingshead, and Barry Gray, all of whom have been very supportive and helpful during my graduate training.

Additionally, I wish to acknowledge the members of the Pritchard and Egan laboratory for all their help. Specifically, I thank Tim R. Willingham, and William F. Averett for excellent technical support. I also thank Brent Limbaugh for stimulating discussions.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

BAP	blood agar plate
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CAMP	Christie-Atkins-Munch-Petersen factor
CD44	hyaluronan receptor
CDM	chemically defined medium
cfu	colony forming unit
Cm	chloramphenicol
dNTP	deoxynucleoside triphosphate
ECM	extracellular matrix
Em	erythromycin
FALGPA	furyl-acryloyl-Leu-Gly-Pro-Ala
GAS	group A streptococcus (*Streptococcus pyrogenes*)
GBS	group B streptococcus (*Streptococcus agalactiae)*
HA	hyaluronic acid (hyaluronan)
HPLC	high pressure liquid chromatography
hylB	group B streptococcal hyaluronate lyase
kb	kilobase(s)
PCR	polymerase chain reaction

pepB	GBS oligopeptidase
PMN	polymorphonuclear leukocyte
PROM	premature rupture of membranes
PVDF	polyvinylidene difluoride
RC	rolling circle replication
RHAMM	receptor for HA-mediated motility
SD	Shine-Dalgarno sequence
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBA	thiobarbituric acid assay
THB	Todd-Hewitt broth

INTRODUCTION

Prologue

Group B streptococci (GBS) are gram-positive cocci that usually grow as long chains or as diplococci. Structurally, GBS have thick, rigid peptidoglycan cell walls and a polysaccharide capsule consisting of covalently bound group- and type-specific antigens. Group B-specific polysaccharide antigen serves as an immunochemical marker to distinguish GBS from other β-hemolytic gram-positive streptococci (41). GBS are further subdivided into several serotypes based upon the presence of different type-specific polysaccharide antigens. Currently, eight serotypes of GBS have been identified (39). The four major serotypes of GBS are Ia, Ib, II, and III. GBS may be further classified based upon the presence of certain protein antigens such as C, R and X surface proteins (78). The R protein antigen is found mainly in serotype II and III GBS and is not present in serotypes Ia and Ib GBS strains (44). In contrast to R antigen, alpha- and beta-component of C protein is usually found on the surface of type I GBS (36).

Although the definitive identification of GBS is based upon the detection of the group B-specific polysaccharide, certain other morphological characteristics and biochemical tests are also used in the presumptive identification of GBS. For example, GBS grown on sheep blood agar plates typically form white flat colonies that are surrounded with a narrow clear zone of hemolysis. A surface-bound hemolysin is

responsible for this effect (21). If GBS are cultured in certain media under anaerobic conditions, most GBS strains produce an orange pigment (59). The ability to hydrolyze sodium hippurate to glycine and benzoic acid is also characteristic of GBS because all strains express cell-associated hippuricase (4, 18, 22). Another presumptive test for GBS is the so-called CAMP test that was named after its developers Christie, Atkins, and Munch-Petersen (11). It is based upon the ability of GBS to secrete a protein (CAMP factor) which in the presence of β-toxin produced by *Staphylococcus aureus*, leads to the hemolysis of sheep erythrocytes, and forms a characteristic arrowhead-shaped clear zone on sheep blood agar plates. Fig.1 illustrates several characteristic features of GBS structure as well as several important molecules produced by the bacteria.

Pathogenesis of GBS infections

Group B streptococci were initially recognized as a causative agent of bovine mastitis and hence were named *Streptococcus agalactiae* (73). In 1938, Fry described 3 cases of fatal puerperal sepsis caused by GBS showing that the organism is also a human pathogen (23). After that, GBS infections had been reported sporadically. During the last 20 years GBS has emerged as a major cause of serious perinatal infections (54, 80). Today, up to 50,000 GBS infections are reported annually in the United States (1). It has become the primary cause of life-threatening infections of newborn babies, causing sepsis, meningitis, and pneumonia (82). Although the mortality rate has been stabilized by effectively antibiotic therapy, over 1,000 babies still die from GBS infections annually (82). The incidence of neonatal infection is estimated to be about 4 per 1,000 live births in our institution (15). These infections are roughly classified as either early- or late-

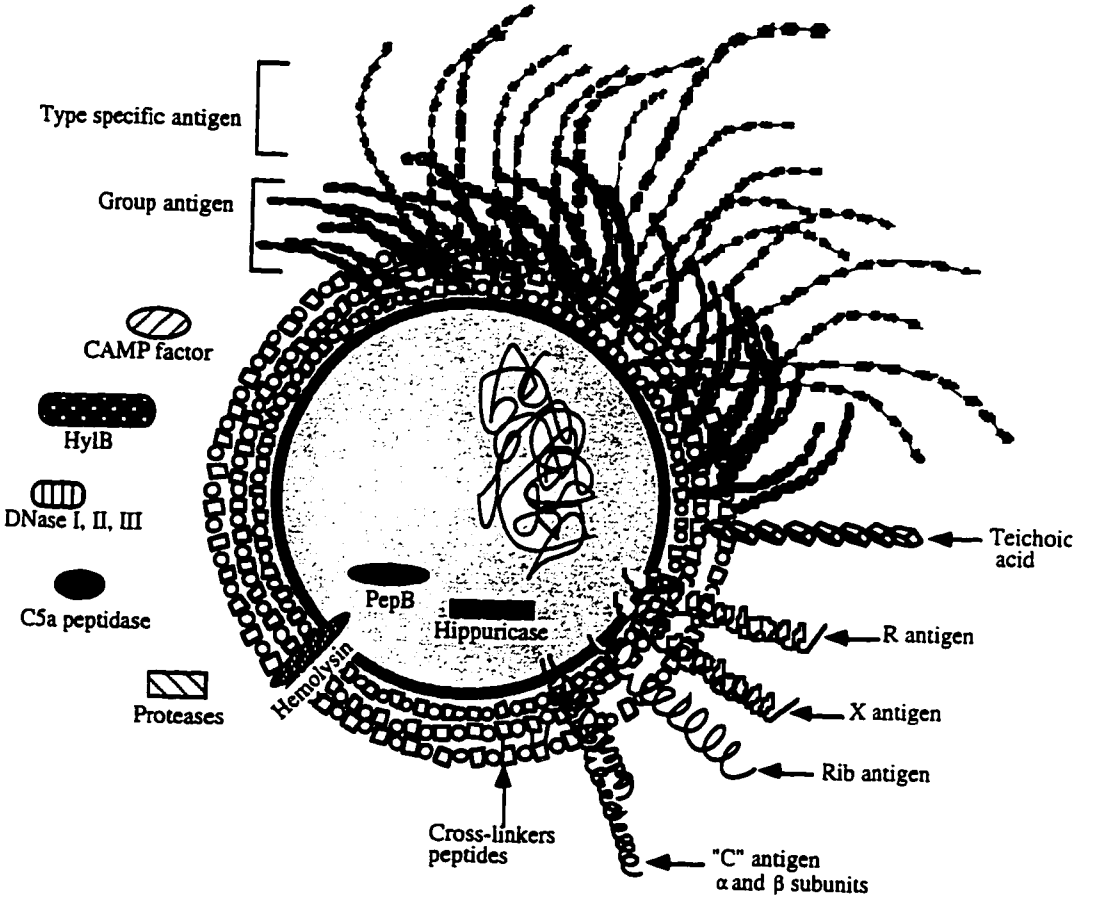


FIG. 1. Group B streptococcus and its products.

onset infections (81). In addition to neonatal infections, GBS also cause serious infections in adults with chronic illnesses, such as diabetes mellitus, or who are otherwise immunocompromised (19, 70).

GBS mainly colonize mucosal membranes of the throat and lower gastrointestinal tract of infants and children (50, 60). After puberty, genitourinary tracts of males and females become common sites of GBS colonization (5, 48). It has been estimated that one third of pregnant women are colonized with GBS in the lower genital tract at some time during pregnancy (14). However, the lower gastrointestinal tract is thought to be the primary human GBS reservoir (15).

GBS colonization, of course, does not inevitably lead to infection and only 1-2% of colonized infants will actually become infected (3). Several maternal factors are believed to increase the risk of neonatal GBS infection. These factors include prematurity (gestation less than 37 weeks), prolonged rupture of membranes (PROM), chorioamnionitis, unusually heavy colonization with GBS, and low serum concentrations of anti-GBS type specific polysaccharide antibodies. The susceptibility of human neonates to systemic GBS infection is also at least partly due to the immaturity of immune system. For example, it has been reported that in human newborns mononuclear and polymorphonuclear leukocytes counts are significantly lower than adult levels (47). In addition, *in vitro* experiments showed that granulocytes isolated from infants exhibit defective chemotactic properties (38). Also, compared with adults, neonatal leukocytes appear to have less intracellular killing efficiency (72).

The detailed pathogenesis of neonatal GBS infection is poorly understood. Neonatal GBS transmission can be roughly classified into two pathways: vertical and horizontal transmission. In vertical transmission, GBS colonizing the maternal genital tract ascend and penetrate the placental membrane to cause *in utero* GBS infections. Early-onset GBS diseases are then thought to result in the fetus following the aspiration of contaminated amniotic fluid during or just before birth. In addition to vertical transmission, horizontal transmission of GBS can occur after birth, often nosocomially acquired, resulting in late-onset GBS disease.

Invasive ability of GBS

GBS are part of the normal bacterial flora of many people, colonizing mucosal surfaces of the throat, lower intestine, and genital tract. However, it is clear that the bacteria also possess the ability to systematically invade a susceptible human host and cause potentially fatal infections. It has been demonstrated that early-onset GBS disease is more likely to occur when the mother's vagina and cervix are heavily colonized (15). On the other hand, when maternal cultures are negative for GBS, the occurrence of neonatal GBS infection is extremely low. The significant association between neonatal infections and premature delivery suggests that GBS can penetrate the amniotic membranes and infect the amniotic fluid and fetus. The critical question is just how this happens.

Amniotic membranes possess a collagen-rich matrix that may be ruptured under certain conditions. Bacterial infection is one of several factors that are thought to contribute to the premature rupture of membranes (PROM) (51). The reasons for

PROM caused by bacterial infection have not yet been elucidated. It is possible that the interaction of pathogens and amniotic membranes may elicit inflammatory factors such as peroxidase and elastase from activated neutrophils. These factors have been shown to damage amniotic membranes (69). Also, bacterial colonization could allow various bacterial enzymes to degrade extracellular matrix components and weaken the amniotic membranes. *Pseudomonas aeruginosa,* for example, has been shown to produce collagenases that degrade collagen fibrils of the chorioamniotic membranes (55). There is also considerable circumstantial evidence that GBS can produce factors that weaken chorioamniotic membranes (29, 58).

Aspiration of GBS contaminated amniotic and vaginal fluid into the lungs seems to be a primary route of infection of infants who develop early onset disease. GBS have been shown to be capable of invading cultured human lung epithelial and endothelial cells (25, 26, 30, 66). This property is probably related to the ability of GBS to traverse the lung to the circulation *in vivo*. This ability has been demonstrated in several different animal systems including piglets, rabbits, rats, and neonatal lambs (10, 49, 63, 72). Rubens and colleagues developed a primate model of GBS sepsis using *Macaca nemestrina* (65). They inoculated a virulent GBS strain (COH-1) into the amniotic fluid of pregnant monkeys. All premature infants showed symptoms of GBS infection that were similar to early onset sepsis in human infants. They also found that GBS were present in pulmonary alveolar spaces, in vacuoles of alveolar epithelial cells, fibroblasts, macrophages, and capillary endothelial cells of infant lung tissue.

Lung infections result in an inflammatory response which includes an influx of neutrophils into the lungs. Additional evidence for the lung being the initial site of GBS infection is the frequent observation that the neutrophils in GBS-infected infants are often depleted in the circulation and accumulate in pulmonary tissue. Toxic products of the neutrophils, as well as those produced by GBS, both presumably contribute to the observed lung injury. Two such bacterial products that appear to be important in GBS pathogenesis are a hyaluronidase and an oligopeptidase. These two enzymes are the primary focus of this dissertation (Fig. 2).

GBS "collagenase" and GBS oligopeptidase

Collagen is one of most abundant proteins in mammalian tissues. It maintains tissue structure and strength. Collagen is an unusual polypeptide with nearly every third amino acid residue being a glycine. Also, the sequence glycine-proline-hydroxyproline occurs very frequently. Collagen fibrils are heterogeneous macromolecules in mammalian tissues where 19 different types of collagen can be found (68). Collagen can be degraded by enzymes called collagenases. The prototypical collagenase cleaves the triple helix region of the native form of collagen. Other collagenases have somewhat different specificity. Methods for measuring collagenase activity were originally very time-consuming and cumbersome. Later, more convenient collagenase assays were developed which used synthetic peptides as substrates. One frequently employed assay uses 2-furanacryloyl-Leu-Gly-Pro-Ala (FALGPA) (76). The 2-furanacryloyl group is thought to have structural similarity with the proline in a native collagen molecule. So, FALGPA is considered to be a peptide analog of collagen. Enzyme activity is routinely measured in

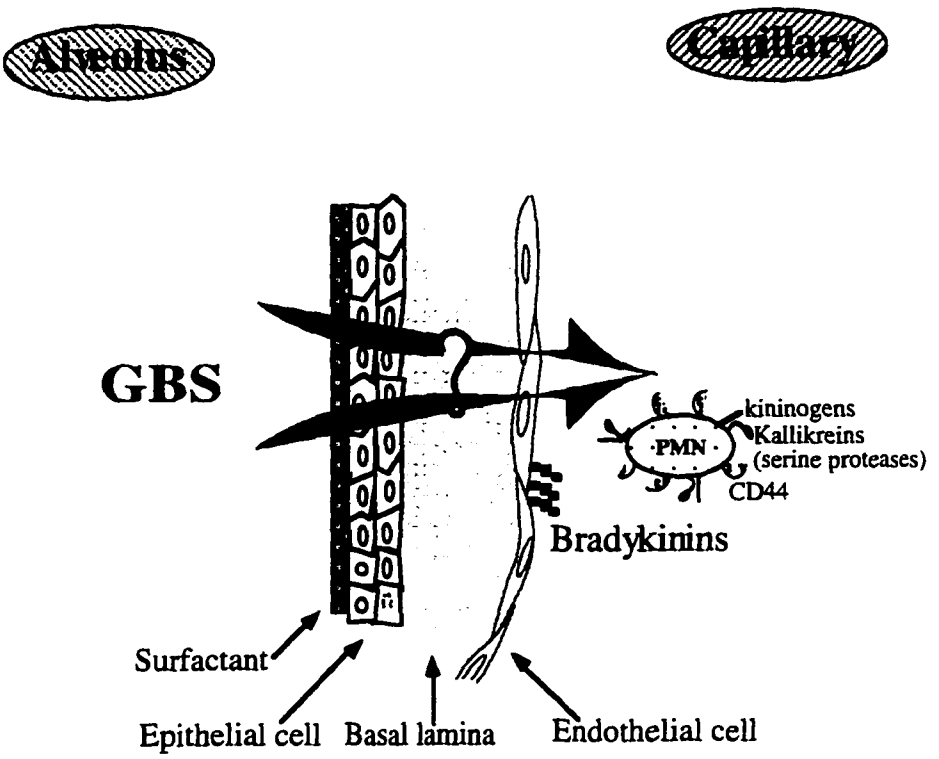


FIG. 2. Group B streptococcal invasion and damage to pulmonary tissues.

a recording spectrophotometer at a wave length of 324 nm. The cleavage of the peptide by collagenase results in a decrease in the absorbance. However, FALGPA hydrolysis is not completely specific for collagenases. Some collagenases do not hydrolyze FALGPA. For example, Bond et al. reported that certain *C. histolyticum* collagenases were unable to hydrolyze FALGPA but did degrade native collagen (9). More important, some enzymes that degrade FALGPA are not collagenases. In a study of *Treponema denticola,* a pathogenic oral spirochete, Makinen isolated a 62 kDa endopeptidase that had very high activity on FALGPA, but did not exhibit any collagenase or gelatinase activity (46).

A so-called GBS collagenase was recently described in a paper by Jackson et al. based primarily on the ability of the enzyme to hydrolyze FALGPA. The authors speculated that the enzyme contributed to the premature rupture of amniotic membranes and to neonatal GBS infections (34). However, our studies showed that neither intact GBS cells nor cell lysates could hydrolyze native or denatured collagen. We therefore decided to investigate the exact specificity of the enzyme on natural substrates. This led to our discovery that the GBS enzyme was actually an oligopeptidase.

Oligopeptidases of various specificities are widely distributed in mammals and in microorganisms (62). Recently, many different bacteria have been shown to produce oligopeptidases. Some of these bacteria are human pathogens. The biological roles of these oligopeptidases are not always obvious. Clearly, they are capable of degrading peptides but they often exhibit striking specificity for certain peptides. We have shown that the GBS oligopeptidase, for example, is highly specific for certain human bioactive peptides involved in normal immune responses. One of these bioactive peptides is

bradykinin (BK), a member of a group peptides generated from kininogens (35). BK can influence a number of biological processes, including blood flow, increase vascular permeability, and smooth muscle contraction (8). It also stimulates the secretion of various secondary immune mediators, such as platelet-activating factor, prostaglandins, substance P (neurogenic inflammation), acetylcholine, and noradrenaline (sympathetic nerves) (8). Kinins can also stimulate monocytes to synthesize the inflammatory cytokines IL-1, and TNF-α (75). As an inflammatory mediator, BK can mediate the migration of neutrophils through the epithelial gap junction. Recently, Maeda et al. reported that bradykinin directly facilitates the systemic dissemination of the pathogens *Vibrio vulnificus* and *Pseudomonase aeruginosa* (45).

Various oligopeptidases are co-localized with kininogens and kallikreins to regulate BK homeostasis *in vivo* (17). The half life of BK is about 30 seconds in human blood (52). It might therefore be expected that any disruption BK homeostasis caused by exogenous bacterial enzymes might have serious consequences. The hydrolysis of BK and other bioactive peptides by GBS oligopeptidase may result in the down regulation of normal immune responses against the bacteria, and therefore enhance their chances of survival on mucosal surfaces. It is also possible that degradation of bioactive peptides by bacterial oligopeptidases might stimulate synthesis of the peptides and this in turn may lead to deleterious consequences. An improved understanding of these processes may eventually lead to chemotherapeutic approaches to specifically inhibiting long-term colonization by GBS and other pathogenic bacteria.

GBS "neuraminidase" and GBS hyaluronate lyase

GBS "neuraminidase". Neuraminidases are widely distributed in the bacterial kingdom. These enzymes remove sialic acid from sialo-glycoconjugates (77). In some cases, removal of terminal sialic acid residues may create receptors for pathogen colonization and invasion (24). In other cases, it may allow bacteria to escape immune defenses by destroying receptors on host phagocytes (13, 64). GBS neuraminidase was first described in 1969 by Hayano and Tanaka and Okuyama (28). They reported that neuraminidase activity was present in streptococci of groups A, B, C, G, and L, as well as in *Streptococcus sanguis*. Milligan and coworkers subsequently demonstrated that the GBS enzyme is highly specific for type I bovine submaxillary mucin prepared by Sigma Chemical Company (57). In studies involving more than 300 clinical GBS strains, Milligan and Mattingly revealed that all types I and II strains had low enzymatic activity. Type III GBS strains, however, could be classified into two groups, high enzyme producers and non-producers (56). They also showed that high neuraminidase activity was more frequently found in serotype III GBS isolated from infected infants compared to asymptotically colonized infants. They therefore speculated that "neuraminidase" is a virulence factor for type III GBS. However, we subsequently found that the enzyme had been completely misidentified and was really a hyaluronate lyase.

GBS hyaluronate lyase. Hyaluronidases are a group of enzymes capable of degrading hyaluronan (hyaluronic acid, HA). These enzymes are widely distributed in microorganisms and animals (40). The first report describing what we now know to be hyaluronidase activity was made in 1933 (16). A so-called "spreading factor" was

detected in the culture filtrates of various bacteria. It caused the spreading of india ink injected just under the skin of rabbits. Since then, hyaluronidases have been identified from microorganisms, animal venoms, and mammalian tissues. The hyaluronidases present in venom are thought to facilitate the diffusion of venom components in the tissues of the victim. Hyaluronidase activity has been found in venom isolated from snakes, fish, bees, wasps, scorpions, and spiders. One of them, bee venom hyaluronidase, has been characterized and cloned (27). Hyaluronidase activity is also found in various mammalian tissues including testes, liver, and serum. A hyaluronidase from mammalian sperm, PH-20, was first identified as a surface protein (61). It showed 36 % amino acid sequence identity with a hyaluronidase isolated from bee venom. PH-20 is capable of degrading the hyaluronan surrounding a mammalian egg (43). Other mammalian hyaluronidases appear to play an essential role in the metabolism of hyaluronan (42).

In 1941, McClean first documented that hyaluronidase activity could be detected in the culture supernatant obtained from certain GBS strains. At that time, it was suggested that the invasive properties of bacteria may be associated with hyaluronidase production (53).

Kjems and coworker confirmed that GBS produced hyaluronidase (37). In 1980, they reported that hyaluronidase-positive GBS were more frequently recovered from infants who had serious infections. It was recently reported that GAS may contain up to three different hyaluronidases. Two of them are bacteriophage associated (32).

Mutagenesis studies of the GAS bacteriophage hyaluronidases suggested that an additional hyaluronidase might also be present on the chromosomal DNA of GAS (33).

Staphylococcus aureus also has been reported to produce hyaluronidase (20). This organism is a gram-positive coccus that normally colonizes the human nose, vagina, skin, and colon. Invasive infections may cause serious diseases including septicemia, meningitis, and wound infections. *Streptococcus pneumoniae* is another human pathogen that produces hyaluronidase (6). It normally colonizes the nasopharynx but is also capable of causing systemic infections including bacteremia and meningitis. All these various bacterial hyaluronidases appear to act as virulence factors and probably are involved in pathogenesis.

Hyaluronic acid (HA, hyaluronan). The substrate of hyaluronidase is hyaluronic acid (HA) or hyaluronan. It is a major component of the extracellular matrix (ECM) of tissues and is usually present along with other proteoglycans and collagen. HA is ubiquitous in the tissues of animals and in various fluids, such as the vitreous of the eye. HA is a polysaccharide with a very high molecular mass of from 100 - 5,000 kDa. Although it is a glycosaminoglycan it is not covalently linked to protein. HA is composed of disaccharide-repeating units consisting of D-glucuronic acid (β1-3)N-acetylglucosamine (β1-4). The polymer forms an expanded random coil in solution. It binds to a large amount of water to form a viscous hydrated gel. Cations associated with the carboxylate groups of HA result in increased osmotic pressure. These physical and chemical properties of HA are essential for homeostasis of the extracellular space.

In addition to its role as a structural component of the ECM, hyaluronan also has several other important biological functions including a role in egg fertilization, embryonic development, cell proliferation, cell differentiation, wound healing, tumor cell growth, and inflammation (42). In many of these functions the interaction between HA and specific protein receptors is critical. CD44, for example, is a member of a group of HA-binding proteins that has been extensively studied. It is expressed in a variety of isoforms on different cell types, including T cells, B cells, macrophages, granulocytes, fibroblasts and epithelial cells (74). Data from several *in vitro* experiments reveal that the interaction of CD44 and hyaluronan is important for selective migration of lymphocytes to human tissues (2). It seems very likely that GBS hyaluronate lyase will interfere with some of these processes. For example, GBS hyaluronate lyase may inhibit neutrophil adherence to capillary endothelial cells because the GBS enzyme will degrade HA on the endothelial cells. This, in turn, may interfere with the normal cascade of inflammatory responses.

Dissertation studies

A common characteristic of bacterial pathogens is their ability to survive and multiply in an infected host. Serious infections are often associated with pathogens that can systemically invade and be widely disseminated in host tissues. It appears that successful pathogens usually produce numerous products (virulence factors) that contribute to pathogenesis. Two factors produced by GBS are strong candidates for important roles in virulence, the hyaluronate lyase and an oligopeptidase. These two enzymes are the subjects of the biochemical and molecular genetic studies described in this dissertation.

CHARACTERIZATION OF PEPB , A GROUP B STREPTOCOCCAL OLIGOPEPTIDASE

BO LIN, WILLIAM F. AVERETT, JAN NOVAK,
W. WINN CHATHAM, SUSAN K. HOLLINGSHEAD, JOHN E. COLIGAN,
MARIANNE L. EGAN, AND DAVID G. PRITCHARD

Based upon Infection and Immunity (1996)
64, 3401-3406

ABSTRACT

Group B streptococci were recently reported to possess a cell-associated collagenase. Although the enzyme hydrolyzed the synthetic collagen-like substrate N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala, we found that neither the highly purified enzyme nor crude GBS cell lysate solubilized a film of reconstituted rat tail collagen, an activity regarded as obligatory for a true collagenase. We cloned and sequenced the gene for the enzyme (*pepB*). The deduced amino acid sequence showed 66.4% identity to PepF oligopeptidase from *Lactococcus lactis*, a member of the M3 or thimet family of zinc metallopeptidases. The group B streptococcal enzyme also showed oligopeptidase activity and degraded a variety of small bioactive peptides, including bradykinin, neurotensin, and peptide fragments of substance P and adrenocorticotropin.

INTRODUCTION

Group B streptococci (GBS) are the most frequent cause of serious, often fatal, bacterial infections of neonates in the United States and, in addition, are a common cause of peripartum maternal sepsis (19). Although GBS are harmless commensals most of the time, the organism clearly possesses the capability to systemically invade a susceptible human host and cause life-threatening infections. This ability appears to be due, at least in part, to the production of a variety of degradative enzymes that facilitate systemic invasion. We have extensively studied one such enzyme of GBS, a secreted hyaluronidase (9, 12) that is capable of breaking down hyaluronic acid, a major component of the extracellular matrix of many tissues.

The recent report by Jackson et al. (8) of a collagenase produced by GBS appeared to be yet another example of the production of an enzyme of GBS capable of

degrading a component of the extracellular matrix and, possibly, facilitating invasion. The investigators speculated that the enzyme might contribute to the premature rupture of membranes frequently associated with neonatal infections. Jackson et al. (8) assayed the collagenase activity of GBS by using the synthetic substrate N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA), which is hydrolyzed by all known collagenases and is thought to mimic the primary structure of collagen. We decided to isolate and purify the enzyme and further characterize it.

MATERIALS AND METHODS

Bacterial strains. Serotype III GBS strain 3502 was isolated from the blood of an infected infant. It was grown in a fermentor as described previously (12) using the chemically defined medium (CDM) described by van de Rijn and Kessler (17) purchased from Hazleton Research Products, Inc., (Denver, Penn). *Escherichia coli* strains XL-1 blue MRF' and XLOLR were purchased from Stratagene corp. (La Jolla, Calif.). *E. coli* INVαF' competent cells were obtained from Invitrogen Corp. (San Diego, Calif.). *E. coli* strains were grown in LB medium or on LB agar plates. Kanamycin (Sigma Chemical Co, St. Louis, Mo.) (50 μg/ml) was added to select for transformants.

Enzyme assays. The rate of hydrolysis of the synthetic collagenase substrate FALGPA (N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala) (Sigma) was measured by a modification of a procedure described by van Wart and Steinbrink (18). A 10 μl sample was added to 300 μl of an enzyme assay mixture composed of 0.45 mg/ml FALGPA, 0.24 M NaCl, 50 mM imidazole, 12 mM $CaCl_2$, and 2.4 mM $ZnCl_2$, pH 7.0. The rate of decrease in absorbance at 345 nm was followed in a recording spectrophotometer at room temperature.

Gelatinase activity was estimated using an agarose plate assay. Samples (10 µl) were placed in wells cut in a 1% agarose gel containing 0.1% gelatin, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5, and 10 mM $CaCl_2$ and incubated overnight at room temperature. The gel was then stained for 20 min with 0.1% Coomassie Blue and destained in water on a rotary shaker. Gelatinase activity was revealed as a clear zone on a blue background.

Collagenase activity was assessed essentially as described by Birkedal-Hansen and Taylor (4). Acid-soluble rat tail tendon collagen (25 mg, Sigma) dissolved in 12 ml of 13 mM HCl was neutralized with 3 ml of 0.3 M HEPES buffer, pH 7.5, poured onto a 10 by 15 cm agarose-coated polyester sheet (Gelbond, FMC BioProducts, Rockland, Maine) and allowed to gel. The collagen gel was dried overnight at room temperature. It was then rinsed under running water for 1 min and again air dried. Test samples (10 µl) were spotted onto the collagen fibril film and incubated for 2 to 16 h in a humid atmosphere. The film was then stained for 20 min in 0.1% Coomassie Blue and rinsed in water. Collagenase activity yielded a clear zone on a dark blue background.

Enzyme purification. GBS cells from a log phase culture (A_{650nm} = 0.8) were harvested by centrifugation, washed in 0.1 M NaCl, and disrupted using a continuous flow cell breaker operated at 15,000 psi. The cell lysate was centrifuged at 11,000 X g for 1 h, and proteins in the supernate were precipitated with 80% saturated ammonium sulfate overnight at 4°C. After centrifugation, the pellet was redissolved in 50 mM imidazole buffer, pH 7.0. Ammonium sulfate was removed by passing the mixture through a column (5 by 25 cm) of Bio-Gel P2 (Bio-Rad, Richmond, Calif.). Material in the void volume was applied to a column (2.5 by 22 cm) of DEAE-Sephacel (Pharmacia

LKB Biotechnology, Inc., Piscataway, N.J.) equilibrated with 50 mM imidazole buffer, pH 7.0. The column was washed with the buffer, and adsorbed enzyme was eluted with a 0 to 1.0 M NaCl gradient in the same buffer. FALGPA-hydrolyzing activity of the fractions was measured by a modification of a procedure described by Van Wart and Steinbrink (18). Fractions containing enzyme activity were pooled and the NaCl concentration adjusted to 2.0 M. This mixture was loaded onto a 1.75 X 20 cm Phenyl Sepharose (Pharmacia) column that had been equilibrated with 2.0 M NaCl in 50 mM imidazole buffer. After washing with 120 ml of the same buffer, the enzyme was eluted with a linear decreasing gradient of 2.0 M to 0 M NaCl in 50 mM imidazole buffer, pH 7.0. The active fractions were pooled and concentrated using a Centricon 30 ultrafiltration device (Amicon Inc., Beverly, Mass.). Final purification of the GBS enzyme was carried out using a Bio-CAD Perfusion Chromatography Workstation (Perseptive Biosystems Inc. Cambridge, Mass.). Briefly, 0.5 ml of the concentrated partial purified enzyme was absorbed on to a Bio-CAD DEAE anion-exchange chromatography column and eluted using a gradient from 0.4 M to 1.0 M NaCl in 50 mM Tris-HCl pH 7.4 buffer. The peak containing the enzyme activity was analyzed by SDS-PAGE under reducing conditions (2).

Amino acid sequence determinations. N-Terminal amino acid sequencing was carried out on protein bands electroblotted to polyvinylidene difluoride membranes following SDS-PAGE as described by Matsudaira (10). Internal amino acid sequences were obtained by digesting 120 μg of purified GBS enzyme with 2 μg of sequencing grade trypsin (Promega Corp., Madison, Wis.) in 75 mM NH_4HCO_3 buffer pH 8.0. After 16 h incubation at 37°C, peptides were separated by reverse phase HPLC on a Hewlett

Packard model 1050 HPLC equipped with a C18 column (Vydak, Hesperia, CA) using a discontinuous acetonitrile gradient in 0.1% trifluoroacetic acid (11). Individual peaks were collected manually. Well-resolved peaks were sequenced using an Applied Biosystems Model 477A protein sequencer with a blot cartridge according to the manufacturer's program, BLOTT-1 (Perkin Elmer, Foster City, Calif.).

PCR amplification of internal gene fragments. Degenerate oligonucleotide primers were obtained from DNA International Corp. (Lake Oswego, Oreg.). The 100 µl PCR reaction mixture consisted of 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 50 pmol of each of the primers. PCR amplifications were done under conditions described previously (6) in which the first three cycles were carried out at 94°C, 45 sec; 37°C, 30 sec; 72°C, 2.5 min, followed by 35 cycles of 94°C, 45 sec; 45°C, 30 sec; 72°C, 2.5 min. PCR amplified fragments were analyzed on 1% agarose gels and cloned into the pCR™ II vector using a TA Cloning Kit (Invitrogen Corp., San Diego, Calif.). Plasmid DNA was isolated using a QIAGEN midiprep kit (Chatsworth, Calif.). The cloned gene fragment was excised from the plasmid by digestion with *Eco*RI. After separation on a low-melting agarose gel, the fragment was purified by phenol-chloroform extraction. It was then labeled with Digoxigenin-11-dUTP by a random primer DNA labeling method (Genius™ system, Boehringer Mannheim Corp., Indianapolis, Ind.).

Cloning procedures. A lambda phage library of GBS 3502 chromosomal DNA fragments constructed previously was used (9). Screening for recombinant phages was carried out using the PCR amplified internal gene fragment as a probe. After secondary screening, *in vivo* excision was performed to convert recombinant phage to pBK-CMV plasmids using the Stratagene protocol.

The single specific primer PCR technique (14) was used to clone the 5' end of the gene. In this method, GBS chromosomal DNA was digested with *Eco*RI and ligated into a similarly cut site in pUC19. After overnight ligation at room temperature, the ligation mixture was used as the template for "Hot Start" PCR amplification with Ampliwax PCR Gem 100 (Perkin-Elmer) in a total volume of 100 μl. The optimized PCR reaction mixture contained 40 pmol of the gene-specific reverse primer, 20 pmol of the pUC19 universal forward primer (F-20), 200 μM dNTPs, 2.5 mM $MgCl_2$, and 50 mM KCl in 20 mM Tris-HCl, pH 8.4 buffer (Gibco BRL, Gaithersburg, Md.). After incubation of the reaction mixture at 80°C for 5 min, 2.5 units of *Taq* polymerase was applied on the solidified wax layer. Initial denaturation at 94°C for 2 min was followed by 35 cycles of 94°C, 30 sec; 58°C, 30 sec; and 72°C, 1.5 min. After remaining at 72°C for 10 min, the samples were stored at 4°C until used. PCR product was cloned into the pCR™ II vector (Invitrogen) and the constructs were transformed into the *E. coli* strain DH5α.

A PCR-amplified fragment containing the full-length gene was cloned into the pCR™ II vector (Invitrogen) and transformed into *E. coli* INVαF' competent cells. Transformants were lysed using a Model 300 Fisher Sonic Dismembrator fitted with a microtip. Approximately 10^8 cells were suspended in 300 μl 50 mM imidazole buffer, pH 7, and sonicated in 10 sec bursts for a total of 60 sec at 30% of maximum power. After centrifugation, supernates were tested for FALGPA-hydrolyzing activity.

Determination of the cleavage sites of peptides. Bradykinin, ile-ser-bradykinin, neurotensin, substance P (2-11), ACTH (1-10), and defensin HNP-2 were obtained from Sigma. Each peptide (100 μg) was incubated with 2 μg of purified enzyme in 50 mM ammonium acetate, 5.0 mM $CaCl_2$, and 5.0 μM $ZnCl_2$, pH 7.0 for 2 h at 37°C.

Digestion was carried out on a smaller scale (10 μg peptide) for defensin HNP-2. Digestion mixtures were analyzed directly using an API III triple quadruple mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) operating in the electrospray mode. Aqueous solutions of the samples were injected into a 10 μl/min flow of 50% acetonitrile in water containing 0.1% formic acid using a syringe pump to deliver the mixture to the electrospray interface.

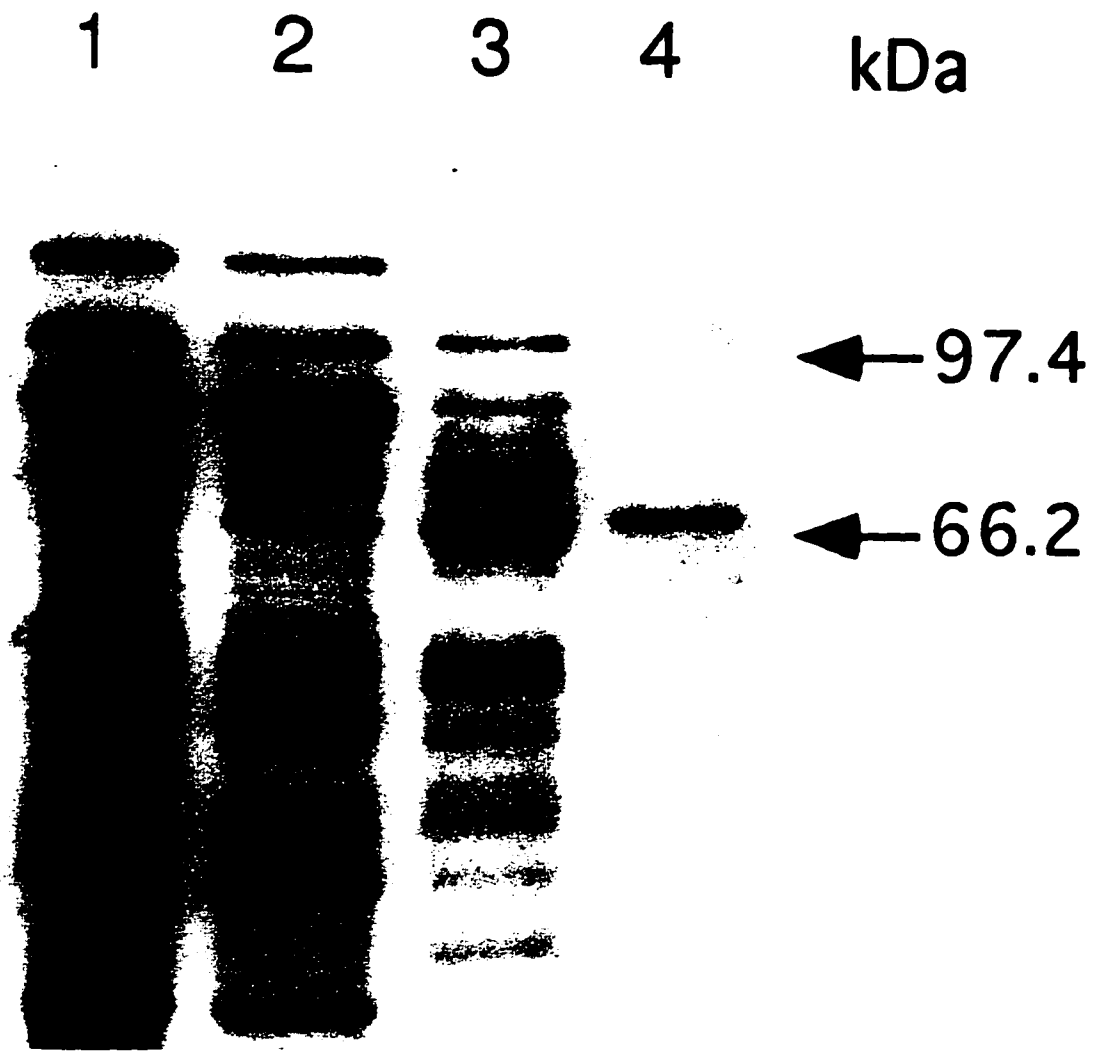
Nucleotide sequence accession number. The nucleotide sequence reported in this paper was submitted to GenBank and has been assigned the accession number U49821.

RESULTS

Enzyme purification. Intact GBS cells were found to exhibit weak FALGPA-hydrolyzing activity, and in agreement with the results reported by Jackson et al. (8), much higher activity was found in cell lysates. Enzyme was therefore purified from the lysate of a cell pellet obtained from a 60 liters of a log-phase culture. Fig. 1 illustrates the progressive purification of FALGPA-hydrolyzing activity by the various chromatographic procedures. The final product gave a single band upon polyacrylamide gel electrophoresis with a molecular weight of approximately 70 kDa.

The pH optimum for FALGPA hydrolysis was found to be 7.4. As reported by Jackson et al., enzyme activity was completely inhibited in the presence of the metal chelators EDTA and 1,10-phenanthroline (10 mM final concentration). A low concentration (2 μM) of Zn^{+2} ions restored activity of purified enzyme preparations and was routinely used in the standard assay mixture. A small increase in enzyme activity was observed in the presence of 10 μM Mn^{+2} or Cu^{+2} ions but the same concentration of

FIG. 1. SDS-PAGE protein profiles of the GBS enzyme preparation at different stages of purification. Lanes: 1, GBS cell lysate; 2, peak of enzyme activity on DEAE-Sephacel column; 3, after Phenyl-Sepharose column; and 4, after purification on a BioCAD. The arrows indicate the migration positions of the mol wt standards, bovine serum albumin (66.2 kDa), and rabbit muscle phosphorylase b (97.4 kDa).

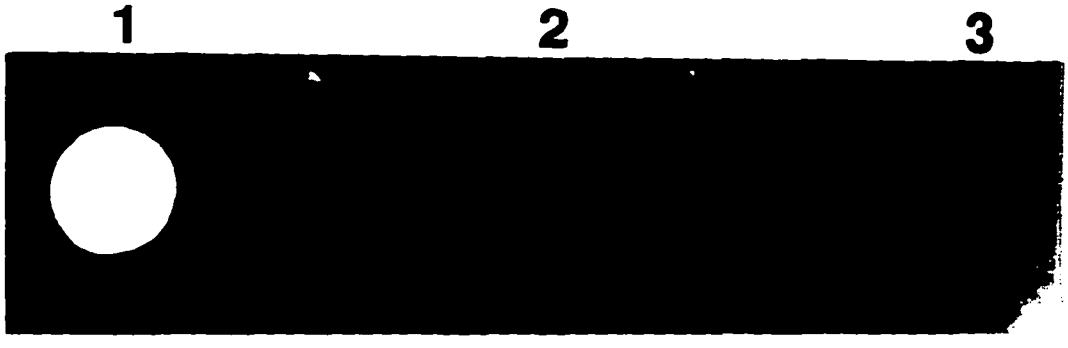


Co^{+2} ions resulted in a large increase in enzyme activity (data not shown). Excess Zn^{+2} ions (10 mM) strongly inhibited, whereas the same concentration of Co^{+2} ions activated the enzyme. We did not observe any increase in enzyme activity in the presence of dithiothreitol (0.2 - 5 mM).

Surprisingly, neither the purified GBS enzyme nor the original cell lysate exhibited any gelatinase activity in our agarose gel assay. This led us to test the ability of the GBS enzyme to solubilize a film of reconstituted collagen fibrils. Fig. 2 shows the results obtained when collagenolytic activity of the purified GBS enzyme and collagenase from *C. histolyticum* were compared. A 100-fold excess of the GBS enzyme (based on FALGPA-hydrolyzing activity) compared to the clostridial collagenase showed no trace of activity on a reconstituted collagen film, even when incubation times were extended to 16 h. This result indicated that the GBS enzyme was not a collagenase. Additional studies were therefore carried out to identify the true specificity of the enzyme.

Cloning and sequencing the gene for the GBS enzyme. The N-terminal amino acid sequence of the purified enzyme, as well as sequences of several tryptic peptides, were determined and compared to sequences in the GenBank data base. All sequences showed high similarity to amino acid sequences present in an oligopeptidase (PepF) isolated from *Lactococcus lactis* (12). Based upon these sequences and a knowledge of their relative positions in the protein, degenerate oligonucleotide primers were designed for use in PCR amplification of internal fragments of the enzyme gene. One pair of degenerate primers (1 and 2) permitted the amplification of a 384 bp fragment which was then cloned and sequenced (PCR #4, Fig. 3). It was capable of coding for a protein fragment that exhibited 58.6% amino acid identity (over 128 amino acid residues)

FIG. 2. Collagenase assay on a film of reconstituted rat tail tendon cartilage. A 10 µl aliquot of *C. histolyticum* collagenase was placed at position 1. Position 2 received 10 µl purified enzyme from GBS with equivalent FALGPA-hydrolyzing activity (measured in arbitrary units), and position 3 received 10 µl of the enzyme from GBS containing 100 times as many units. The film was incubated at 37°C for 2 hrs in a moist environment and stained with Coomassie Blue.



with a sequence in the lactococcal oligopeptidase. The gene fragment was then labeled and used as a probe to screen approximately 10^6 recombinants in a λ phage library of GBS chromosomal DNA fragments. Positive phage clones were obtained and converted to recombinant pBK-CMV plasmids by *in vivo* excision. Clones with different restriction patterns were sequenced. One of them, clone no. 21, which contained a 1.475 kb GBS enzyme gene fragment, was completely sequenced (Fig. 3). Sequence analysis showed that it contained a portion of a single open-reading frame capable of coding for a 422 amino acid segment.

The N-terminal portion of the gene was cloned using the single specific primer technique (14). Chromosomal DNA from GBS strain 3502 was digested with *Eco*RI and ligated into a similarly cut site in pUC19. The ligation mixture served as a template PCR amplification using a gene-specific reverse primer (no. 3) and the universal primer F-20 for pUC19 (Fig. 3). This permitted the PCR amplification of a 3.2 kb fragment, which was then cloned (Plasmid N12) and sequenced. It overlapped 660 bp with plasmid no. 21. Further upstream was a nucleotide sequence capable of coding for an amino acid sequence identical to that obtained by protein sequencing the N-terminus of the purified enzyme.

In order to express the gene for the GBS enzyme in *E. coli*, it was necessary to obtain a clone containing the entire gene. Primers were therefore designed (no. 4 and no. 5 in Fig 3) and used to amplify the gene from GBS chromosomal DNA. A single 1.9 kb PCR product containing the Shine-Dalgarno sequence was obtained and cloned into the pCR™ II vector (plasmid no. 5). After transformation into INVαF' competent cells, transformants were lysed by sonication and tested for enzyme activity to identify those

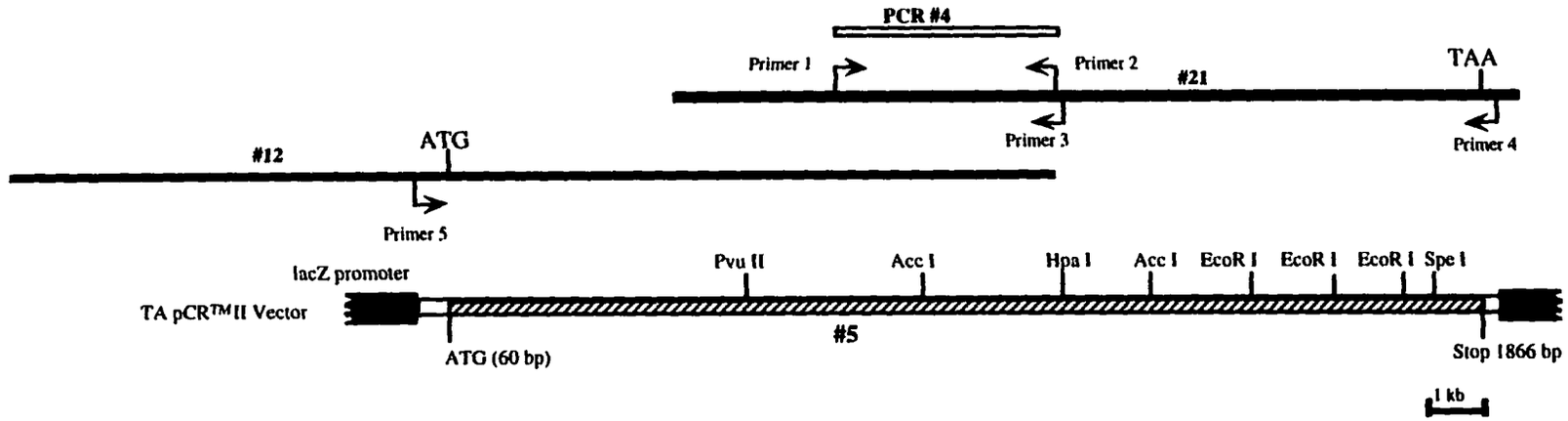


FIG. 3. Diagram of the 1.9 kb region containing the *pepB* gene sequenced in this study. The locations of the PCR amplified gene fragments 4 (used as a probes for library screening) and 12 (containing the 5' portion of the gene) are indicated. A full-length copy of the *pepB* gene was amplified using primers 4 and 5.

possessing a functional gene. A transformant exhibiting very high enzyme activity (no. 5) was selected for further study. The recombinant enzyme was purified from cultures of this clone using the same procedure used to isolate the enzyme from GBS cultures. Like the GBS enzyme, the recombinant enzyme also gave a band upon SDS PAGE with an apparent M_r of 70 kDa (data not shown).

As shown in Fig 4, an open reading frame coding for a 69.6 kDa protein was identified. The deduced amino acid sequence showed 66.4% identity to the oligopeptidase from *L. lactis* for the alignment depicted in Fig. 5. Adjacent to the N-terminus was an ATG-start codon preceded by a putative ribosomal binding sequence GGAGG (Fig. 4) 7 bp before ATG. An inverted repeat, possibly a transcription termination sequence, was observed 23 bp after the stop codon TAA (Fig. 4). A typical Zn-binding motif, His-Glu-X-X-His, was present at amino acid position 385 to 393. An isoelectric point of 4.90 for the GBS enzyme was calculated using the EditSeq program of DNASTAR.

Substrate specificity. The GBS enzyme was tested for its ability to cleave various peptides including several that were reported to be substrates for PepF oligopeptidase from *L. lactis*. The results are summarized in Table 1. Bradykinin, neurotensin, adrenocorticotropic hormone (fragment 1-10), and substance P (fragment 2-11) were all cleaved at the same positions reported for the lactococcal enzyme. However, in two cases there was mass spectroscopic evidence that limited cleavage of some additional peptide bonds had also taken place. In neurotensin, for example, the main cleavage occurred between the two Arg residues but a small amount of cleavage also took place between the Pro and Tyr residues. Similarly, ACTH (fragment 1-10) had a minor

FIG. 4. Features of the nucleotide and deduced amino acid sequences of the gene for GBS oligopeptidase PepB. The N-terminal amino acid sequence of the purified enzyme, as well as sequences of six tryptic peptides, are indicated by dotted underlining. A putative ribosome binding region (SD) is indicated by bold double underlining. An inverted repeat 23 nucleotides after the stop codon (TAA) that may be a transcription termination sequence is indicated by opposing arrows. A typical Zn-binding motif, His-Glu-X-X-His, is indicated in the box.

FIG. 5. Alignment of the amino acid sequence of GBS oligopeptidase (GBS PepB) with that of the oligopeptidase of *Lactococcus lactis* (PepF) illustrating regions of identity. Identical amino acid residues are marked by solid lines. Double dots denote highly conserved amino acids, and single dots indicate moderately conserved amino acids.

Table 1. Substrate specificity of the enzyme from GBS

Substrate	Cleavage Sites[a]
FALGPA	Furanacryloyl-Leu↓-Gly-Pro-Ala
Bradykinin	Arg-Pro-Pro-Gly-Phe↓-Ser-Pro-Phe-Arg
Ile-Ser-Bradykinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe↓-Ser-Pro-Phe-Arg
Neurotensin	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg↓-Arg-Pro↓-Tyr-Ile-Leu
ACTH(1-10)	Ser-Tyr-Ser-Met↓-Glu-His-Phe↓-Arg-Trp-Gly
Substance P (2-11)	Pro-Lys-Pro-Gln-Gln↓-Phe↓-Phe↓-Gly-Leu-Met-NH2
Defensin HNP-2	Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-The-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

[a] Large arrows indicate the primary sites of cleavage, and small arrows indicate minor cleavage sites.

cleavage site between Met and Glu. The 29 amino acid long peptide defensin HNP-2 was not cleaved by the GBS enzyme.

DISCUSSION

A continuous spectrophotometric assay for collagenase based upon the hydrolysis of the synthetic substrate FALGPA was introduced in 1981 (18) and has been widely used by numerous investigators. The method is convenient, rapid, sensitive, and quantitative. However, FALGPA hydrolysis is not completely specific for collagenases. In fact, numerous authors have cautioned against assuming that activity on the synthetic peptide substrate proves that an enzyme is a collagenase (7). The recent report of a GBS collagenase was based primarily on the ability of the GBS enzyme to hydrolyze FALGPA. However, we found that neither the purified enzyme nor a crude cell lysate degraded collagen fibrils, an ability that is regarded as the obligatory criterion of a true collagenase (7). The enzyme also did not exhibit any gelatinase activity.

The high degree of amino acid similarity between the GBS enzyme and oligopeptidase PepF from *L. lactis* prompted us to determine whether the GBS enzyme also displayed oligopeptidase activity. We found that it did cleave a variety of biologically active peptides at exactly the same positions as did the lactococcal enzyme. Like the lactococcal enzyme, the GBS enzyme showed no detectable proteolytic activity. For example, the enzyme did not cleave bovine serum albumin or human complement factor D. The latter molecule was tested because it contained the same LGPA sequence found in FALGPA.

PepF oligopeptidase from *L. lactis* has been placed in the M3 or thimet oligopeptidase family of metallopeptidases (13) and the GBS enzyme almost certainly

belongs in that family. Members of this family exhibit almost identical substrate specificity and also possess highly conserved amino acid sequences around the zinc-binding active site region. The original member of this enzyme family was called thimet oligopeptidase as an acronym for thiol-dependent metallopeptidase (15). The GBS enzyme, however, is not activated by thiols. This, of course, is not surprising since, like PepF oligopeptidase, it contains no cysteine residues. Thimet oligopeptidase cleaves a number of different peptides, including bradykinin and neurotensin, at exactly the same sites that are cleaved by the GBS and lactococcal enzymes (3).

Camargo et al. (5) isolated and characterized endopeptidase 22.19 from rabbit brain which also exhibits similar specificity to that of the GBS enzyme. These authors carried out a detailed study of the structural requirements of bioactive peptides for cleavage by the enzyme. They found that unlike other proteases and peptidases, substrate specificity was not determined by the amino acids flanking the sensitive bonds. Rather, it appeared that the length and flexibility of the peptide were the dominant factors. It was further suggested that the presence of a β-turn in the positively charged peptides may be an important factor for its interaction with the enzyme. Since defensins, abundant bactericidal peptides of neutrophils, are both positively charged and possess β-turns we tested whether they were cleaved by GBS oligopeptidase. A highly sensitive mass spectrometric procedure, however, revealed no evidence of any cleavage of defensin HNP-2.

GBS oligopeptidase is completely inhibited by excess zinc (e.g. 10 mM) as are many other zinc oligopeptidases (1). Like some of these enzymes, the GBS

oligopeptidase is activated by low concentrations (10 μM) of Co^{+2} and is not inhibited by high concentrations (e.g., 10 mM) of the ion.

The biological function of GBS oligopeptidase is not known. If it is involved in the degradation of peptides in its surroundings then the existence of a peptide transport system could be expected. Monnet et al. (11) recently suggested that a report of an oligopeptide transport system in lactococci (16) opens up the possibility that PepF oligopeptidase might be involved in degradation of peptides transported from outside the cell. No such system has yet been identified in GBS. Some authors have speculated that the mammalian peptidases of the M3 family may be biologically important for the conversion and inactivation of bioactive peptides (5). The GBS enzyme is certainly active on many of these peptides, but further studies will be required to determine if this is important in pathogenesis.

ACKNOWLEDGMENTS

This work was supported in part by funds provided by the Office of the Dean of the School of Medicine at the University of Alabama at Birmingham; Grant AI-30634 from the National Institute of Allergy and Infectious Diseases; Public Health Service Program Project Grant HD-17812 from the National Institute of Child Health and Human Development; a grant from the American Heart Association; and funds from the Office of Research and Development, Medical Research Service, Dept. of Veterans Affairs.

Nucleotide sequencing was carried out in the DNA Sequencing Core Facility of the Center for AIDS Research which is supported in part by grant number P30-AI-27767 from the National Institute of Allergy and Infectious Diseases. Electrospray mass spectrometry carried out in this study was performed on an instrument purchased with

funds from a NIH instrumentation grant (S10RR06487). The Mass Spectrometry Shared Facility and the Protein Purification and Microanalysis Facility used in this study are both supported in part by NCI Core research grant P30 CA131148 to the UAB Comprehensive Cancer Center.

REFERENCES

1. **Auld, D. S.** 1995. Removal and replacement of metal ions in metallopeptidases. Methods Enzymol. **248:** 228-242.

2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, pp. 10.2.4-10.2.9, Green Publishing Associates and Wiley-Interscience, New York,

3. **Barrett, A. J., M. A. Brown, P. M. Dando, C. G. Knight, N. McKie, N. D. Rawlings, and A. Serizawa.** 1995. Thimet oligopeptidase and oligopeptidase M or neurolysin. Methods Enzymol. **248:** 529-556.

4. **Birkedal-Hansen, H., and R. E. Taylor.** 1982. Detergent-activation of latent collagenase and resolution of its component molecules. Biochem. Biophys. Res. Commun. **107**: 1173-1178.

5. **Camargo, A. C. M., M. D. Gomes, O. Toffoletto, M. J. F. Ribeiro, E. S. Ferro, B. L. Fernandes, K. Suzuki, Y. Sasaki, and L. Juliano.** 1994. Structural requirements of bioactive peptides for interaction with endopeptidase 22.19. Neuropeptides. **26:** 281-287.

6. **Compton, T.** 1990. Degenerate primers for DNA amplification, p. 39-45. *In* PCR Protocols: a guide to methods and applications. Academic Press, San Diego, CA.

7. **Grubb, J. D.** 1994. Assays for bacterial type I collagenases. Methods Enzymol. **235:** 594-602.

8. **Jackson, R. J., M. L. Dao, and D. V. Lim.** 1994. Cell-associated collagenolytic activity by group B streptococci. Infect. Immun. **62:** 5647-5651.

9. **Lin, B., S. K. Hollingshead, J. E. Coligan, M. L. Egan, J. R. Baker, and D. G. Pritchard.** 1994. Cloning and expression of the gene for group B streptococcal hyaluronate lyase. J. Biol. Chem. **269:** 30113-30116.

10. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:** 10035-10038.

11. **Monnet, V., M. Nardi, A. Chopin, M.-C. Chopin, and J.-C. Gripon.** 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. J. Biol. Chem. **269**: 32070-32076.

12. **Pritchard, D. G., B. Lin, T. R. Willingham, and J. R. Baker.** 1994. Characterization of the group B streptococcal hyaluronate lyase. Arch. Biochem. Biophys. **315:** 431-437.

13. **Rawlings, N. D., and A. J. Barrett.** 1995. Evolutionary families of metallopeptidases. Methods Enzymol. **248:** 183-228.

14. **Shyamala, V., and G. F.-L. Ames.** 1993. Genome walking by single specific primer-polymerase chain reaction. Methods Enzymol. **217:** 436-446.

15. **Tisljar, U., and A. J. Barrett.** 1990. Thiol-dependent metallo-endopeptidase characteristics of Pz-peptidase in rat and rabbit. Biochem. J. **267:** 531-533.

16. **Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman.** 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. **175:** 7523-7532.

17. **van de Rijn, I., and R. E. Kessler.** 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. **27:** 444-448.

18. **Van Wart, H. E., and D. R. Steinbrink.** 1981. A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Anal. Biochem. **113:** 356-365.

19. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morbid. Mortal. Weekly Report. **41:** 25-32.

GROUP B STREPTOCOCCAL NEURAMINIDASE IS ACTUALLY A HYALURONIDASE

DAVID G. PRITCHARD AND BO LIN

Infection and Immunity (1993)
61, 3234-3239

Format adapted for dissertation and errata corrected

ABSTRACT

The extracellular group B streptococcal enzyme described in numerous reports as a neuraminidase is really a hyaluronidase. Over the past 25 years, the enzyme was routinely assayed with bovine submaxillary mucin as the substrate and by the thiobarbituric acid procedure to measure released sialic acid. Characterization of the actual compound released by the enzyme revealed it to be an α, β-unsaturated derivative of hyalobiuronic acid that was derived from hyaluronic acid contaminating the mucin preparation. Previous reports describing an association of elevated levels of extracellular neuraminidase with virulent strains of group B streptococci must be re-evaluated with the recognition that the enzyme is really a hyaluronidase.

INTRODUCTION

A recent epidemiological study of the incidence of group B streptococcal infections in the United States (30) estimated that >15,000 cases and >1,300 deaths due to these infections occur annually. They are the most common cause of neonatal meningitis and sepsis in this country. Several different extracellular products of GBS have been associated with the virulence of the organism. One of these, reported to be a neuraminidase (sialidase), was first described by Hayano and Tanaka (10) in 1969. Similar enzymes were found in streptococcal groups A, B, C, G, and L and in *Streptococcus sanguis*. In that report, as well as in all subsequent studies of the GBS neuraminidase, bovine submaxillary mucin was used as the enzyme substrate. Hayano and Tanaka prepared bovine submaxillary mucin by two different methods and found that one preparation was a much better substrate for the various neuraminidases than the

other. They also studied the nature of the product released by the enzyme and concluded that it was a sialic acid derivative but probably not N-acetylneuraminic acid.

In a study of 74 clinical isolates GBS, Milligan and coworkers (17) reported that serotype III strains of GBS isolated from diseased infants were more likely to produce elevated levels of neuraminidase than serotype III strains from asymptomatically colonized infants or strains of other serotypes. The authors suggested that the ability of certain serotype III strains to produce elevated levels of neuraminidase might be related to the observed high frequency of neonatal infections caused by this serotype. The association of high levels of neuraminidase activity with serotype III isolates was confirmed in a later study (16). Subsequently Milligan and coworkers (18) described the purification and partial characterization of the neuraminidase from serotype III GBS. They reported that the enzyme displayed a very limited substrate specificity, only releasing sialic acid from bovine submaxillary mucin. The authors described a purification procedure involving ammonium sulfate fractionation, affinity chromatography on Affi-Gel blue to remove previously added albumin, ion-exchange chromatography on DEAE cellulose, and gel filtration on Sephacryl S-200. The enzyme had a molecular mass of approximately 105 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), higher than most previously reported bacterial neuraminidases. Brown and Straus (4) compared the neuraminidases produced by various serotypes of GBS. They found that neuraminidases from the different serotypes were immunologically similar but that their molecular masses ranged from 110 to 180 kDa. They also found that regardless of serotype, all neuraminidases tested were active only on bovine submaxillary mucin.

As part of our continuing interest in the virulence factors of GBS, we were interested in determining whether neuraminidase production is subject to phase variation. To facilitate these studies, we prepared to clone the gene for the GBS neuraminidase. Several observations made at the beginning of the project prompted us to re-examine the actual substrate specificity of the enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Serotype III GBS strain 3502, isolated from the blood of an infected infant, was found to produce high levels of the putative neuraminidase, in comparision with several other clinical isolates tested in the mucin assay described below. Strain 1733, also serotype III, was a vaginal isolate from an asymptomatically colonized woman. This strain did not show any enzyme activity and was used as a control strain. Bacteria were grown in the chemically defined medium (CDM) described by van de Rijn and Kessler (28) (purchased from Hazleton Research Products, Inc., Denver, PA) at 37°C without agitation or aeration.

Substrates and reagents. Type I and type IS bovine submaxillary mucins were obtained from Sigma Chemical Co., St. Louis, MO 63178. *Vibrio cholerae* neuraminidase, human umbilical cord hyaluronic acid, fetuin, colominic acid, gangliosides, 3'- and 6'-N-acetylneuramin-lactose, N-acetylneuraminic acid, and other monosaccharides were obtained from Sigma. Type I mucin was exhaustively treated with *Vibrio cholerae* neuraminidase to remove all susceptible sialic acid residues and then fractionated to yield a substrate that reacted specifically with the GBS enzyme. This was accomplished by first de-O-acetylating 300 mg of type I bovine submaxillary mucin in 0.1 N NaOH for 45 min at 0°C as described by Varki and Diaz (29). The pH of the mixture was adjusted to

4.7, and insoluble material was removed by centrifugation. The mixture was then brought to pH 6.0 and was exhaustively treated with an excess of *Vibrio cholerae* neuraminidase (0.5 units for 16 hrs at 37°C). The digestion mixture was passed through a Bio-Gel P-2 column (2.5 by 50 cm; Bio-Rad Laboratories, Hercules, Calif.) to separate high-molecular weight material from released sialic acid. Fractions were analyzed for protein and for free sialic acid. The high-molecular weight material was fractionated by anion-exchange chromatography on a DEAE-Sephacel column (1.5 by 15 cm; Pharmacia LKB Biotechnology, Piscataway, N.J.). The column was eluted with a NaCl gradient (0 to 1.0 M NaCl in 50 mM imidazole HCl, pH 6.0). Fractions were dialyzed to remove salts and were analyzed by the thiobarbituric acid assay following either acid hydrolysis or treatment with the GBS enzyme.

Enzyme purification. GBS strain 3502 was grown at 37°C in 4 L of chemically defined medium until the culture reached an A_{650} of 0.8. Most of the cells were removed by centrifugation at 2000 X g for 30 min. The remaining cells were removed by use of a Minitan ultrafiltration cell equipped with a 0.2 μm-pore-size filter packet (Millipore Corp., Bedford, Mass.). High molecular-weight material in the supernate was concentrated by use of the Minitan ultrafiltration cell equipped with a 10,000-molecular-weight-cutoff filter packet. Before use, filter packets were routinely treated with 0.05% Tween 20 in 10 mM sodium phosphate buffer (pH 6.8) for 30 min and then washed with the same buffer without the detergent to reduce nonspecific adsorption of the enzyme. The retained concentrated retentate (200 ml) was washed with 5 volumes of 10 mM sodium phosphate buffer (pH 6.8) by use of the ultrafiltration cell in the constant-volume mode. A small portion of the retentate was saved for analysis, while the remainder was

passed through a DEAE-Sephacel column (1.5 by 13 cm) that was previously deactivated with Tween 20; elution was down with 10 mM phosphate buffer (pH 6.8). Fractions possessing enzyme activity were pooled and passed through an affinity column (6) that contained 5 ml of N-(p-aminophenyl)oxamic acid linked to agarose (Sigma) and that was also previously deactivated with Tween 20. The column was washed with 30 ml of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5). The bound enzyme was eluted with 50 mM sodium carbonate buffer (pH 9.0), and 0.5-ml fractions were collected in tubes containing 50 μl of sodium acetate buffer (pH 5.6) as described by Den et al. (6).

Analytical procedures. Enzyme assay mixtures consisted of 100 μl of 2 mg of type 1 bovine submaxillary mucin per ml, 20 μl of 10 X buffer (0.5 M ammonium acetate buffer [pH 6.5], 0.1 M $CaCl_2$), the enzyme diluted in 2 mg of bovine serum albumin per ml, and water to make a final total volume of 200 μl. The enzyme assays were carried out for exactly 5 min at 37°C, and the reactions were terminated by placing the tubes briefly in a boiling water bath. Tubes were assayed by the improved thiobarbituric acid assay described by Skoza and Mohos (26). Sialic acid was also released from mucin by acid hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 hr.

Protein content of column fractions was estimated by the Bradford method (3) and dye reagent supplied by Bio-Rad. The glucuronic acid content of fractions was determined by the procedure described by Bitter and Muir (2).

PAGE was carried out by a modification of the basic Laemmli procedure (1). For eventual recovery of enzyme activity, SDS gels were run in the absence of mercaptoethanol and without heating the samples in a boiling water bath prior to loading.

Gels were electroblotted to polyvinylidene difluoride membranes for direct sequencing as described by Matsudaira (15). Protein sequencing was carried out on a model PI 2090E gas-phase microsequencer (Porton Instruments, Tarzana, Calif.).

Thin-layer chromatography was done with the two solvent systems recommended by Schauer (25). Cellulose plates, obtained from EM Science, Gibbstown, N.J., were run in *n*-butanol-*n*-propanol-0.1 N HCl (1:2:1, [vol/vol/vol]) (solvent A), and silica gel plates, also obtained from EM Science, were run in n-propanol-water (7:3, [vol/vol]) (solvent B). After development, the plates were dried and 0.5-cm bands of the adsorbent layer were scraped off the plates, eluted with water, and the effluents were assayed by the thiobarbituric acid assay.

Routine gas chromatographic analysis was carried out on a model 5890 Hewlett Packard gas chromatograph equipped with a wide-bore fused-silica capillary column (30 m by 0.53 mm) coated with an 0.88 mm film of HP-1. Methanolysis of samples was done with Teflon-capped glass vials at 80°C for 16 h. Methanolic HCl was prepared by passing dry HCl gas through methanol until the HCl concentration was 1.5 M, as determined by titration with a standard base. Re-N-acetylation of the amino sugars was carried out as described by Etchison and Holland (8). Trimethylsilylation of the samples was carried out using the Tri-Sil reagent supplied by Pierce Chemical Co., Rockford, Ill.

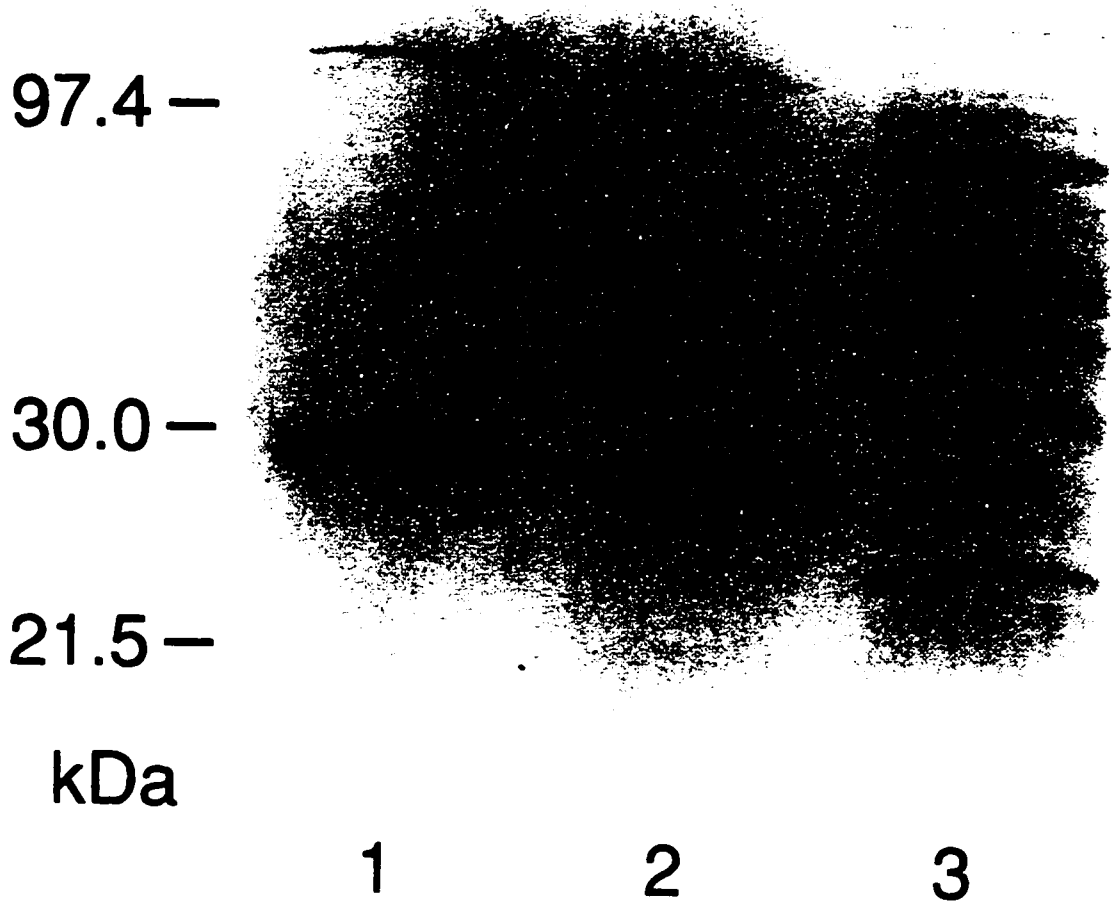
Mass spectrometry was carried out on a model 5985A Hewlett-Packard combined gas chromatography-mass spectrometry system with a 2-m packed column of 2% SP2250 on Supelcoport (Supelco, Inc., Bellefonte, Pa.). Electron impact mass spectra were obtained at an ionizing voltage of 70 eV.

RESULTS

Enzyme purification. GBS strain 3502, a serotype III strain that produced high levels of the enzyme in chemically defined medium, was grown to mid-log phase, and the high-molecular-weight material released into the culture medium was recovered by ultrafiltration. The enzyme was partially purified by DEAE-Sephacel chromatography as described by Milligan et al. (18). As reported by these authors, this step resulted in a major purification, since the enzyme was not bound to the column whereas most contaminating proteins were retained. The results of PAGE of the initial retained material and the enzyme preparation after passage through a DEAE-Sephacel column are shown in Fig. 1.

We found that we could further purify the enzyme by affinity chromatography on a column designed to bind neuraminidases; this column contained N-(p-aminophenyl)oxamic acid linked to agarose. Cuatrecasas and Illiano (5) designed this affinity chromatography support on the basis of the observation that N-substituted oxamic acids are potent reversible inhibitors of influenza virus neuraminidases (7). We eluted the bound enzyme with pH 9.0 buffer. As shown in Fig. 1, PAGE of the bound material yielded two intense bands, one at about 100 kDa and the other at about 25 kDa. An unstained gel was then electroblotted to a polyvinylidene difluoride membrane, which was cut into strips and assayed for enzyme activity. Both bands possessed enzyme activity, although the high-molecular-weight band was by far the most active. Coomassie Blue-stained bands from another electroblotted polyvinylidene difluoride membrane were excised and subjected to gas phase protein sequencing. The N-terminal sequence of the first 16 amino acids of the low molecular-weight band was determined to be

FIG. 1. SDS-PAGE protein profiles of the group B streptococcal enzyme preparation at different stages of purification. Lane 3, the high molecular-weight material present in the culture supernate after concentration by ultrafiltration. Lane 2, the enzyme preparation after passage through a DEAE-Sephacel column to remove most other proteins. Lane 1, material bound to the affinity column of N-(p-aminophenyl)oxamic acid linked to agarose and eluted with 50 mM Na_2CO_3, pH 9.



DQVTTPQVVNHVNSNN. This sequence exactly matched that reported for the N-terminus of the GBS CAMP factor (23). The high-molecular-weight band, which accounted for the bulk of the enzyme activity, failed to directly yield an N-terminal sequence.

Substrate specificity. In agreement with previous reports, we found that only bovine submaxillary mucin was a suitable substrate for the GBS enzyme. No color was obtained in the thiobarbituric acid assay for released sialic acid when α1-acid glycoprotein, fetuin, colominic acid, gangliosides, human secretory IgA, 3'- or 6'-N-acetylneuramin-lactose, porcine submaxillary mucin, and ovine submaxillary mucin were tested as possible substrates for the enzyme. In addition, the GBS enzyme was not active on 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid, a fluorogenic sialidase substrate effectively used in the recent cloning of several bacterial neuraminidases (11, 12, 22, 24). Furthermore, we found that only Sigma's type I bovine submaxillary mucin, produced by the procedure of Nisizawa and Pigman (20), was active in the assay. Sigma's type IS bovine submaxillary mucin, produced by the procedure of Tettamanti and Pigman (27), produced no color in the assay using the GBS enzyme.

For characterization of the actual substrate of the GBS enzyme, type I bovine submaxillary mucin was exhaustively treated with an excess of *Vibrio cholerae* neuraminidase, and released sialic acid was removed. The high-molecular-weight material was then fractionated by anion-exchange chromatography, and column fractions were analyzed in the thiobarbituric acid assay following either acid hydrolysis or treatment with the GBS enzyme. The results are shown in Fig. 2. Fractions assayed after acid hydrolysis produced a small peak that eluted at a low salt concentration. Surprisingly,

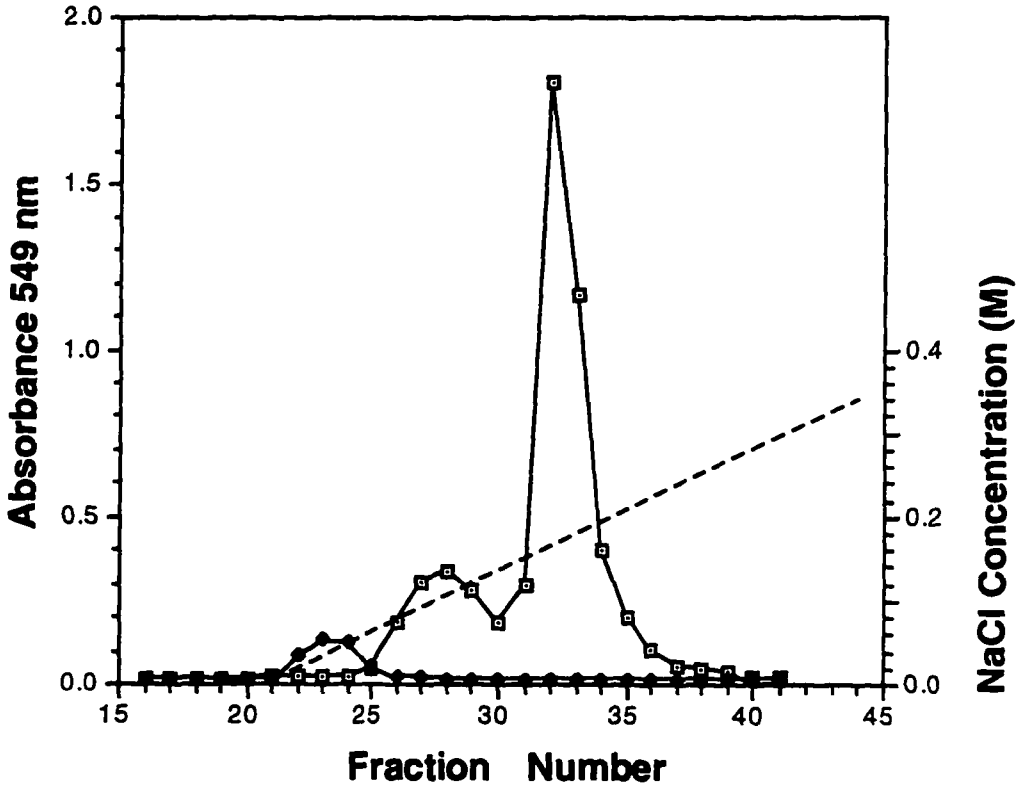


FIG. 2. Anion-exchange chromatography on DEAE-Sephacel of type I bovine submaxillary mucin that was exhaustively treated with *Vibrio cholerae* neuraminidase. The column was eluted with a linear gradient of NaCl (dashed line). Fractions were assayed by the thiobarbituric acid procedure both after acid-hydrolysis (solid diamonds) and enzyme treatment (open squares).

enzyme treatment of the fractions followed by analysis in the thiobarbituric acid assay yielded two peaks eluting at higher salt concentrations; neither of these peaks produced any color in the assay after acid hydrolysis. If the material released by the GBS enzyme was really sialic acid, then it would also have been released by acid hydrolysis and would also have yielded just as much color in the thiobarbituric acid assay. That these results did not occur suggests that the material released by the enzyme was not sialic acid after all.

Characterization of the material released by the GBS enzyme. A portion of the pooled fractions of the largest peak from the above-mentioned DEAE column that contained material susceptible to digestion by the GBS enzyme was exhaustively digested with the enzyme, and the resulting mixture fractionated by gel filtration on a Bio-Gel P-2 column. Column fractions of low-molecular-weight peak of material that produced color in the thiobarbituric acid assay were pooled and passed through a column of AG-50(H^+) (Bio-Red), and the column was then subjected to a water wash. The combined effluents were lyophilized. Like N-acetylneuraminic acid, the unknown material was bound to a DEAE-Sephacel column run in 10 mM pyridine-acetate buffer (pH 5.4) and could be eluted with 0.5 M pyridine-acetate buffer of the same pH. Thin-layer chromatography (TLC) of the unknown in solvent A on cellulose plates yielded an R_f of 0.51, identical to that of N-acetylneuraminic acid. However, TLC of unknown in solvent B on silica gel plates yielded an R_f of 0.67; that of for N-acetylneuraminic is 0.53. Gas-liquid chromatography of the material following methanolysis, re-N-acetylation, and trimethylsilyation only revealed peaks due to N-acetylglucosamine (major peak at 11.94 min). An N-acetylneuraminic acid standard similarly derivatized showed a major peak at

14.79 min. Direct trimethylsilylation of the unknown material yielded a peak eluting very late, at 18.49 min, much later than any standard monosaccharide tested.

Electron impact mass spectrometry of the derivatized unknown compound yielded several very high mass ions characteristic of a disaccharide. This result, the observation that the compound bound to DEAE-Sephacel, and the fact that this compound produced color in the thiobarbituric acid assay suggested to us that the unknown might be an α, β-unsaturated derivative of hyalobiuronic acid. Such a compound was first described by Linker and coworkers (14) in 1956. We therefore tested a pure sample of hyaluronic acid isolated from human umbilical cord as a possible substrate for the GBS enzyme. It proved to be an excellent substrate for the purified GBS enzyme when assayed by the thiobarbituric acid procedure. Furthermore, the product of the GBS enzyme digestion of hyaluronic acid had a retention time and mass spectrum identical to those of the material released from mucin. Only 5.7 μg of hyaluronic acid yielded an A_{549} of 1.00 in the thiobarbituric acid assay, whereas 328 μg of Sigma type 1 bovine submaxillary mucin was required to yield the same absorbance. Finally, an analysis of the type I mucin for glucuronic acid content showed that it was contaminated with approximately 1.86% hyaluronic acid.

DISCUSSION

The above-described results demonstrate that the GBS enzyme previously thought to be a neuraminidase is in reality a hyaluronidase. The enzyme is more precisely termed a hyaluronate lyase since the reaction catalyzed is not a hydrolysis but an elimination; i.e., the glycosidic bond is not broken by the addition of water but is cleaved with the introduction of a double bond. The confusion over the true specificity of the

enzyme has persisted for almost 25 years, in part because the mucin preparation routinely used as a substrate was contaminated with hyaluronic acid. This contaminant resulted in a colored product in the widely used thiobarbituric acid assay for sialic acid. It was well known that 2-deoxyribose also resulted in color in the assay, but 2-deoxyribose had a different absorption spectrum (λmax at 532 nm, unlike that of sialic acid [549nm]). The contaminating hyaluronic acid, however, gave rise to a product that had an absorption spectrum identical to that of sialic acid. This results occurs because both substrates ultimately yield β-formylpyruvic acid, the prechomogen in the thiobarbituric acid assay, as illustrated in Fig. 3. The β-formylpyruvic acid reacts with thiobarbituric acid in the assay to produce a red chromophore that is measured at 549 nm. β-Formylpyruvic acid is not the direct product of the periodate oxidation of sialic acid but is formed as a result of aldol splitting of the direct product by the hot, acidic conditions of the assay (21). In contrast, Hascall and coworkers (9) showed that β -formylpyruvic acid is the direct product of periodate oxidation of the unsaturated glucuronic acid residue in the disaccharide produced by β-eliminative cleavage of hyaluronic acid. These workers also reported that the released β -formylpyruvic acid could be measured using the thiobarbituric acid assay. Linker and coworkers (14) first identified the unsaturated disaccharide shown in Fig. 3 as the product of the incubation of hyaluronic acid with bacterial enzyme preparations obtained from *Staphylococcus aureus*, *Clostridium welchii*, pneumococci, and streptococci. It was the trimethylsilyl derivative of this disaccharide that gave rise to the peak eluting very late in the gas chromatogram of the material released by the enzyme. The reason that only N-acetylglucosamine was seen in the gas

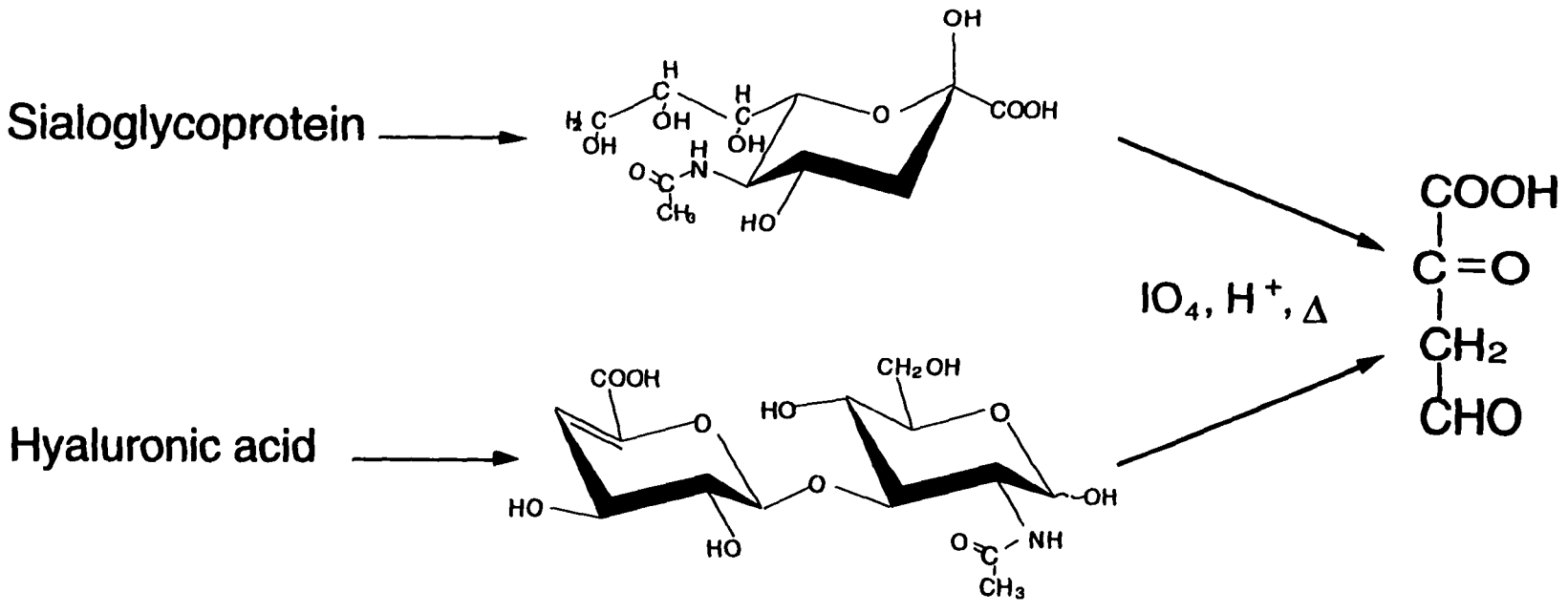


FIG. 3. Simplified illustration of how neuraminidase, acting on mucin, and bacterial hyaluronidase, acting on hyaluronic acid, yield quite different direct reaction products which both give rise to the same intermediate, β-formylpyruvic acid, in the thiobarbituric acid assay. Condensation of β-formylpyruvic acid with thiobarbituric acid in the assay yields a colored product with a λmax at 549 nm

chromatogram following methanolysis of the disaccharide is that the unsaturated glucuronic acid residue was acid labile.

The results shown in Fig.2 provided our first evidence that the compound released by the GBS enzyme was not sialic acid. Exhaustive cholera neuraminidase treatment of the mucin preparation made it possible to separate by ion-exchange chromatography material that did not produce any color in the thiobarbituric acid assay after acid hydrolysis from material that did produce color after enzyme treatment. If bound sialic acid had been present in the fractions, it would have been released by the acid treatment and resulted in color in the assay. This apparent discrepancy is explained by the fact that the two peaks eluting late both contained hyaluronic acid. (The hyaluronic acid present in one of the peaks may have been associated with protein.) GBS hyaluronidase treatment released the unsaturated disaccharide shown in Fig. 3. It is the presence of the double bond in the glucuronic acid residue that leads to the formation of β-formylpyruvic acid in the thiobarbituric acid assay and subsequent color formation. Acid hydrolysis of hyaluronic acid yields products that do not contain the double bond and hence do not react in the thiobarbituric acid assay.

We incorporated the N-(p-aminophenyl)oxamic acid agarose affinity column in our purification procedure at a time when we still thought the enzyme was a neuraminidase. The binding of hyaluronidase to this affinity support is probably explained by the fact that the substrates of both neuraminidase and hyaluronidase are acidic glycoconjugates which are mimicked to some extent by the N-(p-aminophenyl)oxamic acid agarose.

Milligan and coworkers performed PAGE on their stage V purified enzyme (18) and found, in addition to a band at about 106 kDa, several distinct lower-molecular-mass bands and a diffuse band in the lower-molecular-mass region, all of which possessed enzyme activity. Probably because we added the N-(p-aminophenyl)oxamic acid-agarose affinity column purification step, our purified enzyme showed only two intense bands on a polyacrylamide gel, a band at about 100 kDa and one at about 25 kDa. The lower-molecular-mass band yielded an N-terminal amino acid sequence identical to that of GBS CAMP factor. However, we are not sure whether the small amount of enzyme activity observed in this region of the gel was due to the intense CAMP factor band or to a component of the faint zone of material surrounding the band. The observation that GBS CAMP factor bound to the affinity support, however, was unexpected and may provide a clue about the nature of the as-yet-undefined physiological target of the cytolytic factor. Perhaps its ability to cause hemolysis of erythrocytes in the presence of a suitable cofactor depends on its binding to sialic acid or some other acidic sugar on the erythrocyte surface.

Although Milligan and coworkers (17) were misled concerning the actual specificity of their putative neuraminidase, they did demonstrate that elevated levels of the enzyme appeared to be associated with strain virulence. In addition, Musser et al. (19) identified a high-virulence clone of type III GBS that produced high levels of a putative extracellular neuraminidase. Consistent with both of these findings is the report that GBS hyaluronidase is associated with the virulence of GBS strains. Kjems et al. (13) used a turbidimetric procedure to screen 252 GBS stains of various serotypes for hyaluronidase production. They reported that while only 43% of type III GBS strains

hyaluronidase-positive in their assay, such strains were more than twice as likely as hyaluronidase-negative strains to be associated with serious neonatal infections. The recognition that the actual enzyme dealt with in all these studies is a hyaluronidase should facilitate future work on possible roles of the enzyme in the pathogenesis of GBS infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service program project grant HD-17812 from the National Institute of Child Health and Human Development and grant AI-30634 from the National Institute of Allergy and Infectious Diseases. We thank Dr. John R. Baker and the UAB Glycoprotein Sequencing Facility for their assistance with protein sequencing and Dr. Ray Furner of the Department of Psychiatry and Behavioral Neurobiology for his assistance with mass spectrometry. We thank Meg Mosteller Barnum for her able technical assistance.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York.

2. **Bitter, T., and H. M. Muir.** 1962. A modified uronic acid carbazole reaction. Anal. Biochem. **4:** 330-334.

3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

4. **Brown, J. G., and D. C. Straus.** 1987. Characterization of neuraminidases produced by various serotypes of group B streptococci. Infect. Immun. **55:** 1-6.

5. **Cuatrecasas, P., and G. Illiano.** 1971. Purification of neuraminidases from *Vibrio cholerae*, *Clostridium perfringens* and influenza virus by affinity chromatography. Biochem. Biophys. Res. Commun. **44:** 178-184.

6. **Den, H., D. A. Malinzak, and A. Rosenberg.** 1975. Cytotoxic contaminants in commercial *Clostridium perfringens* neuraminidase preparations purified by affinity chromatography. J. Chromatogr. **111:** 217-222.

7. **Edmond, J. D., R. G. Johnston, D. Kidd, H. J. Rylance, and R. G. Sommerville.** 1966. The inhibition of neuraminidase and antiviral action. Br. J. Pharmacol. Chemoter. **27:** 415-426.

8. **Etchison, J. R., and J. J. Holland.** 1975. A procedure for the rapid, quantitative N-acetylation of amino sugar methyl glycosides. Anal. Biochem. **66:** 87-92.

9. **Hascall, V. C., R. L. Riolo, J. Hayward, and C. Reynolds.** 1972. Treatment of bovine nasal cartilage proteoglycan with chondroitinases from *Flavobacterium heparinum* and *Proteus vulgaris*. J. Biol. Chem. **247:** 4521-4528.

10. **Hayano, S., and A. Tanaka.** 1969. Sialidase-like enzymes produced by group A, B, C, G, and L streptococci and by *Streptococcus sanguis*. J. Bacteriol. **97:** 1328-1333.

11. **Henningsen, M., P. Roggentin, and R. Schauer**. 1991. Cloning, sequencing and expression of the sialidase gene from Actinomyces viscosus DSM 43798. Biol. Chem. Hoppe-Seyler. **372:** 1065-1072.

12. **Hoyer, L. L., A. C. Hamilton, S. M. Steenbergen, and E. R. Vimr.** 1992. Cloning, sequencing and distribution of *the Salmonella typhimurium* LT2 sialidase gene, nanH, provides evidence for interspecies gene transfer. Mol. Microbiol. **6:** 873-884.

13. **Kjems, E., B. Perch, and J. Henrichsen.** 1980. Serotypes of group B streptococci and their relation to hyaluronidase production and hydrolysis of salicin. J. Clin. Microbiol. **11**: 111-113.

14. **Linker, A., K. Meyer, and P. Hoffman.** 1956. The production of unsaturated uronides by bacterial hyaluronidases. J. Biol. Chem. **219:** 13-25.

15. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:** 10035-10038.

16. **Mattingly, S. J., T. W. Milligan, A. A. Pierpont, and D. C. Straus.** 1980. Extracellular neuraminidase production by clinical isolates of group B streptococci from infected neonates. J. Clin. Microbiol. **12:** 633-635.

17. **Milligan, T. W., C. J. Baker, D. C. Straus, and S. J. Mattingly.** 1978. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B streptococci. Infect Immun. **21:** 738-746.

18. **Milligan, T. W., S. J. Mattingly, and D. C. Straus.** 1980. Purification and partial characterization of neuraminidase from type III group B streptococci. J. Bacteriol. **144:** 164-172.

19. **Musser, J. M.,. S. J. Mattingly, R. Quentin, A. Goudeau, and R. K. Selander.** 1989. Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B *Streptococcus*) causing invasive neonatal disease. Proc. Natl. Acad. Sci. USA. **86:** 4731-4735.

20. **Nisizawa, K., and W. Pigman.** 1959. The composition and properties of the mucin clot from cattle submaxillary glands. Arch. Oral. Biol. **1:** 161-170.

21. **Paerels, G. B., and J. Schut.** 1965. The mechanism of the periodate-thiobarbituric acid reaction of sialic acids. Biochem. J. **96:** 787-792.

22. **Rothe, B., B. Rothe, P. Roggentin, and R. Schauer.** 1991. The sialidase gene from *Clostridium septicum*: cloning, sequencing, expression in *Escherichia coli* and identification of conserved sequences in sialidases and other proteins. Mol. Gen. Genet. **226:** 190-197.

23. **Ruhlmann, J., B. Wittmann-Liebold, D. Jurgens, and F. J. Fehrenbach.** 1988. Complete amino acid sequence of protein B. FEBS Letters. **235:** 262-266.

24. **Russo, T. A., J. S. Thompson, V. G. Godoy, and M. H. Malamy.** 1990. Cloning and expression of the *Bacteroides fragilis* TAL2480 neuraminidase gene, *nanH*, in *Escherichia coli*. J. Bacteriol. **172**: 2594-2600.

25. **Schauer, R.** 1977. Characterization of sialic acids. Methods in Enzymol. **50C:** 64-89.

26. **Skoza, L., and S. Mohos.** 1976. Stable thiobarbituric acid chromophore with dimethyl sulphoxide: application to sialic acid assay in analytical de-o-acetylation. Biochem. J. **159:** 457-462.

27. **Tettamanti, G., and W. Pigman.** 1968. Purification and characterization of bovine and ovine submaxillary mucins. Arch. Biochem. Biophys. **124:** 41-50.

28. **van de Rijn, I., and R. E. Kessler.** 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. **27:** 444-448.

29. **Varki, A., and S. Diaz.** 1984. The release and purification of sialic acids from glycoconjugates: methods to minimize the loss and migration of o-acetyl groups. Anal. Biochem. **137:** 236-247.

30. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morbid. Mortal. Weekly Rep. **41:** 25-32.

CLONING AND EXPRESSION OF THE GENE FOR GROUP B STREPTOCOCCAL HYALURONATE LYASE

BO LIN, SUSAN K. HOLLINGSHEAD, JOHN E. COLIGAN, MARIANNE L. EGAN, JOHN R. BAKER AND DAVID G. PRITCHARD

Journal of Biological Chemistry (1994)
269, 30113-30116

Format adapted for dissertation and errata corrected

ABSTRACT

Group B streptococci (GBS) are a major cause of serious human perinatal infections. Most clinical isolates of GBS secrete hyaluronate lyase, and production of high levels of the enzyme has been associated with strain virulence. Degenerate oligonucleotide primers, designed on the basis of the amino acid sequences of tryptic peptides prepared from the purified enzyme, permitted the polymerase chain reaction amplification from GBS chromosomal DNA of a 363-base pair internal DNA fragment of the GBS hyaluronate lyase gene (*hylB*). This DNA fragment was used as a probe to screen a λ phage library of GBS chromosomal DNA fragments. Sequence analysis of positive clones identified an open reading frame capable of coding for an 111-kDa protein. Since no single clone was found to contain the entire gene it was necessary to reconstruct the gene from two plasmids containing inserts with suitable overlapping sequences. When this reconstructed gene was transformed into *Escherichia coli*, high level expression of hyaluronate lyase activity was obtained.

INTRODUCTION

Group B streptococci (GBS) are presently the most frequent cause of serious, often fatal, bacterial infections of neonates in the United States and are also a common cause of peripartum maternal sepsis (16). At our institution, the attack rate for neonatal disease is about 4 per 1000 live births (early- and late-onset infections), while maternal sepsis occurs in 1-2 per 1000 deliveries (5). Several different bacterial products are believed to be important in pathogenesis. One of them is an extracellular hyaluronate lyase, which we recently demonstrated (11) had been incorrectly identified as a neuraminidase for many years (9, 10). Strains producing high levels of the enzyme were

frequently found to be the most virulent strains (9, 10). We recently reported that GBS hyaluronate lyase degrades hyaluronan (hyaluronic acid) by a unique mode of action (12). Rather than randomly cleaving hyaluronan chains yielding a mixture of oligosaccharide fragments like other known hyaluronidases, the GBS enzyme appears to processively move along the hyaluronan chains, continuously releasing disaccharide units as it travels. In view of the enzyme's unusual manner of degrading hyaluronan, its potential importance in pathogenesis, and to aid future development of specific inhibitors of the enzyme of possible therapeutic value, we set out to clone, sequence, and express the gene for GBS hyaluronate lyase.

MATERIALS AND METHODS

Bacterial strains. Serotype III group B streptococcal (GBS) strain 3502, isolated from the blood of an infected infant, was grown in chemically defined medium in a fermentor as described previously (12). *Escherichia coli* strains XL1 Blue MRF' and XLOLR, obtained from Stratagene Cloning Systems (La Jolla, CA), were grown in LB medium, which for the XL1 Blue MRF' strain was supplemented with 0.2% maltose and 10 mM $MgSO_4$. One Shot™ INVαF' *E. coli* competent cells were obtained from Invitrogen Corp. (San Diego, CA).

Hyaluronidase assays. GBS and *E. coli* strains were screened for hyaluronidase production by growth on T-Soy agar plates containing 0.4 mg/ml hyaluronan (Sigma Chemical Co., St. Louis, MO) and 10 mg/ml bovine serum albumin (BSA, Sigma) essentially as described by Smith and Willett (14). After overnight growth at 37°C the plates were developed by flooding them with 2 N acetic acid, which results in precipitation of a hyaluronan/BSA complex in the gel. Positive colonies are surrounded

by clear zones on a turbid background. Culture supernatants were routinely assayed for GBS hyaluronate lyase activity by adding 50 µl of the test sample to a cuvette containing 1 ml of a 0.2 mg/ml solution of hyaluronan in 50 mM ammonium acetate buffer, pH 6.5, 10 mM $CaCl_2$. The rate of increase in absorbance at 232 nm was followed in a recording spectrophotometer. One enzyme unit of GBS hyaluronidase activity was defined as the amount of enzyme that catalyzes the release of 1 nmole of the unsaturated disaccharide, 2 - acetamido -2 - deoxy - 3 - O - (β-D-gluco-4-enepyranosyluronic acid)-D-glucose, from hyaluronan per min in 50 mM ammonium acetate buffer, pH 6.5, and 10 mM $CaCl_2$. A millimolar absorption coefficient of 5.5 (15) was used in all calculations.

Purification of GBS chromosomal DNA. GBS cell pellets were digested with 500 units/ml mutanolysin (Sigma) in pH 7.5 buffer (10 mM Tris-HCl, 1 mM EDTA) for 30 min at 60°C followed by the addition of SDS (2% final concentration) and incubation for an additional 10 min. The resulting cell lysate was incubated at 37°C with ribonuclease A (Sigma, 2 mg/ml final concentration) for 30 min and finally digested with proteinase K (Sigma, 0.2 mg/ml final concentration) for 1 h. The mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA precipitated with 2 volumes of ethanol.

Enzyme purification. GBS hyaluronate lyase was purified as described previously (12) . Briefly, this involved concentration of the high molecular weight components in the culture filtrate of GBS strain 3502 by ultrafiltration, passage through a DEAE-Sephacel (Pharmacia Biotech Inc., Piscataway, NJ) column, affinity chromatography on a column of N-(*p*-aminophenyl)oxamic acid linked to agarose (Sigma), and finally gel filtration on a column of Sephacryl S300 superfine (Pharmacia). Fractions

containing the peak of enzyme activity were pooled and concentrated using a Centricon 30 ultrafiltration device (Amicon Inc., Beverly MA). Polyacrylamide gel electrophoresis of the final product gave a single band of approximately 105 kDa.

Peptide sequencing. Tryptic peptides were produced by digesting 0.4 mg of purified enzyme with 5 μg of sequencing grade modified trypsin (Promega) in 75 mM NH_4HCO_3 buffer, pH 8.0, for 16 h at 37°C. Peptides were separated by reverse phase HPLC on an octyl 208TP (4.6 mm x 15 cm) column (Vydac, Hesperia, CA). The gradient employed was from 5% to 70% aqueous acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min. Fractions containing individual peaks were collected manually. Of the more than 30 well resolved peaks, 10 were sequenced and two (peaks 51 and 65) gave sequences longer than 10 residues. The sequencing was performed with an Applied Biosystems Model 477A protein sequencer with a blot cartridge according to the manufacturer's program, BLOTT-1 (Perkin Elmer, Foster City, CA). Amino acid analysis of purified enzyme was carried out on duplicate samples hydrolyzed in 6 N HCl for 2 h at 150°C. The composition was determined on a model 6300 amino acid analyzer using standard conditions and ninhydrin detection.

PCR conditions. Oligonucleotide PCR primers (degenerate and exact) were synthesized by DNA International (Lake Oswego, OR). Standard PCR reaction mixtures contained 50 pmol of each primer, 1 unit of *Taq* polymerase (Perkin Elmer, Norwalk CT), 200 μM dNTPs, 1.5 mM Mg^{2+}, 50 mM KCl, 10 mM Tris, pH 8.3. When degenerate primers were used, the first three cycles were carried out at 94°C, 45 sec; 37°C, 30 sec; 72°C, 2.5 min, followed by 35 cycles of 94°C, 45 sec; 45°C, 30 sec; 72°C, 2.5 min. When exact primers were used, all the annealing steps were carried out at 58°C for 1 min

and only 30 cycles were performed. After remaining at 72°C for 10 min, the samples were stored at 4°C until used.

Construction and screening of a library of GBS chromosomal DNA. The products of a partial *Sau*3A digest of GBS chromosomal DNA were electrophoretically separated on an 0.8% agarose gel. DNA fragments of approximately 3-9 kb were electroeluted and ligated to the *Bam*HI/CIAP predigested arms of the Lambda Zap Express™ vector (Stratagene Cloning Systems, La Jolla, Calif. 92037) and packaged using a Gigapack® II Gold Packaging kit (Stratagene). Recombinant phages (approximately 10^6) were screened for the presence of a desired insert using appropriate radiolabeled probes (9) . Following secondary screening, positive recombinant phages were converted to pBK-CMV plasmids by *in vivo* excision using the Stratagene protocol.

Molecular techniques. Plasmid DNA for probes, restriction digests, and DNA sequencing were prepared using a QIAGEN Midiprep Kit (Chatsworth, CA). The initial gene fragment used as a probe for the *hyl*B gene was amplified by PCR and then cloned using a TA™ Cloning Kit (Invitrogen). The *hyl*B-specific DNA was excised from the plasmids by digestion with *Eco*RI and purified using a "Gene-Clean" kit (Bio 101 Inc., La Jolla, CA). This insert, as well as a second gene fragment PCR amplified with exact primers, was labeled using [^{32}P]dATP by a nick-translation procedure (Life Technologies, Inc.). Reconstruction of a complete *hyl*B gene was accomplished by combining two of the partial gene clones identified in the gene library. A DNA fragment containing the 3' portion of the gene from plasmid #22 was excised with *Eco*RI/*Sal*I and ligated into plasmid #42 cut with *Eco*RI/*Xho*I.

DNA sequencing and analysis. Double-stranded nucleic acid sequencing was carried out using dye-labeled primers on an Applied Biosystems model 373A automated DNA sequencer. Sequence data were assembled and analyzed using the DNASTAR MegAlign, EditSeq, and GeneMan Modules (Madison, WI) and the GCG program (Genetics Computer Group, Madison WI).

RESULTS AND DISCUSSION

Purified group B streptococcal hyaluronate lyase was digested with trypsin and the resulting peptides separated by HPLC. Several of the well-resolved peptide peaks were selected for amino acid sequencing. On the basis of the sequences of two of the peptides, #51 (ESLAWLHQNFY) and #65 (AEGFYADGSYIDHTNVAYT), degenerate oligonucleotide PCR primers were designed. One combination of forward and reverse direction primers permitted the amplification from GBS chromosomal DNA of a 363-base pair DNA fragment, PCR TAC#9 (Fig. 1). This fragment was cloned into the plasmid vector pCR™ II and sequenced. A search of the GenBank data base revealed a high similarity (63% identity over 121 amino acids) with the recently reported (2) amino acid sequence of pneumococcal hyaluronidase. This result convinced us that we had indeed amplified a portion of the GBS *hyl*B gene. A ^{32}P-labeled probe was then prepared by nick-translating the cloned fragment of the *hyl*B gene and used to screen a phage library of GBS chromosomal DNA fragments.

A total of 15 positive phage clones was obtained, and following *in vivo* excision, nucleotide sequences of the ends of the resulting plasmids were determined. Based on the observed sequence overlaps, one of the largest inserts (plasmid #42) was selected for complete sequencing. Reliable sequence data were usually obtained for about 300

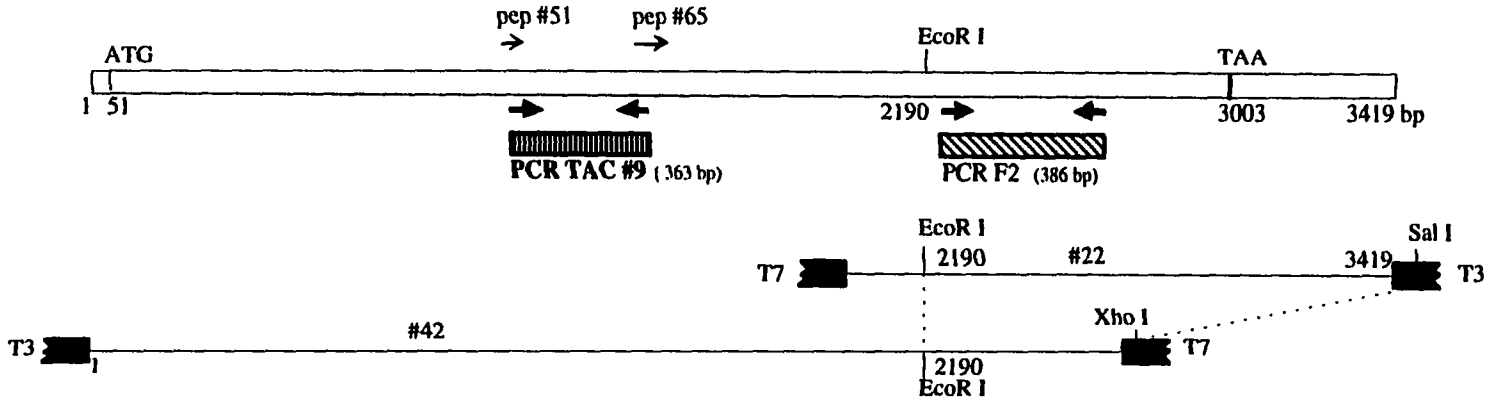


FIG. 1. Reconstruction of a complete GBS *hyl*B gene from the plasmid clones #22 and #42. A 1.229 kb *Eco*R I/*Sal* I DNA fragment corresponding to the 3' end of the gene was excised from plasmid #22 and ligated with a 6.7 kb *Eco*R I/*Xho* I fragment of plasmid #42 containing the vector, pBK-CMV (4.512 kb), and a 2.19 kb fragment from the 5' end of the gene. The relative location of the two PCR amplified gene fragments used as probes is also indicated.

nucleotides in each sequencing reaction. New sequencing primers were then designed, and sequencing continued toward the center of the inserts until the entire insert was sequenced. In order to obtain a clone containing the 3' end of the *hyl*B gene, oligonucleotide primers were designed which permitted the PCR amplification of a 386-base pair insert, PCR F2 (Fig. 1). This PCR fragment was used as a probe to identify two more phage clones containing the 3' end of the *hyl*B gene, one of which (plasmid #22) was completely sequenced.

When the complete sequence of the two overlapping clones, each containing a partial gene fragment (Fig. 1), was determined, a single open reading frame coding for a protein of 111,319 Da was observed (Fig. 2) within the 3419 base pairs sequenced. Amino acid sequences corresponding to those of the two sequenced peptides used to design the original degenerate PCR primers are present and indicated in Fig. 2. An inverted repeat, possibly a transcription termination sequence, occurs 12 base pairs after the stop codon. No promoter or ribosome binding sites were observed but a triple repeat of unknown function is present near the start of the sequence.

A search of the GenBank data base revealed 50.7% amino acid identity between GBS hyaluronate lyase and the pneumococcal hyaluronidase (2) for the alignment depicted in Fig. 3. However, comparison of the protein sequence of the GBS enzyme with that of the hyaluronidases from *Streptococcus pyogenes* bacteriophage H4489 (7) and *Clostridium perfringens* (4) using the Bestfit program in the Wisconsin GCG package found no alignment similarity scores that were more than 1 S.D. above a mean score generated with ten randomized sequences containing the same amino acids. A 12-amino acid motif is present twice in both the GBS enzyme and in the pneumococcal

FIG. 2. Features of the nucleotide and deduced amino acid sequences of the gene for GBS hyaluronate lyase. A triple repeat of unknown function at the beginning of the sequence is indicated by underlining. Amino acid sequences corresponding to the two peptides used to design the original degenerate PCR primers are indicated by dotted underlining. An inverted repeat 12 nucleotides after the stop codon (in bold type) that may be a transcription termination sequence is indicated by opposing arrows.

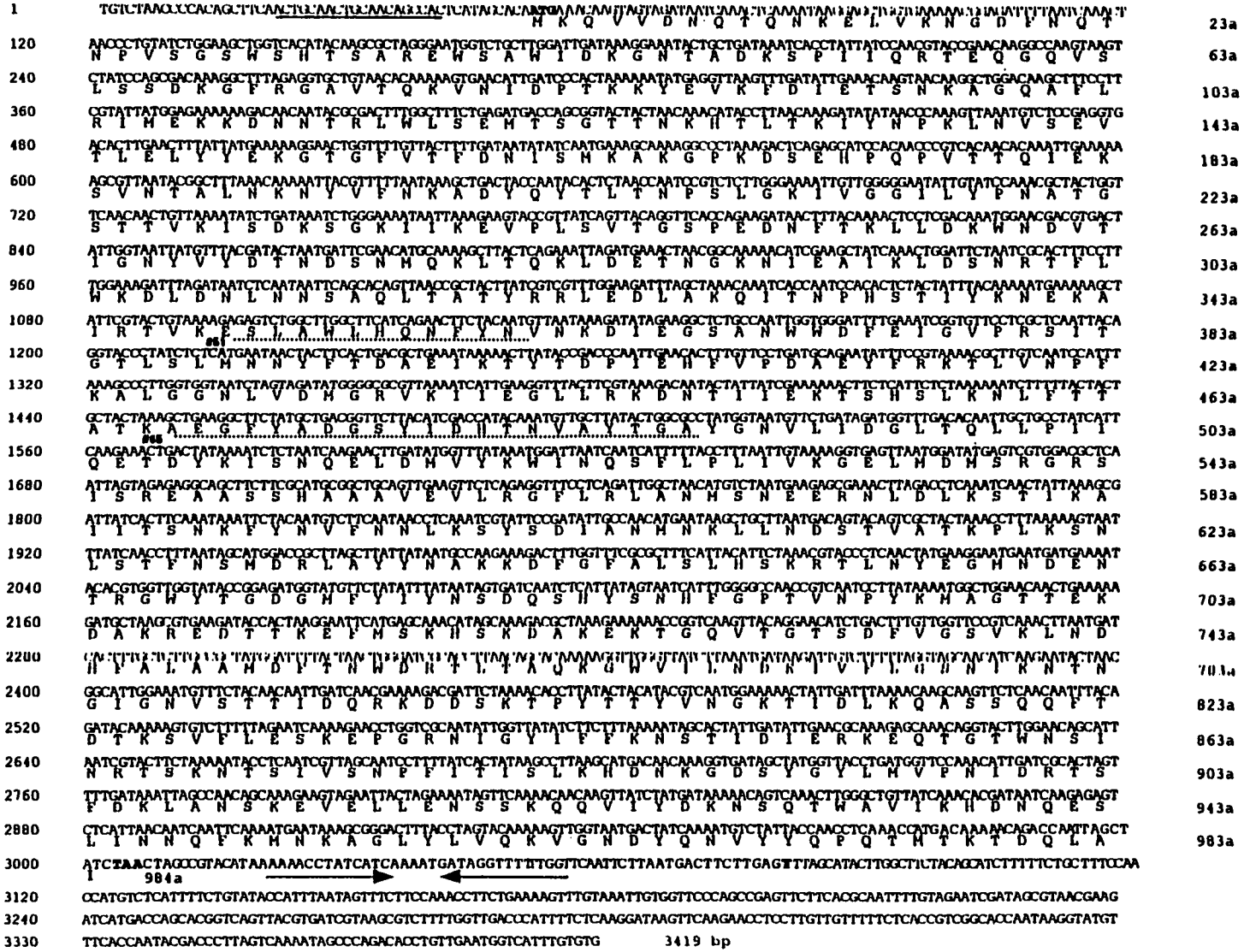
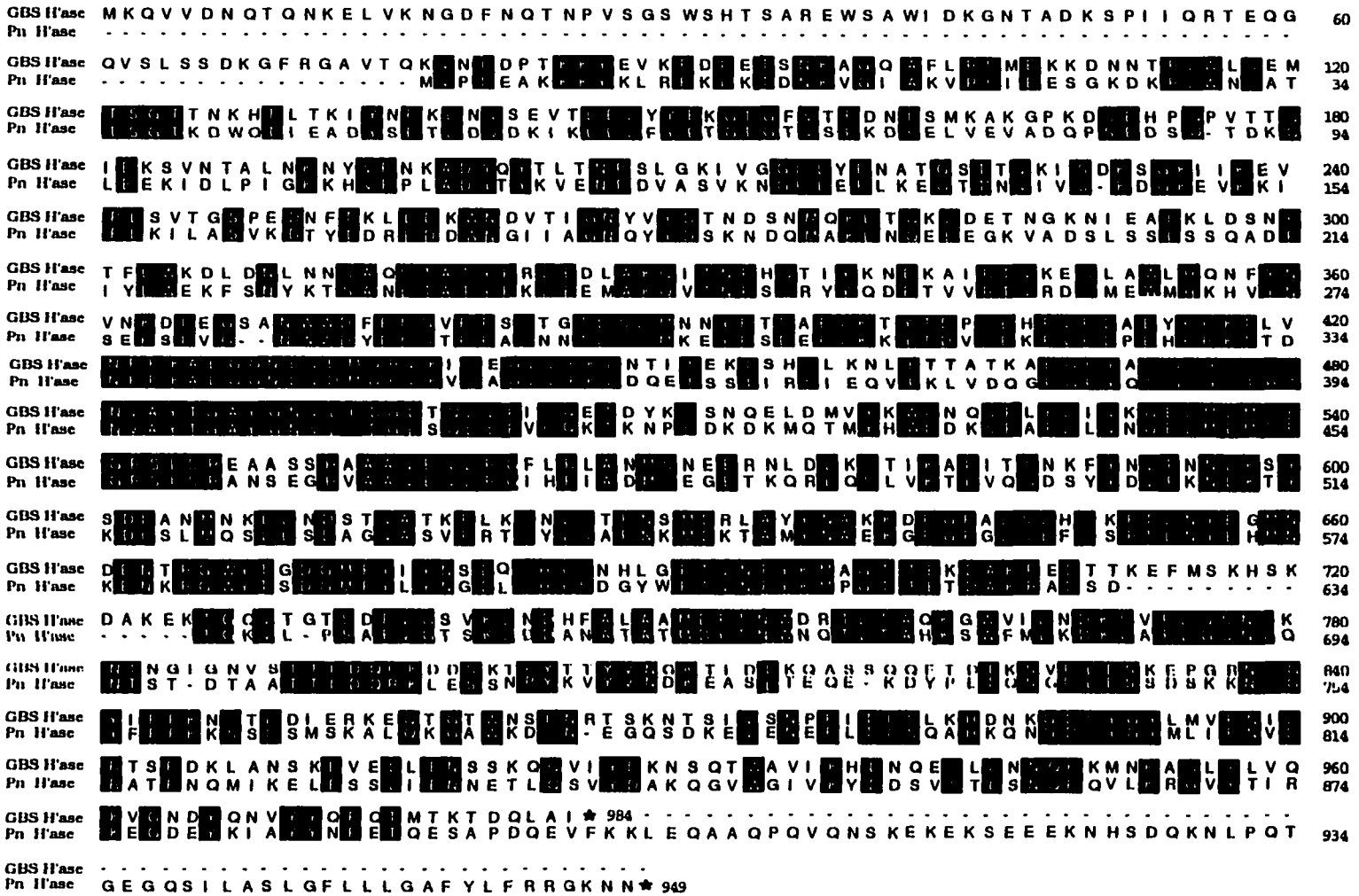


FIG. 3. Alignment of the amino acid sequence of GBS hyaluronate lyase (GBS H'ase) with that of the hyaluronate lyase of *Streptococcus pneumoniae* (Pn H'ase) illustrating regions of identity. Highlighted areas denote conserved amino acids.



enzyme (Fig. 4). In both cases the sequences are separated by 105 amino acids. It is of interest that repeating motifs of unknown function have also been found in a variety of other bacterial enzymes (13).

The deduced amino acid composition of the GBS enzyme agreed well with the amino acid analysis of the purified enzyme (data not shown). The isoelectric point of GBS hyaluronate lyase was calculated (using the EditSeq module of DNASTAR) to be 8.84, which explains why it does not bind to DEAE-Sephacel at pH 6.8 during the purification procedure.

Since no single clone contained the entire gene, it was necessary to reconstruct it from two plasmids containing inserts with suitable overlapping gene fragments. Two such plasmids (#22 and #42) had an *Eco*RI restriction site in their overlapping sequences. As illustrated in Fig 1, a 1.229 kb *Eco*RI/*Sal*I fragment containing the 3' end of the *hyl*B gene excised from plasmid #22 was ligated with *Eco*RI/*Xho*I digested plasmid #42 containing the 5' end of the gene and the expression vector pBK-CMV. This construct was transformed into *E. coli* INVαF' competent cells. Transformants bearing the reconstructed *hyl*B gene produced large clear zones in the hyaluronidase plate assay (Fig. 5). The clear zones were even larger than that of the parent GBS strain 3502. The supernate of an overnight culture of the *E. coli* clone was found to contain 258 u/ml hyaluronate lyase activity. It is not known at this time whether the enzyme found in the culture supernate is actively transported in *E. coli* or whether it comes from lysed cells.

Little is known about the role of GBS hyaluronate lyase in pathogenesis. It may simply facilitate the invasion of tissues by the bacteria. However, there is a strong possibility that the enzyme may also subvert some normal host defense mechanisms.

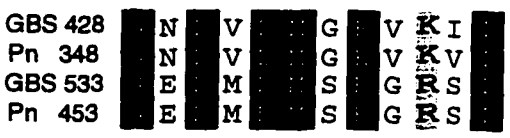
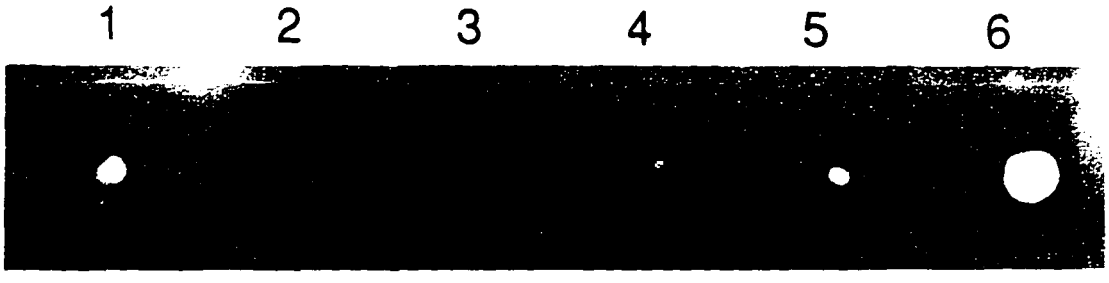


FIG. 4. Amino acid motifs of unknown function present twice in both the GBS hyaluronate lyase and in the pneumococcal hyaluronidase.

FIG. 5. Hyaluronidase agar plate assay of *E. coli* and GBS strains. The diameter of the clear zones surrounding the bacterial colonies is proportional to the amount of hyaluronidase produced. The spotted colonies are 1) the *E. coli* host strain INVαF' containing the plasmid vector pBK-CMV; 2) INVαF' containing the plasmid vector with the reconstructed *hylB* gene ; 3) the parent serotype III GBS strain 3502; 4) the low producer serotype Ib GBS strain 501; 5) the non-producer serotype III GBS strain 1733; and 6) the E. coli host strain INVαF'.



Hyaluronan and molecules that bind to it are involved in numerous immune system functions (3, 6, 8). Neutrophils and macrophages, for example, have high surface concentrations of the hyaluronan receptor CD44 (6) and GBS hyaluronate lyase may well interfere with the functions of these cells.

Our recent evidence that GBS hyaluronate lyase appears to degrade hyaluronan processively suggests the possibility that there may be separate domains of the enzyme involved in processive movement and enzymatic activity. The availability of the cloned gene will greatly facilitate studies of this possibility as well as future X-ray crystallographic structural studies, studies of *hylB* gene regulation, and work on the biological roles of the enzyme.

ACKNOWLEDGMENTS

Nucleotide sequencing was carried out in the DNA Sequencing Core Facility of the Center for AIDS Research and DNA sequence analysis was carried out using the University of Wisconsin Genetics Computer Group (GCG) program. Both were supported in part by grant number P30-AI-27767 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York.

2. **Berry, A. M., R. A. Lock, S. M. Thomas, D. P. Rajan, D. Hansman, and J. C. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *E. coli*. Infect. Immun. **62:** 1101-1108.

3. **Bourguignon, L. Y. W., V. B. Lokeshwar, X. Chen, and W. G. L. Kerrick.** 1993. Hyaluronic acid-Induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. J. Immunol. **151:** 6634-6644.

4. **Canard, B., T. Garnier, B. Saint-Joanis, and S. T. Cole.** 1994. Molecular genetic analysis of the nagH gene encoding a hyaluronidase of *Clostridium perfringens*. Mol. Gen. Genet. **243:** 215-224.

5. **Dillon, H. C., Jr, S. Khare, and B. M. Gray.** 1987. Group B streptococcal carriage and disease: a 6-year prospective study. J. Pediatr. **110**: 31-36.

6. **Haynes, B. F., M. J. Telen, L. P. Hale, and S. M. Denning K.** 1989. CD44 - a molecule involved in leukocyte adherence and T-cell activation. Immunol. Today. **10:** 423-428.

7. **Hynes, W. L., and J. J. Ferretti.** 1989. Sequence analysis and expression in *Echerichia coli* of the hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A. Infect. Immun. **57:** 533-539.

8. **Laurent, T. C., and J. R. E. Fraser.** 1992. Hyaluronan. FASEB J. **6**: 2397-2402.

9. **Milligan, T. W., C. J. Baker, D. C. Straus, and S. J. Mattingly.** 1978. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B streptococci. Infect Immun. **21:** 738-746.

10. **Musser, J. M., S. J. Mattingly, R. Quentin, A. Goudeau, and R. K. Selander.** 1989. Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B *Streptococcus*) causing invasive neonatal disease. Proc. Natl. Acad. Sci. USA. **86:** 4731-4735.

11. **Pritchard, D. G., and B. Lin.** 1993. Group B streptococcal neuraminidase is actually a hyaluronidase. Infect. Immun. **61**: 3234-3239.

12. **Pritchard, D. G., B. Lin, T. R. Willingham, and J. R. Baker.** 1994. Characterization of the group B streptococcal hyaluronate lyase. Arch. Biochem. Biophys. **315:** 431-437.

13. **Roggentin, P., B. Rothe, J. B. Kaper, J. Galen, L. Lawrisuk, E. R. Vimr, and R. Schauer.** 1989. Conserved sequences in bacterial and viral sialidases. Glycoconjugate J. **6:** 349-353.

14. **Smith, R. F., and N. P. Willett.** 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. Appl. Microbiol. **16:** 1434-1436.

15. **Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki.** 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. J. Biol. Chem. **243:** 1523-1535.

16. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morbid. Mortal. Weekly Rep. **41:** 25-32.

INSERTIONAL INACTIVATION OF THE GROUP B STREPTOCOCCAL HYALURONATE LYASE GENE

BO LIN AND DAVID G. PRITCHARD

To be submitted to FEMS Microbiology Letters

ABSTRACT

Group B streptococcal hyaluronate lyase appears likely to contribute to the ability of the microorganism to invade a susceptible host. The production of a mutant GBS strain unable to produce the enzyme would greatly facilitate studies of its actual role in pathogenesis. Commonly employed mutagenesis procedures did not yield such a mutant. The desired mutant was obtained, however, by transforming group B streptococci with a plasmid construct which contained two segments of the hyaluronate lyase gene in opposite orientations to each other which were separated by a promotorless *cat* gene in pMU1328. The resulting mutant was extremely stable and it was not possible to obtain any revertants, even after extensive subculturing in the absence of antibiotics. Molecular analysis of the *hylB*$^-$ mutation revealed that two copies of the part of plasmid pLIN110 containing the *cat* gene and a fragment of the *hylB* gene had inserted into, and thereby inactivated, the chromosomal copy of the *hylB* gene.

INTRODUCTION

Group B streptococci (GBS) produce a hyaluronate lyase capable of degrading hyaluronan and chondroitin sulfate in the extracellular matrix of tissues (13), an activity that appears likely to contribute to the invasive properties of the bacteria. Similar enzymes are also produced by two other human pathogens, *Streptococcus pneumoniae* (2) and *Staphylococcus aureus* (5). We previously cloned and sequenced the gene for GBS hyaluronate lyase and expressed it in *Escherichia coli* (9). We also studied the unusual mode of action of the enzyme and investigated its substrate specificity (13). Unlike other known hyaluronidases, which randomly cleave hyaluronan chains yielding a mixture of oligosaccharide fragments, the GBS enzyme appears to processively move

along hyaluronan chains continuously releasing disaccharide units as it moves. This property significantly contributes to the ability of the enzyme to rapidly degrade very large hyaluronan chains.

Investigations of the pathogenic effects of GBS hyaluronate lyase would be greatly facilitated by the availability of GBS mutants unable to produce the enzyme. Unfortunately, producing such mutants has been difficult. There currently are no published procedures for producing GBS mutants targeted to a specific gene. Several GBS mutants that have been described were made by Tn*916* transposon mutagenesis (14). However, it is now widely recognized that this transposon does not insert randomly into the bacterial chromosome and certain potentially useful mutations are never obtained (15). We screened more than 8000 Tn*916* transconjugates without finding a single hyaluronate lyase-negative mutant.

Another commonly used procedure for specifically mutating selected bacterial genes is insertion-duplication mutagenesis. This method, for example, has been successfully used to insertionally inactivate the genes encoding pneumolysin and autolysin in pneumococci (3). Using this approach, we made several *E. coli*-streptococcal vector constructs containing a segment of the GBS hyaluronate lyase gene (*hylB*). These plasmid constructs were then introduced into GBS cells by electroporation in an attempt to inactivate the hyaluronate lyase gene by an insertion-duplication event. Unfortunately this approach also was unsuccessful in GBS, possibly because of the difficulty of obtaining the desired mutant when the transformation efficiency and the probability of chromosomal integration are both extremely low, and maybe also because such insertions are inherently unstable in GBS. We then made a number of plasmid constructs in which

two segments of the GBS gene were separated by a promoterless chloramphenicol acetyltransferase (*cat*) gene in the hope that we might be able to force a double crossover event resulting in the inactivation of the hyaluronate lyase gene. We describe in this report how in one of these constructs we inadvertently placed the two GBS hyaluronate lyase gene segments in opposite orientation to each other. Electroporation of this construct into GBS produced the desired mutant.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Serotype III GBS strain 3502 was originally isolated from the blood of an infected infant. It was grown in the chemically defined medium (CDM) described by van de Rijn and Kessler (17), in Todd-Hewitt broth, or on solid media composed of Trypticase Soy Agar (Becton Dickinson, Cockeysville, MD) containing 5% sheep blood. *Escherichia coli* competent cells (One Shot™ INVαF') were purchased from Invitrogen Corp. (San Diego, CA). Erythromycin was used at a concentration of 4 μg/ml for GBS and 200 μg/ml for *E.coli.* Chloramphenicol was used at concentrations of either 5 μg/ml or 20 μg/ml for GBS, and 25 μg/ml for *E.coli.*

Plasmids. pMU1328 was originally constructed for use in detecting streptococcal promoters (1). The plasmid can replicate in both *E. coli* and streptococci because it contains a *Col*E1 origin of replication from pBR322 as well as a streptococcal replicon from the gram-positive shuttle plasmid pVA838 (10). An erythromycin resistance gene in the plasmid is expressed in both *E. coli* and streptococci. The plasmid also has a promoterless chloramphenicol acetyl transferase (*cat*) gene obtained from

plasmid pC194 (7). PCR fragments of the *hylB* gene were cloned in the vector pCR II using the TA cloning procedure (Invitrogen Corp., San Diego, Calif.).

Electroporation of GBS. Competent cells were prepared by inoculating 400 ml of Todd-Hewitt Broth with GBS strain 3502 from a blood agar plate. The culture was grown at 37° C without shaking until it reached an absorbance of 0.4 at 600 nm. The cells were harvested by centrifugation, washed twice with 50 mM sodium acetate buffer, pH 6.0, and treated with 100 units/ml mutanolysin (Sigma) at 37° C for 30 min to increase the permeability of the cell walls. The suspension was then centrifuged and the cells were washed three times with ice-cold 10 % glycerol. Finally, the cell pellet was resuspended in 10 % glycerol and 300 µl aliquots were stored at -70° C.

Competent cells (100 µl) were combined with 0.5 - 1.0 µg of plasmid DNA and placed on ice for 5 min. Electroporation was carried out using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) operated at a field strength of 12,500 V/cm, a capacitance of 25 µF, and the pulse controller set for 200 Ohms. After pulsing, 1 ml of THB was immediately added to the electroporation cuvette which was then incubated on ice for 10 min. The cell suspension was then transferred to a tube containing 10 ml THB and incubated at 37° C for 2 hr. The cells were recovered by centrifugation, spread on a blood agar plate containing 4 µg/ml of erythromycin, and incubated overnight at 37° C in a Gas-Pak chamber.

Hyaluronidase assays. GBS strains were screened for hyaluronidase production by growth on T-Soy agar plates containing 0.4 mg/ml hyaluronan (Sigma) and 10 mg/ml bovine serum albumin, essentially as described by Smith and Willett (16). Single colonies were spotted on a plate and incubated overnight at 37°C. A complex of hyaluronan and

bovine serum albumin was precipitated in the agar by flooding the plate with 2 N acetic acid. Hyaluronidase-producing colonies yield clear zones on a turbid background.

Hyaluronidase activity of culture supernatants was measured using a simple spectrophotometric assay. GBS cells were grown in CDM until the absorbance at 600 nm of the culture reached about 0.8. After centrifugation a 10 µl aliquot of the supernatant was added a cuvette containing 300 µl of 0.2 mg/ml hyaluronan, 50 mM ammonium acetate buffer (pH 6.5), and 3 mM $CaCl_2$. The increase in absorbance at 232 nm was followed in a recording spectrophotometer. One enzyme unit of GBS hyaluronidase activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of the unsaturated disaccharide, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose, from hyaluronan per min. A millimolar extinction coefficient of 5.5 was used in all calculations (19).

Isolation of GBS chromosomal DNA. The cell pellet from a 10 ml GBS culture was washed twice with 50 mM sodium acetate buffer (pH 6.0) and then treated with 100 units of mutanolysin (Sigma) in the same buffer for 30 min at 60° C. The cells were lyzed by adding 10 % SDS to give a final concentration of 2% and incubated for 10 min at 60°C. The cell lysate was then treated with 2 mg/ml of ribonuclease A at 37° C for 30 min followed by protease K (0.2 mg/ml final concentration) digestion for 1 hr. The mixture was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and the DNA was precipitated with two volumes of ethanol containing 0.3 M sodium acetate.

Southern blots. Restriction endonuclease digests of DNA were separated on agarose gels and transferred to positively charged nylon membranes (Schleicher & Schuell,

Keene, NH) by vacuum blotting. DNA fragments were fixed on the membranes in a UV cross-linker (Stratagene, La Jolla, CA). DNA probes were labeled with digoxigenin-11-dUTP (5-20 ng/ml) by a random primer DNA labeling procedure (Genius System, Boehringer Mannheim Corp., Indianapolis, Ind.). Hybridization and colorimetric detection were carried out as recommended by the supplier of the labeling system.

PCR conditions. Reaction mixtures (100 µl) contained 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 50 pmol of each of the primers. The mixture was initially heated to 95°C for 60 sec and 1 unit of *Tag* DNA polymerase (Perkin-Elmer) was added. The PCR reaction was accomplished by 30 cycles of 94°C, 60 sec; 60°C, 60 sec; 72°C, 2.5 min. For amplification of DNA fragments larger than 4 kb, 5 units of *Tag* DNA polymerase were used and the extension time was increased to 5.5 min at 72°C. PCR amplified products were analyzed on 0.8% agarose gels. In some cases, the PCR fragments were cloned into the pCR II vector (Invitrogen) and plasmid DNA was purified using a QIAGEN Midiprep Kit (Qiagen Inc., Chatsworth, CA).

Passage in antibiotic-free media to assess stability of the *hylB*⁻ mutant. Todd Hewitt broth (50 ml) was inoculated with about 10^8 *hylB*⁻ GBS and cultured at 37° C for 16 hours. A 0.5 ml aliquot of this culture was then used to inoculate 50 ml of THB which was incubated at 37° C until the absorbance at 650 nm was greater than 1.0. After four more subcultures, aliquots of the bacteria were spread on T-Soy blood agar plates with or without antibiotics and incubated overnight. The numbers of colonies on both plates were compared. Single colonies were spotted onto plates containing 5 µg/ml chloramphenicol and also onto HA-BSA plates to test for hyaluronidase production.

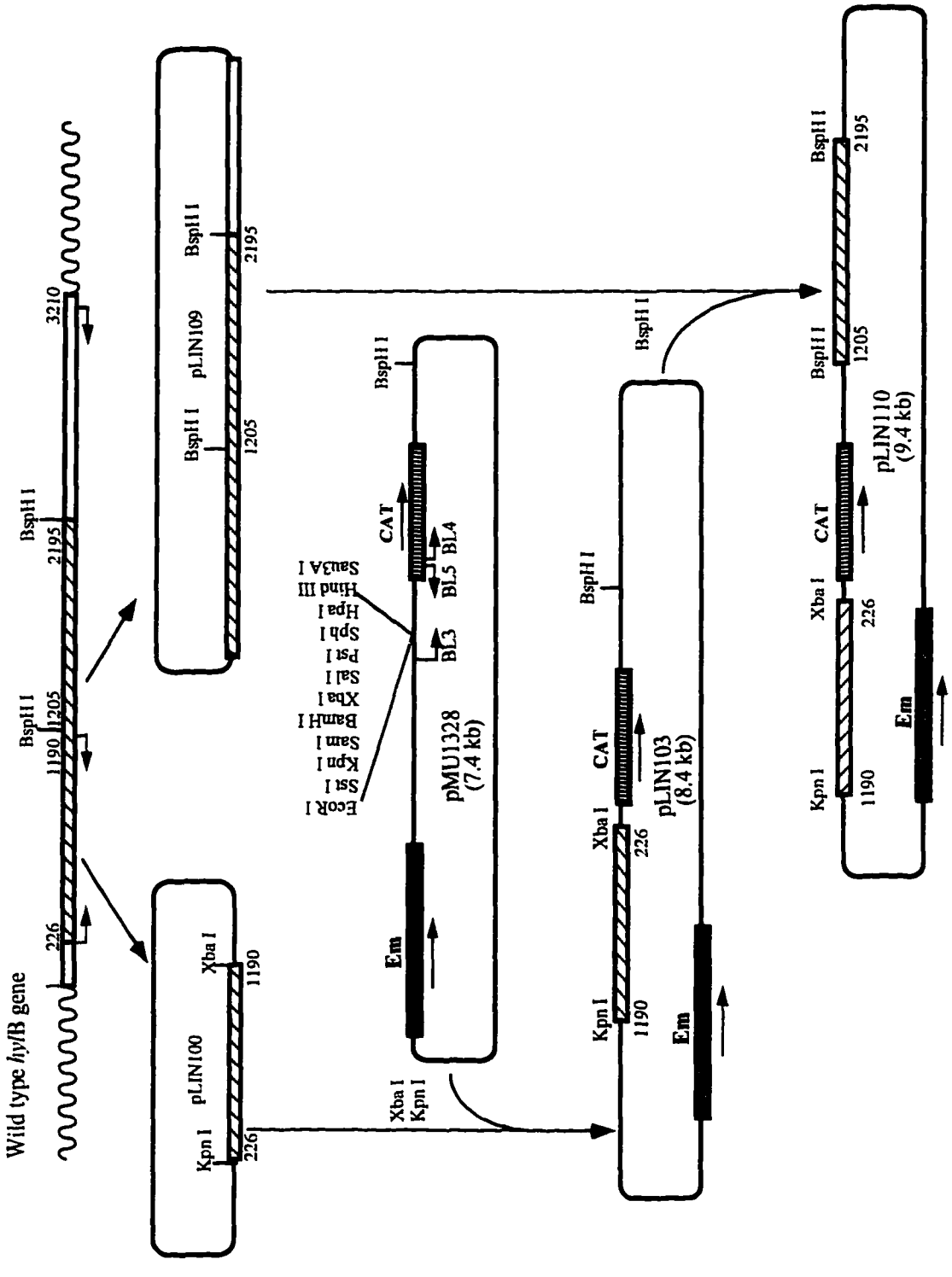
Aliquots (5 μl) of undiluted overnight cultures, containing approximately 10^6 cells, were also placed on HA-BSA plates to detect the presence of hyaluronidase activity.

RESULTS AND DISCUSSION

Plasmid constructions. Our strategy for specifically inactivating the GBS hyaluronate lyase gene (*hylB*) was to first introduce replicating plasmids containing fragments of the hyaluronate lyase gene (*hylB*) into a GBS strain by selecting for erythromycin resistance. The cells would then be grown under non-selective conditions favoring the loss of replicating plasmid, and cells in which the plasmid had integrated into the chromosome would be selected by their ability to grow in the presence of chloramphenicol. The plan involved constructing a special shuttle vector containing two large segments of the *hylB* gene separated by a promoterless chloramphenicol acetyl transferase (*cat*) gene. We started with a plasmid containing the *cat* gene that had been constructed by Achen et al. for use in detecting streptococcal promoters (1). These authors actually made a pair of plasmids, pMU1327 and pMU1328, with the polylinker from M13mp18 in opposite orientations. We inadvertently used pMU1328 rather than pMU1327 in making our initial construct, which resulted in the upstream *hylB* fragment being inserted in the opposite orientation to the *cat* gene and the downstream *hylB* fragment. Surprisingly, this construct was the only one that led to the successful inactivation of the GBS hyaluronidase gene.

Fig. 1 summarizes the steps used in the construction of plasmid pLIN110. The intermediate plasmid pLIN100 was made by TA cloning a 965 bp *hylB* fragment in the vector pCR II. Similarly, pLIN109 was made by subcloning a 2984 bp fragment of the *hylB* gene into the same vector. A 1063 bp *hylB* fragment was then excised from

FIG. 1. Construction of plasmid pLIN110. Intermediate plasmids pLIN100 and pLIN109 were made by cloning PCR-amplified fragments of the wild type *hylB* gene into the vector pCRII. The PCR primers are indicated by small arrows with numbers corresponding to their location on the *hyl*B gene. A 965 bp fragment was cloned to produce pLIN100 and a 2984 bp fragment was used to construct pLIN109. Plasmid pLIN103 was then made by cloning a 1063 bp *Xba* I/*Kpn* I fragment of pLIN100 into a similarly cut pMU1328 plasmid. Finally a *Bsp*H I fragment of pLIN109 was cloned into the *Bsp*H I site of pLIN103 to make plasmid pLIN110.

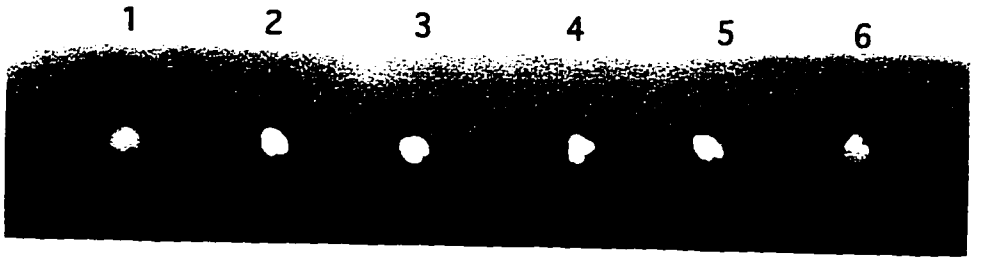


pLIN100 by digestion with *Xba*I and *Kpn*I and cloned into a similarly cut region of the polylinker of pMU1328 to yield pLIN103. Finally a 979 bp DNA fragment corresponding to nucleotides 1213 - 2192 of the *hylB* gene was excised from pLIN109 by digestion with *Bsp*H I and cloned into the *Bsp*H I site of pLIN103 to give pLIN110.

Transformation of group B streptococcus. Plasmid pLIN110 was transformed into serotype III GBS strain 3502 by electroporation. Transformants were selected by their ability to grow on THB-agar plates containing 4 μg/ml erythromycin. Antibiotic-resistant colonies were pooled and used to inoculate 50 ml of THB containing 4 μg/ml erythromycin. Approximately 10^9 cfu were then plated on each of 20 THB-agar plates containing 5 μg/ml chloramphenicol in order to select transformants expressing the CAT gene. Only 2 antibiotic-resistant colonies were obtained after overnight incubation. These were streaked on THB-agar plates containing 20 μg/ml chloramphenicol and, after overnight growth, 72 randomly selected colonies were tested for hyaluronidase production on HA-BSA plates. Four isolates, 110M4, 110M16, 110M21 and 110M26, did not produce any clear zones indicating that they were hyaluronidase-negative (Fig. 2). However, large clear zones, indicative of hyaluronidase production, were obtained for the parental strain 3502 and an erythromycin-resistant transformant (110EM) containing replicating plasmid pLIN110. However, the *hylB*$^-$ mutants no longer exhibited resistance to erythromycin.

In order to determine the stability of *hylB*$^-$ mutant 110M4, it was subcultured several times in THB media in the presence or absence of chloramphenicol. Aliquots of bacteria from each of the subcultures were plated on the T-Soy sheep blood agar plates and on plates containing chloramphenicol. We found that there were negligible differences

FIG. 2. Plate assay for hyaluronidase activity of GBS strains. The diameter of the clear zones surrounding the bacterial colonies is roughly proportional to log of the amount of hyaluronidase produced. The spotted colonies correspond to: 1, the parental serotype III GBS strain 3502; 2 to 5, *hylB* mutant strains 110M4, 110M16, 110M21 and 110M26, respectively; 6, strain 110Em which contains replicating plasmid pLIN110.

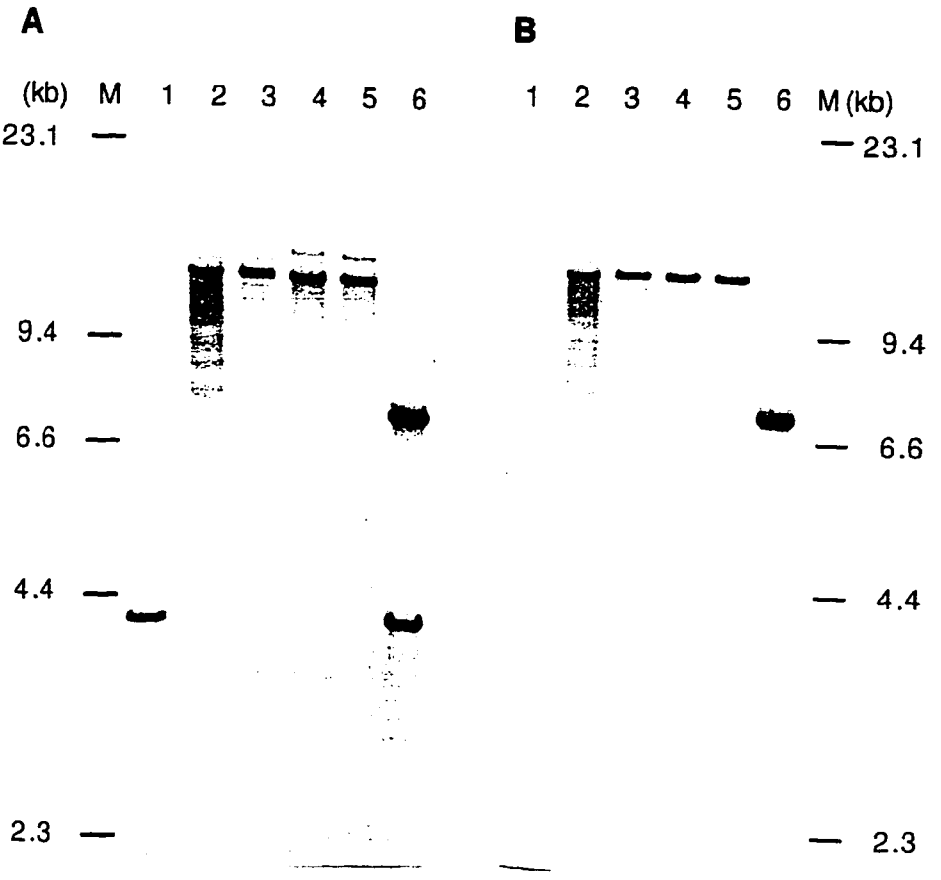


in the number of colonies on either set of plates. More than 10^3 single colonies of clone 110M4 were then spotted on HA-BSA plates to test for revertants producing hyaluronidase. None were found. In addition, culture supernate from mid-log phase cultures of a mutant strain exhibited no detectable hyaluronidase activity in either the HA-BSA plate assay or the spectrophotometric assay.

Evidence that plasmid pLIN110 inserted into the *hylB* gene. Total cellular DNA was isolated from the four *hylB*$^-$ mutant strains, the parental strain 3502, and strain 110Em which contains replicating plasmid pLIN110. DNA was digested with *Hind*III, separated on a 0.7 % agarose gel, and blotted to nitrocellulose membranes for Southern analysis. Two probes were used, a 0.8 kb *Hind* III/*Pst* I *hylB* fragment excised from pLIN101 (probe V) and linearized plasmid pMU1328 DNA. As shown in Fig. 3a, probe V hybridized with a 4 kb *Hind*III fragment of 3502 DNA (lane 1) and 110Em DNA (lane 6). An 8.7 kb band corresponding to *Hind* III digested plasmid pLIN110 was also observed in strain 110Em (lane 6). This probe also hybridized to 15 kb fragments of DNA from the four mutant strains (lanes 2-5). When linearized pMU1328 DNA was used as a probe it also hybridized with the 15 kb *Hind* III fragment of DNA from the mutants and the 8.7 kb band of plasmid DNA from 110Em (Fig. 3b). It did not hybridize with any DNA fragments from the parental strain 3502. These results indicated that plasmid pLIN110 had integrated into the *hylB* gene of the mutants. The absence of an 8.7 kb band in Southern blots of mutant DNA also indicated that free plasmid was no longer present.

Additional evidence that the mutants did not contain replicating plasmid was obtained from Southern blots of non-digested total cellular DNA probed with linearized

FIG. 3. Southern blot analysis of plasmid integration into the *hylB* gene of the GBS 3502 chromosome. *Hind* III-digested samples of total cellular DNA were probed with either a 0.8 kb *Hind* III/*Pst* I DNA fragment (885 bp - 1715 bp of the *hyl*B gene) in panel A, or with linearized pMU1328 in panel B. Lane 1, 3502 DNA; Lanes 2 to 5, 110M4, 110M16, 110M21 and 110M26 DNA respectively; Lane 6, 110Em DNA.



pMU1328. Distinct bands corresponding to free plasmid were observed for strain110Em. The probe did not hybridize with DNA from the parental GBS strain 3502. However, the four mutant strains gave bands near the origin that were larger than the 23 kb standard (data not shown). Since *Hind* III and *Eco*R I digests of DNA from all four mutants gave identical Southern blots when probed with probe V and *Hind* III linearized pMU1328 (data not shown) they were probably identical and only one of them, 110M4, was selected for further characterization.

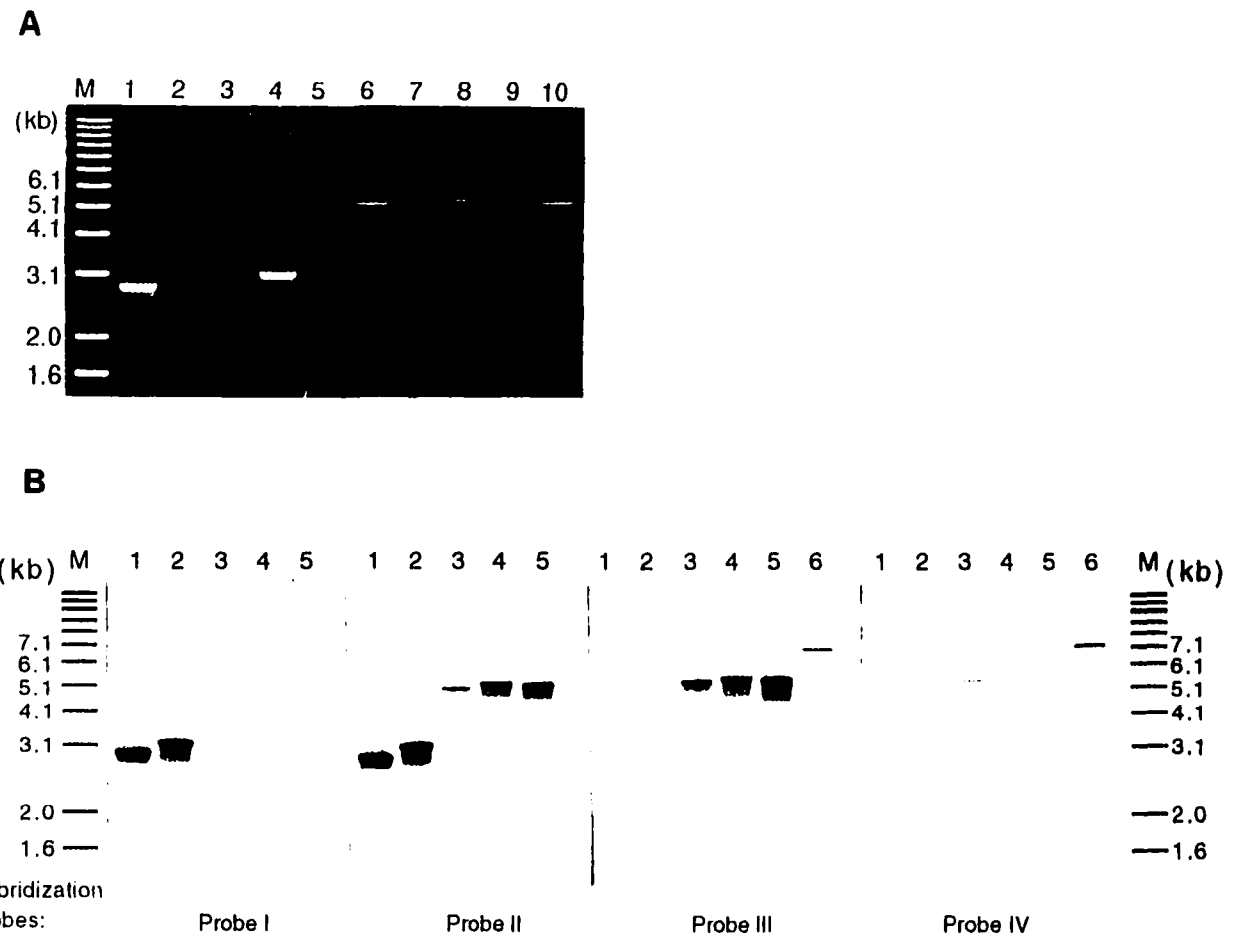
Detailed analysis of plasmid integration into the chromosome. The observed resistance of the mutants to chloramphenicol indicated that the *cat* gene had integrated into the GBS chromosome downstream of an active promoter. The *hylB*$^-$ mutant, as pointed out previously, did not exhibit resistance to erythromycin, and PCR primers specific for the erythromycin resistance gene failed to detect it in the DNA isolated from *hyl*B$^-$ mutant. Similarly, primers BL3 and BL5 specific for vector sequences flanking the *hylB* fragment (nucleotides 226-1194) in pLIN110 amplified the expected 1 kb DNA fragment from DNA extracted from 110Em but not from the mutant (data not shown). Finally, Southern blots obtained for *Hind* III, *Eco*R I, and other restriction endonuclease digests of mutant DNA indicated that the multicloning site of pMU1328 was not present in the mutant chromosome (Fig. 3 and Fig. 6). These experiments are consistent with the deletion a 3.5 kb fragment of plasmid pLIN110 DNA starting upstream of the *cat* gene to downstream of *Em* gene. The apparent size of the insert suggested that two copies of part of plasmid pLIN110 had integrated into the chromosome.

Additional information on the nature of the integration of the plasmid pLIN110 into the GBS chromosome was obtained by selective PCR amplifications using 5 different sets of primers and Southern blot analysis of the amplified products. The first set of primers, 113 and 2995, were chosen since they were located completely outside of the recombination region. They amplified a 2.8 kb DNA fragment from 3502 chromosomal DNA (Fig. 4a) which as shown in Fig. 4b hybridized with a 0.5 kb *hylB* fragment (probe I) and with a 0.5 kb *Pst*I/*Eco*RI *hylB* fragment (probe II) but not with a 1.1 kb *Hpa*I/*Bsp*HI fragment of pMU1328 (probe III) or a PCR amplified 0.8 kb Em gene fragment (probe IV). This set of primers did not yield detectable products when mutant DNA was used as the template. This presumably was because the sequences specific for this set of primers were much further apart in the mutant DNA and under our PCR conditions fragments much larger than 6 kb are not amplified.

The second pair of primers included 113 and BL10. The 22-mer BL10 sequence was specific for plasmid pMU1328 at a position 2 kb downstream of the *cat* gene start codon. When the template was 3502 chromosomal DNA, no bands were obtained (Fig 4a, lane 3), but a 3.1 kb DNA fragment was amplified from mutant chromosomal DNA (Fig. 4a, lane 4). This fragment hybridized with the two *hylB* probes I and II (Fig 4b).

The two primers in the third set, 2010 and 1768, are oriented in opposing directions and therefore do not amplify the intervening section of the *hylB* gene when 3502 DNA was the template (Fig 4a, lane 5). However, they did lead to the amplification of a 5.2 kb PCR fragment from the mutant DNA (lane 6) which hybridized with probes II and III. Since this PCR product overlaps with probe II only 53 bp at the *Pst*I site, the intensity of hybridization band is weaker than other bands on the same blot (Fig. 4b, lane

FIG. 4. PCR analysis of the pLIN110 insertion. (A) Total cellular DNA isolated from parental strain 3502 served as the template in lanes 1, 3, 5, 7, and 9; whereas in lanes 2, 4, 6, 8, and 10 the template was DNA from the mutant strain 110M4. The five pairs of primers used in the PCR experiments are as follows: lanes 1 and 2, primers 113 and 2995; lanes 3 and 4, primers 113 and BL10; lanes 5 and 6, primers 2010 and 1768; lanes 7 and 8, primers BL14 and BL5; and lanes 9 and 10, primers BL9 and 2995. (B) Analysis of the PCR products by Southern blotting. Probe I is a 0.5 kb *Hind* III fragment of the *hyl*B gene. Probe II is a 0.5 kb *Pst* I/*Eco*R I fragment of the *hyl*B gene. A 1.1 kb *Hpa* I/*Bsp*H I fragment of pMU1328 containing the entire CAT gene was probe III. Probe IV, a 0.8 kb *Em* gene fragment, was amplified from pMU1328 by PCR. 10 μl of each PCR product shown in lanes 1, 4, 6, 8, and 10 of panel A were analyzed in lanes 1, 2, 3, 4, and 5 respectively of panel B. Lane 6 contained *Hind* III linearized pMU1328.



3). However, this fragment gave an intense band when probe III, which contains the *cat* gene, was used.

In order to study the possibility that two copies of the *cat* gene had also been inserted into the chromosome, the primer set BL5 and BL14 was used. These primers were specific for sequences on the *cat* gene but, once again, they pointed away from each other. They led to the amplification of a 5.3 kb DNA fragment from mutant DNA (Fig 4a, lane 8) and, of course, no product from parental strain 3502 DNA (Fig 4a, lane 7). The amplified fragment of mutant DNA hybridized with a 1.1 kb *cat* gene fragment from pMU1328 (probe III) as shown in Fig. 4b, lane 4, and also with the *hylB* gene fragment, probe II (Fig. 4b, lane 4).

Primers BL9 and 2995 were used to further define the nature of plasmid integration. As shown in Fig. 4a, lane 10, when mutant chromosomal DNA was used as the template, a 5.2 kb DNA fragment was amplified which hybridized with probes II and III, but not with probe I. This result along with the other PCR results indicate that two copies of part of plasmid pLIN110 had integrated into the *hylB* gene (between nucleotides 1213-2192) of the GBS chromosome as illustrated in Fig. 5.

To further define the exact nature of the plasmid insertion into the *hylB* gene, Southern analysis of chromosomal DNA from mutant 110M4 and the parental strain 3502 was carried out after digestion of the DNA with *Pfi*M I and *Bst*E II, *Eco*R I, and *Eco*R V. Restriction enzyme sites for *Pfi*M I and *Bst*E II flank the site of the plasmid integration in the *hylB* gene but are not present in plasmid pMU1328. Blots probed with a 0.5 kb *Pst* I/*Eco*R I fragment of *hylB* (Probe II), therefore, hybridized with a 2.4 kb *Pfi*M I/*Bst*E II DNA fragment of 3502 DNA (Fig. 6a, Lane 1). However, this probe hybridized

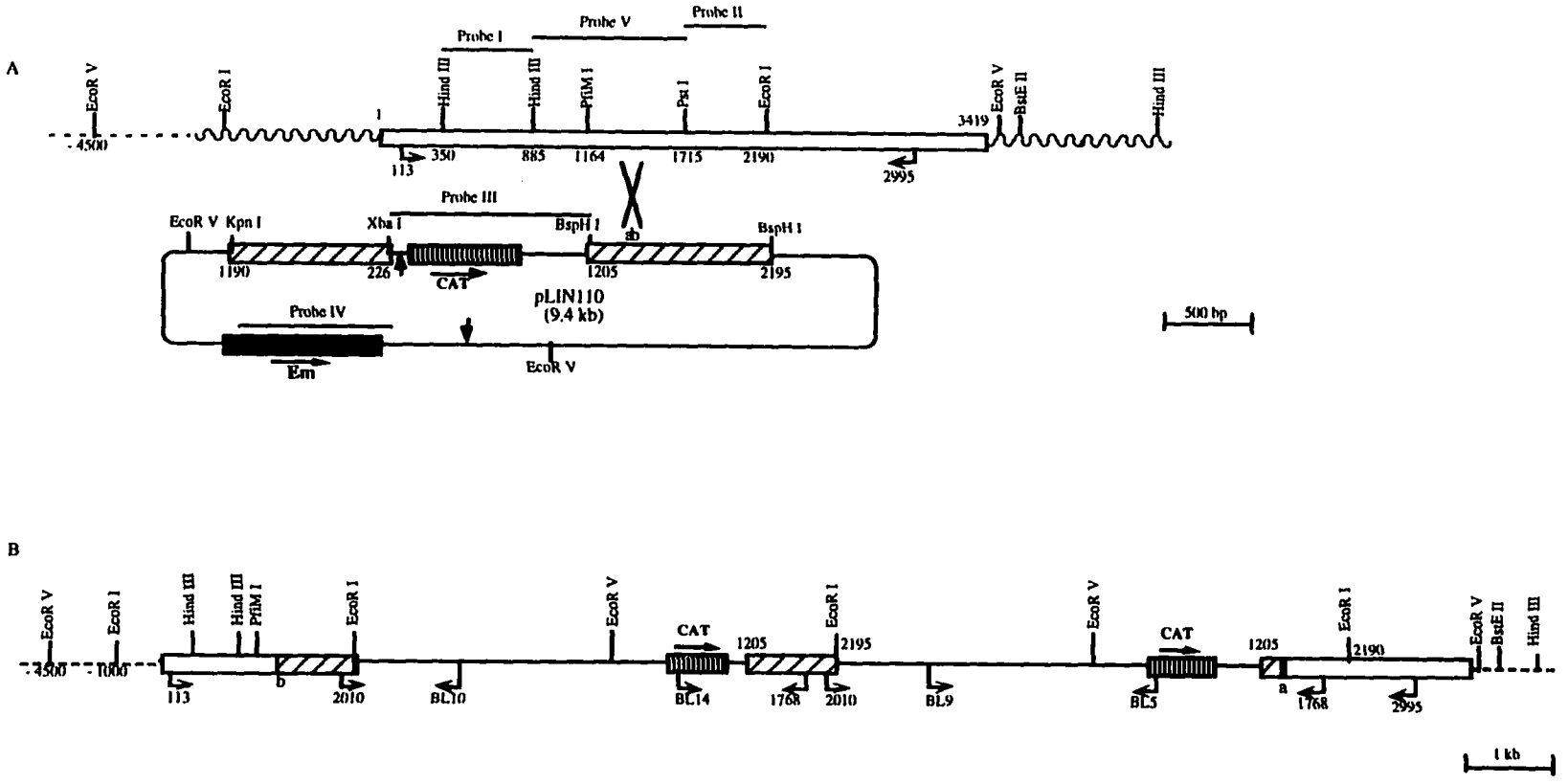
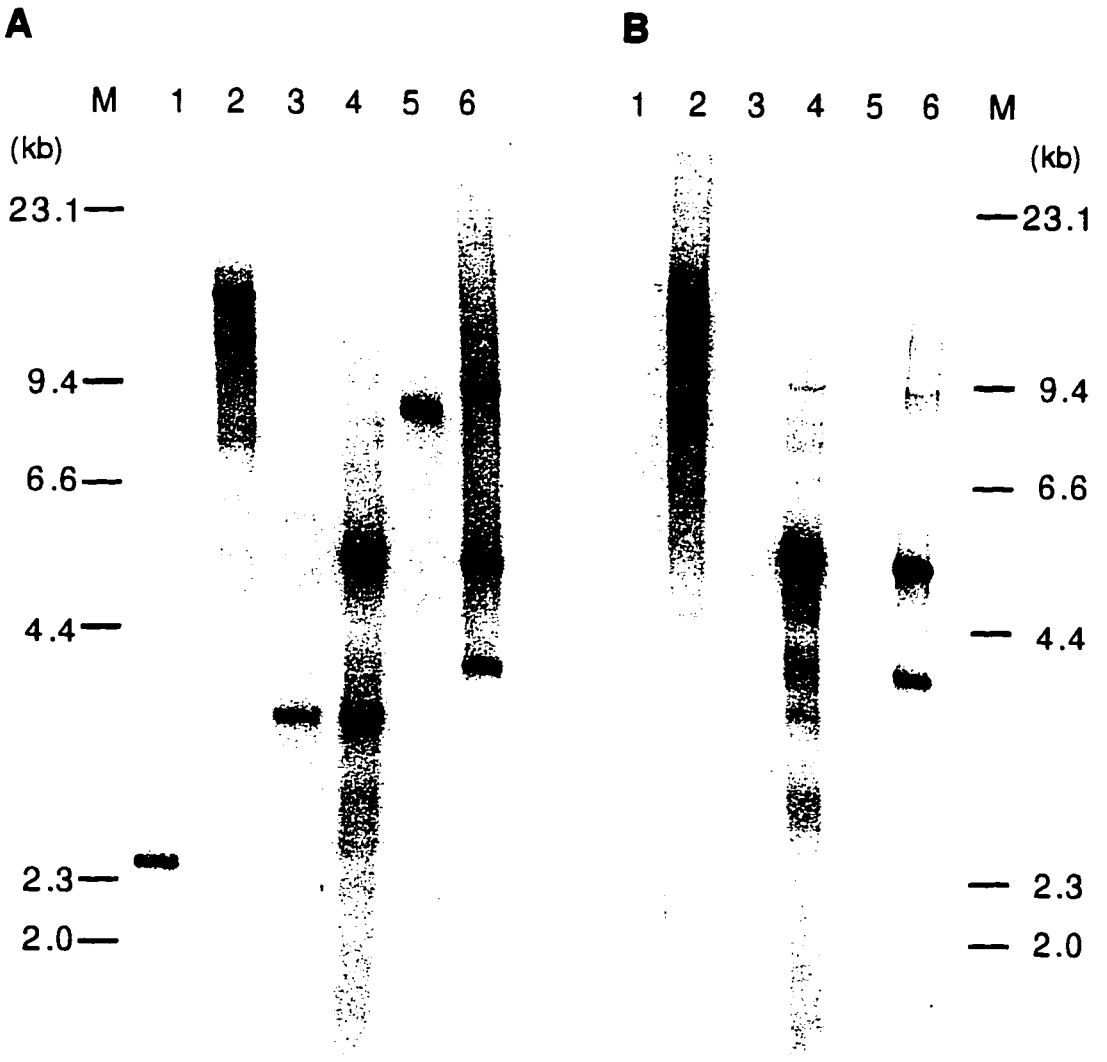


FIG. 5. Integration of plasmid pLIN110 into the chromosome of GBS 3502. The primers used to analyze the nature of the insertion are depicted by small arrows. Two copies of part of the plasmid are shown to have inserted.

FIG. 6. Southern blot analysis of the disrupted *hylB* gene. Lanes 1, 3, and 5 correspond to total cellular DNA from GBS 3502 and lanes 2, 4, and 6 from the *hylB* mutant. DNA samples were digested with *Pfi*M I/*Bst*E II (1,2), *Eco*R I (3,4), and *Eco*R V (5,6). Filters were hybridized with either a 0.5 kb *Pst* I-*Eco*R I fragment of the *hyl*B gene (A) or a 1.1 kb pMU 1328 *cat* gene fragment obtained by PCR amplication (B).



with a 14 kb fragment of the mutant DNA (Lane 2). The 11.6 kb difference is consistent with the above PCR results, indicating again that more than one copy of part of plasmid pLIN110 had inserted into the GBS chromosome.

An *Eco*R I site is present near the 3' end of the *hylB* gene fragment cloned downstream of the *cat* gene in pLIN110. An *Eco*R I digest of genomic DNA probed with a *hyl*B fragment (probe II) specific for this region hybridized with a 3.2 kb fragment from both the parental and mutant strains (Fig. 6a, lanes 3 and 4). In addition, a 5.4 kb fragment of mutant DNA also hybridized with the probe.

*Eco*R V does not cut within the *hylB* gene but it does cut at two sites in the plasmid pMU1328 (1). A 9.5 kb fragment of *Eco*RV-digested 3502 DNA containing the complete *hylB* gene hybridized with probe II (Fig. 6a, lane 5). This probe also hybridized with 10.5, 5.4 and 4 kb DNA fragments of mutant DNA cut with *Eco*R V. This result is also consistent with the model illustrated in Fig. 5 in which one of the two original EcoR V sites in the plasmid has been lost. Similar blots probed with a fragment of the *cat* gene (probe III), detected a 14 kb *Pfi*M I/*Bst*E II fragment, a 5.4 kb *Eco*R I fragment, and two *Eco*R V fragments of 5.4 and 4.0 kb in digests of mutant DNA. These results further confirm that two copies of the part of plasmid pLIN110 containing the *cat* gene and a fragment (1213-2192) of the *hylB* gene inserted at the target site shown in Fig. 5.

The exact mechanism of pLIN110 integration is not clear at this time. However, information available on the nature of the replication of plasmid pMU1328 suggests one possibility. The plasmid possesses a gram-positive replicon originating from plasmid pVA380-1 that replicates by a rolling circle (RC) mode (6, 8). The *Rep* protein (replication protein encoded by plasmid) plays an essential role in the control of

replication (4). *Rep* protein introduces a nick in one of the parental DNA strands to generate a 3' OH end which is then elongated (leading strand synthesis). It subsequently catalyzes the covalent closing of the newly synthesized strand, resulting in a double stranded DNA plasmid and a single stranded circular DNA which serves as a template for the lagging strand synthesis. The formation of the ss-DNA appears to contribute to plasmid instability which may lead to plasmid loss or curing, particularly under non-selective conditions (8, 11). This instability may partially explain why pLIN110 was lost in the GBS *hylB*⁻ mutants. It is also possible that ss-pLIN110 in GBS 3502 might participate in an homologous recombination resulting in the chromosomal integration of the plasmid. In addition, the very nature of RC replication makes it probable that some pLIN110 dimers will be formed which might also undergo recombination. For example, pJRS1039 derived from pWV01, another RC type plasmid, was reported to insert into *Streptococcus pyogenes* chromosome as a dimer (12). Our PCR and Southern blots results demonstrated that two segments of pLIN110 tandemly inserted into the *hylB* gene in the 3502 genome. Similar tandem integrations complicated the interpretation of hybridization patterns obtained in a molecular genetic study of *Streptomyces* (18). Clearly, further studies will be needed to more completely understand the precise mechanism of pLIN110 integration.

We are currently using the *hylB*⁻ mutant, GBS 110M4, in animal studies to investigate the role of hyaluronate lyase in GBS pathogenesis. We anticipate that it will prove to be very useful in this and other studies of GBS pathogenesis.

ACKNOWLEDGMENTS

Nucleotide sequencing was carried out in the DNA Sequencing Core Facility of the Center for AIDS Research and DNA sequence analysis was carried out using the University of Wisconsin Genetics Computer Group (GCG) program. Both were supported in part by grant number P30-AI-27767 from the National Institute of Allergy and Infectious Diseases. We would like to thank Dr. Janet L. Yother and Dr. Susan K. Hollingshead for their extremely helpful comments and suggestions. We thank William F. Averett for his able technical assistance.

REFERENCES

1. **Achen, G. M., B. E. Davidson, and A. J. Hillier.** 1986. Construction of plasmid vectors for the detection of streptococcal promoters. Gene. **45:** 45-49.

2. **Berry, A. M., R. A. Lock, M. S. Thomas, P. D. Rajan, D. Hansman, and C. J. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *E. coli*. Infect. Immun. **62**: 1101-1108.

3. **Berry, A. M., C. J. Paton, and D. Hansman.** 1992. Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3. Microb. Pathog. **12:** 87-93.

4. **Espinosa, M., G. D. Solar, F. Rojo, and J. C. Alonso.** 1995. Plasmid rolling circle replication and its control. FEMS Microbiol. Letters. **130:** 111-120.

5. **Farrell, M. A., D. Taylor, and T. K. Holland.** 1995. Cloning, nucleotide sequence determination and expression of the *Staphylococcus aureus* hyaluronate lyase gene. FEMS Microbiol. Letters. **130:** 81-85.

6. **Gruss, A., and D. S. Ehrlich.** 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol. Rev. **53:** 231-241.

7. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150:** 815-825.

8. **Leblanc, D. J., L. N. Lee, and A. Abu-al-jaibat.** 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. Plasmid. **28:** 130-145.

9. **Lin, B., S. K. Hollingshead, J. E. Coligan, M. L. Egan, and J. R. Baker.** 1994. Cloning and expression of the gene for group B streptococcal hyaluronate lyase. J. Biol. Chem. **269:** 30113-30116.

10. **Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell.** 1982. A cloning vector able to replicate in *Escherichis coli* and *Streptococcus sanguis*. Gene. **19:** 345-353.

11. **Michel, B., and S. D. Ehrlich.** 1986. Illegitimate recombination occurs between the replication origin of the plasmid pC194 and a progressing replication fork. EMBO J. **5:** 3691-3696.

12. **Perez-Casal, J. F., H. F. Dillon, L. K. Husmann, B. Graham, and J. R. Scott.** 1993. Virulence of two *Streptococcus pyogenes* strains (types M1 and M3) associated with toxic-shock-like syndrome depends on an intact *mry*-like gene. Infect. Immun. **61:** 5426-5430.

13. **Pritchard, D. G., B. Lin, T. R. Willingham, and J. R. Baker.** 1994. Characterization of the group B streptococcal hyaluronate lyase. Arch. Biochem. Biophys. **315:** 431-437.

14. **Ruben, C. E., M. R. Wessels, L. M. Heggen, and D. L. Kasper.** 1987. Transposon mutagenesis of type III group B streptococcus: correlation of capsule expression with virulence. Proc. Natl. Acad. Sci. USA. **84:** 7208-7212.

15. **Scott, J. R., F. Bringel, D. Marra, G. V. Alstine, and C. K. Rudy.** 1994. Conjugative transposition of Tn*916*: preferred targets and evidence for conjugative transfer of a single strand and for a double-stranded circular intermediate. Mol. Microbiol. **11:** 1099-1108.

16. **Smith, R. F., and N. P. Willett.** 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. Appl. Microbiol. **16:** 1434-1436.

17. **van de Rijn, I., and R. E. Kessler.** 1980. Growth characteristics of group A *Streptococci* in a new chemically defined medium. Infect. Immun. **27:** 444-448.

18. **Wohlleben, W., and G. Muth.** "*Streptomyces* plasmid vectors." Plasmids, a Practical Approach. Hardy ed. 1993. 145-175, IRL Press. Oxford.

19. **Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki.** 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. J. Biol. Chem. **243:** 1523-1535.

SUMMARY

Group B streptococci (GBS) are gram-positive bacteria that can cause serious, often fatal, neonatal sepsis, pneumonia and meningitis. Despite the fact that GBS remain highly susceptible to antibiotics, over 1,000 babies still die of GBS infections in the United States annually (82). Asymptomatic carriage of GBS is extremely common but under certain circumstances the bacteria can systemically invade a susceptible host and cause life-threatening infections. It is therefore very important to elucidate the nature of the bacterial factors that contribute to the invasive properties of GBS. This dissertation focuses on two such factors of GBS, both degradative enzymes: an oligopeptidase and a hyaluronate lyase.

GBS oligopeptidase (PepB)

The recent report that GBS possess a cell-associated collagenase was based primarily on the ability of the enzyme to hydrolyze the synthetic peptide FALGPA, a widely used collagenase substrate (34). This enzyme was of particular interest because of the possibility that it might act in concert with other degradative enzymes in breaking down the extracellular matrix of host tissues, thereby facilitating GBS invasion. We isolated and purified this enzyme from GBS serotype III 3502 strain. Surprisingly, neither the purified enzyme nor the crude GBS cell lysate could solubilize a film of reconstituted rat tail tendon collagen. This enzyme also did not exhibit any gelatinase activity. It became clear that the enzyme was not a collagenase after all.

Additional experiments were performed to further study the biochemistry and molecular genetics of the GBS enzyme. The enzyme was first purified to homogeneity and tryptic peptides were prepared and sequenced. On the basis of the peptide sequences, degenerate primers were designed and used to amplify an internal gene fragment by PCR. This was then used as a probe to screen a phage library of GBS chromosomal DNA fragments. Finally, the single-specific-primer PCR technique was used to complete the cloning of the full length gene. The striking amino acid sequence similarity between the GBS enzyme and an oligopeptidase (*PepF*) isolated from *Lactococcus lactis* suggested that the GBS enzyme might also exhibit oligopeptidase activity. This possibility was confirmed when it was shown that the purified GBS enzyme cleaved several different bioactive peptides at exactly the same positions as did the lactococcal enzyme. For example, besides hydrolyzing FALGPA, the GBS enzyme also degraded bradykinin, neurotensin, peptide fragments of substance P and adrenocorticotropin. Like PepF, the GBS enzyme did not exhibit any protease activity. Clearly, the previously described GBS collagenase is actually an endo-oligopeptidase. We, therefore, named the GBS enzyme oligopeptidase PepB and the corresponding gene *PepB*.

The biological function of GBS *PepB* is not known. It may serve as a housekeeping gene to metabolize peptide fragments resulting from the degradation of proteins. One intriguing possibility is that GBS oligopeptidase may contribute to GBS pathogenesis by degrading certain bioactive peptides involved in the regulation of inflammatory responses to bacterial infections. If this hypothesis is true, a peptide transport system would be expected to be present since most PepB activity is found

intracellularly. Preliminary evidence that this is indeed the case was obtained from experiments that showed that GBS rapidly removes bradykinin added to a chemically defined medium containing an excess of all 20 amino acids. Further experiments are needed to study this interesting phenomenon and to elucidate the biological roles of oligopeptidase *PepB*.

Biochemical characterization of GBS hyaluronate lyase

Hyaluronidase was first recognized as a potential GBS virulence factor in 1941 by McClean (53). It was suggested that the enzyme facilitates bacterial invasion by degrading hyaluronan, one of the major components of extracellular matrix of tissues. There were no reports describing the biochemical properties of GBS hyaluronidase when we began our initial studies. However, for almost 25 years several investigators incorrectly attributed what was really GBS hyaluronidase activity to a so-called "neuraminidase." The explanation for such a serious misidentification of the enzyme was that the mucin preparations almost universally used as substrates for the enzyme were contaminated with hyaluronic acid. The thiobarbituric acid assay thought to be measuring released sialic acid was actually detecting the unsaturated disaccharide product of hyaluronate lyase digestion.

GBS hyaluronidase is more precisely referred to as a hyaluronate lyase because the reaction it catalyzes is not a hydrolysis but an elimination reaction resulting in the formation of an unsaturated disaccharide product. The GBS enzyme therefore differs from two other types of hyaluronidase that degrade HA by hydrolyzing either the β-1,4 glycosidic bond between 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid (e.g.,

testicular hyaluronidase) (12) or the β-1,3 glycosidic bond between D-glucuronic acid and 2-acetamido-a-deoxy-D glucose (leech hyaluronidase) (40).

Molecular cloning and sequence analysis of the gene for GBS hyaluronate lyase

In order to facilitate studies of the possible pathogenic roles of GBS hyaluronate lyase, the gene for the enzyme was cloned and expressed in *E. coli.* Using an improved enzyme purification procedure, we isolated sufficient enzyme from 150 liters of GBS culture to prepare tryptic peptides and we sequenced several of them. Based upon the amino acid sequences of two selected peptides, degenerate oligonucleotide primers (both forward and reverse) were designed and synthesized. One combination of primers resulted in the PCR amplification of an internal gene fragment of GBS hyaluronate lyase. This was then used as a probe to screen a phage library of chromosomal DNA fragments. Since no single clone was found to contain the entire gene it was necessary to reconstruct the gene from two plasmids containing inserts with suitable overlapping sequences. The recombinant GBS hyaluronate lyase gene was then cloned into an *E. coli* expression vector which resulted in the expression of a high level of enzyme activity.

The *hylB* gene consists of 2952 base pairs coding for a protein with a molecular mass of 111.319 kDa. A typical Shine-Dalgarno (SD) sequence was not present in the cloned gene but a triple repeat of unknown function sequence was located fourteen nucleotides upstream of the start codon. A comparison of the full-length sequence of *hyl*B with *hyl*A, a hyaluronidase isolated from *Streptococcus pneumoniae,* showed 50.7 % amino acid sequence identity (6). On the other hand, *hylB* gene exhibited no significant similarity with the *hyl*P1 and *hyl*P2 hyaluronidase genes encoded by *Streptococcus pyogenes* bacteriophage (31, 32, 33).

Insertional inactivation of the group B streptococcal lyase gene

Investigations of the pathogenic effects of GBS hyaluronate lyase would be greatly facilitated by the availability of GBS mutants unable to produce the enzyme. Unfortunately, producing such mutants has been difficult. There currently are no published procedures for producing GBS mutants targeted to a specific gene. Several GBS mutants that have been described were made by Tn*916* transposon mutagenesis (67, 79). However, it is now widely recognized that this transposon does not insert randomly into the bacterial chromosome and certain potentially useful mutations are never obtained (71) . We screened more than 8000 Tn*916* transconjugates without finding a single hyaluronate lyase-negative mutant.

Another commonly used procedure for specifically mutating selected bacterial genes is insertion-duplication mutagenesis. This method, for example, has been successfully used to insertionally inactivate the genes encoding pneumolysin and autolysin in pneumococci (7). Using this approach, we made several *E. coli*-streptococcal shuttle vector constructs containing a segment of the GBS hyaluronate lyase gene (*hylB*). These plasmid constructs were then introduced into GBS cells by electroporation in an attempt to inactivate the hyaluronate lyase gene by an insertion-duplication event. Unfortunately this approach also was unsuccessful in GBS, possibly because of the difficulty of obtaining the desired mutant when the transformation efficiency and the probability of chromosomal integration are both extremely low, and maybe also because such insertions are inherently unstable in GBS. We then made a number of plasmid constructs in which two segments of the GBS gene were separated by a promoterless chloramphenicol acetyltransferase (CAT) gene in the hope that we might be able to force

a double crossover event resulting in the inactivation of the hyaluronate lyase gene. In one of these constructs, the two GBS hyaluronate lyase gene segments were inadvertently placed in opposite orientation to each other. Electroporation of this construct into GBS produced the desired mutant.

The results of Southern blotting and PCR amplifications with selected primers revealed that two copies of part of plasmid pLIN110 had integrated into the *hylB* gene (between nucleotides 1205-2195) on the GBS chromosome. In addition, a 3.5 kb fragment of the plasmid, including the gene for erythromycin resistance, had been deleted. This hyaluronidase-negative mutant is extremely stable and we were not able to obtain any revertants, even after extensive subculturing in the absence of antibiotics. The mutant is currently being used in animal studies to further investigate the role of hyaluronate lyase in GBS pathogenesis. We anticipate that it will prove to be very useful in this and future studies of GBS pathogenesis.

GENERAL REFERENCES

1. **Agnoli, F. L.** 1994. Group B streptococcus: perinatal considerations. J. Family Practice. **39**: 171-177.

2. **Aho, R., S. Jalkanen, and H. Kalimo.** 1994. CD44-hyaluronate interaction mediates in vitro lymphocyte binding to the white matter of the central nervous system. J. Neuropathol. Exp. Neurol. **53**: 295-302.

3. American Academy of Pediatrics Committee on Infectious Diseases and Committee on Fetus and Newborn. 1992. Guidelines for prevention of group B streptococcal (GBS) infection by chemoprophylaxis. Pediatrics **90**: 775-778.

4. **Ayers, S. H., and P. Rupp.** 1922. Differentiation of hemolytic streptococci from human and bovine sources by hydrolysis of sodium hippurate. J. Infect. Dis. **30**: 388-399.

5. **Baker, C. J., D. K. Goroff, S. Alpert, V. A. Crockett, S. H. Zinner, J. R. Evrard, B. Rosner, and W. M. McCormack.** 1977. Vaginal colonization with group B streptococcus: a study in college women. J. Infect. Dis. **135**: 392-397.

6. **Berry, A. M., R. A. Lock, S. M. Thomas, D. P. Rajan, D. Hansman, and J. C. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *Escherichia coli.* Infect. Immun. **62**: 1101-1108.

7. **Berry, A. M., J. C. Paton, and D. Hansman.** 1992. Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3. Microb. Pathog. **12**: 87-93.

8. **Bhoola, K. D., C. D. Figueroa, and K. Worthy.** 1992. Bioregulation of kinins: kallikerins, kininogens, and kininases. Pharmacol. Rev. **44**: 1-80.

9. **Bond, M. D., and E. V. Wart.** 1984. Characterization of the individual collagenases from *Clostridium histolyticum.* Biochemistry **23**: 3085-3091.

10. **Bowdy, B. D., S. M. Aziz, S. L. Marple, K. Yoneda, T. H. Pauly, D. Coonrod, and M. N. Gillespie.** 1990. Organ-specific disposition of group B streptococci in

piglets: evidence for a direct interaction with target cells in the pulmonary circulation. Pediatric. Res. **27**: 344-348.

11. **Christie, R., N. E. Atkins, and E. Munch-Petersen.** 1944. A note on a lytic phenomenon shown by group B streptococci. Aust. J. Exp. Biol. Med. Sci. **22**: 197-200.

12. **Cramer, J. A., L. C. Bailey, C. A. Bailey, and R. T. Miller.** 1994. Kinetic and mechanistic studies with bovine testicular hyaluronidase. Biochim. Biophys. Acta. **1200**: 315-321.

13. **Crocker, P. R., S. Kelm, C. Dubois, B. Martin, A. S. McWilliam, D. M. Shotton, J. C. Paulson, and S. Gordon.** 1991. Purification and properties of sialo adhesin, a sialic acid binding receptor of murine tissue macrophages. EMBO. **7**: 1661-1669.

14. **Dillon, H. C., E. Gray, A. M. Pass, and M. B. Gray.** 1979. Anorectal and vaginal carriage of group B streptococci in pregnant women. J. Infect. Dis. **145**: 794-799.

15. **Dillon, H. C. Jr, S. Khare, and B. M. Gray.** 1987. Group B streptococcal carriage and disease: a 6-year prospective study. J. Pediatr. **110**: 31-36.

16. **Duran-Reynals, F.** 1933. Studies on a certain spreading factor existing in bacteria and its significance for bacterial invasiveness. J. Exp. Med. **58**: 161-181.

17. **Erdos, E. G.** 1990. Some old and some new ideas on kinin metabolism. J. Cardiovasc. Pharmacol. **15**: 520-524.

18. **Facklam, R. R., J. F. Padula, and L. G. Thacker.** 1974. Presumptive identification of groups A, B and D streptococci. Appl. Microbiol. **27**: 107-113.

19. **Farley, M. M., R. C. Harvey, T. Stull, J. D. Smith, A. Schuchat, J. D. Wenger, and D. S. Stephens.** 1993. A population-based assessment of invasive disease due to group B streptococcus in nonpregnant adults. N. Engl. J. Med. **328**: 1807-1811.

20. **Farrell, A. M., D. Taylor, and K. T. Holland.** 1995. Cloning, nucleotide sequence determination and expression of the *Staphylococcus aureus* hyaluronate lyase gene. FEMS Microbiol. Letters. **130**: 81-85.

21. **Ferrieri, P.** 1985. GBS enzymes, hemolysin, toxins and other products. Antibiot. Chemother. **35**: 57-70.

22. **Ferrieri, P., L. W. Wannamaker, and J. Nelson.** 1973. Localization and characterization of the hippuricase activity of group B streptococci. Infect. Immun. 7: 747-752.

23. **Fry, R. M.** 1938. Fatal infections by hemolytic streptococcus group B. Lancet **1**: 199-201.

24. **Galen, J. E., J. M. Ketley, A. Fasano, S. H. Richardson, S. S. Wasserman, and J. B. Kaper.** 1992. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. Infect. Immun. **60**: 406-415.

25. **Gibson, R. L., M. K. Lee, C. Soderland, E. Y. Chi, and C. E. Rubens.** 1993. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect. Immun. **61**: 478-485.

26. **Gibson, R. L., C. Soderland, W. R. Henderson Jr, E. Y. Chi, and C. E. Rubens.** 1995. Group B streptococci (GBS) injure lung endothelium *In Vitro*: GBS invasion and GBS-induced eicosanoid production is greater with microvascular than with pulmonary artery cells. Infect. Immun. **63**: 271-279.

27. **Gmachl, M., and G. Kreil.** 1993. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. Proc. Natl. Acad. Sci. USA. **90**: 3569-3573.

28. **Hayano, S., A. Tanaka, and Y. Okuyama.** 1969. Distribution and serological specificity of sialidase produced by various groups of Streptococci. J. Bacteriol. **100**: 354-357.

29. **Helmig, R., J. T. Halaburt, N. Uldbjerg, A. C. Thomsen, and A. Stenderup.** 1990. Increased cell adherence of group B streptococci from preterm infants with neonatal sepsis. Obstet. Gynecol. **76**: 825-827.

30. **Hulse, M. L., S. Smith, E. Y. Chi, A. Pham, and C. E. Rubens.** 1993. Effect of type III group B streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. Infect. Immun. **61**: 4835-4841.

31. **Hynes, W. L., and J. J. Ferretti.** 1987. Cloning of the hyaluronidase gene from *Streptococcus pyogenes* bacteriophage H4489A. Streptococcal genetics 150-152.

32. **Hynes, W. L., and J. J. Ferretti.** 1989. Sequence analysis and expression in *Escherichia coli* of the hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A. Infect. Immun **57**: 533-539.

33. **Hynes, W. L., L. Hancock, and J. J. Ferretti.** 1995. Analysis of a second bacteriophage hyaluronidase gene from *Streptococcus pyogenes:* evidence for a third hyaluronidase involved in extracellular enzymatic activity. Infect. Immun. **63**: 3015-3020.

34. **Jackson, R. J., M. L. Dao, and D. V. Lim.** 1994. Cell-associated collagenolytic activity by group B streptococci. Infect. Immun. **62**: 5647-5651.

35. **Jacobsen, S.** 1966. Separation of two different substrates for plasma kinin-forming enzymes. Nature **210**: 98-99.

36. **Johnson, D. R., and P. Ferrieri.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J. Clin. Microbiol. **19**: 506-510.

37. **Kjems, E., B. Perch, and J. Henrichsen.** 1980. Serotypes of group B streptococci and their relation to hyaluronidase production and hydrolysis of salicin. J. Clin. Microbiol. **11**: 111-113.

38. **Klein, R. B., T. J. Fischer, S. E.. Gard, M. Biberstein, K. C. Rich, and E. R. Stiehm.** 1977. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics **60**: 467-472.

39. **Kogan, G., D. Uhrin, B. Jean-Robert, L. C. Paoletti, A. E. Blodgett, D. L. Kasper, and H. J. Jennings.** 1996. Structural and immunochemical characterization of the type VIII group B streptococcus capsular polysaccharide. J. Biol. Chem. **271**: 8786-8790.

40. **Kreil, G.** 1995. Hyaluronidases-a group of neglected enzymes. Protein Sci. **4**: 1666-1669.

41. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57**: 571-595.

42. **Laurent, T. C., and R. E. Fraser.** 1992. Hyaluronan. FASEB J. **6**: 2397-2404.

43. **Lin, Y., K. Mahan, W. Lathrop, D. Myles, and P. Primakoff.** 1994. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. J. Cell Biol. **125**: 1157-1163.

44. **Linden, V., K. K. Christensen, and P. Christensen.** 1983. The occurrence of R-protein among isolates of group B streptococci from human sources. Acta Pathol. Microbiol. Immunol. Scand. **91**: 153-156.

45. **Maeda, H., T. Akaike, Y. Sakata, and K. Maruo.** 1993. Role of bradykinin in microbial infection: enhancement of septicemia by microbial proteases and kinin. Proteases, Protease Inhibitors and Protease-Derived Peptides **42**: 159-165.

46. **Makinent, K. K., P. Makinen, and S. A. Syed.** 1992. Purification and substrate specificity of an endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35405, active on furylacryolyo-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267**: 14285-14293.

47. **Manroe, B. L., C. R. Rosenfeld, A. G. Weinberg, and R. Browne.** 1977. The differential leukocyte count in the assessment and outcome of early-onset neonatal group B streptococcal disease. J. Pediatr. **91**: 632-637.

48. **Manuel, F. R., S. W. MacDonald, and J. A. Embil.** 1980. Prevalence of group B beta-hemolytic streptococci in male urethra. Scand. J. Infect. Dis. **12**: 33-35.

49. **Martin, T. R., J. T. Ruzinski, C. E. Rubens, E. Y. Chi, and C. B. Wilson.** 1992. The effect of type-specific polysaccharide capsule on the clearance of group B streptococci from the lung of infant and adult Rats. J. Infect. Dis. **165**: 306-314.

50. **Mauer, M., M. C. Thirumoorthi, and A. S. Dajani.** 1979. Group B streptococcal colonization in prepubertal children. Pediatrics **64**: 65-67.

51. **Maxwell, G. L.** 1993. Perterm premature rupture of membranes. Obstet. Gynecol. Survey. **48**: 576-583.

52. **McCarthy, D. A., D. E. Potter, and E. D. Nicolaides.** 1965. An *in vivo* estimation of the potencies and half-lives of synthetic bradykinin and kallidin. J. Pharmacol. Exp. Ther. **148**: 117-122.

53. **McClean, D.** 1941. The capsulation of streptococci and its relation to diffusion factor (hyaluronidase). J. Pathol. Bacteriol. **53**: 13-27.

54. **McCracken, G. J.** 1973. Group B streptococci: the new challenge in neonatal infections. J. Pediatr. **82**: 703-706.

55. **McGregor, J. A., J. I. Mdcm, C. French, D. Lawellin, A. Franco-buff, C. Smith, and J. K. Todd.** 1987. Bacterial protease-induced reduction of chorioamniotic membrane strength and elasticity. Obstet. Gynecol. **69**: 167-174.

56. **Milligan, T. W., C. J. Baker, D. C. Straus, and S. J. Mattingly.** 1978. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B streptococci. Infect. Immun. **21**: 738-746.

57. **Milligan, T. W., D. C. Straus, and S. J. Mattingly.** 1977. Extracellular neuraminidase produced by group B streptococci. Infect. Immun. **18**: 189-195.

58. **Newton, E. R., and M. Clark.** 1988. Group B streptococcus and preterm rupture of membranes. Obstet. Gynecol. **71**: 198-202.

59. **Noble, M. A., J. M. Bent, and A. B. West.** 1983. Detection and identification of group B streptococci by use of pigment production. J. Clin, Pathol. **36**: 350-352.

60. **Pass, M. A., B. M. Gray, S. Khare, and H. C. Dillon.** 1979. Prospective studies of group B streptococcal infections in infants. J. Pediatr. **95**: 437-443.

61. **Primakoff, P., and D. G. Myles.** 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. Dev. Biol. **98**: 417-428.

62. **Rawlings, N. D., and A. J. Barrett.** 1995. Evolutionary families of metallopeptidases. Methods Enzymol. **248**: 183-228.

63. **Rojas, J., L. E. Larsson, C. G. Hellerqvist, K. L. Brigham, M. E. Gray, and M. T. Stahlman.** 1983. Pulmonary hemodynamic and ultrastructural changes associated with group B streptococcal toxemia in adult sheep and newborn lambs. Pediatric Research **17**: 1002-1008.

64. **Rosen, S. D., S. I. Chi, D. D. True, M. S. Singer, and T. A. Yednock.** 1989. Intravenously injected sialidase inactivates attachment sites for lymphocytes on high endothelial venules. J. Immunol. **142**: 1895-1902.

65. **Rubens, C. E., H. V. Raff, C. Jackson, E. Y. Chi, J. T. Bielitzki, and S. L. Hillier.** 1991. Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. J. Infect. Dis. **164**: 320-330.

66. **Rubens, C. E., S. Smith, M. Hulse, E. Y. Chi, and G. Van Belle.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect. Immun. **60**: 5157-5163.

67. **Ruben, C. E., M. R. Wessels, L. M. Heggen, and D. L. Kasper.** 1987. Transposon mutagenesis of type III group B streptococcus: correlation of capsule expression with virulence. Proc. Natl. Acad. Sci. USA. **84**: 7208-7212.

68. **Sakai, L. Y.** 1995. The extracellular matrix. Sci. Med. **2**: 58-67.

69. **Schoonmaker, J. N., D. W. Lawellin, B. Lunt, and J. A. McGregor.** 1989. Bacteria and inflammatory cells reduce chorioamniotic membrane integrity and tensile strength. Obstet. Gynecol. **74**: 590-596.

70. **Schwartz, B., A. Schuchat, J. M. Oxtoby, L. S. Cochi, A. Hightower, and V. C. Broome.** 1991. Invasive group B streptococcal disease in adults. A population-based study in metropolitan Atlanta. J. Am. Med. Assoc. **266**: 1112-1114.

71. **Scott, J. R., F. Bringel, D. Marra, G. V. Alstine, and C. K. Rudy.** 1994. Conjugative transposition of Tn*916*: preferred targets and evidence for conjugative transfer of a single strand and for a double-stranded circular intermediate. Mol. Microbiol. **11**: 1099-1108.

72. **Sherman, M. P., J. T. Johnson, R. Rothlein, B. J. Hughes, C. W. Smith, and D. C. Anderson.** 1992. Role of pulmonary phagocytes in host defense against group B streptococci in preterm versus term rabbit lung. J. Infect. Dis. **166**: 818-826.

73. **Stableforth, A. W.** 1938. Incidence of various serological types of *Streptococcus agalactiae* in herds of cows in Great Britain. J. Pathol. Bacteriol. **46**: 21-29.

74. **Stamenkovic, I., A. Aruffo, M. Amiot, and B. Seed.** 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J. **10**: 343-348.

75. **Tiffany, C. W., and R. M. Burch.** 1989. Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. FEBS lett. **247**: 189-192.

76. **Van Wart, H. E., and D. R. Steinbrink.** 1981. A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Anal. Biochem. **113**: 356-365.

77. **Varki, A., and S. Diaz.** 1984. The release and purification of sialic acids from glycoconjugates: methods to minimize the loss and migration of O-acetyl groups. Anal. Biochem. **137**: 236-247.

78. **Wagner, B., M. Wagner, and V. Kubin.** 1980. Immunoelectron microscopic study of the location of group-specific and protein type-specific antigens of group B streptococci. J. Gen. Microbiol. **118**: 95-105.

79. **Wessels, M. R., R. F. Haft, L. M. Heggen, and C. E. Rubens.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect. Immun. **60**: 392-400.

80. **Wilkinson, H. W.** 1978. Group B streptococcal infection in humans. Annu. Rev. Microbiol. **32**: 41-57.

81. **Yagupsky, P., A. M. Menegus, and R. K. Powell.** 1991. The changing spectrum of group B streptococcal disease in infants: an eleven-year experience in a tertiary care hospital. Pediatr. Infect. Dis. J. **10**:801-808

82. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morbid. Mortal. Weekly Rep. **41**: 25-32.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Bo Lin

Major Subject Microbiology

Title of Dissertation Characterization Of Group B Streptococcal Oligopeptidase And Hyaluronate Lyase

Dissertation Committee:

David S. Pritchard, **Chairman**

Susan K. Hollingshead

Director of Graduate Program

Dean, UAB Graduate School

Date 7/16/97

PS-1428