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Transcriptional reprogramming under vancomycin pressure in *Staphylococcus aureus*

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The World Health Organization (WHO) has defined Vancomycin intermediate *Staphylococcus aureus* (VISA) and Vancomycin resistant *Staphylococcus aureus* (VRSA) as high priority pathogens. VISA and VRSA are produced by different mechanisms, hence VISA cannot convert to VRSA. Consequently, upon vancomycin treatment of VSSA isolates, there can be emergence of VISA but not its conversion to VRSA. But we observed that when VSSA (MIC ≤2 µg/mL) isolates, lacking *vanA* or *vanB* genes, are grown under vancomycin stress for 60 days, 4 out of 8 isolates get converted to VISA (MIC = 4-8 µg/mL), and 1 converted to VRSA (MIC ≥16 µg/mL). Further, the VRSA isolate had a vancomycin MIC that was 8-fold higher than that of its sensitive counterpart. Hence, the VRSA has been interpreted as a heteroresistant isolate. To dissect the molecular underpinning of this transient resistance pattern, we analyzed the gene expression profile of these isolates and publicly available datasets. *pbp2* gene was observed to be consistently upregulated in all the VISA isolates except the heteroresistant isolate. Pathway analysis revealed upregulation of peptidoglycan biosynthesis in VISA isolates. However, the distinct transcriptional profile of the heteroresistant isolate (with upregulation of *recR*, *ureC* and *atl*) suggests potential role of increased mutation due to SOS response or biofilm formation in this phenotype.

Keywords: Heteroresistance, Peptidoglycan

Staphylococcus aureus is a Gram-positive opportunistic pathogen responsible for infections ranging from minor skin and soft tissue infections to infective endocarditis, pneumonia, and osteomyelitis[1,2]. According to the World Health Organization (WHO) reports, *Staphylococcus aureus* is placed as a high priority pathogen (MRSA, VISA and VRSA)[3]. *S. aureus* uses various strategies to evade the host immunity as well as the antibiotics used for its eradication[4,5]. The stress response protective of the pathogen regulated by alternative sigma factor B, the alarmones ppGpp and pppGpp and others[6], cause specific differential expression of genes, such as, downregulation of genes functional in translation and upregulation of genes functional in amino acid transport[7]. This stress response acts as a protective barrier, and impacts antimicrobial susceptibility by inducing dormancy, activating antimicrobial efflux pumps or by promoting growth in biofilms[8]. This regulatory system is also known to give rise to increased resistance to β-lactam antibiotics by arresting the growth of the cells and inactivating antibiotic targets[9].

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Antimicrobial resistance (AMR), according to WHO, is one of the top ten public health threats[10]. After the emergence of Methicillin Resistant *Staphylococcus aureus* (MRSA), vancomycin became the last resort antibiotic for treatment of MRSA infections[11]. However, since 1997, non-susceptibility to vancomycin has been reported in vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous VISA (hVISA)[12]. Also, a strain with even lesser susceptibility to vancomycin than VISA, named vancomycin resistant *Staphylococcus aureus* (VRSA) was first reported in 2002 after the emergence of vancomycin resistant *Enterococcus* (VRE) in 1986[13-15]. CLSI guidelines has defined Minimum inhibitory concentration (MIC), i.e., the concentration of antibiotic at which there is no visible growth for different antibiotics used against *S. aureus*[16]. Three levels of vancomycin susceptibility are defined in increasing order of MIC of vancomycin, i.e., Vancomycin sensitive *Staphylococcus aureus* (VSSA) (MIC ≤2 µg/mL), vancomycin intermediate *S. aureus* (VISA) (MIC = 4-8 µg/mL) and vancomycin resistant *S. aureus* (VRSA) (MIC ≥16 µg/mL). Another lower susceptibility level of *S. aureus*, lower than VSSA but higher than VISA, heterogenous VISA (hVISA),

consists of a subpopulation which behaves like VISA and the MIC of the parent strain is in the VSSA range[17]. The molecular and genotypic mechanism for emergence of VISA and VRSA are often considered to be distinct. VISA owes its origin to diverse mutations in distinct genes all of which contribute to common phenotypes such as thickened cell walls, reduced autolysis, and decreased haemolytic activity[18]. VRSA, on the other hand, emerged due to the presence of mobile genetic elements i.e., van gene clusters, such as, *vanA* and *vanB* present on transposons in various plasmids[19]. These van genes modify the native D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser, thereby making them resistant to vancomycin[20]. Vancomycin resistance in *S. aureus* has been known to be derived from vancomycin resistant *Enterococci* (VRE) by Horizontal gene transfer (HGT)[11,21]. Although VISA and VRSA are mechanistically different, Wang *et al*.[22] have shown the conversion of VSSA to VRSA by continuous growth of VSSA strains in the presence of vancomycin for 60 days. However, this VRSA strain lacked the *vanA* gene cluster.

Earlier, Strugeon *et al*.[23], have shown that stringent stress response helps in dissemination of antimicrobial resistance. It is thought that stochastic gene expression in response to stress is responsible for cell-cell heterogeneity[24]. Such heterogeneity of resistance has also been explained by gene amplifications and by mutations in specific genes[25]. Also, this heteroresistance is unstable in nature, i.e., resistance is lost in the absence of antibiotic pressure[25,26]. This reversal is explained via deletion of or reduction in the copy number of amplified regions, or a compensatory mutation[25]. El-Halfawy & Valvano[27] have defined heteroresistance as the presence of a subpopulation of cells having an MIC of at least 8-fold higher than that of the susceptible population.

In the present study, we set out to test if *Staphylococcus aureus* undergoes any significant change in MIC when continuously grown in the presence of vancomycin. If so, we would consider multiple hypothesis for explaining the change. Further, from transcriptomic data, we propose a mechanistic model for emergence of vancomycin non-susceptibility during prolonged exposure of *S. aureus* to the antibiotic.

Materials and Methods

Bacterial strains and growth conditions

Eight clinical isolates of vancomycin sensitive *S. aureus* samples were cultured on Brain Heart Infusion agar (BHI) or Mueller Hinton Agar (MHA). Bacteria were routinely grown at 35±2°C for 15 h and culture was grown in BHI broth or Mueller Hinton Broth (MHB) and incubated at 35±2°C overnight at 220 rpm. All media were purchased from Himedia Laboratories. ATCC 700699[28] strain purchased from Himedia was used as a control for all experiments unless mentioned otherwise.

Antimicrobial susceptibility test (AST) by disk diffusion

Disk diffusion test was performed as described by Hudzicki[32] in 2009. Briefly, an overnight culture of *S. aureus* in Mueller Hinton broth (MHB) (Himedia) was adjusted to a turbidity corresponding to 0.5 McFarland Standard (Himedia). Using a sterile swab, the culture was streaked over the entire Mueller Hinton Agar (MHA) (Himedia) surface thrice by rotating the plate by approximately 60°. Antimicrobial disks (Cefoxitin, co-trimoxazole, gentamicin, linezolid, nafcillin, rifampicin, levofloxacin and amikacin) purchased from Himedia, were then placed on the agar surface. Plates were incubated at 35±2°C for 16-18 h. The zone of inhibition was measured using a ruler and interpreted according to CLSI guidelines[16].

Minimum inhibitory concentration (MIC) determination:

For determination of MIC of daptomycin, the culture was streaked on MHA plate and the daptomycin strip (Himedia) was placed on the plate and incubated. Result was interpreted according to CLSI guidelines[16]. MIC of vancomycin was determined following the protocol by Andrews in 2001[30]. Briefly, an overnight culture of *S. aureus* was diluted in Cation-adjusted MHB (CaMHB) up to 10^8 CFU/mL. The experiment was performed in a 96-well plate. The final culture concentration used was 10^5 CFU/mL. Vancomycin at a concentration of 64 µg/mL was serially diluted in the plate and culture was added and incubated for 16-18 h at 35±2°C and 220 rpm in a shaker. Results were observed by checking the lowest concentration at which there was no visible growth and interpreted according to CLSI guidelines (MIC of VSSA ≤2 µg/mL, MIC of VISA = 4-8 µg/mL and MIC of VRSA ≥16 µg/mL).

Bacterial lysate preparation

For PCR, bacterial lysate was prepared. About 250 µL of an overnight culture was centrifuged at 17,900 g for 2 min and the pellet resuspended in 500 µL autoclaved MilliQ. This step was repeated twice followed by heat shock at 94°C and transfer to ice for 10 min. It was

then centrifuged at 17,900 g for 10 min at 2°C and the supernatant collected and stored at –20°C.

PCR of *vanA* and *vanB* genes

Genes encoding *vanA* and *vanB*, were investigated by polymerase chain reaction (PCR) using the primers listed in Table 1[31,32]. It was performed in a 20 µL reaction mix, using the following steps: an initial denaturation step at 98°C for 2 min, followed by 35 cycles at 98°C for 10 s, 46°C for 1 min, and 72°C for 90 s, and a final extension step at 72°C for 10 min for *vanA* gene. For *vanB* gene, an initial denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed in 1.5% agarose gel by adding ethidium bromide (EtBr) and visualized in UV-transilluminator (Biorad).

Development of vancomycin non-susceptibility

The protocol described by Wang *et al.*[22], was used to develop vancomycin non-susceptibility *in vitro*. Briefly, 8 cultures of vancomycin sensitive (MIC = 2 µg/mL) *Staphylococcus aureus* (VSSA) were grown in the presence of vancomycin (Himedia) at sub-MIC value and the strains were passaged to fresh medium containing the same vancomycin concentration every 24 h. MIC was determined after every 4 days of treatment and the vancomycin concentration in the medium was increased accordingly. This was repeated every 4 days for 60 days. Thereafter, stability of VISA and VRSA was determined by passaging them in vancomycin-free plates every 24 h for 30 days (Fig. 1).

Table 1 —Primers used in the study for PCR and qRT-PCR

Gene	Primer sequence	Annealing Temp.
vanA	F:ATGAATAGAATAAAAGTTGC R:TCACCCCTTTAACGCTAATA	46°C[31]
vanB	F:GTGACAAACCGGAGGCGAGGA R:CCGCCATCCTCCTGCAAAAAA	50°C[31]
recF	F:GATGTGAATGGCATGGATGC R:TGGGATATTCCCCAACTTCG	50°C*
recR	F:GAACCCGAACTTAGAGGGGG R:TCGCCACCTACCGATAACCC	52°C*
ureB	F:GATCGACCAATTCAAGTGGG R:TCAAATCGAACAGCTGCTCC	60°C*
ureC	F:GTAGGATCTATCGAGCCGGG R:CATCGCCATTTACGGCAGAG	51°C*
vraS	F:GCTGTTACATTTAAGACCGC R:CATGCACAACTTTCATTGGC	60°C*
pbp2	F:ACTATGTCAGTGTGGATGGG R:GGACGTTTAAAGTCTTCGCC	50°C*
pbp4	F:GGAAGCTGTAAATAAGGGGC R:GCGATTGTCCATACTTGTCC	60°C*
pflA	F:ACTTTGATGCATCGGGTGGC R:ATCATTAGCACATCCAGCCG	51°C*
pflB	F:ACGTGACCTTAAAGCAGGCG R:GTTACCCAAGTTGGGTCTCC	51°C*
rsbW	F:CTTTTTACGCGAAGGTGGCC R:CGCCATTATTTCGCACCTGC	52°C*
tagA	F:CGTTTAAAGCAACCTCTAGCG R:TGCAATGCATATTGTGCCGC	51°C*
tagX	F:GATCCACATGATAGTGACGC R:GTAATTGTAAGCTCGCTGCC	50°C*
atl	F:TCAGCTGCTAAACCTGCAGC R:TCTTGGTTGTGCTGAAGCGC	60°C*
gyrA	F:CGTGAAGGTGACGAAGTTGTAGG R:TAACTGGCGTACGTTTACCATAACC	52°C[32]

*This study

Retrieval of transcriptomic datasets

National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO)[33] was searched with the query string "Bacterial stress response" or "VISA" and limited to the species *Staphylococcus aureus*. After screening of all the datasets, eight stress datasets and 5 VISA and 1 telavancin resistant dataset was included for analysis (Table 2)[34-46]. The telavancin resistant dataset was considered as VISA, because of the shared mode of action between telavancin and vancomycin.

Data analysis

For the gene-level analysis, gene annotation was downloaded from NCBI or Kyoto Encyclopedia of Genes and Genomes (KEGG)[47]. All gene IDs were mapped to the genome of *S. aureus* COL. Case and control groups were specified for all the studies and t-test was performed to obtain the differentially expressed genes (P <0.05).

For pathway analysis, pathway annotation was downloaded from KEGG for *S. aureus* COL. Pathway score was calculated using the following formula:

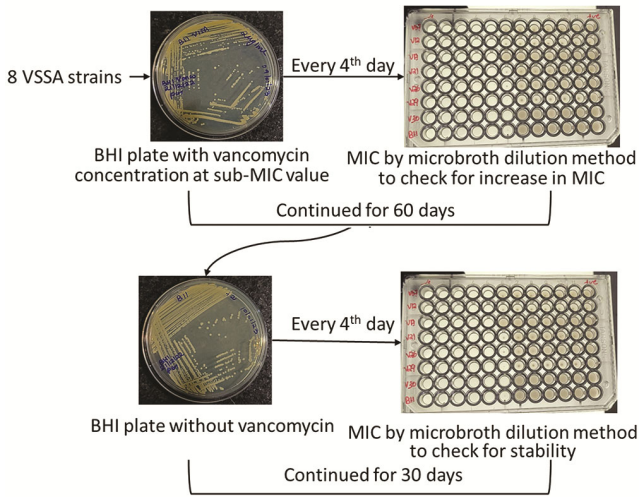

Fig. 1 — Method to develop *in vitro* vancomycin non-susceptibility.

Table 2 — GEO series IDs of datasets analysed with their conditions and no. of samples[61-73]

GEO series ID	Condition	No. of samples
GSE12920[34]	Stress (Temperature)	6
GSE22233[35]	Stress (pH)	6
GSE17391[36]	Stress (Ethanol)	4
GSE31554[36]	Stress (TTO)	4
GSE149013[37]	Stress (GOX)	5
GSE30743[38]	Stress (Mupirocin)	2
GSE9494[39]	Stress (Daptomycin, CCCP, Vancomycin & Nisin)	5
GSE37272[40]	Stress (Vancomycin, telavancin, enduracidin & CCCP)	9
GSE5047[41]	VISA	5
GSE43643[42]	VISA	4
GSE10529[43]	VISA	8
GSE5188[44]	VISA	6
GSE46887[45]	VISA	6
GSE40697[46]	Telavancin Resistant	3

$$Pathway\ score = \frac{\sum t - statistic}{\sqrt{Number\ of\ genes\ in\ the\ pathway}}$$

All analyses were performed using R version (4.1.0)[48].

The pathways were ranked by pathway score. The top up-regulated pathways were drawn using the pathway map from KEGG using the online tool[49].

Data availability

The R codes and metadata are available in figshare named VISA_NIBMG (https://doi.org/10.6084/m9.figshare. 22094861.v1)[50].

RNA isolation

RNA isolation was performed using Qiagen RNA protect bacteria reagent and RNeasy mini kit (Qiagen) as per the manufacturer's instructions with slight modifications. Briefly, overnight bacterial culture, diluted to contain approximately 2×10^8 CFU/mL, was added to RNA protect reagent and centrifuged. For lysis, 200 µL of 15 mg/mL lysozyme (Sigma) in TE buffer, 100 µL of 100 µg/mL lysostaphin (Sigma) in TE buffer and 20 µL of 20 mg/mL proteinase K (Qiagen) was mixed and the solution was added to the pellet, followed by 30 min incubation at 37°C with vortexing at 2 min interval. The remaining lysis protocol and the complete RNA isolation protocol was the same as specified by Qiagen. The concentration of RNA was determined using Nanodrop spectrophotometer (Thermo-Scientific).

cDNA synthesis and quantitative real time Polymerase chain reaction (qRT-PCR)

About 500 ng of total RNA was converted to cDNA in a reaction volume of 20 µL using iScript™ Advanced cDNA Synthesis Kit (BioRad). Real time PCR was performed on Biorad CFX96 Real-Time PCR system (BioRad) using iTaq™ Universal SYBR® Green Supermix (BioRad) in a volume of 10 µL. The gene expression level was normalized using *gyrA* gene as an internal control and plotted as relative normalized expression using CFX96 Maestro software (BioRad). All data were presented as mean expression with the SEM, from experiments performed in triplicate and from three biological replicates. Primers used for qRT-PCR are shown in Table 1.

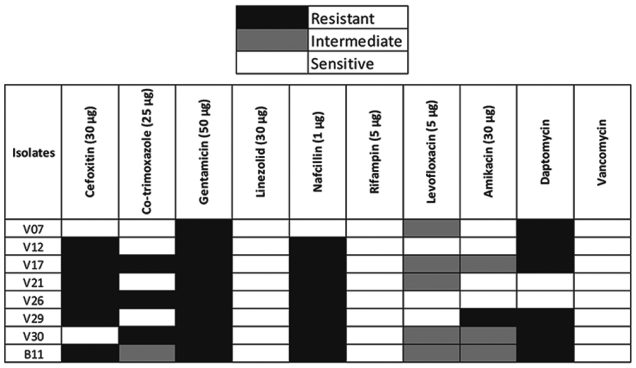

Fig. 2 — Antibiogram of the eight clinical isolates of *Staphylococcus aureus*.

Results and Discussion

Resistance profile of the 8 clinical isolates

Disc diffusion test was performed for cefoxitin (methicillin), co-trimoxazole, gentamicin, linezolid, nafcillin, rifampicin, levofloxacin, and amikacin. MIC of daptomycin and vancomycin was determined for all the 8 clinical isolates to obtain the antibiogram (Fig. 2).

Growing VSSA strains in the presence of vancomycin stress

Eight VSSA isolates were grown in the presence of vancomycin for 60 days and MIC was checked on every 4th day and data were obtained as shown in Table 3. On day 40, based on MIC, 4 (V26, V29, V30 and B11) out of the 8 isolates had changed status from VSSA to VISA. While V26, V29 and V30 showed an MIC of 4 µg/mL, B11 had an MIC of 8 µg/mL. On day 50, B11 became VRSA with an MIC of 16 µg/mL while the other 3 isolates remained VISA. Two out of these 3 isolates, V29 and V30 now had an increased MIC of 8 µg/mL. As the MIC of these isolates changed, they were immediately passaged on BHI media without vancomycin and the MIC was checked after every 4 days, continuously for 30 days. When these 4 isolates were grown in the absence of vancomycin, B11 (VRSA) immediately reverted to

Table 3 — MIC values of eight clinical strains of *S. aureus* after treatment with vancomycin for 60 days. The days shown in bold indicate the days on which MIC was checked.

Day	MIC of Samples (µg/mL)				Day	MIC of Samples (µg/mL)			
	V26	V29	V30	B11		V26	V29	V30	B11
1	2	2	2	2	31	2	2	2	2
2	2	2	2	2	32	2	2	2	2
3	2	2	2	2	33	2	2	2	2
4	2	2	2	2	34	2	2	2	2
5	2	2	2	2	**35**	2	2	2	2
6	2	2	2	2	36	2	2	2	2
7	2	2	2	2	37	2	2	2	2
8	2	2	2	2	38	2	2	2	2
9	2	2	2	2	39	2	2	2	2
10	2	2	2	2	**40**	4	4	4	8
11	2	2	2	2	41	4	4	4	8
12	2	2	2	2	42	4	4	4	8
13	2	2	2	2	43	4	4	4	8
14	2	2	2	2	44	4	4	4	8
15	2	2	2	2	**45**	4	4	4	8
16	2	2	2	2	46	4	4	4	8
17	2	2	2	2	47	4	4	4	8
18	2	2	2	2	48	4	4	4	8
19	2	2	2	2	49	4	4	4	8
20	2	2	2	2	**50**	4	8	8	16
21	2	2	2	2	51	4	8	8	16
22	2	2	2	2	52	4	8	8	16
23	2	2	2	2	53	4	8	8	16
24	2	2	2	2	54	4	8	8	16
25	2	2	2	2	**55**	4	8	8	16
26	2	2	2	2	56	4	8	8	16
27	2	2	2	2	57	4	8	8	16
28	2	2	2	2	58	4	8	8	16
29	2	2	2	2	59	4	8	8	16
30	2	2	2	2	**60**	4	8	8	16

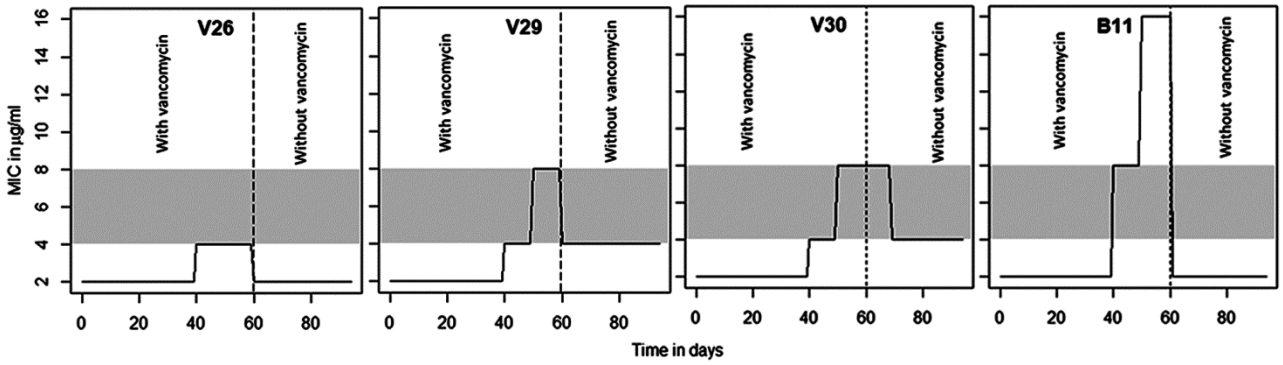

Fig. 3 — Vancomycin MIC profile obtained by growing 8 VSSA isolates in the presence of vancomycin for 60 days showed that 4 isolates (V26, V29, V30, B11) changed status from VSSA (MIC ≤2 µg/mL) to VISA (MIC = 4-8 µg/mL) on day 40. Out of these, 1 isolate (B11) became VRSA (MIC ≥16 µg/mL) on day 50. Upon removing vancomycin pressure, only two isolates (V29 and V30) remained VISA. [The shaded zone represents MIC of VISA according to CLSI guideline, the vertical dotted line denotes the time point when vancomycin was removed from the culture medium]

VSSA. V26 (VISA), as well reverted to VSSA. Only 2 isolates, V29 and V30, continued to be VISA (MIC = 4 µg/mL) even without the presence of vancomycin. Changes in MIC over a period of 60 days of vancomycin treatment followed by growth without any vancomycin for 30 days are shown in Fig. 3.

As already mentioned, the definition of heteroresistance is the presence of a subpopulation of cells having an MIC at least 8-fold higher than the antibiotic concentration at which the dominant population can grow. Since B11 showed growth with an MIC of 16 µg/mL that is 8-fold higher than the

MIC of the sensitive B11 cells (2 µg/mL), we considered B11 to be a heteroresistant isolate.

Selection of studies and analysis

We analyzed the change in gene expression levels in *S. aureus* strains exposed to various stresses, which included physical stress (pH or temperature) and antimicrobial agents (mupirocin, vancomycin, GOX). This was performed as it is already known that certain stress response regulators may lead to the development of resistance to antibiotics. Additionally, we analysed transcriptomes of VISA compared to VSSA, to identify genes differentially expressed in VISA. Eight VSSA stress and 6 VISA studies were retrieved from NCBI GEO. Analysis was performed to detect the differentially expressed genes (P <0.05). The number of genes differentially expressed (DEGs) was variable across the 14 studies. Of the DEGs, two genes (SACOL2282 and SACOL2286) were observed to be upregulated in acid and alkaline stress and 2 VISA studies (GSE43643 and GSE10529). These two genes code for *ureC* and *ureE*, respectively. One downregulated gene (SACOL0204) was common among mupirocin stress and two VISA studies (GSE10529 and GSE43643) while the eight down-regulated genes (SACOL0204, SACOL1244, SACOL0205, SACOL1535, SACOL1694, SACOL0522, SACOL0004, SACOL1280) were observed to be common in mupirocin stress and two VISA studies (GSE10529 and GSE43643). Mean log-fold change of specific genes functional in glycolysis, Krebs cycle, and gluconeogenesis are shown in Fig. 4.

Further, we performed pathway level analysis to measure the degree of modulation of pathways that might explain the changes associated with stress response and of vancomycin non-susceptibility. We observed 9 pathways (each with pathway score >6 in at least two studies). The mean pathway scores of the 9 selected pathways are shown in Fig. 5.

Thus, analysis of transcriptomic data from multiple studies of stress response and VISA reveals an overlap between stress and vancomycin resistance in *S. aureus*. Cell wall synthesis, and teichoic acid biosynthesis were uniformly upregulated in the isolates, both under stress and VISA compared to VSSA.

Based on the pathway scores, it is clear that peptidoglycan biosynthesis (sac00550) and glycolysis/ gluconeogenesis (sac00010) are the pathways that were observed to be uniformly upregulated in all datasets. But the other pathways did not show uniformity in up- or down-regulation. It is already

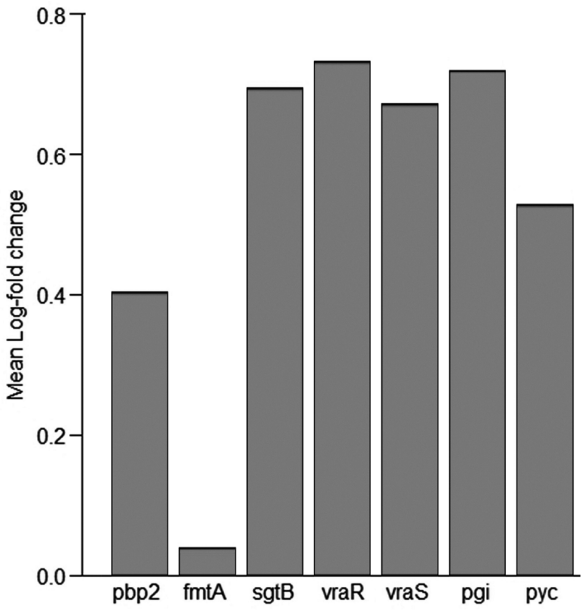

Fig. 4 — Mean log-fold change of genes functional in peptidoglycan biosynthesis, vancomycin resistance, glycolysis, and gluconeogenesis.

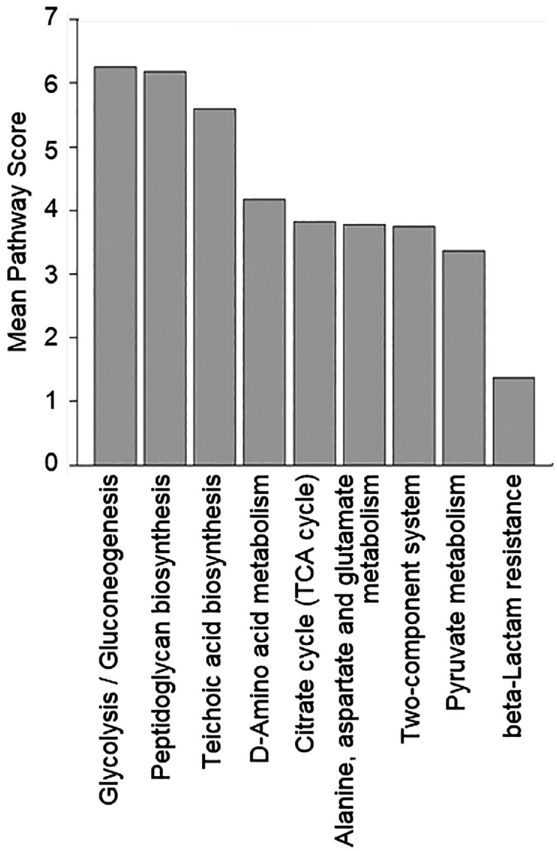

Fig. 5 — Mean pathway score of the nine selected pathways of all the datasets.

known that increase in peptidoglycan biosynthesis in VISA is essential for the bacterium to survive vancomycin treatment[51]. Two representative diagrams showing the upregulation of peptidoglycan biosynthesis are shown in Suppl. Fig. S1. (*All supplementary data are available only online along with the respective paper in IJEB at NIScPR repository at https://nopr. niscpr. res.in/handle/123456789/61124*).

Gene expression changes in response to vancomycin

To further understand the reason behind the stable VISA isolates and the transient resistance in one VISA and one VRSA isolate, we performed qRT-PCR of certain genes obtained from literature review and from the analysed datasets. V26 VISA (Sample at MIC = 4 µg/mL, Day 40), B11 VISA (Sample at MIC = 8 µg/mL, Day 40), and B11 VRSA (Sample at MIC = 16 µg/mL, Day 50) samples were collected from one time point and V29 and V30 VISA (Sample 1 at MIC = 4 µg/mL, Day 40 and sample 2 at MIC = 8 µg/mL, Day 50) samples were collected from two time points. An ATCC 700699 strain, which is *S. aureus* Mu50 VISA isolate, was also analyzed to understand if the VISA isolates showed similar results when compared to the ATCC strain. We observed that *pbp2* gene was upregulated in all the three VISA isolates obtained as well as in ATCC 700699 (supplementary Fig. 2 A-C). One of the genes which is usually upregulated in VISA, *vraS*, which is part of a two-component system and functions in sensing cell wall stress, did not show a consistent upregulation in all the VISA isolates. Similarly, *pbp4*, functional in increasing the cross-linking of peptidoglycan in the surface of the cell and *atl*, an autolysin gene, are observed to be downregulated in the VISA isolates but did not show consistent downregulation in all the studies. Teichoic acid biosynthesis genes *tagA* and *tagX* also did not show upregulation in all the VISA studies as shown. Formate acetyltransferase (*pflB*) gene was consistently downregulated in all the studies (Suppl. Fig. S2 A-C).

B11 isolate that first converted to VISA and then to VRSA showed completely different gene expression changes (Suppl. Fig. 2D). Both B11 VISA (MIC = 8 µg/mL) and VRSA (MIC = 16 µg/mL) showed upregulation of *ureC*, *rsbW* and *atl* genes. Also, upregulation of *atl* was observed, a major autolysis gene in *S. aureus*, which has been reported to mediate biofilm formation[52]. *rsbW* gene however was observed to be upregulated in not only B11 but also in the other VISA samples. Another gene upregulated in B11 VRSA was *recR*, a component of RecFOR homologous recombination pathway, functional in DNA repair[53]. Both, B11 VISA and B11 VRSA showed an overall different transcriptional response as compared to the other VISA isolates which primarily showed resistance by increasing cell wall synthesis. The summary of the results obtained is shown in Fig. 6.

Only some significant changes were observed when the gene expression of these VISA and VRSA samples were compared with that of ATCC 700699 which is a VISA isolate (Suppl. Fig. S2E). Most of the genes did not show any significant change.

AST of VISA and VRSA isolates to check their susceptibility to other commonly used antibiotics

We tried to find out if due to the presence of vancomycin, susceptibility of the isolates to other antibiotics is changing from what it was before antibiotic exposure. The same samples were used as for the qRT-PCR analysis. V30 VSSA isolate was susceptible to cefoxitin (methicillin) but after its conversion to VISA it became resistant to the antibiotic. Also, V30 and B11 VSSA showed intermediate resistance to levofloxacin but after conversion to V30 VISA and B11 VISA and VRSA, respectively, became resistant to levofloxacin. Similarly, resistance pattern changes were observed in response to amikacin as shown in the antibiogram in

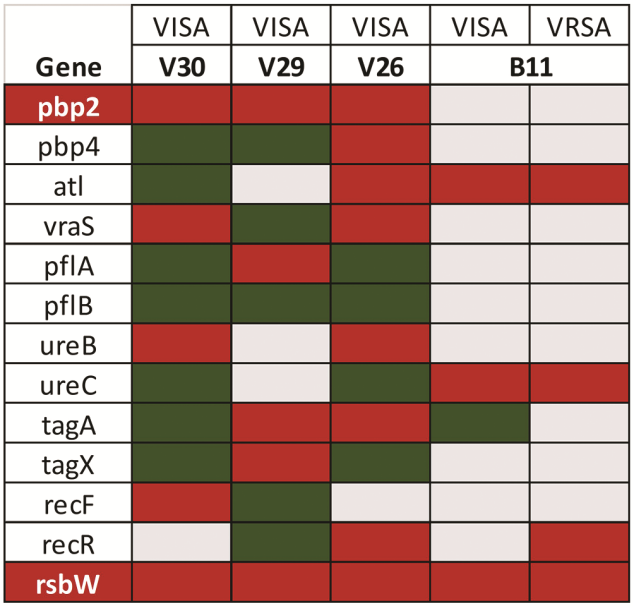

Fig. 6 — Upregulation of *pbp2* gene was observed in three of the VISAs. *rsbW* gene which is implicated in heteroresistance was observed to be upregulated in all of the samples. (Red, Upregulation; Green, Downregulation).

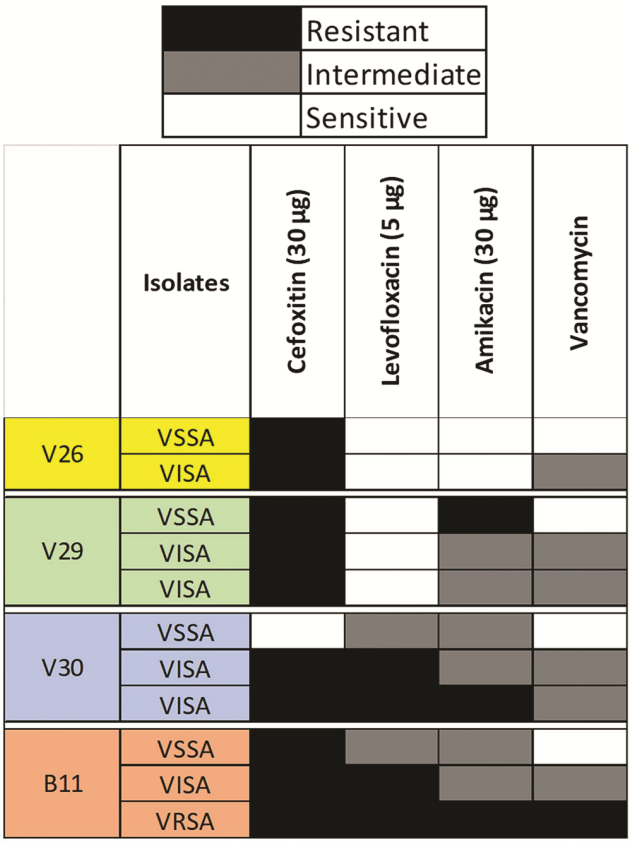

Fig. 7 — Antibiogram of the four *S. aureus* after conversion to VISA or VRSA.

Fig. 7. This is consistent with the result from the transcriptomic analysis where we observed beta-lactam resistance pathway to be upregulated in 10 of the 14 datasets.

Rapid loss of stability of resistance upon removal of vancomycin exposure suggests the existence of a transient transcriptional reprogramming in *S. aureus*: VSSA grown in the presence of vancomycin showed three main phenotypes: (i) a VISA phenotype which remained stable for two isolates (2 of the 8 isolates tested); (ii) a VISA phenotype which reverted to VSSA upon the removal of vancomycin (1 of 8); and (iii) a VRSA phenotype which rapidly reverted to VSSA upon the removal of vancomycin treatment (1 of 8) and which lacked the *vanA*, *vanB* gene cluster as was observed by Wang *et al.*[22]. This transient resistance pattern or this heterogenous behavior maybe explained in terms of transient changes in gene expression, as observed in response to stress[24]. To understand why such a change is occurring in the presence of vancomycin, we sought explanation by re-analyzing multiple *S. aureus* transcriptomic datasets. We observed consistent upregulation of pathways functional in cell wall thickening. This was further corroborated by the results of qRT-PCR that revealed increased expression of genes functional in cell wall synthesis (*pbp2*) in 3 out of 4 laboratory-derived VISA strains. This is consistent with the established notion of higher cell wall synthesis as the underlying mechanism of VISA.

In contrast, B11 VISA and VRSA samples did not show upregulation of *pbp2*. Rather, the VRSA strain showed differential expression of recombination related genes. One of the genes observed to be upregulated in the VRSA is *recR* which is functional in DNA repair. It is part of the RecFOR pathway and helps in binding of RecA to ssDNA[53]. This suggests, SOS response to stress[54], activating *RecR* and hence increasing mutagenesis making it resistant to increased levels of vancomycin. Upregulation of *rsbW* (the anti-sigma factor of *sigB*)[55] as observed in all samples would cause reduction in *sigB* levels, one of the alternative sigma factors functional in stress response[56]. Inactivation of the *rsbW* gene has been reported to lead to a switch in the heteroresistant phenotype to a homogenously methicillin resistant phenotype in *Staphylococcus epidermidis*[57]. Hence, upregulation of *rsbW* might indicate the heterogenous nature in the resistance pattern in the samples. The *atl* and *ureC* genes were also upregulated in both B11 VISA and VRSA. Only a few studies till now have focused on the *ureC* gene[58,59] and one of the interpretations from Sun *et al.*[59] study was that in *Proteus mirabalis*, *ureC* gene expression contributes to biofilm formation. But how this upregulation of *ureC* gene was contributing to the increased resistance was not investigated. Also, *atl* has been implicated to be involved in biofilm formation[52,59,60]. Biofilm-producing *S. aureus* show increased tendency to resist antimicrobials than biofilm nonproducers, as has been reported earlier[61,62]. Hence, increased biofilm formation might also be the reason behind the increased resistance observed in B11.

We hypothesize that due to an increase in cell wall synthesis due to the cell wall stress (vancomycin), in most of the VISA strains, there is an increased demand for N-acetyl glucosamine (NAG), and N-acetyl muramic acid (NAM). Both can come from the glycolytic intermediate fructose-6 phosphate. Also, the Krebs cycle intermediate oxaloacetate can be converted to fructose-6-phosphate via gluconeogenesis. Hence, we checked the differential

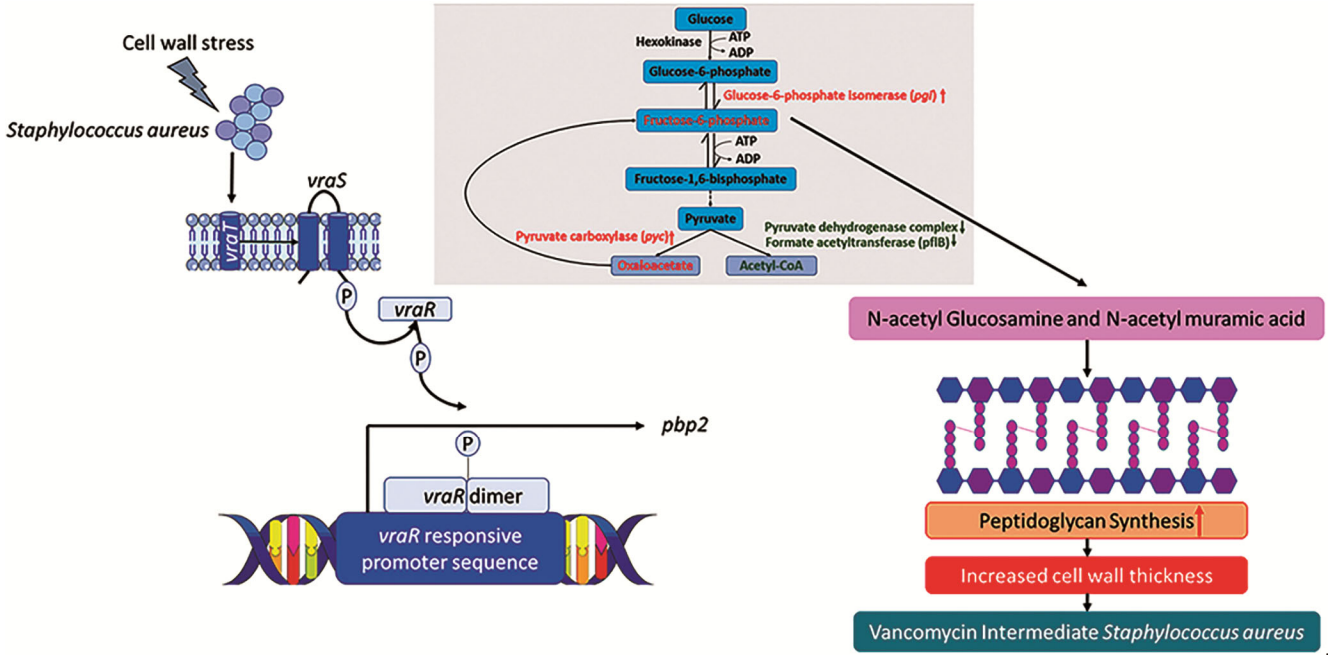

Fig. 8 — Model showing how cell wall stress can lead to increase in peptidoglycan biosynthesis and hence increased cell wall thickness leading to VISA phenotype. [vraTSR three-component system senses any cell wall stress and upregulates the expression of peptidoglycan biosynthesis genes like *pbp2*. The increased peptidoglycan synthesis increases the demand for NAG and NAM, which is fulfilled by metabolic reprogramming]

expression of the genes *pgi* (SACOL0966), and *pyc* (SACOL1123), which are functional in glycolysis and gluconeogenesis. Both *pgi* and *pyc* were upregulated in some stress studies and in three of the six VISA studies (Fig. 4). Genes *pdhA*, *pdhB*, *pdhC* and *pdhD*, members of the pyruvate dehydrogenase complex, were downregulated (data not shown) in most of the studies showing that less acetyl CoA is available to enter Krebs cycle, hence making oxaloacetate available for synthesis of NAG and NAM. Also, by qRT-PCR analysis we observed downregulation of *pflB* gene which converts pyruvate to acetyl CoA and formate[63], which suggests decreased formation of acetyl CoA and increasing availability of metabolites for NAG and NAM synthesis. Based on the level of modulation of gene expression, and pathway score, we propose a model of emergence of vancomycin non-susceptibility in VISA (Fig. 8). The model suggests that when there is some cell wall stress like when *S. aureus* is exposed to vancomycin for a prolonged period, the three-component system vraTSR participates in the upregulation of the gene *pbp2* functional in peptidoglycan synthesis. VraT helps in sensing the stress and promoting auto-phosphorylation of VraS. VraS in turn phosphorylates the response regulator VraR[64-66]. VraR activates or represses transcription of various genes functional in cell wall synthesis[67]. As mentioned, due to an increased demand for cell wall synthesis and hence of N-acetyl glucosamine (NAG), and N-acetyl muramic acid (NAM), there is increased synthesis of fructose-6-phosphate which helps in the formation of NAG and NAM[68].

It was observed that the resistance profile of the samples changed after their conversion to VISA or VRSA. They became resistant to some of the antibiotics to which they were previously sensitive. This indicates that vancomycin treatment for a prolonged period in *S. aureus* can lead not only to reduced susceptibility to vancomycin but also to other antimicrobials.

Conclusion

The VISA evolves from the mutations while VRSA emerges via the horizontal gene transfer (HGT) of mobile genetic elements (*vanA*, *vanB* gene clusters). Our present observation of the evolution pattern of VISA and VRSA, revealed three major phenotypes: One VISA which reverted to VSSA upon vancomycin removal, two stable VISAs and one heteroresistant sample which became VRSA in the course of treatment and reverted when grown without vancomycin. Upregulation of *pbp2* gene involved in transglycosylation reaction during peptidoglycan

synthesis was observed in all VISA except the heteroresistant isolate (B11 VISA). Differential expression of some metabolic genes functional in cell wall synthesis was observed in the VISA isolates (except B11). Therefore, vancomycin stress leads to VISA through transcriptional and metabolic reprogramming in at least 3 of the 4 VISA strains. B11 VISA was observed to be different from other VISA, with no significant difference in pbp2 or metabolic gene expression. Also, the B11 VRSA strain was not associated with *vanA* and *vanB* genes nor with *pbp2* or those metabolic gene expression. B11 VRSA is heteroresistant and most likely caused by transcriptional reprogramming.

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Conflict of interest

Authors declare no competing interests.

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