Streptococcal β-hemolysins: genetics and role in disease pathogenesis

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A zone of β-hemolysis surrounding colonies on blood-agar media is a hallmark phenotypic feature of the pathogens group A *Streptococcus* (GAS) and group B *Streptococcus* (GBS). In each case, lysis of red blood cells reflects the action of a potent protein exotoxin. Although these toxins have been the subjects of numerous investigations over the years, their purification and molecular identification have proven elusive. These difficulties reflect the instability of hemolytic activity, as both toxins function only in the context of the bacterial surface or certain high molecular weight 'stabilizer' molecules. This review highlights the recent discoveries of two markedly distinct genetic loci, necessary and sufficient for the β-hemolytic phenotypes of GAS and GBS, respectively. The generation of isogenic GAS and GBS β-hemolysin-deficient mutants and their analysis using *in vitro* and *in vivo* model systems has shown that both toxins function as virulence factors in the pathogenesis of invasive infections.

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In physicians' offices for more than 50 years, culturing a throat swab on blood-agar media has been the standard for documenting pharyngitis produced by group A *Streptococcus* (GAS) [1,2]. A preponderant growth of small, grey bacterial colonies with a surrounding zone of complete red blood cell (RBC) lysis (so-called β-hemolysis) yields the presumptive diagnosis. A weaker zone of β-hemolysis encircling colonies on selective culture of a rectovaginal swab obtained during pregnancy would probably indicate colonization with group B *Streptococcus* (GBS), and guide intrapartum prophylactic antibiotic therapy to prevent neonatal infection [3,4]. The hallmark β-hemolytic phenotypes of these two important pathogens of humans are produced by the potent exotoxins streptolysin S (SLS) of GAS and β-hemolysin/cytolysin (β-h/c) of GBS.

Beginning with the pioneering studies of Julia Weld [5] and E.W. Todd [6], the streptococcal β-hemolysins have been investigated extensively, and much has been learned about their respective spectra of cytolytic activity, patterns of membrane injury, growth phase and nutrient requirements for production, non-specific inhibitors, and procedures for partial cell-free purification [7–11]. However, neither toxin has been purified to homogeneity and their precise molecular nature remains unknown, owing in large part to the requirement for high molecular weight 'stabilizer' molecules to preserve hemolytic activity in culture supernatants and in the absence of the bacterial cell surface. This review describes how an alternative molecular genetic approach led to the recent discovery of genetic loci necessary and sufficient for the β-hemolytic phenotypes of GAS and GBS. Of interest to molecular microbiologists, these studies revealed that the structural genes and predicted proteins of the GAS and GBS β-hemolysins are entirely distinct. Of interest to the medical community, the generation and testing of isogenic β-hemolysin mutants *in vitro* and *in vivo* has demonstrated the essential role of both toxins in producing the main symptoms of invasive disease.

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Streptolysin S of group A *Streptococcus*

The principal factor responsible for β-hemolysis in GAS is SLS, an oxygen-stable cytolysin that is not immunogenic in the course of natural infection [12,13]. The cytolytic spectrum of SLS is broad, including the membranes of erythrocytes, leukocytes, platelets, tissue-culture cells and sub-cellular organelles such as lysosomes and mitochondria; by weight, it is one of the most potent cytotoxins known [14]. Unlike the numerous exoproducts and enzymes (including SLS) elaborated by GAS during log-phase growth, SLS is unique because it can be synthesized continuously by stationary-phase cells in the presence of a minimal energy source, for example, glucose and Mg^{2+} ions [15]. SLS exists primarily in a cell-bound form, presumably linked to the streptococcal surface by lipoteichoic acid. SLS activity can be stabilized in solution by carrier molecules such as albumin, α-lipoprotein, and the RNAase-resistant fraction of yeast RNA (RNA core) [7,8]; however, the toxin is delivered most effectively to target cells by direct contact with GAS [16]. Insertion of SLS into the RBC membrane results in the formation of transmembrane pores and osmotic cell lysis, reminiscent of complement-mediated cytotoxicity [15,17]. Gel filtration and other biochemical analyses of SLS RNA core preparations estimated that the SLS polypeptide is 2.8 kDa [18] or 30 amino acids [19] in size. Most features of SLS contrast with those of streptolysin O (SLO), a 57 kDa oxygen-labile prototype of the cholesterol-binding, 'thiol-activated' cytolysin family that contributes negligibly to GAS β-hemolysis but elicits antibodies useful for documenting recent exposure to GAS [20,21].

The chromosomal locus for SLS production was first identified upon generation and characterization of SLS-negative GAS serotype M1 and M18 transposon mutants in the laboratory of J. DeAzavedo in Toronto [22]. The transposon insertions in these

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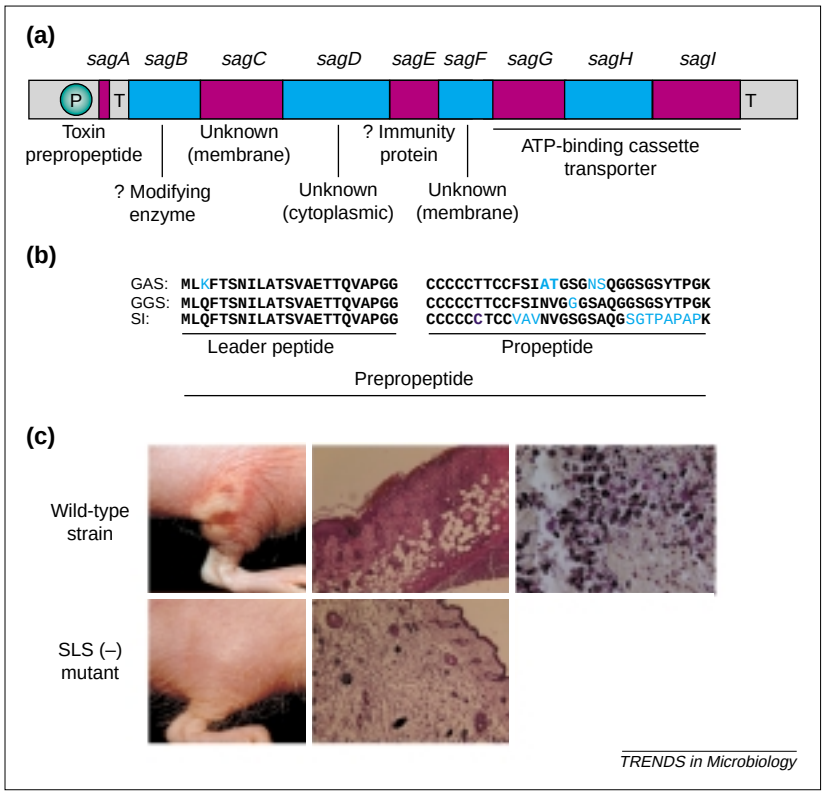

Fig. 1. Genetics and virulence properties of streptolysin S (SLS) of group A *Streptococcus* (GAS). (a) The nine-gene GAS *sag* operon for SLS biosynthesis. (b) Homologies of GAS SLS operons from β-hemolytic GAS, group G streptococcus (GGS) and *Streptococcus iniae* (SI). (c) Decreased virulence of GAS SLS mutants in the mouse model of necrotizing skin/soft-tissue infection. Necrotic ulcer formation with neutrophilic inflammation and tissue destruction is seen in mice inoculated with a wild-type GAS parent strain; these changes are markedly diminished or absent following identical challenge with the SLS-deficient mutant.

mutants mapped to a promoter motif upstream of a unique ORF encoding 53 amino acids that was named *sagA* for 'streptolysin-associated gene A'. Subsequently, B. Beall at the CDC in Atlanta performed chromosome-walking studies on an SLS-negative GAS serotype M49 mutant [23] assisted by the University of Oklahoma GAS genome-sequencing project [24]. These studies revealed eight additional contiguous genes (*sagB–sagI*) immediately downstream of *sagA* (Fig. 1a). Collaborative studies then used integrational mutagenesis to verify the phenotypes of the transposon mutants and define the functional boundaries of the *sag* locus. Targeted integrations in each *sag* gene yielded non-hemolytic GAS, whereas mutations upstream of the *sag* promoter or downstream of *sagI* did not affect SLS production. Cloning of the entire nine-gene *sag* locus in a non-hemolytic, non-pathogenic strain of *Lactococcus lactis* resulted in robust and stable β-hemolytic transformants. The intact *sag* locus, conserved among GAS of various *emm* genotypes, is both necessary and sufficient for SLS production [23].

Examination of the GAS *sag* locus reveals several features characteristic of a bacteriocin biosynthetic operon [23]. The first gene, *sagA*, encodes a 53 amino acid candidate prepropeptide. Within SagA is a typical Gly–Gly proteolytic cleavage motif, separating an amino-terminal 23 amino acid leader sequence from a 30 amino acid propeptide that matches precisely the size of mature SLS calculated from earlier biochemical analyses. The SagA propeptide sequence is highly enriched in amino acids (Ser, Thr, Gly and Cys) that are the precursors for post-translational modification and thioether-bond formation in other cyclical bacteriocin toxins [25]. The *sagG–sagI* genes have strong homology to those of ATP-binding cassette (ABC) transporters commonly required for the export of bacteriocin peptides [26]. The SagB and SagE predicted proteins share very weak homology with a bacteriocin-modifying enzyme and immunity protein, respectively. RT-PCR analysis confirms an operon structure, as the *sagB–sagI* genes use the same promoter as *sagA*. As in other bacteriocin operons, a 'leaky' terminator situated between *sagA* and *sagB* acts as a regulatory mechanism, yielding an abundance of structural gene transcripts (*sagA* alone) and smaller amounts of mRNA for downstream genes involved in modification, processing and export of the mature toxin [23]. We have recently found that precise, in-frame exchange of the *sagA*, *sagB*, *sagC*, *sagD*, *sagF* and *sagG* genes yielded non-hemolytic mutants in which SLS production could be restored by reintroducing the corresponding single gene on a plasmid [V. Datta *et al.* (2002) Detailed mutational analysis of the GAS *sag* operon for streptolysin biosynthesis. Abstract 132, 6th ASM Conference on Streptococcal Genetics, Ashville, NC, USA]. On repeated attempts, non-hemolytic *sagE* allelic exchanges were generated that could not be complemented with *sagE in trans*. Should *sagE* encode an immunity function, we speculate that allelic exchange of *sagE* might force compensatory mutations in other genes to eliminate SLS production.

Carr *et al.* [17] and later others [27] discovered that antibodies generated against synthetic peptides corresponding to the SagA propeptide domain neutralize SLS activity, thereby confirming that *sagA* is the structural gene for the SLS toxin. The expression level of *sagA* is under transcriptional control of the GAS global regulators *covR/covS* (also known as *csrR/csrS*), *mga*, *rofA* and *fas* [28–32]. Other investigations have attributed global regulatory functions to the *sagA* gene itself, applying the designation *pel*, for 'pleiotropic effects locus', to the ORF. A Tn*917* '*pel*' mutant with an insertion in the region of the *sag* promoter was non-hemolytic and showed decreased transcription of the genes for M-protein, the cysteine protease SpeB and streptokinase [33]. Passage of this mutant in mice selected for restoration of *sagA* transcription and β-hemolysis but did not reverse all of the pleiotropic effects [34]. By contrast, an insertion-duplication mutation of the *sagA* gene in an M6 strain was shown to have normal *emm* transcription but to express a truncated version of the M-protein that would not

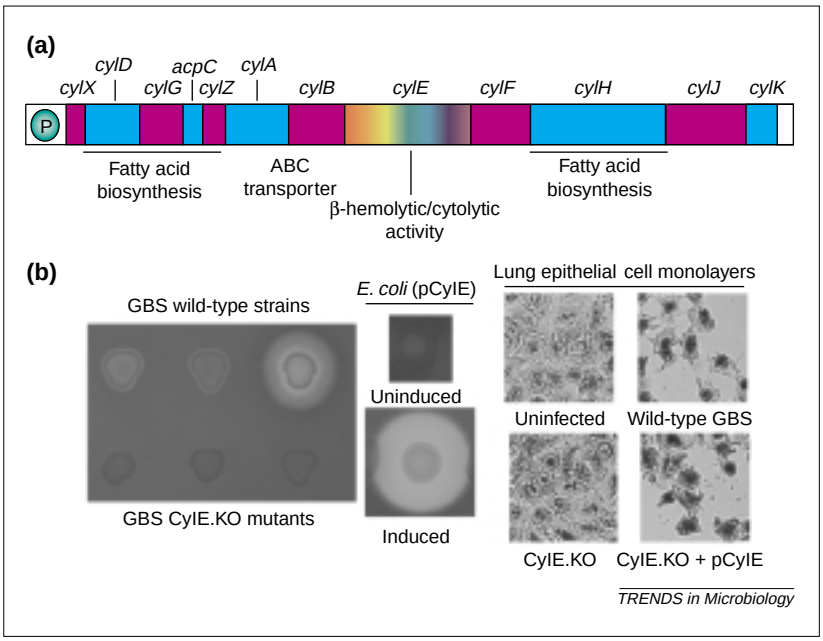

Fig. 2. Genetics of β-hemolysin/cytolysin (β-h/c) of group B *Streptococcus* (GBS). (a) Location of the *cylE* gene encoding β-h/c activity within the GBS chromosome. (b) Targeted mutagenesis, heterologous expression and lung epithelial cell cytotoxicity testing for the GBS *cylE* gene, demonstrating it is necessary and sufficient for β-h/c activity. Modified, with permission, from [43].

anchor to the cell surface [35]. Further investigations are needed to unravel the potentially complex GAS genetic networks that regulate and respond to the levels of SLS production.

Homologs of the GAS *sag* operon for SLS biosynthesis were recently identified in invasive human isolates of β-hemolytic group C and G streptococci [36] and the zoonotic pathogen *Streptococcus iniae* (Fig. 1b) [37]. The contribution of SLS to the pathogenesis of streptococcal necrotizing soft-tissue infection has been examined in a murine model. In this model, wild-type bacteria elicit an ulcer with bacterial proliferation, neutrophilic inflammation and histopathological evidence of vascular injury and tissue necrosis (Fig. 1c). In contrast to the parent strains, isogenic SLS-negative *sag* mutants of GAS, group C *Streptococcus* and *S. iniae* do not develop ulcers, and biopsy of the inoculation site demonstrates bacterial clearance and minimal degrees of inflammation or tissue injury (Fig. 1c) [36–38]. These results suggest SLS is a crucial virulence factor in GAS necrotizing soft-tissue infection. By contrast, SLO is not required for GAS necrotic ulcer formation [39]. *In vitro* studies suggest that SLS can contribute to pathogenesis by direct cytotoxicity, inflammatory activation and by inhibiting neutrophil phagocytosis [15,27,35]. The latter observation could help explain the paradox of decreasing bacterial clearance despite the intense influx of neutrophils seen with wild-type GAS infection. SLS has been shown to interact synergistically with the streptococcal cysteine protease SpeB and host-neutrophil-derived proteases and oxidants to produce cellular injury *in vitro* [15,40], and it is likely such complex processes are involved in the rapid tissue necrosis that is characteristic of invasive GAS soft-tissue infection [8].

β-hemolysin/cytolysin of group B *Streptococcus*

Like SLS, the β-h/c of GBS is an oxygen-stable, non-immunogenic, pore-forming cytolysin that has yet to be fully purified [41,42]. GBS β-h/c activity is stabilized by albumin, starch or Tween 80 and inhibited by phospholipids such as dipalmitoyl phosphatidylcholine (DPPC) [7,8,10]. The chromosomal location of genes encoding GBS β-h/c activity was first discovered by Spellerberg *et al.* upon analysis of non-hemolytic mutants generated using a novel transposition vector [22]. Subsequent investigations independently identified and extended the GBS *cyl* locus (Fig. 2a) by mapping the location of a GBS DNA fragment that conferred β-hemolysis to *E. coli* [43].

Targeted mutagenesis and expression cloning experiments demonstrated that the *cylE* ORF, encoding a predicted 78 kDa protein without GenBank homologies, was by itself necessary for GBS β-h/c activity and sufficient to confer β-hemolysis when expressed as a fusion protein in *E. coli* (Fig. 2b) [43]. The putative GBS β-h/c, CylE, appears anomalously located amid a fatty-acid biosynthesis (*fab*) operon. The β-h/c phenotype is also curiously linked to the production of an orange carotenoid pigment by GBS: non-hemolytic (NH) mutants are invariably non-pigmented and hyper-hemolytic (HH) mutants are invariably hyper-pigmented [44,45].

In vitro studies using isogenic GBS mutants with an NH or HH phenotype have shed light on how the β-h/c toxin could contribute to disease pathogenesis. GBS β-h/c production is correlated with cytolytic injury to lung epithelial cells (Fig. 2b) [46], lung endothelial cells [47], brain endothelial cells [48] and macrophages [49]. Injured cells show the formation of surface blebs, dramatic loss of cytoplasmic density, splitting of the cytoplasmic and nuclear membranes, dilated organelles and clumping of nuclear chromatin, all consistent with a pore-forming mechanism of action [46]. GBS β-h/c stimulates the transcription of intracellular nitric oxide synthase (iNOS) and the production of nitric oxide (NO) in macrophages [49], and can trigger macrophage apoptosis through a MyD88-independent pathway [50]. At sub-cytolytic doses, β-h/c promotes GBS invasion of human lung epithelial cells and triggers release of the neutrophil chemoattractant interleukin (IL)-8 [51]. The β-h/c is also the key factor activating human brain microvascular endothelial cell genes [IL-8, GROα, GROβ, intercellular adhesion molecule (ICAM)-1 and granulocyte–macrophage colony-stimulating factor (GM-CSF)] implicated in the neutrophilic inflammatory response of GBS meningitis [K.S. Doran and V. Nizet (2002) Group B streptococcal factors involved in regulation of gene expression in the blood–brain barrier.

Table 1. Comparison of key features of GAS and GBS β-hemolysins[a]

Feature	Streptolysin S (GAS)	β-hemolysin/cytolysin (GBS)
Spectrum and mode of action	Broad, pore-forming	Broad, pore-forming
Genes required for β-hemolytic phenotype	Entire nine-gene *sag* operon (except *sagE*?)	A single gene (*cylE*)
Genes sufficient to confer β-hemolytic phenotype	Entire nine-gene *sag* operon (to *Lactococcus lactis*)	A single gene (*cylE*) (to *Escherichia coli*)
Predicted size	2.9 kDa	78 kDa
Homologies	Bacteriocin	None
Precise molecular structure	Unknown	Unknown
Stabilizers	RNA-core, lipoteichoic acid, albumin, non-ionic detergents	Starch, albumin, non-ionic detergents
Inhibitors	Trypan blue, phospholipids (e.g. DPPC)	Phospholipids (e.g. DPPC), proteases (e.g. subtilin)
Linked phenotypes	SpeB, M-protein ?	Pigment production
Found in other species?	Yes (GCS, GGS, *Streptococcus iniae*)	Unknown
Naturally immunogenic?	No	No
Experimentally immunogenic?	Yes	Unknown
Proinflammatory effects	Neutrophil activation	Cytokines, nitric oxide
Virulence factor (models)?	Yes (necrotizing cellulitis)	Yes (pneumonia, septicemia)

[a]Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; GAS, group A *Streptococcus*; GBS, group B *Streptococcus*; GGS, group G *Streptococcus*; SpeB, streptococcal pyogenic exotoxin B.

Abstract 38, 6th ASM Conference on Streptococcal Genetics, Ashville, NC, USA].

GBS β-h/c contributes to virulence in animal studies. When administered to neonatal rats via the pulmonary route, NH GBS mutants possess a 1000-fold greater LD_{50} than the wild-type parent strains, confirming a role in the pathogenesis of pneumonia and systemic spread [52]. In an adult mouse model of GBS arthritis, hemolysin expression is associated with higher mortality, increased bacterial loads, greater degrees of joint injury, and release of the proinflammatory cytokines IL-6 and IL-1α systemically and intra-articularly [53]. Challenge of rabbits with isogenic GBS mutants showed that hemolysin production was associated with significantly higher mortality and evidence of liver necrosis with hepatocyte apoptosis [54]. It appears that GBS hemolysin is a pluripotent virulence factor that contributes to disease pathogenesis by cytotoxicity and inflammatory activation.

Several virulence properties of GBS β-h/c can be blocked by DPPC, the major component of pulmonary surfactant. DPPC significantly reduces β-h/c-associated cellular toxicity [46,55], cytokine activation [51,56] and macrophage apoptosis [57]. Lack of DPPC inhibition of β-h/c toxicity might contribute to the increased incidence and severity of GBS pneumonia and sepsis in premature, surfactant-deficient neonates [58]. Experiments in the ventilated premature rabbit model of GBS pneumonia and retrospective analysis of clinical data from human neonates with early-onset GBS infection corroborate the beneficial effects of surfactant therapy against GBS-induced lung injury [59,60].

Questions for future research

- Will the availability of new genetic information and targeted hemolysin-negative mutants allow purification and characterization of the mature β-hemolysin toxin proteins?
- Does streptolysin S possess bacteriocin activity against other human microflora?
- Through what signal transduction pathways (e.g. Toll-like receptors) does the GBS β-hemolysin/cytolysin activate cytokines and other innate immune response factors?
- Will active immunization with unprocessed or inactivated streptococcal β-hemolysin toxins afford protection against subsequent challenge with the infectious organism?
- Do strain differences in regulation of β-hemolysin production *in vivo* contribute to the epidemiology of invasive streptococcal infection?

Conclusion

The β-hemolysins of two major pathogenic streptococci, GAS and GBS, are both broad-spectrum cytotoxins that have long been postulated to play a role in the tissue injury and systemic spread associated with severe human infections. Although these toxins are responsible for a similar colony phenotype on blood-agar and share other phenotypic properties, the discovery of the genetic loci for GAS and GBS β-hemolysin production revealed their molecular basis is fundamentally distinct. Table 1 summarizes some of the known features of these two streptococcal β-hemolysins.

Human leukocytes, epithelial cells and endothelial cells respond variably to the streptococcal β-hemolysins, with release of proinflammatory and/or chemotactic defense factors such as IL-8 (GBS) or cathelicidin antimicrobial peptides (GAS; V. Nizet and R.L. Gallo, unpublished). The ability to detect proportionately and respond to sub-cytolytic levels of these exotoxins is probably adaptive for the host in the vast majority of encounters – serving to orientate the innate immune response and contain the organism on the mucosal epithelial surface. However, in special circumstances such as the overwhelming *in utero* exposure of a premature, surfactant-deficient

neonate to invasive GBS, over-activation of these same response pathways could contribute to massive release of cytokines and the manifestations of septic shock. More puzzling perhaps is the well-documented ability of GAS to cause life-threatening invasive infections such as necrotizing fasciitis and toxic shock syndrome in previously healthy children and young adults. Do these individuals have subtle deficits in their innate immune recognition of GAS SLS production, allowing deeper penetration and multiplication of the organism? Alternatively, do certain GAS strains 'phase-shift' *in vivo* towards constitutive high-level production of SLS with attendant injury to host tissues?

Elucidating the molecular genetic basis of the streptococcal β-hemolysins promises to open a new era of detailed investigation of these unique and potent toxins. The availability of precise isogenic mutants and cloned hemolysin determinants should facilitate purification of these elusive proteins and allow careful analysis of their contribution to disease pathogenesis. Curiously, humans do not appear to generate neutralizing antibodies to either toxin during the course of natural infection. One can speculate that this non-immunogenicity derives from the potent cytotoxicity of these compounds against macrophages and other antigen-presenting cells at the initial steps of the humoral immune response pathways. The recent observations that non-toxic synthetic peptides of the SLS precursor maintain enough sequence features of the mature toxin to elicit neutralizing antibodies [17,27] suggest that subunits or inactivated toxoids of each hemolysin could have significant immunogenicity. Vaccine or chemotherapeutic strategies (e.g. phospholipid inhibitors) designed to neutralize β-hemolysin activity could be beneficial in managing human GAS and GBS infection.

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Environmental regulation of mutation rates at specific sites

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Recent studies on bacterial adaptation to stress suggest that bacteria can regulate the generation of mutations at specific sites in response to environmental conditions. Here, we review these findings and discuss the circumstances under which these mechanisms might prove advantageous.

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Pathogenic bacteria are exposed to continually changing, stressful environments such as transitions between different host environments, repeated exposure to antibiotics or attack by host immune systems. Such stresses impose strong selection for stress-resistant phenotypes, but it is unlikely that any single phenotype will be competitively superior in all environments [1]. Exposure to continuously changing, stressful environments might therefore indirectly impose selection for mechanisms that generate phenotypic variation, as such mechanisms could increase the probability of a successful phenotype being produced in a given environment [2–4]. Note that this selection will be indirect because genes responsible for variation-generating mechanisms will increase in frequency only when linked to a beneficial phenotype [2–4].

The type of mechanism for generating phenotypic variation that is favoured by selection is likely to be critically dependent on the range and repeatability of the environmental variation [1]. These mechanisms can be split into two categories: those that involve differential regulation of gene expression [5,6] and those that involve mutational events [7,8]. In this review, we focus on the latter group, specifically concentrating on recent work suggesting that the environment can regulate the rates of mutation at specific sites, resulting in variation of the rate of switching between a limited number of phenotypes, presumably as an adaptation to regular fluctuations between a fixed set of environments. Other mechanisms of phenotypic variation based on genetic variation, such as elevated genome-wide mutation rates [9], environmentally regulated genome-wide mutation rates [10] and elevated site-specific

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