Staphylococcal phenomics: metabolomic and proteomic responses to environmental stressors

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Staphylococcal species are opportunistic pathogens known to cause an array of acute and chronic infections. The high pathogenicity of these species is thought to be due to their remarkable capacity to rapidly adapt to changes in environmental conditions. Bacterial response mechanisms for survival in changing environments involve metabolomic, genomic, proteomic and structural adjustments within the cell. It has recently become apparent that substantial heterogeneity exists within the bacterial population to maximise chances of survival of the species. This chapter will highlight the role of rapidly changing compositions of metabolites and proteins as potential response mechanisms to facilitate the survival of staphylococcal species following exposures to various environmental stressors. Such advanced understanding of "phenomics" may help in the development of new strategies for antimicrobial chemotherapy.

Keywords Staphylococci; proteomic; metabolomic; environmental stressors; small colony variants; phenomics

1. Introduction

Staphylococcal species are opportunistic pathogens which cause a wide range of acute and chronic infections. These infections lead to high morbidity and mortality worldwide and thus pose a significant threat to animals and humans alike [1]. It has been proposed that this high pathogenicity is due to their ability to rapidly adapt to changes in the environment such as sudden changes in nutrient availability, pH, temperature, osmotic pressure and exposure to toxic chemicals. An example of this adaptation is their ability to survive as fomites on inanimate surfaces such as medical devices to facilitate successful transmission between human hosts [2]. Bacterial survival mechanisms involve alterations in metabolism and protein turnover to optimise cell performance under the prevailing conditions. Adaptations of cells to specific micro-environmental conditions may also lead to heterogeneity within the bacterial population, maximising chances of survival [3-6]. One of the best examples of heterogeneity is the formation of small colony variants (SCVs) that can occur following exposure to antibiotics [3] or environmental stressors such as cold stress [6]. SCV formation is a common phenotypic shift mechanism used by staphylococci species in response to toxic chemical or cold stress resulting in a more resilient phenotype which enhances survival of these species [6, 7]. This phenotypic conversion coupled with the enhanced capacity for biofilm formation offer defence mechanisms for these bacteria against both the host immune system and harsh environments [6, 8].These phenomena are thought to be due to the ability of the organism to alter its metabolism and protein expression [9-12].

Over the past decade, metabolomic and proteomic techniques for investigating bacteria have been substantially advanced, allowing a better understanding of integrated metabolism within cells and further insight into understanding evolutionary and pathogenic mechanisms [13]. These studies have also provided a capacity to investigate the influences of environmental stimuli on the cellular homeostasis via measures of changing profiles of metabolites and protein concentrations under a range of environmental conditions [14]. Metabolomic and proteomic studies indicated that subtle alterations in protein composition provided the foundation for altered metabolism under the various environmental conditions [6, 15, 16]. The analyses have also shown that changes in a small number of proteins and metabolites can help in survivability under stressful conditions and it is apparent in many cases that it is not necessary to have a myriad of changes in protein or metabolites compositions for surviving in a specific environment [11, 17]. As the metabolome and proteome represent the structural and functional operations of living organisms, metabolomic and proteomic investigations have become fundamental to understanding the dynamics of cellular function of any organism [18]. For example, the presence of low molecular weight metabolites such as betaine and proline may be required as osmoprotectants in response to exposure to high osmotic pressure [19]. Specific structural proteins and enzymes may be required to facilitate these increases in cytoplasmic betaine and proline and together would represent homeostatic changes in metabolite and protein inventories [20]. This review will summarise the premise that the survival of staphylococci following perturbations of environmental conditions requires significant alterations in the proteome leading to altered metabolic homeostasis for survival. In as similar manner, alterations in metabolic homeostasis would also underlie the switch to the "virulence mode of existence" when opportunities present to invade host tissues. Traditionally, evolutionary survival over the millennia has been thought to rely on advantageous mutations; however, phenomic switches are now clearly seen to be vitally important.

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2. Environmental stressors and Staphylococci species: metabolomics and proteomics leading to phenomics theory

The growth and the survivability of bacterial cells under both normal and harsh conditions are mainly dependent on the ability of the bacteria to maintain a proper conformation of proteins which is important for their function [21]. Since it has been proposed that as certain environmental stressors can lead to the alteration of the structures of proteins causing aggregation into non-functional proteins, some of these response could threaten the survivability of bacteria [18, 22]. If the bacterial cells do not adapt appropriately in time, then the viability of its proteins will be compromised. The viability and function of proteins must be maintained to optimise survival of the bacterial cells. Bacterial cells have homeostatic mechanisms that assist to refold misfolded proteins or remove those that cannot be refolded [22, 23]. The most popular systems that are involved in coping with many stress conditions are chaperons and proteases. Both of these systems are embedded in stress response regulatory mechanisms via complex pathways. In the presence of stress, chaperons and proteases assist by unfolding proteins and also help in monitoring the expression of many transcriptional factors [24]. In addition to chaperons and protease, proteolysis mechanisms are crucial for the maintenance of cellular protein quality by removing non-functional and sometimes toxic proteins [23].

Cellular response and adaptation to various environmental stresses are essential for organisms to survive in their natural dynamic habitat. For this purpose, cells have developed numerous signal-transducing pathways involving a receptor that receives a signal that is relayed to other components inside the cell. Phosphorylation is frequently utilised by cells for these signal-transducing events [25]. In recent years protein kinases have been found to be involved in the regulation of metabolism, stress, and sporulation [26, 27]. It has also been proposed that protein phosphorylation facilitates the control of pathogenicity of virulent species through the regulation exopolysaccharide production and transport [28]. Different serine/threonine and tyrosine phosphorylated proteins have been identified in *S. aureus*. They have been shown to be involved with regulating central carbon metabolism (enolase, triose isomerise, fructose bisphosphate, aldolase, pyruvate dehydrogenase, phosphate acetyl transferase, and glyoxalase family protein), as well as certain protein synthesis pathways [29].

Environmental stress always leads to the activation of sigma (σ) factor, which is a protein that binds to RNA polymerase, and initiating the recognition of new promoter which is then followed by the responsive protein transcription mechanism [30]. σ^B is crucial in establishing stress response in *Staphylococcus* species. For example, it offers resistance to antibiotics such as Methicillin and Vancomycin by assists in the formation of biofilm [31-33] A set of reactions takes place in response to the stress which activates the sigma factor and consequently, its regulatory gene begins transcription [34]. It has been demonstrated that σ^B controls the transcription of more than 250 genes [35]. Protective functions performed by the translated protein helps the bacterial cell in coping with the environmental stress [31-33].

2.1. Cold stress

Fluctuations in temperature represent a major environmental stress that threatens bacterial survival, and adaptive responses can vary in different microbial systems. There are many parameters which can influence the temperature tolerance in the microbial systems [36]. For example, when the ambient temperature decreases, extended expression of the σ^B factor is required for microbial survival as it assists in maintaining the growth rate of bacteria [37]. It has been shown by Onyango et al., 2012 that some pathogenic species of staphylococci (*S. aureus, S. epidermidis,* and *S. lugdunensis*) were able to survive prolonged exposures to severe cold temperature at 4 °C. This survival was proposed to be mediated by increasing proportions of SCV phenotypes over time with concomitant changes in composition of cell wall-associated proteins [6]. It has also been proposed that the survival of *S.aureus* in cold temperatures was made possible by the expression of cold shock proteins including CspA, CspB and CspC, but to date only the CspB has been shown to be highly expressed in response to cold stress [38]. CspA was shown to play a role in the regulation of pigment expression in *S.aureus* through the mechanism of the pathway of SigB-dependent, which covers roles from non-specific and various stress resistance to the control of pathogenicity [39] .The pigmentation was proposed to play an important role in the survival of bacteria during invasion or exposure to fluctuating environments [40] but this has yet to be elucidated. It was also found that in *S. aureus* RN4220, CspC protein was greatly expressed under normal conditions as well as in response to exposure to antibiotics [41]. The cold shock proteins have been found to be higher in methicillin -resistant *S.aureus* than in sensitive methicillin *S.aureus* [42], suggesting that these proteins may have a role in the antibiotics resistance associated with virulence. These proteins preserve the essential structures of bacteria in a freezing environment which provides a mechanism for transferring of pathogens between hosts and can facilitate transfer via ingestion of food [43].

It has been found that *S. aureus* can reduce membrane fluidity in response to cold stress [44]. This reduction in fluidity of membrane leads to an inefficient membrane associated function (e.g. active transport and protein secretion). Similarly, stabilization of secondary structures of RNA and DNA were observed under cold stress conditions which leads to reduced functionality of ribosomes, and hence reduced level of mRNA translation. It has also been shown that

the cold stress affects the conformation and shaping of some proteins, so the ribosomes must be adapted to cold stress to be fully functional [44].

A well-established temperature-sensing system exists inside many bacteria that helps in recognition of environmental changes and induces an inbuilt defence mechanism against environmental stressors [45]. The Des system of *B. subtilis* has been well studied in this regard and it has been revealed that two major types of proteins are involved in its cold sensing mechanism. One is Des K, a membrane-bound histidine kinase, and the other is Des R, which is soluble and regulates bacterial transcription [46]. Des R is particularly involved in the regulation of Des genes transcription, which encodes Δ5-desaturase. It is an oxygen dependent enzyme which catalyses the reactions involving the desaturation of fatty acid chains. The presence of five transmembrane helices and a C-terminal carrying kinase domain in the cytoplasmic portions give the DesK protein the ability to sense the membrane fluidity [47]. This gene is present in *S. aureus* and may play the same role in response to cold [48].

2.2. Osmotic stress

Bacteria can be exposed to considerable variations in nutrient and ion concentrations that lead to changes in the external osmotic pressure which demand rapid responses in the ultrastructure and biochemistry of the bacterial cells. A number of mechanisms have evolved in bacteria to survive osmotic changes. Staphylococcal species are well known for their adaptability in a variety of salt concentrations [49]. Sudden exposure to osmotic stress can lead to immediate water efflux causing changes in cell turgor, dehydration and consequently cell death. The adaptability to withstand fluctuations in high osmotic stress was proposed to be due to a number of factors such as the osmo-protectants choline glycine, betaine and proline, which can be accumulated and used to restore the intracellular K^+. Two transport routes for proline exist in the *S.aureus* system; a low affinity system encoded by *proP* homology gene and a high affinity system expressed by *putP* gene [50]. These processes prevent the pleiotropic effects in the cell during the exposure to osmotic shock [19, 51]. The genomes of staphylococci have revealed that the cytoplasmic homeostasis is maintained by a set of transports systems, known, as BetT, PutP, ProP, and ProU [51-53]. Exposure to high osmolarity leads to the increasing sodium ions in the cytoplasm which hampers the process of glucosylglycerolphosphte (GgpS) synthase protein binding with DNA that leads to enzyme detachment. The newly produced glucosylglycerol replaces the sodium ions present in cell. This is a self-regulatory mechanism and as the concentration of sodium is dropped inside the cell, the enzyme rebinds to DNA and thus the system will be inactivated [54]. Osmotic stress was shown to cause an increase in the cells size of *S. aureus*; this increase was associated with a shortened interpeptide bridge of the peptidoglycan. This mechanism was proposed to be a defence strategy against fluctuating osmotic conditions [55].

An alteration in the osmolality of external environment immediately surrounding the cells stimulates changes in the composition of membrane phospholipids. Survival of *S.aureus* in the highly hypertonic environment requires the synthesis and inclusion of cardiolipin into the membrane to generate L-form variants [56]. A high threshold for transient hyper osmotic pressure is prevailed in salt tolerant *S.aureus* due to the presence of large sized Ebh protein, which is present in the cell wall. However, the exact mechanism for the activation of the defence system against high osmolality has not yet been explored and little is known of the alterations in metabolic homeostasis [54]. It has also been found that the pyruvate dehydrogenase multienzyme complex was higher in *S.aureus* in response to osmotic stress. This enzyme is required for the synthesis of osmoprotectants elements [57].

2.3. Acid and Alkaline stress

An optimum pH must be balanced for the control of many cellular processes, such as transcription and biochemical pathways for metabolism. For instance, pH is crucial in maintaining protein shape and structure for the proper functioning of proteins. Non-extremophilic bacteria have the ability to survive at a variety of pH conditions ranging from 5.5 to 9, but the cytoplasmic pH is maintained in the range of 7.4 to 7.8 [58].The survival of bacteria is not only defined by the inherent virulence factors but is also dependent on the ability to withstand the host immune system and simultaneously adapt to changes in the environment with parameters such as pH and osmotic shock [59, 60]. Acidification processes are used to preserve food from bacterial contaminations, by making growth conditions sub-optimal, if not inhibitory, for most potential contaminants. However, certain bacteria, such as *S.aureus,* have been shown to be sufficiently adaptable to survive and even proliferate under these adverse conditions leading a restricted shelf life for food products. These staphylococci must therefore have efficient mechanisms for rapid adaptation to survival in alkali and acidic conditions to survive in the fluctuating environments [61, 62]. It has been found that *S.aureus* can reside in the lysosomal compartments of non-phagocytic cells at the pH range of 4.5 to 5.5. This has been shown to occur before it moves into the cytosol of non- phagocytic cells [63]. In human wound sites, the bacteria must sustain a high pH to establish an infection in the host tissues and this suggests a capability of actually modulating the immediate environment [64]. Frequent pH alterations represent a feature of the “life cycle” for *S.aureus* where it travels from host to fomites and various substrates between hosts enabled by a well-developed capacity to deal with pH fluctuations in the environment [65]. One approach used in surviving at high acidity is to pump out cellular protons to reduce intracellular pH [66]. Alternatively, bacteria can augment the alkaline compound concentrations inside the cell to

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neutralize the impact of acidic ambiance and the damaged macromolecules of microbial system needs a repair mechanism [67].

When cells of *S. aureus* were grown at pH7.5 and directly exposed to conditions at pH2 the cells died. However, tolerance to high acidity can be achieved after pre-treatment and acclimatisation of *S. aureus* to sub-lethal acidity pH4 which resulted in greater subsequent survival capacity at pH2. This acidity tolerance been proposed to be due to the activation of the sigma-B factor which induces superoxide dismutase (soda). The role of soda in survival at low pH is supported by the evidence that strains which have mutated soda are susceptible to acidic environments [30]. This implies that oxidative stress can be introduced in the microbial system in response to pH challenge and that oxidative damage might be a potential consequence of pH challenge [51]. This is crucial in comprehending the collective impact of oxidative stress and low pH during the engulfment of bacteria by macrophage lysosome [62].

The combined effects of the bacterial systems to tolerate fluctuating environmental pH provide a powerful adaptive response capacity for the staphylococci. Acid associated damage can be recovered and the pH inside cell can be largely maintained within an optimum range [61]. Some virulence factors are known to be associated with the alkaline stress management system but this is not well understood. In *S. aureus* it has been found that 122 genes were over-expressed after being exposed to alkaline shock conditions suggesting significant impact on the proteome and metabolic homeostasis [68].

2.4. Oxygen availability

The presence of oxygen has a great impact on bacterial growth and survival. Some obligate anaerobic bacteria are sensitive to oxygen requiring complete anaerobic conditions for their survival [69]. Facultative aerobes that can utilize oxygen when available, but also survive under anaerobic conditions would have a selective advantage for surviving fluctuating environments between hosts. For the invading pathogen, the process of invasion and infection may encounter highly variable oxygen tensions where for example conditions within an abscess can be anaerobic [70].

Changing pH can also affect the oxygen availability and it's generally noticed that alkalinisation and acidification of a medium reduces the oxygen concentration of the surroundings [71]. *Staphylococci* species have the ability to survive under reduced oxygen surroundings by using alternative electron acceptors in respiration such as nitrate or by using fermentation [69, 72, 73].In the *S.aureus* infection process, oxygen concentrations vary between skin surfaces, host tissues and fluids, and the bacterium must rapidly adapt to the immediate environment [74]. During reduced oxygen tensions, gene-expressing factors involved in the nitrate respiration and fermentation are activated. Increased glycolytic and fermentation pathway activities were found to overcome the reduced oxygen availability that resulted in loss of NAD+ regeneration and ATP synthesis capacity. Inductions of genes responsible for lactate and formate secretion were also observed in low oxygen tension environment [75].

Staphylococcus respiratory responses SrrB and SrrA is the two-component system that manages the gene expression for the factors that confer pathogenicity and it was found to control citric acid cycle and fermentation enzymes [69, 76]. This system was found to be analogous to the one present in *B. subtilis* [77] where oxygen presence generated the synthesis and utilisation of the aerobic respiratory chain. *B.subtilis* expresses the Rex protein which monitors respiratory genes and enzymes for respiration and metabolism [78]. It has been found in *Staphylococcus carnosus* that nitrate reductase and nitrite reductase operons were regulated by two component system NerB-NerC in response to oxygen [79, 80]. NreB is a cytosolic protein which acts as direct oxygen sensor in the anaerobic environment [80].

2.5. Combinations of Stressors

It is very crucial to explore the effects of various combinations of stresses as the cells are continually challenged with a large number of combinations and permutations of variable parameters. Multiple factors have not been extensively investigated in the literature [71]. Investigations of intracellular growth of *Salmonella typhimurium* within a macrophage displays a protein profile which is completely different from the ones exhibited by the corresponding bacterial strains growing under acidic or oxidative stress conditions [81]. Generally the pH alteration process generates a reduced oxygen environment [71]. Whether in a host wound site or surviving between hosts, the environmental conditions are dynamic resulting in complex combinations of stresses, for which the bacteria must adjust growth and metabolism to maintain homeostasis and survive [82].

Investigations assessing food safety have explored some elements of exposure to multiple stresses with a view to determine optimum strategies for reducing bacterial proliferation for safe storage of food. Inhibition of pathogenic species is generally performed by reducing the pH with organic acids such as citric acid or proprionic acid often in combination with a reduced temperature. Ethanol formation with concurrent organic acid production (reduced pH) in wine and beer products also acts together as an efficient inhibitor of microbial growth. [83].

3. Epigenetics as a factor in stress response in *staphylococci*

In general bacteriology, it is assumed that cells of a pure homogeneous culture, grown under optimal conditions, would be genetically identical with a uniform phenotype. However, recent research has shown that this may not always be the

case and several instances of heterogeneity within clonal microbial cultures have been reported [84, 85]. This heterogeneity has often been manifested as variations in the output phenotype of the bacteria with each variation seeking to promote the survival of the bacteria in a specific niche or under a particular stress condition [86]. However, this heterogeneity is seldom documented as the "assumption of homogeneity" is made to accommodate a reductionist approach to facilitate progress in the testing of hypotheses. The assumption has steered research away from the reality that in nature, bacteria may grow in several different forms responding to micro-environmental conditions which could lead to atypical variants within the population counter to what might be assumed [87]. Heterogeneity of phenotypes in a population would potentially assist the bacteria to survive in a dynamic environment where many parameters are subject to variation [5]. Many other species of bacteria also opt for this "bet-hedging" strategy to survive in the harsh and changing environments where their phenotype profile within the population alters to reflect compatibility within the environment [88]. This heterogeneity was proposed to be due to the alteration in expression of cellular factors like signal transducers, global regulators, non-coding RNAs and transcription factors that bring changes in the genome expression [4].

The mechanism of heterogeneity was first observed by Joseph Bigger in 1944 during his research on penicillin action. In his study, majority of staphylococcal cells were killed by the penicillin lysis. But a few of them escaped and survived and were considered as persister cells [89]. Persistence against a variety of environmental stresses is an epigenetic trait of the sub-population of the bacterial population and although these bacterial cells have retarded growth pattern but they can tolerate antibiotic treatment or high levels of metal ions [90]. Once the ambient stress subsides, bacterial cells revert back to their regular phenotypes with normal metabolic functions. It is believed that antibiotic exposure stimulates the bacteria into a response mode which offers protection and also optimises homeostasis for competitive survival. These persister cells have been referred to as dormant cells as they can't be killed by use of antibacterial agents, but they still maintain a level of metabolism and are not truly dormant as spores [91]. These persister cells are a small number, but they ensure the survival of the bacterial population under harsh conditions. When good conditions prevail these survival cells remultiply and reproduce a full range of phenotypes capable of rapid growth and adaptation[92].

Environmental studies have described bacteria isolated for a specific phenotypic characteristic relevant to survival in that environment that is lost when continually cultured under laboratory conditions [93]. The 'lost' phenotype is termed the adaptive phenotype and its loss has been attributed to several possible mechanisms including plasmid instability and genetic mutations. However, several independent studies disproved both hypotheses showing that adaptive traits were not encoded by plasmids. In addition, the adaptive phenotype was also seen in generations long after the environmental condition thought to give rise to it was no longer present. With adaptive phenotypes being lost at significantly high rates, the latter hypothesis also became seemingly implausible. With both hypotheses nullified, a new epigenetic hypothesis was generated to help explain this extreme phenotypic variability [93]

Epigenetics refers to a heritable phenotypic change that cannot be attributed to changes within the DNA sequence but rather variations of gene expression [94]. Epigenetic systems/phenotypes have been described for a variety of biological organisms and cell types. The result of such variations is the manifestation of a range of stable phenotypes within a bacterial population, each functioning in a unique way that is of adaptive advantage to the species. Genomic imprinting, prion involvement, histone modification, and DNA methylation are some of the mechanisms used by bacteria to orchestrate epigenetic changes [94].

3.1. Small Colony Variants (SCVs)

It has been proposed that many of these phenotypes exist as vital members of diverse populations of cells as the culture grows providing an effective response mechanism for survival mechanism when natural selection pressures are rapidly applied to these populations [66]. It is proposed that the prevailing environmental conditions may actually influence the population diversity of various phenotypes with those most capable of effective survival and proliferation dominating the population diversity. It is difficult to isolate the different phenotypes, but the principle can be demonstrated when both wild type and small colony variants of staphylococci can be observed in plate cultures inoculated from an SCV origin [6, 7, 95]. It has also been demonstrated that storage of *S. aureus*, *S. epidermidis* and *S. lugdunensis* at 4°C over 2 weeks changed the proportions of wild-type versus SCV colonies observed in culture with the latter increasing over time relative to their counterpart [6]. SCVs have been associated with a wide range of diseases of a recurrent form that do not respond to antibiotics. These diseases associated with the clinical isolation of SCVs include chronic infections, such as brain abscess, osteomyelitis, arthritis, and device-related infections. Treatment of SCV-related infections has been a challenge since most of the infections were persistent and have the tendency to recur [96, 97]. Symptoms of the infections start disappearing whilst receiving consistent antibiotic treatment but as soon as treatment is discontinued the infection reoccurs with similar intensity and often will not respond to repeated antibiotics treatments [95].

Studies with SCV mutants have provided insights in regard to the metabolic homeostasis of these phenotypes usually linked to a potential switching off or down regulation of key components in energy metabolism. These mutants have slower metabolic rates, reduced production of virulence factors, and are auxotrophic for some key compounds, including haemin and menadione. Haemin, menadione and thymidine-dependent SCVs all showed reduced alpha (α) toxin production and consequently less cytotoxic damage in host cells [98]. Despite their atypical characteristics, SCV

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were still found to be more resistant intracellularly. This heightened resistance was not attributed to the ability of these bacteria to inactivate host mechanisms as the wild-type does. This may also be linked to a diminished effect of anti-microbial compounds in clinical treatments. Instead, they produce an impressive array of adhesive factors that are associated with intracellular colonisation and survival. Vaudaux *et al.,* [99] demonstrated this by using the *hemB* mutant and reported that this variant expressed more fibronectin-binding proteins that caused it to strongly adhere to endothelial cells therefore facilitating rapid internalization. This increased endocytic uptake of the *hemB* mutant was correlated with an increased expression of the fibrinogen adhesion gene *cflA* and was found to occur independently of the *agr* gene that mainly regulates virulence factors in *S. aureus*. Internalized SCV bacterial cells could thus persist readily due to reduced toxic production and an acquired ability to withstand intracellular defences better [98, 100]. It was therefore hypothesised that the SCV phenotype adapted to the intracellular environment by up-regulating protective mechanisms that were highly effective at maintaining its viability, rather than developing mechanisms that attempted to manipulate or deactivate the host defence mechanisms [98].

Adaptations of *Staphylococci* to environmental stimuli have been extensively studied. However, the roles of various staphylococcal proteins for facilitating survival and mediating pathogenicity have yet to be fully elucidated. Comparisons of *S. aureus* SCV proteins and their corresponding wild type strains have shown elevations in abundance of enzymes associated with glycolytic and fermentation pathways [101]. They also have shown that the SCVs have reduced expression of TCA cycle proteins and those involved in nucleotide and folate metabolism [11]. Besides this, an insensitivity against global regulators was found in SCV's where they were generally regarded as potent effectors which have augmented expression of some extracellular proteins such as α-haemolysin or some serine proteases along with leukocidins [101]. SCV's were also found to be deficient of purine/pyrimidine pathway regulatory proteins. Expression of formate-tetra hydrofolate ligase was found to be reduced in the SCVs which was crucial for the *de novo* deoxythymidine monophosphate synthesis. The proteins required for the translation and transcription processes were also found to be reduced [11].

S. aureus is generally considered as extracellular pathogen but the SCV phenotypes have been shown to be capable of forming an intracellular invasion including replication and survival inside neutrophils, macrophages and epithelia cells which explains persistence of these infections in certain chronic conditions and diseases e.g. Cystic Fibrosis (CF) [102-104]. Secretion systems are used by the pathogens which have developed an invasive strategy by initially stimulating the host cells to engulf them. Engulfment of *S. aureus* by the phagocytes exhibits the engulfment efficiency similar to other dead and alive bacterial cells [105]. But this approach is dependent on the binding of integrin proteins of host and fibronectin binding proteins [105, 106]. Surface proteins of *S. aureus* are also actively involved in its intracellular uptake and persistence e.g. clumping factor A. Host cell Src kinase also assists in the intracellular uptake of *S. aureus* [107]. *S.aureus* can escape the host defence system and it can take refuge inside the cell where it is not exposed to antibiotic pressures and is effectively inaccessible to the host defence system. An adaptation of this strategy by the invading microbe results in relapsing infection after receiving a suitable antibiotic regimen [1, 108].

The sustainability of *S. aureus* inside the host cell after its extracellular infection is induced by a fluctuating environment and it also requires an alteration in the genetic expression of *S. aureus*. Genetic expression of the *S. aureus* was analysed post-extracellular infection and it was observed that it is well adapted to the intracellular environment [109]. Following bacterial uptake inside the cell, its genetic expression is altered and all the metabolic activities re-aligned for a different homeostasis. Later, all the expression and metabolic functions are resumed on exit of the host cell and bacteria divide normally [108, 109].

3.2. Biofilm: A metabolomic and proteomic perspective

Enhanced biofilm formation has been associated with the formation of the SCV phenotype. A biofilm is a structured and layered community of bacteria, often formed at liquid/solid interfaces. Its formation is thought to be regulated by epigenetic switch. For example, SinR protein is considered as the master regulator of switching the planktonic cells to form biofilm [110]. Biofilm has long been regarded as a crucial virulence factor in bacterial pathogenesis as it affords an adaptive advantage in natural and clinical environments and increases persistence of bacteria on indwelling devices [111]. Bacteria housed within biofilms exhibit a high degree of chemical and structural organization. Surface-associated proteins and fibrinogen binding proteins (FBP) are thought to mediate the initial process of adherence. Organic polymers, referred to as extracellular polymeric substances (EPS) are produced and excreted to form a matrix on and around the cells acting like an adhesive that holds the biofilm together. Though most cells within the biofilm are not directly attached to the surface, they are held together by a homo-polymer known as polysaccharide intercellular adhesion (PIA). These substances encase the bacteria and allow them to proliferate while protecting them from the immune system, antimicrobials and environmental challenges [111-113]. The result being that the biofilm acts as a reservoir for the pathogens and increases microbial virulence, especially in cases of formation on the surfaces of indwelling medical devices [10, 114].

Infections by *Staphylococcus* species account for 60% of bacterial infection in developed countries [115]. When encased within a biofilm, this structure affords features responsible for heightened resistance to antibiotics, slow growth rate, spatial heterogeneity, drug-tolerance and resistance. Moreover, these features of SCVs coupled with enhanced capacity for biofilm formation are consistent with the concept of forming persister cell to survive harsh and challenging

conditions cells [116]. Drenkard and Ausubel [117] reported that SCVs of bacteria that produced chronic infections had biofilm that was formed much faster and much thicker than that observed for their WT colonies. The combination of hyper-biofilm formation plus the ability of bacteria to form a SCV phenotype would result in a bacterial mass that is highly adept at adhering to and sustaining relapsing implant-related infections.

Some global alterations in the genetic expression of the biofilms can be stimulated upon introduction of different kinds of stressors such as osmotic shock, temperature changes and low oxygen tension. Treatments with certain chemicals like ethanol and antibiotics can also induce drastic changes in their gene expression and regulation mechanism [118, 119]. These changes in gene expression would subsequently result in significant alterations in the proteome leading to altered metabolic homeostasis for survival [9, 120]. *S. epidermidis* and *S. aureus* have the gene clusters of *ica* which are responsible for the expression of polymers that help in the formation of biofilms. N-acetyl glucosamine (P-NAG) and polysaccharide intercellular adhesion (PIA) are the two examples of such polymers. However, there are a few species of *Staphylococcus* which don't contain the *ica* gene clusters in their genome and can form biofilms. This implies that such strains exhibit alternative molecular mechanisms for the biofilms which don't depend on the *ica* gene expression products [121]. Instead these strains depend largely on large biofilm-associated proteins (Bap) which are required for the mediation of cell to cell and cell to surface communications [122, 123]. Cell to cell disruption mechanisms which can help in the detachment of the developed biofilm have not been identified, yet some surfactants are known to exhibit the ability of disrupting the cell to cell association in biofilms. This feature was observed during the in vitro studies of *Pseudomonas aeuroginosa* and *Bacillus subtilis* [124] where biofilm formation on surfaces can be achieved by the enzymatic degradation mechanisms; studies on *Actinobacillus actinomycetemcomitans* have shown the enzymatic digestion of biofilms [125].

Some of the surfactants known to exhibit properties which assist in the biofilm detachment have been identified as peptides of *Staphylococcus* origin, known as PSMs, phenol soluble modulins, and their expression is strongly affected by the quorum-sensing system *agr* [126]. Such peptides have amphiphilic structure with α-helix conformation for which the surfactant like characteristic of the peptides can be attributed. Some peptides with β- conformation were found to have impact on the maturation and detachment of biofilms in the strain of *S. epidermidis*. The same research study showed that dissemination of biofilms infections can be assisted by these peptides and, they could potentially be employed as tools for in vivo biofilms detachment [123]. An analysis of the biofilm and planktonic cells of *S.aureus* has shown different protein profiles For example, the surface proteins such as fibrinogen-binding proteins and cell wall components such as peptidoglycan have increased in the biofilm cells. Moreover, the enzymes involved in pyruvate and formate metabolism were also up-regulated. However, immunodominant antigen A and staphylococcal secretory antigen were found down-regulated [127].

The response generated in *Staphylococcus* strains against certain stresses such as osmotic shock, heat shock and ethanol is mainly due to the up-regulation of the *icaADBCA* operon. It has already been mentioned that *icaADBCA* operon expresses the polymer necessary for biofilm production [121]. A more firm and viable biofilm is maintained due to the maintenance of the up-regulation process of this operon. There are different pathways for every stress stimulus which aim to induce the *icaADBCA operon* i.e. high salt concentration stimulus will adopt a different pathway for its induction compared with pathway used by ethanol stress [121, 128]. *S. epidermidis icaR* is the gene product which negatively regulates the operon. In this strain, another regulator named *SigB* helps in the production of biofilms by hindering the expression of *icaR* gene [129].

4. Conclusion

Staphylococci can adapt rapidly and effectively to fluctuating environmental conditions which assist in the transfer between hosts as a pathogen and provide the framework to successfully invade and colonise host tissues and fluids. The mechanisms of adaptation involve metabolic and structural adjustments within the cell to help in coping with substantial changes in the surrounding environment with the objective of retaining optimal conditions in the cytoplasm. The adjustments of the cell metabolism and structure lead to a phenotypic (phenomics) conversion to optimise cell function in the changing conditions to maximise survival and proliferation. Phenomics is the study of variability in phenotype of an organism in response to environmental influences. Historically this has been the realm of genetic mutation but with the more recent developments of genomics, metabolomics and proteomics. Phenomics becomes the umbrella concept signifying underlying variability amongst the individuals of a species.

Evidence is emerging that exposure to various changes in environmental conditions leads to changes in metabolic homeostasis, membrane fatty acid composition and cell size. When stressors are removed there is then phenotypic reversion to the "normal" or "wild" type described under optimal growth conditions.

This review found evidence suggesting that the ability of *staphylococci* species to undergo the phenotypic conversion to a more resistant form enhances the survival of the organism. The genotypic changes have been partly characterised but much work is needed on the actual trigger mechanisms that communicate and cause the switch to SCVs phenotype. This phenotypic conversion coupled with biofilm formation and metabolomic changes offer a defence mechanism for *staphylococci* species against both host immune system and harsh environments. Proteins, metabolites and structural alterations represent evolutionary adaptations for survival. The investigations of changing protein and metabolite

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inventories provide great insight in understanding homeostatic responses to environmental challenges. Understanding the mechanisms that allow the bacteria to survive adverse host and environments conditions may help to develop new strategies for antimicrobial chemotherapy. Key to this understanding will be the elucidation of the controlling factors for phenotypic shift within cells of the same colony.

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