Spring 2003

Hyaluronate Lyase Gene of *Streptococcus pyogenes*: Molecular Characterization and Regulation

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HYALURONATE LYASE GENE OF *STREPTOCOCCUS PYOGENES:* MOLECULAR CHARACTERIZATION AND REGULATION

by

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B. A. May 1997, Virginia Wesleyan College

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCE

OLD DOMINION UNIVERSITY
MAY 2003

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ABSTRACT

HYALURONATE LYASE GENE OF *STREPTOCOCCUS PYOGENES:* MOLECULAR CHARACTERIZATION AND REGULATION

Sheryl Lynne Walton
Old Dominion University, 2003
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Streptococcus pyogenes causes an assortment of diseases ranging from pharyngitis to necrotizing fasciitis. This bacterium has the ability to elaborate several extracellular products capable of causing tissue damage; one of which is a hyaluronate lyase. Little information is available regarding the regulation of streptococcal hyaluronidase. As such, the expression of *hyl*A in the hyaluronate lyase-producing strain ATCC 10403 and in strains that do not produce detectable enzyme was analyzed by RT-PCR and quantitative PCR. Hyaluronate lyase transcript was detected throughout growth for all strains; however, expression of *hyl*A in the enzymatically inactive strains, 71698 and SF370, was determined to have a different pattern of expression than that of the active strain 10403.

The effect of various environmental conditions on *hyl*A expression was evaluated. Temperature had little effect on the relative amount of *hyl*A transcript for strain 10403. However, *hyl*A expression was slightly decreased after growth in pH 5.0 and increased under anaerobic and increased carbon dioxide conditions. The presence of hyaluronic acid in the growth media resulted in a two-fold increase in *hyl*A expression. However, no difference was detected in the titer of enzymatic activity.

Expression of *hyl*A and *hasA* were examined for the hyaluronate lyase non-producing strain 71698; and indicated the relative amount of *hasA* transcript was considerably greater than that of *hyl*A.

Also, 176 strains of *S. pyogenes* were screened by PCR for *hyl*A and showed all contained the gene; however, 34 strains showed a decrease in the size of the 3' region. The 3' region was PCR amplified, cloned, and the sequence determined from six of these 34 strains. Comparison of the sequences revealed a deletion of 183 bp in the same location in all six strains.

A preliminary analysis of the role of hyaluronate lyase in streptococcal infection was evaluated. Preliminary data suggests hyaluronate lyase to be an important virulence factor as was determined by a 5.7% reversion of single-recombinant mutant strain 10403::pHAS:*hyl*A from a HylA-negative to a HylA-positive phenotype.

For future virulence studies, an isogenic mutant strain 10403Δ*hyl*A was created by replacing an internal portion of *hyl*A with erythromycin resistance gene (*ermR*). Inactivation of hyaluronate lyase did not affect growth or protease or hemolytic activities.

This dissertation is dedicated to Jan Zajdowicz, for without his constant words of encouragement and comic relief I would have been unable to achieve this goal and still be able to retain a shred of sanity. I would also like to dedicate this dissertation to my dad and mom, who have always given me support and words of wisdom.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Wayne Hynes for mentoring me through my time at Old Dominion University. Additionally, I'd like to thank my committee members, Drs. Keith Carson, Christopher Osgood, and Robert Ratzlaff for providing me with essential guidance through the course of my studies and research.

Also, I would like to thank Drs. Dan Sonenshine and Harold Marshall for their continued zeal and enthusiasm for research. One can only hope to have as much passion and interest after so many years of service.

To Karin Berling, I give thanks for allowing me to bounce ideas off of you and for always having pure optimism.

This work was, in part, supported by a grant from the Public Health Service (1R15AI43029) from the National Institutes of Health to Dr. Wayne Hynes.

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INTRODUCTION

Streptococcus pyogenes. *Streptococcus pyogenes* is a Gram-positive, non-endospore forming, facultatively anaerobic, coccus that is classified in the family *Streptococcaceae*. The term "streptococcos" was initially used by Billroth in 1874 to describe chain-forming, spherical shaped bacteria that he isolated from the wounds of animals (16). The term, however, was merely used as a descriptive term for the congregation in which the bacterial cells were most found. A decade later, *Streptococcus* was first used by Rosenbach as the genus name to describe chain-forming cocci, which he isolated from suppurative lesions in man; Rosenbach named these cocci *Streptococcus pyogenes* (135).

Since the initial naming of *S. pyogenes* by Rosenbach, the classification of the organism as well as other streptococci has taken many turns over the past century. The first definitive classification came in 1906 when Andrewes and Horder (2) classified streptococci into eight separate groups based on the carbohydrate fermentation capabilities, morphological observations, and the characteristics of the streptococci when grown on milk.

Based on Andrewes and Horder's assessment, *S. pyogenes* was classified in a group by itself, and was separate from *S. equinus*, *S. mitis*, *S. salivarius*, *S. faecalis*, and the pneumococci (2). This classification was modified in 1937 by Sherman (141), who divided the streptococci into four main groups:

The model for this dissertation is the journal *Infection and Immunity*.

Pyogenic, Viridans, Lactic, and Enterococcus. *S. pyogenes* was incorporated in the pyogenic division as it adhered to the criteria of being pathogenic to man, β-hemolytic, and contained a group polysaccharide that could be isolated by the Lancefield precipitin technique (84).

Prior to the classification set forth by Sherman, it was determined by Hitchcock in 1924 that a 'residue antigen' was common among all streptococci (68). This antigen was examined further by Lancefield who grouped the streptococci by the presence of specific group polysaccharides or "C" substances, which could be precipitated by fairly simple techniques (91). Based on this finding, Lancefield categorized both hemolytic and non-hemolytic streptococci into groups A to E and N (91). A correlation was seen between the categories set forth by Lancefield and those outlined by Sherman, such that most serological groups could be equated with particular species. For example, group A streptococci were synonymous with strains of *S. pyogenes*. Since Lancefield's initial grouping into groups A to E and N, there have been additional groups (F, G, H, K, L, M, O, P, Q, R, S, T, U, V) formed based on further characterization of the streptococci (84). Lancefield's classification was ideal in its simplicity; however, for purposes of specificity it was also necessary to analyze the biochemical activities for distinguishing the different groups of streptococci.

Among the groups determined through the Lancefield serological grouping and biochemical analyses, several are known to cause disease in humans, including groups A, B, C, D, and G. Group B streptococci are associated with

bacterial endocarditis and neonatal meningitis; groups C and G are associated with pharyngitis (126). Perhaps the most pathogenic to humans and quite possibly the most dynamic of the different groups of streptococci is the group A streptococcus, *S. pyogenes*. *S. pyogenes* is the causative agent for a plethora of inflammatory infections, including pharyngitis, impetigo, scarlet fever, toxic shock-like syndrome, cellulitis, necrotizing fasciitis, as well as the delayed sequelae rheumatic fever and post-streptococcal glomerulonephritis. In recent years, group A streptococci have also been suggested to be associated with Tourette's syndrome, attention deficit disorders, and obsessive compulsive disorders (54).

S. pyogenes has caused disease for centuries, having been documented as causing scarlet fever epidemics as early as the 1600's (152). Although the organism has been ever-present and remains sensitive to penicillin, there has been an unexplained reemergence of rheumatic fever, as well as, the emergence of streptococcal toxic shock syndrome and invasive group A streptococcal skin and soft tissue infections since the 1980's (85). Perhaps the most devastatingly damaging, and possibly the most frightening streptococcal infection that has reemerged in the past few decades is necrotizing fasciitis (151), a severe, invasive soft tissue infection that may result in debilitation or possibly death. The severely invasive forms of streptococcal infection prompted the Weekly World News and The National Examiner to run headlines in 1994 such as "Killer Bug Ate My Face," "Flesh-Eating Virus Invades the US," "Watch for Killer Bug." Headlines such as these, combined with text in the articles such

as "...streptomania hits home...," "...get eaten to death...," and "...strepto-death..." struck fear and panic into the hearts of many. The severity and the bizarreness of the flesh-eating infections has even led such shows as "The X-files" to describe an unknown death in one episode as having been caused by a "...deadly, flesh-eating group A streptococcal infection that resulted in the loss of the victim's face." In 1998, Guinness World Book of Records anointed *S. pyogenes* "the world's most dangerous bug" due to its ability to cause such debilitating and fatal infections.

Due to the increased morbidity and mortality, both general and scientific media suggested that *S. pyogenes* had changed in some way to become more virulent. No evidence exists to date to corroborate these suggestions. The notoriety that developed may in fact be the result of better epidemiological studies, which have increased public knowledge of the number of extreme streptococcal infections that exist, and may not be a result of changes in the bacterium (31).

How *S. pyogenes* can be the etiological agent for such a wide range of diseases is not known; however, studies have suggested that this ability is due to the number of extracellular products the bacterium has the potential to elaborate. To gain a better understanding of streptococcal infections, the numerous products of the bacterium and their role in streptococcal infections must be better evaluated and understood. Group A streptococci elaborate a number of products that are believed to specifically aid in the infectious process (Fig. 1). Although all of these products are potentially important in streptococcal

pathogenesis and their role will be discussed briefly, the characteristics and role in infection of hyaluronate lyase will be the focus of this work.

Streptococcal virulence factors. Many pathogenic bacteria, including *S. pyogenes*, produce extracellular products that have tissue-damaging effects. Some of these products may play roles in the pathogenesis of disease by facilitating the spread of bacteria or their toxins through tissues. Virulence factors secreted by streptococci may act as anti-phagocytic mechanisms, spreading factors (substances that increase the permeability of such components of connective tissues as fibrin, collagen, and hyaluronic acid (39)), or assist in the tissue damage seen during infection.

M protein. M protein is a surface antigen that is anchored in the cell membrane and confers antiphagocytic properties to *S. pyogenes* (48). Studies have shown that absence of the M protein gene (*emm*), resulting in loss of M protein, resulted in rapid phagocytosis of cells (140); however, when an *emm* gene was introduced into an M-negative strain, phagocytosis was greatly impaired (127). M protein gives antiphagocytic properties by binding to complement regulatory protein factor H (129) and fibrinogen (165, 166). The binding to fibrinogen ultimately results in the acquirement and activation of plasminogen, which can later be converted to its active form, plasmin, by streptokinase (103). M protein has also been found to enhance vasoactivity through the binding of human kininogen, which releases a peptide vaso-enhancer, bradykinin (9). Clearly there is an immense role for M protein during

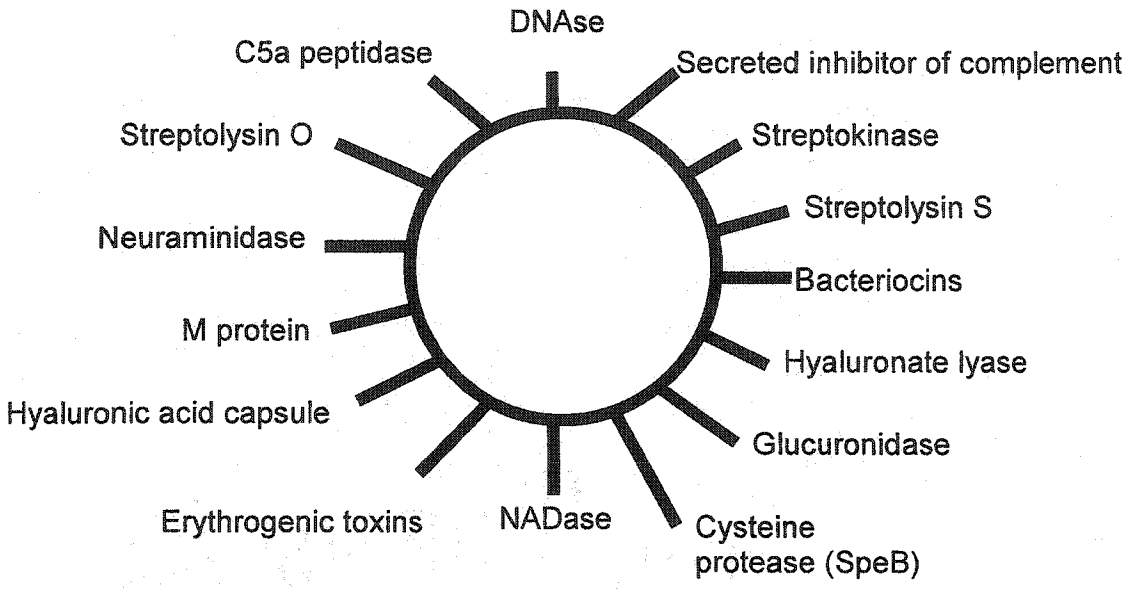

FIG. 1. Some of the potential virulence factors produced by group A streptococci.

the course of streptococcal infection whether it be antiphagocytic, or indirectly as a spreading factor. Studies have also shown that immunity to M protein confers protection against *S. pyogenes* infection (32, 33), further evidence to the important role of this protein in infection.

Extracellular cysteine protease. One of the important secreted virulence factors group A streptococcal (GAS) strains produce is extracellular cysteine protease, otherwise known as pyrogenic exotoxin type B (SpeB). Several pyrogenic exotoxins are produced by GAS that have been implicated in the pathogenesis of the severe invasive streptococcal infections including necrotizing fasciitis. The pyrogenic exotoxins known to be produced by GAS are A, B, C, D, E, F, F, G, H, J and Z (31, 121, 122). These toxins appear to act as superantigens in that they interact with major histocompatibility complex (MHC) class II and Vβ regions of the T lymphocyte receptor to activate T cells, resulting in the release of inflammatory cytokines such as tumor necrosis factor and gamma interferon (44, 62, 122). Although each exotoxin is thought to be important during an infection, many studies have focused on SpeB. Recently, SpeB was implicated in the pathogenesis of invasive infections. Patients having such infections had elevated levels of antibodies against exotoxin B (60). Cysteine protease acts directly and indirectly as a spreading factor. This protease directly cleaves human extracellular matrix components such as fibronectin and virtonectin, which are involved in maintaining the shape of cells and the integrity of tissues (87). Furthermore, Burns et al (19) demonstrated that SpeB indirectly causes tissue damage by activating a human endothelial cell

matrix metalloprotease, a protein involved in the maintenance of proper tissue structure and function. Such activity would help the bacteria invade to the underlying tissues. SpeB has also been shown to cleave human interleukin 1β into its active form (86). Vascular permeability is also affected by the activity of SpeB through the cleavage of plasma kininogen, which results in the release of kinin (104). Kinin increases vascular permeability allowing for the dissemination of bacteria throughout the host. SpeB also affects a few group A streptococcal virulence factors including hyaluronic acid capsule synthesis (167), streptolysin O (130), M protein, protein H, and C5a peptidase (11). Epidemiological and sequence analyses have shown that 3 variants of the cysteine protease gene are common among isolates from streptococcal infections (153). Further analysis of the variants showed a distinct association between one variant of exotoxin B, mSpeB2, and the exceedingly virulent M1 strains connected with invasive streptococcal infections worldwide (153) The action of SpeB provides a mechanism for the streptococci to spread throughout the host, resulting in tissue damage and shock-like manifestations associated with some streptococcal infections.

Streptolysins O and S. *S. pyogenes* produces two distinct cytolysins, an oxygen-labile streptolysin O (SLO) and oxygen-stable streptolysin S (SLS) (156). Streptolysin S is a potent hemolysin that is part of a nine-gene operon that consists of the SLS prepropeptide gene (*sagA*), genes responsible for the proteolytic cleavage of the prepropeptide (*sagBCD*), cellular immunity (*sagE*), and transport (*sagFGHI*) (120). When plated on blood agar, β-hemolysis is seen

surrounding the colony; this hemolysis has been primarily attributed to the presence of SLS (57). Cells lysed by the action of SLS are not limited to erythrocytes, but also include lymphocytes, polymorphonuclear leukocytes, platelets, several tissue culture cell lines, bacterial protoplasts, and intracellular organelles such as lysosomes and mitochondria (57). SLS has been suggested to be required for the formation of necrotic lesions in mouse models as was evidenced by reduced virulence of *S. pyogenes* following inactivation of the gene for SLS, *sagA* (15). Lesion formation was minimal when compared to wild-type strains in a mouse model (15).

Streptolysin O is also a secreted protein that has cytolysin properties and is very similar in action to thiol-cytolysins from other pathogenic bacteria, including the perfringolysin from *Clostridium perfringens*, pneumolysin from *Streptococcus pneumoniae*, and the listeriolysin from *Listeria monocytogenes* (1, 14, 27, 144). SLO has a variety of target cell types including erythrocytes, leukocytes, macrophages, platelets, and various cell culture lines. The lysin acts by interacting with cholesterol in the host cells to form pores, thereby disrupting the osmolar balance (67, 94). When SLO is injected intravenously into mice or rabbits, death of the animal results in minutes (7, 72). Inactivation of *slo* resulted in a loss of virulence, as well, as reduced necrosis and mortality when compared to infection with wild-type *S. pyogenes* in a mouse model for invasive streptococcal infection (101).

Streptokinase. Various strains of streptococci secrete streptokinase (18, 73). The exact role of streptokinase in virulence has not been established, but the presence of antibodies to the protein implies that it is produced during an infection and therefore, involved in the virulence of the pathogen. Streptokinase has been implicated as a spreading factor, though the action as a spreading factor is indirect. Although streptokinase is not an active protease and has no inherent enzymatic activity, it triggers a protease-like reaction (41, 106, 107). The primary activity known to be associated with the molecule is the species-specific activation of human blood plasminogen to plasmin (3). The exact biochemical mechanisms the protein uses is unknown; however, it is known that by stoichiometrically binding plasminogen, a conformational change and self-cleavage of plasminogen at a specific arginine-valine peptide bond results in the formation of the serine protease, plasmin, from plasminogen (3, 103, 106, 107). The formation of plasmin is important in that it is the plasmin that is able to degrade a broad range of mammalian proteins including matrix proteins, fibrin and soluble plasma proteins. During an infection, fibrin clots form to limit the spread of the infection. Since streptokinase has the ability to cause a reaction that ultimately results in the destruction of these clots, a means for the pathogens to spread is provided.

Deoxyribonuclease. Deoxyribonuclease (DNase) is an extracellular endonuclease that is produced by group A streptococci. The fact that antibodies to DNase are commonly found in patients suffering from streptococcal infections (56, 133) gives support that DNase is involved in the pathogenesis of the

infection in some way, and therefore, DNase could be a potential virulence factor. Although no specific role of DNase in pathogenesis has been elucidated, the studies have suggested that the activities of DNase provide a means for the spreading of streptococci by catalyzing the cleavage of the 3'-phosphate bond of native host DNA released on cell lysis, resulting in variable lengths of 5' fragments (56, 133). During a streptococcal infection various virulence factors result in the lysis of cells releasing DNA into the environment, thereby increasing the viscosity of the surrounding areas. Since the activity of DNase is directed towards DNA, the streptococci may produce the enzyme to depolymerize the viscous DNA in its surrounding environment to enable further spreading. By degrading the DNA that may hinder the spread of bacteria, the DNase provides the bacteria with a means to spread throughout the body of the host, not to mention a source of nutrients for survival. The replication of *S. pneumoniae* is known, which secrete DNase, is enhanced by the products formed by DNA degradation (47).

Hyaluronic acid capsule. Group A streptococci have a protective capsule of which the sole component is hyaluronic acid. Hyaluronic acid is comprised of repeating units of glucuronic acid and N-acetylglucosamine (154). Synthesis of the capsule is the result of the hyaluronate synthase operon, which is comprised of three genes: *hasA*, *hasB*, and *hasC* (35). The *hasA* gene encodes hyaluronate synthase, which adds alternating N-acetyl-D-glucosamine and D-glucuronic acid residues to form hyaluronic acid (34, 36); *hasB* encodes UDP-glucose dehydrogenase, which forms glucuronic acid from UDP-glucose

(37); *hasC* encodes UDP-glucose pyrophosphorylase, which froms UDP-glucose from UTP and glucose-1-phosphate (29). Of the three genes, only *hasA* and *hasB* have been found to be required for capsule synthesis (5). The hyaluronic acid capsule of *S. pyogenes* is similar in its make-up to the hyaluronic acid found in many animal tissues, confers resistance to phagocytosis and shields the cells from recognition from the host immune response (163). In addition to providing anti-phagocytic properties to the streptococci, studies have suggested that the capsule acts as an adhesin in the pharynx due to its ability to bind CD44 on epithelial cells (138). Epidemiological analyses have shown a connection between occurrences of rheumatic fever and invasive streptococcal infections as a result of infections caused by mucoid strains of GAS (83), further substantiating the need for hyaluronic acid capsule during a streptococcal infection. Studies have shown a decrease in virulence associated with loss of the capsule (75, 137, 163). Mouse studies in which both capsular and acapsular strains were inoculated into mice intraperitoneally and subcutaneously showed that encapsulated strains produced dermal necrosis, purulent inflammation, and bacteremia, whereas, the acapsular strains produced a mild inflammation (139, 163, 164).

Hyaluronate lyase. Although somewhat paradoxical, *S. pyogenes* also produces hyaluronidase, an enzyme that is found in many kinds of organisms, both eukaryotic and prokaryotic (82). Hyaluronidases have hyaluronic acid, a major constituent of human connective tissues and the sole component of the streptococcal capsule, as their substrate. Hyaluronic acid is a polymer of N-

acetyl glucosamine and glucuronic acid, linked by β 1-3 and β 1-4 glycosidic bonds, that lacks any covalently linked peptide (95). Hyaluronidases are separated into three main types based upon their mode of cleavage of the hyaluronic acid. The first type are the testicular-type hyaluronidases, hyaluronate 4-glycanohydrolase, found in mammalian spermatozoa, lysosomes, and the venoms of stonefish, bees, scorpions, and various other insects and snakes (28, 90, 93). By acting as endo-β-N-acetyl-D-hexosaminidases, these hyaluronidases degrade hyaluronic acid and/or chondroitin sulfate to a tetrasaccharide. The second type of hyaluronidase, hyaluronate 3-glycanohydrolase, is found in leeches and some hookworms (70, 71). This type of hyaluronidase degrades hyaluronic acid to a tetrasaccharide through endoglucuronidase activity. Bacterial hyaluronidases, hyaluronate lyases, comprise the third type of hyaluronidases. These carry out an elimination reaction of the β-1-4 linkage in hyaluronic acid, yielding disaccharide end products (Fig. 2) (59, 82). Since the hyaluronidase from *S. pyogenes* uses this action for hydrolysis of hyaluronic acid (66), streptococcal extracellular hyaluronidase will be referred to as a hyaluronate lyase.

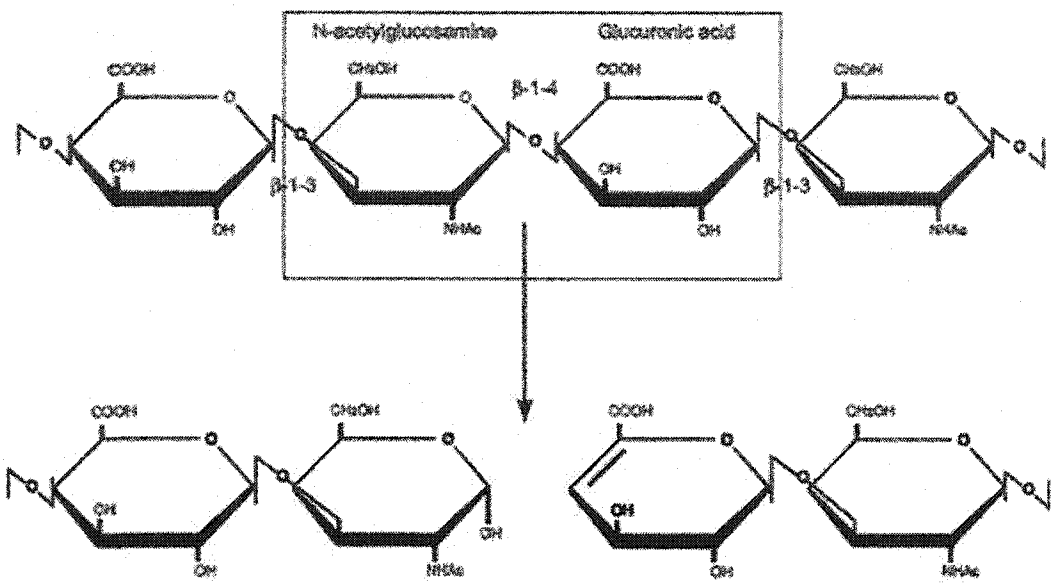

FIG. 2. The structure of hyaluronic acid and the resulting disaccharide products following hydrolysis by bacterial hyaluronate lyases.

Although information regarding other bacterial hyaluronidases exists, most of the literature pertaining to streptococcal hyaluronidases is from studies on streptococcal infections. Hyaluronate lyase production has been found in streptococcal groups A, B, C, F, and G (61). Group A streptococci produce at least two hyaluronidases: [1] secreted as an extracellular product, which is the hyaluronate lyase, and [2] encoded by a bacteriophage that allows for the bacteriophage to penetrate a streptococcal hyaluronic acid capsule (80). Additionally, another putative hyaluronidase, Spy1600, has been identified (46). Although the extracellular hyaluronate lyase is considered to be a putative virulence factor, very little is known about the effects of the enzyme during a streptococcal infection. Since group A streptococci produce a capsule of which its sole component is hyaluronic acid, it appears to be contradictory to produce an enzyme that also degrades the capsule. The relationship between hyaluronate lyase and the hyaluronic acid capsule remains to be determined; however, some form of regulation probably exists between these two streptococcal factors. Early studies suggested that hyaluronic acid capsule is produced early in the growth of the organism, while hyaluronate lyase is found later in the growth cycle (108); however, the time of production for hyaluronate lyase is not known for *S. pyogenes*. Additionally, no information is available regarding the conditions under which the enzyme is produced. However, studies showed that when hyaluronic acid was added to the media, there was an increase in the production of hyaluronate lyase (66).

The streptococcal capsule appears to be utilized by the bacterium as a means of protection from the host defense system (51, 117). Thus, by producing an enzyme that can destroy this protective mechanism, the bacterium is exposing itself to the harsh elements of the host defense. However, by degrading the hyaluronic acid capsule, the bacterium potentially establishes the ability to replicate freely, and by continually producing hyaluronate lyase, institutes the means to degrade the hyaluronic acid constituent of the host's connective tissues for further bacterial or toxin spread. Destruction of the hyaluronic acid of the connective tissues may result in the clinical manifestations, primarily the severe tissue damage seen in streptococcal infections such as necrotizing fasciitis. Because hyaluronate lyase produced by streptococci is capable of degrading hyaluronic acid and may potentially deteriorate the integrity of human connective tissues to allow for better dissemination through the infected organism, the enzyme has been referred to as a spreading factor (39).

Although hyaluronate lyase has been acknowledged as a spreading factor, only approximately 25% of the strains tested using an *in vitro* assay for activity actually produced the enzyme (80). Although *in vitro* analysis indicates only a small number of group A streptococci produce hyaluronate lyase, antibodies directed to both extracellular hyaluronate lyase and the bacteriophage hyaluronidase have been found in patients following streptococcal infection (63, 161, 162). The presence of these antibodies indicates that hyaluronate lyase, in either an active or inactive form, is produced *in vivo* during infection. This finding suggests that there may be some means of regulation by the bacterium that

allows for the expression of hyaluronidase *in vivo*. In other words, some factor that is not present *in vitro* may "turn on" expression of the gene for hyaluronate lyase during infection. Although what that factor may be, or what form of regulation exists for the production of hyaluronate lyase has not yet been determined, studies have shown that *in vivo* conditions may turn on gene expression. McClean (108) in early studies showed that strains that did not appear to produce hyaluronate lyase on initial screening were found to have enzymatic activity after serial passage through mice. The passage of the apparent non-producing strains through the mice "resulted in a considerable increase in the virulence of the strains" (108). This induction of the production of hyaluronate lyase after animal passage supports the suggestion that hyaluronate lyase production is necessary for streptococcal infection. When one considers the potential ability of hyaluronate lyase to allow for the dissemination of the producing organism, as well as its toxins, the enzyme can be considered as an important factor for streptococcal infection. The immense tissue destruction that is elicited during streptococcal infection can also in part be due to the activity of hyaluronate lyase. Therefore, hyaluronate lyase must be further analyzed to establish the exact mechanisms of its activities, and what role this enzyme may have in streptococcal infections.

Hyaluronate lyase production has been suggested to be involved in the virulence of various pathogenic organisms (42, 82) . The role of hyaluronate lyase for bacterial pathogens may be to enable the organisms to spread through the connective tissues from the initial site of infection, more readily giving access

to the underlying tissues. Pathogens capable of causing infection on mucosal areas and on the skin, which is estimated to be comprised of approximately 50% of hyaluronic acid (95), have been speculated to produce hyaluronate lyase as a means of entry through the hyaluronic acid-rich skin into the deeper tissues. To date, little evidence is available to substantiate this claim. Incidentally, information regarding the function of the hyaluronate lyases produced by bacterial pathogens is limited; however, since connective tissues provide a means of defense against bacterial infection, any enzyme capable of deteriorating that line of defense could potentially be important in enhancing the spread of pathogens from the initial site of infection.

Little information is available regarding the genetic composition of hyaluronate lyase. Relatively little information exists regarding sequence analysis of hyaluronate lyase genes. Eleven bacterial hyaluronidase genes and two bacteriophage genes have been sequenced. The sequence for the hyaluronate lyase gene from the ATCC T-type 22 hyaluronidase-producing *S. pyogenes* strain 10403 (*hyl*A) has been determined (76). The gene is 2607 bp in length, encoding a 99.6 kDa protein. Hyaluronidase gene sequences are also available for multiple serotypes of *Streptococcus pyogenes* (10, 46, 145), *Staphylococcus aureus* (43), *Streptococcus agalactiae* (52), *Streptococcus pneumoniae* (13), *Propionibacterium acnes* (148), *Streptomyces griseus* (123), *Streptomyces coelicolor* (123), *Proteus vulgaris* (136), *Streptococcus intermedius* (155), *Streptococcus constellatus* (155) and *Clostridium perfringens* (20). Additionally, *spy1600* a putative hyaluronidase gene was also identified

from the sequence of the streptococcal chromosome (46). The bacteriophage hyaluronidases, *hylP* (78) and *hylP2* (80) that have been sequenced are both derived from phages that infect group A streptococci. Streptococcal chromosomal sequences have identified additional bacteriophages, all of which encode hyaluronidases (10, 145). Having sequence analysis for these various hyaluronate lyase genes has allowed for comparisons between the genes, and has shown significant similarity between some, as well as, distinct differences between others (82). Interestingly, the hyaluronidase, or mu toxin, produced by *C. perfringens* was the most distinct of all the other hyaluronidases, having little similarity when compared to the sequences of the other genes. Spy1600 also had very little similarity when compared to other hyaluronidase genes and shared the most similarity (30%) with the mu toxin from *C. perfringens*. The hyaluronate lyases produced by these bacteria vary in molecular weight from that of *S. pyogenes*. Although the conditions under which hyaluronidases are produced have not been determined, by gaining further knowledge regarding the sequences of the various bacterial hyaluronidase genes and therefore, the respective proteins, a better understanding of the exact enzymatic mechanisms of hyaluronate lyase may be gained and in doing so, its role in streptococcal infections will be better understood.

Streptococcal regulatory mechanisms. By summarizing some of the putative virulence factors produced by group A streptococci, the relationship between the streptococcal products and clinical manifestations of the different streptococcal diseases becomes apparent. However, nearly all strains produce

most, if not all, of the factors described, suggesting some other relationship between the diseases produced by *S. pyogenes* and the organism itself. Also, epidemiological studies suggest that a single strain of *S.pyogenes* can cause more than one type of disease (118). One possibility could be that the various diseases are a result of the organism's reaction to the different stimuli encountered in the human host. *S. pyogenes* can survive and replicate in blood, the gastrointestinal tract, skin, throat, and the female urogenital tract (31). Therefore, the bacterium must adjust its gene expression to acclimate to the surrounding environment for survival. Inasmuch, the regulatory mechanisms possessed by *S. pyogenes*, which control the expression of various genes in response to different environmental conditions, are probably critical for understanding the process of the bacterium during the course of infection. Unfortunately, very little is known about the global regulators present in group A streptococci. However, since the completion of the sequence for the streptococcal chromosome from various serotypes of *S. pyogenes* (10, 46, 145), studies have been designed to ascertain the complex regulatory mechanisms of this bacterium.

One mechanism that *S. pyogenes* uses to modify gene expression as an adaptation to the bacterium's environment involves two-component signaling systems. Two-component systems are comprised of a surface-located histidine kinase protein that becomes stimulated by some external signal, such as pH, temperature, osmolarity, and carbon dioxide; upon stimulation, the kinase is autophosphorylated. The kinase in turn, phosphorylates its cytoplasmic partner,

a response regulator protein (69). The sequence of the group A streptococcal genome from SF370 revealed a total of 13 two-component regulators, suggesting a complex global network of regulation exists; for seven of these pathways a function has been identified (46, 89). Two-component systems are associated with regulating various cell functions, including salivaricin lantibiotic operon and the competence factor response system, which is regulated by ComD/ComE (38). Additionally, virulence factors streptolysin S (*sagA*), hyaluronic acid capsule (*hasAB*), and pyrogenic exotoxin B (*speB*) expression are regulated by another pathway, CovR/CovS (45, 65). Another regulatory mechanism, Mga, regulates M protein (*emm*), C5a peptidase (*scpA*), M-like proteins (*mrp, enn,* and *fcR*), serum opacity factor (*sof*), and secreted inhibitor of complement (*sic*) expression (128, 143). Furthermore, the two-component system, *spy2026/spy2027* (Ihk/Irr (45)), is suggested to be involved in bacterial virulence (46). Additionally, a recent study has reported that the Ihk/Irr system controls expression of genes necessary for evading and surviving phagocytosis (160). One other two-component pathway (SPy0528/Spy0529) that has been identified is thought to be a homolog of two-component systems (YycR-AucG and hk02-rr02) that are essential for growth in *Bacillus subtilis* and *S. pneumoniae*, respectively (92). Yet another pathway, the *fas* regulon, functions to positively regulate gene expression for streptokinase (*ska*) and streptolysin S (*sagA*), while negatively regulating fibronectin (*fbp*) and fibrinogen (*mrp*) binding (89). Of the seven, the Mga and CovR/CovS systems are the most understood.

The most studied regulator in *S. pyogenes* is Mga, the multiple gene

regulator in group A streptococci. The gene (*mga*) has homology to phosphorylation acceptor motifs, suggestive of a role as a response regulator protein of a two-component system; however, the primary protein, ie. the sensor kinase, of the pathway has yet to be identified (128). Initially identified by Simpson et al as *virR* (143) and by Perez-Casal et al as *mry* (128), the gene was established to be a positive transcriptional regulator for M protein gene (*emm*) expression. The gene was later named *mga* to indicate its role in regulation of multiple genes in group A streptococci. Mga functions as a transcriptional activator by binding to the promoter of the genes it regulates (128). Studies involving inactivation of *mga* resulted in the loss of M protein expression, signifying a positive regulation by Mga (147). Since the initial discovery of the involvement of Mga with M protein expression, studies have determined that the Mga system regulates a number of virulence factor genes, including C5a peptidase (*scpA*), M-like proteins (*mrp*, *enn*, and *fcR*), serum opacity factor (*sof*), and secreted inhibitor of complement (*sic*) (25, 26, 109, 112, 131) Mga is also responsible for the regulation of its own gene expression (124). Studies involving *emm* expression have helped to elucidate the role of the *mga* regulon. By investigating *emm* expression under different environmental conditions, such as various concentrations of carbon dioxide (21), osmolarity, temperature, iron, and growth with free exchange of gases (shaking) (110), it was suggested that Mga regulates expression of the various genes under its control in response to environmental stimuli. Although Mga appears to function solely as a positive regulator allowing for rapid production of necessary factors, the response is also

controlled by a negative regulator, Nra (negative regulator of group A streptococcus) (132). Nra is a global negative regulator that was found to regulate *mga* gene expression, as well as expression of the fibronectin-binding protein F2 gene (*prtF2*), and a collagen-binding protein (*cpa*)(132). Interestingly, Nra negatively regulates its own expression; in contrast, it is positively regulated by Mga (132). In contrast, another global regulator, RofA (*rofA*), is an autoregulated, positive-transcriptional regulator of the gene encoding the fibronectin-binding protein (50). Additionally, RofA was shown to suppress *mga* expression (8).

The other two-component signaling pathway found in *S. pyogenes* for which information is available is the CovR-CovS (Cov = control of virulence) regulon (45), also known as the CsrR-CsrS regulon (97). Whereas Mga is a positive transcriptional regulator, CovR-CovS is a negative transcriptional regulator for many virulence genes. CovS acts as the sensor protein, with CovR being the response regulator (97). Inactivation of *covR* has been shown to affect transcription of *hasA*, the first gene in the hyaluronic acid capsule operon, such that expression was increased, which resulted in more mucoid colonies (97). Additionally, inactivation of *covR* resulted in an increase in streptokinase (*ska*), streptolysin S (*sagA*), and mitogenic factor (*speMF*) (45). Furthermore, CovR negatively regulates its own operon's expression (45). Recently studies have shown that CovR directly, or indirectly, affects transcription for at least 15% or 271 genes from *S. pyogenes*, including genes responsible for stress adaptation, gene regulation, as well as the virulence factors already described (58). How

CovR negatively regulates these genes is not known; however, studies have shown that CovR regulates *hasA* transcription by directly binding to the promoter region upstream of the *has* operon (12).

Although the CovR pathway represses a number of genes independent of those controlled by the Mga regulon, CovR neither affects nor is affected by Mga (45). However, *ska*, *sagA*, and *speB*, which are all regulated by CovR, are also influenced by other regulators (8, 89, 149). Kreikemeyer et al (89) found the *fas* operon (*fasBCAX*), a two-component pathway, which controls genes for streptokinase and streptolysin S. The *fas* operon encodes two histidine kinases (*fasBC*), as well as, a response regulator protein (*fasA*). Interestingly, a gene (*fasX*) downstream of the *fas* operon was found to be required for Fas to function (89). Rgg was found to positively regulate expression of *speB* (22, 105). Chaussee et al reported that Rgg also affects the expression of a number of genes associated with the streptococcal stress response (23, 24). Interestingly, another regulator RopA, which was shown to suppress transcription of *mga*, has also been shown to suppress expression of *sagA* (8). An additional global regulator, Pel (pleiotropic effect locus), was found to interact directly, or indirectly, with both negative or positive regulatory systems, such that inactivation of *pel* resulted in loss of streptolysin S, reduction or loss of M protein and cysteine protease (SpeB), as well as affected production of streptokinase, fibronectin binding proteins, and superoxide dismutase (100).

Additionally, genes controlled by the various regulatory pathways, as well as the genes *mga* and *fas*, were affected by growth phase, a third regulatory

mechanism in *S. pyogenes* (45, 111). Gene expression of CovR, however, was not affected by growth phase (45). Regulation of *nra* expression also appears to be influenced in a growth phase-dependent manner as is seen by its late exponential expression (132). Growth phase-dependent regulation is a common form of regulation for many group A streptococcal virulence factors; however, the exact signals responsible have not been identified. Some genes, including *emm*, *scpA*, *hasA*, and *mga*, show increased expression during exponential and late exponential growth phases; however, genes such as *sagA* and *speMF* have elevated expression during stationary phase (15, 30, 111). Another mode of regulation related to growth phase-dependent regulation that *S. pyogenes* may use in response to environmental signals involves the use of secondary sigma factors. The sequence of the genome of *S. pyogenes* revealed a potential secondary sigma factor that had similarity with σ^H, a secondary sigma factor implicated with stationary-phase gene regulation in *Bacillus subtilis* and *S. pneumoniae* (4, 96). Opdyke et al (125) investigated the locus as well as the protein, which they named *sigX* (σ^X) and concluded that σ^X was indeed a secondary sigma factor due to its ability to initiate transcription from two promoters (p*cinA* and p*femB*) the functions of which have yet to be identified. Although σ^X was shown to initiate transcription of *cinA* and *femB*, attempts to express *hasA*, which is dependent on the primary sigma factor (σ^A), were not successful (125). The actual role of this secondary sigma factor at this time is unknown; however, as with that of *B. subtilis*, it may be involved in gene regulation during stationary-phase of growth.

With respect to growth phase-dependent regulation, the response of *S. pyogenes* to amino acid depletion is also important. During stages of infection, as well as in culture, streptococci encounter various niches in which free amino acids may be limited. In response to the lack of amino acids, streptococci restrict RNA synthesis for the corresponding aminoacyl-transfer RNA (tRNA) (40) and consequently protein synthesis to prevent synthesis of elements that may not be needed at the time, such as virulence factors (150). Studies involving amino acid starvation have revealed a complex network of regulatory factors, which were previously unknown. These investigations have shown that gene expression for the stringent response regulator protein RelA (*relA*) are enhanced following the depletion of amino acids (115, 116). As a result, a global regulator, ppGpp, is synthesized and rRNA and tRNA synthesis is inhibited (115, 116). More recent studies, however, have explicated a more intricate regulation pattern resulting from amino acid depletion. Steiner and Malke (150) investigated a *relA*-independent response to limited amino acids and showed that the *fas* and *covR/covS* signal transducers were stimulated (150). Another study involving nutritional stress showed that the virulence regulator, Rgg was stimulated as well (149). While CovR/S negatively regulates the transcription of *ska*, *sagA*, and *speB*, Rgg stimulates expression of *speB*, and Fas positively regulates *ska* and *sagA*. Through this intricate network of regulation, the level of expression for these genes is maintained to prevent loss or over-production of their products.

Hypotheses and specific aims. Although literature regarding many virulence factors of *S. pyogenes* exists, hyaluronate lyase has been neglected. The objective of these studies will primarily focus on developing a better understanding of the role of streptococcal hyaluronate lyase as a virulence factor, and perhaps begin to understand under what conditions the enzyme is produced. Through these studies a preliminary understanding may be attained into the relationship between the streptococcal hyaluronate lyase and hyaluronic acid capsule. In order to complete these objectives, hyaluronate lyase was examined at the molecular level; as well as under conditions to simulate an *in vivo* environment. For this study, there were two main hypotheses: 1) the production of hyaluronate lyase is regulated by some environmental factor, and 2) that a hyaluronate lyase gene is present in all group A streptococci. The ability of group A streptococci to cause a variety of diseases has been linked with its capacity to elaborate numerous extracellular products. Although streptococcal hyaluronate lyase has a potential role in the pathogenesis, there is relatively little information regarding the enzyme, and no information regarding its regulation. By gaining information regarding the production of hyaluronate lyase, it may be possible to better comprehend the role of the enzyme in streptococcal infections.

The Specific Aims of this study were:

SPECIFIC AIM 1- Regulatory studies: Evaluate the regulatory mechanisms of hyaluronidase gene expression by examining the relationship between the

production of hyaluronidase and such factors as temperature, pH, aeration, presence of substrate, and the production of a hyaluronic acid capsule.

SPECIFIC AIM 2- Hyaluronate lyase gene size variability studies: Determine size and location of a deletion seen in some hyaluronidase genes of group A streptococcal strains and examine the importance of these deletions in hyaluronidase activity.

SPECIFIC AIM 3- Inactivation of *hyl*A studies: Inactivate the hyaluronate lyase gene (*hyl*A) and determine its effect on the growth of *S. pyogenes*, as well as the effect on other enzymatic properties of *S. pyogenes*.

GENERAL LABORATORY MATERIALS AND METHODS

Chemicals and equipment. A list of chemicals, equipment, and their respective manufacturers, used throughout this study can be found in either in Appendix I, or where used in the text.

Bacterial strains. One hundred seventy-six strains of *S. pyogenes* were used in this study. Strains were isolated from various geographic locations and from different diseases (Appendix II). *S. pyogenes* strains were generously provided from the culture collection of Professor J.J. Ferretti, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, the Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia, the culture collection of Dr. W. Hynes, Old Dominion University, Norfolk, Virginia, or by Professor J. Tagg, Otago University, Dunedin, New Zealand. *S. pyogenes* cultures were stored at -80°C, plated on blood agar, then subcultured before use. *Escherichia coli* strains used in this study were JM109 (Promega), BHB2600 (a gift from Professor Adwin Vriesema, The Netherlands), DB11 (University of Oklahoma Health Sciences Center), or DH5α (Life Technologies).

Oligonucleotide primers and dual-labeled probes. Oligonucleotide primers and dual-labeled probes used in these studies are outlined in Table 1 and were purchased from Integrated DNA Technologies. Primers and probes were prepared in nuclease-free water as a 100 µM stock concentration and were stored at -20°C. Dual-labeled probes (HylA-Probe, GyrA-Probe, HasA-Probe) were labeled with a 5' 5-carboxyfluorescein (FAM) label and the 3' end with a

black-hole quencher (BHQ). Probes were diluted to a working concentration of 2.0 µM, stored at -20°C, and were used within one week from dilution.

Media preparation. The following media were utilized in these studies. Todd-Hewitt Broth (THB) was prepared by adding 30g Todd-Hewitt medium to a final volume of one liter (L) of water. The pH was adjusted to 7.8 or to an otherwise noted pH. The solution was autoclaved and stored at room temperature. Antibiotics were added aseptically when required.

Todd-Hewitt Agar (THA) was prepared in the same manner as THB; however, following pH adjustment, 1.5% granulated agar was added to the broth. The medium was autoclaved, cooled to room temperature, and if required, antibiotics were added. Twenty milliliters of THA were aseptically aliquoted into a 100 x 15 mm petri dish, allowed to solidify, and stored at 4°C.

2x YT Media was prepared by combining 16g tryptone, 10g yeast extract, and 5g sodium chloride (NaCl) with water to a final volume of 1L. The pH was adjusted to 7.5 and autoclaved. Bottles of broth were stored at room temperature. Antibiotics were added aseptically prior to use if required. 2x YT agar was prepared by adding 1.5% agar prior to autoclaving. Following autoclaving, agar was cooled to room temperature, required antibiotics, or other additives, such as isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration 100 µM) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (final concentration 20 µg/mL) and dispensed to 100 x 15 mm petri dish.

SOB Broth was prepared by adding 10g tryptone, 5g yeast extract, 5g NaCl, 10 mM magnesium chloride ($MgCl_2$), and 10 mM magnesium sulfate

($MgSO_4$) to water to a final volume of 1 liter; pH was adjusted to pH 7.5 and autoclaved. Sterile broths were stored at room temperature.

Brain Heart Infusion-Bovine Serum Albumin-Hyaluronic Acid Medium (BHB) was prepared as previously described (77). Briefly, the medium was prepared by combining 3.7g Brain Heart Infusion Medium (BHI) and 1g purified agar with 60 mL of water. Following autoclaving, the agar was cooled to 55°C. Sterilized 2 mg/mL hyaluronic acid and filter-sterilized 5% bovine serum albumin (BSA), pre-warmed to 55°C were gradually added, in the above order, to autoclaved BHI agar such that the media contained a final concentration of 400 µg/mL hyaluronic acid and 1% BSA. Twenty mL aliquots of BHB were poured into 100 x 15 mm petri dishes.

Hyaluronidase Assay plates were prepared as previously described (76). Briefly, 1g purified agar was resuspended in 60 mL 0.2 M sodium phosphate buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4), pH 6.5 and was autoclaved. Following autoclaving, agar was cooled to 55°C and pre-warmed sterile 2 mg/mL hyaluronic acid and 5% BSA were gradually added, in that order, to give final concentrations of 400 µg/mL hyaluronic acid and 1% BSA. Medium, in 20mL aliquots, was dispensed into 100 x 15 mm petri dishes and stored at 4°C.

Agarose gel preparation. Agarose gels for routine screening of plasmid or PCR products were prepared by adding 0.8% agarose to TBE buffer (Tris; boric acid; EDTA) or TAE buffer (Tris; glacial acetic acid; EDTA). Agarose gels used for the separation of DNA fragments smaller than 200 bp were prepared by adding 3% Super Fine Resolution (SFR) agarose to TBE buffer. The mixture

was microwaved to melt agarose, cooled to 55°C, and poured into the gel chamber. To visualize DNA fragments, ethidium bromide was added to the agarose for a final concentration of 0.5 µg/mL.

TABLE 1. Oligonucleotide primers and dual-labeled probes used in this study.

Primer/Probe	Oligonucleotide Sequence (5'-3')	Reference
Hyl-A	CAATTTCAATATCATTACGG	(76)
Hyl-B	GCATCTCTGAAAGAAGAAG	(76)
Hyl-C	GAACGCCCTCGAGTCATATC	(76)
Hyl-D	CAAAAGTACCCTTACTGTTC	(76)
Hyl-E	GCTTTACAACTGGAGCTTACGG	(76)
Hyl-F	GACGTGCATCATCTGTTGACTATC	(76)
Hyl-G	AGCTATTGTTTATGTAAGGG	
Hyl-H	TGTGCAAAAATAAGTATTCAC	
Hyl-J	AGCAAAGCTAAGGAATAGGTTTGAA	
Hyl-K	GTGAATACTTATTTTTGCAC	
Hyl-L	GGCAGTTCCTTGGCCCATCAT	
Hyl-M	CCATACTCAAGATGGCGATCG	
Hyl-N	CTAAATCCTTAAGTCTTTCT	
Hyl-O	ATGATGGGCCAAGGAACTGC	
GyrA-F	TTATCACGTTCCAAACCAGTCAA	(23)
GyrA-R	CGACTTGTCTGAACGCCAAA	(23)
HasA-F	ACCGTTCCCTTGTCAATAAAGG	(23)
HasA-R	CGTCAGCGTCAGATCTTTCAAA	(23)
pJC2-A	GCCTTTGAGTGAGCTGATAC	
pJC2-B	CACTGACAGAAAATTTGTGC	

TABLE 1. Continued.

Primer/Probe	Oligonucleotide Sequence (5'-3')	Reference
M13 Forward	CGCCCAGGGTTTTCCCAGTCACGAC	
M13 Reverse	TCACACAGGAAACAGCTATGAC	
Gfp-F	ATGAGTAAAGGAGAAGAACT	
Gfp-R	CTATTTGTATAGTTCATCCATG	
HylA-Probe*	CAATTACTACTTTATTCAAACCTATTC	
GyrA-Probe*	CGACGCAAACGCATATCCAAAATAGCTTG	(23)
HasA-Probe*	CGCCATGCTCAAGCGTGGGC	(23)

* 5' FAM-3' BHQ

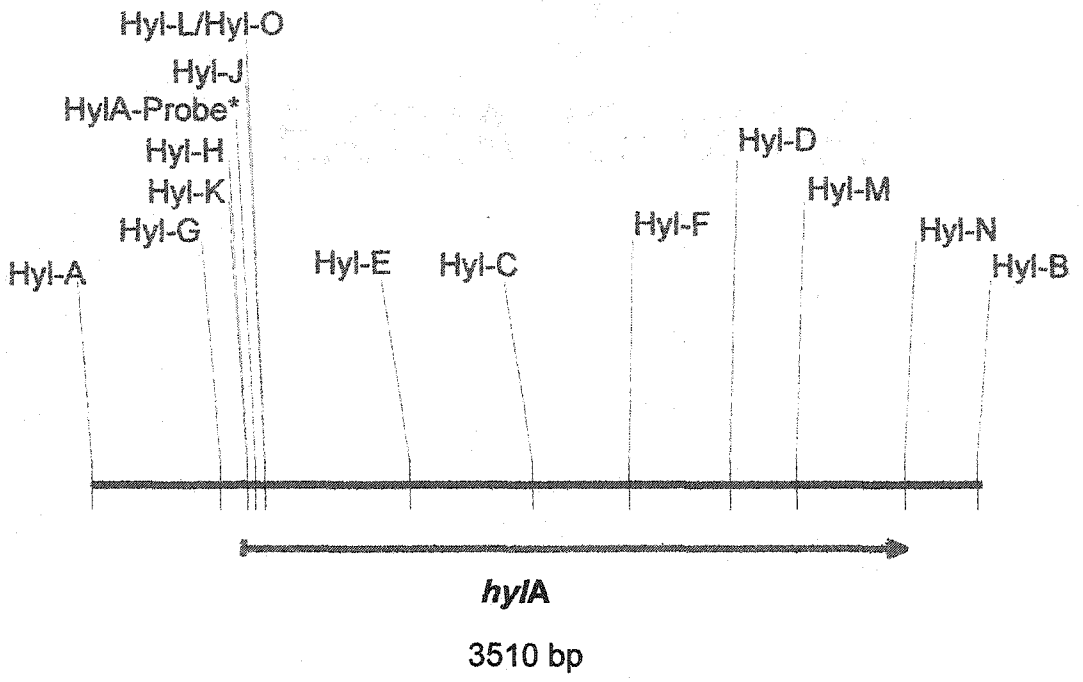

FIG. 3. Location of primers for the hyaluronate lyase gene regions used in this study. The HylA-Probe* was labeled with a 5' 5-carboxyfluorescein (FAM) label and the 3' end with a black-hole quencher (BHQ).

METHODS FOR REGULATORY STUDIES

Bacterial strains and growth for regulation studies. ATCC strain 10403 (M-type 22), strain SF370 (M-type 1), and strain 71698 (M-type 28) were grown on Sheep Blood agar (BBL, MD) at 37°C and subcultured before inoculating into Todd-Hewitt broth (THB) supplemented with 5% horse serum. Broth cultures for examination of *hyl*A expression at different pHs were prepared at pH of 5.0, 7.4, 7.6, and 9.0. These cultures were then grown aerobically at 37°C. For aeration studies, cultures were grown in anaerobic, increased CO_2, and aerobic conditions at 37°C. Cultures were also grown with (shaking) and without (stationary) free exchange of gases at 37°C. Cultures for temperature studies were grown aerobically at 32°C, 37°C, and 42°C. To analyze the effect of hyaluronic acid on *hyl*A expression, 600 µg/mL hyaluronic acid was added to the medium as previously described (66).

Production of hyaluronate lyase. To determine the time of production for hyaluronate lyase by strain 10403, an overnight culture was diluted 1:100 into fresh THB + 5% horse serum and incubated at 37°C. Prior to inoculation, the overnight culture was pelleted and washed with isotonic saline to remove residual extracellular HylA. Samples were taken at 60-minute intervals for determination of optical density at a wavelength of 600nm. A 1 mL aliquot from each time point was transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 16000 x g and the supernatant collected. Hyaluronidase activity in the supernatant was detected by transferring 20 µL of

supernatant to wells in the hyaluronidase assay plates prepared as previously described and incubated at 37°C for 18 hours (77). Following the addition of 2N acetic acid to precipitate the bovine serum albumin-hyaluronic acid complex, hyaluronidase activity was determined by measuring the diameter of the zone of clearing in a plate assay. Hyaluronate lyase titer was determined by serially diluting the supernatant and assaying for activity as above. The titer of hyaluronate lyase is recorded as arbitrary units per mL (AU). The greatest dilution of the supernatant to give detectable clearing was considered to contain one AU.

Polymerase chain reaction (PCR) of potential promoter regions of the hyaluronate lyase gene from *S. pyogenes* strain 10403. To determine the region upstream of *hyl*A on the *S. pyogenes* chromosome that exhibits promoter activity, specific primers were used to amplify the potential promoter-containing fragments by PCR. Primers were developed to amplify a 128 bp fragment upstream of the proposed GTG initiator codon (Hyl-G, Hyl H; Table 1; Fig. 3), a 198 bp fragment upstream of the potential ATG initiator codon (Hyl-G, Hyl-L; Table 1; Fig. 3), and a 90 bp fragment internal to the GTG and ATG codons (Hyl-K, Hyl-L; Table 1; Fig. 3). PCR reactions were prepared according to manufacturer's guidelines for Takara *Ex Taq*™ Polymerase such that the reactions contained final concentrations of 1x Takara *Ex Taq* Buffer, 2.5 mM dNTP Mix, 20 pmol each primer, 200-500 ng DNA template, 1.25 U Takara *Ex Taq* DNA Polymerase, and water to a final volume of 50 µL. PCR amplification

was performed using the following protocol: initial denaturation for 1 minute at 94°C, 30 cycles of denaturation at 94°C for 1 minute, annealing at 49°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. PCR products were electrophoresed on a 3% SFR TBE agarose gel at 75 V for 2 hours. Desired fragments were isolated from the gel using the QIAEX II Gel Extraction Kit in accordance with manufacturer's guidelines. Briefly, the gel slice was weighed and solubilized in the presence of QIAEX II silica particles with Buffer QX1. The QIAEX II silica particles-DNA fragment complex was washed with PE buffer and the purified DNA fragment was eluted from the particles with nuclease-free water.

Construction of plasmids containing potential promoters for *hylA*. Plasmid pJC2 (102), containing promoter-less green fluorescent protein gene (*gfp*), was purified from a transformed culture of *E. coli* strain JM109 using the Wizard® *Plus* SV Mini-prep DNA Purification System as outlined in the manufacturer's instructions. Briefly, an overnight 2 mL culture of JM109:pJC2 was pelleted by centrifugation at 16000 x g. The pellet was resuspended in Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/mL RNase A). Following resuspension, cells were lysed with the addition of Cell Lysis Solution (0.2 M NaOH, 1% SDS). Neutralization Solution was added. Following neutralization, lysate was centrifuged for 1 minute at 16000 x g. Supernatant was transferred to Column, and centrifuged 1 minute at 16000 x g. Plasmid DNA collected on the filter of the column was washed with Column Wash Solution, and plasmid DNA was eluted in nuclease-free water. The

plasmid was linearized by digestion with restriction enzyme *Sma*I. Plasmid digest was electrophoresed on a TBE 1% agarose gel. Linearized plasmid was isolated from the gel using the QIAEX II Gel Extraction Kit as outlined by the manufacturer. As the Taq DNA polymerase used for amplification of the fragments added 3' A overhangs to the amplicon, the addition of T-overhangs to the plasmid was necessary to facilitate ligation between the PCR product and plasmid pJC2. The addition of T-overhangs was accomplished using standard protocols (6). Briefly, a reaction containing 2-5 µg blunt-ended vector pJC2, 1x Taq DNA Polymerase Buffer, 2.5 mM $MgCl_2$, 1 mM dTTP, 5 U Thermophilic Taq DNA Polymerase, and sterile water to a final volume of 100 µL was incubated for 2 hours at 75°C. Ligation between pJC2 and the PCR products was performed using manufacturer's guidelines for DNA ligase. In short, ligation reactions were prepared such that there was a 3:1 ratio of insert DNA to vector DNA, 1x ligation buffer, 2 U T4 DNA ligase, and water to a final volume of 20 µL. Reactions were incubated at 14°C for at least 18 hours. Ligations were assessed by electrophoresing an aliquot of ligation mixture on a 0.8% agarose gel. For simplicity, plasmids containing Hyl-G-Hyl-H fragment, Hyl-G-Hyl-L fragment, and the Hyl-K-Hyl-L fragment were named pSW5-GTG, pSW5-ATG, and pSWGTG-ATG, respectively.

Transformation of plasmids into *E. coli*. Ligation mixes were transformed into *E. coli* strain JM109 by standard protocols (6). Briefly, an overnight culture of *E. coli* strain JM109 was diluted 1:100 into pre-warmed SOB broth and was incubated at 37°C for a period of 2.5 hours (OD_{600}=0.375). Cells

were washed by centrifugation at 16000 x g and resuspended in Transformation Buffer (TFB) (100 mM potassium chloride, 45 mM manganese chloride, 10 mM calcium chloride, 3mM hexamminecobalt (III) chloride ($HACoCl_3$), 10 mM potassium 2-(4-morpholino)-ethane sulfonic acid (K-MES)). After further centrifugation cells were resuspended at 1/12 original volume in TFB with the addition of DnD (1 M diothiothreitol (DTT), 90% dimethyl sulfoxide (DMSO), 10 mM potassium acetate). Competent cells were incubated on ice with ligation mix for 30 minutes, cells heat-shocked at 42°C for 90 seconds, allowed to grow for 60 minutes in SOB, and plated on 2x YT agar containing 100 µg/mL ampicillin. Plates were incubated at 37°C overnight and resulting colonies screened for fluorescence by exposing colonies to ultraviolet light. Fluorescing colonies were picked and screened for presence of promoter fragment by PCR or restriction enzyme digests. Further confirmation of insert was attained by sequencing with primers specific for the plasmid (pJC2-A and pJC2-B).

Sequencing of plasmids containing potential promoter regions.

Sequencing of pSW5-GTG, pSW5-ATG, and pSWGTG-ATG was performed using dideoxy-sequencing method outlined for the ABI PRISM 310 Genetic Analyzer. Briefly, sequencing reactions were prepared as outlined for the Big Dye™ Terminator v3.0 Cycle Sequencing Ready Reaction. Reactions contained 200-500 ng plasmid DNA, 4.0 pmol primer (pJC2-A or pJC2-B; Table 1), 5.4 µL Bioline Half-Dye Buffer, 2.6 µL Big Dye, and sterile water to a final volume 20 µL. The incorporation of the dideoxy-labeled nucleotides was accomplished by using the following PCR reaction as outlined by the manufacturer: twenty-four cycles

of denaturation at 96°C for 30 seconds, annealing at 50°C for 15 seconds, and extension at 60°C for 4 minutes. The ramping speed for each step was 1°C per second. Following the PCR, unincorporated dyes were removed by precipitation with 95% reagent ethanol, 3 M sodium acetate pH 5, and sterile water for one hour. Sequencing reactions were pelleted by centrifugation at 16000 x g, pellets were washed with 70% ethanol, and pellets were dried by heating at 90°C for 1 minute. Pellets were resuspended in ABI PRISM Template Suppression Reagent and were heated to 95°C for 2.5 minutes prior to being loaded onto the ABI PRISM 310 Genetic Analyzer. Sequences obtained were analyzed by using the Vector NTI Suite of programs (Informax, MD).

Insertion of the green fluorescent protein gene (*gfp*) into *S. pyogenes* strain 10403. Once a successful fusion between the potential *hyl*A promoters and *gfp* was achieved, pSW5-GTG, pSW5-ATG, and pSWGTG-ATG were digested with restriction enzymes, EcoRI and XbaI, such that the promoter:*gfp* fusions were excised from the vector. The isolated fragments were inserted into either streptococcal integration vector, p7int (114), or *E. coli-S. pyogenes* shuttle vectors pAT28 and pAT29 (157). Following ligation, the plasmids were transformed into either *E. coli* strain JM109, DB11, or BHB2600. After transformation with the plasmid containing p7int-fusion, the cells were plated on 2xYT agar containing either 1 mg/ml (JM109) or 250 µg/mL erythromycin (DB11, BHB2600). Cells transformed with the pAT28/29 vectors were plated on 2xYT containing 250µg/mL spectinomycin. The presence of the insert was confirmed by PCR using primers specific for the amplification of *gfp*

(Gfp-F, Gfp-R; Table 1) and by sequencing. The plasmids were electroporated into *S. pyogenes* strain 10403 using a previously described technique (142). Briefly, 1 mL of an overnight culture of *S. pyogenes* strain 10403 was diluted in 49 mL pre-warmed THB supplemented with 5% horse serum and incubated for three hours (OD_{600}= 0.200). The cells were pelleted by centrifugation at 15,300 x g at 4°C. The pellet was resuspended in 0.5 M sucrose, transferred to a microcentrifuge tube, and pelleted by centrifugation at 16000 x g. The supernatant was removed and cell pellet was washed in 0.5 M sucrose a total of five times. Following the last wash, the cells were resuspended in 100 µL of 0.5 M sucrose. An aliquot of 40 µL of cells was transferred to a microcentrifuge tube, 1-5 µL of plasmid was added, and the mixture transferred to a 2 mm electroporation cuvette. The cells were exposed to a single pulse of 2.5 kV, 25 µF, 200 Ω on a BTX Transporator™ Plus. Cells were incubated at 37°C for 2 hours for expression. Electroporated cells were then plated on THA containing 2.5 µg/mL erythromycin (p7int) or 250 µg/mL spectinomycin (pAT28/pAT29) for selection of the desired streptococci. Colonies that grew on the plates were analyzed by PCR for the presence of *gfp*. Colonies were directly viewed for GFP on a Nikon fluorescent microscope using an excitation wavelength of 365 nm and an emission wavelength of 470 nm.

RNA isolation. *S. pyogenes* cultures were prepared by inoculating 1 mL of an 18-hour culture into 50 mL pre-warmed THB supplemented with 5% horse serum. RNA was isolated at two hour intervals for an 18 hour period, or from cultures grown under various conditions to an OD_{600}= 0.375 (cfu/mL= 3.0×10^7).

For the environmental conditions, OD_{600}= 0.375 (4 hour growth) was randomly chosen and represents mid-growth. Cells were pelleted and RNA was isolated using a modification of extraction protocols previously outlined (113, 134). Briefly, lysis of cells was executed by resuspending the cell pellet in 1 mL RNA Lysis buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.01 M β-mercaptoethanol). The cell resuspension was transferred to a 1.5 mL bead-beater tube filled 1/3 with 0.1 µm glass beads. The resuspension was subjected to two rounds of bead-beating and the lysate transferred to a 1.5 mL microcentrifuge tube. RNA was purified using acid phenol and chloroform extractions, in which equal volumes of acid phenol and chloroform were added to lysate. Prior to extractions, acid phenol was pre-warmed to 65°C. RNA was separated from phenol/chloroform by centrifugation at 16000 x g. The upper aqueous layer containing RNA was transferred to a sterile nuclease-free microcentrifuge tube. Phenol and chloroform extractions were repeated until a white precipitant interface no longer appeared between the two layers. The upper aqueous layer was transferred to a sterile microcentrifuge tube and precipitation of RNA was achieved by adding 0.1 volume diethylpyrrocarbonate-treated 5 M ammonium acetate and 2.5 volumes 95% reagent ethanol, followed by precipitation at -20°C for 18 hours. Following centrifugation at 16000 x g for 20 minutes, the RNA pellet was washed once with RNAse-free 70% ethanol. Precipitated RNA was resuspended in 200 µL of RNA Storage Solution and, to facilitate resuspension, the RNA was heated to 65°C for 10 minutes. RNA was stored at -20°C for further use. For long term storage, RNA was stored at -80°C.

DNase-treatment of RNA isolate. To remove residual DNA contamination, total RNA was treated with RQ1 RNase-Free DNase in accordance with manufacturer's instructions. Briefly, 20 µL total RNA was diluted in 1X RQ1 RNase-Free DNAse Reaction Buffer, 1U RQ1 RNAse-Free DNase, and nuclease-free water to a final volume of 100 µL. Reactions were incubated at 37°C for three hours. Following incubation, digestion was terminated with the addition of 2 µL of RQ1 DNase Stop Solution followed by incubation at 65°C for 10 minutes. DNase-treated RNA was stored at -20°C or for long-term storage at -80°C.

Reverse-transcription-polymerase chain reaction (RT-PCR). Total RNA was used in RT reaction as outlined by manufacturer's instructions for the Improm-II™ Reverse Transcription System. In brief, 25 ng total RNA was incubated with 20 pmol of reverse primers specific for *hyl*A, *gyrA*, or *hasA* (Table 1) for 5 minutes at 70°C to anneal primers to RNA template. Following incubation, the reaction was immediately cooled at 4°C for 5 minutes. A master-mix containing 3 mM $MgCl_2$, 1X ImProm-II™ Reaction Buffer, 0.5 mM dNTP, 20 U Recombinant RNasin® Ribonuclease Inhibitor, 1 µL ImProm-II™ Reverse Transcriptase, and nuclease-free water was added such that the RT reaction had a final volume of 20 µL. Negative controls, which tested for DNA contamination, were prepared where ImProm-II™ Reverse Transcriptase was not added to the reaction. Synthesis and extension of cDNA was achieved by incubating reactions at 37°C for one hour. Inactivation of the reverse transcriptase was

performed by heating the reaction to 70°C for 15 minutes. Newly synthesized cDNA was stored at -20°C until use in either a standard PCR reaction or in a real-time PCR reaction as described below.

Standard PCR reactions for the amplification of a 90 bp fragment of *hyl*A contained 2 µL cDNA, 1X Taq DNA Polymerase Buffer, 2.5 mM $MgCl_2$, 1.6 mM dNTP, 1 U Taq DNA Polymerase, 20 pmol forward and reverse primers (Hyl-K, Hyl-L; Table 1), and nuclease-free water to a final volume of 25 µL. Amplification was achieved using the protocol previously outlined (p. 38).

Preparation of standard curve for real-time analysis. Chromosomal DNA from *S. pyogenes* ATCC strain 10403 was quantified using an Eppendorf Biophotometer. Chromosomal DNA was serially diluted and an aliquot of each dilution was used in a real-time PCR reaction (TaqMan assay) on a Smart Cycler® system (Cepheid, CA). Real-time PCR reactions were prepared such that each reaction contained 10 µL DNA, 1X Taq DNA Polymerase Buffer, 2.5 mM $MgCl_2$, 1.0 mM dNTP, 20 pmol each forward and reverse primer [specific for *hyl*A (Hyl-K, Hyl-L; Table 1; Fig. 3), *gyrA* (GyrA-F, GyrA-R; Table 1), or *hasA* (HasA-F, HasA-R; Table 1)], 3.0 pmol fluorescein-labeled probe specific for *hyl*A, *gyrA*, or *hasA* (Table 1), 1 U Taq DNA Polymerase, and sterile water to a final volume of 25 µL. The program used to amplify *hyl*A was: initial denaturation at 95°C for 75 seconds without optics, followed by 45 cycles of denaturation at 95°C for 5 seconds without optics and an annealing/extension stage at 49°C for 40 seconds with optics. The programs for *gyrA* and *hasA* were similar to that of *hyl*A with the exception of the annealing temperature, which was adjusted to

55°C and 59°C, respectively. Each dilution was programmed as a standardized sample on the Smart Cycler® system and cycle threshold value, the cycle at which fluorescence becomes significant above background fluorescence, for each was plotted accordingly. Resultant standard curves were used for determination of relative transcript amounts in subsequent experimental reactions.

Quantitative polymerase chain reaction (Real-time PCR). Real-time PCR was carried out essentially as previously described (24). An aliquot of cDNA from a RT reaction, no-RT controls, and fluorescein-labeled probes were used in TaqMan assays on a Smart Cycler® system for detection of *hyl*A, *gyrA,* and *hasA* as described above. The cycle threshold for each sample was plotted against a standard curve and raw abundance values for transcript quantities were determined. The *hyl*A cDNA raw abundance values were normalized against *gyrA* cDNA raw abundance values (*hyl*A cDNA quantity/*gyrA* cDNA quantity) to determine relative *hyl*A expression from each RNA isolate. The same procedures were performed for the determination of *hasA* transcript levels. The process was repeated in triplicate from at least two independently isolated RNA preparations. The mean normalized value from each isolation was used to determine the overall mean normalized value for the sample (mean from all extractions). The mean +/- the standard error of the mean for each sample is reported.

Comparison between intracellular and extracellular hyaluronate lyase. Eighteen hour cultures of *S. pyogenes* strains were diluted 1:100 into pre-warmed THB containing 5% horse serum. The cultures were grown for periods of 12 hours with 1 mL aliquots collected at 1 hour time points. Cells were pelleted by centrifugation at 16000 x g, supernatant was collected and assayed for hyaluronate lyase activity by dispensing 20 µL into wells in a hyaluronidase assay plate as previously described (76). To remove bound/attached extracellular hyaluronate lyase from cells, cell pellets were washed twice by resuspending in 1mL 0.89% saline solution, pelleting by centrifugation at 16000 x g. To release any intracellular hyaluronate lyase, cell pellets were lysed by resuspending cells in 500 µL of Lysis Solution (1X TE Buffer (Tris-HCl, EDTA pH 8.0); 50 mg/mL Lysozyme), followed by incubation at 37°C for one hour. Following incubation, cells were sonicated on ice for 5 one minute blasts at 35% on Virsonic Cell Disrupter. An aliquot of cell lysate was Gram stained to determine cell lysis. Cell debris was pelleted by centrifugation and twenty microliters of cell lysate was transferred into wells on a hyaluronidase assay plate as previously described; plates were incubated for eighteen hours. Following the addition of 2N acetic acid to the assay plate, zones of clearing surrounding the point of inoculation were measured.

METHODS FOR HYALURONATE LYASE GENE SIZE VARIABILITY STUDIES

Bacterial strains and hyaluronate lyase assay. Strains of *S. pyogenes* used in this study are outlined in Appendix II. Strains 350, 422, 872, 1020, 1055, and 94-146, along with ATCC strain 10403 and strain SF370 (M-type 1) were used in the additional analysis described here. All strains were grown on Todd-Hewitt agar or Sheep Blood agar at 37°C. Hyaluronidase activity of the strains was detected using the BHB plates prepared as previously described (77). *Escherichia coli* strains were grown on 2x YT media supplemented with antibiotics as required.

Crude lysis of *S. pyogenes* for PCR. DNA was obtained from 176 strains of *S. pyogenes* using a crude cell lysis procedure previously described (79). In brief, a 2mL overnight culture of *S. pyogenes* was pelleted by centrifugation at 16000 x g. The supernatant was removed and cell walls were weakened by resuspending pellets in 1 mL STE buffer (20% sucrose, 10mM Tris-HCl pH 8, 100mM NaCl, 1mM EDTA) containing 25mg/mL lysozyme and 50U/mL mutanolysin, followed by incubation at 37°C for 1 hour. Cell suspension was centrifuged, supernatant was removed, and cells were lysed by resuspending and incubating the cell pellet in 1 mL lysis buffer (100 mM KCl, 10mM Tris-HCl pH 8.3, 0.1mg/mL gelatin, 0.45% Igepal, 0.45% Tween 20) containing 100µg/mL proteinase K for 1 hour at 60°C. After incubation, proteinase K was inactivated by heating to 95°C for 10 minutes. DNA from each of the 176 strains was analyzed for the presence of *hyl*A by PCR as described

earlier (p. 38). Primers derived from the sequence of *hyl*A were used to amplify the 3' regions (Hyl-B, Hyl-D; Fig. 3) of all tested strains and are listed in Table 1. Additionally, the entire gene (Hyl-A, Hyl-B; Table 1; Fig. 3), the internal region (Hyl-E, Hyl-F; Table 1; Fig. 3), and the 5' region (Hyl-A, Hyl-C; Table 1; Fig. 3) of the gene were amplified. DNA fragments were amplified using Taq DNA polymerase, or Takara Ex Taq. PCR products were electrophoresed on a 0.8% TBE agarose gel at 150V for 1.5 hours.

Cloning of the 3' region of the hyaluronate lyase gene. PCR amplified 3' regions from strains 350, 422, 872, 1020, 1055, 94916, 10403, and SF370 were cloned into *E. coli* using either the pCR 2.1 vector system or the pGEM-T Easy vector system according to the manufacturer's instructions. Briefly, 3' amplicons were ligated with the above vectors using the protocol described above. Ligation mixtures were incubated with competent *E. coli* strain JM109 on ice for 1 hour; cells were heat shocked at 42°C for 90 seconds and allowed to express in SOB at 37°C for 1 hour, then plated on 2x YT agar containing 100µg/mL ampicillin, X-GAL, and IPTG. Blue-white screening allowed for the picking of colonies that contained an insert. Transformants were screened either by restriction enzyme digestion or by PCR using primers specific for the 3' region of *hyl*A. Plasmids containing the cloned 3' region from each of the six strains were sequenced using the dideoxy-sequencing method outlined for the ABI PRISM 310 Genetic Analyzer. Standard M13 sequencing primers (M13-Forward and M13-Reverse; Table 1) and two primers internal to the cloned fragment (Hyl-M and Hyl-N; Table 1; Fig. 3) were used. The fragments were sequenced in

both directions. Alignment and homology analysis was performed using the Vector NTI Suite (InforMax, MD).

Isolation of chromosomal DNA from *S. pyogenes* strains containing the smaller hyaluronate lyase gene region. Chromosomal DNA was isolated from the six strains of *S. pyogenes* using a previously described protocol (73). Briefly, a 50 mL overnight culture of *S. pyogenes* was diluted 1:1 in pre-warmed THB supplemented with 5% horse serum and incubated at 37°C for 1 hour. To weaken the cell wall, 1 g sterile glycine was added to the culture and the cultures incubated for an additional 1 hour at 37°C. Cells were pelleted by centrifugation at 15300 x g for 10 minutes. Pellets were resuspended in 5 mL Lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 50 mg/mL lysozyme, 50 U/mL mutanolysin) and incubated at 37°C with shaking. To inactivate nucleases 200 µg/mL proteinase K and 0.6% sodium dodecyl sulfate (SDS) were added to the lysate. Following incubation at 55°C for 1 hour, 25 mL GES (200 mM guanidium thiocyanate, 100 mM EDTA pH 8, 10% sarkosyl) was added and incubated on ice for 10 minutes. Next, 15 mL 7.5 M ammonium acetate and 25 mL chloroform: isoamyl alcohol (24:1) mix were added. After centrifugation, the upper aqueous layer was collected and DNA was precipitated with 0.6 volume isopropanol at 4°C. Precipitated DNA was pelleted by centrifugation at 15300 x g, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). DNA was further treated one or more times with phenol: chloroform extractions, and then concentrated by precipitation by adding 0.1 volume 3M sodium acetate pH 5 and 2 volumes 95% ethanol. The

chromosomal DNA was digested with *Eco*RI, electrophoresed on a 0.8% TBE agarose gel, and transferred by capillary action to a membrane using a standard protocol for Southern blotting.

Southern blot hybridization using *hyl*A. Southern blot analysis was utilized to confirm the presence of the 3' region of *hyl*A in the chromosomal DNA of strains of *S. pyogenes* that possess a smaller *hyl*A. Southern blot analysis was performed as outlined (6). Following transfer of the DNA to a nylon membrane, the DNA was cross-linked to membrane by baking for 30 minutes at 80°C, followed by ultraviolet-cross-linking. The membrane was pre-hybridized with standard hybridization buffer (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking reagent), rotating at 68°C, for at least 2 hours and was hybridized overnight, rotating at 68°C, using either a PCR amplified 3' fragment or internal region of *hyl*A labeled with digoxigenin (DIG). Un-hybridized probe was removed by subsequent washes. Initially, the blot was washed twice at room temperature with a 2X wash solution (2X SSC, 0.1% SDS), followed by two washes at 68°C in 0.5X wash solution (0.1X SSC, 0.1% SDS) with constant agitation. Following the washes, the membrane was blocked in 1% blocking reagent for 30 minutes and then incubated for 1 hour in 1% blocking reagent containing 150 mU/mL anti-DIG-AP. Unbound antibody was removed by washing twice in 100 mL washing buffer (0.1 M maleic acid, 0.15 NaCl; adjusted to pH 7.5, 0.3% Tween® 20). After washes, the blot was equilibrated in detection buffer (0.1M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). The blot was

developed using the color-substrate solution (detection buffer, NBT/BCIP stock solution).

Preparation of the DIG-labeled probe was achieved by heat denaturing 15 µL purified PCR amplified 3' or internal regions of *hyl*A for 10 minutes. To the denatured DNA, 1X hexanucleotide mix, 1 mM dNTP mix, and 2U Klenow fragment of *E. coli* DNA polymerase I was added. The mix was incubated at 37°C for at least 1 hour. Following incubation, the reaction was stopped by the addition of 0.2 M EDTA, pH 8.0. The labeled DNA was precipitated by incubation in 4M lithium chloride (LiCl) and pre-chilled 95% ethanol at -80°C for 30-60 minutes. Following precipitation, the labeled DNA was pelleted by centrifugation at maximum speed for 15 minutes and the pellet washed with cold 70% ethanol. The DNA was resuspended in 50 µL nuclease-free water and was ultimately added to hybridization buffer for blotting. The probe was stored at -20°C.

METHODS FOR INACTIVATION OF *hylA* STUDIES

Bacterial cultures used in the preparation of a *hylA* knock-out mutant strain 10403:Δ*hylA*. *S. pyogenes* ATCC strain 10403 was grown on Sheep Blood agar at 37°C, or was inoculated into THB containing 5% horse serum grown at 37°C. *S. pyogenes* strain 10403::pHAS:*hylA* (76) was grown on THB or THA containing 2.5 µg/mL erythromycin. *E. coli* strain TOP10F' containing the plasmid pCRNT/T7: *hylA* (from the culture collection of Dr. W. Hynes, Old Dominion University, Norfolk, Virginia) was grown in 2x YT medium supplemented with 100 µg/mL ampicillin and grown at 37°C. *E. coli* strain DB11 containing plasmid pUC:erm (obtained from the culture collection of Professor J.J. Ferretti, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma) was grown in 2x YT medium supplemented with 250µg/mL erythromycin.

Enzymatic digestion of the hyaluronate lyase gene. The plasmid containing *hylA*, pCRNT/T7: *hylA*, was isolated from *E. coli* strain TOP10F' using standard protocols (6) and was digested with the restriction enzyme *Eco*RV. *Eco*RV digests *hylA* in such a way that a 924 bp internal fragment is removed from the gene. The digested plasmid was electrophoresed on a 0.8% TBE agarose gel at 150V for 1.5 hours. The 4484 bp fragment was isolated from the agarose gel using the QIAEX II Gel Extraction Kit as described. To prevent the fragment from re-annealing, the digested fragment was subjected to a calf intestine alkaline phosphate (CIAP) treatment. Briefly, CIAP buffer was added to

the purified fragment to a final volume of 50 µL. CIAP (1U) was added and the reaction mixture was incubated at 37°C for 30 – 60 minutes, followed by heat inactivation at 85°C for 15 minutes. The CIAP-treated plasmid was stored at -20°C.

Isolation of the erythromycin resistance gene from the plasmid pUC:erm. To isolate the erythromycin resistance gene (*ermR*) from the plasmid pUC:erm, the plasmid was digested with restriction enzymes, *Eco*RI and *Hind*III. Digestion of the plasmid with these restriction enzymes removes a 1162 bp fragment, which encodes *ermR*. The digest was electrophoresed on a 0.8% agarose gel in TAE buffer and the 1162 bp fragment was purified from the gel using the QIAEX II Gel Extraction Kit. Following purification, the recessed ends of the digested fragment were "filled-in" using standard protocols (6). Briefly, the digested fragment was incubated in the presence of the Klenow fragment of *E. coli* DNA polymerase I and all dNTPs. Following the blunt-ending, the *ermR* was ligated with *Eco*RV-digested pCRNT/T7:*hyl*A using standard protocols (6); the resulting plasmid was transformed into *E. coli* strain JM109 and plated on 2x YT broth containing 1 mg/mL erythromycin. Selected colonies were screened by restriction enzyme digestion. One clone was chosen for further analysis and was sequenced using the internal *hyl*A primer Hyl-E (Table 1) to confirm the presence of *erm*R. Sequencing reactions were prepared using the dideoxy-sequencing method outlined for the ABI PRISM 310 Genetic Analyzer. Once the presence of *ermR* was confirmed to be contained in the plasmid, primers specific for *hyl*A:*ermR* region (Hyl-N, Hyl-O; Table 1; Fig. 3) were used for PCR

amplification. The amplified fragment was purified and was electroporated into *S. pyogenes* strain 10403 (142) . Following electroporation and expression in THB for 3 hours at 37°C, the culture was plated on THA containing 2.5 µg/mL erythromycin and incubated at 37°C. Colonies were selected for erythromycin resistance and were assayed for loss of hyaluronate lyase activity using the BHB assay plate (77).

Southern blot analysis for the presence of *ermR*. Chromosomal DNA was isolated (73) from colonies that were erythromycin resistant and HylA$^-$, as well as from wild-type *S. pyogenes* strain 10403. The DNA was digested with *Eco*RI and was electrophoresed on a 0.8% agarose gel. The plasmid pUC:erm, digested with *Eco*RI and *Hind*III (drops out *ermR*), was also included on the gel. The DNA was transferred to a membrane for Southern blot analysis using standard protocols (6), and the membrane was hybridized with DIG-labeled probes specific for *hyl*A and *ermR*. The blot was developed using standard methods.

Growth curve comparison between wild-type strain 10403 and mutant strain 10403Δ*hyl*A. To compare the growth of wild-type *S. pyogenes* strain 10403 and mutant strain 10403Δ*hyl*A, an overnight culture of each was diluted into fresh Todd-Hewitt broth (THB) supplemented with 5% horse serum and incubated at 37°C. Samples were taken at 60-minute intervals for determination of optical density at a wavelength of 600nm.

Assay for hemolysin activity. To determine hemolysin activity, a single bacterial colony was stabbed onto Sheep Blood Agar and incubated at 37°C overnight. Hemolysin activity was observed as a zone of clearing around the point of inoculation.

Assay for protease activity. Cysteine protease activity was determined by stabbing a single bacterial colony onto a plate containing Columbia Blood Agar and 3% Carnation Skim Milk (81). Cultures were incubated at 37°C overnight. Protease activity was indicated by a zone of clearing surrounding the point of inoculation.

Preliminary analysis of the role of hyaluronate lyase in streptococcal infection. An overnight culture of a single-recombinant mutant strain 10403::pHAS: *hyl*A (76), which no longer produces hyaluronate lyase, was diluted 1:100 into 99 mL pre-warmed THB supplemented with 5% horse serum. The culture was grown for 6 hours until cells were in late log phase, pelleted by centrifugation at 15300 x g, and washed twice with isotonic saline solution. The pellet was resuspended in 1.5 mL of isotonic saline, and a viable count carried out (53). One hundred µL of cell suspension was injected subcutaneously into each of 5 CD-1 white mice. Mice were observed for formation of lesions or abscesses. Once a lesion was observed, the wound was swabbed and plated on blood agar plates. Beta hemolytic colonies that grew were analyzed for erythromycin resistance, the production of catalase, and for the production of hyaluronate lyase. Mice were observed for a period of two weeks. The entire

procedure was performed on two separate groups of mice. Following experimentation, mice were euthanized by subjection to excessive carbon dioxide. Prior to experimentation to this animal study, protocols used were reviewed and approved by the university's Institution for Animal Care and Use Committee (IACUC) (IACUC Protocol # 00-001).

RESULTS OF REGULATORY STUDIES

Hyaluronate lyase activity from strain 10403 during bacterial growth. Since the time of hyaluronate lyase production by strain 10403 was not known, the point at which HylA activity is detected from this strain was determined. HylA activity was assessed by applying cell-free culture supernatants obtained over a 10 hour period, as well as from an 18-hour culture, to a standard hyaluronate lyase assay plate (76). Hyaluronate lyase activity was evaluated by observing and measuring the diameter of the zone of clearing surrounding the well containing the supernatant (Fig. 4). A zone of clearing, indicating hyaluronate lyase activity, was first observed at hour two, increased in size until hour 10, and remained constant to hour 18.

The time of production for HylA was then correlated with growth. A culture of strain 10403 was grown; turbidity was measured at a wavelength of 600 nm, and assayed for hyaluronate lyase activity each hour for a 10 hour period. HylA activity was detected in the early exponential stage of the growth cycle. To ascertain the titer of hyaluronate lyase for each time point, the supernatant was serially diluted and assayed (Table 2). The titer represents the inverse of the greatest dilution of the supernatant to give detectable clearing on a hyaluronidase assay plate; this dilution was considered to contain 1 AU. HylA titer leveled off at 64 arbitrary units (AU) around hour 7. Culture turbidity and specific enzymatic activity (AU/log cfu/mL) were measured and plotted against time (Fig. 5).

Determination of promoter region for *hylA* from strain 10403. Since HylA activity was detected as early as hour 2, it was of interest to establish how this gene is regulated. The promoter for *hylA* is not characterized. Sequence analysis of the 3510 bp fragment containing *hylA* proposed two potential start sites, a GTG and an ATG initiation codon. Analysis of the sequence upstream revealed a typical ribosome binding site (RBS) 9 bp upstream of the GTG site and further upstream were additional regions that resemble potential promoters. No RBS or similar promoter region was found appropriately placed upstream of the ATG. Since it was only suggestive that promoter activity exists upstream of the GTG site or potentially between the two possible start sites, the promoter activity of these regions was analyzed by cloning upstream of a promoter-less *gfp* gene. A 128 bp region upstream of the GTG site, a 198 bp region upstream of the ATG site (encompassing the GTG site), and a 90 bp region between the two sites were PCR amplified. *E. coli* clones and the plasmids obtained were named pSW5-GTG (Fig. 6), pSW5-ATG, and pSWGTG-ATG, for each of the respective cloned inserts.

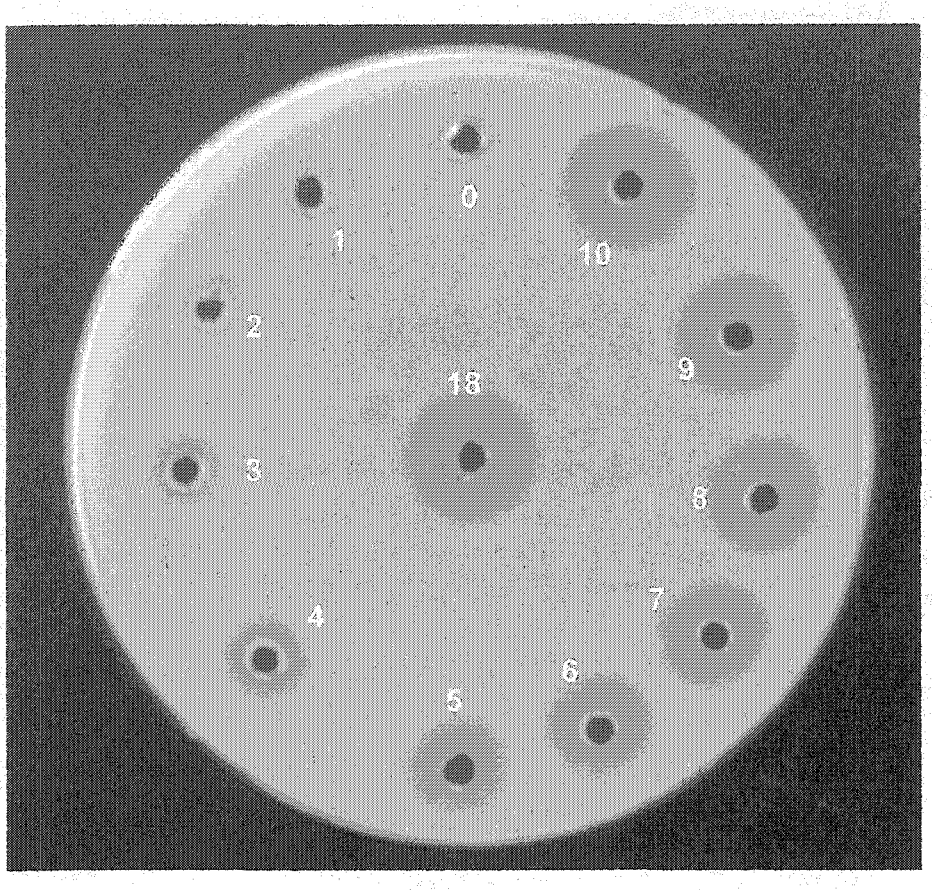

FIG. 4. Hyaluronate lyase activity of strain 10403 over an 18-hour period was determined by the zone of clearing in a plate assay. An increase in the zone of activity for hyaluronate lyase was detected up to 10 hours.

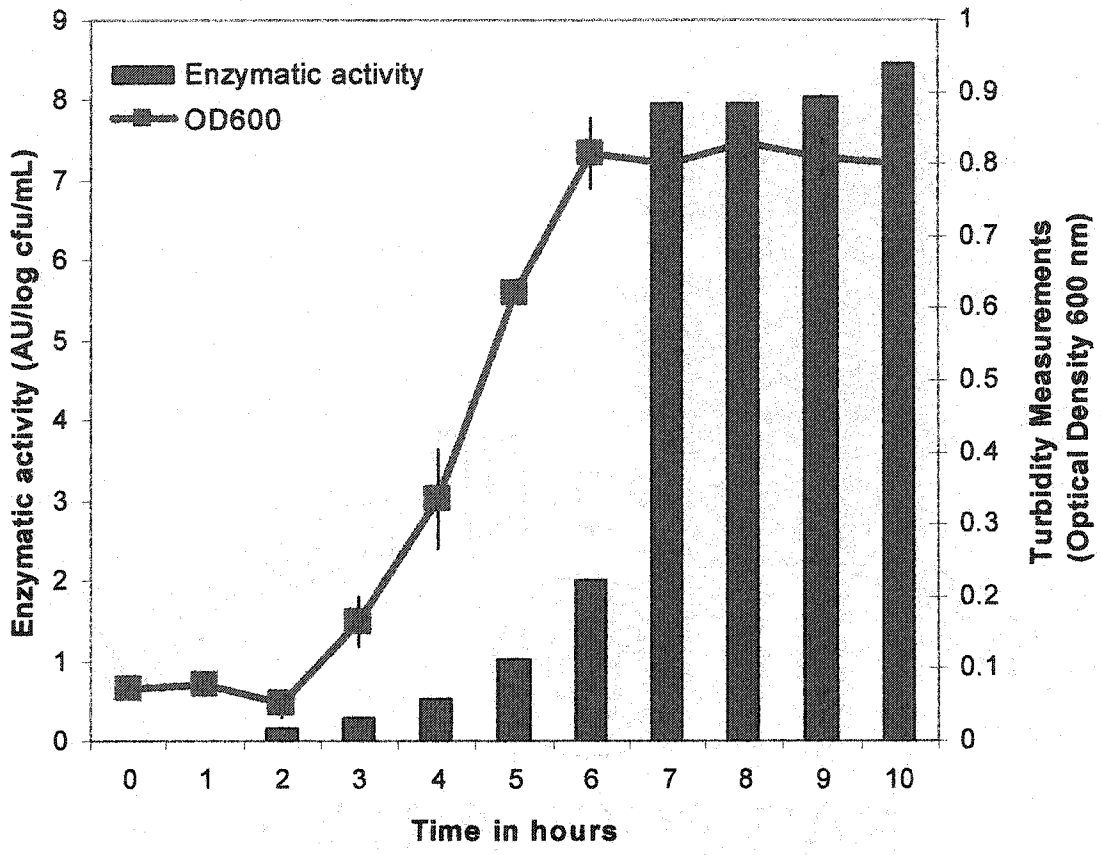

FIG. 5. Growth curve and specific enzymatic activity of strain 10403 over a 10 hour period. Enzymatic activity (representative of hyaluronate lyase activity) plotted with growth indicated that HylA activity is produced in early exponential phase.

TABLE 2. Hyaluronate lyase titer of culture supernatants collected over a 10 hour period

Sample (Timepoint)	Titer [1]	Specific Enzymatic Activity[2]
0	No activity detected	0
1	No activity detected	0
2	1 AU	0.153
3	2 AU	0.284
4	4 AU	0.532
5	8 AU	1.010
6	16 AU	2.015
7	64 AU	7.947
8	64 AU	7.949
9	64 AU	8.028
10	64 AU	8.443

1- Titer recorded as arbitrary units per mL (AU). The greatest dilution of the supernatant to give detectable clearing on hyaluronidase assay plate was considered to contain 1 AU.

2- Specific enzymatic activity is the equivalent of titer/(log cfu/mL).

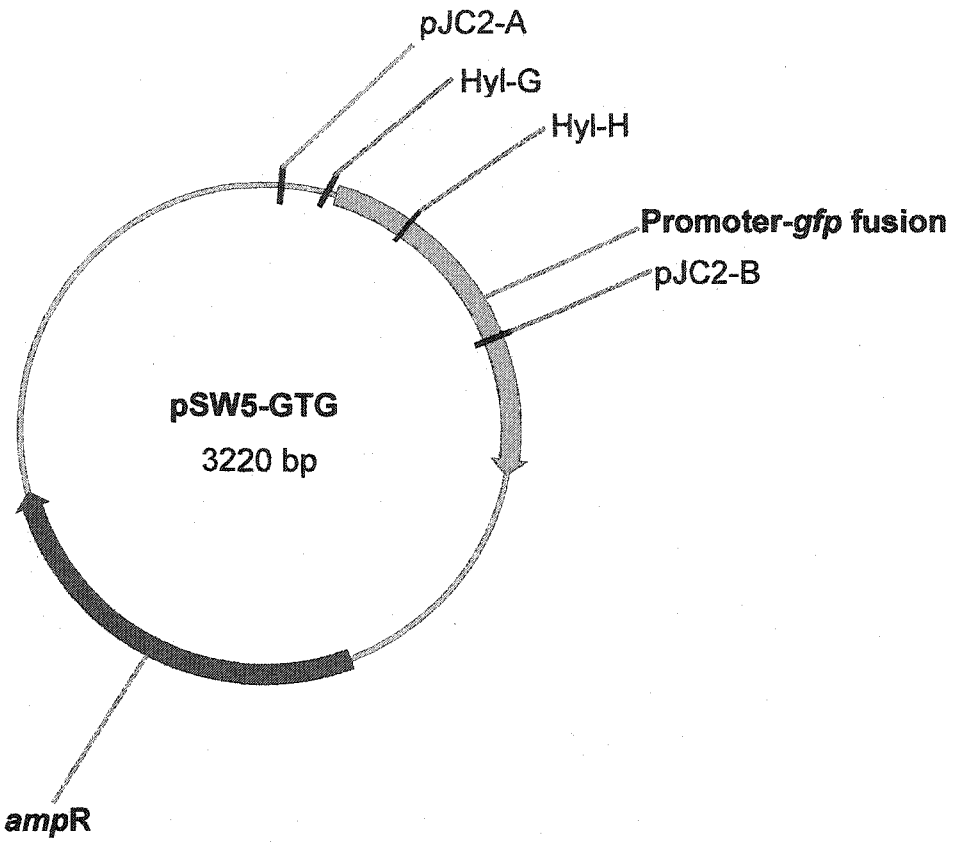

FIG. 6. Plasmid map of construct pSW5-GTG containing the 128 bp fragment of *hyl*A GTG fused to *gfp*. Plasmid pSW5-GTG was prepared such that it contains a *hyl*A promoter-*gfp* fusion. The primers and their locations for sequencing are marked.

Upon exposure to ultraviolet light, fluorescence was observed, indicating promoter activity. However, it was found that some strains of *E. coli* (DB11) were found to fluoresce poorly, whereas other strains (JM109, BHB2600) fluoresced quite well. When *E. coli* strain JM109 was used, two of the three regions possessed promoter activity. *E. coli* containing pSW5-GTG and pSW5-ATG glowed fluorescent green when exposed to ultraviolet light, whereas no fluorescence was observed from *E. coli* containing pSWGTG-ATG. Plasmids were isolated and sequenced to confirm the presence of the fragments and the integrity of the sequence. Sequence analysis confirmed the presence of the fragment and that no mutations were introduced by the PCR.

Since pSWGTG-ATG did not show promoter activity and contained a 90 bp portion of a fragment identical to that of pSW5-ATG, a plasmid having promoter activity, it was anticipated that a transcript for the 90 bp fragment would be present in strain 10403 as part of *hyl*A transcript. RNA was isolated from strain 10403 and analyzed by RT-PCR for the presence of transcript for the three regions screened for promoter activity. RT-PCR analysis, using primers Hyl-K and Hyl-L (Table 1) showed the presence of a band approximately 90 bp in size; however, screening by RT-PCR for transcripts for the region upstream of GTG did not result in any amplicons. These results suggest that the promoter region was contained in a region upstream of the GTG codon.

Insertion of *gfp* into *S. pyogenes* strain 10403. Once the region that contained promoter activity in *E. coli* was determined, the fragment as a *gfp* fusion was excised from the plasmid and inserted into a streptococcal integration

vector, p7int (114). By inserting the *hyl*A promoter region-*gfp* into p7int, it was possible to integrate the construct (Fig. 7) into the streptococcal chromosome without disrupting *hyl*A and would allow for determination of promoter activity. Therefore, both hyaluronate lyase and the *hyl*A promoter-induced GFP would be detected. Following electroporation of the new plasmid into *S. pyogenes* strain 10403, colonies were screened for erythromycin resistance, hyaluronate lyase activity, fluorescence, and β-hemolysis. Fluorescence was not immediately detectable in streptococcal cells. However, using a fluorescent microscope with an excitation wavelength of 365 nm and an emission wavelength of 470 nm, green fluorescent protein production was observable in some clusters of cells. Although green fluorescent protein expression in 10403 was apparent, the fluorescing cells were difficult to visualize, as some cells showed a degree of autofluorescence (Fig. 8). Autofluorescence was particularly noticeable in regions of high cell density.

Since the lack of, or poor, fluorescence in strain 10403 may have been due to there being only one copy of *gfp* in the cells, the same promoter-*gfp* fusion, as inserted into p7int, was inserted into the shuttle vectors pAT28 and pAT29 (157), which can replicate in *S. pyogenes*, thereby resulting in multiple copies of *gfp* in the cells. These plasmid constructs were electroporated into strain 10403 and resulting colonies were screened for spectinomycin resistance, hyaluronate lyase activity, β-hemolysis, and fluorescence. Crude cell lysis was performed on the resulting clones and the DNA was screened for *gfp* by PCR. PCR resulted in amplification of a single fragment of the approximate size of *gfp*

(Fig. 9). Although *gfp* was inserted into strain 10403, poor fluorescence was still observed.

Standard RT-PCR analysis for the detection of *hyl*A transcript in strain 10403. Since the *gfp* reporter assay proved a difficult assay for hyaluronate lyase expression in *S. pyogenes*, an alternative methodology was utilized in which RNA transcript levels for *hyl*A were followed. Because HylA was detected throughout the growth cycle of strain 10403, *hyl*A expression was anticipated to be constitutive. Alternatively, gene expression may have occurred early and the protein then being detected throughout growth. To assess the presence of *hyl*A transcript, RNA was isolated from strain 10403 every two hours, beginning at hour zero, for a period of 10 hours. Reverse transcriptase reactions, containing equal concentrations of total RNA, were used to synthesize cDNA from any *hyl*A transcript and the resultant cDNA was utilized in PCR for amplification of a 90 bp region of the *hyl*A transcript. This region corresponds to the 90 bp between the potential start sites, GTG and ATG, which was amplified using specific primers (Hyl-K, Hyl-L; Table 1; Fig. 3). Electrophoresis of the resultant cDNA indicated the presence of a transcript at all time points, suggesting constitutive expression (Fig. 10).

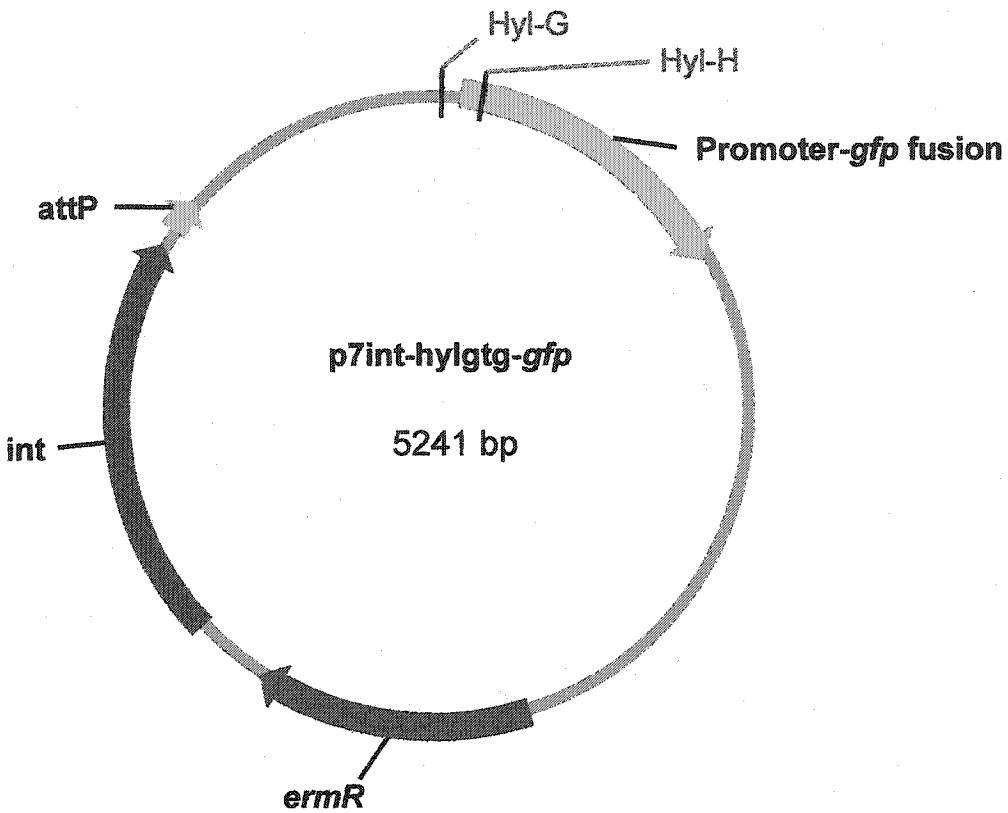

FIG. 7. Plasmid construct p7int-hylgtg-*gfp*. This construct was prepared for the integration of *gfp* fused to the *hyl*A promoter into the chromosome of *S. pyogenes* strain 10403.

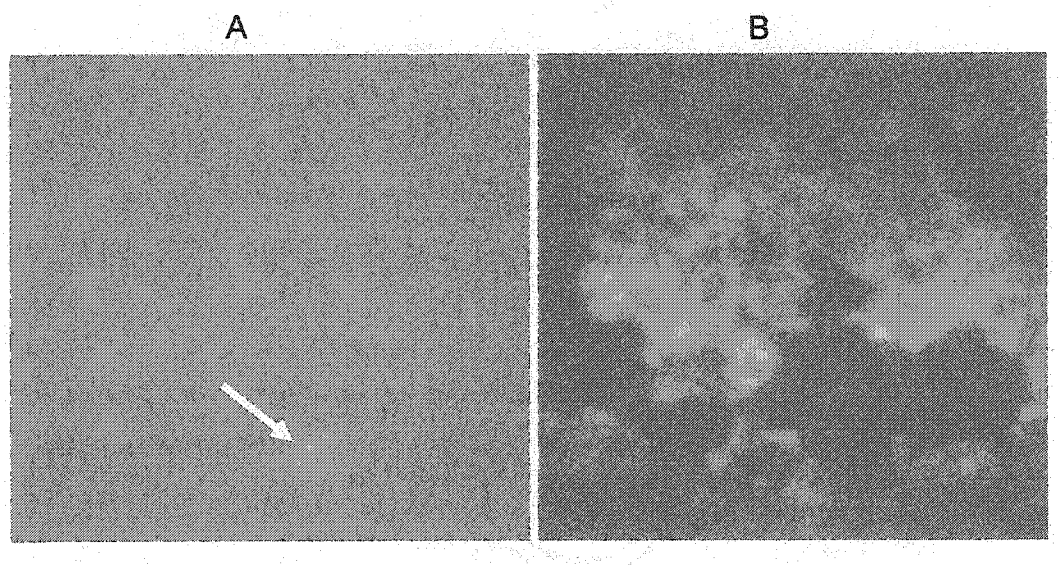

FIG. 8. Fluorescence of *S. pyogenes* strain 10403. Cells containing *gfp* were directly viewed by fluorescent microscopy with an excitation wavelength of 365 nm and an emission wavelength of 470 nm. Frame A shows cells exhibiting GFP, as indicated by the arrow, following electroporation, whereas frame B contains wild-type strain 10403 and exhibits only auto-fluorescence.

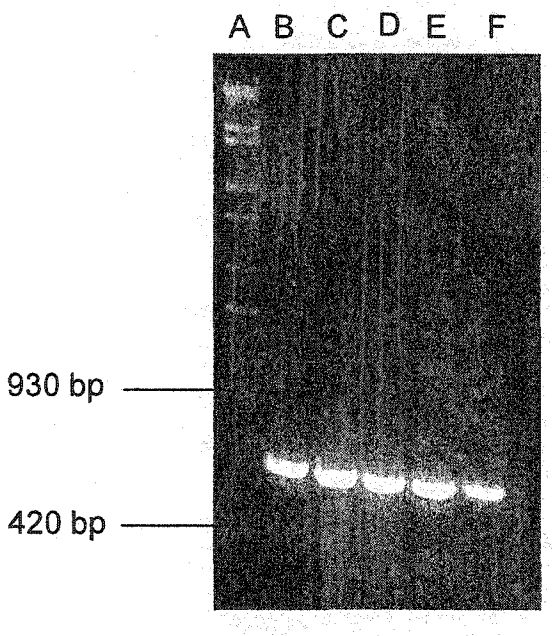

FIG. 9. PCR results showing the presence of *gfp* in *S. pyogenes* strain 10403. Primers specific for *gfp* were used in PCR and amplified a fragment approximately 750 bp in size from 4 colonies of *S. pyogenes* strain 10403 (Lanes B-E), which had been electrotransformed with the plasmid pAT28:*gfp*. Lane A: λ-sty MW ladder; lane F: pJC2 (contains *gfp*).

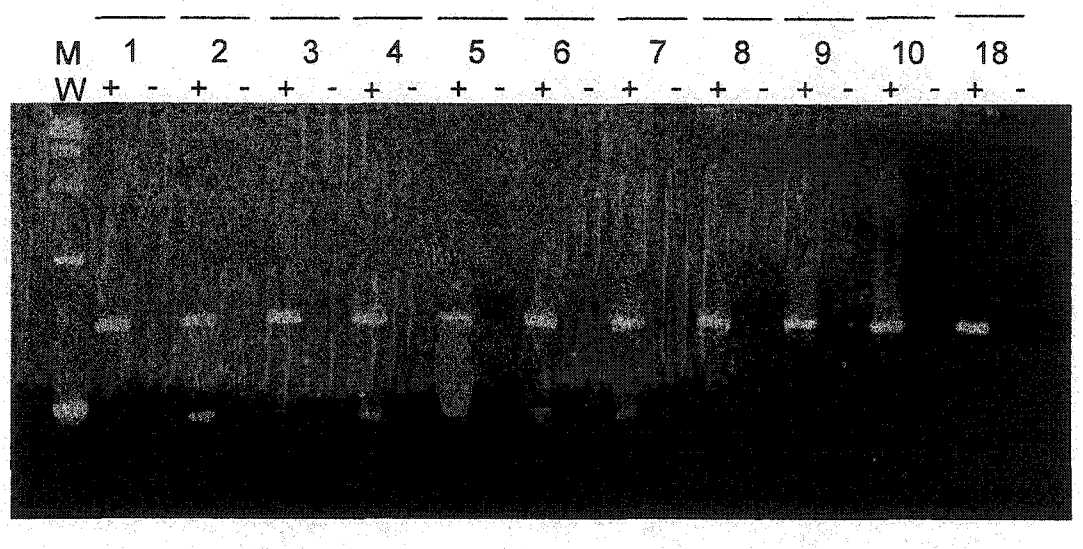

FIG. 10. Gel showing results of standard RT-PCR indicating the presence of *hyl*A transcript throughout the growth of strain 10403. Using primers specific for a 90 bp region of the *hyl*A transcript, the desired transcript was detected over eighteen hours. The samples are annotated as a RT-PCR sample (+), where reverse transcriptase was included in the reaction or a PCR sample (-), where no reverse transcriptase was added.

Real-time quantitative PCR for the quantitation of *hyl*A transcript in strain 10403. Because standard RT-PCR does not represent the amount of *hyl*A transcript for each time point, real-time quantitative PCR was utilized. Equal amounts of total RNA were used in reverse transcription for the synthesis of cDNA for *hyl*A and an internal control *gyrA*, which is constitutively expressed in *S. pyogenes* strain NZ131 (24). Reverse transcription and real-time PCR analysis of RNA from strain 10403 confirmed that *gyrA* expression was also constitutive in strain 10403. Prior to real-time analysis, genomic DNA from strain 10403 was used to generate standard curves for *hyl*A and *gyrA*, which were used for the relative quantification of the cDNA. For real-time PCR analysis, an aliquot of cDNA was used in reactions for the amplification of *hyl*A and *gyrA* cDNA. Any DNA contamination that may have been contained in the RNA sample following DNase-treatment, as determined by the reverse transcriptase negative control, was subtracted from each value. The quantity of cDNA for *hyl*A, representative of the amount of *hyl*A transcript, was normalized to that of *gyrA* present in each RNA sample from the various time points. The results for strain 10403 revealed an increase in the quantity of *hyl*A transcript during early to mid-exponential phase (2-4 hours) of the growth cycle, followed by a decrease at hours 6-8. The pattern of expression for *hyl*A in strain 10403 showed a bi-modal pattern, in which hour 8 had a very low level of *hyl*A transcript, whereas hour 18 had an increase in transcript amounts. Analysis of the hours between 8 and 18 indicated an increase in *hyl*A transcript at hour 10 that remained steady through to hour 16, which was followed by another increase at hour 18 (Fig. 11).

Comparison of *hylA* transcript levels between different strains of *S. pyogenes*. Different strains of *S. pyogenes* were analyzed for the presence of *hylA* transcript. Transcript levels of strain SF370, an acapsular and non-hyaluronate lyase producing strain, and strain 71698, an encapsulated and non-hyaluronate lyase producing strain, were investigated and compared to strain 10403. Cultures of each strain were grown for up to 8 hours with RNA isolated every two hours, as well as from an 18-hour culture. Reverse transcriptase reactions were carried out for *hylA* and *gyrA* cDNA for each sample. The *hylA* raw abundance cDNA values were normalized against the respective *gyrA* value (Appendix III). Neither strain produces active HylA, but both had detectable *hylA* transcript. Analyzing the transcript levels over time for the two strains showed a gradual increase over the early to mid-exponential phase (4 hours) of the growth cycle, followed by a gradual decrease (Fig. 12). The two non-producing strains were found to have a single peak of *hylA* expression, as compared to the bimodal pattern seen in strain 10403. Expression of *hylA* by strain 71698 was greater than that seen in the other two strains, having a peak in transcript level at hour 4, followed by a decline. The levels of transcript for strain 71698 were at least two times greater than that observed for strain 10403 over the first eight hours of growth.

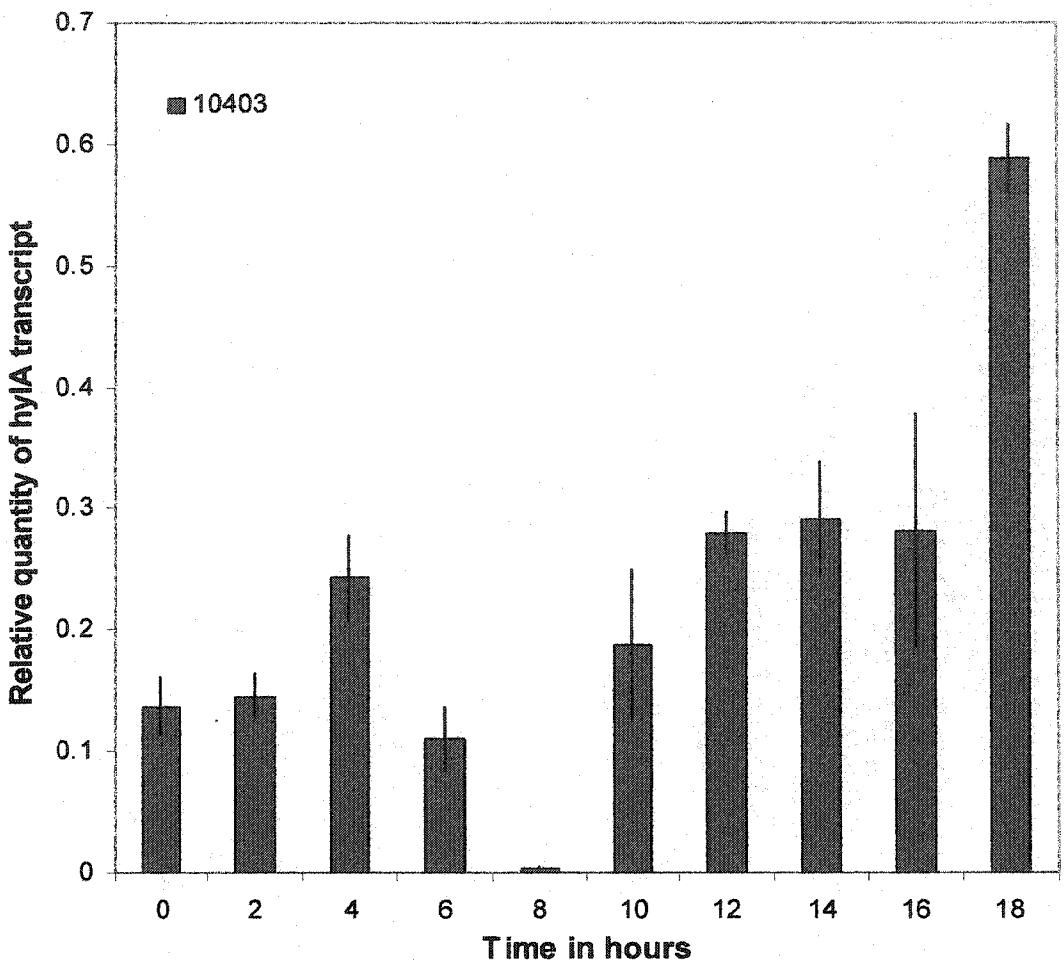

FIG. 11. Relative quantity of *hyl*A transcript determined over an 18 hour growth for strain 10403. The *hyl*A cDNA values were normalized to *gyr*A cDNA values. The results shown are representative of at least two independently isolated RNA preparations analyzed in triplicate and represent the mean +/- the standard error of the mean for each sample.

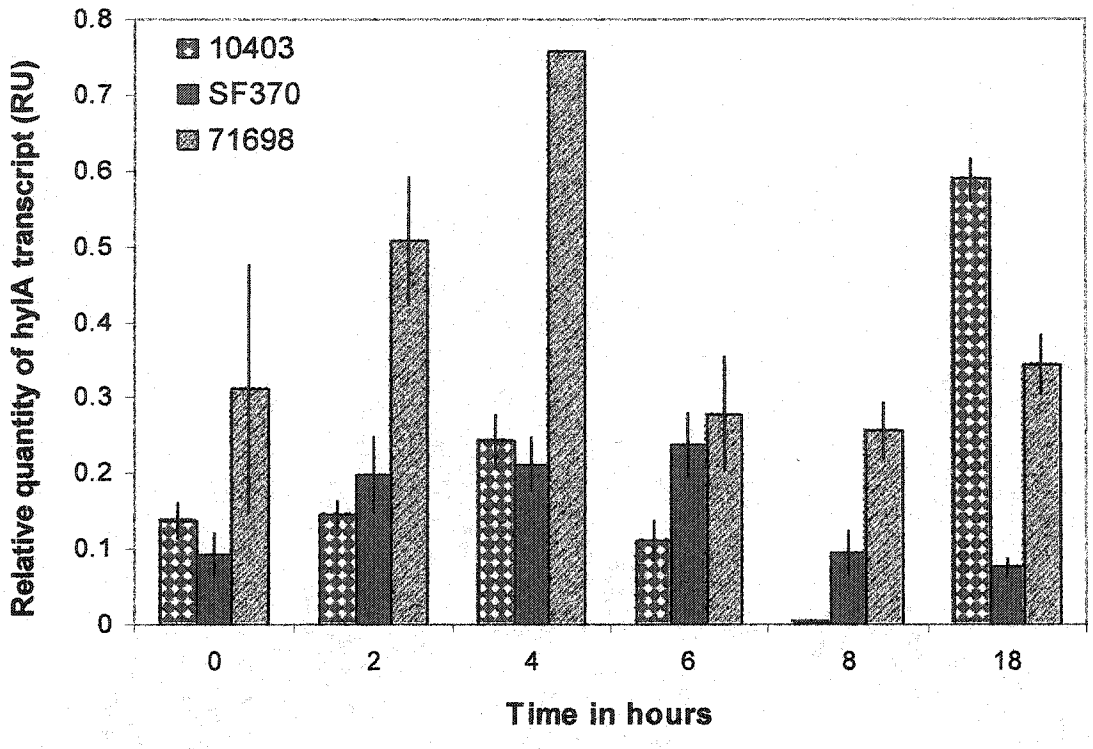

FIG. 12. Comparison of the level of *hyl*A transcript from hyaluronate lyase-producing and non-producing strains. Transcript levels for strains 10403 (acapsular, hyaluronate-lyase producer), SF370 (acapsular, non-hyaluronate-lyase producer), and 71698 (encapsulated, non-hyaluronate lyase producer) were determined. The cDNA values for *hyl*A were normalized against that of *gyrA*. The experiment was performed using RNA prepared from at least two independent RNA isolations for each strain analyzed in triplicate. Each strain was independently isolated. The results are representative of the mean relative quantity of *hyl*A transcript ± the standard error of the mean.

Determination of intracellular and extracellular hyaluronate lyase in strain 10403. Due to the bi-modal pattern of expression for *hyl*A in strain 10403, it was possible a feedback inhibition mechanism may be responsible, such that the level of intracellular HylA was inhibiting expression of *hyl*A at hour 8. To investigate this possibility, a culture of strain 10403 was grown for 18 hours with 1 mL aliquots collected at each hour. Culture supernatants and cell lysates were assayed for hyaluronate lyase activity. To remove residual extracellular HylA, the pellets were washed with isotonic saline prior to lysis by sonication. The supernatant samples showed a gradual increase in the diameter of the zone of clearing surrounding around the well (Fig. 13). The cell lysate revealed a small zone of clearing for all time periods (Fig. 14). To determine that the zone of clearing was potentially due to bacteriophage hyaluronidase and not representative of intracellular HylA, the isogenic mutant strain 10403Δ*hyl*A1 was assayed in the same manner. Since expression of *hyl*A in strain 10403Δ*hyl*A1 is interrupted and does not produce extracellular hyaluronate lyase, any zone of clearing would not be HylA. Assaying revealed the same small zone of clearing as seen from strain 10403.

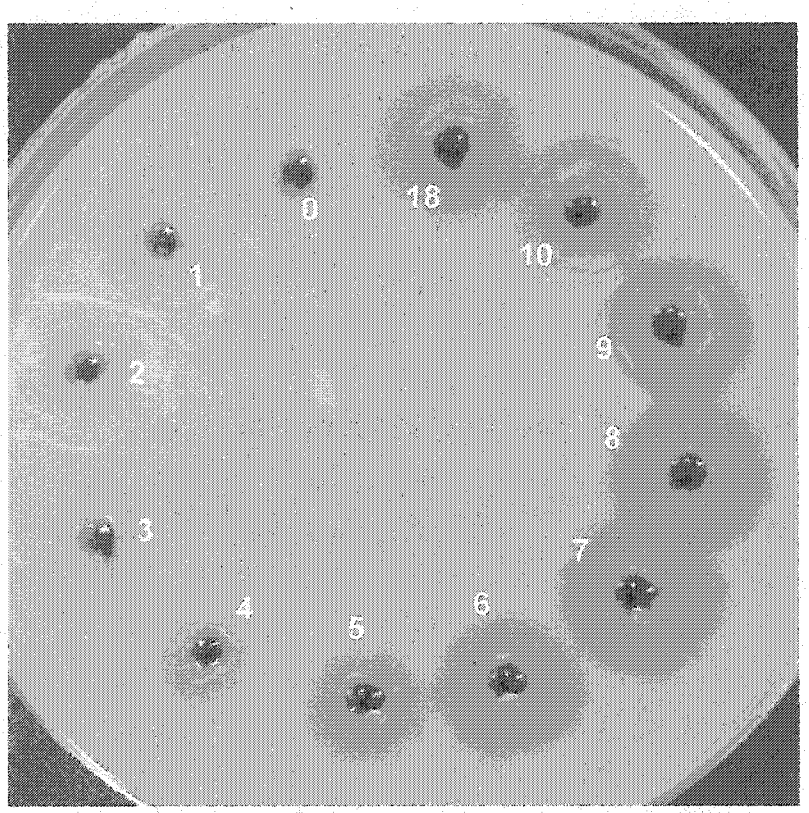

FIG. 13. Assay of strain 10403 for extracellular hyaluronate lyase activity over hours 0-10 and 18. Strain 10403 was grown for 18 hours with aliquots collected at each hour. Supernatant was assayed for hyaluronate lyase activity, seen as a zone of clearing surrounding the well.

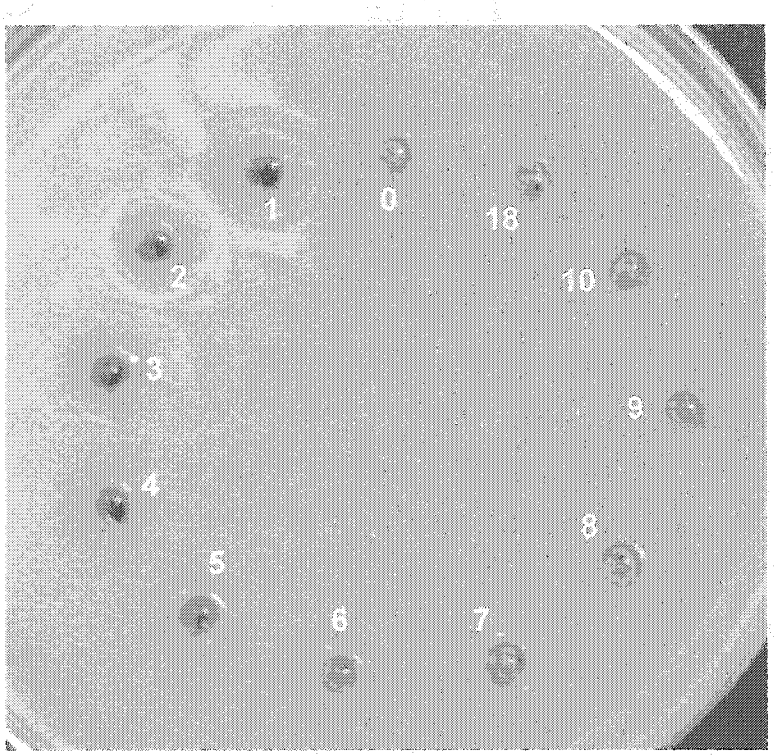

FIG. 14. Assay of intracellular hyaluronate lyase from strain 10403. Following sonication, assaying of cell lysate for HylA shows small zones of clearing. Lysates from hours 0-10 and 18 are shown.

The effect of temperature on *hylA* expression. The effect of various temperatures that may be encountered *in vivo* during infection on the relative amount of *hylA* transcript over time was examined. Temperatures were chosen based on the normal temperatures that may be found on the skin (32°C), physiological temperature (37°C), and an elevated temperature representative of fever conditions encountered during streptococcal infection (42°C). An overnight culture, grown at each of the above temperatures, was inoculated into fresh media and incubated for 4 hours (OD_{600}= 0.375/ cfu/mL= 3.0×10^7) at the specified temperatures. To determine the effect of temperature on *hylA* expression, cDNA was synthesized and quantified by real-time PCR. The raw absorbance values for *hylA* were normalized against *gyrA* values to determine the relative transcript amount of *hylA* at the different temperatures. The assay was repeated in triplicate from at least two independently prepared RNA isolates for each environmental condition. The mean +/- the standard error of the mean is reported for each sample. No difference in the level of relative transcript for *hylA* at the different temperatures was seen, with each having approximately 0.196 RU (Fig. 15). Additionally, no difference was seen in the HylA titer at any of the temperatures.

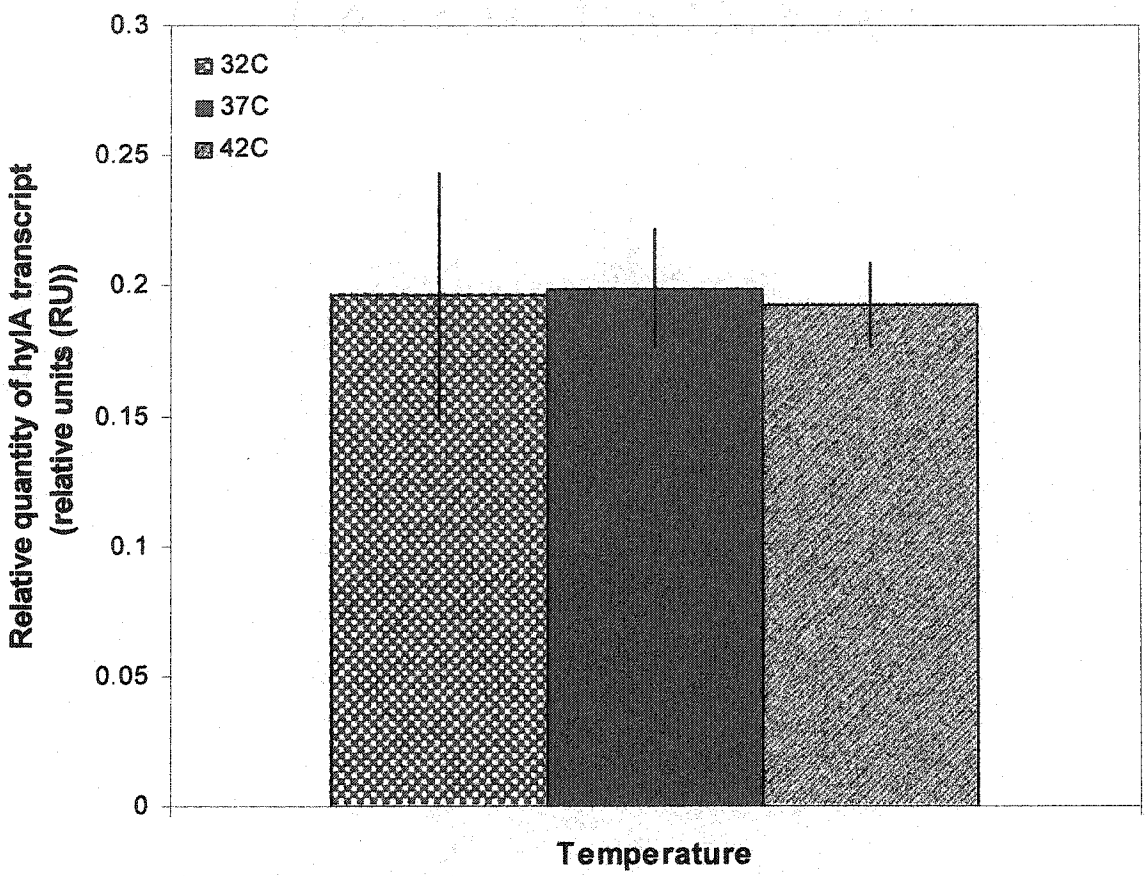

FIG. 15. The effect of temperature on the level of *hyl*A transcript in strain 10403. RNA was isolated from cultures grown for 4 hours at temperatures (32°C, 37°C, and 42°C). The *hyl*A cDNA values were normalized to *gyr*A cDNA values. The results shown are representative of at least two independently isolated RNA preparations for each strain and represent the mean +/- the standard error of the mean.

Effect of pH on *hyl*A expression in strain 10403. The influence of different pH levels on *hyl*A expression by strain 10403 was examined. The levels of pH were chosen based on the various pH levels that may be encountered during streptococcal infection. An aliquot of overnight culture grown at the specified pH was inoculated into fresh media (at the same pH) and incubated for 4 hours (OD_{600}= 0.375/ cfu/mL= 3.0 x 10^7). The pH of culture supernatant was analyzed and did not show any change in the level of pH following growth. Bacterial growth was inhibited at pH 9.0 and therefore, was not included in this analysis. Transcript levels for each sample were quantified using real-time PCR and the values obtained for *hyl*A were normalized against that of *gyrA*. A comparison of transcript levels between the samples showed little difference in the relative amount of *hyl*A transcript between cultures grown at pH levels 7.4 and 7.6, 0.216 RU and 0.200 RU respectively; however, at the acidic pH (pH 5) a two-fold decrease was seen when compared to values obtained from pH 7.4 and 7.6 (Fig. 16). No difference was seen in the HylA titer at the different levels of pH.

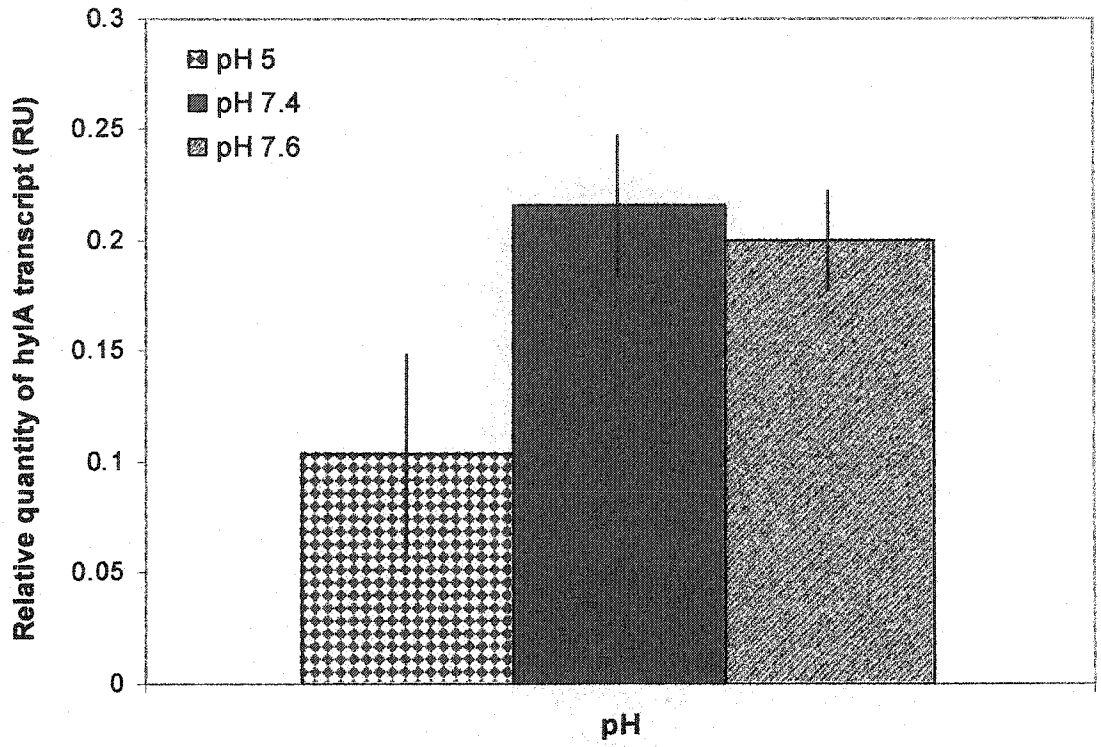

FIG. 16. The effect of pH on *hyl*A transcript levels in *S. pyogenes* strain 10403. To assess the effect of various pH conditions that may be encountered *in vivo*, RNA was isolated from cultures grown 4 hours under various pH conditions (pH 5, 7.4, 7.6) and real-time quantitation was performed. The values for *hyl*A cDNA were normalized against cDNA values for the internal control, *gyrA*. The results shown are representative of the mean of at least two independent RNA isolations run in triplicate ± the standard error of the mean.

The effect of aeration on *hyl*A expression. To establish the effect of different types of aeration on *hyl*A expression, *S. pyogenes* strain 10403 was grown under aerobic, anaerobic, and increased carbon dioxide conditions. Anaerobic conditions were attained by making media anaerobic by incubation in an anaerobic jar. Increased carbon dioxide levels were obtained by placement of media in a candle jar. An aliquot of 18-hour cultures of *S. pyogenes* strain 10403 grown under the various aeration conditions was inoculated into fresh media and incubated for 4 hours (OD_{600}= 0.375/ cfu/mL= 3.0×10^7) accordingly. Cultures grown under anaerobic and increased carbon dioxide conditions had approximately the same relative quantity of *hyl*A transcript, 0.349 RU and 0.361 RU respectively; whereas, cultures grown aerobically had approximately 1.8-fold less (Fig. 17). The HylA titer representing active protein was determined to be the same for all three aeration conditions, 16-32 AU.

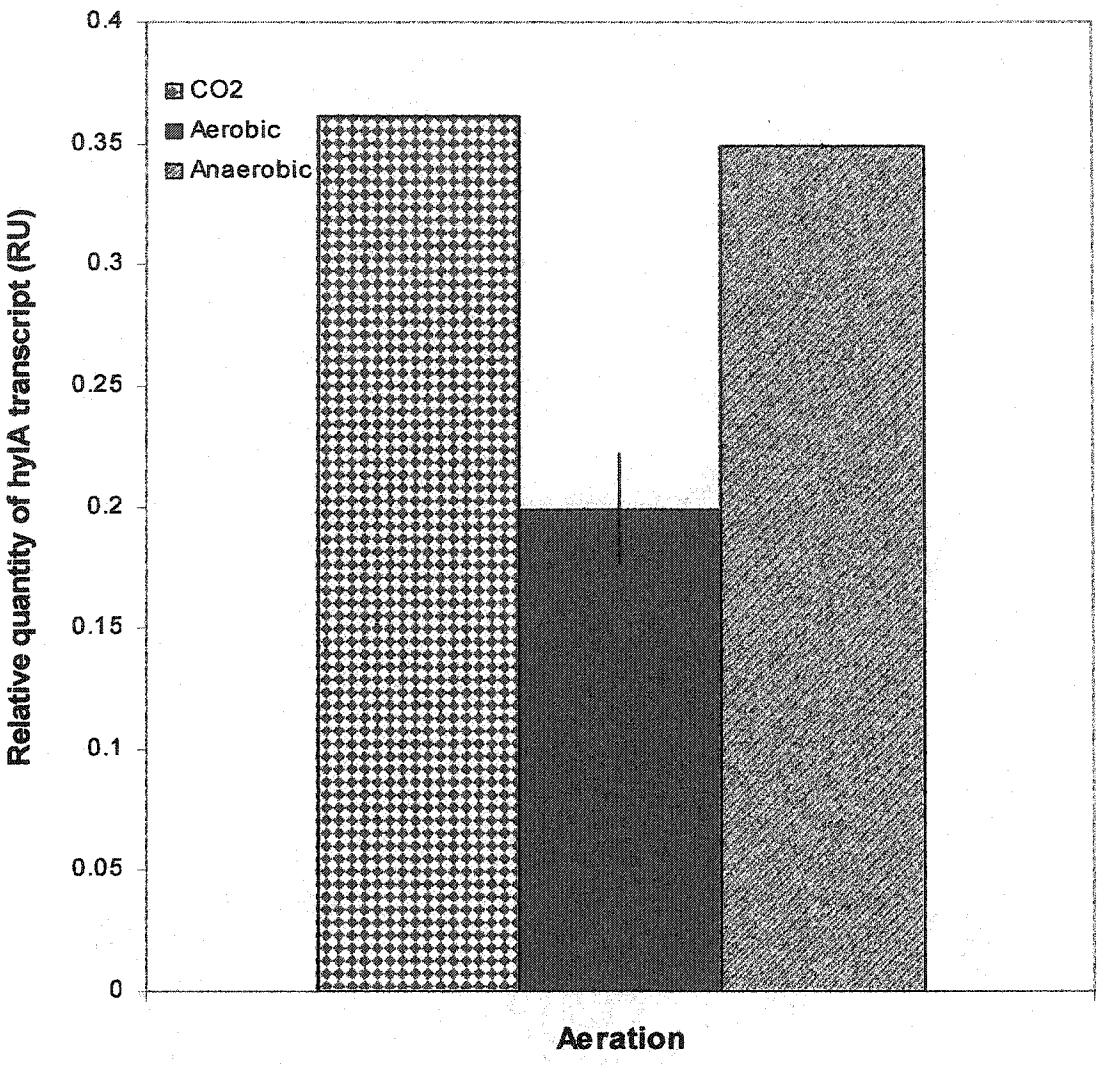

FIG. 17. The effect of aeration on *hylA* expression in strain 10403. RNA was isolated from *S. pyogenes* strain 10403 grown under aerobic, anaerobic, and increased carbon dioxide conditions. Relative amounts of *hylA* transcript are reported, which were obtained by normalizing *hylA* cDNA values against those of *gyrA*. The experiments were repeated using at least two independent RNA isolations; results are representative of the mean of the experiments ± the standard error of the mean.

The effect of gaseous exchange on *hylA* expression in strain 10403. To assess the effect of gaseous exchange (shaking) on *hylA* transcript levels, RNA was isolated from cultures grown for 4 hours at 37°C with and without shaking. Real-time PCR was utilized to quantify the amount of cDNA in each sample and the relative amount of *hylA* transcript was determined by normalizing the cDNA value for *hylA* against that of *gyrA*. No difference was seen between the two conditions when either relative transcript levels (Fig. 18) or HylA titer, 16-32 AU, was determined.

The effect of hyaluronic acid on *hylA* expression in *S. pyogenes* strain 10403. The effect of the addition of 600 µg/mL hyaluronic acid on *hylA* expression by strain 10403 was examined. Cultures were inoculated into fresh media containing the same components, incubated for a period of 4 hours, and RNA isolated at two hour intervals. There was an approximately a 2-fold elevation in the amount of *hylA* transcript amount for cultures grown with the addition of 600 µg/mL hyaluronic acid at 2 and 4 hours, 0.304 RU and 0.527 RU, when compared to those grown without hyaluronic acid, 0.145 RU and 0.245 RU respectively (Fig. 19). No difference was seen in the titer of HylA produced by strain 10403 grown with or without hyaluronic acid.

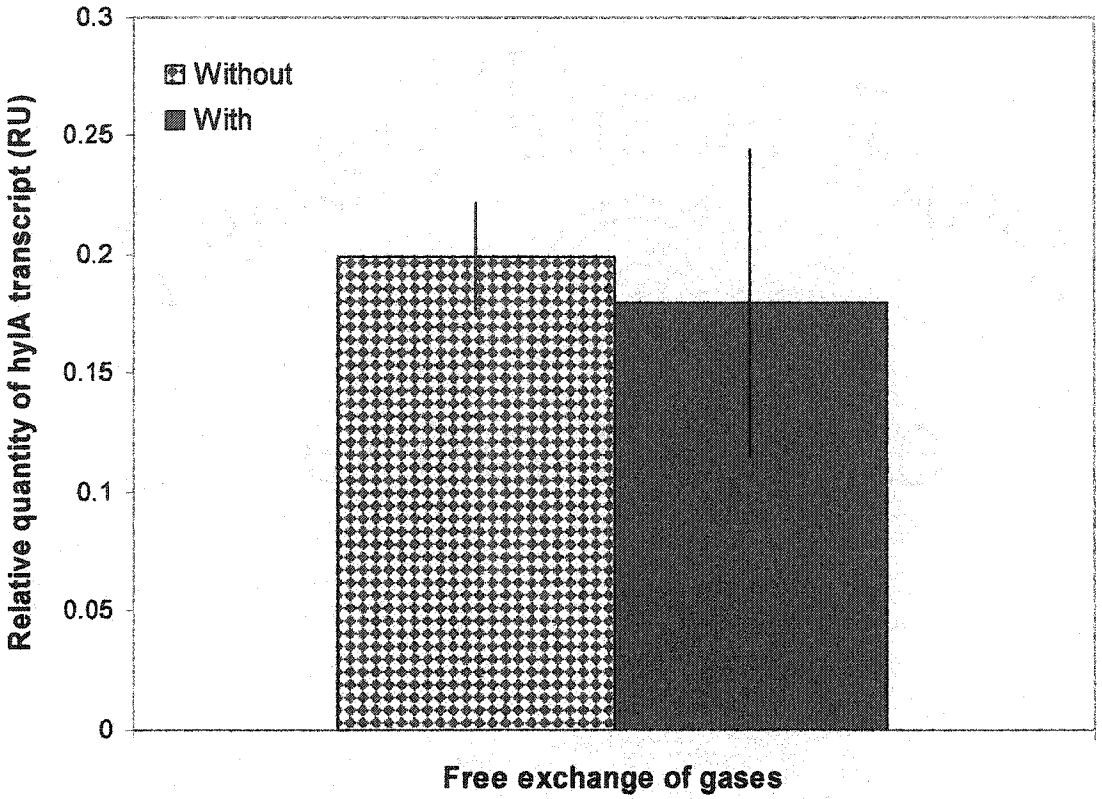

FIG. 18. The effect of gaseous exchange on *hyl*A transcript levels in *S. pyogenes* strain 10403. The relative amount of *hyl*A transcript was determined from RNA isolated from cultures of *S. pyogenes* strain 10403 grown at 37°C with and without exchange of gases (shaking). The values were determined by normalizing *hyl*A cDNA values against *gyrA* values. The experiments were performed from at least two independent RNA preparations analyzed in triplicate. Results are representative of the mean relative quantity of *hyl*A ± the standard error of the mean.

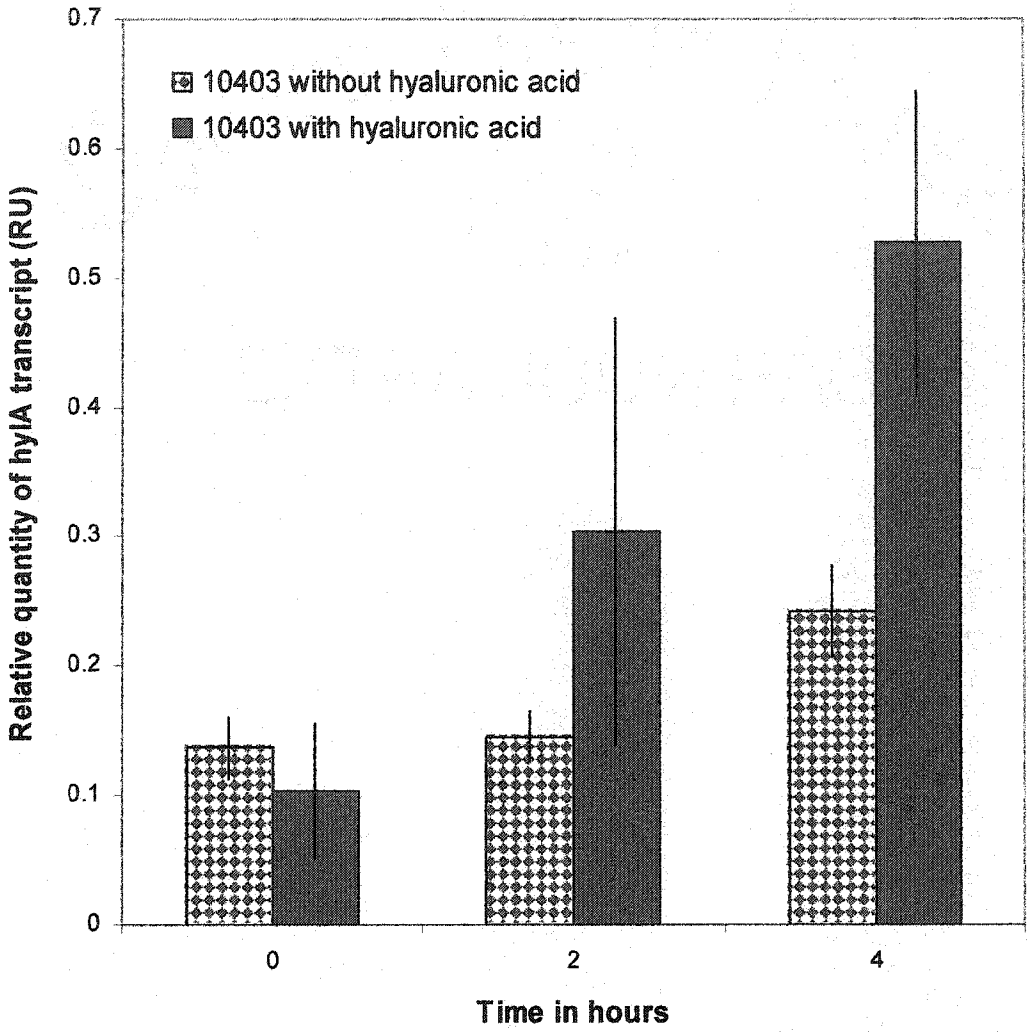

FIG. 19. The effect of hyaluronic acid on *hyl*A expression in *S. pyogenes* strain 10403. The relative amount of *hyl*A transcript was determined from RNA isolated from cultures grown over a period of 4 hours with or without the addition of 600 µg/mL hyaluronic acid. Relative quantity of *hyl*A transcript was determined by normalizing *hyl*A cDNA values against *gyrA* values. The experiment was performed using RNA prepared from two independent isolations and analyzed in triplicate. Results are representative of the mean quantity of *hyl*A transcript ± the standard error of the mean.

A comparison between *hyl*A and hyaluronic acid synthase (*hasA*) expression. Strain 10403 is acapsular and does not have a *hasA* transcript. Because of this, it was necessary to use a strain known to be encapsulated as well as having *hyl*A transcript present. Unfortunately, an encapsulated, hyaluronate lyase-producing strain was not identified; as a result a strain that was a non-producer was utilized. Strain 71698 was used for the comparison of *hasA* and *hyl*A expression. The relative amounts of transcript for *hyl*A and *hasA* were determined by normalizing each cDNA value against that of *gyrA*. The relative amounts of *hasA* transcript in this strain were much greater than that of *hyl*A, making it impossible to compare the pattern of expression for both genes in the same graph. The relative quantity of *hasA* transcript plotted against time is shown in Fig. 20 and *hyl*A transcript levels are shown in Fig. 12. In strain 71698, *hasA* expression occurs primarily during the early to mid-exponential phase of growth, having the most transcript during hours 2 and 4, and is diminishing at later times. Between hours 4 and 6, a 50-fold decrease in the amount of *hasA* transcript is seen. Evaluation of the overall pattern of expression between *hasA* and *hyl*A indicates that they follow the same pattern such that each increase to hour four then decrease, suggestive of a normal pattern of distribution. When comparing the amount of transcript between the two genes, a minimum of a 25-fold difference and maximum of 1800-fold difference between the relative amounts of transcript is observed.

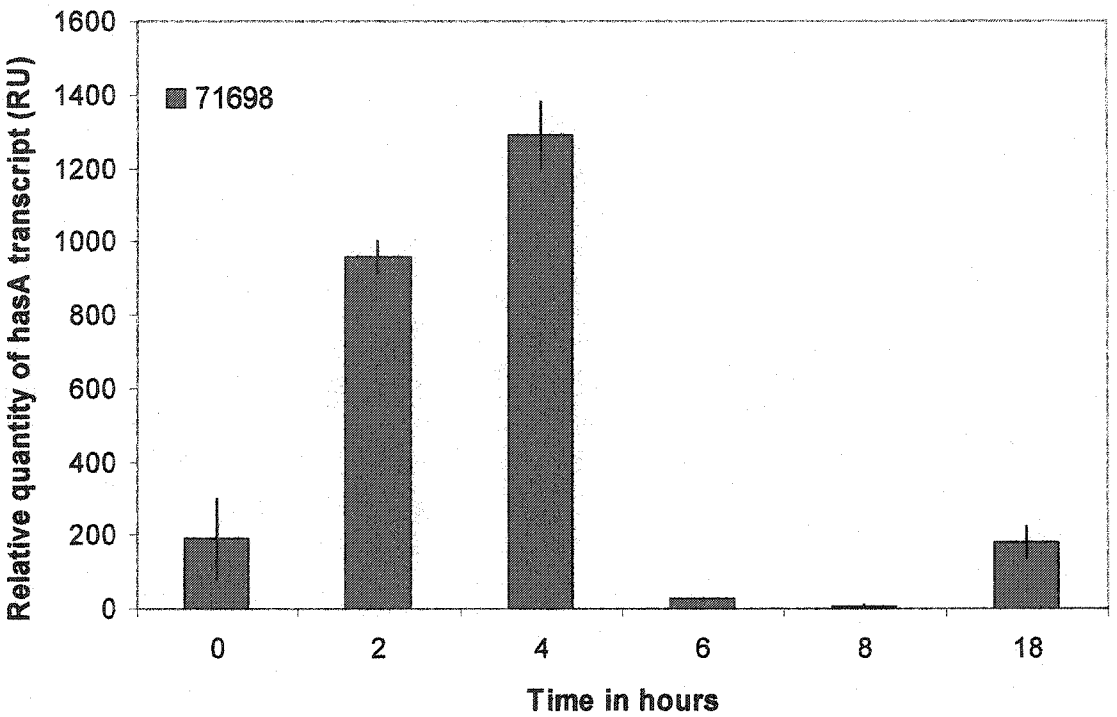

FIG. 20. Relative quantity of *hasA* transcript from strain 71698 grown over 8 hours, as well as from hour 18. Relative quantity of *hasA* transcript was determined by normalizing *hasA* cDNA values against *gyrA* values. The experiment was performed using RNA prepared from two independent isolations and was analyzed in triplicate. Results are representative of the mean relative quantity of *hasA* transcript ± the standard error of the mean.

RESULTS OF HYALURONATE LYASE GENE SIZE VARIABILITY STUDIES

PCR analysis of *S. pyogenes* strains for hyaluronate lyase gene.

PCR analysis of the 3' region of the hyaluronate lyase gene region from 176 strains of *S. pyogenes* showed that all strains contained a region with size similarity to *hyl*A; however, 33 strains (20%) gave a smaller amplicon when compared to *hyl*A of strain 10403 (Fig. 21). A comparison between *hyl*A from strain 10403 and the PCR amplified hyaluronate lyase gene region (3510 bp) from these strains, which contain the smaller gene region, indicated a small difference in size, confirming the 3' results (Fig. 22). PCR amplification of the 5' region of the gene and an internal region from the strains resulted in an amplicon of the same size, 1.758 kb or 1.356 kb respectively, as that found in *S. pyogenes* strain 10403 (Fig. 23 and Fig. 24). Assaying those strains containing the smaller hyaluronate lyase gene for hyaluronate lyase activity revealed that there were both hyaluronate lyase-producing and non-producing strains (Fig. 25). A list of the 33 strains, which showed a smaller sized hyaluronate lyase gene relative to *hyl*A, and their hyaluronate lyase activity, is in Appendix V.

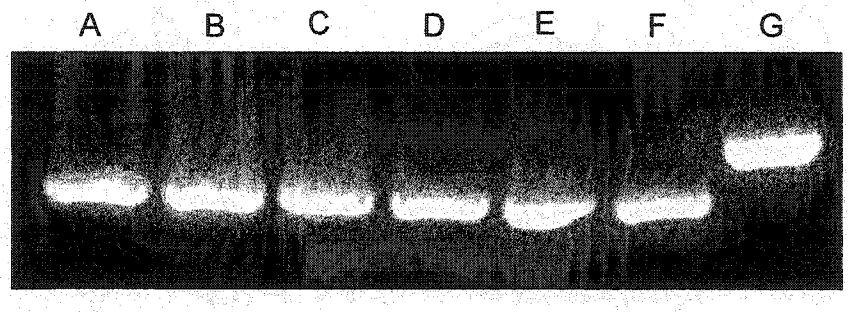

FIG. 21. PCR amplification of DNA from strains of *S. pyogenes* showing a decrease in the size of the 3' region of a putative hyaluronate lyase gene relative to *hyl*A. Lanes: A, strain 350; B, strain 422; C, strain 872; D, strain 1020; E, strain 1055; F, strain 94-146; G, strain 10403.

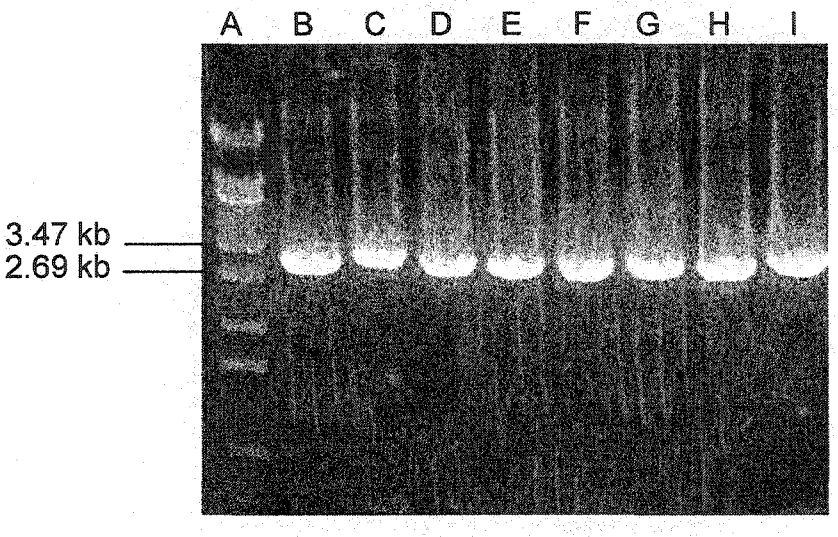

FIG. 22. PCR amplification of the entire hyaluronate lyase gene of *S. pyogenes.* Using primers specific for the hyaluronate lyase region, all strains amplified a fragment; however, some strains showed a slight decrease in size when compared to strain 10403 (Lane I). Lane C is the same size as lane I. Lanes: A, λ sty MW ladder; B, strain 350; C, strain SF370; D, strain 422; E, strain 872; F, strain 1020; G, strain 1055; H, strain 94-146, I, strain 10403.

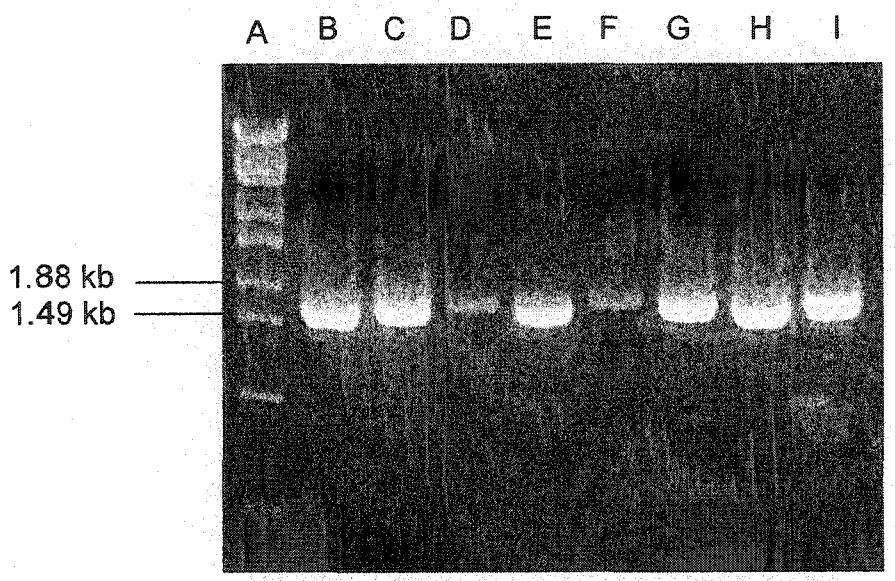

FIG. 23. PCR amplification of the 5' region of the hyaluronate lyase gene. Primers specific for the amplification of the 5' region of the hyaluronate lyase gene resulted in an amplicon of 1758 in size. Lanes: A, λ sty MW ladder; B, strain 350; C, strain SF370; D, strain 422; E, strain 872; F, strain 1020; G, strain 1055; H, strain 94-146, I, strain 10403. Due to the amount of DNA loaded, lanes D and F appear different in size.

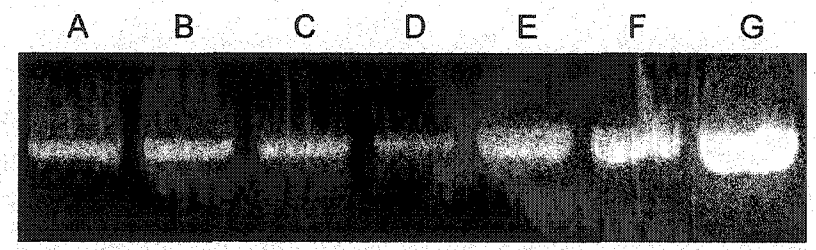

FIG. 24. PCR amplification of the internal region of the hyaluronate lyase gene from *S. pyogenes*. PCR analysis of 176 strains for the detection of *hylA* using primers specific for the internal region indicated the presence of an approximately 1356 bp DNA fragment in each of the strains. The amplified DNA did not differ in size from the internal fragment of *hylA* obtained from 10403. Lanes: A, strain 350; B, strain 422; C, strain 872; D, strain 1020; E, strain 1055; F, strain 94-146, G, strain 10403.

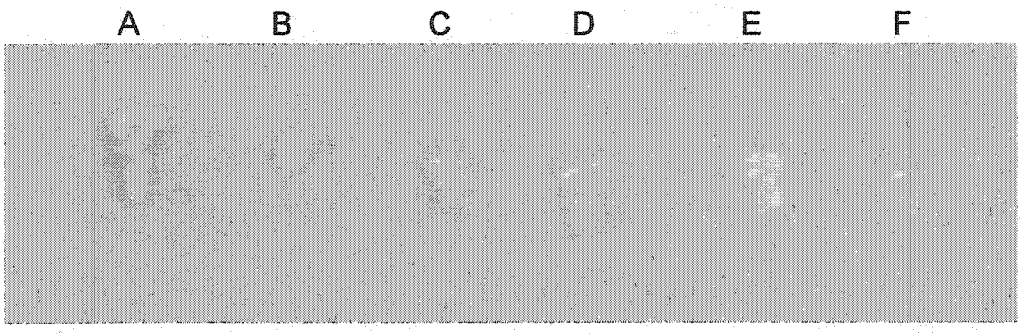

FIG 25. Hyaluronate lyase activity of six strains using the brain heart infusion-bovine albumin-hyaluronic acid assay plates. Hyaluronate lyase activity appears as a zone of clearing and was found in strains 422 (B), 1020 (C), and 1055 (D). Strains 350 (A), 872 (E) and 94-146 (F) did not produce any obvious hyaluronate lyase activity.

Southern blot analysis. To confirm that the region being amplified was the hyaluronate lyase gene region, Southern blot analysis was performed. Chromosomal DNA was isolated and digested with *Eco*RI (Fig. 26). Although it is not known where the enzyme *Eco*RI digests the chromosome of strain 10403, there are no sites within the 3.5 kb fragment sequenced. The sequence of strain SF370 indicates that following *Eco*RI digestion the hyaluronate lyase gene would be contained in fragments approximately 14.6 kb and 4.7 kb in length, as the *hyl*A gene contains a single *Eco*RI site. However, by comparing the 3510 bp hyaluronate lyase gene regions from strains 10403 and SF370, it was found that *hyl*A from SF370 contains an *Eco*RI site, whereas the region from strain 10403 does not, suggesting that *hyl*A may be contained in a fragment approximately 19 kb in length. Sequence analysis (Appendix V) indicated that an *Eco*RI site was contained in the 3' region of the hyaluronate lyase gene of the strains containing the smaller gene. Once transferred to a membrane, the DNA was hybridized with either a 1.356 kb internal fragment of *hyl*A or with a 992 bp 3' region of *hyl*A. Following development of the blot, hybridization with the 3' region of *hyl*A was observed (Fig. 27). The strains containing the smaller gene showed hybridization to the two fragment lengths predicted, while strain 10403 showed one fragment. In all of the samples, an extra band of similarity to the probe, approximately 2 kb in size, is evidenced and can be explained as non-specific hybridization. Hybridization of the internal probe to a fragment 19 kb in size was also observed.

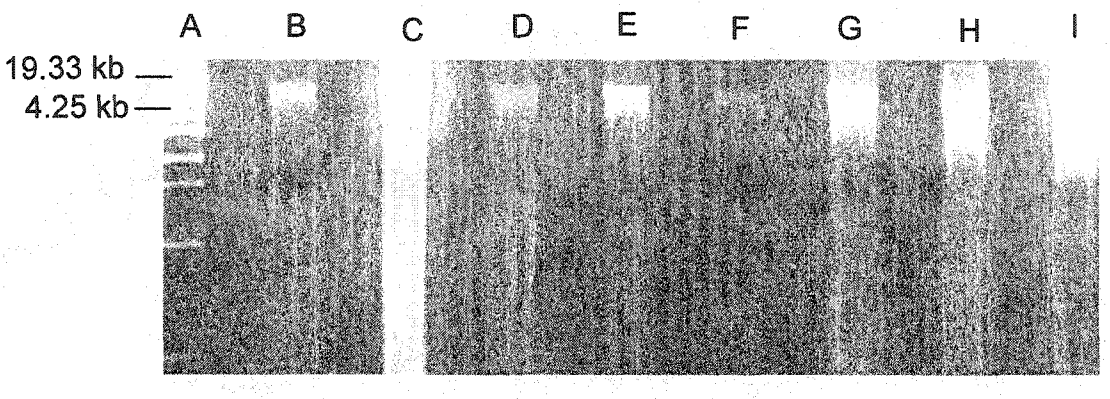

FIG. 26. *Eco*RI digested chromosomal DNA from strains containing a smaller hyaluronate lyase gene relative to *hyl*A. Lanes: A, λ sty MW ladder; B, strain 350; C, strain SF370; D, strain 422; E, strain 872; F, strain 1020; G, strain 1055; H, strain 94-146, I, strain 10403.

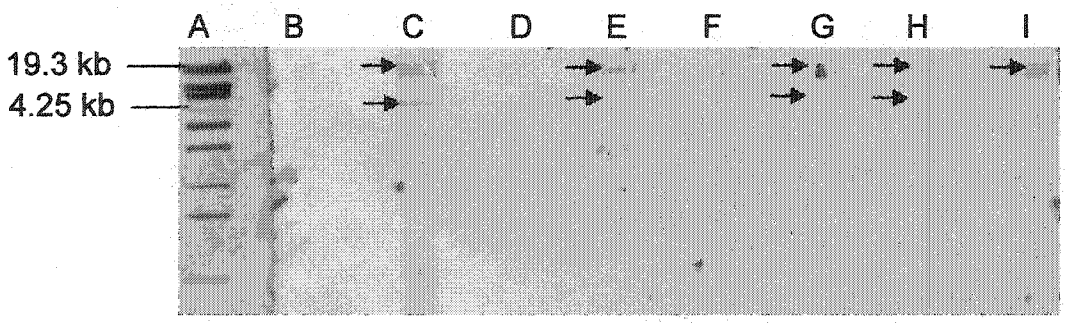

FIG. 27. Southern blot analysis showing similarity of the hyaluronate lyase gene from strains containing a smaller gene, relative to *hyl*A. Although bands of hybridization can be seen only in samples C, G, H, and I due to photographic insensitivity, bands of the same sizes were present in all samples. The anticipated bands are marked with arrows. Lanes: A, λ sty MW ladder; B, strain 350; C, strain SF370; D, strain 422; E, strain 872; F, strain 1020; G, strain 1055; H, strain 94-146, I, strain 10403.

Nucleotide sequence of the 3' region for *S. pyogenes* strains containing a smaller hyaluronate lyase gene region relative to *hyl*A. Six strains having the smaller 3' region (350, 422, 872, 1020, 1055, and 94146) were used for sequence analysis. The six strains included apparently both hyaluronate lyase-producing and non-producing strains. The strains were isolated from different infections, as well as geographic locations (Appendix V). The 3' region of the hyaluronate lyase gene from these strains was cloned, sequenced, and compared to the sequence of *S. pyogenes* strains 10403 (76) and SF370 (46). Sequencing of the 3' region indicated that the fragment was 809 bp in length for these all strains; this is 183 bp shorter than the 992 bp fragment of *hyl*A. Sequences of the 3' region from these strains can be found in Appendix VI.

Alignment of the sequences, with that of *hyl*A, revealed very few differences in the nucleotide sequence between the different strains relative to *hyl*A, with the exception of a deleted region. Analysis indicated that a deletion of 183 bp occurred in the same location in all the strains. This was located between positions 2273-2455 of *hyl*A from strain 10403 (Fig. 28). Additional analysis of the nucleotide sequence indicated that the deletion introduced an *Eco*RI site into this region; *hyl*A from strain 10403 does not contain an *Eco*RI site. Further analysis of the nucleotide sequence in the area of the deletion revealed a 9 bp repeated sequence (AAAACAATG) that marks the boundaries of the deletion. Only one copy of the repeat is found in those strains that show the smaller gene, while two copies of the repeat are found in strain 10403 (Fig. 29).

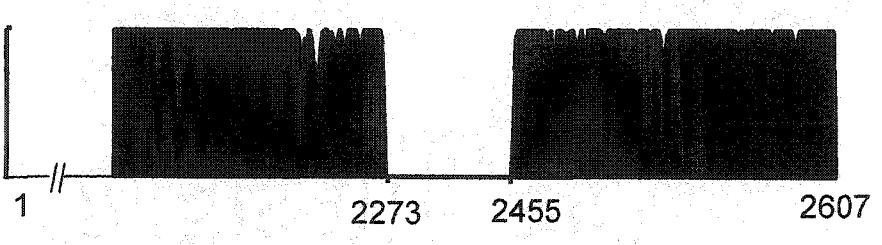

FIG. 28. Graphical view of the alignment of the sequences of the 3' region of the hyaluronate lyase gene from six *S. pyogenes* strains showing a 183 bp deletion. The deletion spans from position 2273 to 2455 as numbered from the first nucleotide in the open reading frame of *hyl*A of strain 10403.

```
350    AAAACAATG----------//----------CT
422    AAAACAATG----------//----------CT
872    AAAACAATG----------//----------CT
1020   AAAACAATG----------//----------CT
1055   AAAACAATG----------//----------CT
94146  AAAACAATG----------//----------CT
10403  AAAACAATGACAAACTGGCCGCTGT//TTGCTCATCAAGGAATTGCAGCTAAAAACAATGCT
```

FIG. 29. Abridged sequence alignment of the 3' region of six strains showing the deletion relative to *hyl*A. The alignment revealed a 9 bp repeat that (highlighted in gray) marks the boundaries of the deletion.

RESULTS OF INACTIVATION OF *hyl*A STUDIES

Preliminary assessment of the role of hyaluronate lyase in streptococcal infections. The role hyaluronate lyase plays in the growth of the bacterium or its role in infection is not known. The enzyme's role in streptococcal infection as a virulence factor is merely speculative. To begin to understand the role that hyaluronate lyase plays in infection, mice were inoculated with the hyaluronate lyase-producing strain 10403 and a single recombinant mutant strain 10403::pHAS:*hyl*A, which no longer produces hyaluronate lyase. Initially, the flanks of five male CD-1 white mice were subcutaneously injected with 100 µL (2.0×10^7cfu) of a 6 hour culture of 10403::pHAS:*hyl*A that had been pelleted and washed with isotonic saline. The mice were observed for a period of 2 weeks for abscess and/or lesion formation. Abscess formation became apparent at about day 4 as a small raised red bump. The swelling expanded and lesions became noticeable about a week following injection (Fig. 30). Purulent lesions were swabbed, plated on blood agar or antibiotic-containing media. Colonies were assayed for hyaluronate-lyase production. Approximately 5.7% of the colonies assayed had reverted from hyaluronate lyase non-producing mutants to the wild-type phenotype. The colonies that showed reversion were still erythromycin resistant. PCR analysis indicated that reversion had occurred, as primers specific for *hyl*A resulted in an amplicon approximately 1 kb in size. Had the plasmid still been incorporated in the chromosome at the *hyl*A location, no fragment would have amplified using the chosen PCR conditions.

Inactivation of *hylA*. Because the preliminary animal studies showed that the single recombinant hyaluronate lyase non-producing mutant strain 10403::pHAS:*hyl*A appeared to revert to its wild-type phenotype, a double recombinant mutant was created to "knock-out" *hyl*A expression in strain 10403. The sequence of *hyl*A from strain 10403 revealed two internal *Eco*RV sites that would excise a 924 bp fragment from the gene (76). Following removal of the 924 bp internal fragment of *hyl*A from plasmid pCRNT/T7: *hyl*A, the region was replaced by an erythromycin resistance gene, *ermR*. The presence of *ermR* in *hyl*A was associated with loss of hyaluronate lyase activity, and expression of a erythromycin resistance phenotype of the *E. coli* colonies. Sequencing of a plasmid (Fig. 31), using an internal primer, confirmed the presence of *ermR* in *hyl*A. Following PCR amplification, using primers Hyl-O and Hyl-N (Table 1; Fig. 3), the *hyl*A:*ermR* fragment was introduced into *S. pyogenes* strain 10403 by electrotransformation. After electroporation, colonies of strain 10403 were obtained that exhibited erythromycin resistance; the loss of hyaluronate lyase activity confirmed the interruption of the gene (Fig. 32). Two mutant strains, 10403Δ*hyl*A1 and 10403Δ*hyl*A2, were selected and showed a small amount of hyaluronate lyase activity surrounding the inoculum. Since the zone of clearing was small, it was most likely due to a bacteriophage associated hyaluronidase found in strain 10403.

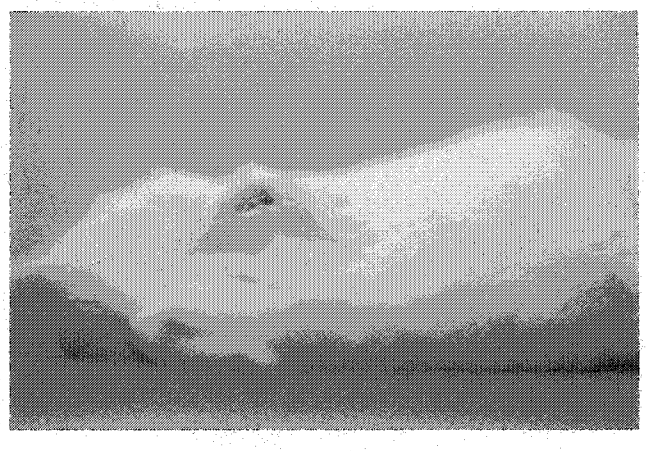

FIG. 30. Lesion formation on a CD-1 mouse following subcutaneous injection of *S. pyogenes* mutant strain 10403::pHAS:*hyl*A. Inactivation of *hyl*A did not result in a loss of the ability to cause infection as seen by the lesion present on the day 12 following subcutaneous injection.

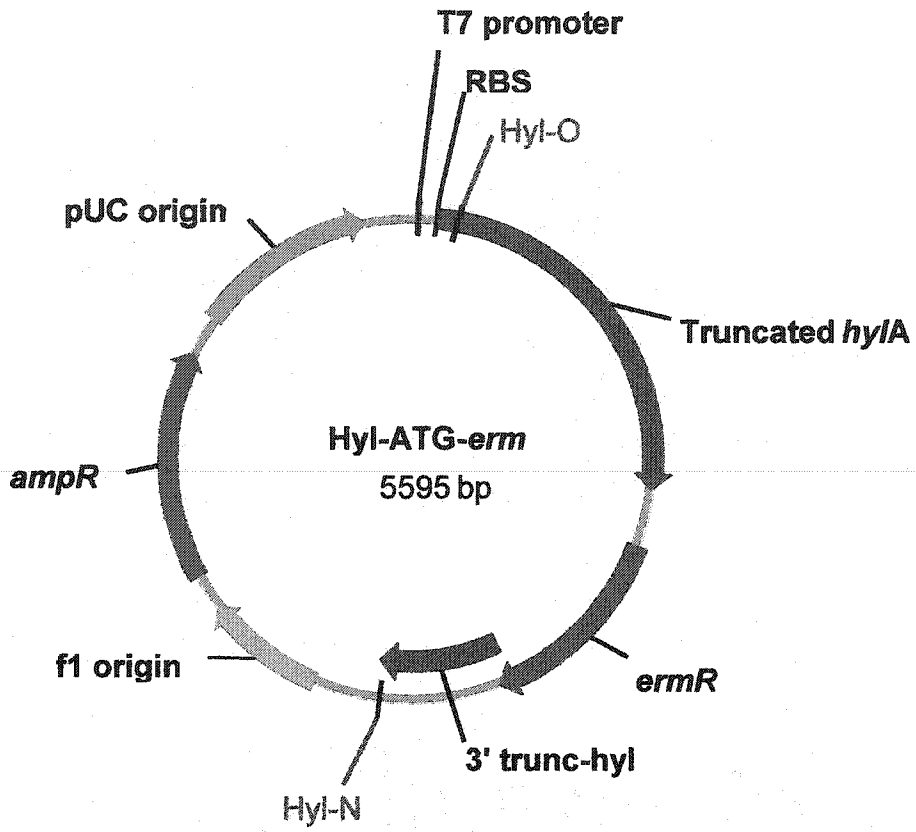

FIG. 31. Plasmid construct Hyl-ATG-*erm*. This construct was constructed to inactivate *hyl*A. Digesting *hyl*A with *Eco*RV dropped out a 924 bp fragment which was replaced with *ermR*. The hyl-ATG-*erm* fragment was PCR amplified using the primers Hyl-N and Hyl-O and was inserted into *S. pyogenes* strain 10403.

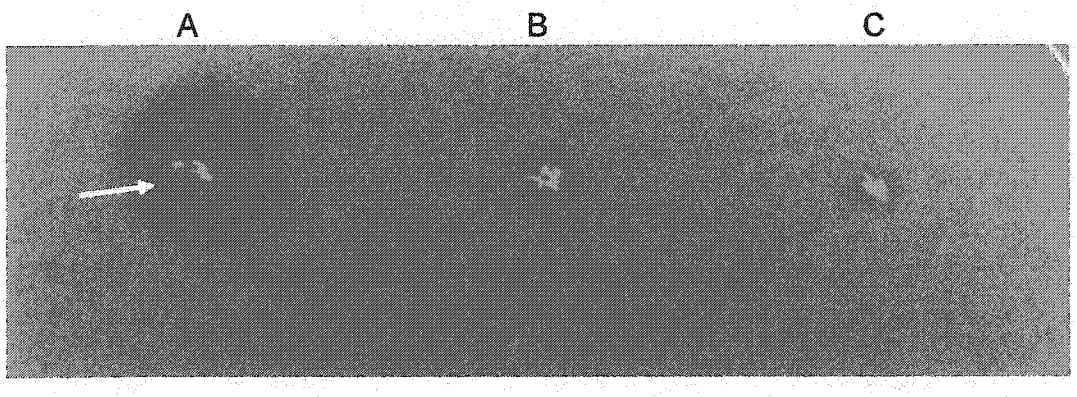

FIG. 32. BHB assay for the detection of hyaluronate lyase from wild-type strain 10403 and mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2. Hyaluronate lyase activity is evidenced by the zone of clearing surrounding the point of inoculation (marked with an arrow). Samples were as follows: A, 10403^{wt}; B, 10403Δ*hyl*A1; C, 10403Δ*hyl*A2.

Chromosomal DNA isolated from the mutants, was digested with *Eco*RI (Fig. 33) and analyzed for the presence of *ermR* and *hyl*A by Southern blotting. *Eco*RI digests the streptococcal chromosome such that *hyl*A is contained in an approximately 19 kb fragment (Fig. 27). Since *ermR* is not digested with *Eco*RI, it too should be contained within such a large-sized fragment. Southern blot analysis confirmed the presence of *ermR* integration into *hyl*A (Fig. 34). Strains lacking hyaluronate activity contained an inserted *erm*R in the size fragment predicted. There was no hybridization apparent between *ermR* and the wild-type strain 10403. The 3' region of *hyl*A hybridized to 10403Δ*hyl*A1 and 10403Δ*hyl*A2, but not to the plasmid pUC:erm. Hybridization with the 3' region of *hyl*A did result in non-specific binding within the chromosome; the non-specific fragments were present in all three streptococcal preparations.

Growth curve comparison between wild-type strain 10403 and mutant strain 10403Δ*hyl*A. To determine the effect, if any, that *hyl*A inactivation has on the growth of strain 10403, a comparison was made between the growth of the wild-type strain 10403 and mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2. Cultures of wild-type strain 10403 and mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2 were grown and turbidity was measured at a wavelength of 600 nm each hour for ten hours and was plotted (Fig. 35). No apparent difference in the growth of the strains containing the inactivated *hyl*A compared to that of the wild-type 10403 was seen.

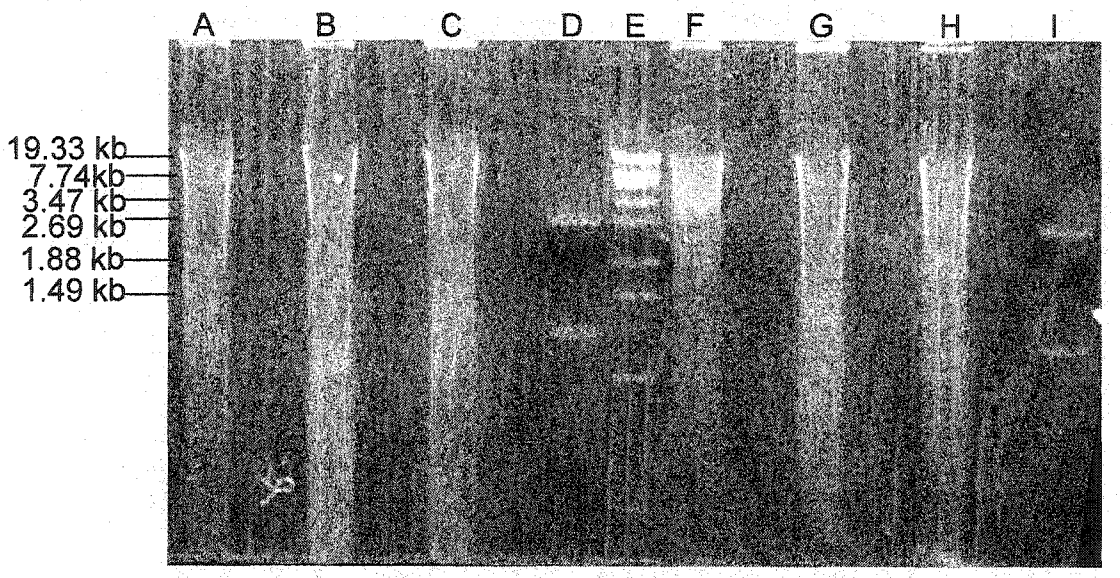

FIG. 33. Chromosomal DNA from wild-type strain 10403 and mutant strain 10403Δ*hyl*A digested with *Eco*RI for Southern blot analysis. *Eco*RI/*Hind*III digested pUC:erm was included as a positive control for *ermR*. Lanes: A, 10403^{wt}; B, 10403Δ*hyl*A1; C, 10403Δ*hyl*A2; D, pUC:erm; E, λ sty MW ladder; F, 10403^{wt}; G, 10403Δ*hyl*A1; H, 10403Δ*hyl*A2; I, pUC:erm. Sizes of the λ sty MW ladder bands are shown on the left of the figure.

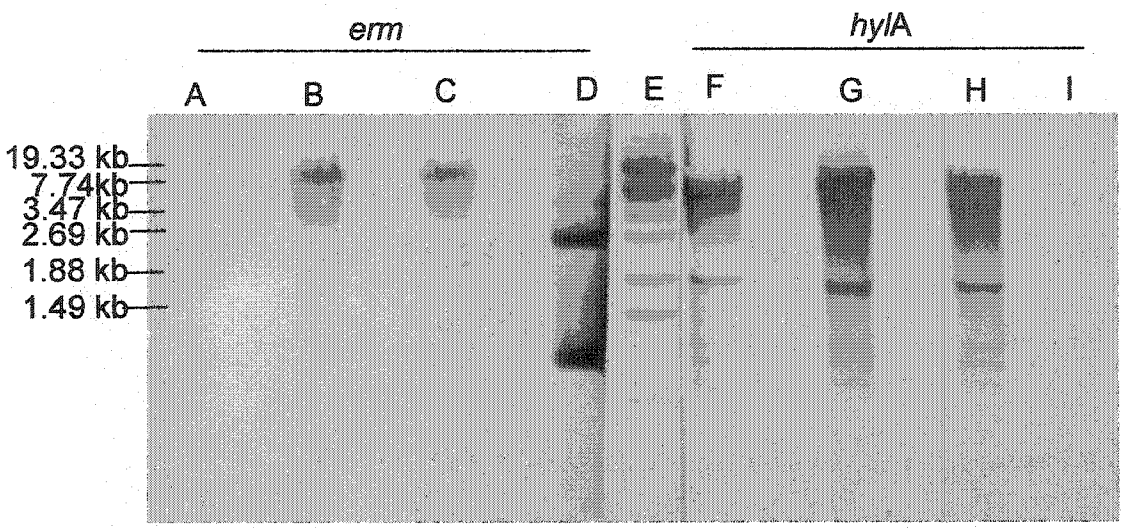

FIG. 34. Southern blot analysis of wild-type strain 10403 and mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2 for the presence of *hyl*A and *ermR*. Lanes A, B, C, and D were hybridized with DIG-labeled pUC:erm. Lanes F, G, H, and I were hybridized with DIG-labeled 3' region of *hyl*A. Lanes: A and F, 10403^{wt}; B, and G, 10403Δ*hyl*A1; C and H, 10403Δ*hyl*A2; D and I, pUC:erm; E, λ sty MW ladder. Sizes of the λ sty MW ladder bands are shown on the left of the figure.

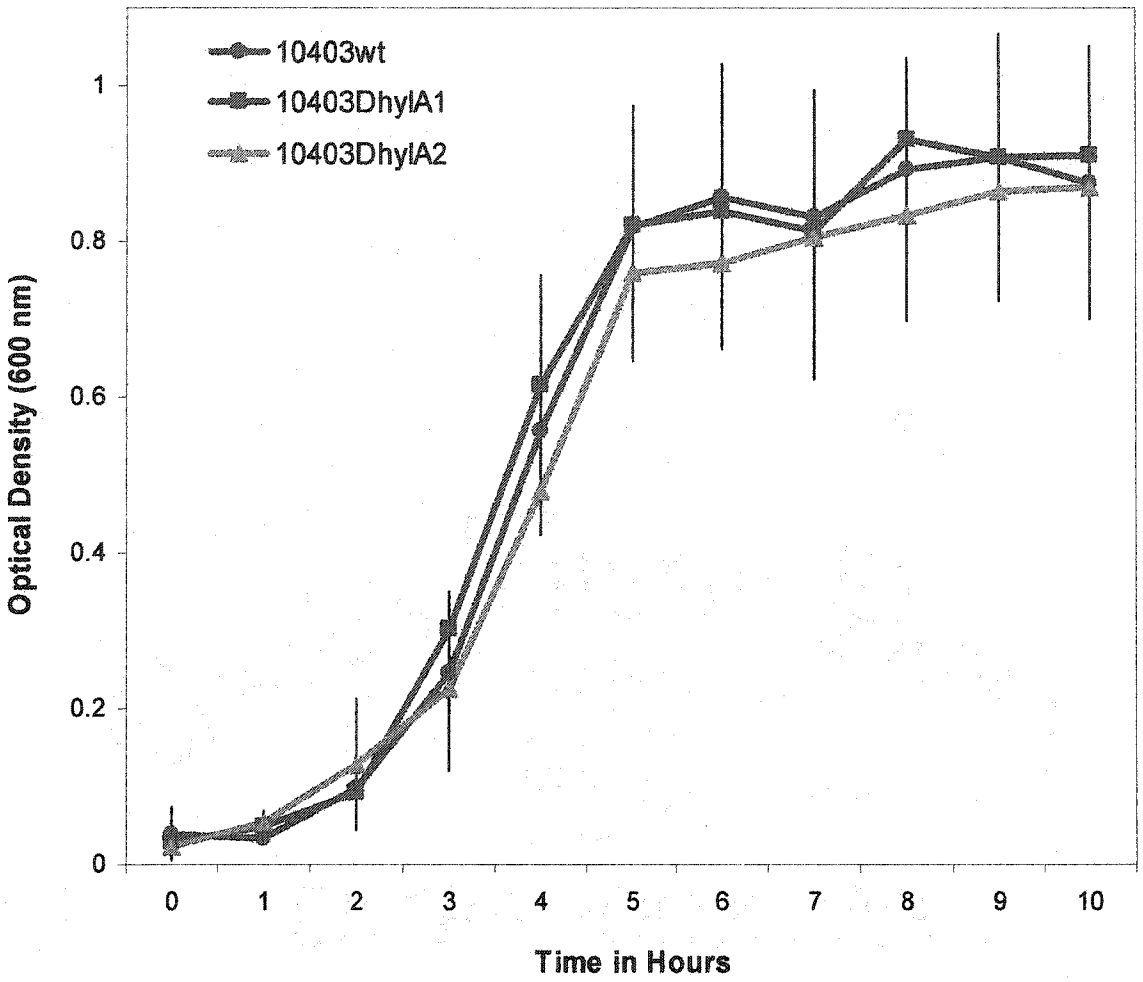

FIG. 35. Growth of wild-type strain 10403 and mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2. Results are representative of the mean ± standard deviation of the mean for turbidity measurements taken at a wavelength of 600 nm for each sample.

Effect of inactivation of *hyl*A on hemolysin and protease activities. Since the strains containing the inactivated *hyl*A may be used in pathogenesis studies designed to establish the role of hyaluronate lyase in streptococcal infection, it was necessary to determine the effect, if any, that the inactivation of *hyl*A may have on other cellular activities in the cell. For this purpose, strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2 were assayed for hemolysin and protease activities. Following inoculation of strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2, as well as wild-type strain 10403 on to blood and milk plates, large zones of clearing surrounding the point of inoculation were seen on both assay plates (Fig. 36, Fig. 37). Due to the obvious hemolytic and protease activities that were observed on the assay plates, inactivation of *hyl*A was determined to not have an obvious effect on these activities.

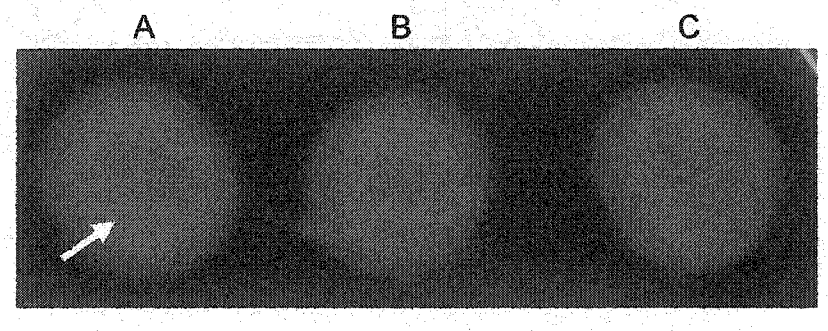

FIG. 36. Effect of inactivation of *hyl*A on hemolysin activity. No obvious difference was seen between the hemolysin activity (as indicated by the zone of clearing surrounding the colony) of wild-type strain 10403 (A) and mutant strains 10403Δ*hyl*A1 (B) and 10403Δ*hyl*A2 (C). An arrow is pointing to a zone of hemolysis.

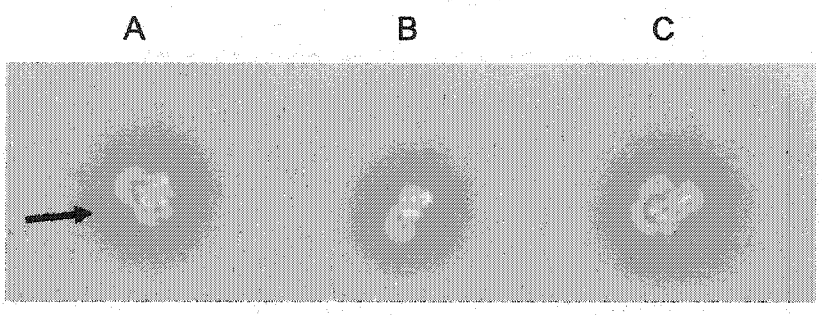

FIG. 37. Effect of *hyl*A inactivation on protease activity. Wild-type 10403 (A) and 10403Δ*hyl*A1 (B) and 10403Δ*hyl*A2 (C) were stabbed onto milk plates to assay for protease activity and detected a zone of clearing surrounding the colony. An arrow is pointing to a region of protease activity.

DISCUSSION

Production and expression of hyaluronate lyase in *S. pyogenes* strain 10403. Early studies suggested that hyaluronic acid capsule is produced early in the growth of the organism, while hyaluronate lyase is found later in the growth cycle (108); however, prior to this study, an in depth molecular analysis on the time of production of the hyaluronate lyase from *S. pyogenes* had not been conducted. Hyaluronate lyase activity was detected much earlier than previously suggested (108), being present in the early exponential stage of growth. One possible explanation for this observation is the lack of hyaluronic acid capsule production by strain 10403. This study made an assessment of the levels of hyaluronate lyase gene expression during *S. pyogenes* growth by examining both mRNA levels and enzymatic activity.

Sequence analysis from a 3510 bp fragment containing *hyl*A of strain 10403 indicated three potential translational initiation codons, a GTG at position 614, an ATG at position 683, or another ATG at position 686 (76). Analysis of the sequence upstream of these sites revealed a typical ribosome binding site (RBS) 9 bp upstream of the GTG (valine) site; further upstream of the RBS were the -10/-35 regions suggestive of a potential promoter region (76). No RBS site was found directly upstream of the ATG sites, nor were there -10/-35 regions immediately upstream of these sites. Initial analysis, in *E. coli*, using a *gfp* reporter assay of the regions upstream of the three potential initiation codons showed a region capable of functioning as a promoter, which was located upstream of the GTG site. This was subsequently confirmed by RT-PCR

analysis, suggesting that the GTG is the translational initiation codon for HylA. Interestingly, another streptococcal virulence factor, the protein involved in hyaluronic acid capsule synthesis, HasA, also has a GTG initiation codon (36).

RT-PCR results indicated the presence of the *hyl*A transcript at each time point of the eighteen-hour growth, signifying constitutive expression of the gene in strain 10403, as well as in strains SF370 and 71698, neither of which produce detectable active enzyme. Although there is no detectable hyaluronate lyase activity associated with strains SF370 and 71698, these results suggest an inactive form of the enzyme may be produced, the enzyme may require some post-transcriptional modification, or alternatively there is no translation of mRNA. Only approximately 25% of the strains tested with an *in vitro* assay for hyaluronate lyase activity actually produced detectable active enzyme (80). Although such *in vitro* studies suggested only a low percentage of group A streptococci produce hyaluronate lyase, antibodies directed to both extracellular hyaluronate lyase, as well as bacteriophage hyaluronidases, have been found in patients following a streptococcal infection (63, 161, 162). The presence of these antibodies indicates that hyaluronate lyase is produced *in vivo* during infection. Perhaps those strains not showing active enzyme *in vitro* may be stimulated to produce active enzyme *in vivo*. Alternatively, these strains may produce an inactive protein that still stimulates an antibody response despite the lack of enzymatic activity. Such a possibility would still explain the presence of the antibodies post-streptococcal infection. Cloning and expression of hyaluronate lyase genes from strains that do not appear to produce active HylA,

followed by western blot analysis, would be one way to assess production of an inactive protein.

Growth phase-dependent regulation of virulence factors is a common theme in *S. pyogenes*. In order to assess potential growth phase-dependent regulation of *hyl*A expression, *hyl*A transcript was measured using quantitative PCR over an 18 hour period. Strains 71698 and SF370 showed a gradual increase in relative transcript amounts during the early to mid-exponential phase (2-4 hours) of growth which then gradually decreased as growth entered the late exponential and stationary phases, suggestive of a single transcriptional peak of production with more production during exponential growth. A similar pattern of expression has been identified for other group A streptococcal genes, including *emm*, *scpA*, *hasA*, and *mga*, all of which were determined to have maximal gene expression during exponential growth (15, 30, 111). In contrast, the pattern of *hyl*A expression for strain 10403 was the same as that seen with the other two strains initially; however, after dropping to almost undetectable amounts of transcript at hour 8 (early stationary phase), the relative amount of *hyl*A transcript increased as stationary phase progressed. The pattern of distribution for *hyl*A transcript was therefore bi-modal, having two peaks of increased transcript amounts.

Although a bi-modal pattern of gene expression has not been identified in the literature for *S. pyogenes* genes, other genes that show elevated levels of gene expression during stationary phase have been identified and include *sagA, speA, speB,* and *speMF* (15, 30, 111, 158). Why there is a difference in the

pattern of expression between the tested strains is unknown at this time. One possibility is that the transcript is synthesized early, gradually degrades or is translated to protein and once levels are low, gene expression is stimulated a second time. Another possibility may be that the bi-modal expression is a result of strain 10403 producing an active enzyme, whereas, strains 71698 and SF370 do not. During the processing and/or secretion of HylA, there may be some unknown factor that results in the inhibition of transcription at that particular stage of growth. Alternatively, some factor may inadvertently result in the stimulation of transcription and subsequent protein production for strain 10403, which may be an explanation for the increased levels of *hyl*A transcript seen in strain 10403, while the other two strains have decreased levels. Conversely, perhaps the active enzyme is present because of this bi-modal pattern of transcript. However, since transcript and HylA are detected early in the growth, this possibility seems unlikely. If this bi-modal expression were the reason for the active protein, it would be anticipated that active protein would be detected later in growth. Preliminary analysis of another *S. pyogenes* M-type 22 strain, 622401, which produces active hyaluronate lyase, showed a decrease in *hyl*A transcript at hour 8, followed by an increase at hour 18, perhaps suggesting a similar bi-modal pattern of expression as that seen in strain 10403. These results may give support to a relationship between active protein production and gene expression patterns.

Since HylA is a secreted protein, the bi-modal pattern of gene expression in strain 10403 may be due to a feedback inhibition mechanism. Perhaps a

build-up of intracellular HylA over the early stages of growth negatively regulates gene expression, thereby resulting in a decline in the relative amount of *hyl*A transcript at hour 8. To evaluate this possibility, a comparison was made between intracellular and extracellular HylA levels. If feedback inhibition did occur, it would be expected that there would be a difference in the titer between the two different fractions. Unfortunately, such a relationship could not be determined as the intracellular hyaluronate lyase appears to be inactive. Any zone of clearing observed was minimal and most likely due to a bacteriophage hyaluronidase associated with strain 10403 since the same zone size was found with the mutant strain 10403Δ*hyl*A1. The finding that intracellular HylA was apparently inactive suggests that HylA does not become enzymatically active until it is secreted. Other streptococcal products require additional processing before becoming active, including SpeB. SpeB is initially produced as a 43 kDa protein that is proteolytically processed to its final active 28 kDa protein (64).

The sequence of *hyl*A from strain 10403 indicates the presence of a typical type II signal peptide (76), suggesting *S. pyogenes* utilizes a type II secretion pathway for secretion of this protein. The signal peptide ensures that the precursor for HylA will be transferred, by way of a signal recognition particle pathway or with the assistance of a chaperone protein, from the cytoplasm to the cytosolic membrane (159). The positively charged N-terminus makes contact with the membrane lipid bilayer, after which SecA, a precursor-stimulated ATP-ase, drives the translocation through an aqueous channel or translocase (formed by SecY)(159). For translocation to be complete, the signal peptide of the

precursor protein must be cleaved by a signal peptidase to release the protein into the extracellular side of the translocase. Following translocation, the protein is processed into its native, potentially active conformation and translocated across the cell wall (159). The putative signal peptide cleavage site for HylA is located between amino acid positions 32 and 33, which would result in an extracellular molecular size of 95.9 kDa (76). Attempts to purify HylA from *S. pyogenes* strain 10403 have resulted in an active protein with a molecular size between 55 kDa and 70 kDa (Hynes, personal communication). Clearly, this differs from the anticipated molecular size; however, a 90 kDa protein has been reported also (76). Further analysis of the amino acid sequence for HylA from strain 10403, using a signal peptide predictive program (http://genome.cbs.dtu.dk/), indicated another potential cleavage site between amino acid positions 625 and 626. Cleavage at this site, as well as the cleaving of the signal peptide, would result in a protein of about 67 kDa, a size similar to that previously identified. Alternatively, the possibility of another start site for HylA exists. If the ATG site, and not the GTG, were the site of initiation, a predicted signal peptide cleavage site would be located between amino acid positions 601 and 602, which although is far too distant from the ATG site to be considered a signal peptide, would yield a protein of 68 kDa. However, the possibility of an alternative start site seems unlikely since RT-PCR analysis done in this study indicated GTG as the initiation codon. Previous studies reported that a hyaluronate lyase from a type 4 strain of *S. pyogenes* was determined to be 50 kDa by SDS-PAGE and 70 kDa by gel filtration (66). Such data suggests

that proteolytic processing in addition to the cleavage of the signal peptide may occur during the production of active HylA. Other hyaluronate lyases have been found to exhibit variation in the size of the hyaluronate lyase. The nucleotide sequence for the hyaluronate lyase gene from *Streptococcus agalactiae* (*sagHL*) predicted a protein of 121.2 kDa having a 30-amino acid signal peptide; however, SagHL was reported to exist as one of three polypeptides of ~118, ~110, or ~94 kDa following purification (52). Additionally, an enzymatically active 89 kDa polypeptide was identified from *S. agalactiae*. This small product was believed to be a degradative product of the native SagHL (52). Also, the hyaluronate lyase from *Streptococcus pneumoniae* (SpnHL) had two identified forms, of 89 kDa and 107 kDa (13).

Although processing of hyaluronate lyase has been suggested in other strains, the hyaluronate lyase from *S. pyogenes* requires further investigation. By PCR amplifying partial regions of *hyl*A, cloning, and assaying for hyaluronate lyase activity, it may be possible to gain a better understanding as to what sort of proteolytic cleavage may occur for production of the final active protein. Through this type of analysis, it may be possible to establish whether the N-terminus is cleaved at a site other than that predicted, or if there is an additional site of cleavage in the C-terminal region of the precursor peptide. Additionally, through the development of anti-hyaluronate lyase antibodies, investigations into the relationship between intracellular and extracellular HylA may be conducted to obtain an explanation for the production of HylA by strain 10403. By comparing

the size of intracellular to secreted HylA, it may be possible to ascertain the differences in size, as well as, the site of protein cleavage.

The effect of various environmental conditions on *hylA* expression. Temperature regulation of virulence factor genes, which are required for infection, of bacterial pathogens is a widely distributed mechanism of gene control. Temperature regulation of gene expression has been found in *Yersinia pestis, Shigella, Listeria monocytogenes* (88), *Borrelia burgdorferi* (17, 55), and *Streptococcus pyogenes* (146). Although information regarding the regulation of streptococcal iron homeostasis and streptolysin S in response to temperature is available (146), no information was available for *hylA* expression. The effect of various physiological conditions on *hylA* expression was examined. No difference was seen in the expression levels of *hylA* for cultures grown at the different temperatures that may be encountered *in vivo*, sugggesting *hylA* is not regulated by temperature. Similarly, *hylA* expression at pH 7.4 and pH 7.6 showed no difference. However, the quantity of *hylA* transcript from cultures grown at pH 5.0 showed a two-fold decrease in transcript compared with cultures grown at pH 7.4 and pH 7.6. Hughes et al (74) reported that less than a 2-fold change in the levels of gene transcript can be biologically significant. Smoot et al (146) used a threshold of 1.5-fold difference as being biologically significant. Using these parameters for significance, the two-fold decrease seen in the relative amount of *hylA* transcript from cultures grown at pH 5.0 (relative to pH 7.4 and pH 7.6) may be biologically significant to the organism during infection. Although cultures grown at pH 5.0 had a decrease in the amount of expression,

no difference between the HylA titer compared to the other pH levels was observed. *S. pyogenes* undergo fermentation, which through the production of an acid would decrease the pH level. One possibility may be that at the low pH, increased translation to HylA occurs, thereby resulting in a decrease in the amount of transcript but no concomitant loss in the amount of protein at the low pH.

S. pyogenes is considered an "indifferent anaerobe" due to the use of fermentative metabolism regardless of the aeration conditions (119). Analysis of the relative amounts of *hyl*A transcript from cultures grown under various aeration conditions indicated that *S. pyogenes* grown under anaerobic and increased carbon dioxide conditions had approximately the same quantity of *hyl*A transcript, 0.349 RU and 0.361 RU respectively. Cultures grown aerobically had approximately a 1.8-fold decrease in relative amount of transcript. Under anaerobic conditions, fermentation would result in a decrease in pH. Because of this decrease in pH, one would expect that there would be a similarity between the amounts of transcript detected under anaerobic and pH 5.0 conditions. Conversely, a 3.4-fold increase in the amount of transcript detected in cultures grown anaerobically in comparison to transcript amounts at pH 5.0 was observed. Since an anaerobic environment would also be acidic, the increase in transcript may be due to the effect of an anaerobic environment on other streptococcal factors that influence *hyl*A. Under anaerobic conditions, an increase in the production of streptococcal proteases may occur, which may degrade hyaluronate lyase, necessitating an increase in the amount of

expression of *hyl*A so that more protein synthesis may occur. Alternatively, perhaps more post-translational modification occurs that results in a decreased effect on transcript levels. Aeration conditions have been shown to influence expression of other streptococcal virulence factors, including *mga, emm* (M-protein), and *prtF* (fibronectin binding protein). Expression of both *mga* and *emm* is positively stimulated in the presence of carbon dioxide and shows a slight decrease when grown in the presence of oxygen (110, 124). Anaerobic conditions stimulate the expression of *prtF* (49). The effect of free exchange of gases (shaking vs. stationary) on the relative amount of *hyl*A transcript level indicated very little difference, suggesting no effect. Assay of the supernatant of cultures grown under the various conditions showed very little difference, if any, in the titer of hyaluronate lyase activity.

Early studies showed that when hyaluronic acid was added to the media, there was an increase in the production of hyaluronidase as detected by enzymatic assay (66). The effect that the addition of hyaluronic acid had on *hyl*A expression in strain 10403 over a period of four hours was in agreement with these early studies; cultures grown in the presence of hyaluronic acid showed a two-fold increase in the relative amount of transcript when compared to those grown without the substrate. However, although a two-fold increase in the amount of transcript was detected, no difference was found in the titer of hyaluronate lyase activity, suggesting that a two-fold change in the amount of transcript is not important. However, the appropriate statistical analysis was not

performed to assess significance due to the weak power of the experimental design, which suggested a 99.9% chance of error.

Although this investigation into the effect of changing environments, which may be encountered during an infection, on *hyl*A expression revealed some changes in the transcript levels, the studies were performed *in vitro* and cannot simulate the extreme response of the host immune system during a streptococcal infection. Future studies involving animal models may allow for a true assessment of the effect of *in vivo* conditions on *hyl*A expression. Graham et al (58) have recently reported studies in which the level of transcript for genes under the control of CovR/CovS was assessed from tissue excised from soft-tissue infection. Such studies may promote future possibilities for the study of expression levels of different genes *in vivo*.

The relationship between hyaluronic acid capsule and hyaluronate lyase gene expression. Faber and Rosendal (42) reported there were four main categories of *S. pyogenes*: 1) hyaluronate lyase-producing, acapsular; 2) non-hyaluronate lyase-producing, acapsular; 3) non-hyaluronate lyase-producing, encapsulated; 4) hyaluronate lyase-producing, encapsulated. Investigation of 176 strains of *S. pyogenes* identified the first three of these categories; however, our lab has not identified any strain from the fourth category, encapsulated hyaluronate lyase-producing strains. One explanation for this is that the strains may have lost virulence due to repeated subculturing on media, such that they are no longer as virulent as when first clinically isolated. In order to do a comparison between *hyl*A and *hasA* expression, it was

necessary to use a strain that produced a transcript for both genes. Strain 10403 does not produce a transcript for *hasA*; however, the encapsulated strain 71698 does produce both transcripts. Comparison of the expression patterns for the relative amounts of *hyl*A and *hasA* indicated a single peak of transcription for both genes, showing greatest expression in the early to mid-exponential phases of growth. The results obtained for *hasA* agree with earlier findings from other strains, which suggested that hyaluronic acid capsule was produced early in growth (30, 108). McClean (108) suggested that hyaluronic acid capsule is produced early in the growth of the organism, while hyaluronate lyase is found later in growth. In contrast, the results obtained from this study indicate that the genes for each are expressed simultaneously, perhaps indicating similar regulatory mechanisms. Expression of *hasA* is under the control of the CovR/CovS two-component regulatory pathway (97) as well as some form of growth phase-dependent regulation (30). Graham et al (58) recently reported inactivation of the CovR/CovS system resulted in at least a five-fold increase in the amount of *hasA*, whereas the relative amount of *hyl*A transcript was insignificantly changed, suggesting that *hyl*A is not regulated by CovR/CovS. However, whether the strain used in their study produced active hyaluronate lyase is unknown. Some factor that has not yet been identified may be produced during growth that controls the expression of both genes in the strains used in this study. The relative amounts of transcript for *hasA* in strain 71698 were much greater than that of *hyl*A, which may explain the predominance of hyaluronic acid capsule and lack of detectable hyaluronate lyase in this strain.

Additionally, the level of *hyl*A transcript from strain 71698 was two times greater than that of strain 10403. Since active HylA is not produced in strain 71698, the amount of transcript found may be due to the transcript being long-lived, having not been processed and degraded. Alternatively, the increase in the amount of *hyl*A transcript may be due to the presence of a hyaluronic acid capsule possessed by this strain. As shown through studies involving the addition of hyaluronic acid to growth conditions, approximately a two-fold increase in the amount of *hyl*A transcript was observed. The presence of capsular hyaluronic acid seems like a more appropriate reason as strain SF370's amount of transcript is similar to that found in strain 10403 since both strains are acapsular.

Although a comparison was made between *hyl*A and *hasA* expression in strain 71698, whether the same results would be found in a hyaluronate lyase-producing strain such as strain 10403 is uncertain. In order to better establish the relationship between the two genes, a hyaluronate-lyase producing strain that also produces a *hasA* transcript, or is actually encapsulated, should be examined. Furthermore, animal studies may also help to resolve this streptococcal paradox. Through such studies, it may be possible to determine expression of the necessary genes, and in the process, evaluate any relationship between expression between *hyl*A and *hasA* over the course of streptococcal infection.

Size variability of the hyaluronate lyase gene of *S. pyogenes*. The sequence for the hyaluronate lyase gene from the ATCC T-type 22 hyaluronate lyase-producing strain 10403 (*hyl*A) was previously determined (76). The gene

is 2607 bp in length, encoding a 99,636 Dalton protein. PCR analysis of the hyaluronate lyase gene region from 176 strains of *S. pyogenes* indicated that all strains contained a gene region exhibiting similarity to *hyl*A. PCR amplification of the 3' region of the hyaluronate lyase gene from the 176 strains revealed the presence of a deletion within 33 (20%) of the tested strains. No apparent association between the size variability and disease, hyaluronate lyase activity, or serotype was observed. The 3' regions of six of these strains were analyzed by DNA sequencing. All six strains contained a smaller gene, which had an in-frame deletion of 183 bp relative to *hyl*A. Alignment of the sequences from the 3' region of the shorter clones, with that of *hyl*A from strain 10403, indicated that the deletion occurred in the same location within all the strains and was located between positions 2273-2455 of the *hyl*A gene open reading frame. Since hyaluronidase activity was detected among some of the strains possessing the deletion, it was putatively determined that the deleted fragment has no involvement in enzymatic activity; however, this could not be completely determined due to the presence of bacteriophage hyaluronidases. By cloning and expressing the genes from these strains, any question regarding the hyaluronate lyase activity would be answered.

Further evidence that the 3' region is not involved in hyaluronate lyase activity was obtained by comparing expression of the cloned gene from the M1 strain SF370, a non-hyaluronidase producing strain, to that of expression from *hyl*A from strain 10403. The gene from SF370 contains a termination sequence at position 2416-2418 of the *hyl*A open reading frame. Termination at this site

may result in the loss of enzymatic activity seen in this strain. Introduction of a premature termination codon into *hyl*A of strain 10403, at the site where termination occurs in SF370, did not result in loss of hyaluronate lyase activity when cloned into *E. coli* (Hynes, unpublished data).

A streptococcal bacteriophage hyaluronidase gene, *hylP2*, also shows a deletion when compared to the streptococcal bacteriophage hyaluronidase gene, *hylP* (80). Alignment studies showed that a collagen-like repeat region of ten Gly-X-Y repeats in *hylP* was deleted in *hylP2*. Analysis of the 183 bp region deleted from the 3' region did not show any motifs similar to those found in the deleted region in the bacteriophage hyaluronidase gene *hylP2*. However, a 9 bp sequence (AAAACAATG) repeat was found to mark the boundaries of the deleted region. Only one copy of the sequence is found in those strains that have the smaller gene, while both repeats are found in the larger gene of strain 10403. Whether this sequence is the site of insertion of a fragment in strain 10403, or associated with the deletion of the fragment in the other strains is unknown. A search of the nucleotide databases did not suggest any association of this sequence with an insertion sequence or transposon. A search of the streptococcal chromosome from SF370 for the AAAACAATG repeat sequence found 57 identical copies of the sequence; however, apart from the repeats found within the hyaluronate lyase gene no other repeat pairs were in close proximity to one another. Chromosomal sequence analysis of strains MGAS8232 (M-type 18) and MGAS315 (M-type 3) showed 59 and 48 identical repeats. Additionally, the sequence from *S. pyogenes* MGAS8232 (145) showed

that the *hyl*A gene is similar to that of strain 10403, in that it has both copies of the repeat sequence. On the other hand, analysis of the sequence from strain MGAS315 (10) showed the *hyl*A gene is shorter relative to strain 10403, and has only one copy of the repeat. Additionally, the *hyl*A from strain MGAS315 had a much smaller 3' region of the gene, suggesting a deletion. A search for similarity to the 183 bp fragment missing from the shortened genes indicated no similarity other than to that found within the hyaluronate lyase gene, suggesting the fragment is not a known insertion sequence.

Although information is available regarding certain aspects of hyaluronate lyases, little is known about what part of the protein molecule is needed for enzymatic activity. Reports on the active site of hyaluronate lyases from *Streptococcus agalactiae* (SagHL) and *Streptococcus pneumoniae* (SpnHL) suggest that the catalytic region contains conserved histidine, tyrosine, and asparagine residues (98, 99). Comparison of the amino acid sequences from SagHL and HylA indicates that the asparagine, histidine, and tyrosine residues associated with activity are present in HylA, and that the active site region is between amino acid residues 257–327, corresponding to nucleotide positions 769-981 of *hyl*A. This is outside the area associated with the deletion in the 3' region. Whether the same active region of the group A streptococcal hyaluronidase gene (*hyl*A) is necessary for hyaluronate lyase activity is not known, but it would be likely based on the similarities of the genes. Although this study has suggested that the deletion may play no role in hyaluronate lyase activity, cloning and sequencing entire genes from some of

these strains having a smaller *hyl*A, assaying for hyaluronate lyase activity, and comparing them to strain 10403 may enable a more definitive answer. Additionally, by looking at different regions of the hyaluronate lyase gene and at how mutations introduced into those regions affect activity, and by cloning and expressing recombinant forms of the shorter gene, a better understanding of the extracellular hyaluronate lyase of *S. pyogenes* at the molecular level will be gained.

Preliminary assessment of the role of hyaluronate lyase in a streptococcal infection. The role of hyaluronate lyase in streptococcal infections is unknown. Because of its ability to degrade a major constituent of connective tissues (hyaluronic acid) it has been speculated to be a spreading factor. The current investigation examined the possible role of hyaluronate lyase as a virulence factor. Following subcutaneous injection of a single-recombinant mutant strain 10403::pHAS:*hyl*A (76) into CD-1 out-bred mice abscess and lesion formation were observed. The tissue damage suggests that strains lacking an active hyaluronate lyase are able to initiate an infection. Pus taken from the lesions and abscesses revealed β-hemolytic streptococci. Colonies were then screened by a hyaluronidase assay (77). Colonies recovered from the wound showed two phenotypes, $HylA^+$ and $HylA^-$. Approximately 5.7% of the colonies screened showed hyaluronate lyase activity, indicating the potential reversion of the mutant to the wild-type form. This occurrence may be indicative that hyaluronate lyase is required for S. *pyogenes* to cause infection as was found to be the case for the hyaluronic acid capsule. Following inoculation of a

mutant strain in which *hasA* was disrupted by a non-replicative plasmid, only revertant encapsulated colonies were recovered (75). The inactivation in strain 10403::pHAS:*hyl*A was by a single recombination event resulting in insertion of the entire plasmid into the *hyl*A gene. Reversion to wild-type could be accomplished by a reverse recombination event within the cell that results in expulsion of the plasmid. Although the colonies screened were both $HylA^+$ and $HylA^-$, all colonies were still erythromycin resistant and $HylA^+$ colonies amplified *hyl*A, suggesting the plasmid still resided within the cell.

Because 10403::pHAS:*hyl*A apparently reverted to its wild-type phenotype, an accurate assessment of the loss of hyaluronate lyase on infection could not be assessed. Because of this, isogenic mutant strains 10403Δ*hyl*A1 and 10403Δ*hyl*A2 were created by replacing an internal portion of *hyl*A with an erythromycin resistance gene (*ermR*). Reverse recombination similar to what appears to have occurred in the single recombinant mutants would result in a gene containing a deletion. Inactivation resulted in a loss of hyaluronate lyase activity, but did not appear to affect the growth of the organism. Furthermore, inactivation did not result in a loss of two other activities important for streptococcal virulence, that being the cysteine protease and hemolysin activities. Hence, these double recombinant mutant strains should be useful for future animal studies to investigate the role of hyaluronate lyase as a virulence and/or spreading factor of *S. pyogenes*.

CONCLUSIONS

This study has provided an in-depth evaluation into the hyaluronate lyase gene of *S. pyogenes.* Hyaluronate lyase activity was detected in the early exponential phase of growth and was detected throughout the growth of strain 10403. Additionally, it was ascertained that *hyl*A is expressed not only in hyaluronate lyase-producing strains, but also in those that do not produce detectable enzymatic activity. Expression of *hyl*A in the enzymatically inactive strains, 71698 and SF370, was determined to have a single peak of expression, in which the relative levels of transcript were greatest in the early to mid-exponential with little in the stationary stages of growth. In contrast, strain 10403 showed a different pattern of expression, having increased relative amounts of *hyl*A transcript in the early to mid-exponential growth followed by a decrease at hour 8, then another increase in late stationary growth.

The effect of various environmental conditions on *hyl*A expression was evaluated by determining the relative amount of *hyl*A transcript present in the cell. Temperature had little effect on the relative amount of *hyl*A transcript for strain 10403. However, *hyl*A expression was slightly decreased after growth in pH 5.0 and was slightly increased under anaerobic and increased carbon dioxide conditions. Additionally, the presence of hyaluronic acid in the growth media resulted in a two-fold increase in *hyl*A expression. Although a difference was seen in the levels of mRNA, no difference was detected in the titer of enzymatic activity.

This study also determined that the time of expression for *hyl*A and *hasA* was the same in strain 71698. The amount of transcript increased until hour 4 then declined over the rest of growth; however, the relative amount of *hasA* transcript was considerably greater than that of *hyl*A.

All of the streptococcal strains screened had a region with similarity to the hyaluronate lyase gene (*hyl*A) of strain 10403 as determined by PCR. However, variation in the size of the 3' region of the hyaluronate lyase region was seen in some strains of *S. pyogenes*. The decreased size results from an in-frame deletion of 183 bp compared to *hyl*A, with the boundaries of the deleted region being marked by a nine base pair repeat (AAAACAATG).

Finally, a preliminary study into the role of hyaluronate lyase as a putative virulence factor during a streptococcal infection was evaluated. Growth *in vivo* resulted in the reversion of a single-recombinant mutant strain from a HylA-negative to a HylA-positive phenotype. Also, an isogenic mutant strain 10403Δ*hyl*A was created by replacing an internal portion of *hyl*A with erythromycin resistance gene (*ermR*). Inactivation of hyaluronate lyase did not have any obvious affect on growth, protease, or hemolytic activities.

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APPENDIX I

MATERIALS AND EQUIPMENT

Materials and equipment used are listed under the respective manufacturers.

1. Fisher Scientific, New Jersey.
    i. Yeast Extract
    ii. Agarose Low EEO, Electrophoresis Grade
    iii. Agar, Granulated Solidifying Agent
    iv. Sodium Chloride, Enzyme Grade
    v. Tris Crystallized Free Base, Molecular Biology Grade
    vi. Sodium Phosphate, Dibasic, Anhydrous, Enzyme Grade
    vii. Sodium Phosphate, Monobasic, Anhydrous, Enzyme Grade
    viii. Sodium Citrate, Enzyme Grade
    ix. Sodium Hydroxide, Molecular Biology Grade
    x. Sodium Acetate, Anhydrous, Enzyme Grade
    xi. 3-(N-Morpholino) Propane-Sulfonic Acid (MOPS), Enzyme Grade
    xii. Magnesium Chloride (Hexahydrate), Enzyme Grade
    xiii. Maleic Acid, Reagent Grade
    xiv. Guanidium Thiocyanate, Molecular Biology Grade
    xv. Glycine, Tissue Culture Grade
    xvi. EDTA, Disodium Salt, Electrophoresis Grade
    xvii. Dextrose, Anhydrous, Molecular Biology Grade
    xviii. Boric Acid, Electrophoresis Grade

xix. Sarkosyl, Molecular Biology Grade

xx. Bovine Serum Albumin (Fract V), Biotechnology Grade

xxi. Saturated Phenol pH 6.6 ± 0.2, Biotechnology Grade

xxii. Saturated Phenol pH 4.3 ± 0.2, Biotechnology Grade

xxiii. Tween®20, Enzyme Grade

xxiv. Ethidium Bromide, Electrophoresis Grade

xxv. 2-Mercaptoethanol, Electrophoresis Grade

xxvi. 2-Propanol, Certified ACS

xxvii. Chloroform, Molecular Biology Grade

xxviii. Iso-Amyl Alcohol, Biotechnology Research Grade

xxix. Light Mineral Oil (Paraffin Oil, Light)

xxx. Reagent Alcohol, Anhydrous Ethyl Alcohol

xxxi. Potassium Phosphate, Monobasic, Enzyme Grade

xxxii. Potassium Chloride, Enzyme Grade

xxxiii. Ammonium Acetate, Enzyme Grade

xxxiv. Diothiothreitol (DTT), Molecular Biology Grade

2. Sigma, Missouri

i. Hyaluronic Acid (from Human Umbilical Cord)

ii. Lauryl Sulfate (Sodium Dodecyl Sulfate)

iii. Erythromycin

iv. Igepal CA-630

v. Ampicillin (D (84)-α-Aminobenzylpenicillin)

vi. Fusidic Acid

vii. Rifampin (3-[4-Methylpiperazinyl-iminomethyl]rifamycin SV)

viii. Mutanolysin (from *Streptomyces globisporus* ATCC 21553)

ix. Nylon Membrane, BioBond

3. Ambion, Texas

i. The RNA Storage Solution

ii. RNAseZap

4. Promega, Wisconsin

i. Taq DNA Polymerase in Storage Buffer B

ii. PCR Markers

iii. Improm-II Reverse Transcription System

iv. RQI DNase

v. Wizard Plus SV Miniprep Plasmid Purification Kit

vi. pGEM-Teasy Vector System

vii. Klenow Fragment of *E. coli* DNA Polymerase I

5. Becton-Dickinson, Maryland

i. Bacto™ Tryptone, Pancreatic Digest of Casein

ii. Bacto™ Todd Hewitt

iii. BBL GasPak Plus Anaerobic System Envelopes with Palladium Catalyst

iv. BBL Dry Anaerobic Indicator Strips

v. TSA containing 5% Sheep's Blood

6. Amresco, Ohio

i. Agarose SFR, Biotechnology Grade

ii. Diethylpyrocarbonate (DEPC), High Purity Grade

iii. Dimethyl Sulfoxide (DMSO), ACS Grade

7. United States Biological, Massachusetts

i. Lysozyme, Chicken Egg White Muramidase, Molecular Biology Grade

8. MBI Fermentas, New York

i. Restriction Enzymes

ii. DNA Ligase

9. Bioline USA, Incorporated, New Jersey

i. Bioline Half-Dye™ Buffer

10. Invitrogen, California

i. pCR 2.1 Vector System

11. Roche, Germany

i. DIG DNA Labeling and Detection Kit

12. Applied Biosystems, California

i. ABI PRISM Template Suppression Reagent

ii. Big Dye™ Terminator v3.0 Cycle Sequencing Ready Reaction

iii. ABI PRISM 310 Genetic Analyzer

13. Cepheid, California

i. Smart Cycler™ System

14. The Virtis Company, New York

i. Virsonic Cell Disrupter

15. Sequoia-Turner Corporation, California
    i. Model 690 Spectrophotometer
16. Eppendorf, Germany
    i. Eppendorf Biophotometer
17. BTX, California
    i. BTX Transporator™ Plus

APPENDIX II

S. PYOGENES STRAINS USED IN THIS STUDY

Strain	M(T)-type	Disease	Hyaluronate lyase activity
7	T-11		Negative
39	PT2841		Positive
51	22	Scarlet Fever	Positive
58	T8/25	Scarlet Fever	Negative
61	4	Scarlet Fever	Positive
73	T4/49	Scarlet Fever	Positive
99	T2/28		Negative
114	3		Positive
125	78		Negative
163			Positive
170			Negative
175			Negative
199	3	Scarlet Fever	Positive
203	T8/25	Scarlet Fever	Negative
208	T2	Scarlet Fever	Negative
209	6	Scarlet Fever	Negative
210	T3/13	Scarlet Fever	Positive
218	1	Scarlet Fever	Negative
220	T12/B3264	Scarlet Fever	Positive
227	1		Negative
250	T3	Carrier	Positive
258	8	Nasopharyngitis	Negative
260	60	Nephritis	Positive
264	49	Acute Glomerulonephritis	Negative
275	1	Scarlet Fever	Negative
287	1	Tonsilitis	Negative
300	T4/28	Acute Glomerulonephritis	Positive
302		ulcerative	Negative
307		abscess	Positive
315		p. anricular abscess	Negative
322		Empyema	Positive
329		Impetigo	Positive
335		Acute Glomerulonephritis	Negative
350		Scarlet Fever	Negative
364	5	Cellulitis	Positive
SF370	1	Wound Infection	Negative
380	3	Scarlet Fever	Positive
389		Scarlet Fever	Positive
402	18	Acute Rheumatic Fever	Negative
410	T4/28	Scarlet Fever	Positive
422	T3/B3264	Scarlet Fever	Positive
430	T4/28		Positive
440	T9		Negative
448	T22		Positive
451	1	Acute Rheumatic Fever	Negative

APPENDIX II. CONTINUED.

Strain	M(T)-type	Disease	Hyaluronate lyase activity
462	30	Scarlet Fever/Acute Rheumatic Fever	Negative
470		Acute Rheumatic Fever	Negative
478		Acute Rheumatic Fever	Negative
486		Acute Rheumatic Fever	Positive
499			Negative
505		Rheumatoid Infection	Negative
519	T11/12		Negative
530	T11/12		Negative
537			Negative
545	T1		Negative
552			Negative
561			Positive
573	31	Septicemia	Positive
576	12	Septicemia/tonsilitis	Negative
581	T12	Septicemia	Negative
585	4	Bacteremia	Positive
593			Negative
600	T22		Negative
606			Negative
625	12		Negative
629			Positive
638	1		Negative
650	T4		Positive
670	T3/13/B		Negative
675			Negative
695	Col type I	Necrotizing fasciitis	Negative
696	Col type II	Necrotizing fasciitis	Negative
703		Rheumatic Fever	Positive
704	1	Rheumatic Fever	Positive
709		Rheumatic Fever	Positive
714	1	Severe Invasive	Negative
722	1	Severe Invasive	Negative
740	1		Negative
745			Negative
749	1	Pharyngits	Negative
759	12	Acute Post-streptococcal Glomerulonephritis	Negative
760	56	Acute Glomerulonephritis	Positive
772	9		Negative
775	14		Negative
777	17		Negative
785	28		Negative
788	32		Negative
793	36		Positive
795	39		Negative
801	49		Positive
805	53		Negative
808	55		Negative
811	57		Positive

APPENDIX II. CONTINUED.

Strain	M(T)-type	Disease	Hyaluronate lyase activity
817	60		Positive
821	66		Positive
825	47		Negative
835	49	Skin Infection	Positive
845	1	Toxic Shock-like Syndrome	Negative
850	1	Toxic Shock-like Syndrome	Negative
862	1	Blood	Negative
863	1	Necrotizing fasciitis	Negative
864	T28	Blood	Negative
869			Negative
871		Blood	Positive
872		Wound	Negative
875		Toxic Shock-like Syndrome	Negative
879		Toxic Shock-like Syndrome	Negative
880		Toxic Shock-like Syndrome	Negative
909			Negative
922			Negative
944			Positive
950			Positive
960			Positive
972			Positive
979			Positive
1007			Negative
1013		Foot	Negative
1020		Throat	Positive
1025		Lung	Negative
1030		Blood	Negative
1045		Blood	Positive
1055		Blood	Positive
1059		Hand	Negative
1074		Throat	Positive
1080			Positive
1084			Positive
10403	22	Throat	Positive
13218			Negative
74-755	49		Negative
77-588	49		Negative
80-003	49		Negative
81-071	49		Negative
81-081	49		Negative
81-185	49		Positive
84-404	49		Positive
85-069	49		Negative
86-715	49		Negative
869A			Negative
869B			Negative
87-263	49		Negative
87-300	49		Positive
90-025	49		Negative

APPENDIX II. CONTINUED.

Strain	M(T)-type	Disease	Hyaluronate lyase activity
90-131	49		Negative
90-195	49		Negative
90-214	49		Negative
90-223	49		Negative
90-225	49		Negative
92-362-0637			Negative
93-B15539			Negative
94-146			Negative
94-163-1367			Positive
94-175-1578			Positive
94-321-1885			Negative
95-10369			Positive
95-10497			Negative
95-12682			Negative
95-12725			Negative
95-17664			Positive
95-279-3671			Negative
95-30375			Positive
95-4383			Positive
96-05396			Negative
96-012-3884			Negative
96-070-0918			Negative
96-096-2862			Negative
96-241-3401-2			Negative
97-087-1138			Negative
BL-46-02469			Negative
CGH-97-1			Positive
GT6266	49		Positive
GT6267	49		Positive
GT6479	49		Positive
GT8760	49		Positive
GT9538	49		Positive
71698	28		Negative
FF22	52		Negative

APPENDIX III

COMPARISON BETWEEN STRAINS: MEAN RAW ABUNDANCE VALUES

Strain	Time	Extraction	Mean *hylA* abundance value*	Mean *gyrA* abundance value*
10403	0	1	0.243	1.34
10403	0	2	0.067	0.452
10403	2	1	0.027	0.36
10403	2	2	0.0355	0.325
10403	4	1	1.78	3.879
10403	4	2	0.019	0.459
10403	4	3	0.043	0.238
10403	6	1	0.209	10.473
10403	6	2	0.011	0.873
10403	8	1	0.003	1.627
10403	8	2	0.006	1.04
10403	10	1	0.045	0.18
10403	10	2	0.02	0.141
10403	12	1	0.645	2.546
10403	12	2	0.293	1.03
10403	14	1	0.156	0.79
10403	14	2	0.394	1.125
10403	14	3	0.029	0.089
10403	16	1	0.065	0.022
10403	16	2	0.0085	0.021
10403	18	1	0.389	0.694
10403	18	2	0.44	1.79
SF370	0	1	0.056	0.6
SF370	0	2	0.05	0.578
SF370	2	1	5.51	43
SF370	2	2	0.063	0.278
SF370	2	3	0.055	0.206
SF370	4	1	0.673	15.13
SF370	4	2	0.026	0.5
SF370	4	3	0.144	0.81
SF370	6	1	6.48	9.13
SF370	6	2	0.096	0.829
SF370	6	3	0.08	0.232
SF370	8	1	0.013	0.144
SF370	8	2	0.011	0.321
SF370	8	3	0.017	0.123
SF370	18	1	0.017	0.2
SF370	18	2	0.008	0.029
SF370	18	3	0.0035	0.047

APPENDIX III. CONTINUED.

Strain	Time	Extraction	Mean *hylA* abundance value*	Mean *gyrA* abundance value*
71698	0	1	0.215	0.449
71698	0	2	0.525	0.509
71698	2	1	0.643	1.034
71698	2	2	0.024	0.056
71698	4	1	0.341	0.452
71698	4	2	0.033	0.044
71698	6	1	0.023	0.025
71698	6	2	0.012	0.034
71698	8	1	0.025	0.085
71698	8	2	0.009	0.041
71698	18	1	0.084	0.221
71698	18	2	0.086	0.278

*Represents the mean raw abundance value from triplicate real-time analyses.

APPENDIX IV

EFFECT OF ENVIRONMENTAL CONDITIONS ON MEAN RAW ABUNDANCE VALUES

Treatment	Extraction	Mean *hylA* abundance value*	Mean *gyrA* abundance value*
32C	1	0.498	3.452
32C	2	0.189	2.216
32C	3	0.17	0.68
37C	1	0.25	0.656
37C	2	0.202	1.24
37C	3	0.187	0.486
42C	1	1.1175	10.93
42C	2	0.139	0.695
42C	3	0.193	0.828
Aerobic	1	0.25	0.656
Aerobic	2	0.202	1.24
Aerobic	3	0.187	0.486
Increased CO2	1	0.147	0.411
Increased CO2	2	0.791	1.938
Anaerobic	1	0.316	0.911
Anaerobic	2	0.043	0.124
With Gaseous Exchange	1	0.016	0.145
With Gaseous Exchange	2	0.277	1.13
pH5.0	1	0.09	0.6
pH5.0	2	0.009	0.106
pH5.0	3	0.007	0.054
pH7.4	1	0.661	2.667
pH7.4	2	0.412	2.243
pH7.6	1	0.232	0.877
pH7.6	2	0.25	0.656
pH7.6	3	0.187	0.486
With Hyaluronic Acid 0	1	0.002	0.0425
With Hyaluronic Acid 0	2	0.62	0.407
With Hyaluronic Acid 2	1	0.022	0.0533
With Hyaluronic Acid 2	2	0.055	0.3
With Hyaluronic Acid 4	1	0.155	0.441
With Hyaluronic Acid 4	2	0.185	0.28

*Represents the mean raw abundance value from triplicate real-time analyses.

APPENDIX V

S. PYOGENES STRAINS HAVING SIZE VARIABILITY IN THE 3' REGION OF THE HYALURONATE LYASE GENE REGION AS COMPARED TO STRAIN 10403

Strain	M(T)-type	Disease	Hyaluronate lyase activity
7	T-11		Negative
58	T8/25	Scarlet Fever	Negative
114	3		Positive
250	T3	Carrier	Positive
350*		Scarlet Fever	Negative
364	5	Cellulitis	Positive
380	3	Scarlet Fever	Positive
389		Scarlet Fever	Positive
422*	T3/B3264	Scarlet Fever	Positive
440	T9		Negative
462	30	Scarlet Fever/Acute Rheumatic Fever	Negative
561			Positive
600	T22		Negative
703		Rheumatic Fever	Positive
777	17		Negative
788	32		Negative
795	39		Negative
871		Blood	Positive
872*		Wound	Negative
880		Toxic Shock-like Syndrome	Negative
944			Positive
1013		Foot	Negative
1020*		Throat	Positive
1045		Blood	Positive
1055*		Blood	Positive
1074		Throat	Positive
869A			Negative
869B			Negative
19617		Tissue	Positive
92-362-0637			Negative
94-146*			Negative
95-4383			Positive
CGH-97-1			Positive

*Indicates strains in which the 3' region of the *hyl*A gene was sequenced (Appendix VI)

APPENDIX VI

SEQUENCES FOR THE 3' REGION OF THE HYALURONATE LYASE GENE REGION FROM STRAINS USED IN SPECIFIC AIM 2

Strain 350 3' region of hyaluronate lyase

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAA AAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGTTATTAAAGACAAAAAACTGGAAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 422 3'region of the hyaluronate lyase gene region

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGTTATTAAAGACAAAAAACTGGGAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 872 3'region of the hyaluronate lyase gene region

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGTTATTAAAGACAAAAAACTGGAAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 1020 3'region of the hyaluronate lyase gene region

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGTTATTAAAGACAAAAAACTGGAAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 1055 3'region of the hyaluronate lyase gene region

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATACTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGCTATTAAAGACAAAAAACTGGAAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 94146 3'region of the hyaluronate lyase gene region

```
  1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
 51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
151 AGCATAAGTAAGGCACTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAAATACCTTTATCACTA
251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
301 CCAAATATGACTCGTCAAGAATTTGAAACCTATATTAGCAAGCTTGATAT
351 CGACTTGTTAGAAAACAATGCTCTTAACTCTCATAAAATTCCCCACAAAA
401 GACAACGACGCTTACCGCATACTGGGTATCAGTCCTCCTCTTTAGAATTC
451 TTAGGTGGAGCTTTAGTTGCTTCTTTTAACCATATCACAAAACCCTTTCG
501 TAAGAAAGACTTAAGGATTTAGCTGTTATTGGAAAGTAAGGGTTAACAAT
551 GAAACGTTATTAAAGACAAAAAACTGGAAACTGCACTGAGCCCCAAAAGT
601 TGGCTACTCTATTTGAAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTA
651 AGTCCTTTTCGTTTTATTTTGATACGGTTATTGGTGCAATAATCGATGAT
701 AATAGTAGGTAGAACGAGTCACTTTAGCCGTCCTTAAAAGAAAATCTAAT
751 CAGACTCGTCTTTGTCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTT
801 CAGAGATGC
```

APPENDIX VI. CONTINUED.

Strain 10403 3'region of the hyaluronate lyase gene region

```
   1 CAAAAGTACCCTTACTGTTCTTATGTTAACAATCAACCCGTTGACTTGAA
  51 TAATCAGCTAGTTGATTTTACAAACACTAAAAGTATTTTCCTTGAAAGTG
 101 ATGATCCCGCTCAAAATATTGGTTACTACTTCTTCAAGCCAACAACACTT
 151 AGCATAAGTAAGGCGCTTCAAACAGGGAAATGGCAAAACATAAAAGCTGA
 201 TGACAAATCACCAGAAGCCATCAAAGAAGTTTCAGATACCTTTATCACTA
 251 TCATGCAAAACCATACTCAAGATGGCGATCGTTATGCCTATATGATGCTT
 301 CCAAATATGACTCGTCAGGAATTTGAAACCTATATTAGCAAGCTTGATAT
 351 CGACTTGTTAGAAAACAATGACAAACTGGCCGCTGTCTACGATCATGATA
 401 GTCAACAGATGCACGTCATTCACTATGAAAAAAAAGCAACGACGTTTTCA
 451 AATCATAATCTTTCTCATCAAGGCTTTTATAGTTTTCCTCATCCTGTCAA
 501 GCAAAATCAGCAACAAAAGTTTGCTCATCAAGGAATTGCAGCTAAAAACA
 551 ATGCTCTTAACTCTCATAAAATTCCCCACAAAAGACAACGACGCTTACCG
 601 CGTACTGGGTATCAGTCCTCCTCATTAGAGTTCTTAGGTGGAGCTTTAGT
 651 TGCTTCTTTTAACCATATCACAAAACCCTCTCGTAAGAAAGACTTAAGGA
 701 TTTAGCTGTTATTGGAAAGTAGGGGTTAACAATGAAACGTTATTAAAGAC
 751 AAAAAAGACTGGAAACTGCACTGAGCCCCAAAAGTTGGCTACTCTATTTG
 801 AAGTAAAGGATTTAGTTCTGTATTGTAGAGGACTAAGTCCTTTTCGTTTT
 851 ATTTTGATACGTTTATTGTTGCAATAATCGATGATAATAGTAGGTAGAAC
 901 GAGTCAATATAGCCGTCCTTAAAAGAAAATCTAATCAGAACTCGTCTTTG
 951 TCCATTCTTTATAATGGTTTTTTGTCTTCTTCTTTTCAGAGATGC
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EXPERIENCE

Old Dominion University
Graduate Research Assistant
- Spring 2001 to present
- Develop and utilize molecular assays for the detection of *Pfiesteria* sp. and other harmful dinoflagellates in water samples

Tidewater Community College- Norfolk Campus
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- Anatomy and Physiology (Fall 1998, Spring & Summer 1999, Fall 2000)
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PUBLICATIONS

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Hynes, W.L, A.R. Dixon, **S. Walton**, and L.J. Aridgides. 2000. The extracellular hyaluronidase gene (*hylA)* of *Streptococcus pyogenes*. FEMS Microbiol. Lett.. **184**:109-112.

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