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Coregulation of Type 12 M Protein and Streptococcal C5a Peptidase Genes in Group A Streptococci: Evidence for a Virulence Regulon Controlled by the *virR* Locus

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Group A streptococci express at least two surface-associated virulence factors, the antiphagocytic M protein and the antichemotactic streptococcal C5a peptidase (SCP). Preliminary evidence suggested that the biosynthesis of these two proteins is coordinately controlled and subject to simultaneous phase variation. To explore this possibility further, a series of phase-switching and phase-locked M^- variants were assayed for SCP by enzyme-linked immunosorbent assay inhibition and for SCP-specific mRNA by dot blot hybridization. All M^- cultures produced diminished amounts of SCP antigen and specific mRNA, whereas revertants produced quantities equivalent to those of the wild-type M^+ culture. A phase-locked strain that harbors a deletion in a region upstream of the M12 and SCP genes, termed the *virR* locus, failed to produce SCP antigen or SCP-specific transcripts. The SCP-specific transcript produced by M^+ bacteria was shown by Northern (RNA) blot hybridization to be 4 kilobases in size, distinguishing it from the transcript which encodes M protein. These data demonstrate that phase switching of both SCP and M12 proteins is at the transcriptional level and that expression is under the control of the upstream *virR* locus. We propose that the genetic determinants of these proteins and of colony morphology comprise a virulence regulon.

The survival and multiplication of bacterial pathogens in a susceptible host depend on their abilities to acquire appropriate nutrients, interact with tissue receptors, and resist host defenses. A recent review (10) noted that the expression of multiple virulence determinants by gram-negative pathogens is a finely tuned process which is responsive to environmental changes and the general physiological state of the bacterial cell. It is now apparent that these virulence genes are globally controlled by a master gene which couples external stimuli to response pathways (10).

The avoidance of human immunological defenses by group A streptococci is dependent on the following surface components: a diffuse hyaluronic capsule which mimics the ground substance of animal tissues (25); immunoglobulin G Fc receptors, which may disrupt recognition by antibodies (6b); a C5a peptidase which destroys chemotactic signals (23); and M protein, which interferes with the deposition of C3b opsonin to prevent phagocytic uptake (8, 9). M protein, thought to be the key determinant of virulence, has been the subject of intensive investigation. This dimeric coiled-coil molecule protrudes from the cell wall, where it both blocks the deposition of C3b opsonin and limits the interaction of bound C3b with receptors on polymorphonuclear leukocytes (8, 9). As a result, M^+ streptococci are resistant to phagocytosis in the absence of type-specific M protein antibody and complement.

Group A streptococcal cultures have long been recognized as genetically unstable (6, 17, 20). The expression of M protein on their surfaces, colony morphology, and virulence were reported to vary dramatically both in laboratory cultures (6, 17) and in streptococci isolated from convalescing patients (16). Concurrent with the transition from opaque to transparent colony morphology is diminished M protein expression (M^-) (17). The high frequency and pleiotropic nature of this variation prompted us to search for a programmed genetic switch that controls colony morphology and M protein expression. A systematic analysis of M^- variants from an $M12^+$ culture, CS24, revealed that this strain undergoes true phase variation and that the on-off states of M protein expression are controlled at the transcriptional level (4, 10, 17). A few stable (phase-locked) M^- variants which were isolated in this study harbored deletions 5′ to the M12 protein-coding sequence. On the basis of these results and deletion studies of a cloned M12 gene (*emm*12) and flanking DNA (4, 15, 17, 18), we proposed that at least one additional gene, henceforth called *virR*, was required for efficient transcription of the M protein gene. Our conclusion was later confirmed by transposon Tn*916* mutagenesis by Caparon and Scott (1).

Wexler et al. discovered that group A streptococci avoid the phagocytic defenses at yet another level (24). These bacteria eliminate the chemotactic signal, C5a, which directs leukocytes to the site of infection (13). C5a is cleaved in the leukocyte-binding site by a highly specific peptidase (23). This enzyme, the streptococcal C5a peptidase (SCP), is also located on the surfaces of virulent streptococcal cells (12). Although M^- streptococci were not studied in detail at the time, we recognized in preliminary experiments that they also produced less SCP (3, 24). More recent experiments showed that the SCP gene, *scpA*, is located downstream from the M protein gene in two different serotypes, M12 (2) and M49 (6a), and is transcribed in the same direction as the M protein genes.

The findings described above are consistent with the possibility that the genes which encode these surface proteins and determine colony morphology belong to a common regulon or operon. Here we test whether *emm*12 and *scpA* compose a regulon which is under the control of *virR* and subject to coordinated phase variation.

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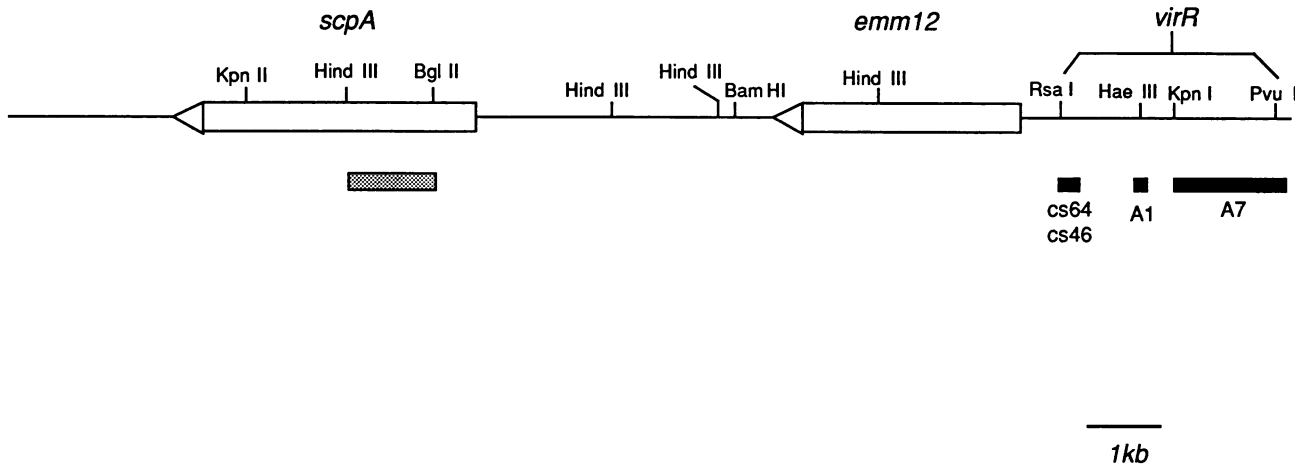

FIG. 1. Genetic map of *scpA*, *emm*12, and *virR*. Symbols: ◁□, boundaries of *scpA* and *emm*12; ■, deletions which define the *virR* locus; ▩, *scpA* insert carried by plasmid pTT45, which was used as the probe for *scpA*-specific mRNA.

MATERIALS AND METHODS

Bacterial strains. The M12 group A streptococcus, strain CS24; its M^- derivatives, strains A7, A13, A14, A17 and C14; and an M^+ revertant of strain A14, strain B14, have previously been described (17). The following additional group A streptococcal strains were used in this study: strain CS101 is type T14, M49 (9); strain CS159 is type M14 SCP^- from our collection; strain CS215-14 is an SCP^- strain produced by nitrosoguanidine mutagenesis (13).

Streptococci were grown to stationary phase in Todd-Hewitt broth supplemented with 2% neopeptone (Difco Laboratories, Detroit, Mich.). *Escherichia coli* cultures harboring recombinant plasmids were grown to late log phase in Luria broth containing 50 μg of ampicillin per ml. Phase-switching strains were enriched for cells which form either opaque (Op^+) or less-opaque (Op^-) colonies by culturing cells on Islam medium under anaerobic conditions (17).

Nucleic acid analysis. Genomic DNA from streptococci and recombinant plasmid DNA were prepared by standard methods (17). Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and digestions were performed as recommended by the supplier. The SCP-specific DNA probe was a restriction fragment corresponding to an internal *Bgl*II-*Hin*dIII fragment of the SCP gene (2). The fragment was isolated from agarose gels by electrophoresis onto NA45 paper (Schleicher & Schuell, Inc., Keene N.H.) and labeled with [α-^{32}P]dATP by nick translation (Nick Translation Kit; Bethesda Research). The extraction, purification, and analysis of total RNA from streptococci by Northern (RNA) blot and dot blot procedures were performed as previously described (17).

Extraction and immunoblot analysis of M and SCP antigens. SCP antigen associated with intact M^+ and M^- cells was quantified by an enzyme-linked immunosorbent inhibition assay (EIA) (13) with rabbit anti-SCP serum, as previously described (23). The M phenotype of streptococcal strains was previously determined (17). Streptococcal cell-associated antigens were released under conditions which prevent protoplast lysis and processed for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as follows. Cells from 100-ml stationary-phase cultures were washed twice in 0.2 M sodium acetate (pH 6.0) and suspended in 5 ml of TEG buffer (100 mM Tris [pH 7.0], 10 mM EDTA, 25% [wt/vol] glucose). Mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 0.2 mg/ml, and the cells were digested for 1 h at 37°C. Protoplasts were removed by centrifugation, and released supernatant proteins were concentrated by lyophilization. Total protein was quantitated by the BAC method (Pierce Chemical Co., Rockford, Ill.).

Western blots (immunoblots) of proteins fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were assayed for the appropriate antigens, as previously described (13). Membranes were treated with either M12- or SCP-specific antiserum followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase and were developed with the Nitro Blue Tetrazolium–5-bromo-4-chloro-indolyl-phosphate substrate system.

Controls showing the absence of nonspecific binding of either the primary or the conjugated antibodies were routinely performed.

RESULTS

Expression of SCP by M^+ and M^- variants. The expression of M12 protein exhibits phase variation (17) and depends on a genetic locus, *virR*, located 5′ to the M12 protein-coding sequence (Fig. 1) (4, 15, 17). Previous studies reported that M^- cultures expressed reduced levels of SCP antigen, suggesting that the antigen may be coregulated with M protein biosynthesis (3, 24). To test the possibilities that SCP undergoes phase variation in concert with the M12 antigen and that full expression also depends on the *virR* locus, the amounts of SCP antigen associated with isogenically related M^- and M^+ variants of strain CS24 were determined (Table 1). Strains A14 (M^-), B14 (M^+), and C14 (M^-) represent an M12 phase-switching lineage serially isolated from wild-type strain CS24 (17). The levels of SCP antigen associated with variants A14 and C14 were below the sensitivity of our assay. On the other hand, strain B14, which is an $M12^+$ revertant of strain A14, expressed levels of SCP equivalent to those of wild-type cells. These data indicate that SCP expression in strain CS24 switches on and off in phase with M protein expression.

In contrast to strain A14, strains A1 and A13 are M^- phase-locked variants of strain CS24; i.e., they do not revert to the M^+ state (17). Strain A7 harbors a deletion in the putative *virR* regulatory locus (Fig. 1) and does not produce detectable quantities of SCP antigen (Table 1). Strain CS215-

TABLE 1. Quantitation of SCP antigen associated with M^- or M^+ phase variants of strain CS24 by EIA

Strain	Phenotype	Deletion in *virR*	SCP ($ng/10^8$ cells)[a]
CS24	$M12^+$	–	22.5
A14	$M12^-$	–	<4.5
B14	$M12^+$	–	21.3
C14	$M12^-$	–	<4.5
A7	$M12^-$	+	<4.5
A13	$M12^-$	–	<4.5
CS101	$M49^+$	–	35.7
CS215-14	$M18^+$	–	<4.5

[a] Amounts of less than 4.5 $ng/10^8$ cells could not be reliably detected in this assay.

14, an SCP^- M^+ mutant produced by nitrosoguanidine mutagenesis, was included as a negative control.

Crude mutanolysin extracts of strains CS24 and A7 (Fig. 2) were analyzed by Western blot to ensure the specificity of the rabbit anti-SCP serum used in the SCP assays described above. Anti-M12 serum reacted with a 60-kilodalton protein approximately the size of the M12 protein (21), whereas anti-SCP serum reacted primarily with a protein of 140 kilodaltons, the SCP protein (23). Extracts from M^- cells revealed only faint bands corresponding to these two proteins. This experiment confirmed that the amount of SCP antigen associated with M^- cells is considerably reduced and that the SCP-specific rabbit serum used in the EIA described above does not react with M12 protein. Control blots with rabbit and goat alkaline phosphatase conjugates alone did not react with proteins on the blot, indicating that the antigens detected by EIA (Table 1) or Western blot (Fig. 2) resulted from specific interactions with antibody rather than from nonspecific interactions with immunoglobulin G Fc receptor (data not shown).

Quantitation of SCP-specific mRNA. In previous reports, we established that the M^- state, whether the consequence of phase variation or inactivation of the *virR* locus by

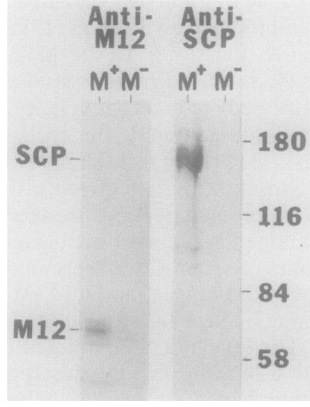

FIG. 2. Western blot analysis of mutanolysin extracts of $M12^+$ and $M12^-$ streptococci. Mutanolysin extracts (total protein, 50 μg) from strains CS24 (M^+) and A7 (M^-) were subjected to electrophoresis in sodium dodecyl sulfate–12.5% polyacrylamide gels at 15 mA for 5 h. Proteins were blotted onto nitrocellulose at 30 mA for 12 h. The nitrocellulose was then cut into strips, and each strip was exposed to a primary antibody, as follows: rabbit anti-M12 serum, 1:400 (18); rabbit anti-SCP serum, 1:2,000 (13, 23); goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase, 1:1,000. Molecular size markers (right) are in kilodaltons.

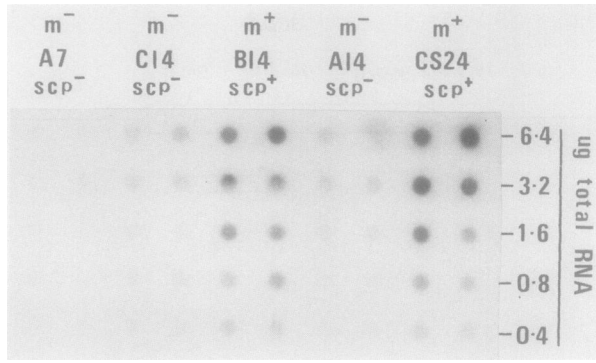

FIG. 3. Quantitation of SCP mRNA in M^+ and M^- phase variants. Duplicate serial dilutions of total RNA were spotted onto nitrocellulose and probed with radiolabeled DNA specific for *scpA*. This probe was a 1.3-kb *Hin*dIII-*Bgl*II fragment internal to *scpA* which was cloned from strain CS24.

deletion mutation, reflects reduced transcription of the *emm*12 gene (15, 17). To determine whether the SCP^- phenotype also results from down-regulated transcription of *scpA*, total RNA from M^- bacteria was assayed for SCP-specific mRNA by dot blot analysis (Fig. 3). The DNA probe used to detect *scpA*-specific mRNA in dot blot and Northern blot assays was a 1.3-kilobase (kb) *Hin*dIII-*Bgl*II fragment carried by plasmid pTT45, which is internal to *scpA* (Fig. 1) (2).

The M^+ strains, CS24 and B14, produced nearly equivalent amounts of SCP mRNA. In contrast, the unstable M^- strains, A14 and C14, produced 10- to 20-fold less *scpA* mRNA. The small quantity of *scpA* mRNA detected is presumed to be from M^+ revertants habitually present in these unstable M^- cultures (17). In contrast, strain A7, which harbors a deletion in the *virR* locus and does not revert to the M^+ state at a detectable frequency, produced an undetectable amount of SCP-specific mRNA under these conditions.

Northern blot analysis of SCP mRNA. The data reported above strongly suggest that *emm*12 and *scpA* are coregulated at the transcriptional level by the *virR* locus. The close linkage of *emm*12 and *scpA* is compatible with the possibility that both are transcribed from a common promoter into a single polygenic mRNA. To test this possibility, the size of the *scpA* transcript was determined by Northern blot (Fig. 4). The 1.3-kb *Hin*dIII-*Bgl*II fragment, internal to *scpA*, hybridized to three RNA species; the predominant species is approximately 4.0 kb in size. This size is consistent with the estimated molecular size of the SCP protein (140 kilodaltons) and indicates that the SCP message is most likely monocistronic. The two other hybridizing species, 3.2 and 1.6 kb in size, more than likely correspond to breakdown products of *scpA*-specific mRNA physically trapped by rRNAs banding at these positions. As expected, the $M12^-$ SCP^- strain, C14, lacked detectable specific mRNA. The larger size of SCP mRNA clearly distinguishes it from that of *emm*12, which has been shown to be 2 kb (15). These findings suggest that *scpA* and *emm*12 are components of a virulence regulon which is controlled by the *virR* locus in some unknown manner.

DISCUSSION

Our laboratory discovered that virulent group A streptococci express an inhibitor of serum-mediated chemotaxis

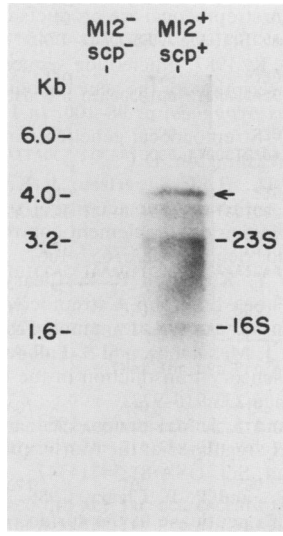

FIG. 4. Northern blot of M12$^+$ and M12$^-$ total RNAs. RNA from M$^+$ cells was from strain CS24 variant B14; RNA from M$^-$ cells was from strain CS24 variant C14 (17). The probe was the same as that used for Fig. 3. RNA molecular weight markers were obtained from Bethesda Research Laboratories, and their mobilities are indicated on the left in kilobases.

(SCP) (3, 23, 24). SCP is a cell surface-associated peptidase which specifically cleaves C5a at the polymorphonuclear leukocyte-binding site, thereby eliminating the chemotactic gradient on the bacterial surface where it has formed (12). Thus, these streptococci can block the phagocytic response at two levels: SCP precludes surveillance (13), and M protein prevents opsonization (8, 9).

The genetic instability of the M$^+$ phenotype has been documented elsewhere (6, 20), and more recently, M$^+$ bacteria have been shown to undergo a high frequency of reversible transitions between the M$^+$ and M$^-$ states (17). Furthermore, the transcription and full expression of *emm*12 (15, 17) and *emm*6 (1) have been shown to depend on a linked genetic locus which spans 0.4 to 1.4 kb of DNA 5′ to the protein-coding sequence. The mechanism by which this locus, which we have termed *virR*, promotes the transcription of M genes and its role in phase variation are not known.

In a preliminary study, we reported that M$^-$ strains of group A streptococci produced less SCP than their M$^+$ counterparts and suggested that SCP may be coregulated with M protein (3). Chen and Cleary showed that *scpA* and *emm*12 are nonoverlapping genes which map within 2 kb of each other on the streptococcal chromosome (2). The present study was initiated to explore the possibility that *scpA* and *emm*12 belong to a common regulatory unit which is subject to phase variation and dependent on the *virR* locus for transcriptional activation (Fig. 5).

To investigate this possibility, we analyzed well-characterized M protein phase variants of the serotype M12 strain, CS24 (17). Mirroring the expression of M protein, the M$^+$ cells had surface-associated SCP protein, whereas M$^-$ cells had significantly reduced quantities of this protein. The frequency of transition between the on and off states ranged from 10^{-4} to 10^{-3}, depending on the strain and direction of transition (17); therefore, unstable M$^-$ cultures are persistently mixtures of M$^+$ and M$^-$ cells. This is reflected in the quantitative data, since phase-switching M$^-$ cultures always produce low but detectable M (17) or SCP antigens and mRNAs.

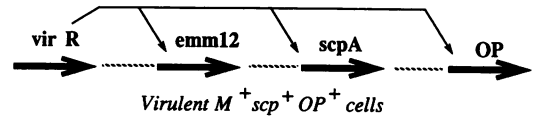

FIG. 5. Genetic model for coordinated expression and phase variation of group A streptococcal surface proteins. A protein product of the *virR* locus is postulated to be required for the expression of antiphagocytic M proteins, antichemotactic peptidase, and other products of undefined genes which determine colony morphology.

Like transcription of the *emm*12 gene (15, 17), transcription of *scpA* was found to be down-regulated in M$^-$ cells (Fig. 3). The size of the *scpA* transcript was found to be 4.0 kb (Fig. 4), which is twice the size of the *emm*12 transcripts (15) and consistent with that expected for a protein of 140 kilodaltons. These results suggest that M and SCP proteins are translated from separate mRNAs but do not rule out the possibility that both RNAs are products of a processed larger RNA species. Moreover, our data do not eliminate the possibility that diminished levels of M12- and SCP-specific mRNAs reflect a shortened half-life in M$^-$ cells.

For the activities of SCP and M genes to vary simultaneously between two states at such a high frequency (10^{-4} to 10^{-3}), they must share a common regulatory circuit. The deletion of DNA upstream of *emm*12 reduced the expression of both M12 and SCP antigens. A previously described M$^-$ variant, strain CS64, has a 50-base-pair deletion in the *virR* locus (4, 15, 18) and was reported to be deficient in SCP production (3). The M$^-$ phase-locked strain, A7, which harbors a deletion in *virR* (Fig. 1), also did not produce detectable levels of SCP mRNA. Another phase-locked M$^-$ variant, strain A13, had no detectable rearrangement in the DNA upstream of *emm*12, yet this strain also produced diminished quantities of SCP antigen. On the basis of these results, we propose that *emm*12 and *scpA* compose a regulon which is controlled by the *virR* locus. Moreover, we suggest that this locus couples the expressions of these genes and possibly others, such as determinants of colony opacity (17) and hyaluronic acid biosynthesis (25), to the phase switch (Fig. 5).

Experiments to determine whether the *virR* locus acts in *cis* or encodes a *trans*-acting gene product are in progress. The *virR* locus was first defined by deletion mutations in phase-locked variants of strain CS24 (4, 15, 17, 18) and is probably identical to the *mry* locus more recently defined by a single Tn*916* mutation (1). To conform to established convention in other systems, however, we suggest that this locus be termed *virR*, because it controls the expression of at least two virulence factors. In other bacteria, products of *virR* genes or their equivalent control the expression of a variety of extracellular proteins in response to changes in their chemical environments (10). The effects of changes in ionic strength or other physical parameters on the expression of M protein or SCP have not been studied. For most bacterial pathogens, virulence depends on surface-associated or secreted macromolecules or both. Although studies of gram-negative bacterial pathogens provide many examples of a single genetic locus which coordinately controls multiple virulence factors (11, 22, 26), evidence for the global regulation of virulence in gram-positive bacteria is limited. In gram-positive bacteria, the *agr* locus in *Staphylococcus aureus* regulates several exoproteins associated with virulence (14).

Covariation of SCP and M protein expression is likely to

be a general property of group A streptococci and not limited to strain CS24. The quantity of SCP antigen associated with three other serotypes and their M^- derivatives was compared in an earlier study (3). In that study, M^- variants were selected by their glossy colony morphology and were shown by their sensitivities to phagocytosis and lack of extractable M antigen to lack M proteins (3). In each case, the M^- variants had less SCP than their M^+ counterparts.

With regard to group A streptococci, it may be fortuitous that *virR*-dependent genes encode virulence factors. Their physical locations on the cell surface may, in fact, be more important. Cell wall growth and the deposition of surface-associated proteins in streptococci are rigidly controlled and localized to the septa of dividing cells (19). Therefore, biosynthesis of SCP and M proteins may have to be coordinated with cell wall growth.

An important unanswered question is whether phase variation contributes to the infectious process. In early studies, it was shown that convalescent patients often shed M^- streptococci for a long time (16). Perhaps it is advantageous for streptococci to be free of surface proteins at later stages of infection for survival in an immune host or to infec' a particular niche which is essential for their continuous dissemination in human populations. Advances in our understanding of the molecular basis of phase variation in a variety of pathogenic bacteria may provide the answer to this question.

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