

Minireview

Adaptation beyond the Stress Response: Cell Structure Dynamics and Population Heterogeneity in *Staphylococcus aureus*

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Staphylococcus aureus, a major opportunistic pathogen responsible for a broad spectrum of infections, naturally inhabits the human nasal cavity in about 30% of the population. The unique adaptive potential displayed by *S. aureus* has made it one of the major causes of nosocomial infections today, emphasized by the rapid emergence of multiple antibiotic-resistant strains over the past few decades. The uncanny ability to adapt to harsh environments is essential for staphylococcal persistence in infections or as a commensal, and a growing body of evidence has revealed critical roles in this process for cellular structural dynamics, and population heterogeneity. These two exciting areas of research are now being explored to identify new molecular mechanisms governing these adaptational strategies.

Key words: *Staphylococcus aureus*, nucleoid, membrane, variant, population heterogeneity

Introduction

Staphylococcus belongs to the Gram-positive *Firmicutes* *Bacillales* group that also includes *Bacillus*, and *Listeria* spp. *S. aureus* is a commensal bacterium that naturally inhabits the nasal cavity of mammals, but it is also an opportunistic pathogen responsible for a broad spectrum of infections ranging from food poisoning and superficial skin abscesses to more serious diseases such as pneumonia, meningitis, osteomyelitis, septicemia and toxic shock syndrome. It has acquired resistance to a wide variety of antibiotics (39), and methicillin-resistant strains (MRSA), the most common cause of nosocomial infections, are now spreading into the community (12).

S. aureus has evolved multiple characteristics allowing it to colonize the outer surfaces of the host, including the anterior nares. In the nasal cavity, lysozyme, the first barrier in our defense systems, is secreted to destroy invading bacteria, but staphylococci have acquired resistance to lysozyme by modifying their peptidoglycan structure through *O*-acetylation (6, 35). Drastic changes in osmotic pressure are also a major threat to bacterial survival on the outer surface, though the ability of staphylococci to grow over a broad range of salt concentrations is well documented. These characteristics combine to allow staphylococci to predominate on the host's outer surfaces.

This highly successful pathogen is also equipped with a variety of tools for survival during infection, such as factors to colonize host tissues (18), to evade the innate immune system (27), to acquire iron (49), or to form biofilms (69).

Categorized as virulence factors and extensively reviewed elsewhere, they likely play a part in facilitating transmission and sustaining a commensal relationship under proper regulatory conditions, which *S. aureus* might have evolved to prevent causing an infection in a healthy host (10).

One prominent feature of *S. aureus* adaptation to stress is its ability to form 'variants' both *in vivo* and *in vitro*. Studies on some biochemical characteristics of variants have explained the mechanisms of their successful adaptation (see below), but the specific mechanisms involved in generating such population heterogeneity or variants remains poorly understood for the most part.

In this review, we start with 1) an overview of the cellular stress responses, and then focus on two recently emphasized concepts, namely 2) the dynamics of cell structure/components, and 3) population heterogeneity or the formation of variants. The interplay in multiple systems including the well established cellular responses are also discussed, in a search for clues to expand our understanding of the uncanny adaptational capabilities of *S. aureus*.

Classical stress response, at a glance

The ability to cope with stress has been studied as the cellular response to stress and the resultant expression of stress-responsive factors. Many studies helped establish the general concept that signal transduction and the regulation of gene expression play key roles in stress response/adaptation (19).

Sigma factors. As in most bacteria, sigma factors are master regulators of transcription. Sigma factors are a subunit of RNA polymerase involved in the recognition of specific promoter sequences to initiate site-specific transcription (55, 90). Generally, bacteria have multiple sigma factors each of which regulates a series of genes often constituting

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the cognate biological system (33). So far, four sigma factors have been identified in *S. aureus*. SigA is the primary sigma factor. It is conserved in all bacteria and responsible for the expression of house-keeping genes (23). SigB is the alternative sigma factor for general responses to stress (24). Its levels are constant throughout the growth phase (38), but SigB can be activated under unusual pH conditions, heat shock (transient), high osmolarity, and later growth phase through the 'partner switching' mechanism, although the precise mechanism for signal transduction to the SigB regulators in *S. aureus* is not known (86). Two additional sigma factors, SigH (56) and SigS (79), were identified later, but they are only weakly expressed under ordinary growth conditions, and their physiological roles remain elusive.

Two-component systems (TCSs). Two-component systems (TCSs) elegantly combine sensing, transducing and transcriptional activation modules within two proteins. The common phosphotransfer mechanism of signal transduction is reflected by the high degree of conservation within the transducing modules, whereas the sensing and response modules display the variability required for signal/response specificity. The first component, a histidine protein kinase, is autophosphorylated at a conserved histidine residue in an ATP-dependent reaction. In a second step, the phosphoryl group is transferred to an aspartate residue in the conserved amino-terminal domain of the second component, the response regulator. Regulatory pathways involving TCSs are often highly interconnected and form complex signal transduction networks (59). Most *S. aureus* strains are endowed with sixteen sets of genes encoding two-component systems, with an additional one present in the staphylococcal cassette chromosome *mec* of MRSA, linked to induction of methicillin resistance. This sophisticated arsenal of environmental monitoring proteins could, in part, explain the highly adaptive nature of *S. aureus*. Many of these systems remain to be characterized, although several have been shown to play a role in virulence (AgrC/AgrA, SaeS/SaeR) (8), resistance to antibiotics (VraS/VraR, GraS/GraR) (35, 43), or adaptations to environmental changes such as oxygen pressure (SrrA/SrrB) and as targets of drug development (26, 30). The best-studied *S. aureus* TCS is *agrAC* that constitutes a peptide quorum-sensing system, a key regulatory system for virulence gene expression.

SarA. SarA (staphylococcal accessory regulator) and its homologues are extensively studied regulators (16). They share a winged helix DNA-binding domain and participate in the regulation of many genes. SarA prefers to bind AT-rich DNA, but lacks a clear consensus target sequence. It was recently found that SarA is present at ~50,000 copies per CFU (29), and was suggested that SarA is the functional counterpart of *Escherichia coli* global regulators such as Hu and IHF, rather than a classical transcription factor.

Specific regulatory systems. Classical transcription factors constitute many of the unique staphylococcal regulatory systems. The heat shock-evoked response of chaperone gene expression controlled by CtsR and HrcA is among the best-documented systems regarding their molecular mechanisms and evolutionary aspects (13, 25, 42, 68). In *Bacillus subtilis*, heat shock genes are classified into four groups according to the regulators responsible for their expression: Class I is

controlled by the HrcA repressor, Class II by SigB, Class III by CtsR, and Class IV by others. The HrcA repressor recognizes the CIRCE operator sequence. CtsR recognizes a tandem heptanucleotide direct repeat. Class I genes including classical chaperone genes (*dnaK*, *groES*, *groEL*) and Class III genes including *clp* genes are distinct in *B. subtilis*. However, in *S. aureus*, the entire HrcA regulon is embedded within the CtsR regulon, with the synthesis of HrcA itself controlled by CtsR (13). Indeed, the CtsR protein of *S. aureus* binds specifically to the *clpP*, *clpC*, *clpB*, *dnaK* and *groESL* regulatory regions, and both CtsR and HrcA bind to the *S. aureus dnaK* and *groESL* promoter regions (13, 28). Other examples, described below, involve evolutionary distinct transcription factors to regulate the same gene or phenomenon: PerR/OxyR-based regulation of *dps* family genes, and ComK/SigH-based regulation of *com* genes. Thus, bacteria have evolved species-specific regulatory systems that differ even among close relatives such as *S. aureus* and *B. subtilis*.

Through such master/global regulators or transcription factors, the gene expression profile is thought to be modulated to adapt to given environments. For example, oxidative stress regulons include enzymes that detoxify or reduce the level of oxidative stress (20), and the *agr* quorum-sensing system induces the expression of exo-enzymes that degrade host tissues to utilize them as nutrients (15). In addition to the direct function of virulence factors, additional layers of adaptation include the dynamic change of cell architectural components and the resultant conversion of the cell's physical characteristics, which are the focus of the next chapter.

Cell architecture dynamics: Cell membrane components

Although staphylococci are immobile and cannot form spores, the cell architecture including the nucleoid and cell membrane undergo dynamic changes when responding and adapting to environmental stress.

Cardiolipin. A general strategy among bacteria is to change phospholipid composition in response to growth phase or environmental stress such as osmolality (74), pH (31, 51), temperature and the presence of organic solvents (7, 73). In the 1970's, the molecular mechanism of staphylococcal salt resistance was studied, focusing on a phospholipid, cardiolipin (CL), which constitutes about 30% of the cell membrane in the stationary phase of growth (80). Two phosphatidylglycerols are enzymatically connected through the glycerol backbone to form CL, which carries four fatty acid tails and two negative charges (77). CL can stabilize a membrane against changes of osmotic stress when included in liposomes (60). Recently, CL was demonstrated to be important for *E. coli* and *B. subtilis* to grow at high salinity (47, 72). In contrast, *S. aureus* does not require CL to grow in high salinity medium (Tsai *et al.*, submitted). Instead, CL is important for long-term survival under high salinity or sudden hypertonic shock. Two genes were found to encode functional CL synthases. The physiological significance of the differential usage of the two homologous enzymes is still unclear, but we recently found that one of them functions specifically under conditions of stress such as high salinity, high temperature, and high pH. Further detailed analysis

focusing on the distinct roles of the two CL synthases is required to understand the role(s) of CL dynamics in *S. aureus*.

Carotenoid and others. The cell membrane also contains a carotenoid pigment, staphyloxanthin, conferring on *S. aureus* its characteristic yellow or orange color. *S. aureus* was named after this characteristic ('*aureus*' stands for golden in Latin), although recent clinical isolates often lack the pigment. The genes for staphyloxanthin biosynthesis (*crtM*, and *crtN*) (87) are under the control of SigB and the yellow color develops through prolonged growth under aerobic conditions. Liu *et al.* indicated that staphyloxanthin contributes to resistance to oxidative stress produced by host phagocytes (46).

Changes in other cell membrane/cell wall components (e.g., lysyl-phosphatidylglycerol, teichoic acid, and cell wall thickness) are also important for adaptation to a variety of agents such as cationic antimicrobial peptides (70) and the clinically important antibiotic vancomycin (21).

Cell architecture dynamics: Nucleoid

Bacterial genomic DNA with a size of ~1 mm is packed in a cell of ~1 μm diameter together with nucleoid-associated proteins (NAPs), RNA, and others (66, 67). This complex, termed the nucleoid, is the site of a series of events important for maintaining genome functions, such as duplication, segregation, gene expression, repair, and insertion/excision of mobile genetic elements. There exist several hundred NAPs and our recent proteomic analysis found that the majority detected in the nucleoid fraction vary among genera (Ohniwa *et al.*, in preparation). Furthermore, they are highly variable depending on growth phase: roughly 70% are replaced from the log to stationary phase in *S. aureus*. The nucleoid from lysed cells is usually a fibrous structure varying in thickness when observed by the 'on-substrate lysis' method with atomic force microscopy regardless of the growth phase (84), but the physical characteristics of the nucleoid change in response to oxidative stress, where the MrgA protein plays a key role (57, 58).

Species-specific expression of Dps/MrgA. MrgA, encoded by *mrgA* (metal ion responsive gene A) (14), is a homologue of *E. coli* Dps. Dps is a member of the Fe-binding protein family that forms dodecameric complexes (3, 32). Dps can bind DNA and protect it against oxidative stress (50), nuclease cleavage, UV damage, thermal shock (62) and acidic environments (17). Dps is one of very few nucleoid proteins that are conserved in many bacterial species (84), including *S. aureus*. However, the manner in which Dps is induced to express depends on the bacterial species. Fig. 1 summarizes the difference in Dps expression between *S. aureus* and *E. coli*. The *E. coli* Dps is the most abundant protein in the tightly condensed nucleoid at the stationary phase (41, 89), whereas the staphylococcal MrgA is not expressed towards the stationary phase and the nucleoid sustains the fibrous structures (57). The nucleoid of *S. aureus* undergoes clogging following molecular genetic manipulation (*mrgA* overexpression or *perR* knockout) but it allows cell proliferation, indicating that the MrgA-dependent nucleoid clogging can permit gene expression and genome

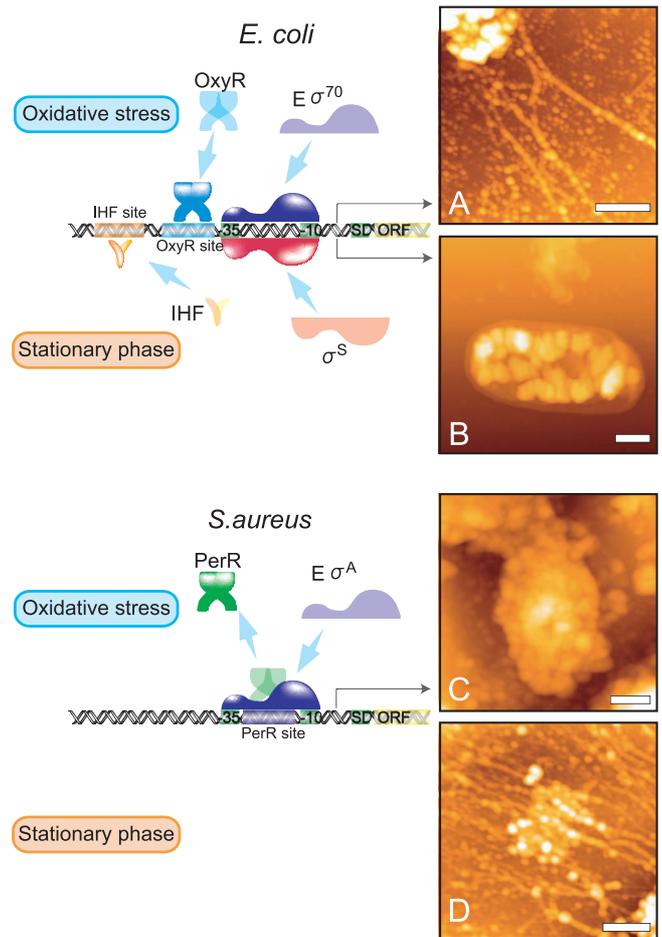


Fig. 1. Regulatory mechanisms of *dps/mrgA* and the dynamics of nucleoid structure in *E. coli* and *S. aureus*. In *E. coli*, *dps* gene expression is induced towards the stationary phase by IHF, which binds to the -50 region together with the σ^S -containing holoenzyme ($E\sigma^S$). OxyR stimulates *dps* gene expression under oxidative stress. In *S. aureus*, only the PerR pathway exists due to the lack of the stationary phase pathway. OxyR and PerR are evolutionary distinct, and OxyR acts as an activator, while PerR is a repressor whose release under oxidative stress induces *mrgA* expression. The fibrous nucleoid in the log phase undergoes tight compaction towards the stationary phase in *E. coli* (panel B), but not under oxidative stress (panel A) due to the presence of the *E. coli*-specific Fis that interferes with Dps-dependent compaction. The nucleoid of *S. aureus* is clogged under oxidative stress through induction of MrgA protein synthesis (panel C), but not in the stationary phase (panel D). Scale bar=500 nm.

duplication/segregation (57). Both *E. coli* *dps* and *S. aureus* *mrgA* are induced to express by oxidative stresses via evolutionary distinct transcription factors, both of which can sense oxidative stress directly. The oxidative stress then causes nucleoid clogging through the induction of MrgA in *S. aureus* but not in *E. coli*, due to the presence of *E. coli*-specific Fis proteins that inhibit the Dps dependent genome condensation (65).

MrgA-dependent oxidative stress resistance: Fe binding or DNA binding? The *S. aureus* nucleoid clogged by MrgA is biologically active in contrast to the *E. coli* nucleoid compacted by Dps in the stationary phase, or more strikingly, the genome protected by acid soluble proteins in spores of *B. subtilis* (54, 78). Consequently there is a need to evaluate whether staphylococcal nucleoid clogging has DNA protec-

tive roles similar to the physical protection provided by *E. coli* Dps (or acid soluble proteins in spores). The fact that *mrgA* is essential to cope with oxidative stress suggests that one or both of its Fe-binding and DNA-binding activity could contribute to oxidative stress resistance. Scavenging free iron is important to reduce the Fenton reaction that generates the hydroxyl radical from ferrous iron (Fe²⁺) and hydrogen peroxide (37). In *Streptococcus mutans*, where the Dps-family Dpr protein can bind iron but not DNA, Fe-binding activity was demonstrated to be critical to cope with oxidative stress (91). However, no Dps family members exhibit DNA-binding activity without Fe-binding activity, leaving open to question the relevance of physical nucleoid clogging and oxidative stress resistance. Currently, we assume that Fe-binding by MrgA is the primary requirement to cope with oxidative stress, and its DNA-binding activity would keep it close to the genome, thereby reducing the Fe concentration around the DNA. This assumption is based on the fact that nucleic acids bind iron well, and the hydroxyl radical is unlikely to spread far from its origin in cellular environments (37). Our recent preliminary results indicated that a mutated MrgA derivative that can bind DNA but cannot bind Fe was unable to protect *S. aureus* cells against oxidative stress (unpublished result). Although it is currently difficult to demonstrate our assumption, a detailed structural analysis of the complex of MrgA-Fe-DNA might provide a clue.

Population heterogeneity

Phenotypic heterogeneity. Diverse subpopulations confer to cells a better chance of surviving a variety of conditions or stress than a homogeneous population. A heterogeneous subpopulation can be generated by diversified gene expression through changes in a variety of cellular factors such as signal transducers, transcription factors, nucleoid proteins including global regulators, and non-protein-coding RNAs (1). The existence of heterogeneous cell populations is well documented regarding antibiotic resistance in *S. aureus*, and a ‘population analysis’ is often used to evaluate the pattern of the successive distribution of resistance in a genetically identical cell population (36). On the other hand, the cell population from a single clone can also exhibit completely distinct phenotypes. In *B. subtilis*, for example, the competence-related genes are expressed in a stochastic manner. The molecular basis for this stochastic expression is the regulatory network controlling the ComK transcription factor, where the positive and negative feedback loops are interconnected (48, 82). Although environmental signals are integrated into this regulatory network, some ‘noise’ can shift the mode of this network to switch on ComK activity in a stochastic manner. The competence gene homologues in *S. aureus* also undergo stochastic expression, although these genes are regulated by the secondary sigma factor SigH, which is evolutionary distinct from ComK (Morikawa *et al.*, in preparation).

Genetic variation. In contrast to the phenotypic heterogeneity in genetically homogeneous cell populations, heterogeneity in bacterial populations is often generated through genetic changes. The genetic change can be spontaneous or

Table 1. Examples of population heterogeneity in *S. aureus*

Types	Notes	References
<i>Antibiotic resistance</i>		(36)
<i>Variant</i>		
small colony variants (SCVs)	deficient in electron transport	(5, 71)
L-form	w/o cell wall, stable or unstable	(2)
others	variants resistant to e.g. antibiotics	(11)
<i>Phase variation*</i>		
hyperhemolytic phenotype		(92)
length of MapW protein	poly(A) tract in coding sequence	(9)
biofilm formation	IS256 insertion in <i>ica</i> or <i>sarA</i>	(40, 85)
protease activity		(85)
<i>Other</i>		
SJ duplication in <i>sigH</i>	see Fig. 2	unpublished

* a comprehensive list of phase variation in bacteria is available in reference (88).

inducible, which is sometimes difficult to distinguish. *S. aureus* has multiple methods of generating heterogeneity as described below (Table 1). One method is the formation of variants under stress, and among the best documented variants are small colony variants (SCVs) (5, 71) and L-form variants.

Small colony variants (SCVs). Small colony variants (SCVs) can be recovered from patients with cystic fibrosis and chronic/persistent infections, and are thought to be an adaptive form to sustain an infection in the host. SCVs are able to persist within non-professional phagocytes, being protected against host bactericidal factors. It is also possible to select or generate SCVs *in vitro* by using aminoglycoside and the *Pseudomonas aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (53): *P. aeruginosa* is also often isolated from cystic fibrosis patients. In many cases, SCVs are deficient in the electron transport system, such as the pathways for producing hemin, menadione and thiamine, and thereby exhibit slow growth. Indeed, the disruption of genes involved in hemin or menadione biosynthesis induces the SCV phenotype. Here it should be noted that aminoglycoside is transported into cells via a proton gradient generated by the electron transport system (83), and HQNO specifically blocks electron transport in Gram-positive bacteria (44). The mutator phenotype increases the frequency with which SCVs form. In addition, it is likely that the formation of SCVs is regulated by environmental signals. Mitchell *et al.* have reported that the general stress response sigma factor, SigB, plays important role(s) in the formation of SCVs induced by aminoglycoside (52) and by HQNO (53), suggesting interplay between the stress response system and the variant forming process.

L-form. The L-form is another special variant generated by *S. aureus* as well as by other bacteria (2). It lacks cell walls, and requires proper osmotic pressure to be isolated from patients. L-forms can also be generated *in vitro* by long-term (several days) incubation with cell wall-perturbing agents such as β -lactams and lysostaphin. The membrane of L-form cells is thought to be equipped with certain features

that can support cell growth and survival without rigid cell wall. A previous study found that a certain L-form strain increased CL content (34). Our recent molecular genetic study found that one of the two CL synthase genes (*SA1891*) is important for generating the L-form, but we found that a *cls1/cls2* double mutant lacking CL can still generate L-form cells albeit with reduced frequency (Tsai *et al.*, submitted). This suggests the existence of CL-independent mechanisms. It is reasonable to think that multiple mechanisms cooperate to generate the L-form variant. It is worth noting that biochemical characteristics such as catalase, coagulase, lipase, fibrinolysin, haemolysin, and enterotoxin production can change during L-form generation and reversion (22, 75, 76, 81), but none of these is a common phenotype for L-form variants, suggesting that the L-form is generated concomitant with drastic phenotypic conversions, where the increase in CL content may be an effective, but dispensable, way to stabilize the membrane. It should also be noted that the membrane of L-forms contains cholesterol (34, 63). Since the *S. aureus* genome lacks any apparent genes for cholesterol synthesis, it has not been concluded whether *S. aureus* can develop a sterol producing capability. It is interesting that certain inhibitors for human cholesterol synthase can prevent the function of CrtM responsible for carotenoid synthesis (45). It is not known whether the *crtM* gene or CrtM enzyme is altered in cholesterol-producing L-form variants.

Phase variation. *S. aureus* also exhibits population heterogeneity through ‘phase variation’. Phase variation is a phenomenon whereby the expression of a certain phenotype is converted at frequencies higher than 10^{-5} per cell per generation, usually in a reversible manner, and is thought to be a major adaptative strategy in bacteria (4, 88). Phase variants are generated by a variety of interesting genetic (mutation, site-specific recombination, homologous recombination, simple sequence DNA repeat tracts) or epigenetic changes [reviewed in (4)]. Reported or postulated phase variations in *S. aureus* include a hyperhemolytic phenotype produced by an unidentified mechanism (92), variable lengths of the MapW protein (major histocompatibility complex MHC class II analogue) (9), and the on/off switch for the formation of a biofilm (40).

The Map-like protein genes are categorized into two groups, one of which, including the *SA1751* gene in strain N315, has a poly(A) tract within the coding sequence. The length of the poly(A) tract in *SA1751* is 10 bases, introducing a stop codon immediately after the tract. On the other hand, other strains have variable lengths of the poly(A) tract, and 8 or 11 adenines result in the full-length translation of the protein. The shift in the poly(A) tract’s length is considered to be brought about by the slipped-strand mispairing mechanism.

Phase variation in the biofilm switch is mediated by the reversible insertion/excision of the insertion sequence, IS256, in biofilm essential genes such as the *ica* locus (40) and *sarA* (85). The insertion of IS256 was suggested not to be random in the genome, because the formation of variants is observed only for the biofilm and protease phenotypes but not for others such as coagulase, hemolysin, and lipase (85). It is interesting that SigB regulates the frequency of IS256

transposition (85), though the precise mechanism is not known. In general, transposition is regulated/affected by a variety of host factors that differ depending on the mobile element (61).

Population heterogeneity by SigH. Recently, a new system generating population heterogeneity was found involving SigH activation (Fig. 2) (Morikawa *et al.*, in preparation). As mentioned above, SigH activity is undetectable when the population is analyzed as a whole, *e.g.*, by examining SigH protein levels by Western blotting (56). This is because SigH production is restricted to a minor cell population. SigH is activated by two distinct mechanisms: one involves post-transcriptional regulation, and the other is transient genetic

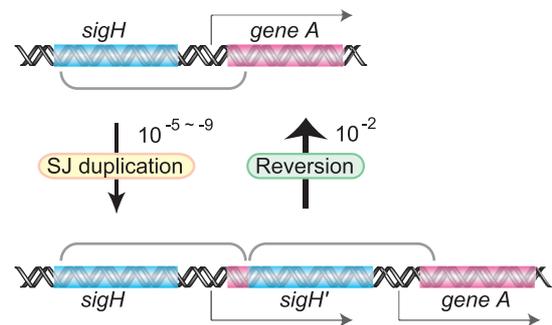


Fig. 2. The activation of *sigH* by Short Junction (SJ) duplication. SJ-duplication occurs at the *sigH* locus and the region encompassed by short direct repeats is duplicated at low frequencies (10^{-5} – 10^{-9}). The resultant new chimeric *sigH* gene, that is the in-frame fusion of the downstream gene and *sigH*, is expressed and the SigH protein is produced so as to induce its regulon. The duplication ceases at a frequency of about 10^{-2} . SigH can be also produced through post-transcriptional regulation without the SJ duplication system, but in both cases, SigH activation remains restricted to a minor cell population.

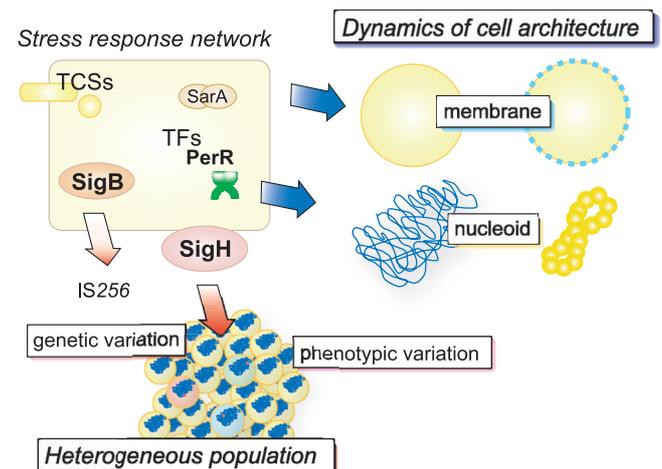


Fig. 3. Schematic representation of the adaptational strategies detailed in this review. Stress signals are sensed and transduced through general or species-specific regulatory networks so as to modulate gene expression. A part of the stress regulon plays an important role in cell architectural dynamics. On the other hand, generating a heterogeneous cell population is beneficial according to the ‘insurance hypothesis’. Heterogeneity is generated either by uneven/stochastic gene expressions or by genetic conversions. Alternative sigma factors, which are generally responsible for stress responses, can affect population heterogeneity. Variants of cell architecture include SCVs, L-form, biofilm variants, antibiotic-resistant variants, etc.

change. In the latter, short-junction (SJ) duplication occurs at the *sigH* locus so as to generate a new in-frame fusion gene at frequencies of 10^{-5} – 10^{-9} in a spontaneous manner. The resultant *sigH* fusion gene is expressed under the control of promoter and translation initiation signals originating from the partner gene. However, this state is genetically unstable, and the duplication is cured at a frequency of $\sim 10^{-2}$, probably helping to maintain the original state (without the duplication) in the majority of the population. This is similar to phase variation, but the frequency of the duplication is lower. It will be of great interest to examine whether the SJ duplication can randomly occur in the genome at direct repeat sites, and if so, to examine how much this mechanism contributes to the adaptive ability of *S. aureus* or other bacteria, which will be the focus of our future work.

Conclusions

In this review, two recently developed aspects of stress adaptation were highlighted. The findings on ‘cell architecture dynamics’ reveal the importance of structural/physical features in understanding stress adaptation. The ‘population heterogeneity’ indicates the necessity for further analysis of their still obscure molecular mechanisms. We emphasize that there is interdependency or crosstalk among the distinct systems: *e.g.*, relevance of the CL content and L-form variant formation, SigB regulated formation of IS256 dependent variants, and the direct generation of population heterogeneity by SigH (Fig. 3). Such multi-layered interconnected survival strategies, together with the general or specialized horizontal gene transfer systems (7, 64), are key features in understanding how *S. aureus* has developed as such a successful pathogen.

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