

## Metabolic Profiles of Clinical Strain of *Staphylococcus aureus* to Subtle Changes in the Environmental Parameters at Different Phases of Growth

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### Abstract

*Staphylococcus aureus* is an important pathogen that can lead to high number of infections worldwide. The present study was aimed to investigate cytoplasmic metabolites of *S. aureus* at mid-exponential and stationary phases following growth in a combination of conditions including variations in pH and temperature. The result of metabolic analysis demonstrated that most of the metabolites measured at stationary phase under optimal conditions were significantly altered in comparison to equivalent cells harvested at mid-exponential phase. The major alteration was mainly observed in nitrogenous bases and organic acids, which were relatively decreased in cells grown to stationary phase, while mannitol, galactonic acid and adenosine were relatively increased in the cultures harvested at stationary phase. At both phases, the metabolic profiles were substantially different between cultures grown under ideal control conditions compared with those grown under more acidic and alkali conditions with lower temperature of 35°C. The analyses of cells harvested from control and treatment samples at mid-exponential phase showed that nitrogenous bases including uracil and adenine were significantly decrease in treatment groups compared with reference controls. However, these metabolites were relatively increased in the treatment cells grown to stationary phase. It was evident that variants in environmental parameters led to differential profiles of cytoplasmic metabolites during adaptation processes to applied conditions. These results provided robust evidence supporting the hypothesis that specific alterations in cytoplasmic metabolites were essential for *S. aureus* to adapt and hence survive under changes in the environmental parameters.

**Keywords:** Cytoplasmic metabolites, *S. aureus*, metabolic homeostasis, stress responses

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## INTRODUCTION

Infections due to *Staphylococcus aureus* have been increased at a remarkable rate in the past era worldwide. The extraordinary feature of this bacterium is its ability to adapt to a wide range of environmental conditions<sup>1-3</sup>. However, the processes involved in the adaptations of *S. aureus* in response to combination of environmental stresses are still not clear. It has been suggested that alterations in the profiles of cellular compositions are vital for bacterial adaptation and infection occurrence<sup>4-6</sup>. It has been reported that staphylococcal adaptation mechanism in response to limited nutrients occurred through a significant changes in the patterns of enzymes and metabolites<sup>7</sup>. When *S. aureus* was grown under sub-optimal conditions, significant changes in the cytoplasmic amino acid, fatty acid and protein patterns were observed<sup>6,8</sup>. Thus, it was assumed that characteristic and specific changes in the amino acid and fatty acid metabolites as well as protein composition led to the survival of *S. aureus* when exposed to confined nutrition in combination with higher concentrations of NaCl<sup>9</sup>. A recent study indicated that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) combined with various temperatures and pH induced alterations in the amino acid profiles of clinical isolate of *S. aureus*<sup>10</sup>.

*S. aureus*, have been shown to adapt, survive and even proliferate under these adverse conditions encountered in wound site, leading to a serious infections<sup>11</sup>. One adaptive response in *S. aureus* towards conditions present on human skin or within a wound site is the alterations in metabolites and proteins toward obtain the optimum homeostasis<sup>12,13</sup>. A lower temperature of 35°C combined with either lower or higher pH induced significant changes in the amino acid levels and protein compositions<sup>6</sup>. However, till now the same experimental design has not been adapted to investigate nucleotides, nucleosides, organic acids and sugar alcohol abundances in response to the conditions of more acidic and alkali combined with lower temperature of 35°C. Thus, this study was aimed to determine the metabolic alterations that could occur as a result of alterations in pH and temperature. It was hypothesized that the exposure of *S. aureus* to conditions of more acidic and alkali with lower temperature would lead to remarkable changes in the metabolic profiles.

## MATERIAL AND METHODS

### *S. aureus* strain and Growth conditions

*S. aureus* used in the current study was isolated from patients who were suffering from chronic muscle pain<sup>14</sup>. This bacterium was used in following investigations to study metabolic and proteomic adaptation to alterations in the environmental factors<sup>5,6,9</sup>.

*Staphylococcus aureus* was cultured in a sub-optimal of environmental parameters including alteration in pH and temperature for analyses of cytoplasm metabolites following growth in tryptic soy broth media (TSB) (Oxoid Ltd-Australia). The control included cells grown under ideal conditions of pH7 at 37°C in tryptic soy broth medium (TSB) and two sets of experimental conditions were applied with (1) 35°C and pH6 with and (2) 35°C and pH8.

### Metabolites extraction

Washed cells were lysed using snap-frozen and thawing technique 3 times and placed in a dryer machine overnight. Approximately 10-12 mg of lyophilized cells were resuspended with 10ml of 1:1(v/v) of cold methanol/water stored at -20°C and mixed thoroughly. The methanol/water lyophilized cell slurries were snap frozen in liquid nitrogen and placed in freezer at (-20°C) for 30 min for a process of slow thawing. Metabolites were then separated from the cell debris by centrifugation at 6,500 xg for 25 min. The supernatants containing the metabolites were dried using a centrifugal vacuum drier (CentriVap, LABCORNC, VWR)<sup>5</sup>.

### Metabolites identification and quantification

Dried cytoplasmic metabolites including carbohydrates and purine and pyrimidine metabolites were evaluated by forming the methoxy-amine-trimethylsilyl (TMS) derivatives by reacting the dry extracts with Methoxyamine-HCL (MOX) and Bis(trimethylsilyl) trifluoroacetamide (BSTFA). Lyophilised metabolites were mixed with 50µl of MOX vortexed very well, and then heated at 60°C for 30 min. Samples were then allowed to cool and subsequently 150µl of BSTFA was added to each sample, vortexed and heated at 100°C for 60 min. Derivatised samples were analysed by auto-sampler gas chromatography (Agilent, Hewlett-Packard 5973) coupled with mass spectrometry. The injection volume was 1µl/sample and flow rate was 0.5ml/min. Metabolites

in the chromatogram were identified on the basis of matching their mass spectra and retention time indices with data in use-generated mass spectral libraries generated from reference standards.

#### GC-MS data processing and statistical analysis

The acquired metabolite data obtained from GC-MS were exported to an Excel® (Microsoft®) sheet. The exported data were then imported to STATISTICA (6, StatSoft) (ANOVA) to find the cytoplasmic metabolites that were considerably different between control and treatment groups. Multivariate analysis including principal component analysis (PCA) was then performed utilizing STATISTICA (6, StatSoft) to further the analysis and have a better understanding.

## RESULTS

### Metabolites analysis of cells grown under normal conditions harvested at mid-exponential and stationary phases

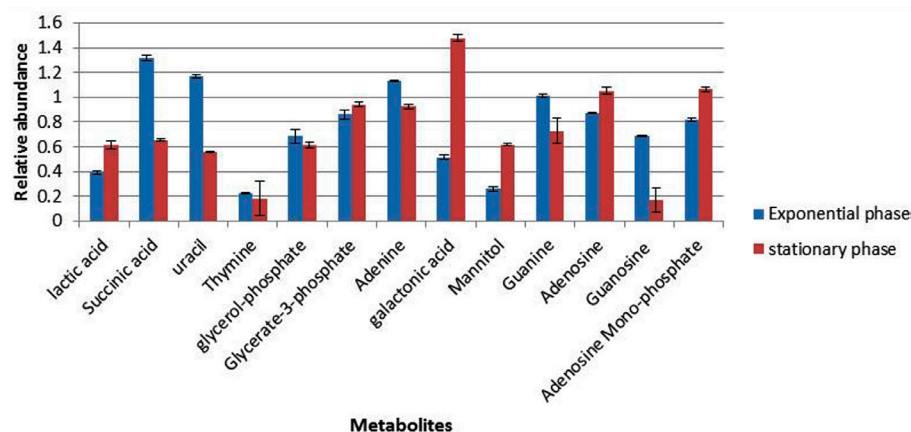
Cultures of *S. aureus* were grown in TSB to mid-exponential and stationary phases of growth under optimal conditions (pH7, 37°C and no added NaCl). The analyses of the cytoplasmic metabolites revealed the presence of 13 metabolites that could be identified by the GC-MS method based on their retention time indices and mass spectra as shown in Fig. 1. The cytoplasmic metabolite profiles were assessed as percentage abundances from stationary phase for comparison with mid-exponential phase. The analysis indicated that significant changes in the relative abundances of

many metabolites of cells grown to the stationary phase compared with those analysed at mid-exponential phase. For instance, lactic acid and galactonic acid were relatively increased in cells harvested at stationary phases compared with mid-exponential phase cells, but the succinic acid was substantially decreased when bacterial cells grown to stationary phase. Purine bases including guanine and adenine were relatively reduced in cells grown to stationary phase in comparison to mid-exponential phases. On other hand, adenosine was relatively increased, but guanosine was significantly decreased.

The cytoplasmic metabolites were further investigated through multivariate analysis using PCA. The result revealed that the metabolites analysed from both phases were very well clustered and reproducible with each phase. However, the analysis clearly showed that metabolic profiles of cells harvested at mid-exponential phase was well separated from those collected at stationary phase, indicating that a significant difference between the two phases. Mid-exponential phase metabolites were clustered at the negative side of component 1 whereas stationary phase metabolites were spread along positive side.

### Metabolites analysis at mid-exponential phase

Metabolite responses from cells collected at mid-exponential phase following exposure to changes in pH and temperature were determined using GC-MS (Fig. 3). The results indicated that nitrogenous bases including adenine, guanine,



**Fig. 1.** The relative abundances of cytoplasmic metabolites extracted from clinical isolate of *S. aureus* harvested at mid-exponential and stationary phases grown under ideal conditions

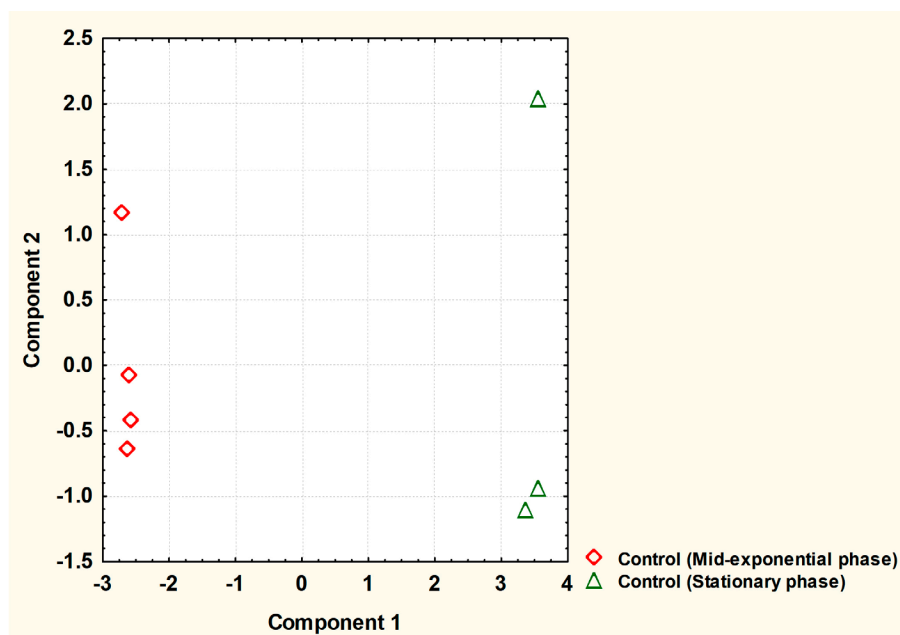
thymine and uracil were relatively decrease in the cultures grown with either more acidic or alkali conditions, with exception of thymine that did not display a change in response to more alkali conditions with lower temperature of 35°C. Lactic acid and mannitol were relatively increased in cells grown in altered conditions compared to cells grown in normal conditions. Adenosine and guanosine were substantially decreased in cells grown under more acidic conditions (pH6), but were significantly increased in the cells grown with more alkali conditions in comparison to reference control.

Detected metabolites were subjected to multivariate analysis such as PCA to further investigate the dimensionality of these metabolites following exposure to changes in pH and temperature. The PCA analysis demonstrated that each treatment regimen resulted in a significant metabolic profile, with great reproducibility within each group. As depicted by PCA plot the treatment groups were very well separated from the control samples. The cells grown under normal conditions (A1-A4) positioned in the negative side of PCA plot while the cells exposed to lower pH of 6 (B1-B4) or higher pH of 8 (C1-C4) with lower temperature placed in the positive side of the plot.

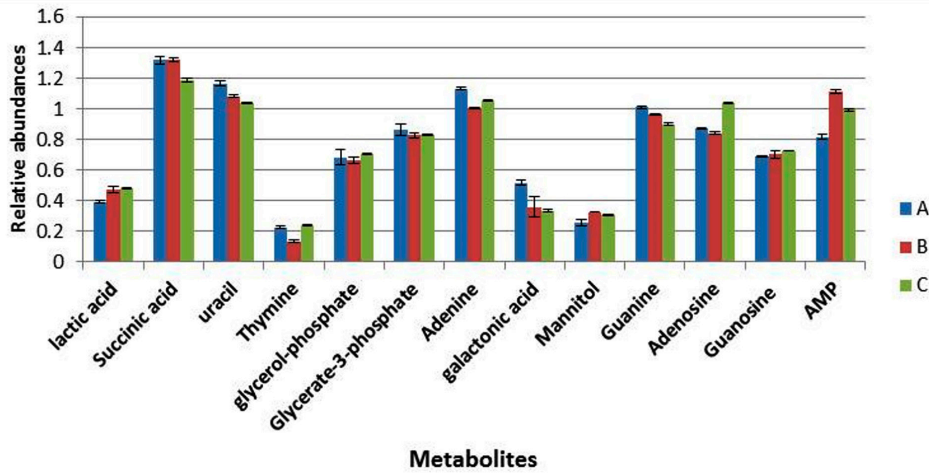
### Metabolites analysis at stationary phase

The cells were grown further to the stationary phase under the same conditions designated as (A to C) before extraction of cytoplasmic metabolites and the analysis by GC-MS. The results indicated that lactic acid, galactonic acid and succinic acid were relatively decreased in cells grown in altered environmental parameters with exception of galactonic acid which did not exhibit any change in response to more alkali condition (Fig. 5). The nitrogenous bases include uracil and adenine were remarkably increased in both treatment regimens (B and C) compared with control samples (A), but thymine and guanine remained consistent in cells exposed to lower pH and higher pH. Both adenosine and guanosine were greatly decreased in cells grown under more acidic (pH6) and alkali (pH8) conditions. Adversely, adenosine mono-phosphate was significantly increased in response to altered environmental conditions.

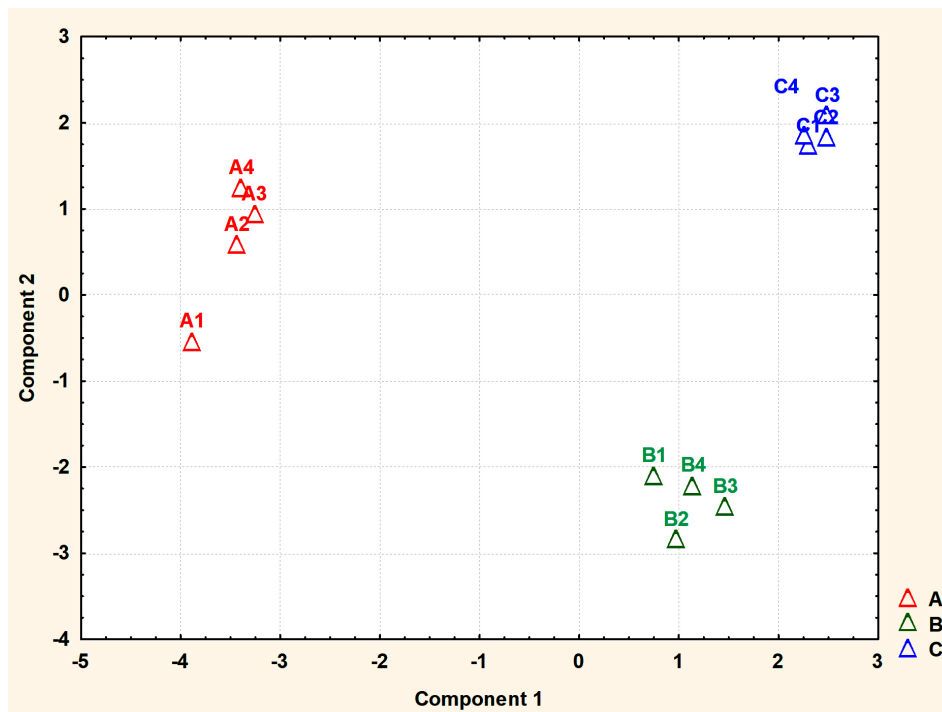
The metabolite profiles were further analysed using principal component analysis (PCA). The plot of PCA revealed that the metabolite profiles were again very distinct from each other as shown for metabolites analysed at mid-exponential phase. The results also displayed that



**Fig. 2.** The principal component analysis (PCA) of *S. aureus* cytoplasmic metabolite profiles derived from cells grown to mid-exponential and stationary phases under normal conditions.



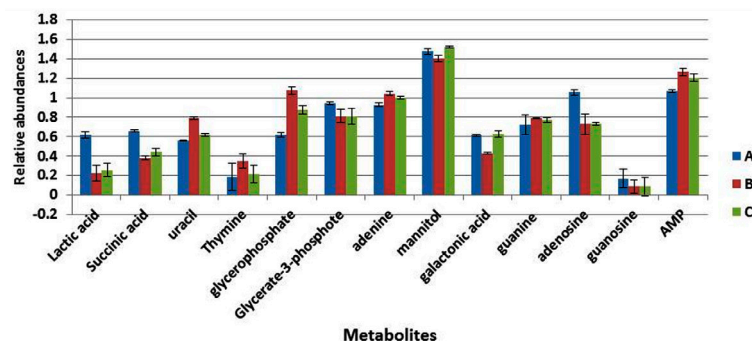
**Fig. 3.** The relative abundances of cytoplasmic metabolites extracted from clinical isolate of *S. aureus* harvested at mid-exponential from cells grown under normal conditions (A), , pH6, 35°C (B) and pH8 at 35°C (C).



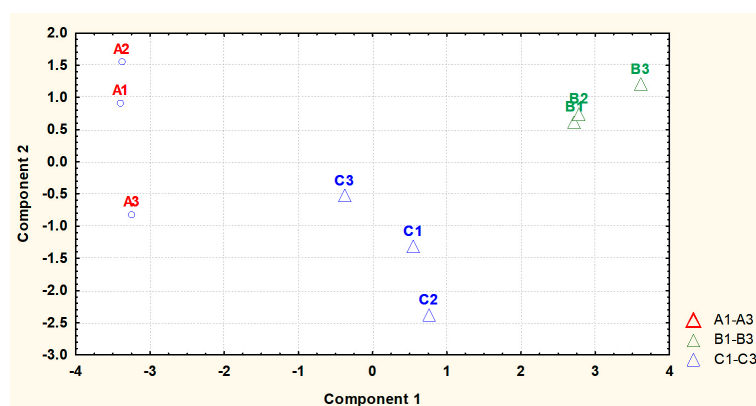
**Fig. 4.** Principal component analysis (PCA) scores (component1 versus component 2) plotted from cytoplasmic metabolite data of *S. aureus*. The *S. aureus* cultures were grown under (A1-A4) ideal conditions at pH7\_37°C (control), pH6 at 35°C, (B1-B4) and pH8 at 35°C (C1-C4) to mid-exponential phase of growth before extraction of cytoplasmic metabolites and analysis by GC-MS.

control and treatment samples were very well separated. Control replicates were resolved along the negative axis, while the cells exposed to more acidic and alkali conditions were spread along

the positive axis. These analyses showed that the metabolite profiles were distinct between each of treatments and control.



**Fig. 5.** The relative abundances of cytoplasmic metabolites extracted from clinical isolate of *S. aureus* harvested at stationary phase from cells grown under normal conditions (A), pH6, 35°C (B) and pH8 at 35°C (C).



**Fig. 6.** Principal component analysis (PCA) scores (component1 versus component 2) plotted from cytoplasmic metabolite data of *S. aureus* harvested at stationary phase. The *S. aureus* cultures were grown under (A1-A4) ideal conditions at pH7\_37°C (control), (B1-B4) pH6 at 35°C, and (C1-C4) pH8 at 35°C to stationary phase of growth before extraction of cytoplasmic metabolites and analysis by GC-MS.

## DISCUSSION

The results from this investigation indicated that the cytoplasmic composition of selected metabolites of control cultures of *S. aureus* grown to mid-exponential phase had a different metabolites composition compared with cultures harvested at the stationary phase. These substantial alterations in the metabolites in the stationary phase could be a mechanism to combat the complex environments present in the stationary phase including the exhaustion of nutrients and accumulation of toxic waste from metabolic products<sup>15,16</sup>. Cells entered the stationary phase had a lower nitrogenous bases relative to active cells at mid-exponential phase. Previous studies have shown that lactic acid bacteria reduced the production of DNA and proteins during the growth in the stationary phase<sup>17,18</sup>. The increase observed in organic acids such as lactic and galactonic acids in the cells

grown to the stationary phase possibly due to the catabolism of the carbohydrate in the cells. The reduction in cytoplasmic metabolites in the cells grown to stationary phase could be attributed to reduced metabolic rates to preserve energy during undesirable conditions<sup>19-21</sup>. The great increase in the mannitol level in the stationary phase cultures could be due to the requirement of this molecule to help the bacteria to cope with stationary phase conditions. It has been shown that mannitol was important to enhance the survivability of *S. aureus* against antimicrobial fatty acids present on human skin<sup>22</sup>.

Metabolic analysis of cells harvested at mid-exponential phase after being exposed to changes in pH and temperature, revealed different metabolic profiles. For example, a significant alteration in the nitrogenous bases was observed, this possibly a mechanism to encounter the stressful condition present in the



growth conditions. Metabolic studies displayed a significant increase in the concentrations of many cytoplasmic metabolites in *Salmonella* in response to altered environmental conditions<sup>23,24</sup>. Earlier studies have shown that *S. aureus* altered many genes in order to combat changes in pH and hence facilitate survival<sup>25-28</sup>. An identical study showed that amino acid and protein composition of *S. aureus* were remarkably altered following exposure to either pH6 or pH8 combined with lower temperature of 35°C<sup>6,29</sup>. Another study determined significant changes in the fatty acid composition and cell sizes of *Staphylococcus lugdunensis* in response to alterations in environmental conditions including changes in pH and temperature<sup>8</sup>. A recent study exhibited that hydrogen peroxide combined with other environmental factors such as altered pH and temperature led to very different metabolic profiles of a clinical isolate of *S. aureus*<sup>10</sup>, suggested these alterations in amino acids were essential for survival under changes in the environmental conditions. Moreover, a new study revealed that *S. aureus* taken up and released different levels of amino acids following growth in altered environmental conditions<sup>29</sup>.

Cells harvested at stationary phase produced significant alterations in their metabolite abundances in comparison to reference controls. This probably a strategy used by bacteria to cope with stressful condition, once this stress removed cells will restart replicate. An earlier study showed that extreme cold stress led to a significant reduction in the amino acid metabolites<sup>5</sup>. The higher abundances of purine and pyrimidine bases in cells grown in altered environmental conditions could be associated with protein synthesis. It has been shown that *Escherichia coli* produced high quantity of proteins at stationary phase<sup>30</sup>. The synthesis of proteins in high concentrations could be a mechanism to enhance the adaptation and hence the survivability of *S. aureus* at complex environment present in stationary phase. *S. aureus* was shown to consumed different levels of amino acids in response to various environmental conditions at stationary phase<sup>29</sup>, suggesting that different amino acid uptakes were required for adaptation under various temperature and pH.

## CONCLUSION

*Staphylococcus aureus* remains one of the challenge pathogens in hospital due to its remarkable ability to adapt to wide range of environmental conditions. This study determined that different growth phases of *S. aureus* led to significant adjustments in metabolic homeostasis under normal conditions. In additions, the exposure of *S. aureus* to more acidic or alkali conditions with lower temperature of 35 °C resulted to specific metabolic profiles associated with each treatment regimen. The outcome of this study was interpreted to show that alterations in metabolic homeostasis were required to the different growth conditions. The differential responses elicited by the bacterium under each treatment led to characteristic metabolic profiles for adaptation to achieve a homeostasis for survival.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

Not applicable.

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