

Virulence News & Views

The function of the bacterial cytoskeleton in *Salmonella* pathogenesis

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Recent studies have identified cytoskeletal elements in bacteria which play important roles in cellular morphology, cell division, DNA segregation and the establishment of cell polarity. However, our understanding of the contribution the bacterial cytoskeleton makes toward virulence is lacking. The MreB protein is a bacterial homolog of eukaryotic actin and interacts intimately with MreC and MreD. We provide evidence that the Mre-based cytoskeleton directly influences pathogenicity in *Salmonella*. Disruption of MreC and MreD caused the downregulation of the *Salmonella* pathogenicity island 1 (SPI-1) type 3 secretion system (T3SS) essential for host cell-invasion, and also flagella-mediated motility. These phenotypic effects were mediated by RcsC, the sensor kinase of the Rcs phosphorelay two-component system and a major envelope stress regulator. Curiously, the SPI-2 T3SS remained viable. Our results demonstrate the importance of the integrity of the bacterial cytoskeleton for virulence, highlighting the impact of associated global regulatory mechanisms on pathogenicity.

Structural homologs of all three major eukaryotic cytoskeletal protein classes are now known to exist in bacteria. These homologs include MreB and ParM (actin), crescentin (intermediate filaments) and FtsZ (microtubules). Ongoing research seeks to understand the mechanisms by which the bacterial cytoskeleton and associated proteins control cell shape, through their control of synthesis of the stress-bearing peptidoglycan cell wall (Shih and Rothfield, *Microbiol Mol Biol Rev* 2006).

To date six essential cell shape-determinant proteins have been identified in non-spherical bacteria: MreB, MreC, MreD, penicillin binding protein 2 (PBP2), RodA and RodZ. The chemical or genetic inactivation of any of these proteins causes a loss of rod-shape and, in the absence of compensatory mutations, eventual cell-lysis. The cell shape-determinant proteins are thought to form a large multi-protein complex together with the peptidoglycan synthetic

enzymes, to coordinate longitudinal cell wall synthesis and hence cell shape (Bendezú and de Boer, *J Bacteriol* 2008; van den Ent et al., *EMBO* 2010). The cytoskeletal MreB protein was believed to form long helical scaffold-like filaments along the length of the cell, directing the positioning of the peptidoglycan synthetic enzymes to spatially regulate cell wall synthesis. However, recent studies that used a sensitive total internal reflection fluorescence microscopy technique (TIRFM) to study MreB filament dynamics suggested that MreB and associated proteins in fact form short dynamic filaments that move around the cell perpendicularly to its long-axis (Dominguez-Escobar et al., *Science* 2011; Garner et al., *Science* 2011).

Bacterial cytoskeletal proteins are recognized for their roles in defining cellular morphology, division and polarity (Shih and Rothfield, *Microbiol Mol Biol Rev* 2006). However, we have little knowledge on how the bacterial cytoskeleton, peptidoglycan-synthetic complexes and cell shape may influence pathogenicity. Bacterial pathogens employ many crucial cell envelope-associated virulence factors, since this forms the initial contact with the host. Such factors include macromolecular cell wall-spanning organelles such as flagella or type 3 secretion systems (T3SSs) and multi-protein needle complexes that actively inject effector proteins into the host cell cytoplasm (Cornelis, *Nat Rev Microbiol* 2006; Stecher et al., *Infect Immun* 2004). It is reasonable to suggest that the bacterial cytoskeleton itself, or the peptidoglycan sacculus, may function in directing the assembly, localization and ultimately function of cell wall-spanning virulence organelles.

Many cell wall-spanning virulence organelles exhibit specific localization patterns, which may be influenced by cytoskeletal elements. MreB has been implicated in regulating the polar or side-wall localization of virulence-related proteins, including secretion system complexes, type IV pili and motility proteins

(Shih and Rothfield, *Microbiol Mol Biol Rev* 2006; Mauriello et al., *EMBO* 2010; Cowles and Gitai, *Mol Microbiol* 2010).

The extent to which the bacterial cytoskeleton is involved in the assembly, function and localization of virulence organelles remains to be examined. We characterized the effects of disrupting the bacterial cytoskeleton on the pathogenicity of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), investigating the effects on major virulence factors (Bulmer et al., *PLoS Pathogens* 2012).

Salmonella enterica serovars remain major human pathogens, causing disease ranging from gastroenteritis to life-threatening systemic typhoid fever (Haraga et al., *Nat Rev Microbiol* 2008). Challenges associated with *Salmonella* include the spread of multidrug-resistant strains and the emergence of serious systemic “non-typhoidal salmonellosis,” associated with immunocompromised patients (Chuang et al., *Epidemiol Infect* 2009; Gordon, *J Infect* 2008). *Salmonella* infections are acquired upon ingestion of contaminated food or drink. Virulence is mediated by a variety of factors including two pathogenicity island-encoded T3SSs: “SPI-1” and “SPI-2.” The SPI-1 T3SS is required for the initial invasion of intestinal epithelial cells and macrophages; SPI-2 T3SS is essential for intracellular survival, enabling *Salmonella* to subvert host cell defense mechanisms and replicate within modified “*Salmonella*-containing vacuoles” (SCVs) (Haraga et al. *Nat Rev Microbiol* 2008). Flagella/chemotaxis systems also assist invasion (Asten and van Dijk, *FEMS Immunol Med Mic* 2005).

The MreB, MreC and MreD cell shape-determining proteins are encoded contiguously within the *mre* operon in *Salmonella*. The cytoplasmic membrane proteins, MreC and MreD, may function to bridge MreB and the peptidoglycan synthesis machinery. Evidence suggests that MreC directly interacts with both MreB and MreD (Kruse et al., *Mol Microbiol* 2005; van den Ent et al., *EMBO*

2010). Furthermore, the localization patterns of MreB, MreC and MreD were shown to be similar and interdependent, and the inactivation of MreB, MreC or MreD results in the formation of phenotypically indistinguishable spherical cells (Divakaruni et al., Proc Natl Acad Sci USA 2005; Bendezú and de Boer, J Bacteriol 2008; Bulmer et al., PLoS Pathogens 2012). These observations demonstrate the relation and interdependency in function between the Mre proteins, suggesting that inactivation of any one protein disrupts both the cytoskeleton and longitudinal peptidoglycan synthesis.

In many bacteria *mreB* appears to be essential and viable $\Delta mreB$ mutants can only be generated through acquiring compensatory mutations (Kruse et al., Mol Microbiol 2005; Shih and Rothfield, Microbiol Mol Biol Rev 2006). To study the role of the Mre proteins in *Salmonella* pathogenicity, precise knock-out mutants of both $\Delta mreC$ and $\Delta mreD$ were generated in *S. Typhimurium* and maintained by expressing the *mre* operon in trans from an inducible promoter. Spherical cells were formed upon the depletion of *mre* expression, as expected. These and all other observed mutant phenotypes were fully completable upon restoration of MreC and MreD expression (Fig. 1). We were unable to construct a viable $\Delta mreB$ mutant. However, the chemical inactivation of MreB caused wild-type *S. Typhimurium* to form spherical cells that were phenotypically identical to the $\Delta mreC$ and $\Delta mreD$ mutants. The chemical disruption of MreB was achieved using A22, a drug that specifically binds MreB, inhibiting polymerization (Bean et al., Biochemistry 2009).

Phenotypic screening of *S. Typhimurium* $\Delta mreC$ and $\Delta mreD$ demonstrated that both motility and the SPI-1 T3SS were almost completely downregulated in these mutants; cells were non-motile and did not express the structural or secreted proteins of either the flagella or SPI-1 organelles (Fig. 1). In addition, transepithelial resistance (TER) assays showed that the ability of $\Delta mreC$ cells to disrupt the tight junctions between polarized Caco-2 cells, a feature which is dependent upon SPI-1 functionality (Boyle, Cell Microbiol 2006), was impaired. Fundamentally, expression of both flagella and SPI-1 genes was repressed at the level of the master-regulators, *flhDC* and *hilA* respectively (Apel and Surette, Biochim Biophys Acta 2008; Ellermeier and Slauch, Curr Opin Microbiol 2007).

Few studies have documented or explored virulence defects resulting from the inactivation of cell shape-determinants or the bacterial cytoskeleton. *E. coli* $\Delta rodZ$ cells were previously noted to be non-motile, the genes encoding the flagella structural proteins and flagellin being downregulated. *flhDC* expression, however, remained unaffected (Niba et al., FEMS Microbiol Lett 2010). Another study reported that *flhDC* expression was decreased in *E. coli* cells treated with mecillinam, an antibiotic that specifically inhibits the longitudinal-transpeptidase penicillin-binding protein 2 (PBP2) (Laubacher and Ades, J Bacteriol 2008). However, little is known about the mechanisms responsible for the downregulation of motility in spherical *E. coli* or *Salmonella*.

Surprisingly, the SPI-2 T3SS was expressed and appeared functional in round-cell $\Delta mreC$ and $\Delta mreD$ mutants. SseB is a SPI-2-secreted protein that forms part of the SPI-2 T3SS “translocon” at the needle tip (Nikolaus et al., J Bacteriol 2001). This protein was detected in membrane fractions and visualized at the cell surface, in both wild-type and $\Delta mreC$ cells. These results were reflected in vivo; the $\Delta mreC$ mutant was inoculated intravenously into C57/BL6 mice, to bypass the SPI-1-dependent stages of infection. Although clearly attenuated compared with the wild-type, the $\Delta mreC$ mutant was able to survive and replicate within the host, demonstrating the retention of some virulence and a functional SPI-2 T3SS.

Several hypotheses were formulated to explain mechanistically the observed virulence phenotypes in $\Delta mreC$ and $\Delta mreD$ mutants. For instance, the bacterial cytoskeleton or peptidoglycan layer may physically support multi-protein wall-spanning organelles. Thus an inability of Δmre cells to correctly assemble and localize cell wall-spanning organelles, either directly from the loss of a functional cytoskeleton or through resulting peptidoglycan structural defects, may cause the downregulation of motility and SPI-1. In this case, retention of SPI-2 functionality could relate to the different spatial organization of this T3SS, its localization potentially being controlled by different factors. Alternatively, the activation of stress response pathways resulting from cytoskeletal or cell wall defects, may lead indirectly to the downregulation of virulence factors whose expression is controlled by global stress regulators, or may cause the cell to specifically repress non-essential energetically expensive processes.

In order to determine whether the flagella and SPI-1 defects were due, at least in part, to the physical inability of $\Delta mreC$ mutants to assemble and localize such structures, we attempted to recover SPI-1 and flagella expression in these cells. *flhDC* and *hilA* were therefore each individually expressed in trans under the control of an inducible promoter, releasing the flagella and SPI-1 genes from upstream regulatory mechanisms, and causing the recovery of flagella or SPI-1 gene and protein expression. Surface expression of both the SPI-1 T3SS and flagella organelles was observed in $\Delta mreC$ cells when *hilA* or *flhDC* were constitutively expressed, respectively (Fig. 1). Furthermore, TER experiments and motility assays demonstrated the functionality of these respective organelles. Our results demonstrate that round-cell mutants lacking a functional cytoskeleton, and/or harboring significant cell wall defects, retain the ability to support functional cell wall-spanning organelles.

These insights led us to examine the regulatory mechanisms responsible for the observed $\Delta mreC$ and $\Delta mreD$ virulence phenotypes. Many global regulators responding to environmental stress in *Salmonella* comprise two-component systems. These systems consist of an integral membrane histidine kinase coupled to a response regulator, which upon activation by phosphorylation, directly regulates transcription (Stock et al., Annu Rev Biochem 2000). Upon generating and screening a panel of mutants of major two-component systems in *S. Typhimurium* $\Delta mreC$, we identified a $\Delta mreC$ $\Delta rcsC$ mutant in which significant motility and SPI-1 effector protein expression and functionality were recovered. The restoration of *rcsC* expression in trans in *S. Typhimurium* $\Delta rcsC$ $\Delta mreC$ resulted in a subsequent loss of flagella and SPI-1 expression.

Thus RcsC is an important regulator of virulence in response to the inactivation of *mreC*. The Rcs phosphorelay regulates gene expression when activated by envelope-associated stress. The sensor kinase RcsC activates the response regulator RcsB via the RcsD protein (Laubacher and Ades, J Bacteriol 2008; Huang et al., Res Microbiol 2006). This complex phosphorelay is well known to activate colanic acid biofilm synthesis. However, it also regulates virulence, repressing motility and SPI-1, and activating SPI-2 expression in *Salmonella* (Arricau et al., Mol Microbiol 1998; Garcia-Calderon et al., J Bacteriol 2007). Interestingly,

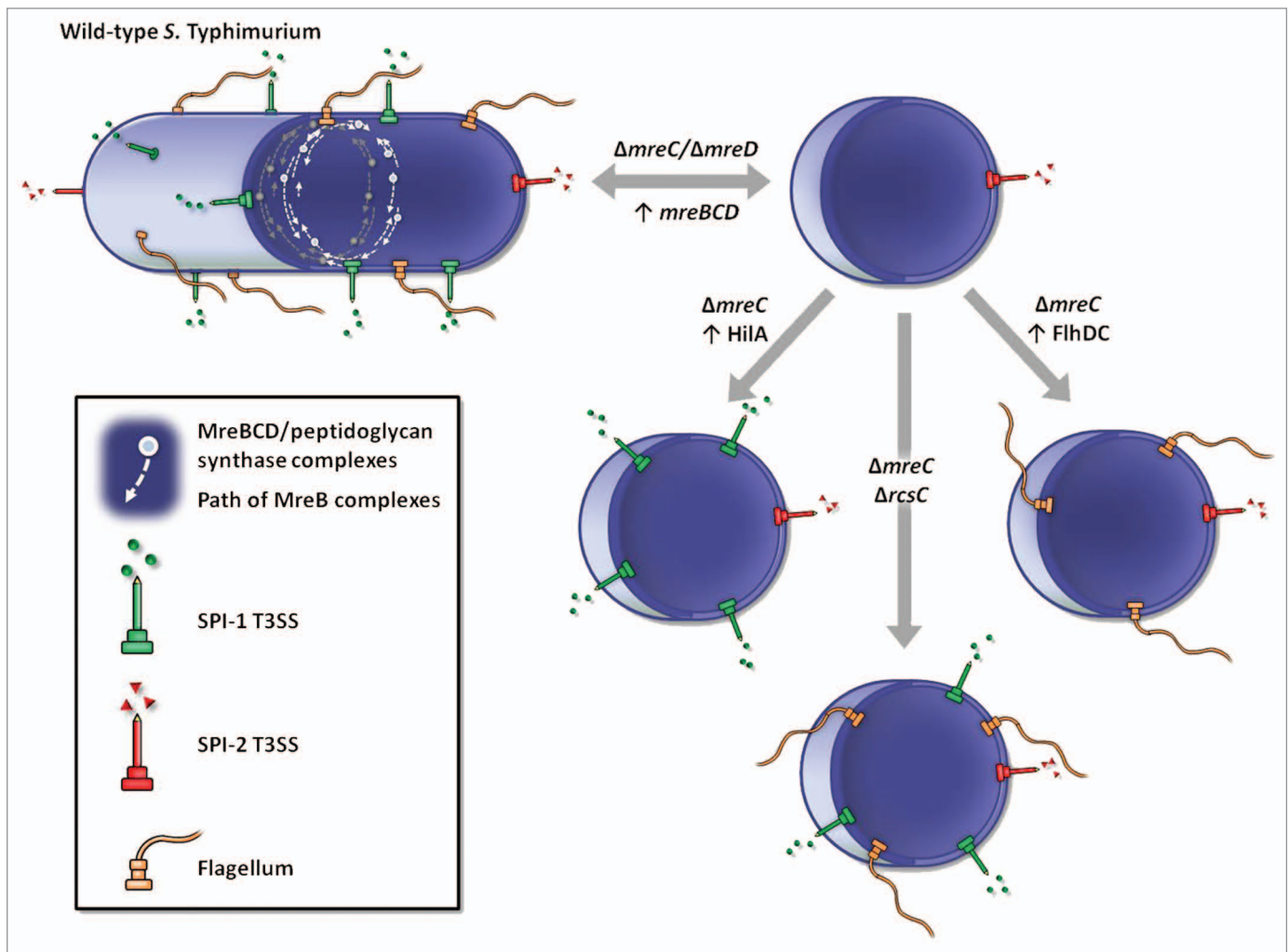


Figure 1. Generation and phenotypic characterization of $\Delta mreC$ mutants of *S. Typhimurium*. Schematic representation of wild-type *S. Typhimurium* expressing the invasion-associated SPI-1 T3SS, flagella for motility, and the SPI-2 T3SS essential for intracellular survival. Wild-type *Salmonella* cells express 1–2 polar SPI-2 needles and approximately 6–8 flagella and SPI-1 needles, distributed predominantly along the lateral cell wall (Bulmer et al., PLoS Pathog 2012). Putative peptidoglycan synthetic complexes comprising the MreBCD cytoskeletal proteins, cell shape-determinants and peptidoglycan synthetic enzymes, are shown as short dynamic complexes moving perpendicularly to the long-axis of the cell. Upon MreC or MreD depletion *S. Typhimurium* formed spherical cells, with a loss of SPI-1 T3SS and flagella expression; SPI-2 expression remained active. SPI-1 T3SS and flagella expression were restored upon recovery of either *mre*, *hilA* (SPI-1 master regulator) or *flhDC* (flagella master regulator) expression respectively, or with the inactivation of *rcsC*, sensor kinase of the Rcs phosphorelay envelope stress response.

meccillinam treatment of wild-type *E. coli* also resulted in a reduction in *flhDC* expression and the activation of several stress response pathways, including the Rcs phosphorelay (Laubacher and Ades, J Bacteriol 2008).

On identifying RcsC as a candidate global regulator of SPI-1 and motility in $\Delta mreC$ mutants, ongoing work aimed to characterize the roles of other members of the Rcs phosphorelay. However, SPI-1 functionality and motility were not recovered in $\Delta rcsB$, $\Delta rcsD$, $\Delta rcsDB$ and $\Delta rcsF$ double mutants of *S. Typhimurium* $\Delta mreC$. This was surprising since the inactivation of any of these genes would be expected to disrupt the system,

preventing RcsB activation and the RcsB-dependent repression of motility and SPI-1 T3SS. RcsF, an outer membrane lipoprotein that activates RcsC in response to peptidoglycan defects, would also be expected to specifically activate RcsC in $\Delta mreC$ mutants.

That a $\Delta rcsCBD$ mutant was unable to restore motility or SPI-1 T3SS in the $\Delta mreC$ mutant suggests that the presence of RcsB and/or RcsD is essential for the restoration of their expression, in the absence of RcsC. An explanation for these results could relate to the complex nature of the RcsC protein, which possesses both kinase and phosphatase activity, and is therefore capable of activating

or deactivating RcsB. Unlike $\Delta rcsD$ or $\Delta rcsB$ mutants, $\Delta rcsC$ mutants may retain residual Rcs phosphorelay activity (García-Calderón et al., Microbiology 2005; Girgis et al., PLoS Genet 2007). Hypothetically, low-level RcsB activation may be necessary for the downstream recovery of *flhDC* and *hilA* expression, while higher levels of activated RcsB lead to their repression. However, in *E. coli* the inactivation of *rcsB*, *rcsD* or *rcsF* was shown to restore motility in non-motile mutants, while *rcsC* inactivation did not restore motility as effectively (Girgis et al., PLoS Genet 2007).

It appears that the inactivation of the bacterial cytoskeleton brings about the

RcsF-independent activation of RcsC. However, the downstream regulatory mechanisms that repress SPI-1 T3SS, motility and activate SPI-2 T3SS are potentially complex and atypical. The regulation of SPI-1 T3SS and flagella expression in $\Delta mreC$ mutants is potentially multifaceted, involving several global regulators to provide tighter control over virulence—especially given that the inactivation of *rscC* alone was not sufficient to completely restore wild-type levels of motility or SPI-1 T3SS activity.

Furthermore, it cannot be excluded that cell wall defects may also directly influence motility/SPI-1 functionality in these mutants; hence the reduced motility in $\Delta mreC \Delta rcsC$ cells (compared with wild-type cells) may be caused at least partially by some dependency of these organelles upon the structural integrity of the cell envelope/peptidoglycan layer. The extent to which cell shape and the robustness of the peptidoglycan cell wall directly affects the assembly and function of the SPI-1 and motility systems is unclear. Studies in Gram-negative bacteria, including *Salmonella*, have demonstrated that mutations in the peptidoglycan-binding regions of structural proteins of both flagella and various secretion system organelles reduced their function (Scheurwater and Burrows, FEMS Microbiol Lett 2011; Pucciarelli and García-del Portillo, Mol Microbiol 2003). Peptidoglycan structural changes also affected virulence in *Salmonella*, causing a reduction in the number of SPI-1 T3SS needles per cell (Pucciarelli and García-del Portillo, Mol Microbiol 2003). Together these studies suggest that alterations to cell shape and peptidoglycan structural integrity may both directly and significantly affect virulence in $\Delta mreC$ mutants.

The activation or overexpression of envelope stress response regulators would be expected in $\Delta mreC$ mutants, where the peptidoglycan structural integrity may be significantly compromised. Surprisingly, within a panel of two-component system mutants including mutants of two major envelope stress response pathways, $\Delta cpxAR$ and $\Delta baeSR$, only $\Delta rcsC$ mutants restored SPI-1 T3S or motility to the $\Delta mreC$ cells (Raivio, Mol Microbiol 2005). It is possible that these regulators only play a minor regulatory role, or that some redundancy exists in the regulatory mechanisms. However, only the Rcs phosphorelay has so far been recognized to respond to peptidoglycan-associated stress specifically (Laubacher and Ades, J Bacteriol 2008).

We have characterized the role of the bacterial cytoskeleton in *S. Typhimurium*, investigating the effects that inactivation of the Mre cytoskeleton had upon the assembly and function of major cell wall-spanning virulence organelles (Bulmer et al., PLoS Pathog 2012). While the SPI-1 T3SS and flagella systems were downregulated in round-cell $\Delta mreC$ mutants, the SPI-2 T3SS remained functional. Interestingly, this phenotype is not due to an inherent inability of round-cell mutants to express and assemble functional wall-spanning virulence organelles. This suggests that the bacterial cytoskeleton does not play an essential role in directing the assembly and localization of such organelles, but affects the expression of these systems. Furthermore, expression of the *flhDC* and *hilA* master regulators in trans in the $\Delta mreC$ mutants, resulted in expression and assembly of flagella and SPI-1 T3S needles, respectively.

The Rcs phosphorelay, a global envelope stress-response pathway, appears to be largely

responsible for the repression of the SPI-1 T3SS and motility in response to the inactivation of the bacterial cytoskeleton. However, it remains unknown what role RcsB and RcsD play in this regulation. The precise nature of the signal(s) responsible for activating the Rcs phosphorelay in round-cell mutants also remain(s) to be identified. The bacterial cytoskeleton itself may be involved in activating global regulators; it has been suggested that the integrity of the bacterial cytoskeleton is utilized for monitoring the bacterial cell's physiological state, as is observed in eukaryotic cells (Chiu et al., Appl Environ Microbiol 2008). Future work aims to further elucidate of the roles of the Rcs phosphorelay, alternative global stress regulators and post-transcriptional regulators, in controlling virulence in *Salmonella* upon disruption of the bacterial cytoskeleton and longitudinal cell wall synthesis.

It is now clear that the integrity of the bacterial cytoskeleton can attenuate the virulence of *S. Typhimurium*, by downregulating the expression of important virulence genes. This regulation is mediated by the Rcs two-component system. The broader importance of the cytoskeleton in virulence needs to be investigated in greater detail in a variety of other bacterial pathogens. These observations highlight the potential of the bacterial cytoskeleton serving as a novel target for a new generation of antimicrobial therapeutics.

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Unveiling malaria's "cloak of invisibility": The role of PfSET10 in the genetic control of *P. falciparum* virulence

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Adherence of *Plasmodium falciparum*-infected erythrocytes to the endothelium is associated with morbidity and mortality from malaria in humans. The parasite expresses a variant protein on the surface of infected erythrocytes called PfEMP1 responsible for adherence.

Monoallelic transcription and expression of one of 60 *var* genes encoding this family in each parasite genome, and the ability to switch expression between them, provides a mechanism of antigenic variation. In a recent study, we reported the identification

of a histone methyltransferase, called PfSET10, which localizes to the perinuclear active *var* gene transcription zone. PfSET10 is required for *var* gene repression and maintenance of the active *var* gene in the poised state during division for activation in daughter parasites.

The identification of the PfSET10 molecule is the first step toward understanding how the expression of PfEMP1 protein is regulated and produced by the parasite. With improved insight into the systems, including the molecules that are involved in controlling antigenic variation, this opens the possibility of targeted treatments that would be more effective in preventing malaria infection.

The malaria parasite *Plasmodium falciparum* infects humans and is responsible for the majority of infection and fatal outcomes occurring in sub-Saharan Africa. The major virulence factor is the highly polymorphic *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a protein expressed by the parasite on the surface of the red blood cell, where it mediates adherence of *P. falciparum*-infected erythrocytes to receptors on endothelial cells (Baruch et al., Cell 1995; Su et al., Cell 1995). Sequestration of infected erythrocytes to receptors on the endothelial lining of blood vessels, erythrocytes and platelets prevent clearance through the spleen (Barnwell, Exp Parasitol 1989; Berendt et al., Nature 1989; Cooke and Coppel, Parasitol Today 1995; Ockenhouse et al., J Exp Med 1992; Rogerson et al., J Exp Med 1995; Wahlgren et al., Parasitol Today 1994). Moreover, this can lead to obstruction of the microvasculature in organs, playing an important role in malaria pathogenesis (Leech et al., J Exp Med 1984).

Each parasite genome contains approximately 60 *var* genes that encode the PfEMP1 family (Gardner et al., Nature 2002). They are located at the subtelomeric or central regions of chromosomes and their transcription is mutually exclusive; with one being transcribed at any given time and the remaining copies are silenced. Antigenic variation is maintained through switching of expression as a result of activation of a different *var* gene, resulting in the production of variant PfEMP1 molecules with alternating receptor-binding properties (Baruch et al., Cell 1995; Smith et al., Cell 1995; Su et al., Cell 1995).

Much of our current knowledge on *var* gene regulation is based on the study of parasites selected for the expression of one PfEMP1 molecule transcribed from a single *var* locus. The analysis of one selected *var* locus in its active and silenced state has provided insights into histone modification of chromatin and the discovery of chromatin binding and/or modifying molecules involved in *var* gene regulation. Despite some important progress

our current understanding on the molecular players and events determining *var* gene activation, poisoning, silencing and switching is limited (Duffy et al., Cell Microbiol 2012).

In an attempt to discover novel regulators for *var* gene expression, we localized a number of putative epigenetic factors to the *P. falciparum* nucleus (Volz et al., IJP 2010). The Plasmodium-specific SET-domain protein PFL1010c, now called PfSET10, showed an intriguing localization exclusively to one spot within the nuclear periphery. Studies in yeast previously showed the association of functional compartments within the nuclear periphery with active genes (Akhtar and Gasser, Nat Rev Genet 2007) and further analysis into the PfSET10 localization revealed its association with euchromatin, characterized by the presence of histone marks H3K4me1, me2, me3 and H3K9ac. *Var* gene expression is initiated in the early ring stage parasite 4 h post invasion and involves *var* locus translocation to an expression site (Duraisingh et al., Cell 2005; Freitas-Junior et al., Cell 2005; Ralph et al., Proc Natl Acad Sci USA 2005, Dzikowski et al., EMBO Rep 2007), which consists of a defined zone of relaxed euchromatin at the nuclear periphery free of the heterochromatic histone mark H3K9me3 and heterochromatin protein 1. With the discovery of PfSET10 we were able to associate the first epigenetic regulator to the *var* gene expression site (Fig. 1).

During transcription, the *var* promoter is enriched in histone marks H3K9ac and H3K4me3 as well as the histone variant H2A.Z (Lopez-Rubio et al., Mol Microbiol 2007; Bártfai et al., PLoS Pathog 2010; Petter et al., PLoS Pathog 2011). As the parasite enters the trophozoite stage, *var* gene transcription is deactivated. This step is consistent with the depletion of the histone variant H2A.Z by the histone deacetylase PfSir2A at the active *var* promoter (Petter et al., PLoS Pathog 2011). After parasite DNA replication and differentiation, canonical histones such as H2A and H3 are deposited, which can be already acetylated prior to nucleosome assembly (Corpet and Almouzni, Trends Cell Biol 2009), while lacking methylation marks (Loyola et al., Mol Cell 2006). Thus, the incorporation of fresh nucleosomes at the *var* gene promoter consisting of a new set of core histones provides the opportunity of silencing of the active *var* gene and switching expression to another family member. *Var* gene silencing is concurrent with the methylation of the histone H3K9 (Lopez-Rubio

et al., Mol Microbiol 2007), subsequent recruitment of Heterochromatin Protein 1 to the *var* locus (Flueck et al., PLoS Pathog 2009; Pérez-Toledo et al., Nucleic Acid Res 2009) and translocation out of the “expression site” (Ralph et al., Proc Natl Acad Sci USA 2005). In contrast, the poised *var* gene continues to be enriched in its promoter for histone marks H3K4me2, H3K4me3 and H3K9ac during division to ensure transcription in daughter cells and further remains within the “expression site” marked by the presence of PfSET10. Based on our recent findings, our current model envisages that PfSET10 is responsible for setting the H3K4me2 and H3K4me3 mark at the active *var* gene to maintain it in its poised state. PfSET10s specific localization to a small and defined zone within the nuclear periphery implies the presence of a very specialized and controlled mechanism of PfSET10 recruitment. Initial clues were provided by the analysis of the histone-binding properties of the PfSET10 PHD finger domain. When presented with differently modified and unmodified histone H3 peptides in an in vitro set-up, the PfSET10 PHD finger displayed a distinct affinity to the un-methylated and mono-methylated form of histone H3K4. These findings imply that one mode of PfSET10 recruitment lies in the PHD finger binding of lysine 4 of a histone H3, which has been freshly in-cooperated into a new nucleosome. After setting the methyl-marks on lysine 4 of histone H3, PfSET10 withdraws from its site of action, possibly through the rapid decrease of its PHD finger affinity to the methylated forms of histone H3K4. A further potential PfSET10 recruitment factor, which is deposited into a newly formed nucleosome, is histone H2A. Histone H2A replaces H2A.Z after *var* gene transcription is turned off and is a characteristic feature of poised or silenced *var* gene promoters (Petter et al., PLoS Pathog 2011). While PfSET10 recruitment to H2A mediated via its PHD finger appears likely, this hypothesis requires further attention. It is tempting to speculate that a combination of specific histone H2A, H3 and H4 core residues are required for PfSET10 binding as this has recently been shown to be a prerequisite for histone methyltransferase recruitment (Du and Briggs, J Biol Chem 2010). While a specifically formed nucleosomal surface may serve as a PfSET10 recognition site, DNA-specific binding factors may also contribute significantly to its recruitment. Interestingly, we detected PfActin-1 as a PfSET10 interacting factor and

as such a component of the *var* gene expression site. PfActin-1 has recently been shown to be directly involved in *var* gene positioning and a spatial chromosome organization (Zhang et al., Cell Host Microbe 2011). Based on these observations, PfActin-1's role is similar and appears to be conserved to what has been observed in other eukaryotes. Whether PfActin-1 recruits PfSET10 directly or if it is responsible for positioning the active *var* gene into the transcription site and by this forms an interaction with PfSET10 remains to be elucidated. Besides PfActin-1, our protein-interaction analysis identified further potential PfSET10 interacting partners, such as putative RNA and DNA/RNA binding proteins. While these interesting candidates for PfSET10 interaction still await functional characterization, it is an exciting thought, that with the discovery of PfSET10, we have found a stepping stone, from which the further molecular and functional dissection of the *var* gene expression is feasible.

However, the physically small size of the *var* gene expression site and the concentrated but scarce PfSET10 protein amount per nucleus clearly present technical challenges in terms of molecular analyses. *Var* gene expression is a highly dynamic process and it may be difficult to catch the players in the act. Several attempts to chromatin-immunoprecipitate PfSET10 failed. With no enrichment observed at the active *var* gene (ChIP-PCR), or at any other parts of the genome (ChIP-seq) points once more to the generally temporal and spatial association of histone modifying enzymes with their target sites.

In recent years, histone modifying enzymes have been intensively studied, since their potential as drug targets for a range of diseases has been recognized. Large chemical libraries have been screened to identify specific histone methyltransferase inhibitors. To date, two selective inhibitors of mammalian histone lysine methyltransferase SU(VAR)3-9 and G9a, respectively, have been discovered (Greiner et al., Nat Chem Biol 2005; Kubicek et al., Mol Cell 2007). With the emergence of drug resistance in *P. falciparum*, drug companies have only recently started to contribute

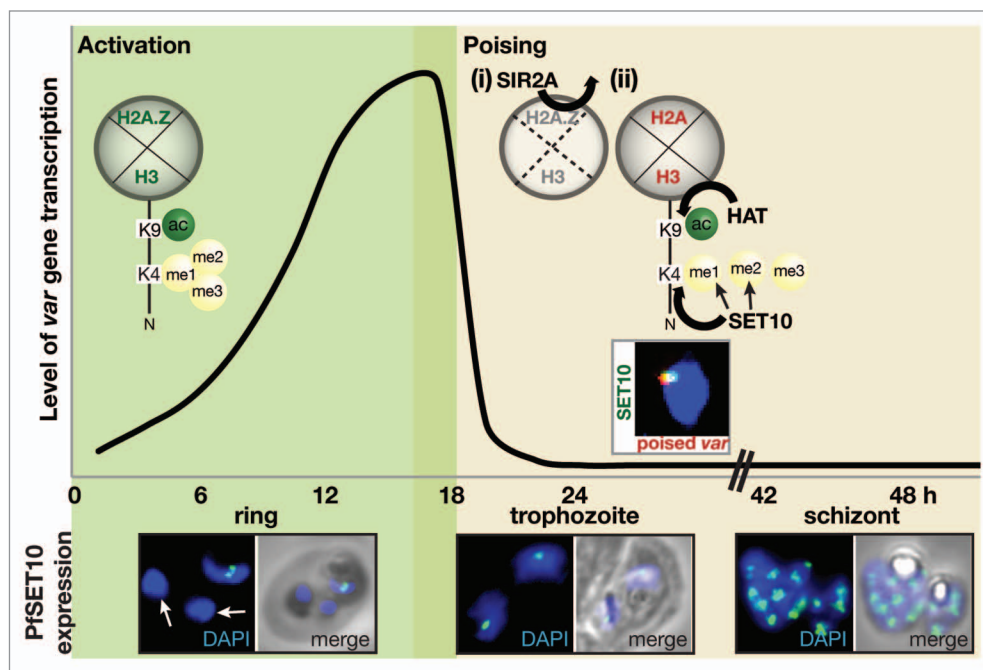


Figure 1. A proposed model of PfSET10 regulation of the active *var* gene in a perinuclear compartment in *Plasmodium falciparum* (see text for details).

chemical libraries, which has led to detection of two new promising anti-malarial compounds, OZ439 and NITD609 (Charman et al., Proc Natl Acad Sci USA 2011; Rottmann et al., Science 2010). PfSET10 is a conserved gene within the Apicomplexan lineage and based on its sequence and structural information its SET domain does not show any similarity to other known histone lysine methyltransferases. This feature places PfSET10 as an interesting candidate for drug screening since an inhibitor would probably display high specificity for PfSET10 binding with a potential to additionally target PfSET10 homologs of other *Plasmodium* species. Further, PfSET10 is essential to *P. falciparum* survival. Several attempts to disrupt the *pfset10* locus or destabilize PfSET10 protein expression were unsuccessful. Interestingly, this observation also implies that the three other *P. falciparum* SET-domain proteins, PfSET1, PfSET4 and PfSET6, which have been predicted to target histone H3 lysine 4 (Cui et al., Int J Parasitol 2008) are unlikely to be functionally redundant with that of PfSET10. This hypothesis is further supported by the apparently different localization of PfSET6 and PfSET4 within the nucleus (Volz et al., Int J Parasitol 2010). Nevertheless, additional

enzymes or co-factors may be required for proper PfSET10 activity.

The essentiality of PfSET10 to the parasite indicates that it may not merely be responsible for *var* gene regulation per se, but instead harbors a transcription site probably utilized by additional genes. The removal of PfSET10 from its transcription site may likely compromise its functionality and subsequently result in altered or abolished expression of potentially essential proteins. Also, a PfSET10 deficiency and resulting lack of preservation of the local euchromatic environment, may in turn affect overall chromosome organization and function.

Targeting unique components of the *var* gene expression site and by this interfering with the parasite's virulence and antigenic variation program would ideally leave the parasite with a compromised ability to evade protective mechanisms of the host, enabling the immune system to take charge. We have identified a histone H3 lysine 4 methyltransferase, PfSET10, which is the first protein shown to regulate variant PfEMP1 expression. It is unique and essential to the parasite, thus representing a potential target for intervention and thereby control of this devastating disease in humans.

A novel approach to develop anti-virulence agents against group A streptococcus

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The emergence of antibiotic resistance among human pathogens is a major cause of concern for public health. In recent years, an alternative approach has been proposed to target virulence of pathogens instead of their growth to generate new antimicrobial agents. We discovered a chemical series of novel anti-virulence agents against group A streptococcus (GAS), a common human pathogen. The anti-virulence agents were able to inhibit the gene expression of multiple virulence factors and also protect mice against group A streptococcal infection. This proof of concept study supports the new paradigm of developing novel therapeutic approaches targeting gene expression of bacterial virulence factors.

The discovery and wide use of antibiotics have greatly attenuated the threat of infectious diseases and contributed to the increase in lifespan since the 1940s. However, the alarming rise of antibiotic resistance among almost all major human bacterial pathogens has become a serious public health problem, which is further aggravated by a diminished developmental pipeline for new antibiotics.

The antibiotics currently in clinical use generally target essential bacterial functions, leading to bacterial death or inhibition of growth. As a result, strong selective pressure will favor bacterial strains that have developed resistance. Antibiotic resistance is now a serious problem for nearly all major bacterial pathogens, including resistance to multiple antibiotics, which has developed in large part due to inappropriate and excessive use of antibiotics.

Recently, a novel approach has been proposed aiming to block pathogen virulence without inhibiting bacterial growth, thereby minimizing selection for the resistance. Several proof-of-concept studies have been published. Hung et al. reported discovery of a novel small compound that inhibited the gene expression of two critical cholera virulence factors, cholera toxin and toxin coregulated pilus. This compound demonstrated *in vivo* efficacy, protecting infant mice from intestinal colonization by *Vibrio cholerae* (Hung et al., Science 2005). A

small compound was also identified that can inhibit the membrane-embedded sensor histidine kinase QseC and protected mice against infection by *S. Typhimurium* and *Francisella tularensis* (Rasko et al., Science 2008).

In our previous studies, we demonstrated that group A streptococcus utilized the host hemostatic system to facilitate invasion (Sun et al., Science 2004). GAS, also known as *Streptococcus pyogenes*, is a major human pathogen, causing over 700 million infections each year worldwide. GAS can cause tonsillitis, impetigo and invasive diseases such as streptococcal toxic shock-like syndrome and necrotizing fasciitis. Impetigo is endemic among children of developing countries (Parks et al., Curr Opin Infect Dis 2012). Post-infection complications of impetigo and streptococcal pharyngitis include rheumatic fever and rheumatic heart disease, causing 15.6–19.6 million and 282,000 new cases, respectively, and resulting in 233,000 to 294,000 deaths each year worldwide. Approximately 663,000 cases of invasive GAS disease occur annually with approximately 25% mortality (Carapetis et al., Lancet Infect Dis 2005).

GAS produces streptokinase (SK) that can activate human plasminogen to form plasmin, which is the central enzyme of the fibrinolysis system. We reported that the interaction of SK with human plasminogen was critical for GAS pathogenicity. GAS is a strict human pathogen and the host specificity of GAS was speculated to be due to the species-specific interaction between human plasminogen and SK. We established a murine model in which human plasminogen was expressed by a transgene. The transgenic mice expressing human plasminogen demonstrated significantly increased susceptibility to GAS infection compared with wild-type mice. When the transgenic mice were infected with a GAS mutant in which SK had been inactivated, this increased susceptibility was largely abolished. These data suggested that both human plasminogen and SK were required for susceptibility to GAS infection. SK was thus considered as a potential therapeutic target for anti-GAS agents.

We developed a simple growth-based turbidometric high throughput screening approach to search for low molecular weight compounds that inhibit expression of the SK gene (Sun et al., Proc Natl Acad Sci USA 2012). A modified GAS strain SKKanGAS was generated carrying a kanamycin resistance gene driven by the SK gene promoter in an extra-chromosomal plasmid. A control GAS strain UMAA2641 with the same kanamycin resistance gene driven by a different promoter was used for counter screening. A total of 55,000 small molecules were screened to identify compounds capable of inhibiting the growth of the SKKanGAS strain under kanamycin selection without interfering with UMAA2641 growth. Positive hits from this screen were tested in a secondary screen for inhibition of wild-type GAS SK protein expression without growth inhibition.

Compounds that inhibit SK expression with minimal interference of GAS growth were selected. A lead compound (CCG-2979) was selected based on its ability to inhibit SK expression with little inhibitory effect on GAS growth. A commercially available analog of CCG-2979 also demonstrated potency at inhibiting SK expression. The global effect of this SK expression inhibitor on GAS gene expression was characterized by a mRNA microarray analysis.

Surprisingly, a wide range of genes was affected (Fig. 1). As expected, SK was down-regulated. A number of other key virulence factors, including multiple adhesins, anti-phagocytic factors and cytolytic toxins, were also found to be affected at the mRNA expression level (Fig. 1). In addition to virulence factors, the SK expression inhibitor also changed the expression of genes involved in metabolism and energy production.

Gene regulation has been extensively studied in GAS. Gene regulatory systems in GAS include “stand-alone” response regulators and two component systems (TCS) (Kreikemeyer et al., Trends Microbiol 2003). “Stand-alone” regulators are transcriptional regulatory proteins that regulate expression of multiple

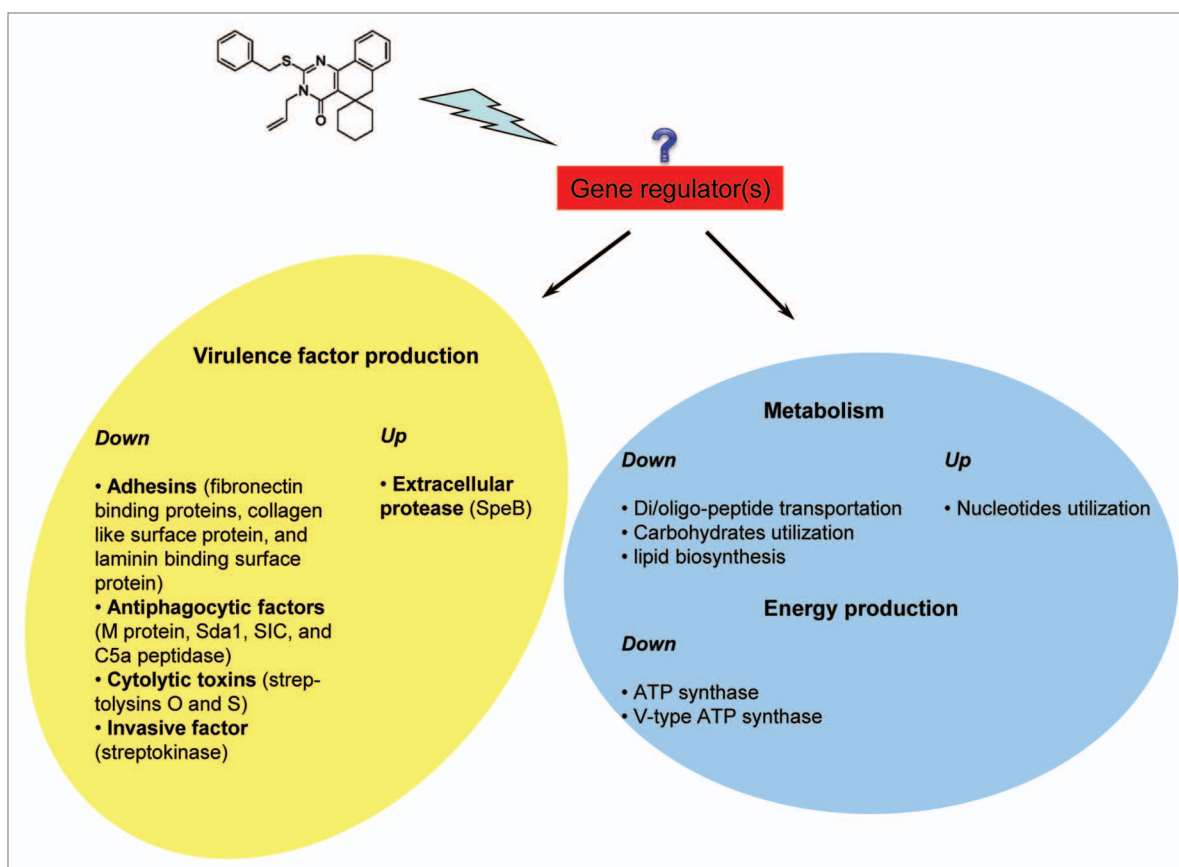


Figure 1. Novel small compound affected expression of *Streptococcus pyogenes* genes involved in virulence, metabolism and energy production.

genes in response to the environment. Two component systems are used by bacteria to sense and respond to environmental stimuli. They consist of a membrane-bound sensor and cytoplasmic response regulator and control the up or downregulation of gene expression of multiple virulence factors (Kreikemeyer et al., Trends Microbiol 2003). The gene expression profile changes we observed suggest that these compounds target a major regulation pathway that affect multiple genes involved in virulence and potentially other cellular functions.

Both compounds decreased GAS resistance to phagocytosis. The in vivo efficacy of these two compounds was tested in our previously established human plasminogen

transgenic mouse model (Sun et al., Science 2004). Mice were treated with the small compounds one day after infection with GAS and a significant improvement in survival was observed in mice treated with the lead compound CCG-2979. It is worth noting that the streptokinase gene is conserved in some pathogenic streptococci such as *Streptococcus dysgalactiae* and *Streptococcus equi*. *Streptococcus equi* is an important horse pathogen, causing strangles, which is one of the most frequently encountered equine diseases, accounting for almost 30% of infectious cases. Thus, this class of compounds could also inhibit gene expression of virulence factors of these important human and domesticated animal pathogens.

In summary, our current study demonstrates the feasibility of developing agents against GAS infection by targeting regulators of gene expression of SK and other virulence factors. The novel anti-virulence agents function through a different pathway from common antibiotics. As a result, this class of anti-microbial agents may complement the current antibiotics and increase the efficacy of the antibiotic treatment (Waldor, N Engl J Med 2006).

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On the evolution of virulence during *Staphylococcus aureus* nasal carriage

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In a recent paper appearing in the *Proceedings of the National Academy of Sciences of the United States of America*, Young and colleagues report the case of a persistent carrier of *Staphylococcus aureus* who went on to develop a virulent bloodstream infection (Young et al., Proc Natl Acad Sci USA 2012). By sequencing the genomes of bacteria sampled from the nose and bloodstream over an extended period, they pieced together the evolutionary changes that accompanied the transition from asymptomatic carriage to invasive disease. Among an unusual cluster of knockout mutations that coincided with the beginning of a period of declining health in the patient, they identified the truncation of an *AraC* family transcriptional regulator as a likely modifier of virulence, demonstrating that genetic changes that occur in bacterial genomes during prolonged carriage could play a role in pathogenesis. Here we discuss the alternative evolutionary hypotheses for the observed excess of knockout mutations and review the evidence in favor of a causal role for regulatory gene dysfunction in bacterial virulence. We consider the prospects for systematic studies into the evolution of virulence in bacterial carriage populations.

Introduction

Many bacterial pathogens are, for the most part, commensal organisms that cause disease infrequently compared with their high rates of asymptomatic carriage in the population. *Staphylococcus aureus* is one such example. *S. aureus* is a major healthcare-associated pathogen, responsible for an estimated 17,000 deaths in the United States between 1999 and 2007, making it one of the two most frequently identified bacterial causes of mortality, second only to *Clostridium difficile* (WHO Mortality Update 2011). Yet the frequency of asymptomatic carriage of *S. aureus* is high. In studies of nasal carriage, 27% of healthy adults have been found to carry *S. aureus* (Wertheim et al., Lancet Inf Dis 2005). Fatal disease is therefore a rare occurrence in the lifecycle of the bacterium. Epidemiological studies have

investigated the risk factors that predispose to invasive disease, but the role of novel genetic changes caused by bacterial evolution within the host has been understudied. However, that is about to change because advances in DNA sequencing make it increasingly feasible to characterize the genomes of large numbers of bacteria rapidly and inexpensively.

In an early example of the potential that high-throughput whole-genome sequencing has for unraveling the genetic basis of bacterial virulence, Young and colleagues charted the evolutionary changes that accompanied the transition from nasal carriage to invasive bloodstream infection in one persistent carrier. The subject was enrolled in a longitudinal study of *S. aureus* carriage. *S. aureus* was cultured from six of eight nasal swabs collected over a 12 mo period. Fifteen months after enrolling in the study, the participant was admitted to hospital with a highly virulent *S. aureus* bloodstream infection.

Whole genome sequencing showed that despite negative swabs in month 10 and at the time of bloodstream infection, all the bacterial colonies isolated from the nasal swabs and from the bloodstream infection were extremely closely related, representing a single cohesive population. A total of 34 genetic variants were observed across 68 colonies, comprising 30 single nucleotide polymorphisms (SNPs) and four insertions or deletions (indels). This represents extremely sparse genetic variation in a genome that is nearly three million nucleotides long. Genome annotation revealed a typical methicillin-sensitive *S. aureus* (MSSA) profile, with no evidence for the acquisition of additional virulence factors or toxin genes beyond the normal repertoire found in carried strains.

Although carriage is a known risk factor for *S. aureus* infection (von Eiff et al., N Engl J Med 2001) and invasive disease may stem from ingress of commensal flora into the bloodstream through compromised epithelia, no source for the bloodstream infection was found: there were no endovascular catheters or

evidence of endocarditis or surgical-site infection associated with a previously fitted pacemaker. However, genetic analysis revealed that the bacterial population formed three distinct clades: colonies isolated from the early nasal swabs prior to any medical intervention (early nasal cultures, ENC), colonies isolated from the late nasal swab, post-antibiotic treatment (late nasal culture, LNC), and colonies isolated from the bloodstream infection (late blood culture, LBC). These clades were distinguished by multiple mutations, leaving open the possibility that evolution in the bacterial population during carriage had resulted in the spontaneous emergence of a more virulent strain.

Further investigation revealed unusual patterns of evolution between the clades. First, the two branches that separated bacteria isolated early during asymptomatic nasal carriage (ENC clade) from bacteria isolated from the bloodstream infection (LBC clade) were significantly longer than expected, meaning that these groups were surprisingly differentiated. Second, half of the eight mutations that occurred on these two branches resulted in premature stop codons that were predicted to knock out protein function. This was a highly statistically significant excess of premature stop codons given that none were observed among the other 26 mutations. Further evidence that these patterns were highly unusual was provided by an analysis of longitudinal bacterial evolution in two other participants from the same carriage study that did not go on to develop invasive disease.

One of the eight premature stop codons was singled out as a potential modifier of virulence phenotype. It occurred in a gene belonging to the *AraC* family of transcriptional regulators (AFTRs) that are regulators of carbon metabolism, stress response and virulence, and which respond to environmental conditions such as antibiotic use and oxidative stress (Yang et al., Trends Microbiol 2011). Knockout mutations in genes such as AFTRs could play an important role in bacterial pathogenesis because of their ability to quickly

effectuate functional change. In what follows we discuss the possible evolutionary processes underlying the observed patterns of mutation between carriage and invasive bacteria, and the evidence in favor of a role for regulatory gene dysfunction in bacterial pathogenesis.

Evolutionary Explanations for Observed Patterns

The significant excess of mutations separating the early asymptotically carried nasal bacteria from the late invasive bloodstream bacteria may be consistent with a number of evolutionary scenarios that have different implications for how we view the evolution of virulence within hosts, and we take each of these in turn (Fig. 1).

Cryptic populations of differentiated bacteria. Unexpectedly high genetic differentiation within a population that is assumed to be homogeneous may be due to cryptic population structure. For example, *S. aureus* is routinely isolated from the nose, axilla and groin of asymptomatic individuals, with concurrent colonization of multiple sites common. If multiple sub-populations evolve mostly in isolation but with limited gene flow between body sites, genetically heterogeneous populations may be observed. Other forms of population structure can also lead to genetic heterogeneity. If the sub-populations are ultimately descended from a single founding colonization event, the degree of differentiation is likely to be slight.

Re-seeding by a latent population of ancestral genotypes. It has been suggested that *S. aureus* can survive intracellularly, possibly within privileged sites such as the phagosomes of neutrophils, and this may contribute to relapsing infections (Thwaites and Gant, Nat Rev Microbiol 2011). This model would allow for the sporadic re-seeding of the *S. aureus* carriage population with latent bacteria. If rates of mutation are retarded during latency, latent bacteria would more closely resemble ancestral genotypes than the contemporary population. Such a process would lead to an apparent excess of mutations between bacteria because some genotypes would be relics from the past.

Relaxed selection associated with a population bottleneck. Neither population structure nor re-seeding by latent bacteria can, by themselves, explain the excess of premature stop codons observed on the branches separating the early nasal bacteria and late invasive bacteria compared with all other branches of the tree. Premature stop codons are generally much rarer than expected under strictly

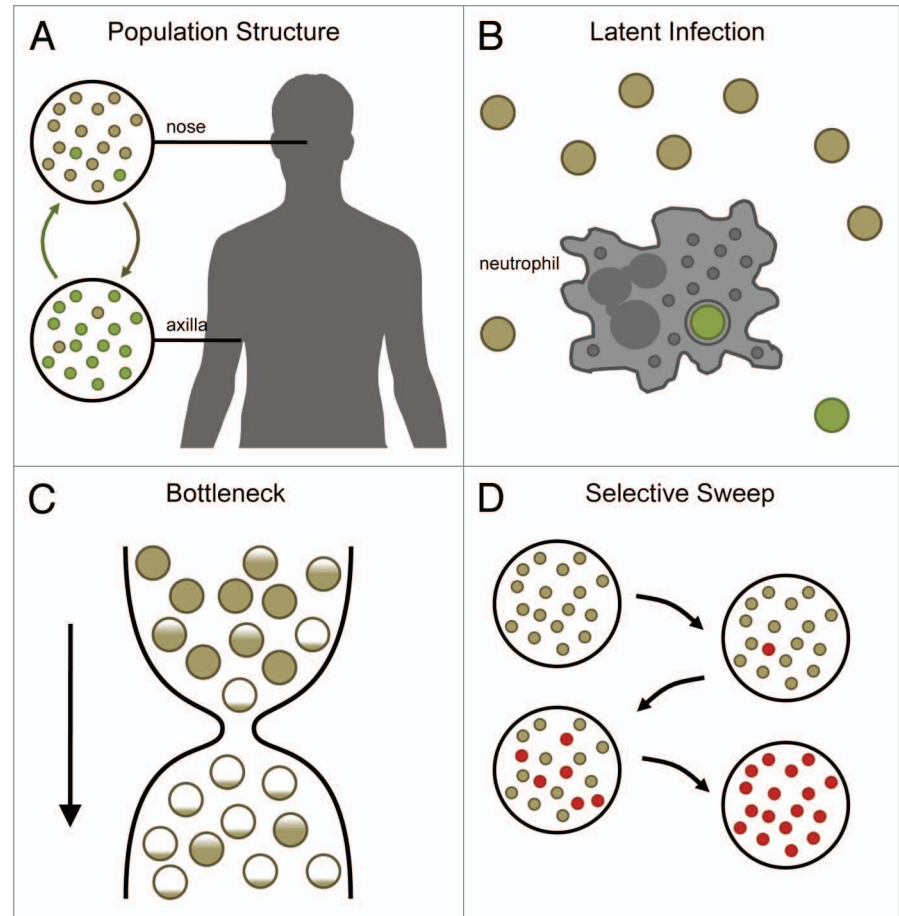


Figure 1. Evolutionary processes that may underlie observed patterns of mutation between carried and invasive bacteria. (A) Gene flow between bacterial sub-populations may generate genetic heterogeneity. In the example, bacteria normally resident in the nose (gold cocci) and axilla (green cocci) occasionally migrate between sites, leading to mixed populations. (B) Latent bacterial populations may persist in privileged sites only to re-emerge later. For example, the contemporary population (gold) may be re-seeded with ancestral bacteria (green) lying dormant in neutrophil vesicles. (C) During a population bottleneck natural selection is weak, allowing an increase in frequency of deleterious mutants. Prior to the bottleneck the bacterial population contains a fitness gradient ranging from the optimal genotype (solid gold) to deleterious mutants (partially gold). Survival during the bottleneck is random, and deleterious mutants may rise to high frequency by chance. (D) Mutants that have a selective advantage may sweep through the population. In the example, a single mutant (red) out-replicates the original genotype (gold). Over time, the fitter mutant replaces the original genotype.

neutral evolution because they are disfavored by natural selection as a result of their drastically deleterious effect on protein function. An apparent burst of mutations inducing premature stop codons could be explained by a temporary relaxation of selection pressures. Such a phenomenon could be caused by a transient but severe reduction in population size (Otto and Whitlock, Genetics 1997). There is some evidence to support this theory: nasal swabs at months 10 and 15, both of which followed antibiotic treatment, showed no growth although the bacterial population was later shown not to have been completely eradicated.

Adaptive evolution. Intense competition for resources between bacteria within the host may result in selective sweeps if spontaneously occurring mutants can out-replicate non-mutant bacteria. The potential for such mutants to arise might be considerable. For example, any trade-off between the exploitation of the host and transmissibility will lead to a situation in which strains that successfully colonize new hosts are susceptible to out-competition by spontaneously arising mutants that better exploit host resources at the cost of future transmission. Alternatively, mutants that are able to exploit sterile sites in the body such

as the bloodstream will quickly multiply in the absence of competition. An important difference between relaxed selection associated with bottlenecks and adaptive evolution is that in the former, spontaneously arising virulent bacteria are less fit than their predecessors, whereas in the latter they are more fit. Since the evolution of highly virulent genotypes is likely to end badly for the pathogen population as well as for the host, in the former they might be viewed as an accelerated form of mutational meltdown while in the latter they could be viewed as a tragedy of the commons.

Evidence for a Role of Regulatory Genes in Pathogenesis

There is increasing evidence that mutations in key regulatory genes play an important role in bacterial pathogenesis. Recent work has provided examples of this phenomenon in *S. aureus* and other major bacterial pathogens.

The *agr* locus of *S. aureus*. The accessory gene regulator (*agr*) locus is a quorum-sensing system responsible for the downregulation of *S. aureus* cell surface proteins and the upregulation of toxin genes during the switch from exponential to stationary growth phase (Gilot et al., J Clin Microbiol 2002). An independent association has been demonstrated between dysfunction of the *agr* locus and 30 d mortality in severely ill patients with *S. aureus* bacteremia: a cohort study demonstrated a 91% increase in mortality with loss of *agr* function (Schweizer et al., Antimicrob Agents Chemother 2011). Loss of *agr* function is believed to result in diminished cell autolysis, and thus impaired killing of *S. aureus* by bactericidal antibiotics. This association was only seen in the most unwell quartile of patients, suggesting interplay between host vulnerability and pathogen dysregulation. Other studies have shown an association between *agr* dysfunction and increased duration of bacteremia as well as decreased vancomycin sensitivity (Fowler et al., J Hosp Infect 2007). While methicillin resistance and toxin production have previously been the focus of much research in *S. aureus* virulence, these findings highlight the importance of regulatory proteins in the pathogenesis of *S. aureus* disease and open new directions for investigation.

AFTR-mediated response to environmental conditions. The *AraC* family of transcriptional regulators (AFTRs) have been implicated in the pathogenesis of bacterial disease (Yang et al., Trends Microbiol 2011; Fantappiè et al., Microbiology 2011). Yang and colleagues

review the ways in which AFTRs respond to small molecules in the environment by upregulating gene transcription. For example, the intestinal pathogen *Vibrio cholerae* responds to bicarbonate secretion in the small intestine by the ToxT mediated transcription of cholera toxin. AFTRs have been demonstrated to play a similar role in numerous Gram-positive and Gram-negative bacterial pathogens of humans, animals and plants. These organisms respond to stimuli specific to the environment in which they are suited to pathogenesis (bicarbonate within the intestine, urea in the urinary tract and cellobiose in plants) by altering gene transcription to enable them to colonize and survive. The Oxfordshire case study represents the first time the role of AFTRs has been associated with the development of virulence in *S. aureus* infection.

Phylogenetic association between AFTR knockouts and hypervirulence. There is evidence from other species that truncation of AFTRs is associated with altered pathogenicity. One of three AFTRs identified in *Neisseria meningitidis*—*MpeR*—has been recently shown to be part of the Fur pathway: a system that is responsive to iron limitation in the environment (a metabolic pathway key to microbial survival within the host), as well as regulating toxin expression and transcriptional activation (Fantappiè et al., Microbiology 2011). A frameshift mutation resulting in loss of the *MpeR* protein was detected in all sequenced isolates of a hypervirulent clonal complex of *N. meningitidis* (ST32) which were selected from different outbreaks in disparate countries, but not in any other isolates. Like *N. meningitidis*, *S. aureus* is well adapted to a biological niche (in this case, the anterior nares). The loss of a gene known to be subject to regulation by environmental stimuli has in the case of *N. meningitidis* been associated with increased likelihood of causing meningitis or septicemia. It is possible that the loss of regulation by environmental stimuli can likewise drive a transition to the bloodstream for *S. aureus*.

Future Studies

The study by Young and colleagues demonstrates the promise that high-throughput whole genome sequencing holds for understanding the evolutionary dynamics of bacteria both during asymptomatic carriage and in disease. These tools might be profitably applied to a number of bacterial pathogens such as *N. meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* that are

generally carried by human hosts without adverse effect, but which can cause life-threatening illnesses. Systematic studies are now needed to yield deeper insights into the genomic underpinnings of pathogenesis.

A major challenge to the design of systematic studies is the unpredictability of the bacterial-host interaction. Of more than 1,100 individuals in a cohort of healthy adults, a single *S. aureus* carrier developed invasive disease during the period of the study to date (just over three years). Very large numbers of people are therefore required for systematic follow-up in order to capture significant numbers of cases. Evolution over shorter time periods may be easier to capture with large cohort studies in areas of high *S. aureus* disease prevalence. An alternative but equally important route that we have not explored here are studies that focus on comparing the frequency of gene acquisition or knockout in large numbers of disease-associated cases and non-disease controls. A genome-wide association study, testing for non-random distribution of changes between these groups, faces several analytic challenges: most importantly, it must adequately detect and control for both relatedness and deeper population structure in a clonal organism like *S. aureus* (Falush and Bowden, Trends Microbiol 2006).

Functional follow-up is required to investigate what phenotypic changes, if any, are induced by the knockout of regulatory genes such as AFTRs in *S. aureus*. It is critical to establish whether changes to biological correlates of virulence can be observed as a direct effect of mutations that occur in vivo. Animal studies using knockout and knock-in strains would be an obvious route for investigation, but if the repertoire of candidate genes identified by whole genome studies becomes large, alternatives that do not require laboratory animals would be preferable. There is a need for in vitro assays that reliably replicate the host environment, both that of nasal epithelium and the bloodstream, with their variable conditions and host defenses. There is much work yet to do in this area, but the early signs are that evolutionary changes that occur within the host may play an important role in tipping the fine balance between asymptomatic carriage and invasive disease.

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Role of phospholipid scramblase 1 in type I interferon-induced protection from staphylococcal α -toxin

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Staphylococcus aureus is a leading cause of community-acquired and health care-associated pneumonia. The virulence of many clinical isolates of *S. aureus* correlates with production of α -toxin. This pore-forming toxin contributes to pathogenesis of pneumonia through cytolysis of lung epithelial cells, disruption of lung epithelial barrier, and induction of inflammation. We recently reported that type I IFNs protect lung epithelial cells from the cytolytic action of α -toxin by reducing leakage of cellular ATP into extracellular space. The effect is dependent on induction and protein palmitoylation of phospholipid scramblase 1 (PLSCR1). In IFN α -pretreated cells, PLSCR1 co-localizes with endocytosed α -toxin and associates with cytoskeleton. Depletion of PLSCR1 negates IFN-induced protection from α -toxin and enhances sensitivity to inhaled α -toxin and an α -toxin-producing strain of *S. aureus*. Here, we discuss the potential implications of these findings.

Induction and Roles of IFNs and IFN-Regulated Genes during Staphylococcal Infections

The complex roles of IFN γ , the only type II IFN, in pathogenesis of staphylococcal infections have been previously well established. IFN γ facilitates bacterial clearance by professional phagocytes and promotes acquired immunity during systemic infections but increases inflammation and tissue injury during localized infections (Zhao et al., *Immunology* 1998; Sasaki et al., *FEMS Immunol Med Microbiol* 2006). Recent studies uncovered the diverse and important roles of type I IFNs in bacterial infections. Several distinct mechanisms initiate type I IFN induction during staphylococcal infections. Recognition of lipoteichoic acid from *S. aureus* by TLR2 results in IRF2-dependent production of IFN α from macrophages (Liljeroos et al., *Cell Signal* 2008). In the presence of anti-staphylococcal IgGs, plasmacytoid dendritic cells recognize endocytosed bacterial DNA and RNA by TLR9 and TLR7, respectively, and secrete massive amounts of IFN α (Parcina et al., *J Immunol*

2008). Although the functional significance of IFN α from these sources has not been clearly established, activation of *Ifnb* gene and IFN-signaling is detrimental in a mouse model of staphylococcal pneumonia caused by prevalent methicillin-resistant *S. aureus* (MRSA) strain USA300 (Martin et al., *J Clin Invest* 2009). Apparently, recognition of Xr domain in staphylococcal protein A by lung epithelial cells increases *Ifnb* gene transcription, activates STAT1 and STAT3 and induces IL-6. This results in increased inflammation and mortality in wild-type, but not in IFNAR1^{-/-} mice, which are deficient in the receptor for type I IFNs. Another report described the detrimental role of type I IFNs in the context of post-viral secondary bacterial pneumonia: induction of type I IFNs by influenza virus inhibits Th17-mediated host defense against *S. aureus* and impairs bacterial clearance (Kudva et al., *J Immunol* 2011). These studies showed that the roles of type I IFNs are distinct from the roles of IFN γ despite a substantial overlap in the range of their target genes. They also highlighted the need to dissect the roles of specific subsets of IFN-regulated genes in pathogenesis of staphylococcal infections.

Since we previously observed that pretreatment of host cells with exogenous type I IFNs protects them from α -toxin-induced cell death, we were interested in identifying IFN-regulated pathways and genes with potentially beneficial roles. Type I IFNs exert most of their functions through activation of JAK1 and TYK2 tyrosine kinases and signal transducers and activators of transcription (STAT), which regulate transcription of several hundred genes. In addition, some of the metabolic and antiviral effects of type I IFNs are mediated by p38 MAP-kinase, PI3-kinase, protein kinase C δ and their substrates. To determine which signaling pathways are involved in protection of lung epithelial cells from α -toxin, we used a screening assay using intracellular ATP as readout for cell viability and a panel of well-characterized pharmacological inhibitors. We found that inhibition of p38 MAP-kinase, PI3-kinase

and fatty acid synthase activity significantly affected IFN-induced protection from α -toxin, which is consistent with previously described roles of these pathways in cellular defense from pore-forming toxins. Remarkably, inhibition of protein palmitoylation wiped out the protective effects of IFN α . Taking advantage of the previous gene expression studies, which listed IFN-regulated genes in the cell line that we used in the screening, and proteome-wide analyses of protein palmitoylation, we identified *PLSCR1* as the leading candidate gene. Although we cannot exclude the possibility that regulation of other genes may contribute to the protective effects of type I IFNs, *PLSCR1* is the first identified IFN-inducible gene associated with cellular defense against α -toxin.

Involvement of PLSCR1 in Host Responses to Staphylococcal α -Toxin

Using shRNA-mediated knockdown and PLSCR1-knockout mice, we validated that PLSCR1 plays a protective role after exposure to purified α -toxin or α -toxin-producing strain of *S. aureus*. Prior studies implicated PLSCR1 protein in translocation of membrane phospholipids and amplification of transcriptional responses to type I IFNs. However, increased expression of PLSCR1 does not correlate with changes in plasma membrane phospholipids after exposure to α -toxin. In addition, overexpression of nuclear localization mutant PLSCR1, which is excluded from the nucleus, is sufficient to increase cell resistance to α -toxin. Thus, the effects of PLSCR1 on gene transcription are not likely to contribute to protection from α -toxin. Since PLSCR1 co-localizes with endocytosed α -toxin and filamentous actin, it is possible that PLSCR1 participates in removal of α -toxin pores from the cell surface. This would be consistent with the previous studies showing that endocytosis, “detoxification” and exocytosis of α -toxin are critical for cellular defense against α -toxin.

Our study provides initial evidence that type I IFN-induced PLSCR1-dependent protection of lung epithelial cells is downstream of ADAM10 (a disintegrin and metalloprotease

10). This zinc-dependent metalloprotease acts as a protein receptor for α -toxin and mediates α -toxin-induced cytotoxicity, cleavage of E-cadherin and disruption of the lung barrier functions (Inoshima et al., Nat Med 2011). Pretreatment with IFN α does not have significant effects on α -toxin binding or oligomerization, loss of intracellular potassium, activation of p38 MAP-kinase or cleavage of E-cadherin. Furthermore, IFN α pretreatment or deletion of PLSCR1 does not have any significant effect on inflammatory responses to α -toxin or α -toxin-producing *S. aureus*. However, pretreatment of cells with IFN α or overexpression of PLSCR1 significantly reduces leakage of intracellular ATP into extracellular space. This finding raises the following questions: What is the mechanism of ATP release into extracellular space after exposure to α -toxin? Does extracellular ATP (eATP) contribute to α -toxin-induced cytotoxicity? Are type I IFNs or IFN-regulated genes involved in regulation of ATP release or responses to eATP? Below, we will contemplate involvement of eATP in responses to α -toxin.

ATP Release and Purinergic Receptor Signaling in Responses to α -Toxin and *S. aureus*

Intracellular ATP is rapidly depleted from host cells after exposure to α -toxin due to reduction of ATP biosynthesis, increased consumption of ATP and release of intracellular ATP into extracellular space, albeit the relative contribution of each of these processes has not been precisely determined. Moreover, it is not known whether ATP escapes the cells through the channels in α -toxin pores or by other mechanisms. Lung epithelial cells use connexin and pannexin hemichannels, maxi-anion channels and exocytosis to release ATP into extracellular space in response to mechanical stress, osmotic shock, fluxes of calcium or proteolysis. In addition, early apoptotic cells release ATP into extracellular space through

pannexin hemichannels as “find-me” signals for recruitment of phagocytes. Since α -toxin triggers changes in intracellular sodium and potassium, calcium fluxes, proteolysis and apoptosis, it is likely that ATP is released into extracellular space via multiple mechanisms. A recent study showed that pharmacological inhibition of pannexin channels inhibits α -toxin-induced hemolysis (Skals et al., Pflugers Arch 2011). Our preliminary unpublished data suggest that vesicular exocytosis and connexin hemichannels contribute to ATP release after exposure to α -toxin and that IFN α pretreatment reduces ATP release through connexin hemichannels. We are currently working to determine whether inhibition of ATP release through exocytosis or connexin hemichannels may preserve intracellular ATP and reduce α -toxin cytotoxicity against lung epithelial cells.

Extracellular ATP is rapidly hydrolyzed to ADP, AMP and adenosine by phosphatases and host ectonucleotidases such as CD39 and CD73. Even transient increases of ATP and ADP are readily sensed by P2 purinergic receptors, which are usually associated with proinflammatory and prothrombotic effects of extracellular nucleotides. Seven P2X receptors are ATP-gated membrane ion channels and eight P2Y receptors are G-protein coupled receptors with diverse ligand sensitivity (ATP, ADP, UTP, UDP and UDP-glucose). Signaling through P2X1 and P2X7 receptors apparently contributes to hemolysis by various pore-forming toxins, including staphylococcal α -toxin. In addition, stimulation of P2X7 receptor with extracellular ATP results in activation of NLRP3 inflammasome, which initiates innate immune responses and promotes inflammation. Our own data suggest that oxidized ATP (a non-selective antagonist of P2 receptors) significantly inhibits α -toxin-induced cell death in vitro and reduces the toxic effects of inhaled

α -toxin in vivo. Thus, extracellular ATP and its metabolites may contribute to pathogenesis of staphylococcal infections by increasing inflammation and tissue injury.

Sequential hydrolysis of ATP, ADP and AMP by host phosphatases and ectonucleotidases may increase extracellular adenosine. One of the key enzymes in this pathway is CD73 ecto-5'-nucleotidase, which produces adenosine from AMP. Although type I IFNs induce the expression and activity of CD73 expression, it remains to be determined whether upregulation of CD73 occurs during staphylococcal infections. Importantly, pathogenic strains of *S. aureus* express adenosine synthase, which may utilize extracellular ATP, ADP and AMP as substrates for adenosine synthesis (Thammavongsa et al., J Exp Med 2009). Since adenosine exerts potent anti-inflammatory effects through P1 adenosine receptors (mainly A2A and A2B), accumulation of adenosine may allow *S. aureus* to escape from phagocytic clearance and killing. Thus, *S. aureus* may utilize pore-forming toxins to increase the availability of substrates for host ectonucleotidases and staphylococcal adenosine synthase, which may result in excessive accumulation of extracellular adenosine and subsequent impairment of bacterial clearance.

Concluding Remarks

We identified PLSCR1 as a mediator of type I IFN-induced protection from staphylococcal α -toxin. Our study uncovered new functions for PLSCR1 and a mechanism for potentially beneficial effects of type I IFNs during staphylococcal infections. Evidently, PLSCR1 acts downstream of ADAM10 and reduces leakage of cellular ATP into extracellular space, which may have broad implications on pathogenesis of staphylococcal pneumonia.

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