Microarray analysis of the genomic effect of eugenol on methicillin resistant Staphylococcus aureus

Sunday Ayuba Buru (sunday.buru@kasu.edu.ng)
Kaduna State University
Mallikarjuna Rao Pichika
International Medical University
Kavitha Mohandas
MAHSA University College

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Ayuba Sunday Buru*[1], Vasanthakumari Neela[2],
Kavitha Mohandas[3], Mallikarjuna Rao Pichika[4],

[1]Genomic Research Lab, Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences, College of Medicine, Kaduna State University, Kaduna, Nigeria

[2] Department of Medical Microbiology and Parasitology,
Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia

[3]Department of Microbiology, Faculty of Medicine, MAHSA University College, Kuala Lumpur, Malaysia.

[4]International Medical University, No.126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.

Corresponding author*, e-mail: sunday.buru@kasu.edu.ng

Abstract

Background: *Staphylococcus aureus* is a highly adaptive human pathogen responsible for serious hospital and community acquired infectious diseases ranging from skin and soft tissue infections to complicated and life - threatening conditions such as endocarditis and toxic shock syndrome (TSS). The rapid resistance of this organism to available antibiotics over the last few decades has necessitated a constant search for more efficacious antibacterial agents. Eugenol [4-Allyl-2-methoxyphenol] belongs to the class of chemical compounds called phenylpropanoids. It is a pure to pale yellow oily liquid substance mostly extracted as an essential oil from natural products such as clove, cinnamon, nutmeg, basil and bay leaf. Eugenol has previously been shown to have antimicrobial activity against methicillin resistant *Staphylococcus aureus*. However, the mechanism of *S. aureus* has not, as yet, been elucidated – hence, the expediency of this study.

Results: Global gene expression outlines in response to sub - inhibitory concentrations of eugenol were analysed using the agilent DNA microarray system to identify gene targets, most importantly essential genes involved in unique metabolic pathways. Transcriptomic analysis of fluctuating genes revealed those involved in Amino acid metabolism, fatty acid metabolism, translation and ribosomal pathways. In Amino acid metabolism for instance, the *argC* gene encodes for N-acetyl-gamma-glutamyl-phosphate reductase. The *argC* gene plays an important role in the biosynthesis of arginine from glutamate in the amino acid metabolic pathway. It is the enzyme that catalyses the third step in the latter reaction and without this process, the production of N-acetylglutamate 5-semialdehyde will not be complete from the NADP-dependent reduction of N-acetyl-5-glutamyl phosphate, which is essential for the survival of some microorganisms and plants.

Conclusion: This study has enabled us to examine complete global transcriptomal responses in MRSA against eugenol. It has revealed novel information with the potential to further benefit the exploratory quest for novel targets against this pathogen, in view to the development of efficacious antimicrobial agents for the treatment of associated infections.

Keywords: DNA microarray, Gene transcription, Eugenol, methicillin resistant *Staphylococcus aureus* (MRSA)

Background

Methicillin resistant *Staphylococcus aureus* infections and related clinical complications are on the increase. It is a highly adaptive human pathogen causing benign soft skin infections, life threatening endocarditis and toxic shock syndrome (TSS) [1]. Also a major nosocomial pathogen, it is implicated in both hospital and community type staphylococcal infections that are difficult to treat and control, due to a multifactorial combination of toxin - mediated virulence, invasiveness and antibiotic resistance [2]. There has been a wide increase in *S. aureus* resistance

to antibiotics over the past decade, and this has posed a great challenge for the development of effective therapeutics against this opportunistic pathogen [2].

Antibiotic resistance in *Staphylococcus aureus* is associated with staphylococcal cassette chromosome *mec* (SCC*mec*) known to encode the *mecA* gene, which confers resistance to *S. aureus* due to gene transfer and consequently, is responsible for methicillin and other beta-lactam antibiotic resistance [1]. The occurrence of resistance within and across different antibacterial classes has reinforced the urgent need for the discovery of new and potent compounds aiming at novel cellular metabolic functions hitherto not targeted by current antibacterial agents [2].

According to a 2007 CDC report, the rate of mortality and morbidity due to MRSA in the U.S.A is on the increase. As such, the significance of this organism and its infections in relation to public health issues cannot be underscored [3]. Therefore, there is a significant need for more research into alternative and more efficacious antimicrobials against this pathogen, including those emerging from natural products [2]. The current strategy for novel antibacterial discovery from natural products involves screening and expression analysis of promising targets against *S. aureus*. These targets encompass multifarious functionalities which include fatty acid metabolism, peptidoglycan and protein biosynthesis, cell division, DNA replication and biosynthesis [4].

Plants and derived natural products have been the source of secondary metabolites such as terpenoids, pentacyclic triterpenoids, flavonoids and glycosides that have shown promising anti-staphylococcal activities [5, 2, 6, 7, 8]. Eugenol, a propenylphenol,, is found abundantly in clove oil, *Cinnamomum* oil, nutmeg, basil, apanese, star anise, Lemon balm, Dill, *Pimenta racemosa* and bay leaf [9]. Eugenol is used in perfumeries, flavourings, essential oils and in medicine as a local antiseptic and anaesthetic. Previous studies have shown that eugenol has antimicrobial, antioxidant and anti - inflammatory, anti – carminative and antispasmodic activities and kills

certain human colon cancer cell lines *in vitro* [10]. Similar studies on other plant derived compounds showing anti - staphylococcal activities have been reported. In a preliminary study, betulinaldehyde has been reported to exhibit antibacterial activity against *S.aureus* with an MIC value of 512 mg/ml. This compound potentiates the activity of the cell wall inhibitors methicillin, vancomycin [11] and *S.aureus* (ATCC 25923) treated with the compounds berberine chloride [12], cryptotanshione [13] and rhein [14].

This study had the objective of ascertaining the transcriptional profile of genes expressed due to exposure of MRSA to sub - inhibitory concentrations of eugenol and to fully understand its possible mechanisms of action. The novel genes identified may then be explored as potential drug targets for the development of therapeutics against MRSA.

Methods

Bacterial strains and growth conditions

Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 700698 was used as the reference strain in this study. The bacterial strain was cultured on Mueller - Hinton Agar (MHA) for 18-24 hours after which pure, single colonies were maintained at 37ºC with shaking at 200 rpm in Mueller-Hinton Broth (MHB). For growth inhibition, sub - inhibitory concentrations of 0.0012 mg/ml (0.00195mM) of eugenol obtained from Sigma-Aldrich (USA) was prepared in 1% dimethyl sulfoxide (DMSO) and added immediately to the broth culture after the OD_{600} reached 0.7, a turbidity comparable to that of a 0.5 McFarland standard and a bacterial count equivalent to approximately 10^6 cfu/ml. The OD_{600} was measured using a Lambda 25 spectrophotometer (PerkinElmer, Inc, MA).

Total RNA isolation and purification

Total RNA was isolated after 1 hour with and without (control) sub - inhibitory concentrations of eugenol using the simple phenol method as described by Salman [15]. The quality and quantity of RNA obtained were determined using the the NanoDrop 2000 Spectrophotometer (Thermo Scientific Nano Drop products Wilmington, USA) and the RNA 6000 Nano Chip on Chip-Electrophoresis (LOT#:QC29BK20) (Agilent Technologies, Inc.Waldbronn, Germany) with the Agilent 2100 Bioanalyser (Agilent Technologies, Inc. Santa Clara, CA).

cDNA synthesis, labeling, hybridisation, staining and scanning

cDNA synthesis, cDNA labeling, cDNA purification, hybridisation, staining and washing steps were performed according to the manufacturer's protocol for the Agilent MRSA customised oligonucleotide microarray (Order 0304399531, Content G4102A) (Agilent Technologies Inc. USA).

Microarray analysis

The microarray utilized for this study comprised 8 arrays with 60 thousand features per array giving rise to a total of 8 292 features. The microarray had a specific Agilent identifier called the Agilent MicroArray Design Identifier (AMADID) which was used to identify the type of array being used. The AMADID had the design ID 045256. The arrays were scanned with the DNA Microarray Scanner with SureScan High Resolution Technology. The features extracted from the scanner were analysed using Genespring 12 (Agilent Technologies, Santa Clara, CA, USA) with the parameters, *p-value* >0.05 and fold change >2.

Validation of genes expression by quantitative real - time PCR

To determine the validity of the array data, transcriptional levels obtained using the microarray were compared with those from quantitative real - time PCR. Genes and primer sequences employed for the real - time PCR analysis are listed in Table X. The house - keeping genes *arc*

and *yqiL* were used as endogenous reference genes. The experiment was performed using the iCycler iQ5 Real Time PCR Detection System (Bio-Rad Laboratories, USA) with Quantitect SYBR Green PCR Kit (Qiagen). For each gene amplified, two biological replicates with three technical replicates were employed. The reaction mixtures were initially incubated for 3 min at 95.0 ºC, followed by 40 cycles of 10 s at 95ºC, 30s at 55 ºC and 20s at 72.0 ºC. The PCR efficiencies results were derived from standard curve slopes in the iCycler software v. 3.1 (BioRad Laboratories, Hercules, CA, USA).

Results and Discussion

To determine the sub - inhibitory effect of eugenol on MRSA, we first exposed exponentially growing cells of MRSA to eugenol and concurrently demonstrated that eugenol dissolved in 10 % DMSO at 0.0012 mg/mL caused growth inhibition of MRSA at 60min.

Table 1 List of genes expressed in response to eugenol treatment of methicillin-resistant *Staphylococcus aureus*, their fold changes, *p*-values and functional classifications

ProbeName	Gene ID	FC (abs)	Regulation	Gene Symbol	Protein Name	Pathway description	Functional class
CUST_3705_PI428639740	SAHV_0568	2.5	up		hypothetical protein 3-hexulose-6-phosphate synthase	Pentose phosphate pathway	Carbohydrate metabolism
CUST_5451_PI428639740	SAHV_1404	-5.3	down		hypothetical protein		complement
CUST_1524_PI428639740	SAHV_1631	3.8	up	*obg*	GTPases	GTP-binding protein	Ribosome biogenesis
CUST_1991_PI428639740	SAHV_2118	-2.0	down	*luxS*	S-ribosylhomocysteinase	Cysteine and methionine metabolism	Amino acid metabolism; Cysteine and methionine metabolism
CUST_5450_PI428639740	SAHV_1404	-4.5	down		hypothetical protein		complement
CUST_2569_PI428639740	SAHV_0045	-2.1	down		hypothetical protein		complement
CUST_6663_PI428639740	SAHV_1949	-2.9	down		hypothetical protein		complement
CUST_193_PI428639740	SAHV_0183	2.0	up	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	Arginine and proline Metabolic pathways Biosynthesis of secondary metabolites 2-Oxocarboxylic acid metabolism	Amino acid metabolism; Arginine and proline metabolism
CUST_2081_PI428639740	SAHV_2209	2.2	up	*rpsK*	30S ribosomal protein S11	Ribosome	Genetic Information Processing; Translation; Ribosome
CUST_4653_PI428639740	SAHV_0973	-2.6	down		hypothetical protein		complement
CUST_2112_PI428639740	SAHV_2219	2.3	up	*rplF*	50S ribosomal protein L6	Ribosome	Genetic Information Processing; Translation; Ribosome
CUST_23_PI428639740	SAHV_0015	2.2	up	*rplI*	50S ribosomal protein L9	Ribosome	Genetic Information Processing; Translation;

CUST_3538_PI428639740	SAHV_0480	2.4	up	hypothetical protein	Ribosome complement

Functional classifications analysis

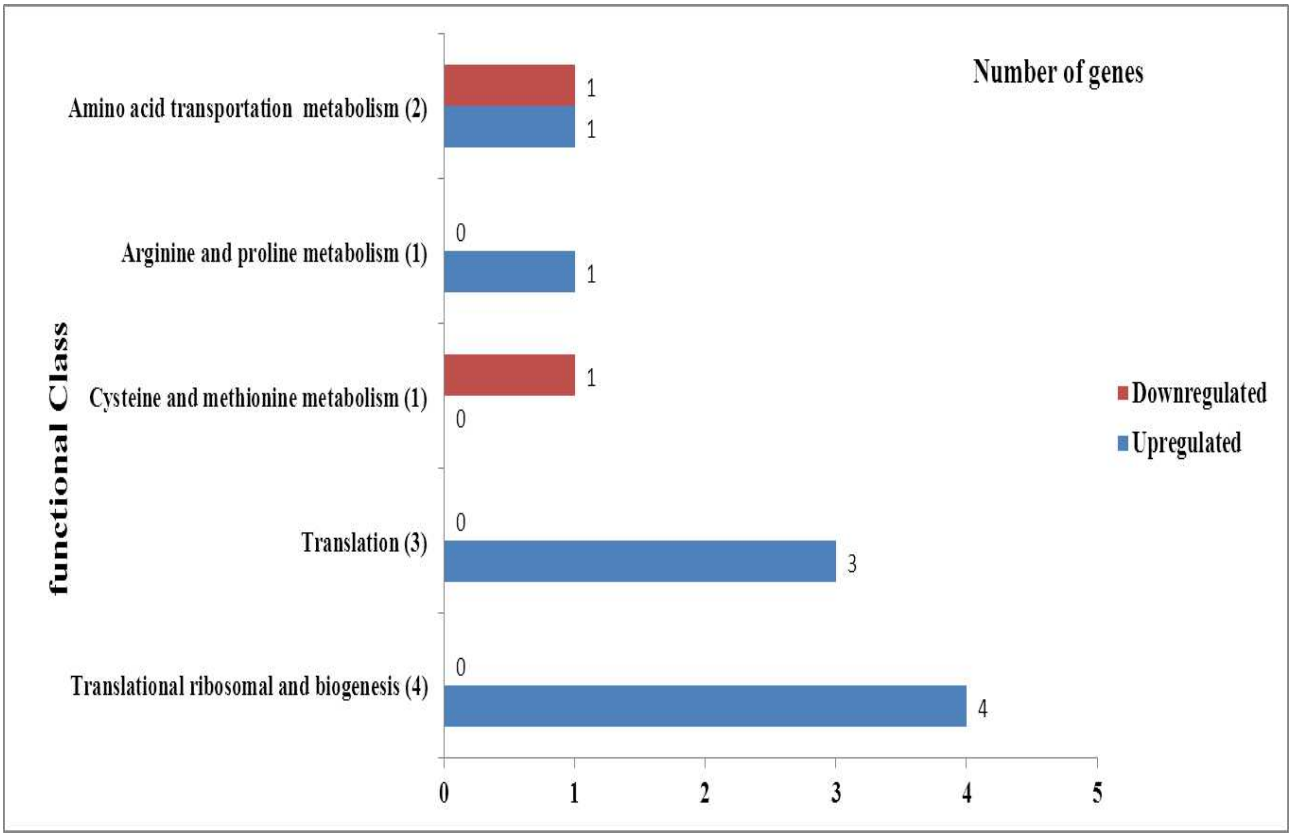

Figure 1: Functional classification of the genes expressed with eugenol treatment of MRSA based on their metabolic pathways. A total of 11 genes fluctuated

Table 2 Functional classes with associated up – regulated and down – regulated genes in Control (C) vs Eugenol (E)

Functional class(es)	Gene(s) symbol	Up-regulated	Down-regulated
Translation	*rplI, rplF*, *rpsK*	*rplI, rplF, rpsK*	
Amino acid transportation metabolism	*luxS, argC*	*argC*	*luxS*
Translational ribosomal and biogenesis	*Obg, rplI, rplF, rpsK*	*Obg, rplI, rplF, rpsK*	
Arginine and proline metabolism	*argC*	*argC*	
Cysteine and methionine metabolism	*luxS,*		*luxS*

Table 3 Control (C) vs Eugenol (E) genes for validation by qRT-PCR (5)

S/NO	Gene ID	Gene symbol	Fold Change	Regulation
1.	SAHV_2118	*luxS*	2.0	down
2. .	SAHV_0183	*argC*	2.0	up
3.	SAHV_2209	*rpsK*	2.2	up
4.	SAHV_2219	*rplF*	2.3	up
5. .	SAHV_0015	*rplI*	2.2	up

Table 4 A comparison between microarray and qPCR results for E (Eugenol) versus C (Control) using an average of 4 housekeeping genes (*glpF, arc, gmk, tpiA*) to normalise data

	qPCR		Microarray		
Gene	**Fold change (Abs)**	**Regulation**	**Fold change (Abs)**	**Regulation**	**Match with Microarray**
rpIF	-1.6	down	2.3	up	×
Rpll	-1.2	down	1.2	up	×
luxS	-2.4	down	-2.0	down	✓
argC	2.5	up	1.3	up	✓
rpsK	-1.1	down	2.2	up	×

Table 5 A comparison between microarray and qPCR results for E (Eugenol) versus C (Control) using the *gmk* gene to normalise data

	qPCR		Microarray		
Gene	**Fold change (Abs)**	**Regulation**	**Fold change (Abs)**	**Regulation**	**Match with Microarray**
rpIF	-1.9	down	2.3	up	×
rpll	-1.4	down	1.2	up	×
luxS	-3.0	down	-2.0	down	✓
argC	2.0	up	1.3	up	✓
rpsk	-1.3	down	2.2	up	×

Table 6 RT-qPCR percentage of correlation with microarray data

Treatment	Percentage correlation with four housekeeping genes (*glpF, arc, gmk, tpiA* HKG)	Percentage correlation with one house keeping gene (*gmk* HKG)
E *vs* C	60	53.3

Gene transcriptional responses in MRSA upon treatment with eugenol

Treatment of MRSA with sub - inhibitory concentrations of eugenol revealed that a total of 13 genes had fluctuated - 7 genes were up - regulated whilst 6 were down - regulated in the transcriptional changes that had occurred. Of the total 13 genes, 6 are hypothetical genes, 2 genes are involved in amino acid metabolism, 4 are involved in genetic information processing i.e. ribosomal and translational biogenesis metabolism, whilst one is involved in carbohydrate metabolism.

Amino acid metabolism

The *argC* gene encodes for N-acetyl-gamma-glutamyl-phosphate reductase. The *argC* gene plays an important role in the biosynthesis of arginine from glutamate in the amino acid metabolic pathway. It is the enzyme that catalyses the third step in the latter reaction and without this process, the production of N-acetylglutamate 5-semialdehyde will not be complete from the NADP-dependent reduction of N-acetyl-5-glutamyl phosphate [16,17]. This reaction therefore is part of the arginine biosynthetic pathway that is essential for some microorganisms and plants [18]. When MRSA was challenged with eugenol, the *argC* gene was shown to be upregulated. In bacteria, the argC protein is a monofunctional unit of 35 to 38 kD. In contrast, in fungi, it is bifunctional and part of a mitochondrial enzyme complex (gene *arg5, 6, arg11* or *arg6*) with an N-terminal acetylglutamate kinase domain and a C-terminal AGPR domain [16,17]. In *Escherichia coli*, the enzyme, a cysteine has been associated with catalytic activity around the residue region which is well conserved and can serve as a signature pattern [16,17].

Microbial secondary metabolites such as antibiotics, pigments and toxins are produced by microorganisms as self-defence mechanism [19]. It is thus understandably expected that challenge of MRSA with eugenol would cause upregulation of the *argC* gene, which in turn would enhance

biosynthesis of secondary metabolites which would include pigments and stress related proteins that are normally expressed when the bacterium is subjected to physiological stress.

The *lux*S gene encodes for *S*-Ribosylhomocysteinase (*lux*S) which is also involved in the amino acid metabolic pathway. It was downregulated when treated with eugenol. *lux*S being a Fe^{2+}-dependent metalloenzyme, plays a catalytic role in *S*-ribosylhomocysteine (SRH) - thioether bond cleavage to produce homocysteine (Hcys) and 4,5-dihydroxy-2,3-pentanedione (DPD) [20][668]. The enzyme S-(5-deoxy-D-ribos-5-yl)-L-homocysteine L-homocysteine-lyase [(4S)-4,5-dihydroxypentan-2,3-dione-forming] belongs to the class of carbon - sulfur lyases and the family of lyases [21] and is also commonly referred to as S-ribosylhomocysteinase and *lux*S [22]. The enzymes are involved in methionine metabolism [22] which is a key step in the biosynthetic pathway of the type II autoinducer (AI-2) which plays a vital role in the quorum sensing mechanism of both Gram-positive and Gram-negative bacteria [23]. AI-2 functions as a signaling molecule in interspecies communication through regulation of niche-specific genes that have diverse functions in various bacterial species, which is often in response to a change in the population density [22]. This reaction serves a dual purpose, i.e. for the detoxification of S-adenosylhomocysteine and also for the production of a type 2 quorum sensing molecule [24]. Cell population density - dependent regulation of gene expression is a key contributing factor for bacterial pathogenesis. There are two quorum-sensing (QS) systems in the staphylococci [25], namely, the accessory gene regulator (*agr)* and *luxS*. In staphylococci, upregulation of *luxS* reduces biofilm formation and virulence rather than induces it, especially during biofilm-associated infection. The *agr* gene has been shown to enhance biofilm detachment through up - regulation of detergent - like peptides, whilst *luxS* decreases cell – to - cell adhesion through down - regulation of biofilm exopolysaccharide expression [25]. The metabolically inert mode of growth of biofilms appears to be characterised somewhat by overall low activity of the staphylococcal QS systems [24]. In this study, when MRSA was challenged with eugenol, *luxS* expression was reduced

suggesting induction of heightened biofilm production and heightened virulence of the bacterium [24], which are typical immediate self - defence responses against aggressive threat. As such, inducers of *luxS*, or metabolites that neutralise inhibitors of *luxS*, would enhance *luxS* expression and reduce biofilm buildup and bacterial virulence, and could be novel targets for antibacterial drug design and development.

Translational ribosomal biogenesis

The genes *Obg, rplI, rplF* and *rpsK* were upregulated upon challenge with eugenol. These genes encode for ribosomal proteins which play a very vital role in genetic information processing. Bacteria live in an environment with recurrently changing conditions; to be able to acclimatise rapidly, they have evolved a wide - ranging array of mechanisms to regulate every cellular process from transcriptional initiation to protein inactivation and degradation. The regulation of gene expression on the transcriptional level relies on signals transferred to the RNA polymerase (RNAP) that alters the enzyme's activity or specificity. It has been observed that different regulatory proteins, molecules and small RNAs can modulate the RNAP. The ribosomes are known to determine the capacity of translation, which is directly linked to cellular growth and control of rRNA transcription. As such, ribosome biogenesis is of great importance for the adjustment to environmental fluctuations. Upregulation of ribosome biosynthesis upon challenge with eugenol is likely to have occurred as a direct response to cellular injury and the need for amelioration by upregulation of protein biosynthesis.

The ribosome is a nucleoprotein particle responsible for one of the key processes in every cell, the decoding of mRNA into protein. Although much research has been focused on the molecular mechanisms behind each step of translation, ribosome biogenesis *per se* and feedback signaling from ongoing protein synthesis to the biogenesis machinery have been somewhat neglected. Formation of the ribosomal particle involves a complex series of processes, i.e., synthesis, processing and modification of both rRNA and ribosomal proteins, and assembly of the components. The quality of

the particle must also be checked and the number of active ribosomes monitored. All of these events must be tightly regulated and coordinated to avoid energy losses and imbalances in cell physiology. Prokaryotic ribosomes sediment as 70S particles and are formed by two subunits, 30S and 50S. The small subunit, 30S, is made of 16S rRNA (1,542 nt) and 21 ribosomal proteins (rproteins), while the large subunit, 50S, is composed of two rRNAs, 23S (2,904 nt) and 5S (120 nt) rRNA, and 33 proteins [26]. The ribosomal subunits have different functions in translation. The 50S particle is a hemisphere, with three structures protruding from its top. The view of the 50S interface, the side that interacts with the 30S particle, shows the central protuberance in the middle, which includes the 5S rRNA and its associated proteins, while the two arms that extend to the left and right are formed by protein L1 and protein L11/rRNA, respectively. The two main functions of the small subunit are to initiate the interaction with mRNA during translational initiation and to decode the message. The 30S particle is approximately half the molecular weight of the 50S subunit and its structure can be divided into an upper part, the head and a lower, larger body. Upon translational initiation, the two subunits associate and become connected via a complex network of molecular interactions along the interface [27]. This region is relatively free of ribosomal proteins and provides binding surfaces for many substrates and ligands [28,29]. The intersubunit RNA bridges have been shown to rearrange, or even break, as part of the translation elongation cycle [30], demonstrating high elasticity of the rRNA that is essential for both signal transmissions between the ribosomal subunits and coordination of their relative movements during translation. The assembly and maturation of the ribosomal subunits are very complex and involve a series of events, such as processing and modification of rRNA, ordered binding of ribosomal proteins and metal ions and sequential conformational changes.

In vivo this takes approximately 2 min at 37°C [31,32]. The biogenesis of ribosomes begins with transcription of the 16S, 23S, and 5S rRNA, which are synthesized as one primary transcript. Maturation of the transcript begins before transcription is completed, with instant formation of local

secondary structures and, as soon as their binding sites emerge from the poly- merase, binding of ribosomal proteins. Simultaneously, rRNA becomes chemically modified at a number of positions and is processed by several RNases to generate mature rRNA species [33].

Conclusions

From our genomic transcriptomics data, eugenol was shown to affect targets / pathways that are involved in amino acid metabolism and protein translation, both of which are very essential for development and transcriptional processes in the survival of bacteria. The functional roles and mechanisms of these target genes are present in both Gram positive and Gram negative bacteria. Eugenol has a high level of penetrability into the bacterial periplasm and cytoplasm, mechanistically through the disruption of the cell wall and complex matrices of the cytoplasm.

Although, the in - depth understanding of the physicochemical properties of eugenol entry into MRSA is limited, the structure activity relationship through high throughput screening (HTS), molecular docking should be explored to further our understanding of the molecular mechanism. -

The emergence and continuous rise of resistance in bacteria to antibacterial agents is currently a global challenge. Eugenol may be used in combination with currently available antibacterial agents as combination therapy to address this global pandemic of bacterial resistance. Antibiotics such as methicillin, vancomycin and teicoplanin are known to disrupt the peptidoglycan complex and can be used in combination with other antibacterial agents to have multiple target efficiency, thereby improving their antibacterial activity. An example in this case, is the study conducted on the combinatory effect of betulinaldehyde with methicillin which reduces the minimum inhibitory concentration (MIC) value in MSSA [34].

In summary, this paper describes the first genome - wide transcriptional analysis of upregulated and downregualated genes in MRSA in response to eugenol, utilising whole genome microarray. The

global transcriptomic profile of eugenol treated MRSA shows fluctuations of major functional classes involved in amino acid metabolism, genetic information processing i.e. ribosomal and translational biogenesis metabolism, complement and carbohydrate metabolism. Genes involved in amino acid metabolism and ribosomal and translational biogenesis metabolism were upregulated suggesting increased protein biosynthesis to ameliorate the effects of cellular injury.

These genes that fluctuated in expression upon challenge with eugenol are important for the survival of MRSA and show promise as novel targets for antibacterial drug design and development.

Conflict of Interest

The authors have no conflict of interest.

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Figures

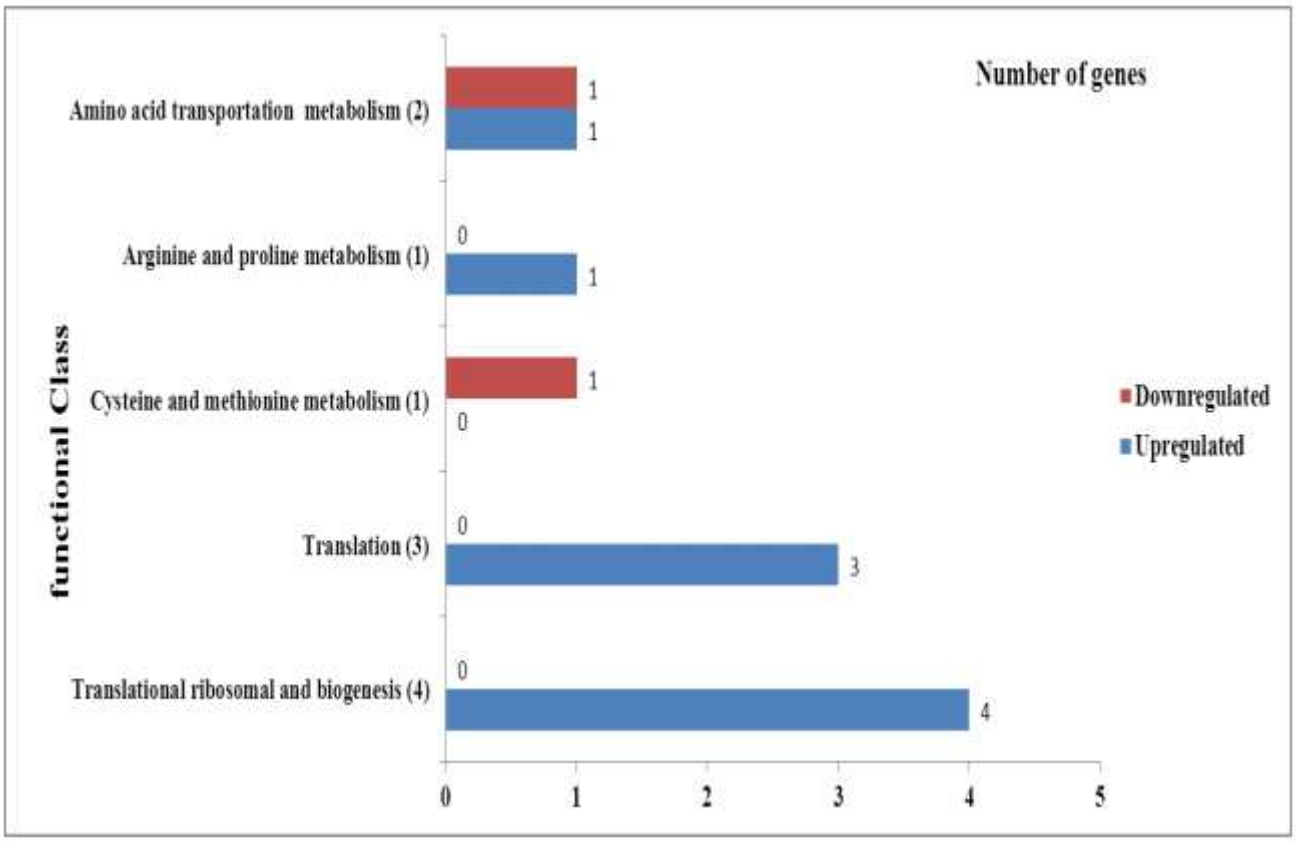

Figure 1

Functional classification of the genes expressed with eugenol treatment of MRSA based on their metabolic pathways. A total of 11 genes fluctuated