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VIRULENCE FACTORS OF THE GROUP A STREPTOCOCCI AND GENES THAT REGULATE THEIR EXPRESSION

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1. ABSTRACT

Group A streptococci produce a wide variety of extracellular (cell-associated and released) virulence factors. The function of these factors varies and includes roles in adhesion, spreading, tissue destruction, immune system evasion, and cell toxicity. How these factors are regulated with regard to one another is important for this organism's ability to bring about the variety of diseases this microbe is capable of causing. Therefore, along with the multitude of virulence factors, there are a number of regulatory systems that regulate, either directly or indirectly, the production of these factors and therefore influence the pathogenesis of group A streptococcal infections.

2. INTRODUCTION

Group A streptococci (*Streptococcus pyogenes*) have an extensive arsenal of virulence factors, comparable to or exceeding that of many other pathogenic microorganisms. The virulence factors include those that are extracellular (e.g. protease, streptokinase, etc.), in that they are released from the cell, as well as cell associated factors (e.g. M-protein, hyaluronic acid capsule, etc.). How these proteins are secreted is still unknown in many instances, although the streptococci have a variety of secretion mechanisms. Some of the factors involved in secretion and maturation of proteins, in particular SpeB, are beginning to be examined. Factors found to be involved include trigger factor (1) and HtrA, a DegP ortholog (2). Recently, it has been shown that secretion by the general (Sec) secretory pathway occurs through a single microdomain, known as the ExPortal, in the cell membrane dedicated to protein export (3). Another mechanism involves the direct transfer of material from the bacterial cell to the cytoplasm of a host cell using cytolysin-mediated translocation (4).

Discussion of many of the virulence factors here is limited to information gained over the past few years to avoid repeating information provided in other reviews (5-7). Also discussed are proteins that regulate the virulence factors of this organism. Many unanswered questions remain regarding the virulence and pathogenesis of group A streptococci and the various diseases this organism causes. To cause disease the organism has to be able to become established in a host, avoid destruction by the host immune response, and then either by design or 'accident' have a particular adverse effect on the host ranging from inflammation ('strep' throat) to tissue destruction (necrotizing fasciitis) and various stages in between.

2.1. Group A streptococci

Group A streptococci are beta-hemolytic, gram positive, catalase negative, chain-forming, extracellular cocci that colonize the throat and skin of their host, and are often considered the most pathogenic member of the genus *Streptococcus*. Streptococci were first differentiated on the basis of hemolytic activity (8). Subsequently the presence of a specific carbohydrate in the cell wall allowed for further division into groups (9). Sherman divided the streptococci into 4 divisions, one of which was the pyogenic division that includes those with the group A antigen (10). Additional differentiation of group A streptococcal strains was accomplished by serological typing using the M-protein (11). Over the years since the development of the M-typing scheme the number of types has risen to more than 80 with the inclusion of new and provisional M-types (12). Recently, molecular typing methods have been developed based on the sequence of the *emm* allele (12-14), a method that closely parallels the serotyping scheme. Such genetic typing has added additional types, such that there are now 124 different *emm* types recognized (12). Because of the chance of variation between the different schemes, the terms "M-types" and "*emm* types" are used to signify the serotype and *emm* sequence typing respectively (12).

With the large number of types (serological or *emm*) of organisms, investigators have, over the years, looked for different ways to classify these important organisms; but so far no method has been totally satisfactory. Other schemes for typing have included T-typing (15, 16), disease association (rheumatogenic vs. nephritic) (17, 18), location (skin vs throat) (19, 20), and opacity factor (presence and type) (21, 22), as well as a number of molecular techniques (23-27).

2.2 Streptococcal diseases

S. pyogenes causes a wide variety of diseases, the pathogenesis of which have been reviewed by Cunningham (5), and will not be addressed here. The severity of disease ranges from mild to severe and include "strep throat", impetigo/pyoderma, scarlet fever, streptococcal toxic shock, septicemia, necrotizing fasciitis, the delayed sequelae of rheumatic fever and glomerulonephritis, and streptococcal reactive arthritis (5). In addition, streptococcal infections have been linked to obsessive-compulsive disorder and Tourette's syndrome (28-30). Other diseases that have also been linked to group A streptococcal infections include pneumonia (31) and endophthalmitis (32). Although most streptococcal infections are the result of inhalation or direct contact, occasionally this organism has caused outbreaks of pharyngitis following ingestion of contaminated foods (33, 34). This wide array of diseases and multiple mechanisms of spread indicate a very complex organism that has the ability to survive and become established in various host sites. It also suggests the need for not only the coordinated expression of the multiple virulence factors but also the ability to switch phenotypes as the organism responds to different environmental cues.

2.3. Genomic sequencing

The complete genomic sequence of a *S. pyogenes* strain was deciphered in 2001 (35). The strain sequenced was a M-type 1 strain; since then complete genome sequences have been determined for one M-type 18 strain and two M-type 3 strains (36-38). The sequence of a M-type 5 strain is nearing completion (http://www.sanger.ac.uk/Projects/S_pyogenes). The availability of such sequence information has changed the approach to the study of pathogenic mechanisms and

virulence factors. We now recognize potential virulence factors, or at least genes that encode factors that could aid in the establishment or maintenance of a disease state. The genomic approach has indicated many proteins thought to be virulence factors based on their similarity to proteins produced by other organisms. In many instances it is not known if the streptococci produce these proteins or if they have the predicted activity. One of the ways in which the sequence availability has affected pathogenesis studies is by the simultaneous examination of multiple genes, using techniques such as microarrays, rather than by studying individual genes or gene products. This approach allows an examination of the coordinate expression of multiple genes, and therefore virulence factors, as well as a comparison of gene expression in different strains (39-42). By examining multiple genes on a genome-wide scale a more complete picture of gene expression, and therefore pathogenesis, will become apparent. The various genomic-scale approaches have shown that there are still many unanswered questions, both at a general as well as at a specific factor level (41). Many studies on bacterial factors have been conducted independently of the host and these new methods provide tools that allow the direct study of interactions between the bacterium and the host. New methods will also allow for both qualitative and quantitative analysis of *in vivo* gene transcription (43), and give us advantages in understanding microbe-host relationships.

Within the published genomes, variation in gene content and allelic diversity is observed; common coding regions between the different strains range from 88 to 93.2% (35-38). The genomes of the strains from the different serotypes vary in size (1,852,442 to 1,900,521 bp) and at least part of this difference is due to the presence or absence of bacteriophages. These phages or phage-like elements may actually account for much of the variation seen in the gene content of the different strains. The presence of different bacteriophages may also affect virulence of the organism, since phages are known to encode different virulence factors (36-38). Chimeric phages in *S. pyogenes* may result in different mixtures of toxins, or other genes, being introduced into different strains. Such phages could change disease frequency or severity, or even generate new M-protein serotypes (38). A number of different potential virulence factors are encoded for by the bacteriophages, particularly the superantigen or superantigen-like proteins (36, 38). Other potential virulence factors encoded for by bacteriophages are hyaluronidases (35) and a phospholipase A_2 (38). The role of these bacteriophage genes and their products is unknown. However, it should be noted that co-culture of streptococcal cells with pharyngeal cells resulted in induction of lysogenic bacteriophages and expression of various phage proteins (44, 45). Such induction would alter the potential virulence factors produced by the cell, suggesting a role for phage proteins in bacterial pathogenesis.

At the regulatory level, genomic analysis has indicated that group A streptococci have at least 13 two component regulatory systems and more than 36 transcriptional factors, with most being common to the different strains (36). Some of these regulators have been assigned functions, while for others the function remains to be determined. The location and sequence of the genes encoding these regulators varies within the chromosome (36), indicating plasticity of the genome that may be responsible for at least some of the variation in virulence seen between strains of *S. pyogenes*.

Although considerable progress has been made in determining which factors affect virulence, and how these factors are regulated, at both the global and specific level, much still remains to be determined. This review will summarize information on the various virulence factors and then discuss the various regulators that have been shown to influence production or expression of these factors.

3. VIRULENCE FACTORS

For the purpose of this review, virulence factors are defined as those factors or agents that allow an organism to become established in a host or to maintain the disease state once an infection has been established. This definition therefore includes those factors responsible for activities ranging from adherence of the organism to appropriate tissue, to those that cause tissue damage or destruction. Many of these factors have only been studied *in vitro*, independent of the host; therefore some of this information may change as the role of the host in disease pathogenesis is fully examined. The virulence factors have been grouped into two broad categories; those that remain predominantly cell associated and those that are secreted and released from the cell. Rather than discuss all the literature pertaining to these agents, this review will concentrate on recent information. For background and more in-depth analysis of the numerous streptococcal virulence factors the reader is directed to the several reviews and publications that cover these topics (5-7, 46-49)

3.1. Cell-associated virulence factors

Many of the virulence factors expressed by Group A streptococci are cell-associated, being either loosely or firmly attached to the cell wall of the bacterium. These cell associated factors play a role in adhesion (e.g. collagen binding proteins, fibronectin binding proteins, lipoproteins, plasminogen binding proteins), a critical step in the establishment of a streptococcal infection or following establishment of the organism, a role in evasion of the host immune response (e.g. M-protein, Emm-like proteins, C5a peptidase, hyaluronic acid capsule, CD15s-related antigen). Whether these factors only play a role in bacterial virulence, or perhaps have some other role for the cell is still to be determined.

3.1.1. M-protein superfamily

Perhaps the best known of the group A streptococcal virulence factors is the M-protein. This protein is one of a group of proteins that make up the M-protein superfamily; all have a similar structure and bind a wide range of host proteins, but differ in a number of important properties. The genes for the M-protein family are flanked by the regulator gene *mga* at the 5' end, and the

C5a peptidase gene (*scp*A) at the 3'end (50). The *scp*A gene (see below) is not a member of the M-protein superfamily, although it is regulated by *mga*. Members of this family, of which there are more than 20 genes, have a conserved cell-associated portion of the molecule. This region or domain consists of domain H that may anchor the protein to the membrane, the peptidoglycan domain, and the cell wall-associated domain (50, 51).

3.1.1.1. M-protein

The gene, protein structure, and antiphagocytic properties of M-protein have been known for some time and have been extensively reviewed elsewhere (5, 6, 52, 53). The M-protein consists of a dimer of two polypeptides arranged in a coiled-coil structure that is held in the cell membrane by the LPSTGE anchor motif (5, 6, 52, 53). The molecule consists of 4 repeat regions (A, B, C and D), and a proline/glycine rich region most likely located in the peptidoglycan layer of the cell. The N-terminal portion of the molecule (A repeats) is hypervariable and confers serotype specificity. To date, 124 M-genotypes have been recognized (12). Antibodies to the variable region confer type-specific immunity following an infection. However, within certain M-types protective immunity is strain-specific rather than serotype-specific (54). This may be due to distinct subpopulations of bacteria within a serotype (55, 56), perhaps giving the organisms a survival advantage; when the immune response recognizes the parent strain, these variants are able to avoid phagocytosis. Another observed variation within serotypes is the size of the M-protein. The variation is due to changes in the number of repeat units in the A and B portions of the M-protein molecule (53, 57), or other types of mutational effects (58, 59).

Since anti-M-protein antibodies are protective, the protein has been considered a likely candidate as a vaccine (60, 61), although development of such a vaccine comes with risk. Antibodies against M-protein, while being protective, are also able to react with host tissue (5, 62-65) and probably play a role in the development of rheumatic fever. Until these cross-reactivity problems can be addressed, the use of M-protein as a streptococcal vaccine will remain questionable. Alternative strategies for vaccine development would be to use a vaccine against the variable region of multiple serotypes, rather than a conserved region of all serotypes. Such a strategy may be just as difficult as trying to avoid the host cross-reactivity, because of the variability seen among M-proteins.

The important function of M-protein, for group A streptococci, is its antiphagocytic ability, resulting from interference with opsonization (6). Various mechanisms for the ability to inhibit phagocytosis have been reported, but all are associated with the ability of the protein to bind host proteins (5, 6), although not all M-proteins appear to do so equally (66). Binding of the complement regulatory protein H and binding of fibrinogen to M-protein inhibits opsonic function (67). Fibrinogen binding also leads to plasminogen binding, which is in turn activated by streptokinase to active plasmin (68). A set of skin strains was shown to express M-proteins that have a high affinity for binding plasminogen (69). The plasminogen could form a cell-bound complex with streptokinase that would act as a plasminogen activator; the plasmin would provide a surface associated protease that could degrade barriers designed to limit bacterial spread and in doing so this could facilitate bacterial spread (69). A factor H-like protein (FHL-1) and human C4b-binding protein also binds to M-protein; the effect of binding of these proteins would be to impede phagocytosis (70, 71). Binding of IgA and C4b-binding protein by the M22 protein conferred resistance to phagocytosis (72). M-protein also binds kininogen, and releases the vasoactive peptide bradykinin (73, 74). The streptococcal cysteine proteinase has also been shown to release active kinins from kininogen (75); perhaps the binding by M-protein allows for the cleavage of the precursor by the protease with release of the active component. The exact mechanism(s) by which all of these proteins bind and interfere with the host response to the pathogen is still unclear. The local release of bradykinin in potentially large amounts could lead to increased vascular permeability with the leakage of plasma. This effect may aid in the spread of the organism. In addition it could explain some of the symptoms associated with streptococcal infections such as fever, pain, edema, and in severe cases hypovolaemic shock (74). The binding, activation, spreading, and antiphagocytic effects of these proteins probably occur at different times or in different tissues during an infection, again adding to the diverse nature of streptococcal virulence factors. M-protein is also involved in the binding of glycosaminoglycans such as dermatan sulfate and heparan sulfate. Full binding of the glycosaminoglycans requires both the amino terminal portion and C-repeat region of the M-protein (76). The effect of binding these sugar derivatives would be to aid in bacterial adherence to human cells, in particular epithelial cells and skin fibroblasts. Recently, M3 protein has been shown to be involved in both adherence and internalization (77). Inactivation of the *emm*3 gene resulted in decreased adherence and internalization, an effect that could be reversed by augmentation with fibronectin (77), indicating the effect of fibronectin is not related to intact M3 protein.

Recently it has been found that inactivation of the *sag*A gene (required for streptolysin S production, see below) results in a reduction in the amount of cell associated M-protein (78). The effect of the *sag* locus was not at the level of transcription, but on localization of the protein to the bacterial cell surface. This was most likely by loss of the anchoring domain or perhaps by cleavage of the M-protein by a protease that is normally repressed by *sagA* (78).

3.1.1.2. Emm-like proteins

Members of the M-protein gene superfamily include the M-proteins, M-related proteins and the immunoglobulin binding proteins that possess antiphagocytic or immunoglobulin binding properties. Members of this family of proteins are structurally similar, particularly in the domains associated with the cell wall-associated region. A more in depth discussion of the overall structural aspects of these proteins is provided in previous reviews (5, 6, 50).

3.1.1.2.1. Immunoglobulin binding proteins

Cunningham described the immunoglobulin binding proteins of group A streptococci as being encoded for by M-protein or M-like protein genes which are controlled by Mga (5). It has been proposed that the heterogeneous family of immunoglobulin binding proteins and M-proteins are variants of a common molecule that has been duplicated from an ancestral gene (5, 50). The immunoglobulin binding proteins are classified based on the type of immunoglobulin (IgG or IgA) they bind, as well as the subclass of the immunoglobulin (5, 50). A number of proteins have been identified that bind IgG and belong to the M-protein superfamily, although there is doubt over the classification and function of the immunoglobulin binding proteins. A more in depth analysis of the M-protein like immunoglobulin binding proteins is found in earlier reviews (5, 50).

The function of the immunoglobulin binding proteins in bacterial virulence remains unclear. Inactivation of M-related genes, in particular those associated with IgG binding, resulted in loss of virulence in skin infections, but not following intraperitoneal injection (79). Recently, a model describing the role of IgA binding in virulence was proposed. In this model, binding of the Fc portion of IgA to the binding protein interferes with IgA effector functions, allowing the bacterium to evade elimination by processes that would normally occur when IgA binds to Fc-alpha receptor CD89 on phagocytic cells (80).

3.1.1.2.2. Non M-related immunoglobulin binding proteins

Two non M-related immunoglobulinbinding proteins produced by group A streptococci have been described. However, the role, if any, in pathogenesis or virulence remains to be determined. The first, SbfI, is an IgG binding protein, which is also a fibrinogen binding protein (81). SbfI bound IgG isotypes 1- 4 with the binding being mediated through the Fc portion of the molecule.

The second non M-protein-like immunoglobulin binding protein is a secreted immunoglobulin binding protein that binds IgG, IgM and IgA (82). Binding is through either the Fc or Fab fragments, with a preference for the Fc fragment. The gene (*sib*A) is found in most strains of *S. pyogenes* from a variety of types. The deduced protein has a signal peptide but no membrane-anchoring region, confirming its secreted status (82). Of interest was the observation that the protein is analogous to a protein involved in cell wall separation in group B streptococci, perhaps indicating SibA is a multifunctional protein (82). The role in pathogenesis is unknown, but its widespread occurrence in various types suggests it has some important or essential function.

3.1.1.2.3. Collagen binding protein

A collagen binding protein has been identified as a possible adhesin for group A streptococci, using collagen on the host cell as the receptor molecule (83). The authors described a 57 kDa soluble form of a collagen receptor, which specifically bound the four types of collagen tested (83). However, no evidence for the role of this protein in virulence has been forthcoming. The sequence of the collagen binding protein reported by Visai *et al* (83) is almost identical to an IgG binding protein reported by Boyle *et al* (84). That protein size was 59.7 kDa, close to the size reported for the collagen binding protein. Searching available genomic databases for the sequence of the IgG binding proteins with similarity to the collagen binding protein indicated similarity with the M-protein gene. These results suggest that the collagen binding protein may have, at one time, been part of the streptococcal M-protein family. Recently, it was shown that M-type 3 and M-type 18 *S. pyogenes* strains isolated during a rheumatic fever outbreak were able to bind and aggregate collagen (85). Binding of collagen to the M-type 3 strains was found to be via the M-protein, while for the M-type 18 it was via binding to the capsule. These results show yet another function of the M-protein and capsule of these strains, and suggest an important role for collagen binding in streptococcal infections.

3.1.2. C5a peptidase

C5a peptidase (*scp*A) is a surface-associated endopeptidase that is specific for the complement component C5a (86). Examination of the 3' terminus of *scp*A genes from different serotypes of group A streptococci revealed differences in the size of the gene. This variation results from changes in the number of repeat units found in the gene (87), ranging from three to six. The function of ScpA in virulence may be to act on and destroy C5a, thereby eliminating the chemotactic signal from the site of infection (49, 86, 88) and protecting the organism from immune detection. However, the exact role and importance in virulence is difficult to ascertain. Husmann *et al* suggested that the contribution of C5a peptidase might be strain dependent as they were unable to find a difference between mutant and wild type strains in their ability to colonize the throat or to cause pneumonia in mice (89). Evidence that C5a peptidase plays a role in virulence includes the increased clearance from subdermal infection sites and nasopharyngeal mucosa in *scp*A mutants and a strong antibody response to the protein in adults but not in uninfected children (88, 90, 91). This later response may, in part, explain why children are more susceptible to streptococcal infections than adults.

3.1.3. Hyaluronic acid capsule

Group A streptococci produce a capsule, the sole component of which is hyaluronic acid. A number of functions for the capsule have been proposed. One of the early proposed roles was to protect the organism from toxic oxygen metabolites by shielding the organism from oxygen by creating a barrier around the cell (92). However, more important functions of the capsule are its role in colonization, phagocytic killing, and infection (89, 93-96). The capsule is involved in attachment to receptors on epithelial cells by binding to CD44, a hyaluronate-binding protein (97); such binding can induce cytoskeleton changes which can lead to cellular invasion (98). Strains lacking a capsule, or having some deficiency in production are not as pathogenic as encapsulated strains; however, this can be overcome by streptolysin S activity in a manner that does

not appear to involve resistance to phagocytosis (99). Epidemiological studies suggest encapsulated strains are more associated with severe invasive infections and the delayed sequelae of rheumatic fever, while strains associated with uncomplicated streptococcal infections tend not to be encapsulated (6, 100).

Synthesis of the hyaluronic acid capsule is encoded by a three gene operon consisting of *has*A, *has*B, and *has*C (101) which is negatively regulated by the CovR/CovS two component regulatory system (102-104). Of the three genes in the operon, *has*A (hyaluronan synthase) and *has*B (UDP-glucose dehydrogenase) are sufficient for capsule expression. Inactivation of *has*C (UDP-glucose pyrophosphorylase) indicated it is not required for capsule expression (105).

Capsule synthesis is regulated, at least in part by CovR, and therefore is influenced by Mg levels (see regulation section). Increased capsule production is observed at $MgCl_2$ levels found in human extracellular fluids (106). The same studies showed that at levels of Ca^{2+} found in human extracellular fluids there was an increase in capsule gene expression. These results suggest that capsule expression is enhanced under conditions associated with an infection.

3.1.4. Fibronectin binding proteins

Adherence to host tissue is the first step in establishing an infection. To accomplish this, *S. pyogenes* has a number of genes that encode proteins that could be classified as adhesin molecules. Among these are the surface-associated fibronectin binding proteins, including SfbI or PrtF1, SfbII, PrtF2, Fbp54, and Pfbp (6). Other proteins, such as M-protein and serum opacity factor are also capable of binding fibronectin. On the other hand SpeB, the cysteine proteinase, alters proteins on the streptococcal surface in such a manner that abrogates fibronectin binding (107).

3.1.4.1. Protein F

Protein F (*prt*F, *sfb*) has been shown to not only bind fibronectin, but also fibrinogen (108). Binding of these two molecules occurs at different locations on the protein; fibrinogen bound to the N-terminal portion of the molecule, fibronectin to the C-terminal portion. In addition, the protein also binds the Fc segment of IgG using the fibronectin binding domains (81). This binding potentially inhibits antibody-mediated events such as phagocytosis and antibody-dependent cell cytotoxicity (81). Additional evidence for the importance of this gene in virulence comes from the high incidence of occurrence of the *prt*F gene in clinical isolates. In a study of clinical isolates from Japanese children the gene was present in 77.3% of isolates (109). Sequence variation is seen among the different *prt*F genes, with most of the variation occurring in the amino terminal domain of the protein. The 3' end of the gene was more conserved, varying in the numbers of proline-rich and fibronectin-binding repeats (110). Despite the variation, the overall structural properties of the protein were conserved. However, the variability may suggest a selective pressure *in vivo* with implications for streptococcal pathogenesis (110).

Immunization (intranasal) studies in mice show that a response to PrtF provides protection from intranasal challenge with heterologous strains of *S. pyogenes* (111). Both the humoral and cellular immune responses were stimulated following vaccination. Such a response suggests Prtf could be a useful candidate for an anti-*S. pyogenes* vaccine. Epitopes recognized by B- and T-cells were found to be clustered in a 30 amino acid fragment of the fibronectin-binding repeats, suggesting this region as a candidate for use in polyepitope-based vaccines (112).

Another role of PrtF in pathogenesis is in the binding and/or adherence of *S. pyogenes* to epithelial cells (113-115). Adherence to epithelial cells is environmentally regulated with repression of protein F expression during growth under reduced oxygen concentrations. Expression of protein F is positively regulated by RofA, a regulatory protein that has both positive and negative affects on the expression of other virulence factors (116, 117). Also, expression appears to be inversely related to M-protein expression; conditions favoring M-protein expression repress expression of protein F (118). In addition to enhancing the binding of the streptococci to epithelial cells, the fibronectin-binding protein also appears to play a role in invasion of eukaryotic cells (119-121). Interestingly, the region of the protein involved in attachment is different from that portion which triggers bacterial cell internalization. Internalization of streptococcal cells, however, is not limited to the use of fibronectin-binding protein, as clinical isolates lacking this protein have been shown to invade non-phagocytic cells (122). Removal of protein F, by the action of the streptococcal proteinase SpeB, results in reduced internalization of bacterial cells. Addition of plasma proteins did not protect PrtF from the action of SpeB, whereas the M-protein (M-1) and protein H were protected from proteolytic degradation (123). These authors speculated that since SpeB production is dependent on the bacterial nutritional status, then production of protease when nutritionally stressed would result in decreased adhesion and internalization. This would facilitate spread of the organisms (123).

3.1.4.2. SfbII

The *sfb*II gene was identified as a protein that exhibited characteristics of a surface-associated protein that was able to bind fibronectin (124). This protein was both genetically and antigenically distinct from SfbI. Subsequently the protein was found to have apoproteinase activity, thereby classifying it as a serum opacity factor (125).

3.1.4.3. PrtF2

Protein F2 is a fibronectin binding protein found in strains of *S. pyogenes* that lack the *prt*F1 gene, but still binds fibronectin. As with the other fibronectin binding proteins, protein F2 most likely plays a role in the adherence of the organism to epithelial cells and perhaps internalization. The protein has two fibronectin binding domains with each able to bind fibronectin independently

of the other (126). Protein F2 production is regulated by Nra (127), and in a manner similar to the effect seen with protein F (118, 128), PrtF2 is expressed in response to alterations in the concentration of atmospheric oxygen (126). Since *nra* transcription appears to be unaffected by aeration conditions (127), the regulation of the fibronectin binding proteins by oxygen levels must be by some other as yet unknown regulatory mechanism. Why fibronectin binding proteins (Protein F and PrtF2) would be regulated by oxygen levels is unknown, but it may represent a mechanism by which expression would aid in adherence and internalization by epithelial cells. Such a mechanism would also explain why increased PrtF2 expression, and hence fibronectin binding, by a Nra mutant was found to result in increased adherence and internalization rates of bacterial cells compared with wild type *S. pyogenes* (120). As these results were obtained from a study using a *nra* mutant, it was not possible to determine whether the effect was due to changes in protein F2 expression or some other factor (120) as other virulence factors also showed increased expression in the *nra* mutant strain, including SpeA, SpeB and SLS (120). These factors could potentially affect the results of adherence/internalization assays. However, some of these proteins are more likely to have an effect on host cell death rather than the adherence or internalization role proposed. Although as discussed, SpeB plays a role in degrading proteins involved in fibronectin binding and therefore internalization; whether such an effect is seen with PrtF2 was not reported.

3.1.4.4. Fbp54

Fbp54 is a 54 kDa fibronectin binding protein found on the surface of group A streptococci (129). This protein, like protein F, appears to be involved in the binding of streptococcal cells to epithelial cells (130). The protein is expressed *in vivo*, as determined by the presence of antibodies to the protein in sera of patients with rheumatic fever or glomerulonephritis (130). Fbp54 antibodies were also detected at higher levels in patients with pharyngitis and impetigo than in controls (131). Surface expression and fibronectin binding suggest that like the other fibronectin binding proteins Fbp54 acts as an adhesin for group A streptococci. Additional evidence for the importance of Fbp54 is its presence in all strains tested, from a variety of serological types. Also the sequence of Fbp54 is highly conserved among the various serotypes (131); again, such conservation suggests an important role for the protein.

3.1.4.5. Pfbp

Pfbp is a 127.4 kDa surface attached fibronectin binding protein, attached by a typical gram-positive LPXTGX motif (132). This protein is able to bind both soluble and immobilized fibronectin. The C-terminal region of the molecule is conserved among many of the fibronectin binding proteins, while the 105 amino acid N-terminal portion shows variability from other fibronectin binding proteins (132). The gene encoding Pfbp is found in a variety of *S. pyogenes* serotypes; however it is not present in all serotypes (132). In those strains that contain *pfbp*, transcription of more than one fibronectin binding protein gene (*pfbp* and *prt*F) does occur. However, it is unclear whether both proteins were expressed on the cell surface at the same time or whether both were contributing to the fibronectin binding activity of the strain (132).

3.1.4.6. FbaA

A surface-associated fibronectin binding protein, FbaA (originally reported as Fba), was present in a limited number of M-types of *S. pyogenes*, in particular M-types 1, 2, 4, 22, 28 and 49, but not in other types (133). The fact that Mga regulates the gene points to its importance in the virulence of *S. pyogenes* (133). Inactivation studies indicated that FbaA, along with M-protein, is required for binding of fibronectin, as well as adhesion and invasion. Fibronectin binding does not solely depend on FbaA, since mutants still retain some binding activity (133). In addition, mortality studies following mouse skin infection models indicate that *fba*A contributes to virulence and may be associated with spreading of the organism (133).

3.1.4.7. FbaB

Another fibronectin binding protein gene, designated *fba*B, was found in all M-types 3 and 18 strains, that do not possess the *fba*A gene, but not in other serotypes (134). In addition the protein was found to be expressed on the surface of some group A streptococci isolated from toxic-shock like syndrome patients, but not on strains isolated from patients with pharyngitis. Inactivation of the gene resulted in a decrease in fibronectin binding, as well as the ability to adhere to and invade epithelial cells (134). Of note in this study was that SpeB, the cysteine proteinase produced by *S. pyogenes*, cleaved FbaB. The role of this modification in virulence is not clear, but it could contribute to the ability of the organism to evade the host defense system (134) and may increase virulence of the organism.

3.1.5. Streptococcal protective antigen

Streptococcal protective antigen (Spa) is a surface protein produced by M-type 18 group A streptococci that is expressed *in vivo* as determined by the presence of antibodies in sera following an infection (135). This protein, along with the M protein and hyaluronic acid capsule, was required for full expression of virulence in the M-type 18 streptococci (135). The protein has a signal peptide, a membrane anchor, and hydrophilic tail similar to the M-like proteins but it does not have other sequence components found in these proteins (135).

3.1.6. Heme binding proteins

Group A streptococci express a surface protein that associates with heme (136) and heme compounds (137). This protein is expressed on the surface of the cell throughout the exponential and early stationary phases of growth, and is enhanced under iron-restricted conditions. This suggests that heme is an important source of iron for the group A streptococci, and that proteins in the *shp* region may be involved in obtaining and transporting heme into the bacterial cell (136). The *shp* gene is cotranscribed with gene homologues of an ABC transporter involved in iron uptake. Bates *et al* described the same iron acquisition operon, however they included an additional gene, *shr* (streptococcal hemoprotein receptor) (137); this gene

preceded *shp* (*spy*1796) in the operon. The genes *spy*1795, *spy*1794, and *spy*1793 comprise a three-gene transporter operon designated *sia*ABC. The exact composition and makeup of the components of this operon requires further investigation. of This operon are conserved, being present in strains of M5, M3 and M18, as well as M1 (136). Furthermore, anti-Shp antibodies were capable of inhibiting growth of the organism *in vitro* (136), indicating the potential for the operon or one of its components to be useful in therapeutic regimens. Still unknown is how these heme-binding proteins are regulated. One possible mechanism may be through the use of regulators involved in iron homeostasis, since heme could act as a source of iron for the cell. Strains in which the peroxidase regulator PerR was inactivated showed a reduction in incorporation of iron (138). Whether this regulator is able to influence expression of heme-binding proteins requires further investigation.

3.1.7. HtrA protease

The DegP protease ortholog HtrA has been reported to be required for full virulence of *S. pyogenes*. In most gram-negative bacteria DegP is found in the periplasm, however in the case of *S. pyogenes* it is probably membrane bound, as the *htr*A gene appears to encode a membrane-spanning region. The exact role of this protein in *S. pyogenes* is still unclear, but is required for thermal stability and resistance to oxidative stress (139). A *htr*A knockout showed reduced virulence, following intraperitoneal injection, in mice with the LD_{50} for the mutant strain being 35-fold higher than the wild type (139). This suggests a role in virulence of the organism in this assay. However, insertional inactivation mutants were not attenuated in subcutaneous mouse infections (2). Of particular interest in these studies was the observation that the mutants showed other phenotypic changes; the mutant failed to process the SpeB zymogen to an active protease, and there was an overexpression of streptolysin S (2).

3.1.8. Protein G-related alpha$_2$-M-binding protein

S. pyogenes produces proteolytic enzymes and is often found in environments containing proteolytic enzymes. As such the organism has developed ways to prevent proteolytic degradation of some, but not all, of its virulence factors. Some virulence factors remain susceptible to proteolytic cleavage in order that they can be regulated by the action of various proteases in a manner that removes or decreases the activity of these proteins during the pathogenic process. The human protease inhibitor alpha$_2$-macroglobulin is bound to the surface of *S. pyogenes* by the surface protein GRAB (protein G-related alpha$_2$-M-binding protein) and may be involved in the regulation of proteolytic activity at the streptococcal surface (140). In doing so it would protect important virulence factors from proteolytic degradation by either the streptococcal proteinases or host proteases. Binding of GRAB to the surface of group A streptococci has been shown to require the sortase gene *srt*A, which is also required for anchoring M-protein, the fibrinogen binding protein F, and C5a peptidase (141). The *grab* gene is found in most strains of *S. pyogenes*, and is highly conserved (140), perhaps indicating an important role in virulence of the organism. The ability to bind alpha$_2$-macroglobulin means that GRAB could also play a role as an adhesion molecule by binding to the alpha$_2$-macroglobulin on the host cell surface. Virulence studies found that strains in which the *grab* gene was inactivated were attenuated in skin infections (140, 142). The mice infected with the mutant showed less dissemination of the organisms and developed smaller lesions.

3.1.9. Streptococcal collagen-like surface proteins

At least 2 collagen-like surface proteins, Scl1 (SclA) and Scl2, can be produced simultaneously by group A streptococci (143-145). These proteins contain an anchor region, a spacer region, and a collagen-like domain consisting of a variable number of Gly-X-Y triplet amino acids. The bacterial proteins, like human collagen appear to have a triple helix structure (146). The number of repeats in Scl1 varied from 14 to 62, while for Scl2 the repeat number varied from 33 to 116 (144, 145). Size variation was observed both between serotypes as well as within a single serotype (144, 145). The variable region of the molecule, although showing considerable sequence differences, appears to maintain structural similarity, suggesting an important role for the structure in the function of the protein.

The role of these proteins in bacterial virulence is not fully understood but isogenic *scl*1 mutants were less virulent following subcutaneous injection (145). Also, a decrease in the ability of the bacteria to adhere to epithelial cells was observed following *scl*1 inactivation, suggesting that this collagen-like protein may play a role in bacterial adherence.

Further evidence that *scl*1 potentially plays a role in virulence is its control by Mga (143, 145), a global regulator of M-protein and other possible virulence factors. However, *scl*2 is regulated differently, being controlled by the number of CAAAA pentanucleotide repeats located downstream of the GTG start site (144); failure to produce Scl2 is due to premature termination of the gene due to the variable numbers of the repeats. The different modes of regulation may allow for expression of the two collagen-like proteins either simultaneously, or under different conditions in the host.

3.1.10. Lipoprotein of *Streptococcus pyogenes*

Lsp or lipoprotein of *S. pyogenes*, is a surface associated protein found on the surface of cells. The gene (*lsp*) was identified by a genome walking study, which examined the region downstream of the *mga* regulon, and upstream of the dipeptide permease housekeeping gene (147). A similar arrangement of genes to the *lsp* operon has been observed in *S. agalactiae*, and it was proposed that the operon might have been transferred to *S. pyogenes* from a pathogenic *S. agalactiae* by a mobile genetic element (147).

Lsp appears to have a role in matrix glycoprotein interaction and for bacterium-host interactions; however, the exact role of Lsp is difficult to determine. A *lsp* mutant showed less adhesion to eukaryotic cells, at least in part

due to the decreased fibronectin binding. The effect on fibronectin binding was not a direct result of the *lsp* mutation, but was affected indirectly through the regulation of the fibronectin binding protein genes, *prtF2* and *sof* (147). In addition, inactivated *lsp* mutants have decreased transcriptional rates for three of the important global regulators in *S. pyogenes*, Mga, CovR and Nra. Another important factor that is influenced in the *lsp* mutant is the cysteine protease, SpeB; transcription of this protein is decreased in these mutants (147). This decrease in transcription may be associated with an effect on the appropriate regulator.

3.1.11. CD15s-related antigen

S. pyogenes expresses a number of surface antigens that may act as adhesins. One of these is the surface-associated CD15s-related antigen (148). The CD15s antigen is expressed by neutrophils and monocytes (149) and plays a role in the binding of the leukocytes and monocytes to the vessel wall and therefore passage of these cells through vessel wall into the tissue. Such an effect, with binding of the antigen to the selectin receptor, supports a role for such proteins in streptococcal adherence. It may also suggest a way by which the organism can spread; by binding to appropriate receptors the bacterial cell may result in host changes that allow for dissemination from the site of infection. In addition, expression of an antigen that mimics this immune system antigen may be a means of camouflage such that the bacterium would not be detected. Alternatively the antigen may act as an adhesin molecule. Another possibility is that since CD15s antigen is involved in leukocyte trafficking, maybe the presence of this protein on bacterial cells in some way interferes with this trafficking process. The exact nature of this antigen and any role in the disease process is unknown at this stage.

3.1.12. Plasmin(ogen) binding proteins

Three surface bound proteins that bind plasminogen have been reported. These are plasmin binding receptor (also known as gylceraldehyde-3-phosphate dehydrogenase or streptococcal surface dehydrogenase), alpha-enolase or streptococcal surface enolase, and plasminogen binding group A streptococcal M-like protein (150-155). These proteins potentially function in binding of the streptococcus to a host cell, since eukaryotic cells also express receptors capable of binding plasmin(ogen). In addition, the presence of the plasmin can be exploited by the bacterial cell to facilitate tissue invasion (156). Recently, an epidemiological study showed that acquisition of plasminogen was associated with the ability to cause invasive disease with the invasive isolates binding more plasminogen than isolates from non-complicated infections (157). The presence of plasmin on the cell surface may be involved in breaking down fibrin barriers allowing the organism to spread or alternatively to decrease the level of plasminogen available to the host during the immune response.

3.1.13. Serum opacity factor

Certain serotypes of group A streptococci produce a type-specific factor, known as opacity factor, which produces an opacity in serum. This protein is found both as a released protein and bound to the cell surface (21, 158) by a LPXSGX motif (159). The opacity reaction results from cleavage of apoprotein A1 that leads to aggregation and insolubility of high-density lipoprotein. This factor was designated as an apoproteinase (160), more specifically an aspartic proteinase (161). Serum opacity factor appears to be a bifunctional protein bound to the cell membrane. The two activities are serum opacification and fibronectin/fibrinogen binding (125, 159, 162-164), with the C-terminal region being involved in fibronectin binding and the remainder of the protein being responsible for opacity factor activity (125, 159, 163). Fibronectin binding by opacity factor is about one order of magnitude lower than seen with the other fibronectin-binding proteins (165). Opacity factor is produced as a part of a two-gene operon, with the second gene's product, SfbX, also being able to bind fibronectin (166). However, the opacification reaction is encoded for by the *sof* gene. The role of opacity factor in virulence is difficult to determine due to the bifunctional nature of the enzyme. However, inactivation of the *sof*2 gene, from an M-type 2 strain, resulted in a reduction in the virulence of the mutant following intraperitoneal injection in mice (162). SOF has recently been shown to be involved in adherence of streptococcal cells through a fibronectin-mediated process (165). Differences in the diseases associated with opacity factor positive or negative serotypes may be associated with one or both of the activities of this protein. Opacity factor positive serotypes tend to be associated with skin diseases (167), whereas those serotypes that cause invasive type diseases tend to be opacity factor negative. Recently it has been shown that serum opacity factor evokes a protective antibody response that protects against infection by opacity factor positive organisms. This protein should therefore be considered as a vaccine candidate for protection against at least some streptococcal infections (168).

3.2. Released extracellular virulence factors

One reason that group A streptococci are so pathogenic and cause an extensive range of diseases is that they produce and secrete a multiplicity of virulence factors into the external mileu. These factors are in addition to those already described that remain cell associated and include: (i) degradative enzymes such as proteases, DNases, and hyaluronate lyases; (ii) toxins such as the streptolysins and superantigens; and (iii) proteins with other functions including inhibition of complement and inference with phagocytosis and killing.

3.2.1. Cysteine protease

Group A streptococci produce, or at least have the potential to produce, a number of different proteolytic enzymes including SpeB (cysteine protease), C5a peptidase, opacity factor (aspartic apoproteinase) and a serine proteinase. In addition, the organism has the potential to utilize proteases produced by the host (169). Along with the hyaluronic acid capsule, perhaps the most widely studied of the streptococcal virulence factors is the streptococcal cysteine proteinase also known as pyrogenic (erythrogenic) toxin B (SpeB). Originally described as two different proteins, the use of molecular techniques indicated that the two molecules were the same. SpeB is produced as

a zymogen that undergoes autocatalytic cleavage to give the mature peptide (170, 171). The gene for SpeB is conserved and found in virtually all strains of group A streptococci (172, 173) suggesting an important role in the survival of this organism.

Proteases have long been considered as virulence factors for organisms producing them, with virulence being attributed to the ability to allow for spread of the organism. In the case of the streptococcal protease a number of different roles in the disease process have been investigated with effects on producer strain proteins, the host immune system, and degradation of host extracellular matrix material (5, 6, 49, 169, 174). Insertional inactivation of the gene decreased mouse lethality (175-177), decreased resistance to phagocytosis (178), enhanced internalization by human cells using a fibronectin–dependent process (107, 179), eliminated fibrinogen cleavage (180), and in a strain specific manner affected expression of the hyaluronic acid capsule (181-183). Other results, again using an insertional mutant, suggested that SpeB has no effect on virulence (182, 184). The reason for the contradictory results is unknown, but may be due to strain-to-strain variation of different (unrelated) effects following gene inactivation, although the same parental strain (AM3) was utilized (175, 182). It is possible that the insertional inactivation resulted in differences that were not detected, especially when one considers the number of potential virulence factors produced by this organism. Perhaps one mutant was able to compensate for the loss of SpeB activity in a manner that the other was not. Another proposed function of SpeB is that of having strepadhesin activity; a surface-associated factor able to mediate the binding of *S. pyogenes* to laminin and other glycoproteins (185). *In vivo*, inverse relationships have been reported for expression of SpeB, and M-protein and SpeA (186, 187), with suppression of SpeB expression resulting in surface M-protein expression (187) and SpeA expression (186). Recently, SpeB was found to affect the ability of certain streptococcal superantigens to stimulate the appropriate T-cells (188). Superantigens stimulating cells expressing T-cell receptor V-beta-4 (TCRBV4), TCRBV7 and TCRBV8 were affected by SpeB, while those stimulating TCRBV2 were not (188). SpeB also degrades immunoglobulins, not only IgG but also IgA, IgD, IgE and IgM (174). Cleavage of IgG into the Fc and Fab fragments enhances survival of the organisms in the presence of opsonic antibodies, most likely by contributing to the ability to escape antibody-mediated phagocytosis (49, 174, 189, 190).

Obviously, the function of SpeB is still uncertain as new functions are ascribed to the protein, and in some cases contradictory results are obtained in different laboratories, albeit sometimes with different serotypes and strains. These results may indicate a varied role for the protein, however which function(s) is/are the most important or essential is still to be determined. One interesting result shows the interplay between important virulence factors of *S. pyogenes*; M-protein is required for the production of mature proteinase. The M-protein is involved in the unfolding of the proteinase zymogen, and production of the mature enzyme (191). Such an effect that would not be expected since the mature proteinase has also been implicated in cleavage of the M-protein from the bacterial cell surface. How these two effects are regulated remains to be determined since too much active proteinase would result in the destruction of M-protein. Another factor essential for the maturation of the protease is trigger factor, encoded by the *rop*A gene (1, 192). The effect of RopA is on the maturation process that occurs outside the cell, rather than the targeting step required for secretion. The activity of RopA is directed to ensure the correct conformation of the prodomain of the protein (1).

The role of proteolytic enzymes, for many if not all cells, is the breakdown of proteins. The breakdown products of the proteolytic digestion are transported into the cell for use as an energy source. Two transport systems found in bacteria, including *S. pyogenes*, are the dipeptide transporters and the oligopeptide transporters. The *opp* operon contributes to both production and export and/or processing of SpeB as well as other secreted proteins (193). Expression of *spe*B was reduced in *dpp* operon mutants in a manner that suggested a link between nutritional pathways and virulence factor expression (194). The link between these two pathways is not understood, but probably involves, either directly or indirectly, the streptococcal multigene regulator Mga (see regulation section). Mga has been shown to regulate, at least partially, expression of the *opp* and *dpp* operons (194).

3.2.2. Streptokinase

Streptokinase (Ska) has long been considered a virulence factor of the streptococci because of it ability to lyse clots that may limit the spread of infection (195). The protein is an activator molecule that converts plasminogen to its active proteolytic form plasmin, in a non-enzymatic manner. The streptokinase-plasmin complex has the potential to degrade fibrin clots as well as the extracellular matrix, to allow for spread of the organism or its toxins, and to activate the complement cascade. In addition to activating plasmin, the active complex is not inhibited by factors that are normally able to inhibit plasma plasmin (196). This gives the producing organism additional spreading factor activity. Streptokinase production is not limited to group A streptococci, but is also produced by other streptococci although the proteins produced by these different organisms show a high degree of similarity (197). It has been shown that there is variation within two regions of the molecule in group A streptococci, most likely a result of recombination events (198). This may be important in the diversification of other virulence factors as well.

One consequence of streptokinase production is its role in the development of acute post-streptococcal glomerulonephritis. This sequelae may be initiated by deposition of streptokinase in the glomeruli of certain patients that leads to local activation of the complement cascade (199, 200). Two lines of evidence suggest a role of streptokinase in the development of nephritis. The first was that streptokinase production was a prerequisite for glomerulonephritis induction in mice; non-nephritic strains failed to show the deposition of streptokinase or C3 in the

kidney (200). Second, only certain types of organisms can cause nephritis, and this effect could be associated with the finding that not all *ska* allelic variants are able to induce nephritis in an experimental mouse model (201).

3.2.3. DNases

Group A streptococci produce four antigenically distinct DNases (A, B, C, and D) capable of degrading DNA. Whether any or all of these are important as virulence factors is unknown (202), although they are produced during an infection because anti-DNase antibodies have been used as a diagnostic tool (203, 204). A gene (*sda*D) identified as a DNase gene from *S. pyogenes* was reported to be similar to the group C streptococcal DNase, with the highest level of homology to the portion of the protein suggested to be the active site (205). Recently, it was shown that of 17 genes studied in cases of acute pharyngitis the *sda*D gene was expressed at the highest level (206).

Genomic sequencing did not reveal similarity to genes with motifs associated with DNase activity, although sequence similarity between mitogenic factors MF1 (SpeF), MF2, and MF3 and proteins with nuclease activity suggested that these might represent the streptococcal DNases (35). DNase B or mitogenic factor MF (207) are immunologically identical to SpeF, although epitopes responsible for the two effects (DNase activity and mitogenic activity) are separate (208). Other DNase proteins have also been identified as mitogenic factors, with Spd1 being the same as MF2, although no mitogenic activity has been associated with the molecule (45). Of interest, was the observation that while this DNase is now phage encoded, it may have been originally chromosomally encoded. This is based on the presence of a signal peptide region in the protein, something not expected for a phage gene product. This report also suggests that nearly all the DNases of group A streptococci may be phage encoded, and that some soluble factor produced by pharyngeal cells is capable of inducing gene expression. Such effects suggest that the phage encoded DNase may play a role as a pathogenic determinant, particularly since it also appears to be linked to SpeC (a phage encoded superantigen) production (45).

3.2.4. Superantigens or pyrogenic exotoxins

Streptococcal pyrogenic exotoxins, or mitogenic factors, were originally discovered and named based on their presumptive role in the development of scarlet fever, and included erythrogenic toxins A, B (now known to be the cysteine proteinase), C and the poorly defined exotoxin D. These toxins are able to act as superantigens by nonspecifically activating large numbers of T-lymphocytes, resulting in the release of interleukins and cytokines. This family of bacterial superantigens has grown, with the availability of genomic sequence analysis, and now also includes SpeF (has nuclease activity (209, 210)) SpeG, SpeH, SpeJ, SpeK, SpeL, SpeM, SSA, SMEZ, and SMEZ-2 (5, 6, 211). Phylogenetic analysis of the superantigen-like proteins identified in the sequence of SF370 suggested horizontal transfer might have distributed the toxin genes (35). The properties, structure, and function of many of these superantigens have been reviewed in the last few years and readers are directed to these reviews (5, 6, 211). Recent data has been associated with confirming the superantigenicity and mitogenicity of SpeF (210), SpeJ (212), SMEZ (213), SpeL and SpeM (214, 215). Another avenue of investigation has involved determining the site of interaction between SpeC, major histocompatibility complex and the T-cell receptor. Like many investigations of *S. pyogenes* virulence factors, these studies show conflicting results. One group stated that the zinc-dependent major histocompatibility complex binding site was critical for the function of SpeC (216), while other results indicated that zinc coordination was not essential for the biological activity of SpeC (217).

The role of these toxins/superantigens in the disease process requires additional investigation as no clear role has been established, and it is possible that the effect may be as much host dependent as toxin dependent. As evidence of this, Chatellier *et al* showed that expression of different toxins varied among clinical isolates of M1T1 strains, but no association with disease severity was seen (218). Obviously, more work on the role of the numerous superantigens in streptococcal diseases is needed to clarify the function of each toxin.

3.2.5. Streptococcal inhibitor of complement

Contained within the *mga* regulon of the M1 strain of *S. pyogenes* is the gene encoding SIC, the streptococcal inhibitor of complement (219), a multifunctional protein able to interact with various host cell proteins and components of the immune system (220, 221). SIC incorporates into the membrane attack complex of complement and inhibits complement-mediated lysis, by binding to the C5b-C7 complex (222). In addition to inhibiting the action of complement, SIC also inactivates human neutrophil alpha-defensin and LL37, two antibacterial peptides of the innate immune response (221). Strains of *S. pyogenes* tested for their ability to induce another defensin associated with cutaneous defense, human beta defensin 2, were very poor at inducing the defensin gene (223). However, these organisms were sensitive to the killing action of the defensin, and it was proposed that the lack of induction was a way of evading the innate immune response. Combined with the effect of SIC inhibiting the alpha-defensin, the streptococcus has a means for avoiding important components of the innate defense system. In addition to the inhibition of antibacterial activity, SIC also decreases colonization of the mucosal surface as well as internalization and killing, thereby enhancing bacterial survival by avoidance of the intracellular environment of PMNs (224, 225). SIC binds to ezrin and moezin proteins involved in the interaction of the actin cytoskeleton with the plasma membrane. The binding of SIC to ezrin may result in alterations of cellular processes required for streptococcal binding, internalization and killing (224). Two other components of the innate response, lysozyme and secretory leukocyte proteinase inhibitor, were also bound to SIC (220). The antibacterial activity of the proteinase inhibitor was inhibited by SIC, although the antiproteinase activity was not. In the case of lysozyme, both the enzymatic and antibacterial activities were inhibited.

The gene for SIC, *sic*, was reported in M-types 1 and 57 suggesting the gene may be restricted to certain serotypes (219). However, within these serotypes there is a high degree of sequence variation (in-frame insertions and deletions, and nucleotide substitutions) probably driven by natural selection (226, 227). Recently the presence of *sic* was reported in 8 other M-protein genotypes, although with different frequencies (109). A gene, *drs* (distantly related to *sic*) has been detected in M-genotypes 12 and 55 (228), and like *sic* shows gene polymorphisms, at least within the M-12 genotype (229). This protein does not appear to inhibit complement, although it does bind to the complement proteins C6 and C7 (230). The authors suggested that since this protein is expressed by serotypes M12 and M55, glomerulonephritis associated strains, which the binding to the complement components gives it the potential to co-localize in the glomeruli. This could lead to formation of antigen-antibody complexes and result in glomerular injury (230).

3.2.6. Streptolysins

Group A streptococci secrete two hemolysins, streptolysin O and streptolysin S and encode three other putative hemolysins based on similarities to other known and theoretical hemolysins (35).

3.2.6.1. Streptolysin O

Streptolysin O (SLO) is an oxygen sensitive, thiol activated toxin that is produced by nearly all *S. pyogenes* isolates (47). SLO interacts with membrane cholesterol, forming pores following aggregation in the membrane of target cells (231). The target cells include erythrocytes, macrophages, leukocytes, platelets and a number of different cell culture types. The toxic effect of SLO, although recognized in *in vitro* cell assays, is also seen following injection into animals (232). The effects, and possible ramifications, of SLO during an infection were proposed using purified SLO, however, the role of the hemolysin in the infectious process *in vivo* has only recently been examined. SLO is important for invasive disease in a mouse skin infection model (233), where mice injected with a *slo* mutant were more likely to survive than those infected with a *slo* wild-type strain. In addition, infection with strains able to revert from SLO^- to SLO^+ suggested an important role in virulence since reversion was much more common in a host than in culture (233). The role of SLO is still not understood, but it does not appear to be involved in the formation of necrotic lesions or spread of the organism from the lesion (233). Recent studies suggested another aspect of SLO activity; that is, enhancement of virulence in poorly encapsulated strains of *S. pyogenes* (99). No effect on virulence was seen in soft-tissue infection; however, the effect was observed using a sepsis model of infection. The authors suggested that in strains deficient in the hyaluronic acid capsule, SLO both protected the streptococci from phagocytic killing as well as enhancing virulence. Further evidence that SLO plays a smaller role than originally thought was obtained by Fontaine *et al* who showed no difference between a wild type and *slo* mutant in either intraperitoneal or subcutaneous infections (234). These results show the importance of *in vivo* assays, since the *in vitro* evidence does not agree with *in vivo* experiments; obviously additional experiments are needed to determine the role of SLO in streptococcal infections.

A recent observation suggests an interesting role of SLO in streptococcal infections. The hemolysin can deliver proteins from the bacterial cytoplasm directly into the cytosol of a host cell in a manner similar to that described for type III secretory pathways (4). *S. pyogenes* was shown to translocate an effector molecule, NAD glycohydrolase, through a SLO pore. The NAD glycohydrolase translocated into the host cell is able to produce the second messenger molecule cyclic ADP ribose that contributes to a cytotoxic effect in keratinocytes (4). Furthermore, the translocation process required bacterial adherence to the cells; cells deficient in their ability to adhere were not able to transfer the glycohydrolase despite producing equivalent amounts of SLO and NAD glycohydrolase. Formation of a pore by the hemolysin is not sufficient for cytolysin-mediated translocation, with the SLO playing a more active role in the process than merely forming a pore (235). This type of effect opens up a new and potentially important role of SLO in streptococcal pathogenesis.

3.2.6.2. Streptolysin S

The second hemolysin produced by most strains of *S. pyogenes* is an oxygen stable non-immunogenic toxin that is released from the cell only in the presence of an appropriate carrier molecule (6, 47, 236). As well as its extracellular location, SLS also exists in intracellular and cell bound forms. Like SLO, SLS can lyse the membranes of a variety of other cells types in addition to erythrocytes. Cell membranes damaged by SLS include lymphocytes, neutrophils, and platelets, as well as subcellular organelles (232, 237). Lysis occurs by insertion of a lysin complex into the membrane leading to the formation of transmembrane pores, similar to the mechanism by which complement lyses bacterial cells (238). SLS is produced by a nine gene locus, of which the first gene, *sag*A, encodes a 53 amino acid peptide responsible at least in part for the hemolytic activity (239, 240). This peptide appears to be related to the bacteriocins, and is probably produced as a prepropeptide which undergoes modification and cleavage prior to, or during, secretion (240). The operon encoding SLS is conserved among the different serotypes of *S. pyogenes* (240), which suggests an important role for the peptide in the survival or virulence of the organism. But expression of SLS is not essential for virulence as some non-hemolytic mutants are capable of causing disease.

The promoter region of the *sag*A gene, when interrupted by transposon mutagenesis, was found to affect not only SLS production, but also SpeB, streptokinase and M-protein (241). This effect was reportedly due to the regulator *pel* (241). Subsequently, using a *sag*B deletion mutant, no such effect was seen, with transcription of all the genes being detected (234). Transposon mutants, in which production of SLS was disrupted but other virulence factors were unaffected, were not able to induce necrotic lesions in mice (239). Using an air sac model Eberhard *et al* found that SLS transposon mutants had a delayed lethal

effect (242). Using an isogenic *sag*B mutant, no difference in the ability of the organism to multiply or change the LD_{50} following intraperitoneal infection was observed. However, a log-fold decrease in the LD_{50} following subcutaneous infection was seen (234). Another study indicated that SLS played a role in the virulence of acapsular strains, having only a modest influence on pathogenicity, an effect not associated with resistance to phagocytosis (99). Obviously the role of SLS in the virulence of group A streptococci has not been completely determined and still remains controversial, and it may be that the effects are strain associated rather than group or serotype associated.

3.2.7. Immunogenic secreted protein

Immunogenic secreted protein (Isp) is encoded by a gene upstream of *mga*. However, neither this gene nor the small open reading frame immediately downstream of *isp* have an effect on the transcription of *mga* or *emm* (243). The gene is conserved among members of the group A streptococci and appears to be post-translationally processed as the protein in culture supernatants is smaller than predicted from the gene sequence. The protein is produced *in vivo*, since antibodies are detected during a streptococcal infection. This indicates Isp is either secreted or at least associated with the bacterial surface (243) during *in vivo* growth. The function of Isp is unknown, but it has domains characteristic of surface proteins, as well as proteins involved in carbohydrate transport/metabolism or cell wall metabolism. BLAST searches indicate some similarity to proteins associated with transfer functions. What function this protein has in *S. pyogenes* is still to be determined.

3.2.8. Immunoglobulin G-degrading enzyme of *S. pyogenes*

Mac was originally described as spot 22 in studies examining the proteins present in *S. pyogenes* culture supernatants (244). Sequence data showed no homology to other bacterial proteins, but regions of identity to a subunit of the human leukocyte adhesion receptor Mac-1 were found (244). Subsequently, a gene denoted *ide*S (immunoglobulin G-degrading enzyme of *S. pyogenes*) was reported which also has similarity to human Mac-1. The gene product of *ide*S is a novel proteinase with specificity for IgG (245, 246). Mac and IdeS appear to be the same protein that is regulated by CovR (42). Two genetic complexes (I and II) were identified among 36 serotypes of *S. pyogenes*; within each complex the Mac variants were closely related, but there were differences between the complexes, with most of the variation occurring in the middle portion of the protein (247).

The function of Mac in *S. pyogenes* pathogenesis appears to be to inhibit opsonophagocytosis and killing by PMNs by interfering with the production of reactive oxygen species (248). Mac inhibits molecular processes that result in reactive oxygen species, and inhibits phagocytosis, through interaction with the Fc receptor CD16. When group A streptococci are taken up by phagocytic cells the gene encoding Mac is upregulated (249), an effect not surprising since it is associated with interference of the oxygen radical production. The other function of Mac, that of an immunoglobulin G endoprotease, may be involved in pathogenesis by inhibiting complement activation. The protease cleaves surface bound IgG at the hinge region, preventing contact with phagocytic cells (245).

3.2.9. Hyaluronidases

Group A streptococci produce a number of different hyaluronidases, including a chromosomally encoded hyaluronate lyase (250, 251), bacteriophage hyaluronidases (35, 36, 38, 250, 252) and Spy1600 identified in the genome of the sequenced M-type 1 strain of *S. pyogenes* (35), as well as in the other sequenced genomes (36, 38). Hyaluronidase has generally been considered to be spreading factor for the producing organism, either for spread of the organism or its toxins or enzymes.

3.2.9.1. Chromosomally encoded hyaluronate lyase

Certain strains of group A streptococci produce and secrete an extracellular hyaluronidase, more specifically a hyaluronate lyase (253). Originally reported as being produced by only a limited number of serotypes (254), it was subsequently discovered that production was a strain associated property rather than a serotype property (255). Hyaluronidase has long been proposed as a virulence factor, in particular a spreading factor, because of its ability to break down the hyaluronic acid present in the ground substance of host connective tissues (253). Recent results suggest that hyaluronate lyase may be more important for the diffusion of proteins or toxins rather than the organism itself (256).

Genomic analysis indicated that the gene for hyaluronate lyase is present in the genome of all strains so far sequenced (M1, M3, M18, and the incomplete M5), and an internal portion of the gene was present in all strains tested by PCR (251). However, less than 25% of strains actually produce the enzyme *in vitro* (250). In terms of the infectious process, the protein is produced, since anti-hyaluronidase antibodies are detected following a streptococcal infection (257). The reason for this difference, enzyme activity vs. antibody occurrence, is currently unknown but may either be due to production of an inactive protein or that enzyme production is induced *in vivo* in those strains not showing activity *in vitro* (251). One of the strange observations with the group A streptococci is that of the apparent contradictory processes that occur – the organism produces an anti-phagocytic capsule as well as an enzyme that degrades that protective component. The physiological consequences of this are unknown, but could be associated with production of different protective factors at different times of growth with the hyaluronic acid capsule produced early, followed by hyaluronate lyase production which destroys the capsule. At this switch time the antiphagocytic activity provided by M-protein and other surface associated proteins would become more effective. To date, no studies have shown a direct relationship between disease and hyaluronate lyase activity.

3.2.9.2. Bacteriophage encoded hyaluronate lyase

Bacteriophages of *S. pyogenes* carry a hyaluronidase gene, the most likely function of which is to degrade the streptococcal capsule and allow the phage access to the cell surface so they can infect encapsulated cells (258, 259). The protein encoded for by the bacteriophage lacks a signal peptide suggesting it is not secreted (250, 252), and at least some of it is associated with the phage tail (260). Such a location would indicate a role in the infection of a streptococcal cell by a bacteriophage. Like the chromosomally encoded hyaluronate lyase, the bacteriophage protein has been shown to be a hyaluronate lyase (261). The hyaluronate lyase genes found in the bacteriophage genomes are similar to each other, and belong to either the *hyl*P-type which contain a collagen-like repeat sequence or the *hyl*P2–type which lacks this region (250, 252). However, allelic variation, the result of recombinational processes, is seen within the phage hyaluronate lyase genes (262). Whether the phage hyaluronate lyase plays a role in infection of a host by the streptococcus is unknown, however antibodies to the protein are detected following streptococcal infections (263, 264). It is possible that the phage hyaluronate lyase adds to the virulence of the *Streptococcus* by functioning as an additional spreading factor enhancing other chromosomally encoded spreading factors such as streptokinase, protease and the extracellular hyaluronate lyase.

3.2.9.3. Spy1600

Analysis of the *S. pyogenes* genomic sequence found a gene designated *spy*1600, that had similarity to meningioma-expressed antigen 5 and the Mu toxin expressed by *Clostridium perfringens* (35), and was therefore designated as a possible hyaluronidase. Recently a gene with similarity to *spy*1600 was reported to be present in *Enterococcus faecium* (265). Neither the gene, nor the putative translated product of *spy*1600, show similarity to any of the other streptococcal hyaluronidases. The similarity reported to those other hyaluronidases is low, being only 32% and 26% respectively. Of interest is the observation that expression of *spy*1600 is upregulated in response to phagocytosis (249). Whether Spy1600 is a really a hyaluronidase, or has some other function perhaps associated with the stress induced by phagocytosis remains to be determined.

3.2.9.4. Neuraminidase

Neuraminidases have been implicated in the pathogenesis of infections by facilitating the colonization of the host mucosa. Early reports indicated that *S. pyogenes* produced a neuraminidase (266). However, Savic and Ferretti (267) found no evidence that *S. pyogenes* produces a neuraminidase, and suggested that the earlier reports were due to the action of hyaluronidase (hyaluronate lyase) as was reported with the group B streptococcal neuraminidase (268).

3.2.10. CAMP factor

For a long time group A streptococci were considered to be CAMP factor negative, with the lack of this activity used to differentiate group A from group B streptococci. Recently the gene for CAMP factor (*cfa*) was identified in the genome of *S. pyogenes* (269). Not only does *S. pyogenes* possess the gene; it is also capable of producing an extracellular CAMP factor able to participate in the CAMP reaction. The reasons for suggesting *S. pyogenes* was CAMP negative were that the level of CAMP activity was generally lower than seen with the group B streptococci and lysis zones were probably masked by streptolysin activity (269). Although no role for CAMP factor in pathogenesis has been reported, the variable expression levels observed and the finding that the gene is common (99 of 100 strains tested) in *S. pyogenes* suggests that it may have a role in the disease process or be required for some physiological function.

4. REGULATION OF VIRULENCE FACTORS

Gene regulation in group A streptococci is a complex series of integrated and intertwined proteins and signals, the full nature of which is still being unraveled. Genome sequencing has revealed that group A streptococci have approximately 100 genes that could encode transcriptional regulator homologues; these include alternative sigma factors, transcription factors, and two-component regulatory systems (35, 36). Within the genomic sequence of the M-1 streptococcal strain SF370, two identical open readings with homology to a secondary sigma factor were identified (35). These were shown to function as a sigma factor *in vitro* by promoting transcription from an appropriate promoter known as the cin-box (270). Strains MGAS8232 (M-type 18) and SF370 (M-type 1) share 11 two-component regulatory systems and more than 36 transcription factors (36). Of the two-component systems, some have been assigned a function (35), but the role in the regulation of factors associated with virulence is not clear. The sequence of the M18 strain reveals the presence of an additional two component regulatory system, not present in the sequenced M1 strain, which has homology to a peptide-inducible system of *S. pneumoniae* (36).

Two of the sensor-regulator pairs appear to regulate small peptide signaling systems (35). The ComD/ComE system has been suggested to regulate competence using a quorum sensing type regulatory mechanism similar to that seen in *Streptococcus pneumoniae* (271); gene homologues of other components of the competence system are also present in this organism (35). A five gene locus, known as the streptococcal invasion locus (*sil*) includes a putative two component regulatory system in group A streptococci and has homology to the quorum sensing regulons of *S. pneumoniae* (272). This locus was found to be associated with virulence as well as DNA uptake in *S. pyogenes*. Inactivation studies indicated the *sil* locus is involved in invasive infections, but not directly in the survival of the microbes in blood (272). Competence could be important in virulence because it allows the organism to change its genetic makeup by acquisition of new pieces of DNA from other bacteria, some of which could encode new or

Table 1. Regulators of *S. pyogenes* and the genes they control

Regulator	Genes positively regulated	Genes negatively regulated
CovR		*has*ABC, *sag*A, *spe*MF, *ska*, *spe*B, *msp*A, *mac*/*ide*S, *cov*R
FasA-FasX	*ska*, *sag*A, *rgg*, *mga,* fibronectin binding proteins	
Irr	*mga*, *has* operon	*sod*, *rgg*
Mga	*emm*, *scp*A, *sof*, *mrp*, *arp*, *scl*1, *sic*, *fba*A, *nra*, *mga*, *opp*/*dpp*. Collagen-like proteins	
Rgg (RopB)	*spe*B, *cov*R/S, *ihk*/*irr*, *fas*BCAX, *isp*1, lysozyme, autolysin, *clp*B, adhesins	*emm*, *scp*A, *cpsX*, *yufM*, *lyt*R, *mac*, *sag*A, *scl*I, *slo*, *spe*H, *grab*, *mac*, *mf*, *cov*R/*cov*S, *mga* regulon,
RALPs: RofA/Nra	*prt*F, *rof*A, *emm*, *has* operon	*prt*F2, *cpa*, *sag*A, *nra*, *mga* regulon, *spe*A, *spe*B
Sag/Pel	*spe*B, *ska*, *slo*, *emm* and *emm*-related genes, *fbp*,	
Rel-independent	*cov*R/S, *fas*BCAX, *slo*	
RocA	*cov*R	*roc*A
AmR	*mga* regulon	
Lsp	Mga, CsrR, Nra, SpeB	
SpeB		*rgg*

See text for relevant reference information

different virulence factors. Within the group A streptococci, there is relatively little variation between the genomes (35, 36, 38), and much of the variation appears to be due to bacteriophages and insertion sequences (38). However, additional variation could be enhanced by the uptake of free DNA by the cells.

The second two-component system associated with small molecule signaling is the *srt*R/*srt*K pair associated with regulation of the salivaricin (*Sal*AI) lantibiotic operon. Salivaricin was first described in *S. salivarius* (273), but has been found in 90% of group A streptococci (274). Lantibiotics are antimicrobial peptides produced by gram-positive bacteria that undergo posttranslational modification (275, 276). These agents could aid in the establishment of the organism during the initial stages of infection by inhibiting the growth of normal flora. The SalA1 lantibiotic operon is autoregulated, with regulation occurring through the action of the sensor/kinase regulatory system similar to that described for quorum sensing in gram-negative organisms (277). Whether regulated antimicrobial agents are important for streptococcal virulence is a matter of conjecture, since not all strains produce the antimicrobial activity. However, their regulatory factors could modulate other factors that have a direct role in virulence, with particular genes being turned on at certain stages of an infection, or in the different niches in which the organism finds itself. A similar effect is seen during infections with *Pseudomonas aeruginosa* (271, 278).

Many of the virulence factors of *S. pyogenes* are controlled by two-component regulatory systems and/or transcription factors including Mga, CovR/CovS, RofA, Nra, Rgg, SagA and RocA (Table 1). These regulatory factors, and others potentially associated with virulence factor production, are discussed individually, however it must be noted that regulation in group A streptococci is not a case of one protein regulating one gene. The interactions that occur between the various regulatory factors are important, and it is this aspect of streptococcal virulence factor regulation that still requires clarification. Kreikemeyer *et al* recently published a review article on the regulatory networks associated with virulence factor regulation (279). They divided the regulatory factors into two groups: the 'stand-alone' response regulators and two component signal transduction systems. A similar convention has been followed here in the organization of the regulators, with discussion of the two component systems (CovR/CovS, FasBCAX, Ihk/Irr) first, then the stand-alone regulators (Mga, Rgg, RALPs). Other regulatory agents that do not fit neatly into these two categories (Sag/Pel, RelA-independent regulation, RocA) are also discussed.

4.1. CovR/CovS

One of the best characterized of the streptococcal regulatory systems is the CovR/CovS system, also known as CsrR/CsrS. Originally named CsrR for capsule synthesis regulator it was subsequently renamed CovR (control of virulence genes) when it was found that additional genes were also under the control of this regulator (280). The CovR/CovS system is also found in the group C streptococci (281). Microarray analysis indicates that CovR/CovS either directly or indirectly influences expression of 15% of the chromosomal genes of *S. pyogenes* (42). Of note is that CovR was able to repress transcription of some genes (*has*) even in the absence of the sensor kinase, CovS, although phosphorylation was required (282).

Initial studies reported an increase in hyaluronic acid capsule production following Tn916 mutagenesis, with insertion of the transposon into the promoter region upstream of *cov*R (102). Inactivation of *cov*R resulted in the same phenotype, and in animal studies, an increase in virulence was observed (103). Subsequent studies found that CovR inactivation also derepressed transcription of *sag*A, *spe*MF and *ska*, but did not affect expression of *cpa*, *mga*, *mac*, *scp*A, or *sic* (103, 248, 280). CovR also represses transcription from its own promoter (280). Expression of *spe*B, with respect to *cov*R, varied depending on the strain; in the M1 strain MGAS166 (103) *cov*R mutations resulted in derepression of *spe*B, while in the M-type 6 strain JRS4 expression of *spe*B was not affected (280). Another study also indicated that CovR is not involved in transcriptional regulation of *spe*B with inactivation of *cov*R resulting in less proteolytic activity, despite the fact that transcription was unaffected (283). MspA (284) or "protein 27" (244) was identified in culture supernatants of *cov*R mutants. The appearance of this protein in the supernatant of the mutant strain indicated that

in the wild type, expression of *msp*A is repressed by CovR (284). The genes regulated by this system reflect those produced in log or in stationary phase, indicating that *cov*R mediates repression in both these growth phases (280) despite *cov*R transcript levels being maximal during the exponential phase of growth.

CovR binds to the promoter region of *has*A, the first gene in the operon required for synthesis of the hyaluronic acid capsule (104). Binding of CovR to the promoter of the *has* operon is phosphorylation-dependent, with the phosphorylation resulting in complexes consisting of multimers of CovR (104). Phosphorylation also leads to the stabilization of the DNA-regulator complex (285). CovR binds to the promoters of a number of regulated genes including *has*A, *sag*A, *spe*B, *ska*, *spe*MF, and *cov*R, albeit with different affinities (285); however, phosphorylated CovR does not bind to its own promoter. A common feature of the promoter sequences is that they are AT-rich with runs of As or Ts; a common motif of 16 bp [5'-T(T/A)ATTTTTAA(A/T)AAAA (C/A)-3'] was found conserved among all five promoters that bound CovR (285). An interesting observation was that inactivation of *has*A resulted in an increase in the levels of *cov*R mRNA in both the exponential and stationary phases of growth, suggesting that *has*A somehow negatively regulates transcription of *cov*R (283). A mechanism for this regulation has not yet been determined.

CovR/CovS responds to environmental Mg^{2+}; this is the first specific signal shown to interact with a two component regulatory system in *S. pyogenes*. The effect appeared to be specific for Mg^{2+} since Ca^{2+}, Mn^{2+}, and Zn^{2+} did not have the same effect; in fact Ca^{2+} was found to increase capsule gene expression. The authors propose that Mg^{2+} triggers the phosphorylation of CovR by CovS; this phosphorylation then results in the repression of Cov-regulated genes (106). Growth in the presence of Mg^{2+} resulted in repression of capsule production and streptokinase expression in exponential growth and streptolysin S expression when the cells reach stationary phase. Previous studies into the role of CovR in expression of *spe*B and *cov*R/*cov*S gave conflicting results (102, 280, 285). The level of Mg did not affect the level of transcription of these genes; neither were genes regulated in a Cov-independent manner (106). The authors speculated that the use of Mg^{2+} is an appropriate signal for an extracellular pathogen as it provides information to the pathogen to ensure maximal expression of the virulence factors in the host.

A recent study indicated that growth of *S. pyogenes* under stress conditions was dependent on CovS. The authors suggested that genes required for growth under stress conditions were repressed by CovR and that the stress activated the phosphatase activity of CovS (282). In this manner CovS inactivated CovR, thereby allowing for expression of stress related genes. Thus, the CovR/CovS system appears to act as an alternative sigma factor system; this may be important for survival of the organism since group A streptococci lack a general stress response sigma factor (282).

4.2. FasBCAX

The *fas*BCAX operon was named because of its ability to regulate fibronectin/fibrinogen binding, hemolytic activity and streptokinase transcription (286). The operon, which appears to be widely conserved among streptococcal serotypes, encodes two potential histidine protein kinases and one response regulator, with peak activities during the late exponential phase of growth (286). The presence of two sensor kinases is of interest and suggested two possible explanations; the sensors detect different signals or alternatively the sensor is a heterodimer rather than being a characteristic homodimer (286). Group C streptococci also have a *fas* operon, consisting of *fas*CAX in which the second kinase protein (FasB) is not part of the operon (281).

FasX was proposed to be an effector molecule for the operon, exerting growth phase control over certain streptococcal virulence factors (286). Expression of *fas*X, which is located downstream of the *fas*BCA operon was originally reported to be dependent on FasA (286); however, expression as part of the polycistronic mRNA containing *fas*BCAX, under conditions of amino acid starvation, suggest that expression of *fas*X is not absolutely dependent on FasA (287).

Mutation of the *fas*BCAX operon, by inactivation of *fas*B gene, increased expression of fibrinogen binding through late stationary phase; the wild type showed a decrease in binding as the cells entered the stationary phase. Additionally, streptokinase and streptolysin S both showed decreased activity in strains in which the *fas* operon was inactivated (286). However, the decrease in hemolytic activity was not related to a decrease in *sag*A/*pel* transcription, as the levels of this transcript increased in the mutants. Changes in *spe*B, *emm*, *has*B, *slo* or *mga* expression were not observed, although transcription of *sod* (superoxide dismutase) increased (286). A slight increase in *rop*B/*rgg* transcript levels was also observed; it would have been expected that this should result in an increase in the level of *spe*B transcript (288). Whether the increase in transcription of *rop*B/*rgg* was sufficient to increase transcription of *spe*B was not addressed, but since *spe*B is regulated by so many factors small changes in the levels of certain regulators may be difficult to detect. This effect also has to be reconciled with the observation that SpeB production occurs late in growth (289). These authors speculated on a temporal factor involved in the regulation of SpeB production; whether *fas*X plays this role is uncertain since the *fas*BCAX operon does not appear to function in a manner similar to other quorum sensing regulators (286).

Expression of *mga*, *emm*, *scp*A, and *has*A decreases as the cells enter stationary phase, with no transcript for these genes being detected (290), suggesting a growth phase regulator that acts directly on *mga* and that Mga then regulates the expression of other genes. It was noted that the native promoter or upstream region for *mga* was required for growth phase regulation (291). Since *emm* expression was not altered by mutations in the *fas* operon (286), this regulatory operon does not appear to be

responsible for the growth phase effect. These results support the suggestion that in addition to the *fas*BCAX system, other regulatory factors are required for the growth phase dependent regulation of streptococcal virulence factors. In addition, the question of whether there is strain-to-strain or serotype-to-serotype variation in the expression of virulence factors needs to be addressed. Reid *et al* noted variation in the time of maximal expression of *spy*0747 between different serotypes of *S. pyogenes* (292). Whether this adds another degree of complexity to the regulatory mechanisms of streptococcal virulence factors is currently unknown. It may be that certain strains lack regulatory elements present in other strains; such a possibility could result in variation in the increase or decrease of transcription levels.

4.3. Ihk/Irr

Recently, an essential role for the Ihk-Irr ("*isp*-adjacent histidine kinase" and "*isp*-adjacent response regulator" respectively) two-component system in the survival of *S. pyogenes* following phagocytosis was reported (249). It is located immediately upstream of the *isp* gene encoding the immunogenic secreted protein (35). The Ihk-Irr system identified as Spy2026 and Spy2027 (35), SpyM3_1732/1733 (38) or SpyM18_ 2083/2084 (36) was suggested to be involved in virulence regulation because of its location near the Mga regulated regulon.

At least eleven genes previously reported associated with virulence are upregulated during phagocytosis, including extracellular and surface-associated virulence factors. In phagocytic uptake studies gene expression was followed using DNA microarray analysis as well as TaqMan Real-Time PCR (249). Genes were differentially regulated during the phagocytic process, with some upregulated, others downregulated. Upregulated genes would provide the organism with the ability to avoid killing by polymorphonuclear leukocytes, as well as enhance phagocytic cell lysis (249). The *mga* and *ihk-irr* regulatory systems were upregulated during phagocytosis suggesting these may be important in regulating genes associated with evasion of the organism from the host defense system (249). Of note was that although *mga* was upregulated, the M-protein gene itself was down regulated; consistent with the role of the protein in preventing phagocytosis. Once phagocytosed, M-protein is of little benefit to the cell. On the other hand, the downregulation of the superoxide dismutase appears to be an anomaly; why downregulate a gene that would increase the chances of survival when exposed to the internal environment of the phagocytic vacuole? The downregulation may indicate the organism avoids exposure to the toxic oxygen radicals long enough to escape the vacuole.

Recently, it was been shown that *S. pyogenes* is not only able to survive phagocytic uptake, but also to escape from the vacuole into the cytoplasm of PMNs (293). In addition, intracellular bacteria appear to undergo a phenotypic switching, which alters morphology and capsule expression, as well as increasing virulence (293). Whether other Ihk-Irr regulated genes are involved in this increased virulence was not determined, but capsule expression was one factor that was enhanced.

4.4. Mga

The first virulence factor regulator found in *S. pyogenes* was the gene now known as *mga*. Originally reported to regulate production of M-protein, with inactivation of *mry* resulting in a ≈50-fold decrease in M-protein levels (294). Other studies found a region upstream of the *emm* gene that regulated production and was associated with phase variation (295, 296). Subsequently, not only was *emm* regulated by this upstream region, but also C5a peptidase. Hence, the term *vir*R was coined to indicate a multi-virulence factor regulator (297). It was later found that *vir*R and *mry* were the same gene, and this gene regulated expression of a number of potential virulence factors. It was renamed *mga*, for multigene regulator in group A streptococci (298). Mga activates transcription of a number of virulence factor genes, predominantly those that are surface-associated. These factors include the M-family of proteins, C5a-peptidase, serum opacity factor, secreted inhibitor of complement, a glycoprotein binding protein, and a collagen-like protein (143-145, 294, 297, 299-302). In addition to these virulence factors, Mga also positively regulates expression of the *opp* and *dpp* operons (194). These operons are associated with transport of small peptides into the cell and probably play a role in nutrition rather than virulence. However, expression of SpeB appears to be linked to these operons. The affect of Mga on a nutritional pathway and the decreased expression of SpeB in transporter mutants indicates a link between these two aspects of streptococcal survival; the need to obtain nutrients and the production of required virulence factors.

Expression of *mga* is environmentally regulated, with aeration conditions being at least one signal that influences expression (303-305). An additional factor that plays a role in the regulation of *mga* is growth phase, with expression of *mga*, and Mga-regulated genes being expressed maximally in the exponential phase and shut off as the bacteria enter stationary phase (291).

When the complete gene was identified and sequenced, *mga* was found to have homology to the regulator protein of two-component regulatory systems (306); to-date, even with the sequence of a number of streptococcal chromosomes being available, the sensor component for this protein has yet to be found. The regulator binds to the promoter regions of regulated genes, allowing for the description of the Mga binding element (298). It also regulates it own transcription (304, 307), a process that involves the binding of Mga to its own promoter, but by a different mechanism to that associated with activation of other genes (307). Transcription of *mga* may be initiated from two promoter regions (304, 307), with binding of Mga required for activation of the proximal, but not the upstream site (307). Mga binds to two sites within the regulatory region upstream of *mga*, with binding to site I being required not only for activation of the proximal promoter, but also for the repression of the distal promoter (307). Mga has a structure that contains four potential helix-turn-helix motifs; only two of these are required for direct activation of the *mga*-regulated virulence regulon. One of these motifs is able to interact

with all the promoter sites, while the other augments these interactions (307). Full autoactivation requires Mga to bind within its own promoter region and also requires both of the DNA-binding domains (308).

An integral membrane protein with similarity to transporters involved in cell wall synthesis, AmrA (activation of Mga regulon), was found to be required for the maximal activation of the Mga regulon in a M-type 6 strain of *S. pyogenes* (309). The *amr*A locus was conserved in the genomes of the M1, M3, M5 and M18 strains (309). How AmrA functions in the group A streptococci to regulate the Mga regulon is unknown, but two possibilities are that AmrA and Mga directly interact, or alternatively, AmrA signals are picked up by other mediators which in turn affect *mga* regulated genes (309). A third possibility links *mga* regulon expression to cell wall turnover, with rapid growth, therefore high wall turnover, being a indicator signal functioning in a manner similar to signals detected in quorum sensing systems (309). This ties the ability to monitor growth phase to the regulation of virulence factors controlled by Mga.

4.5. Rgg

Initially reported as RopB, for regulator of proteinase (192), this gene has a high degree of homology to Rgg, a transcriptional activator of the glucosyl transferase from *Streptococcus gordonii* (192, 288). The presence of the *rop*B gene immediately upstream of the target gene (*spe*B) is similar to the arrangement in *S. gordonii*; however in *S. pyogenes* the regulator is transcribed divergently from the target (192, 288). Inactivation of the *rgg* gene results in the loss of proteolytic activity indicating the gene product acts as a positive regulator for the streptococcal proteinase (192, 288); the loss of proteolytic activity could be complemented by introduction of the *rgg* on a plasmid (288).

Streptokinase, streptolysins O and S, and DNase activities were not affected by the inactivation of *rgg*, suggesting Rgg does not regulate these proteins (288). Lysozyme, autolysin and ClpB expression during stationary phase, was lower in a *rgg* mutant when compared to the wild type; however the nuclease MF-1, DNA entry nuclease, and ORF953 expression was enhanced (310). The regulation of the nucleases suggests that *rgg* plays an important role in nuclease as well as in protease production (310). Whether the change in expression of these proteins in the stationary phase was due to alteration in quantity or stability of the transcripts is unknown. Interestingly, when *spe*B was inactivated, more *rgg* transcript was observed; suggesting SpeB represses expression of *rgg* (310). Using DNA microarray assays, transcripts for *emm*, *scp*A, *sag*A, *slo*, the adhesin *scl*I, the superantigen *spe*H, *grab*, and *mac* were increased in abundance in mutants in which *rgg* was inactivated. However, when assayed by RT-PCR, *grab* transcripts were less abundant; the reason for the variation in results from different assays is currently unknown. The fact that other regulatory pathways regulate many of these genes suggested that Rgg might exert its effect by regulating other regulatory pathways. Rgg appears to repress expression of two regulatory systems, the *mga* regulon, as well as *cov*R/*cov*S (311). The *fas*BCA operon and *ihk*/*irr* system showed decreased expression levels following *rgg* inactivation (311). The two-component regulatory genes *spy*0875, *cps*X, *yuf*M, and *lyt*R all increased in abundance. Alterations in these regulatory genes result in changes in the expression of a number of different virulence factors including: *emm*, *scp*A, *scl*I, *slo*, *sag*A, *ska*, *spe*H, *grab*, *mac*, *mf*-1, and *mf*-3 (311). Whether *rgg* acts directly or indirectly in influencing expression of these other regulatory factors remains to be determined, but it is clear that *rgg* does have a genome wide effect on the expression of virulence factors.

4.6. RofA-like protein (RALP) type regulators

The first gene described in this group of regulatory proteins was *rof*A; the term RofA-like proteins (RALP) was adopted to group together these proteins that show a similar function. A number of these RofA-like regulators exist in the group A streptococci with similarities around 52%, at the amino acid level (312). Multiple forms of these regulators are found in a single strain (35, 127, 312), suggesting an important role in gene regulation. The *nra* gene has 60% similarity to *rof*A and is considered part of this family. Eighty three percent of strains tested were found to contain a *rof*A gene, while only 27% had the *nra* gene; of these 41% also carried the *rof*A gene (313). RALPs appear to regulate expression of important bacterial adhesins such as *prt*F, *prt*F2, and *cpa*, although no correlation between the type of RALP and the microbial surface components recognizing adhesive matrix molecules has been found (313).

4.6.1. RofA

RofA (regulator of F) was originally identified in a strain of *S. pyogenes* in which expression of protein F (*prt*F) was constitutive, rather than being oxygen regulated as found in most other strains (128). The genes for *rof*A and *prt*F are adjacent to each other, but divergently transcribed. Whether oxygen regulated or constitutively expressed, the same regulatory region was used for expression of *prt*F and *rof*A (117) with the host genetic background determining the expression phenotype. Increased levels of RofA acting in trans (117) could overcome the host genetic effect. Depending on the strain, the regulator can have differing effects; negatively regulating some genes, positively regulating other genes, or having no effect on genes that appear to be RofA-regulated in other strains (313). The *rof*A gene is autoregulated, with RofA binding to the intergenic promoter regions for *rof*A and *prt*F. RofA binds to two sites in this region, a 17bp site adjacent to the *rof*A promoter, and a 40bp region, containing two inverted sites, adjacent to the *prt*F promoter (312). These sequences were used to construct a consensus RofA binding site of TTTTCACCAAAAANCAT. The smaller 17bp site contributes to *rof*A regulation, while the larger 40bp binding site was required for expression of *prt*F. This site was suggested to be a common portal for regulation by multiple pathways and environmental signals (312). Different strains demonstrate differences in regions of the larger 40bp site for expression of *prt*F. These differences may reflect the contribution of other elements in the regulation of *prt*F (312). Nra (below) has a

regulatory binding site (GCTTCTAAACTT) that differs from that of the RofA binding site (127), indicating a different regulatory mechanism.

Inactivation of the gene encoding RofA showed this regulator is involved in the negative regulation of a number of virulence-associated factors, although by a different regulatory pathway than that used by the negative regulator, Nra (116). However some genes, and other regulators, are regulated by both these RALPs. Of interest is that promoter regions of genes negatively regulated by RofA do not show a conserved consensus binding sequence, suggesting RofA may act indirectly to control their expression (116). In addition to the direct effect on regulating *prt*F expression, and hence fibronectin and fibrinogen binding, attachment to and internalization by eukaryotic cells was decreased in mutants in which *rof*A was inactivated; also, increased killing of host cells was observed. These effects may be due to the increased production of other virulence factors (116). In a widespread screening, there was a strain-specific variability in the size and makeup of the regulons controlled by RofA or RofA2 (the gene from an M-type 2 strain). (313). Of interest was that RofA2 regulated aminoglycoside resistance (313). The relationship between regulating expression of surface proteins and aminoglycoside resistance is interesting, with changes in the surface characteristics leading to alteration of antibiotic sensitivity. Whether this is a look into the future with regards to antibiotic resistance in the streptococci remains to be determined. The association between antibiotic resistance and regulation of virulence factors suggests that these organisms may undergo changes, the effects of which are not yet determined.

4.6.2. Nra

Nra (negative regulator of group A streptococci) has 62% homology to RofA, and is considered to be in the family of RALPs (127). Like RofA, Nra regulates expression of surface proteins including fibronectin-binding protein F2 and the collagen binding protein, Cpa (127). However, unlike RofA, Nra is a negative regulator of its own expression, two operons adjacent to the gene (one of which includes the collagen binding proteins), as well as *mga* and *prt*F2 (127). Nra regulated genes play an important role in streptococcal virulence. Inactivation of *nra* resulted in decreased host cell viability and increased adherence and internalization, as well as the ability to escape phagocytic vacuoles quicker than *nra*-positive strains (120).

Transcription of *nra* is highest during the early stationary phase, and unlike RofA is unaffected by aeration conditions (127). While Nra is a negative regulator of *mga*, *mga* appears to be a positive regulator of *nra* expression; this would therefore make *mga* an indirect suppressor of *nra*-regulated genes (127). This may be important in the transition between the exponential and stationary phases of growth (127). Such interactions suggest a role for certain factors during growth, with an equally important role for other proteins as the cells enter stationary phase.

4.7. Sag/Pel

Transposon mutagenesis identified a region on the chromosome of a M-type 49, opacity factor positive, strain of *S. pyogenes* that showed altered expression of both surface and secreted proteins (241). The insertion disrupted the transcription of the *pel* RNA (pleiotropic effect locus) located within the promoter region of *sag*A, hence the designation of the region as *sag*A/*pel*. *In vivo* analysis indicated the insertion was in a positive regulatory site, rather than just insertion into the promoter region (242). Examination of other strains indicated the *pel* region was present in different serotypes, and in both opacity factor positive and opacity factor negative isolates. Disruption of the region resulted in phenotypic changes, involving both secreted (SpeB, Ska and hemolysin) and surface-associated proteins (M- and M-related proteins, fibrinogen-binding and IgG-binding proteins). Transcription of *spe*B and *emm* genes was absent in *pel* mutants, *sod* (superoxide dismutase) transcript levels decreased to half, while *ska* was reduced 2-4 fold. However, the levels of transcript for *prt*F2 were unchanged. Introduction of the *pel* gene into a wild type strain resulted in an increase in the level of *emm* transcript (241) indicating a positive regulatory effect. This suggests that either *pel* RNA or the protein encoded by this gene acts as a global regulator, or it at least interacts with some other regulatory system.

In vivo studies using a *sag*A/*pel* mutant indicated that such a mutant was less virulent in a mouse infection model (242). Of note in this study was that some isolates obtained from the kidney of an infected mouse had reverted back to being beta-hemolytic, and showed some fibrinogen and fibronectin binding activity. In these 'hemolytic revertants' the transposon insertion was still present, and the strains still lacked the ability to produce streptokinase and SpeB (242). A similar result, reversion without a change in the position or orientation of the insertion, had been observed with an *mga* mutant (79). Such results suggest a complex regulatory network in *S. pyogenes* in which the organism is still capable of causing disease despite mutations in key locations.

4.8. RelA-associated regulation

Amino acid starvation triggers a series of events that leads to the modulation of gene expression. This response, known as the stringent response, is mediated by the accumulation of ppGpp (guanosine 5'-diphosphate 3'diphosphate) produced by either RelA or SpoT ((p)ppGpp synthetase). Whether ppGpp plays a role in the virulence or regulation of virulence associated gene in *S. pyogenes* is not known, but this compound was shown to accumulate following amino acid starvation (314).

The milieu in which group A streptococci multiply is a rich source of protein although the concentration of amino acids may be low. *S. pyogenes* appears to have evolved a response network that enables it to respond to this protein-rich environment of the host. In contrast to the synthesis of ppGpp in the stringent response, the *rel*A-independent response to amino acid deprivation involves regulation of a number of different genes,

including some virulence factors (287, 315). These studies were conducted by comparing expression of wild-type strains with those in which the *rel*A gene involved in the stringent response [(p)ppGpp-mediated] is inactivated.

Virulence associated factors upregulated by amino acid starvation included the regulators *cov*R/*cov*S and the *fas* operon, the streptolysin S operon and the gene encoding *lux*S, the autoinducer-2 production protein gene (287). By contrast, the *mga* gene was not differentially affected by amino acid starvation (315). A common feature seen in the *fas* and *sag* operons was the production of full-length transcripts under amino acid starvation conditions. For the *sag* operon, amino acid starvation resulted in the production of full-length transcripts containing all the genes associated with SLS production, without altering the level of *sag*A transcript (287). This suggested that stimulation of read-through transcription during starvation is a feature of the *sag* terminator.

The *fas*BCAX operon regulates fibronectin and fibrinogen binding, expression of SLS and streptokinase in a growth dependent manner that shows differential transcription modes dependent on the availability of amino acids. CovR/CovS, another regulatory system, is also upregulated by amino acid starvation with increases of 3-5 fold over unstarved controls. This up-regulation resulted in increased repression of *ska* as well as *spe*B (315). In addition to being negatively controlled by CovR, *spe*B is also positively regulated by Rgg (which is also known as RopB) (192, 288). RopB expression is also increased, albeit moderately, in starved cells. The exact mechanism of action of these two competing systems is not fully understood, but the effect of RopB is to minimize the up-regulation of *cov*R during starvation. However, since the overall effect of amino acid starvation on *spe*B expression is repression, this suggests that *cov*R/*cov*S transcription is stimulated more than *rgg* transcription (315).

The interrelationships that exist between different regulators and amino acid starvation suggest a system that allows *S. pyogenes* to make the most out of nutrient rich environments, but also to be able to adjust synthesis of macromolecules in relation to the supply of amino acids (287). It is also probable that the use of multiple mechanisms allows for the modulation of virulence factors for maximal benefit to the invading organism in the different environments in which it is going to find itself.

4.9. RocA

A complex regulatory network controls expression of CovR/CovS with a number of factors affecting expression. To add to this complex, another regulator known as RocA (regulator of CovR) has recently been described (316); this gene identified as *spy*1605 from the chromosome sequence has some homology to sensor kinases. This gene product activates *cov*R/*cov*S, such that inactivation of *roc*A results in a decrease in the levels of CovR and subsequently an increase in the expression (derepression) of *has* transcription. This regulation appears to be by some indirect route since the RocA sequence does not appear to be a DNA binding protein. This indirect regulation is dependent on the presence of functional CovR. In addition to activating *cov*R expression, RocA also negatively regulates its own expression, the manner (directly or indirectly) of which is still to be determined (316).

5. SUMMARY AND PERSPECTIVE

S. pyogenes has in its arsenal a diverse variety of extracellular products, the functions of which are still, in many cases, under investigation. We may know and understand the biochemical properties of some of these proteins and even have insight into their role in streptococcal virulence. However, the manner in which many of the different factors relate to one another is still unclear. Similarly, we still lack a complete understanding of how these virulence factors are regulated, not only *in vitro* but also *in vivo*. It is unknown whether every factor is necessary for the organism to survive and cause infection and what stimuli from the host environment are responsible for the up and down regulation of expression.

Virulence factors of *S. pyogenes* can be divided into broad categories based on their possible roles such as adhesion (collagen, plasmin/plasminogen and fibronectin binding proteins, GRAB, Lsp, and CD15s-related antigens), host response avoidance (M-protein, immunoglobulin binding proteins, C5a peptidase, hyaluronic acid capsule, Sic, and IdeS), spread and tissue destruction (SpeB, Ska, DNases, hyaluronidases), and those associated with toxicity (streptolysins O and S, and superantigens and pyrogenic exotoxins). Some of these factors may be involved in the disease process at discrete steps, yet others (e.g. SpeB) could have effects at multiple stages. *S. pyogenes* potentially comes in contact with a range of cell types and tissues during an infection. In addition some bacterial cells are able to survive inside host cells following invasion. We do not know what happens to the bacterial cell in these different environments, therefore a better understanding of what this organism does under these different conditions is needed.

How does *S. pyogenes* switch phenotypes (with respect to virulence factors produced) and what triggers the switch? We know that organisms of the same serotype are able to cause diseases ranging from an asymptomatic throat infection to necrotizing fasciitis and the delayed immunological sequelae of rheumatic fever and glomerulonephritis. Which virulence factors are involved in the various protean manifestations of a streptococcal infection still requires further investigation; some are better understood that others. It is likely that only certain factors are produced at one particular time. Information is now being obtained on the expression of some factors *in vivo*, but a lot still remains to be determined.

The genomic approach to the study of bacterial pathogenesis has opened up new avenues of investigation by allowing us to look at genome wide expression. However, some of the proposed virulence factors still need to be examined at a single protein level in order to ascertain any role in pathogenesis before their precise place in

streptococcal virulence can be determined. Beyond the basic scientific interest in a particular factor and its involvement in disease pathogenesis, studies into the virulence of *S. pyogenes* will have the added benefit of providing alternative ways to treat streptococcal infections. Currently *S. pyogenes* is still sensitive to most of the commonly used antibiotics. However what will happen to treatment regimens when this is no longer the case? A greater understanding of what aspects about the organism allow it to so successfully cause disease is needed, and in doing so alternative mechanisms for preventing colonization, disease, or at least the adverse effects induced by *S. pyogenes* may be found. Perhaps we can stop the organism from progressing beyond causing a sore throat, thereby eliminating the more severe diseases. But the microbe alone does not define the nature of streptococcal infections since the host also plays a key role in the process, particularly in those delayed sequelae. Even when we understand the virulence factors it will still be necessary to determine what role the host has in this process, and how or if the organism uses the host response to its advantage.

The host response to contact with, or infection by, *S. pyogenes* is a key element in determining what genes are turned on or off in the bacterial cell. It is no surprise that host cells influence expression of virulence factors (206, 249). The details of the overall effect must still be elucidated. Does the host affect result in the turning on or off of one key element or does it influence a variety of pathways? To date, a number of studies have looked at the regulation of the virulence factors, but there are still many outstanding questions that need to be addressed (279), including what cues the bacterial cell is detecting from the host. In addition to those directly involved in determining how the organism regulates its important virulence factors, other questions regarding the big picture of an integrated network of regulators and virulence factors must be answered. What is at the core of this integrated network? Is there one global regulatory molecule that somehow influences all the different networks, or is each network controlled independently? So far, there is no evidence for a single regulatory control protein. If anything the opposite effect is seen, with multiple regulators controlling a number of different factors. In some cases the enzyme product (e.g. SpeB) of one regulated gene affects expression of other regulators, as well as virulence factors. When one looks at the regulated virulence factor genes, and some of the regulators themselves, many are negatively regulated. Does this suggest that *S. pyogenes* has an evolutionary interest in trying to repress synthesis of these factors so it can live in harmony with us as a host, or is the organism waiting for the opportunity to detect some as yet unknown signal that unleashes its full pathogenic potential? Alternatively, this could represent a situation where the cell is trying to find the most hospitable environment for its survival with the regulated virulence factors being involved in balancing opposing pressures in the environment. One example of this paradox is in cells producing a hyaluronic acid capsule as well as carrying the gene for hyaluronate lyase production. The benefit to the organism of producing an antiphagocytic capsule and then later producing an enzyme that destroys this protective mechanism is not readily apparent. This suggests the need for some type of coordinated regulatory control. However, hyaluronate capsule synthesis is lost during the stationary phase of growth, an effect due to absence of synthase activity at this stage of growth rather than production of hyaluronate lyase (317). Earlier results had suggested a role for a membrane bound hyaluronate lyase (318). It could be that during the early stages of infection the capsule plays an antiphagocytic role, whereas later in the infectious process this protective mechanism is met by M-protein exposed following loss of or destruction of the capsule. This suggests that the lyase, if not involved in capsule turnover, could function as a spreading factor for bacterial products. Other conflicts also occur such as the production of virulence-associated proteins that are cleaved by another virulence factor, the protease. One example would be the need for M-protein in production of the mature protease, with the protease then acting on and cleaving the M-protein. This may represent a way of 'confusing' the immune system; antibodies to M-protein may coalesce on the protein released from the cell therefore not giving other aspects of the immune response (phagocytic cells, complement) the opportunity to destroy the bacterial cell.

Despite years of study, by many investigators, there are many unanswered questions regarding the pathogenesis and virulence of the group A streptococci. Although great progress has been made in understanding this organism, there is still a lot of information to be determined in terms of the virulence factors of the organism, their regulation, and how all these various components relate to one another. Understanding these relationships will provide information on the pathogen, whose sole host is man, and its ability to cause disease, and ultimately how to control infections by *S. pyogenes*.

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