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MiniReview

Hyaluronidases of Gram-positive bacteria

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Abstract

Bacterial hyaluronidases, enzymes capable of breaking down hyaluronate, are produced by a number of pathogenic Gram-positive bacteria that initiate infections at the skin or mucosal surfaces. Since reports of the hyaluronidases first appeared, there have been numerous suggestions as to the role of the enzyme in the disease process. Unlike some of the other more well studied virulence factors, much of the information on the role of hyaluronidase is speculative, with little or no data to substantiate proposed roles. Over the last 5 years, a number of these enzymes from Gram-positive organisms have been cloned, and the nucleotide sequence determined. Phylogenetic analysis, using the deduced amino acid sequences of the Gram-positive hyaluronidases, suggests a relatedness among some of the enzymes. Molecular advances may lead to a more thorough understanding of the role of hyaluronidases in bacterial physiology and pathogenesis.

Keywords: Hyaluronidase; Hyaluronate lyase; Pathogenesis

1. Introduction

Many pathogenic bacteria produce extracellular products that have tissue-damaging effects. Some of the diverse armamentarium of products from pathogenic bacteria serve as virulence factors in the pathogenesis of disease by facilitating the spread of bacteria or toxins through tissues; these are commonly referred to as spreading factors. For many years, the term hyaluronidase has been synonymous with spreading factors. Although all extracellular hyaluronidases are probably spreading factors, not all spreading factors are hyaluronidases. Hyaluronidase is a general term used to describe enzymes that are able to breakdown the substrate hyaluronate (hyaluronic acid, hyaluronan), however, some of these enzymes are also able to cleave chondroitin sulfate [1], albeit at a slower rate. The hyaluronidases can be subdivided into three types [1]. Hyaluronate-4-glycanohydrolases (EC 3.2.1.35) are the testicular-type hyaluronidases found in mammalian spermatozoa, lysosomes and the venoms of various insects and snakes. The second group are hyaluronate-3-glycanohydrolases (EC 3.2.1.36) produced by leeches and some hookworms. Both of these groups of hyaluronidases degrade hyaluronate with the formation of tetrasaccharides as the end product. The third group, the bacterial hyaluronidases or hyaluronate lyases (EC 4.2.2.1 or EC 4.2.99.1), act as endo-*N*-acetylhexosaminidases by elimination across the β-1-4 linkage (Fig. 1). Unlike the other hyaluronidases, the products of hyaluronate lyases are unsaturated disaccharides. These hyaluronidases are the focus of this review. Other polysaccharide lyases and a more detailed examination of the mechanisms of action have been the subject of other reviews [1,2] and will not be addressed here.

Hyaluronate is a linear unsulfated glycosaminoglycan polymer, with an average molecular mass greater than 10 000. The polymer is made up of alternating *N*-acetylglucosamine and glucuronic acid residues linked by glycosidic bonds (Fig. 1), but unlike other glycosaminoglycans, it lacks a covalently linked peptide [3]. Hyaluronate is found in many body tissues and fluids of higher organisms such as umbilical cord, synovial fluid, cartilage, brain, muscle, and is a major component of the extracellular matrix especially in soft connective tissue [3]; 50% of the hyaluronate in the body is found in the skin. It is also present in high concentrations in rooster combs and is produced by certain bacteria such as the streptococci [3]. The hyaluronate isolated from all these various sources has an identical chemical structure.

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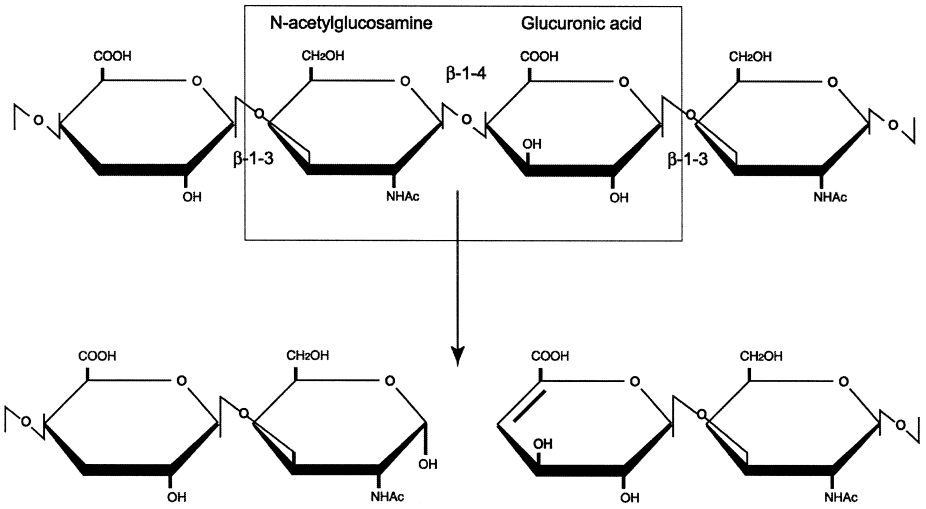

Fig. 1. Structure of hyaluronate and the products after eliminative cleavage by a bacterial hyaluronate lyase. The boxed region shows the repeating unit of *N*-acetylglucosamine and glucuronic acid linked β-1-4 that constitutes hyaluronate. The repeating disaccharide is linked β-1-3 to the adjoining disaccharide.

2. Organisms producing hyaluronidase

A wide variety of microorganisms produce enzymes capable of degrading hyaluronate. Enzymes produced by the Gram-negative organisms are periplasmic rather than being excreted to the extracellular milieu, and so are less likely to play a role in pathogenesis. Some of these enzymes are chondroitin lyases, as they are also capable of degrading substrates such as chondroitin sulfate. Chondroitinase activity has been reported in the genera *Aeromonas*, *Vibrio*, *Beneckea* and *Proteus*. *Bacteroides fragilis*, *Bacteroides vulgatus*, *Bacteroides ovatus*, *Bacteroides melaninogenicus*, *Bacteroides asaccharolyticus* and *Fusobacterium mortiferum* are also reported to produce hyaluronidase [2]. *Treponema pallidum* and *Treponema pertenue*, both pathogens, produce a surface-associated hyaluronic acid degrading enzyme, whereas the non-pathogenic *Treponema denticola* and *Treponema vencentii* do not produce such an enzyme [4]. Anti-hyaluronidase sera, prepared against bovine hyaluronidase, cross-reacted with both the treponemal hyaluronidase and testicular hyaluronidase, suggesting the treponemal hyaluronidase is similar to the hyaluronate-4-glycanohydrolase type enzymes and may not be a hyaluronate lyase; definitive classification would require isolation and characterization of the enzyme. Production of hyaluronidase has also been reported in different species of *Candida*, including *C. albicans*, *C. tropicalis*, *C. guillermondii*, *C. parapsilosis* and *C. krusei* [5], although the type of hyaluronidase is currently unknown.

Gram-positive organisms capable of producing hyaluronidase include various species of *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium*, *Streptomyces* and *Clostridium*. Hyaluronidase production has been reported in streptococci groups A, B, C and G [6], as well as *Streptococcus pneumoniae* [7], members of the viridans streptococci (*Streptococcus intermedius* and *Streptococcus constellatus*) [8], *Streptococcus dysgalactiae* [9] and *Streptococcus uberis* [10]. Among the staphylococci, hyaluronidase production has been shown for pathogenic strains of *Staphylococcus aureus* and *Staphylococcus hyicus* subsp. *hyicus* [11]. *Clostridium perfringens* (Mu toxin) [12], *Clostridium difficile* [13], *Clostridium septicum* (γ toxin) and *Clostridium chauvoei* [14], all potential pathogens, produce hyaluronidase. Two cutaneous Propionibacteria, *Propionibacterium acnes* and *Propionibacterium granulosum*, produce hyaluronidase [15], with the enzyme from *P. acnes* being characterized as a hyaluronate lyase. *Streptomyces hyalurolyticus, Streptomyces coelicolor* and *Streptomyces griseus* produce enzymes capable of degrading hyaluronate [16]. A hyaluronidase purified and characterized from a species of *Peptostreptococcus* also has activity against chondroitin sulfate [17].

Most, if not all, of these Gram-positive genera capable of elaborating hyaluronidase are able to cause infections initiated at a mucosal or skin surface of either humans or animals. The exceptions to this may be the *Streptomyces*; however, some *Streptomyces* species are capable of causing infections, often by gaining access through skin abrasions.

Bacteriophages from two species of streptococci, *Streptococcus pyogenes* [18] and *Streptococcus equi* [19], encode hyaluronidase. A hyaluronic acid capsule can surround the host cells of both of these phages; hence the bacteriophage may require hyaluronidase for penetration of this capsular material. No hyaluronidase activity was detected in the extracellular milieu of either *S. pyogenes* or *S. equi* infected with the temperate bacteriophages [18,19], suggesting the enzymes may be part of the bacteriophage particle.

3. Sequence information

Eight complete bacterial hyaluronidase genes, and two

bacteriophage genes, have had their nucleotide sequence determined. The bacterial hyaluronidases, along with their accession number, are from *S. aureus* (U21221) [20], *Streptococcus agalactiae* (Y15903) [21], *S. pneumoniae* (L20670) [7], *S. griseus* (AB028210), *S. coelicolor* (AL031124), *P. acnes* (U15927) [22], *C. perfringens* (P26831) [12], *Proteus vulgaris* (1095454) [23], along with a partial sequence of the lyase from *Bacteroides thetaiotaomicron* (L42367) [24]. Two sequenced bacteriophage hyaluronidases are both derived from temperate phages that infect group A streptococci (M19348 and U28144) [18].

Sequence analysis of the hyaluronidase genes provides important information on the hyaluronidases. As the number of enzymes sequenced increases, more information will become available that will be valuable in determining similarities and differences between the hyaluronidases. Among those hyaluronidases of which the deduced amino acid sequence is known, there is a wide variation in the molecular masses. The streptococcal bacteriophage enzymes are the smallest, ranging between 36 and 40 kDa, depending on the presence or absence of the collagenous motif [18]. The deduced proteins of the other hyaluronidases are much larger: *S. agalactiae* 121 kDa, *S. pneumoniae* 107 kDa, *C. perfringens* 114 kDa, *S. aureus* 92 kDa, *P. acnes* 82 kDa, and the two *Streptomyces* sp. 77 and 84 kDa. Molecular masses of other (non-sequenced) hyaluronidases also vary considerably, ranging from 50 to 160 kDa [2].

Similarity alignments were conducted on these hyaluronidases as the sequence information became available. Some enzymes show significant similarity, while little or no homology is suggested among other hyaluronidases. This raises the question, how closely related or how similar are these enzymes? Fig. 2 shows the results of a phylogenetic analysis (AlignX, Informax, North Bethesda, MD, USA) using the deduced amino acid sequences of the bacterial hyaluronidases. Proteins from the Gram-positive organisms, with the exception of the clostridial hyaluronidase, appear to be related. Two subdivisions are seen within the main group of Gram-positive hyaluronidases. The *S. agalactiae* and *S. pneumoniae* enzymes appear closer to the *S. aureus* hyaluronidase than to the *Streptomyces* enzymes. Similarly, the two *Streptomyces* hyaluronidases appear related, with the proteins appearing more similar to the hyaluronidase from *P. acnes* than to *S. aureus*. The Gram-negative hyaluronidases, the two bacteriophage proteins, and the clostridial protein appear more distantly related to the main group of Gram-positive hyaluronidases. Not surprisingly, the two Gram-negative proteins group together, as do the two phage hyaluronidases.

The bacteriophage hyaluronidase genes show a high degree of similarity; one major region of difference is the deletion (or addition) of a collagen-like Gly-X-Y region [18,25]. Sequence homology alignments indicate that these hyaluronidases show little homology to other hyaluronidase genes in the databases, a point evident from the phylogenetic tree (Fig. 2). Marciel et al. [25] indicated substantial levels of allelic polymorphism among the streptococcal bacteriophage hyaluronidases and suggested that molecular variation was the result of recombination processes. Such an analysis has not been carried out yet with other hyaluronidases.

Among the remaining Gram-positive organisms, the hyaluronidases from *S. aureus*, *S. agalactiae* and *S. pneumoniae* show high degrees of similarity, with global similarities above 65% and local similarities around 80%. In terms of identical amino acids, the *S. agalactiae* and *S. pneumoniae* appear more closely related than either is to the *S. aureus* protein. The complete sequence of the *S. agalactiae* hyaluronidase contains a promoter region, ribosome binding site and signal peptide [21]. The *S. aureus* gene, although smaller than the *S. agalactiae* and *S. pneumoniae* genes, may be complete as putative promoter, ribosome binding site, and signal peptide regions are present in the sequence [20]. Such is not the case with the *S. pneumoniae* gene, which lacks a signal peptide [7]. Gase et al. [21] indicated that the initially reported sequence of the hyaluronidase gene from *S. agalactiae* was not complete and suggested that the same is true for the *S. pneumoniae* gene.

Like the *S. aureus* hyaluronidase, the *P. acnes* hyal-

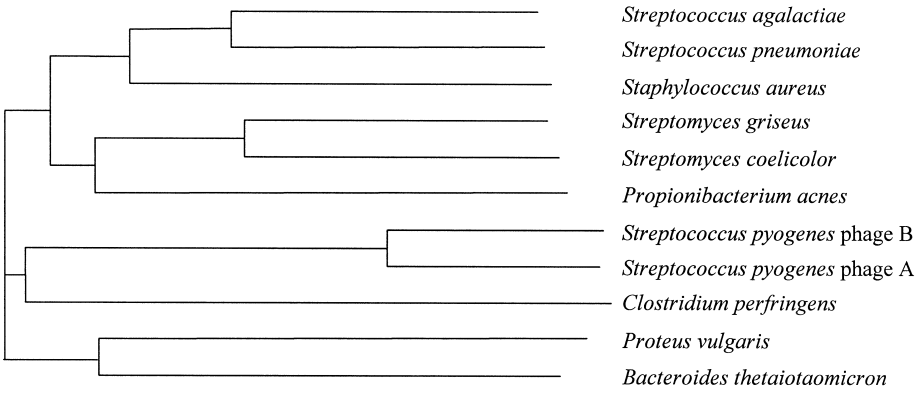

Fig. 2. Phylogenetic tree based on the deduced amino acid sequences, showing the relatedness of the bacterial and phage hyaluronidases.

uronidase gene sequence encodes a signal peptide [22], suggesting a complete gene sequence. Analysis using the sequence of a proposed hyaluronan binding site in *P. acnes* (RKVASSSTK) [22] revealed similar sequences in all the hyaluronidases from the Gram-positive bacteria. Although the location of the conserved region within the protein varies, all are in the more conserved C portion of the protein. The enzymes from the bacteriophages do not appear to have a sequence similar to that of the proposed hyaluronate binding site of *Propionibacterium*, perhaps suggesting a different mechanism of action or at least a different site of substrate binding.

4. Role in disease

Typically, during an infection of any type, the ground substance secreted by connective tissues provides a mechanism of defense against infectious agents. The viscous consistency of ground substance usually provides resistance to penetration of infectious agents and their extracellular products; however, some bacteria have adapted ways to weaken the restraints of connective tissues. Many pathogenic bacteria able to establish infections at the mucosal or skin surface produce the enzyme hyaluronidase. Since hyaluronate is a major constituent of the ground substance of most connective tissues, particularly the skin, hyaluronidase may be an essential component in enabling the spread of the pathogens from an initial site of infection.

The ultimate products of hyaluronidase degradation of hyaluronate are disaccharides (Fig. 1). These disaccharides can be transported and metabolized intracellularly to supply needed nutrients for a pathogen as it replicates and spreads. The role of providing nutrients for the cell may be the main function of hyaluronidases in Gram-negative organisms [24]. In *C. difficile*, Seddon et al. [26] suggest that hyaluronidase may play a role in releasing nutrients thereby promoting establishment of the organism in the gut. A strong association between the production of purulent abscesses and hyaluronidase produced during infections with organisms of the '*Streptococcus milleri* group' including *S. intermedius* has been observed [27]. Isolates from internal abscesses and purulent lesions frequently produce hyaluronidase, and the deeper the abscess the higher the frequency of hyaluronidase production. The authors suggest that products of hyaluronidase degradation might be utilizable as a nutrient source for the organism. In *S. intermedius* [28] and *B. thetaiotaomicron* [24], the hyaluronidase, or proteins associated with the enzyme, are induced in the presence of substrate. Addition of hyaluronate to growth media also results in increased levels of hyaluronidase in group A streptococci [29]. Production of the enzyme in response to the presence of substrate may provide a way of regulating enzyme production to environments (in vivo?) which enable the organism to proliferate and use the substrate.

Perhaps the more significant result of the enzymatic depolymerization of hyaluronate is a decrease in the viscosity of the ground substance. Decreased viscosity results in increased permeability of the connective tissues, and potentially increased spread of microorganisms and toxins through the connective tissues.

C. perfringens, the causative agent of gas gangrene, produces many extracellular proteins during an infection that are potential virulence factors. One of these, the Mu toxin, is a hyaluronidase that by itself is a non-lethal toxin of *C. perfringens*. However, the enzyme facilitates the spread of the major tissue-damaging α-toxin, thereby potentiating its cytolytic activity [12]. The decreased viscosity may also increase the spread of the pathogen, although it is currently unknown how much of an effect hyaluronidase is likely to have on such proliferation. Alternatively, the clostridial hyaluronidase may degrade hyaluronate cell surface coatings, thereby allowing direct contact between the bacterium and specific receptors on the cell surface.

Hyaluronidase production by oral pathogens belonging to *Peptostreptococcus* sp. may play a role in the pathogenesis of periodontal disease [17]. The ability to hydrolyze the ground substance of gingival tissue may lead to periodontal destruction by enabling the bacteria to spread to the roots of the residing teeth. Increased permeability of the gingiva connective tissue may increase spread of bacterial toxins causing even more damage. Damage from the host immune system due to exposure of antigenic determinants, normally hidden, following partial breakdown of the ground substance may cause additional damage.

Two important veterinary pathogens, *S. uberis* and *S. dysgalactiae*, capable of causing mastitis have been shown to produce hyaluronidase [9,10,30]. *S. dysgalactiae* hyaluronidase, along with fibrinolysin, may have an important role in promoting dissemination of the producing organism into host tissue [9]. The enzyme from *S. uberis* has been suggested to play a role in the spread of infection, although no evidence is available [31]. One interesting observation indicated that *S. uberis* hyaluronidase is able to inhibit proliferation of a mammary epithelial cell line [30], suggesting that proliferation could be inhibited by bacterial virulence factors in vivo during differentiation and growth of mammary tissue.

S. pneumoniae causes a number of different infections, including meningitis and pneumonia. During infections, the hyaluronidase may function as a spreading factor, while the pneumolysin acts as a toxin. The sequence of the gene suggests that pneumococcal hyaluronidase is cytoplasmic, released only when the pneumolysin causes lysis of the pneumococci [7]. It is possible that release of hyaluronidase following cell lysis aids in the spread of the toxin from the infection site. Studies on strains of *S. pneumoniae* isolated from patients show that all strains isolated

from meningitis patients were hyaluronidase-positive, while only 15% of strains isolated from healthy patients produced the enzyme [32]. These authors also found that strains lacking or producing low levels of hyaluronidase produced brain infections only when inoculated with exogenous hyaluronidase. Such studies suggest a significant role for the enzyme in bacterial growth or spread. Recently, using signature-tagged mutagenesis, the in vivo role of hyaluronidase in *S. pneumoniae* infections of mice was investigated [33]. These studies provide evidence that the hyaluronidase has a role in pneumonia, but little effect in septicemia. Such results indicate that the enzyme plays a role in adhesion or colonization rather than survival in the bloodstream; not surprising considering the location of hyaluronate in body tissues.

Group A streptococci appear to have a 'conflict of interest' with regard to their production of hyaluronidase. These streptococci produce an anti-phagocytic capsule of which the sole component is hyaluronate. It therefore seems reasonable to hypothesize that the hyaluronidase produced is going to deplete this protective capsule leaving the organisms potentially susceptible to phagocytosis, as the capsule is susceptible to degradation by the hyaluronidase. However, continued production of hyaluronidase establishes a means for the organism to degrade host connective tissues allowing for bacterial spread. Perhaps other anti-phagocytic factors such as M-protein provide sufficient protection for *S. pyogenes* during hyaluronidase production. A similar argument may be made for the role of hyaluronidase production in *S. uberis*, which also produces a hyaluronate containing anti-phagocytic capsule [31].

Some strains of *S. pyogenes* show an acapsular phenotype, while encapsulated strains often lose their capsule when entering or during the stationary phase of growth. One possible explanation for this would be breakdown of the hyaluronate due to hyaluronidase activity. However, by looking at the transcript of the *has* operon, it has been suggested that the unencapsulated phenotype was not due to production of hyaluronidase, but due to loss of mRNA transcript [34]. This conclusion was in part based on the inability to detect hyaluronidase activity in these strains in vitro. Although hyaluronidase is often reported as a spreading factor in group A streptococci, less than 25% of strains tested actually produce hyaluronidase in vitro [18]. While few strains produce demonstrable hyaluronidase in vitro, anti-hyaluronidase antibodies are often present following streptococcal infections [35]. An important aspect that needs examination is whether strains that do not produce hyaluronidase in vitro may produce it in vivo. Because of this, the regulatory relationship between hyaluronidase and the hyaluronic acid capsule still remains to be determined. It seems possible that in vitro loss of capsule due to transcriptional regulation [34] may only be part of the story. What occurs in those strains in which the capsule and hyaluronidase are produced in vivo? This question still needs to be addressed. The destruction of hyaluronate in connective tissues could potentially result in the tissue damage seen in some streptococcal infections, as well as allowing for dissemination of toxins.

Some *S. pyogenes* exhibit two distinct hyaluronidases, an extracellular hyaluronidase and a bacteriophage hyaluronidase [18]. Hyaluronidase associated with streptococcal bacteriophages may allow phages possessing such enzymes to gain access to appropriate bacterial cell receptors. Halperin et al. [36] report finding antibodies to streptococcal bacteriophage hyaluronidase following streptococcal infections, indicating that the host was exposed to the bacteriophage enzyme. This most likely occurs following cell lysis and release of the phage particles. It appears unlikely that the hyaluronidase plays a significant role in streptococcal disease, since the bacteriophage-encoded enzyme is not secreted from the cell [18], although an indirect role may be envisioned. The enzyme, some of which is not associated with phage particles, may degrade the hyaluronate, allowing for dissemination of the phage-encoded erythrogenic toxin which is responsible, at least in part, for the rash of scarlet fever. The hyaluronidase important in toxin spread, phage or bacterial, remains undetermined.

Hyaluronate plays important roles in immune system functions [3]. One possible role for the hyaluronidases of pathogenic organisms may be the breakdown of hyaluronate leading to modulation of the immune system, potentially making a more 'bacteria-friendly environment'. Such a role has been postulated for the group B streptococcal hyaluronidase, although no evidence is yet available [37]. Group B streptococcal infections may be enhanced by the ability of hyaluronidase to degrade hyaluronate on cell surfaces and in tissues. A role for the hyaluronidase in pneumonia associated with some group B streptococcal infections may be the result of increased tissue permeability following exposure to hyaluronidase [37].

5. Outlook

Kreil [1] described hyaluronidases as a group of neglected enzymes. Since this description, much has been accomplished in the study of bacterial hyaluronidases, but more information is necessary to answer important questions regarding the role of these enzymes in bacterial pathogenesis. Such questions apply equally to both human pathogens (e.g. *S. pneumoniae* and *S. aureus*) and animal pathogens (e.g. *S. uberis* and *S. dysgalactiae*). Sequence information on the hyaluronidase genes from many important pathogens still must be elucidated. With the increasing number of pathogens for which chromosomal sequencing is currently underway, one aspect to be explored is whether some of these pathogens produce, or have the ability to produce, hyaluronidase. Sequence information may be used as a guide to look for these enzymes in

organisms for which such activity has not previously been associated. With this information in hand, it should be possible to design experiments to determine if the enzyme is only produced under certain in vivo conditions. This knowledge may be particularly beneficial in understanding disease pathogenesis.

While sequence information provides important information, in vitro studies need to be paired with in vivo analysis. Just what role does the hyaluronidase have for an organism? Is hyaluronidase involved in spread, or is it a means of obtaining nutrients? Perhaps these two roles are not mutually exclusive; organisms may use the enzyme to spread, and in so doing release nutrients useful for bacterial proliferation. How does the organism determine when to produce hyaluronidase, or is it a constitutively expressed enzyme? Some of the enzymes are upregulated in the presence of the substrate, suggestive of a mechanism whereby the cell displays some environmental recognition. Many virulence factors are regulated in such a manner, but the factor(s) responsible for turning genes on or off are often unknown.

Another important issue to be addressed is the ability to modulate the immune system. For pathogenic microorganisms, the ability to avoid being killed by the host defenses is obvious, but how the organism achieves this is often poorly understood. Do bacterial hyaluronidases enhance or inhibit the host immune response to a bacterial infection?

With the current tools available in molecular biology, many of these questions can begin to be approached, but they cannot be fully answered without in vivo models. In vivo, we must look at different aspects, not limiting ourselves to just one component of the response. Survival and growth of the organism, dissemination both of organisms and toxins, tissue destruction and immune modulation may all play a role in pathogenesis of hyaluronidase producing organisms. If we examine only one point, we may miss the overall picture; only by examining all the different aspects will a full understanding for the role of this enzyme be forthcoming. Beyond the need to answer such basic questions, a potential benefit also exists; understanding the regulation and role in disease may lead to drugs effective in preventing or decreasing spread of infections or toxins. Let us not neglect this important group of enzymes any longer.

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