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Studies on the Effect of Amino Acids to Curb the Pathogenesis of Multi Drug Resistant *Staphylococcus aureus*

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The amino acids have been known for their stimulatory and inhibitory effects on the growth and expression of virulent characters such as toxin production, biofilm formation and colony spreading. If the complete mixture of natural amino acids is added to the basal medium, the accumulation of toxins increases, while *Staphylococcus aureus* was unable to produce toxins in an amino acid deprived medium. Present study suggests that the phenylalnine in all cases inhibits toxin production whereas methionine and serine reduces the biofilm disposition in *Staphylococcuss aureus*. Employing chemically modified medium with certain amino acids to scrutinize mechanisms of pathogenesis gives rise to the impending for additional clinical benefits beyond providing new insights in this medically pertinent field of study. It is desirable that using biostable peptides that target virulence pathways instead of microbial viability will reduces the chance of adaptability of a pathogen and therefore reduces the chance of resistance developing to a treatment.

Key Words: *Staphylococcus aureus*, Amino acids, Virulent characters, Biofilm disposition, Biostable peptides.

INTRODUCTION

Staphylococcus aureus, a member of Micrococcaceae family, is an expedient pathogen that antecedent diseases diverging from suppurative wound infection to life threatening bacteria. *Staphylococcus aureus* beget a multitude of extracellular products such as α, β, γ and δ-haemolysin, enterotoxin, toxic shock syndrome toxin *etc*., that display a wide range of biological activities and epidemiologically associated with severe infections[1-3]. It also produces certain enzymes that facilitate the spread of infection to adjoining tissue[4].

These toxins and enzymes in *S. aureus*are regulated by quorum sensing, where signaling pheromone molecules produced by accessory gene regulator (agr) locus accumulate as a function of cell density. When the concentration of autoinducers reaches threshold level, they bind to its cognate receptor and activate the signal cascade, leading to the expression of virulence gene expression responsible for the production of toxins[5]. The *S. aureus*quorum sensing system has diverged such that there are four distinct AIP, each AIP capable of activating its cognate receptor (AgrC) but inhibiting the AgrC of the other groups[6]. Amino acid is an important factor that affects quorum sensing mechanism and hence, virulent production by *Staphylococci* and there is currently great interest in the relationship between amino acids and toxin production in chemically modified media devoid of certain amino acids.

A considerable amount of research has provided important information regarding nutrient availability, environmental factors and a variety of stresses that result in the alteration of virulence, cell growth and biofilm disposition in bacteria using in a chemically defined medium[7-9]. Despite this being a basic requirement for all metabolic functions in bacteria, the relationship between amino acids and pathogenesis is poorly defined and this relationship is particularly important in the era of peptidomimetics due to emergence of multiple drug resistant *S. aureus*.

This study was undertaken to address the role of amino acids in the pathogenecity of *S. aureus*. In the first set of study, an 'aminoswapping' strategy was used to demonstrate that specific amino acids are required for cell growth, colony spreading, biofilm formation and toxin production. A chemically modified medium would provide a system in which the requirement for growth and toxins by *S. aureus* could be studied.

In the next phase of the study, the data presented define the key amino acid residues involved in AIP/AgrC interactions leading to virulent production and imply a role for amino acids in cell protein synthesis and export. A combination of amino acids in chemically modified medium was used to study the mechanism of both activation and inhibition of the agrresponse for production of biofilm and toxins.

EXPERIMENTAL

Bacterial isolates and Screening of multi drug resistance *Staphylococcus aureus*: *S. aureus* clinical isolates were obtained from Trichy Medical College (Tamil Nadu, India) and all these 25 *S. aureus* isolates were recovered from patients with invasive *S. aureus* disease. Pure cultures of the test organism were prepared by growing aerobically at 37 ºC in nutrient agar medium and maintained on agar slants. Each *S. aureus* isolate was screened for expression of Haemolysin and extracellular protein. Antibiotics were purchased from Himedia and were used in following concentration clindamycin 2 µg/mL, gentamycin and penicillin-G 10µg/mL individual, vancomycin 30 µg/mL and linezolid 10 µg/mL. *S. aureus* 762 clinical isolate (SA762) was resistant from all given antibiotics chosen for further analysis[10].

Bacterial cell culture in chemically modified medium and growth conditions: The basal medium used in all amino acid deletion studies was chemically modified broth as shown in Table-1. It contained a carbon source (glucose) and inorganic salts except nitrogen source into which was added various numbers of L-amino acids. The organism was grown for 12, 24, 48 and 72 h at 37 ºC.

Measurement of *S. aureus* cell mass from fluid culture: The density of SA762 in liquid culture media was determined by measuring the optical density at 600 nm after construction and calibration of a standard curve. The optical density of the culture was related to the concentration of viable cells by plate counts. Direct physical measurement of cell dry weight and wet weight were carried out after centrifugation at 10,000 rpm.

Measurement of haemolytic activity: *S. aureus* strain was grown in chemically modified medium devoid of single amino acid from the pool of 18 natural amino acids for amino swapping experiment and different groups of effective amino acids in the later experiment as given in the Table-1. The hemolytic activity of *S. aureus* strain was tested on blood agar plates supplemented with 5 % sheep blood. SA762 culture was spotted on blood agar plates and incubated[11] at different time intervals [12, 24, 48 and 72 h] at 37 ºC.

Protease assay: *S. aureus* strain was grown as described earlier and protease activity was determined by an agar plate assay. The test agar contained 1 % skimmed milk and 1.5 % agar in chemically modified medium[12]. Bacterial strains were grown at different time intervals on the agar plates at 37 ºC.

Colony spreading experiment: Chemically modified media supplemented with 0.24 % agar was autoclaved and poured into plates (90 mm diameter). Overnight cultures of SA762 as describe earlier, was spotted (10 µL) on onto the center of the plates and incubated at 37 ºC for different time intervals[13].

Biofilm assay: SA762strain was grown overnight in chemically modified broth at 37 ºC describe earlier. 100 µL of cell culture was used to inoculate sterile 96-well polystyrene microtiter plates for bacterial attachment *in vitro*[14]. After different time intervals, the wells were gently washed three times with 200 µL of phosphate buffer saline and cells were air dried and fixed with 100 % ethanol. The cells were dried again and then stained with filtered 0.4 % gentian violet diluted in 12 % ethanol for 2 min. Wells were gently washed five times with phosphate-buffered saline to remove stain. Then, 100 µL of 1 % SDS was added to solubilize the cells and optical density was measured at 595 nm in an enzyme-linked immunosorbent assay plate reader.

RESULTS AND DISCUSSION

During investigation of the amino acid mixtures on the accumulation of extracellular proteins, when one amino acid removed at a time from the experimental mixture, it was found that absence of isoleucine, tyrosine and alanine enhances the rate of accumulation (data not shown) and the degree of reduction was increased almost 87 % when the same amino acids added together in the medium in the second phase of the study (Fig. 1).

The deletion of the aspartic acid and isoleucine from the chemically modified medium results in an increase in the

TABLE-1
COMPOSITION OF CHEMICALLY MODIFIED SYNTHETIC MEDIUM USED IN EXPERIMENTS

Chemically modified medium [First phase]		Chemically modified medium [Second phase]	
Basal Medium[#]	Amount (g/100mL)	Name of the medium	Amino acids
NaCl	0.50	CMM + CDI	E, G, I, F, T, Y
K_2HPO_4	0.25	CMM + BI	M, S
Glucose	0.25	CMM + HI	D, G, K, F
		CMM + ECPI	I, Y, A
		CMM + GF	C, V, R, P
L-Amino acids[a]	Amount (mg/100 mL)	L-Amino acids[a]	Amount (mg/100 mL)
Arginine [R]	0.2	Lysine [K]	0.2
Aspartic acid [D]	0.2	Methionine [M]	0.2
Alanine [A]	0.2	Phenyl alanine [F]	0.2
Cysteine [C]	0.2	Proline [P]	0.2
Glycine [G]	0.2	Serine [S]	0.2
Glutamic acid [E]	0.2	Tryptophan [W]	0.2
Histidine [H]	0.2	Tyrosine [Y]	0.2
Isoleucine [I]	0.2	Threonine [T]	0.2
Leucine [L]	0.2	Valine [V]	0.2

[a]Amino acids are listed in order of their subsequent deletion from the medium. [#]Basal medium of second phase of experiment was similar to the first phase. 18 amino acids and tryptic soy broth were used as positive control for entire study. Abbreviations in Table-1: CMM + GF = chemically modified media along with amino acids responsible for growth, BI = Biofilm inhibitors, HI = Haemolysin inhibitors, ECPI = Extracellular protein inhibitors.

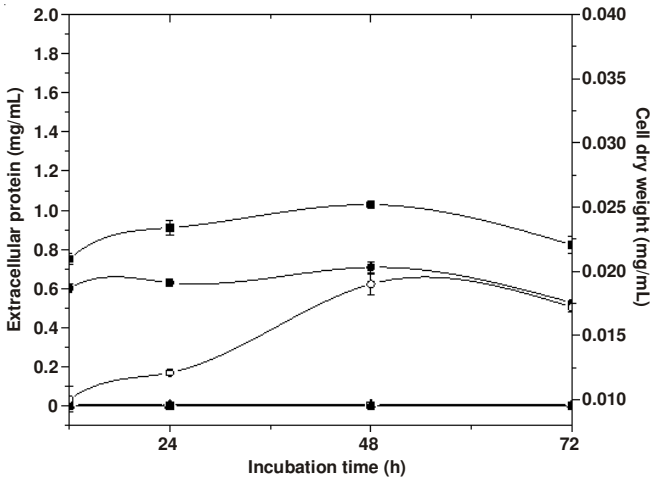

Fig. 1. Production of extracellular protein in relation to bacterial cell dry weight at different time intervals for clinical isolate SA762. -o- cell dry weight in CMM + GF medium, -■- culture grown in tryptic soya broth, -●- culture grown in chemically modified broth supplimented with 18 amino acids, -□- expression of extracellular protein in CMM + BI medium, -▼- CMM+ECPI medium and -▲- CMM + HI medium

protease activity in amino swapping experiment, while a significant reduction was observed in the CMM + HI and CMM + ECPI medium in the second phase of the study (Fig. 2). This result indicates that the presence of phenyl alanine reduces the protease production but cannot inhibit completely.

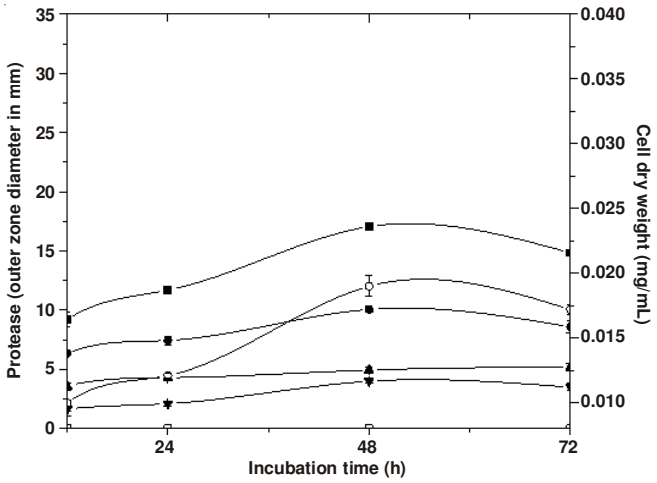

Fig. 2. Effect of amino acids on protease activity. The graph shows the relationship between the growth and proteolytic activity at different time intervals for clinical isolate SA762. -o- cell dry weight in CMM + GF medium, -■- culture grown in tryptic soya broth, -●- culture grown in chemically modified broth supplimented with 18 amino acids, -□- proteolytic activity in CMM + BI medium,-▼- CMM + ECPI medium and -▲- in CMM + HI medium

Under the conditions defined in the Table-1, the concentration of valine, cystine, proline and arginine are required to produce maximal growth and toxins of *S. aureus* clinical strain and the similar results were obtained in the previous studies[15-17]. The deficient medium shows poor growth even after 48 h. Since the inhibition was obtained with the identical concentrations of the same amino acids in group, it is assumed that these amino acids promote the growth of the *S. aureus*. Onou and Mori[18] found that the valine in all cases was required for growth. Another observation in the second phase of the study showed that medium containing tyrosine, phenylalanine, glycine, isoleucine, glutamic acid and threonine inhibit the *S. aureus* growth by 99.91 %. It is of interest, that when testing different mixtures of amino acids in the medium for the inhibition of *S. aureus*, tyrosine is responsible for delayed growth as well as it was essential for inhibition of extracellular protein production.

When the culture was spotted onto soft agar plates (0.24 % agar) devoid of either histidine, threonine and serine, the average diameter of colonies were 17.75, 23.5 and 15.25 mm, respectively after 72 h of incubation (Fig. 3). This result indicates that *S. aureus* has an ability to spread on the soft agar plates and these amino acids are responsible for colony spreading. Colony spreading was enhanced when the period of incubation prolonged.

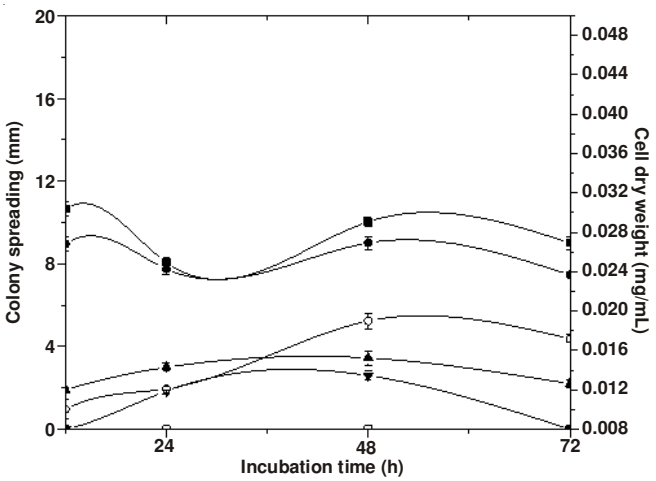

Fig. 3. Qualitative analysis of colony spreading in chemically define medium. The graph shows the relationship between the colony spreading and cell dry weight of SA762. -o- cell dry weight in CMM + GF medium, -■- culture grown in tryptic soya broth, -●- culture grown in chemically modified broth supplimented with 18 amino acids, -□- colony spreading in CMM + BI medium, -▼- CMM + ECPI medium and -▲- represents the colony spreading in CMM + HI medium

It was observed that *S. aureus* strain produces haemolysis on sheep blood agar on chemically modified medium lacking aspartic acid, glycine, lysine and phenyl alanine while tryptophan and proline was responsible for the increased hemolytic activity in the first phase of experiment. In the remainder, cysteine, arginine, proline and valine deleted medium does not showed any haemolytic activity (data not shown). The results were comparable with previous findings where tryptophan analogues inhibit the production of haemolysins and proline is required for *Styphylococcal* pathogenesis where chemically defined media were used varying only in amino acid composition[15,19,20]. Completely haemolyzed or semihemolyzed zones of different diameters were produced around the growth regions when the plates were incubated for different time intervals at 37 ºC. In most of the cases the haemolysin activity was seen only after 24 h. When *S. aureus* cells were incubated in MTB-HI medium, no haemolytic activity was observed which coheres with the first phase, that shows these amino acids interferes the pathway responsible for the haemolysin production.

In the amino swapping experiment, the biofilm disposition was enhanced in CMM-M and CMM-S medium deficient of methionine and serine, respectively. It is evident from the results of the second phase that methionine and serine containing medium (CMM + BI) inhibits the biofilm formation upto 70 folds (Fig. 4).

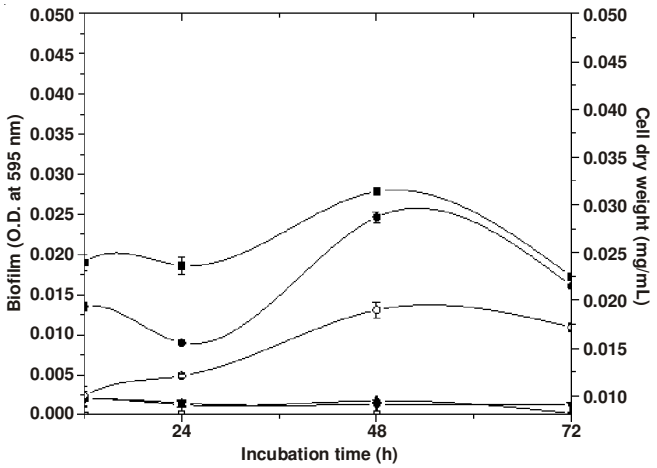

Fig. 4. Biofilm formation in chemically define medium. SA762 strain was grown in chemically defined medium at different time intervals in 96 well plate and optical density was measured at 595 nm. The graph shows a decrease in biofilm formation in chemically modified medium containing diffrernt groups of amino acids. There was no significant decrease in cell dry weight with respect to biofilm formation. -o- cell dry weight in CMM + GF medium, -■- culture grown in tryptic soya broth, -●- culture grown in chemically modified broth supplimented with 18 amino acids, -□- biofilm formation in CMM + BI medium, -▼- CMM + ECPI medium and -▲- represents the colony spreading in CMM + HI medium

These studies were initiated to examine the effect of 18 different amino acids in growth and virulence of *Staphylococcus aureus*. The results clearly showed that phenyl alanine in all the cases required for inhibition of virulent gene expression, while aspartic acid reduces the haemolysin and protease activity. Glycine, phenyl alanine, isoleucine and tyrosine play a crucial role in *S. aureus* growth as well as virulent inhibition.

Conclusion

As the pathogenesis of *Staphylococcus aureus* is dependent on quorum sensing and regulated by small diffusible signal pheromone peptides, these investigations have illuminated the role of amino acids and set the stage for the design of biostable peptidomimetic molecule. The mechanism of agr (accessory gene regulator) activation and amino acid requirement for the formation of AIP is correlated with the data obtained from the present study and due to high inhibition index. These amino acids can be used for peptidomimetics to produce potent antivirulent drugs.

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