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Complete List of Authors:	Pando, Jasmine M.; New Mexico State University, Biology Pfeltz, Richard F.; BD Diagnostics Cuaron, Jesus A.; New Mexico State University, Biology Nagarajan, Vijayaraj; University of Southern Mississippi, Biological Sciences Mishra, Mukti N.; Oklahoma State University, Biochemistry and Molecular Biology Torres, Nathaniel J.; Oklahoma State University, Biochemistry and Molecular Biology Elasri, Mohamed O.; University of Southern Mississippi, Biological Sciences Wilkinson, Brian J.; Illinois State University, Biological Sciences Gustafson, John E.; Oklahoma State University, Biochemistry and Molecular Biology
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Ethanol-induced stress response of *Staphylococcus aureus*.

Jasmine M. Pando¹, Richard F. Pfeldt², Jesus A. Cuaron¹, Vijayaraj Nagarajan⁴, Mukti N. Mishra⁵, Nathaniel J. Torres⁵, Mohamed O. Elasri⁴, Brian J. Wilkinson³, and John E. Gustafson^{1,5*}

¹Department of Biology, New Mexico State University, Las Cruces, NM.

²BD Diagnostic Systems, Microbiology R&D Department, Sparks, MD.

³Microbiology Group, School of Biological Sciences, Illinois State University, Normal, IL,

⁴Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS.

⁵Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK.

*Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Oklahoma State University. Phone: (405)-744-6189. Fax: (405)-744-7799. Email: john.gustafson@okstate.edu.

Abstract

Transcriptional profiles of two unrelated clinical methicillin-resistant *S. aureus* (MRSA) isolates were analyzed following 10 % v/v ethanol challenge (15 min), which arrested growth but did not reduce viability. Ethanol-induced stress (EIS) resulted in differential gene expression of 1091 genes, 600 common to both strains, of which 291 were up-regulated. With the exception of the down-regulation of genes involved with osmotic stress functions, EIS resulted in the up-regulation of genes that contribute to stress response networks, notably those altered by oxidative stress, protein quality control in general, and heat shock in particular. In addition, genes involved with transcription, translation and nucleotide biosynthesis were down-regulated. *relP*, which encodes a small alarmone synthetase (RelP), was highly up-regulated in both MRSA strains following ethanol challenge and *relP* inactivation experiments indicated that this gene contributed to EIS growth arrest. A number of persistence-associated genes were also up-regulated during EIS, including those that encode toxin-antitoxin systems. Overall, transcriptional profiling indicated that the MRSA investigated responded to EIS by entering a state of dormancy and altering the expression of elements from cross protective stress response systems in an effort to protect preexisting proteins.

Key words: *Staphylococcus aureus*; ethanol; stress response; stringent response; growth inhibition.

Introduction

Staphylococcus aureus expresses an extensive suite of virulence factors, causes a broad range of significant diseases, and is a leading cause of healthcare-associated infections (HAI) (Morell and Balkin, 2010; Tang and Stratton, 2010). Methicillin-resistant *S. aureus* (MRSA) is of particular concern due to its propensity to become resistant to multiple antibiotics (Morell and Balkin, 2010; Tang and Stratton, 2010). Although viable MRSA can be recovered from disinfected hospital room surfaces, it is the carriage of MRSA amongst patients and on the hands of health care providers that has been a primary focus of HAI control efforts for several decades (Huang et al. 2006; McLaws 2015; Siegel et al. 2007). Hand hygiene is considered the single most effective approach to controlling the spread of infectious agents including MRSA within healthcare environments. Hand hygiene encompasses both hand-washing with plain or antiseptic soap, as well as the use of alcohol-based hand rubs (ABHR). ABHR use reduces both MRSA transmission within health care facilities and MRSA HAI, and ABHR are routinely employed as a convenient alternative to hand washing in the community (Gordin et al. 2005; Siegel et al. 2007; Todd et al. 2010; World Health Organization 2009). ABHR also conveniently evaporate soon after application without requiring towels for drying hands. However, the volatility of alcohols can also allow for the subsequent re-growth of surviving microorganisms on the skin following recovery from alcohol-induced sub-lethal injury (Lilly et al. 1979). The bactericidal activity of alcohols is attributed to a rapid denaturation of protein and membrane structures, which subsequently leads to the loss of the membrane barrier, loss of the proton motive force

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and transport functions, and the inhibition of protein synthesis and cell division (Chatterjee et al. 2006; McDonnell and Russell 1999).

A number of studies have investigated the effects of low ethanol concentrations on *S. aureus*. Although 0.1 % (v/v) ethanol does not affect *S. aureus* growth rate, it does significantly delay post-stationary phase recovery and return to active growth, as well as the transition from primary to secondary metabolite catabolism, even after the ethanol had been completely depleted from the growth medium (Chatterjee et al. 2006). Ethanol at concentrations as low as 0.03 % v/v can also induce the expression of *S. aureus* heat shock proteins (Qoronfleh et al. 1998). Korem et al. (2010) reported gene expression changes in *S. aureus* following 24 hr growth in agar medium with 3 % v/v ethanol that included the up-regulation of oxidative stress responses, secreted and surface virulence factors, and biofilm production capability.

Growth cessation is the common feature of survival strategies employed by microorganisms to remain viable during nutrient starvation and other inhospitable environmental conditions. These strategies, grouped under the term dormancy, are associated with non-replicative, reversible physiologic states that arise without need for genetic alterations, have interrelated mechanisms, and include persistence, biofilms, viable but non-culturable (VBNC) cells, antimicrobial and/or stress tolerance, and the stringent response (Ayrapetyan et al. 2015; Wood et al. 2013).

The stringent response is initiated by the synthesis of nucleotide guanosine penta and tetraphosphates ((p)ppGpp), referred to as alarmones, in response to limited nutrient availability, particularly amino acids. Alarmone accumulation results in a dramatic down-regulation of genes involved with protein and nucleotide biosynthesis

and many transport functions, while up-regulating amino acid biosynthesis and uptake systems genes, as well as select virulence determinants (Dalebroux et al., 2010). In most bacteria, the alarmone concentration is primarily determined by the activities of RelA, a bifunctional (p)ppGpp synthetase/hydrolase. Firmicutes also possess genes for two small alarmone synthetases (SAS), designated RelP and RelQ in *S. aureus*, which have weaker synthetase activities and lack the N-terminal hydrolase and C-terminal ribosome-interacting sensory domains. *S. aureus* SASs are induced by alkaline or oxidative stress conditions, and by cell wall-active antimicrobials such as vancomycin or ampicillin (Gaca et al. 2015; Kriel et al. 2012).

The stringent response is also involved with a wide range of stress responses and dormancy or persister states (Cohen et al. 2013; Gaca et al. 2015). Other stress systems besides the stringent response that support persistence include the SOS-, heat-, and oxidative stress- response, as well as toxin-antitoxin (TA) modules (reviewed by Zhang 2014). A common objective of all of these stress response mechanisms is the protection and repair of key cellular components such as proteins and nucleic acids (Cohen et al. 2013; Gaca et al. 2015; Zhang et al., 2014).

Earlier studies with *S. aureus* and ethanol exposure focused on the organism's adaptation to and subsequent growth in the presence of sub-inhibitory ethanol levels. The purpose of this study was to understand the basis of the survival of this important pathogen during an ethanol treatment capable of inducing bacteriostasis, information whose absence is surprising given the importance of ABHR to HAI control.

Materials and Methods

S. aureus strains and ethanol susceptibility and viability assays

Unless otherwise noted all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO) and growth media were purchased from Becton, Dickinson and Company (Sparks, MD). Ethanol used for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations (AAPER Alcohol and Chemical Co., Shelbyville, KY) was first filter-sterilized with a 0.22 μm nylon membrane.

MICs and MBCs to ethanol were determined with 1 % v/v increments in tightly-capped tubes as described by Davis et al. (2005) for a panel of 106 well-characterized clinical MRSA (O'Brien et al. 2005, Delgado et al. 2007). Two unrelated strains representing different Smal-PFGE profiles, LP9 and MM66 (Delgado et al. 2007, Matyi et al. 2014), were selected from this panel and utilized for the ethanol challenge studies and transcriptional profiling experiments.

To determine the effects of ethanol on LP9 and MM66 viability, 1 % v/v inocula from overnight MHB cultures were added to fresh MHB and grown to an OD_{625} of 0.5 at 37°C with shaking at 200 rpm. Ethanol was then added to a final concentration of 10 % v/v and at subsequent time points the OD_{625} was recorded, each culture was diluted in fresh MHB, and aliquots of each dilution were plated onto MHA. CFU/mL values for each dilution were calculated based on colony counts following overnight growth at 37°C.

Microarray and qRT-PCR analysis

S. aureus DNA microarrays (version 6) were used for transcriptional profiling of LP9 and MM66 (MHB cultures $OD_{580} = 0.5$, 37°C) following 10 % ethanol challenge for 15 min using protocols provided by the Pathogen Functional Genomics Resource Center (PFGRC) (<http://pfgrc.tigr.org>) as described in Muthaiyan et al. 2008. Duplicate microarrays for LP9 and MM66 were hybridized and analyzed, and ORFs altered 2.0-fold and above were determined to be significant ($P < 0.05$). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO series accession number GSE17391 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17391>). An ethanol challenge time of 15 min was selected to ensure uniform *S. aureus* populations prior to transcriptional profiling and minimize intra-experiment variation.

ORF IDs were mapped to the N315 genome when possible, otherwise they were from the six *S. aureus* genomes on the microarray. Gene functional categories were tailored to facilitate the analysis of this study's results, including segregation of stress-responsive genes into a distinct category, identifying growth arrest-specific genes, placing transcriptional regulators in function-specific categories, and dividing several functional categories into sub-categories.

Primers listed in Table 1 were employed to validate by quantitative real-time PCR (qRT-PCR), as previously described (Riordan et al. 2007), selected genes from the

microarray studies whose expression was altered in response to 10 % v/v ethanol (Table 2).

Staphylococcus aureus Transcriptome Meta-Database (SATMD) (Nagarajan and Elasri 2007) was used to compare the LP9 and MM66 ethanol gene expression profiles to 250 *S. aureus* transcriptional profile datasets publicly available when SATMD was accessed, of which 158 profiles were from environmental factor studies and 92 from regulatory gene mutants.

Construction and characterization of *relP* mutants

relP deletion mutants of strain JE2 were produced utilizing pKOR technology (Bae and Schneewind 2006). Briefly, upstream (1006 bp) and downstream (991 bp) flanking regions of *relP* were amplified using the attB1-*relP*-up:F and *relP*-up:R:SacII, and *relP*-down:F:SacII and attB1-*relP*-down:R primer sets, respectively (Table 1). The amplicons produced were then digested with SacII, ligated together and cloned into pKOR1 utilizing the BP clonase system (Invitrogen) to produce plasmid pKOR1- Δ *relP*. *S. aureus* strain JE2 was electroporated with pKOR1- Δ *relP*, and the *relP* gene was then deleted following the protocol previously described (Bae and Schneewind 2006) producing strain JE2 Δ *relP*. The deletion of *relP* was confirmed by amplification of the genomic DNA of JE2 and JE2 Δ *relP* utilizing *relP* specific primers (Table 1). JE2 genomic DNA produced an amplicon of 700 bp, while JE2 Δ *relP* DNA did not produce any amplicon, confirming *relP* deletion in this mutant. To complement this mutant the *relP* gene was first amplified with its native promoter using the primers *relP*:F:KpnI and

relP:R:XbaI (Table 1) and cloned into KpnI/XbaI digested pLI50 (Lee et al. 1991) to produce pLI50::*relP*. This plasmid was then electroporated in JE2 Δ *relP* to produce strain JE2 Δ *relP*-pLI50::*relP*. Strains JE2, JE2 Δ *relP*, JE2 Δ *relP*-pLI50::*relP* and appropriate pLI50 controls were characterized in an ethanol growth inhibition assay in triplicate. Briefly, overnight MHB cultures (37°C with shaking at 200 rpm) were used to inoculate 20 mL of fresh MHB to an OD_{580nm} of 0.05. After subsequent growth to an OD_{580nm} of ~ 0.30, the 20 mL cultures were equally divided between two 50 mL flasks, of which one culture was challenged with the addition of 9 % v/v ethanol. Growth was then monitored by measuring OD_{580nm} at various time intervals.

Results and Discussion

MRSA susceptibility to ethanol

Ethanol MICs of the 106 clinical MRSA ranged from 6 % to 11 % v/v and the MBCs ranged from 8 % to 15 %, with 9 % and 13 % as the most common MIC and MBC values, respectively (Fig. 1, Table S1¹). Since 94 % of the MRSA demonstrated an MIC below 10 % v/v ethanol and 92 % of the MRSA had an MBC above this concentration, 10 % v/v ethanol was chosen as the test condition to study the ethanol-induced stress (EIS) response. Based on their high and low ethanol susceptibilities, strains LP9 and MM66 were selected for microarray analysis. MRSA MM66 was more ethanol-sensitive than most of the MRSA tested, with an MIC of 7 % and MBC of 10 % v/v ethanol. MRSA LP9, conversely, was more ethanol-tolerant than most of the 106 clinical MRSA, with an MIC of 9 % and MBC of 15 % v/v ethanol.

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The addition of 10 % v/v ethanol to MM66 and LP9 cultures halted the growth of both strains (Fig. S1¹), however the number of cells from both strains surviving at 15 min post-ethanol addition (4.3×10^7 CFU/mL for MM66 and 4.1×10^7 CFU/mL for LP9) was not appreciably reduced compared to time 0 (6.0×10^7 CFU/mL for MM66 and 4.8×10^7 CFU/mL for LP9). In fact, both strains survived quite well up to three hr in 10% v/v ethanol (3.5×10^7 CFU/mL for MM66 and 6.6×10^7 CFU/mL for LP9) (Fig. S1¹). These results demonstrated that RNA isolated from cultures after a 15 min exposure to 10 % v/v ethanol for microarray and qRT-PCR studies were from cells that were viable, but not dividing. We define the transcriptional response we are describing as the *S. aureus* ethanol-induced stress (EIS) response.

The *S. aureus* EIS transcriptional profile

The differences in the EIS transcriptional response of MM66 and LP9 are attributed to the strains unique genetic background (Delgado et al. 2007) and different ethanol susceptibilities (this study). MM66 also has a *graS* mutation which affects the activity of the two component GraRS system and supports the hetero-vancomycin-intermediate phenotype expressed by this strain (Matyi et al. 2014).

Gene expression changes induced during EIS are listed in Table S2¹. A total of 1691 expression changes occurred between strains LP9 and MM66, involving a total of 1091 genes (Table S3¹). Three up-regulated genes altered by 10 % ethanol were confirmed by qRT-PCR in both MM66 and LP9 (Table 2). Six hundred of the 1091 genes were differentially regulated in both strains (Fig. 2A), of which 291 were up-

regulated (Fig. 2B) and 307 were down-regulated (Fig. 2C) in both strains. All but two (SA0012 and SA0922) of the 600 genes common to both strain data sets were differentially regulated in the same direction in both strains (Tables S2¹).

All expression changes in both strains challenged with ethanol are presented graphically by gene functional category in Figure 3. The overall ratio of increased to decreased gene expressions of all 1091 altered genes was 1:1.1 (Table S3¹), and genes in the central metabolism and growth arrest and virulence functional categories were evenly distributed between up- and down-regulation (Fig. 3, Table S3¹). Overall, genes in the DNA replication, recombination and repair, hypothetical proteins/unknown function, and stress response functional categories were predominantly up-regulated (Fig. 3, Table S3¹). In contrast, genes in the amino acid and protein metabolism, cell envelope metabolism and resistances, nucleotide metabolism, transcription, cofactors and secondary metabolites and especially transport functional categories were predominantly down-regulated (Fig. 3, Table S3¹). Ethanol challenge induced an expression change of ≥ 15 -fold in 33 genes in LP9 and/or MM66 (Table 3). One of these genes (SA2308) was only up-regulated in LP9 and another (*cwrA*) was only up-regulated in MM66 (Table 3).

During EIS, there was almost a 2:1 ratio favoring up-regulation for genes in the hypothetical protein and unknown function categories (Fig. 3), also reported by Anderson et al. (2006) for heat shock. These categories accounted for 34.2 % of the total EIS transcriptional profile (Table S3¹), typical of the 30-40 % expected from genomic and transcriptomic studies (Prosser et al. 2014).

The 97 genes assigned to the ad hoc stress response functional category were segregated into six sub-categories (Fig. 4, Table S3¹). Differential gene expression was equally divided between up- and down-regulation for the 14 cold shock sub-category genes (Fig. 4, Table S3¹). In contrast, genes involved with stress responses to heat shock, oxidative shock, and those assigned to the general stress response and other stress response sub-categories were predominantly up-regulated (Fig. 4, Table S3¹). Included in the general stress response sub-category were genes encoding protein quality control functions and global transcriptional regulators with broad functions well beyond heat shock, such as those of the *c/pC* operon (Graham et al. 2013), as well as genes induced by multiple stresses that encode proteins of unknown function such as *asp23* (Campbell et al. 2012; Senn et al., 2005; Shrihari and Singh 2012).

Heat shock activates protein quality control functions that protect protein integrity and functionality by means of protein chaperones which mediate proper folding of nascent proteins and assist proteases that degrade accumulated or improperly folded proteins (Anderson et al. 2006, Graham et al. 2013). The heat shock sub-category contained nine genes, all up-regulated in ethanol challenged LP9 and MM66 (Fig. 4), for an up-regulation to down-regulation ratio of 18:0, the most lopsided of any gene categorization examined.

Oxidative stress response mechanisms are often credited as an underlying mechanism leading to antimicrobial tolerance during growth-arrested conditions such as stringency and persistence (Geiger et al. 2014; Zhang 2014). Genes of the oxidative stress category (Fig. 4, Table S3¹) are predominantly up-regulated during the EIS response. Oxidative stress response and DNA replication, recombination and repair

genes had a 1.8 - 2:1 ratio of up- to down-regulation during EIS (Fig. 3 and 4, Table S3¹). These two responses are interrelated, as strong oxidative stress can trigger the SOS response (Chang et al. 2006, Gaupp et al. 2012). The SOS response protects against the effects of DNA damage via DNA recombination and repair systems. In contrast with these overall increases in the expression of stress response genes, the genes involved with osmotic stress displayed a ratio of 8:1 in favor of down-regulation (Fig. 4).

As evidence of a general stress response activation by ethanol challenge, some of the highest up-regulated genes include eight characterized genes involved with stress responses, listed in Table 3, including genes involved directly with the heat shock (*grpE*, *groES*) (Campbell et al. 2012) and stringent responses (*relP*) (Geiger et al. 2014). The remaining five include *clpB*, which encodes a molecular chaperone (Graham et al. 2013); and the entire *clpC* operon which consists of *ctsR* (encodes a negative regulator of the *clpC* operon and other stress response genes), *mcsA* (encodes a redox sensitive activator of the CtsR activity modulator McsB) and *mcsB* (encodes a protein required for various stress responses), which are involved with the regulation of the *clpC* operon. *clpC*, which encodes the ATPase subunit of the ClpC protease required for stress resistance (Elsholz et al. 2011; Wozniak et al. 2012) (Table 3), and four genes involved with protein fate were also up-regulated in both strains when challenged with ethanol (Table 3).

While genes in the amino acid metabolism and the protein fate sub-categories were generally up-regulated (Fig. 4), the 88 genes encoding protein synthesis functions were differentially regulated by a ratio of 8:1 in favor of down-regulation (Fig. 4, Table

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S3¹). Forty-nine genes encoding ribosomal proteins for instance yielded a total of 89 expression changes, 86 of which were down-regulations and only 3 were up-regulations (Table S4¹). Consistent with the dramatic down-regulation of protein synthesis machinery, the *rpoA* (Table 3), *rpoB*, *rpoC*, and *rpoE* genes that encode components of the core RNA polymerase, and the *rpoD* gene encoding the primary sigma factor SigA that directs transcription initiation in actively growing cells (Morikawa et al. 2012), were all down-regulated during EIS (Table S2¹). Collectively, this demonstrates that under EIS conditions, synthesis of mRNA and protein are down-regulated and factors involved with maintaining existing protein functions are up-regulated.

Genes assigned to the stringent response sub-categories were generally down-regulated (Fig. 4), and genes encoding all three *S. aureus* alarmone synthetases were differentially regulated in one or both of the strains investigated following ethanol challenge. The bifunctional GTP synthetase/hydrolase-encoding *relA* was down-regulated three-fold in LP9 (Table S2¹), the SAS-encoding *relQ* was up-regulated two-fold in MM66 (Table S2¹), and the gene encoding the SAS RelP was highly up-regulated in both LP9 and MM66 (Table 3).

A considerable amount of evidence links both RelP and RelQ to cell envelope stress responses (Geiger et al. 2014). Both are highly up-regulated in response to cell wall-active antimicrobials and the deletion of *relP* and *relQ* leads to diminished survival rates when *S. aureus* is challenged with these drugs (Gaca et al. 2015). The magnitude of *relP* induction in this study prompted an examination of the effect of *relP* inactivation on *S. aureus* survival after an ethanol challenge. In unchallenged MHB cultures, JE2, JE2Δ*relP*, JE2Δ*relP*-pLI50::*relP* and the appropriate pLI50 control strains all grew at an

identical rate for up to 12 hr (data not shown). Eight hr after the addition of 9 % v/v ethanol, the JE2 Δ *relP* and JE2 Δ *relP*-pLI50 cultures grew faster than those of the other strains (Fig. 5). Upon complementation of the *relP* deletion in trans, JE2 Δ *relP*-pLI50::*relP* then grew more slowly than either JE2 or JE2-pLI50 (Fig. 5). A reduced growth rate was observed for JE2 Δ *relP*-pLI50::*relP* compared to the parent strain that is likely associated with a plasmid copy number effect. These results suggest that RelP plays a role in curtailing the active growth state when cells are challenged with ethanol under the conditions of this study.

Of the ten genes assigned to the persistence sub-category (Fig. 4), only the menaquinone pathway gene *menA* that has been linked to persistence in *Mycobacterium tuberculosis* (Zhang et al. 2012) was down-regulated in MM66 (Table S2¹). Up-regulated in both LP9 and MM66 were persistence-associated genes encoding an inorganic phosphate transporter component (*pstB*), trans-translation element 30S ribosomal protein S1 (*rpsA*), and a homolog of a GntR family transcriptional regulator up-regulated during *M. tuberculosis* persistence (SA1748) (Donegan et al. 2014; Overton et al. 2011; Zhang et al. 2012) (Table S2¹). Five of the genes assigned to the persistence sub-category encode components of three *S. aureus* TA modules. *yoeB-sa1* and *yefM-sa1* which encode the YefM-YoeB-sa1 TA module were both up-regulated in LP9 and MM66, *yoeB-sa2* and *yefM-sa2* which encode the YefM-YoeB-sa2 TA module were up-regulated in MM66, and *mazF* which encodes the MazEF TA-module toxin was also up-regulated only in MM66 (Larson and Hergenrother 2014; Schuster et al. 2015) (Table S2¹). TA modules are up-regulated in response to treatment with protein synthesis inhibitors (Reiss et al. 2012; Donegan et al. 2010) or

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heat shock (Anderson et al. 2006), and Schuster et al. (2015) reported that *mazEF* participates in tolerance to cell wall active antibiotics.

The 43 genes assigned to the virulence functional category had an up- to down-regulation ratio of 1:1 (Fig. 3, Table S2¹). Some two-thirds of the genes with reduced expression in the virulence factor cell surface sub-category (Fig. 4, Table S2¹) were attributable to genes encoding capsule biosynthesis functions for strain MM66. Furthermore, a number of genes encoding virulence factor gene regulators (Bronner et al. 2004; Chueng et al., 2004; Flannagan and Clewell, 2002; Meier et al., 2007) were down-regulated in either LP9 (*sarS*, *spoVG*, and *arlR*) or MM66 (*saeR*, *camS*, *agrA*, *icaR* and *agrD*) or both strains (*agrC*) following ethanol challenge (Table S2¹), indicative of cell surface alterations during EIS.

Discounting capsule biosynthetic genes down-regulated in MM66, genes encoding secreted virulence factors were predominantly up-regulated by EIS. It has been widely reported that environmental stress responses such as the stringent response (Reiss et al. 2012), heat shock (Anderson et al. 2006), cell wall active antimicrobials (Song et al. 2012), and sub-inhibitory ethanol concentration (Korem et al. 2010) also up-regulate virulence. Anderson et al. (2006) postulated that the up-regulation of extracellular virulence determinants in response to stress allows distressed cells to lay the groundwork to resume growth by degrading host tissues so nutrients would be available when environmental conditions were once again hospitable. This explanation is consistent with the up-regulation of secreted virulence factors by LP9 and MM66 challenged with ethanol at a time of compromised membrane function and minimal metabolic activity.

Comparison of the EIS transcriptional profile to other *S. aureus* stress response profiles

SATMD transcriptional profiles were interrogated with respect to the 291 up-regulated genes common to both strains during EIS in an effort to identify transcriptional responses similar to that of EIS. Four growth-arrested transcriptional profiles had at least 40 % of their up-regulated genes in common with the 291 EIS up-regulated genes. The top up-regulated high-overlap comparator transcriptional profiles were: heat shock at 43°C for 10 min (25 genes in common, Fleury et al., 2009); the lipoglycopeptide enduracidin at 10 X MIC for 60 min (96 genes in common, Song et al., 2012); vancomycin at 10 X MIC for 60 min by (69 genes in common, Song et al. 2012); and 5 min pH 4.5 acid shock (69 genes in common, Bore et al., 2007).

Table S5¹ is constructed from a list of 85 up-regulated genes present in three or four of the high-overlap transcriptional profiles and the EIS profile. No single gene was up-regulated in all five profiles, and stress response, hypothetical proteins/unknown function, and central metabolism and growth arrest were the predominant functional categories in terms of common up-regulated genes (Fig. 6). These three categories and cell envelope metabolism and resistances were the only functional categories that included genes from all five profiles (Fig. 6). Protein quality control genes assigned to general and heat shock stress response sub-categories, along with oxidative stress response genes, comprised the majority of the stress response functional category members (Table S5¹). Eleven of the 33 EIS high expression-change genes (Table 3) were also up-regulated in two or three of the four high-overlap comparator profiles

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(Table 4). Present among these 11 genes (Table 4) were *relP*, and seven stress response genes from the CtsR regulon including the entire *clpC* operon (Michel et al. 2006). These genes also included those encoding a hypothetical translocase subunit (SA1215), a virulence factor, and a hypothetical xenobiotic response element (XRE) transcriptional regulator. XRE family regulators are involved with mobilizing genetic elements, (Ibarra et al. 2013) and have also been associated with alarmone control, growth arrest and oxidative stress in *Rhizobium etli* (Vercruysse et al. 2011). SA1215 encodes a hypothetical protein predicted to be a homolog of the SecD/SecF preprotein subunit of the staphylococcal general Sec preprotein translocase complex, and the adjacent gene SA1216 encodes a second putative protein fate effector, oligoendopeptidase F. Recently it was reported that increased expression of *yajC*, which encodes a different subunit of the staphylococcal general Sec translocase complex, is associated with 10% ethanol tolerance in *Lactobacillus buchneri* (Liu et al. 2016). Collectively, the 11 genes in Table 4 encoded protein quality control and growth arrest functions, and one virulence factor.

Conclusions

E. coli adapts to growth in liquid culture containing 5 % v/v ethanol by reducing the lipid-to-protein ratio and modifying the fatty acid composition of the plasma membrane to increase membrane rigidity (Barker and Park 2001, Chiou et al. 2004, Dombek and Ingram 1984). Previously Fried and Novick (1973) reported that growth arrest of *E. coli* by carbon starvation imparted protection from the lethal effects of a

subsequent 9.3% v/v ethanol challenge. Chatterjee et al. (2006) reported that *S. aureus* completely metabolized very low levels of ethanol (≤ 0.1 % v/v) in liquid culture over 24 hr. Korem et al. (2010) evaluated the transcriptional profile of *S. aureus* 8325-4 after 24 hr growth with 3 % v/v ethanol and reported a relatively modest 95 up-regulated and 18 down-regulated genes. No protein quality control, heat shock-specific, or alarmone synthesis genes were induced, and overall results suggested a relatively mild stress condition to which *S. aureus* readily adapted to by means of membrane lipid modification, ethanol catabolism, and generally minor metabolic adjustments.

In stark contrast to the 3 % ethanol adaptation transcriptional profile (Korem et al. 2010), the EIS profile did not indicate any attempt to metabolize ethanol or counter altered membrane fluidity. Instead there was a dramatic down-regulation of genes in the amino acid and protein synthesis, cell envelope metabolism and resistances, cofactors and secondary metabolites, nucleotide metabolism, and transport functional categories that indicated general metabolic shutdown in response to an overwhelming ethanol assault. The EIS transcriptional profile analysis also revealed that genes in the DNA replication, recombination and repair and stress response functional categories were predominantly up-regulated. Furthermore, the overall growth arrested profile exhibited by LP9 and MM66 during EIS relied on the selective deployment of stress responses and metabolic regulatory elements (SAS RelP and TA modules) associated with the persister phenotype. Under EIS conditions where new protein synthesis was inhibited and the integrity of existing proteins faced a specific and serious threat, *S. aureus* concentrated its efforts on protecting existing catalytic potential, presumably to retain the capability for later resumption of growth. At the same time, defense of the

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membrane barrier and osmoregulatory functions were abandoned in the face of the ethanol assault.

The unique aspect of this study was the use of ethanol at a concentration high enough to halt *S. aureus* growth. This was far below the 70-85% recommended for ABHR products to be effective, but ABHR exposure times are only 15-30 seconds while for EIS the exposure time was greatly extended to 15 min. *S. aureus* exhibited an impressive display of resilience and metabolic resourcefulness, resulting in an ethanol tolerance just below that of lactic acid bacteria used in the fermentation industry (Lee et al. 2012). The practical consequence of this study is reinforcement of the importance of full compliance by health care workers with ABHR recommendations with respect to ethanol content, application volume, treatment time, thoroughness of hand coverage, and ensuring hands are otherwise clean and dry before application, if ABHR are expected to be effective against MRSA.

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Tables

Table 1. Primers utilized in this study.

Table 2. qRT-PCR results for four genes differentially regulated in *S. aureus* following ethanol exposure.

Table 3. High expression-change genes, differentially regulated by at least 15-fold in LP9 and/or MM66 by ethanol exposure.

Table 4. The 11 up-regulated genes from the four high-overlap SATMD transcriptomes also among the ethanol exposure high-expression change genes.

Figure Captions

Figure 1. MICs (A) and MBCs (B) of 106 clinical MRSA to ethanol (% v/v).

Figure 2. Venn diagrams of LP9 and MM66 for (A) all genes differentially regulated, (B) genes up-regulated, and (C) genes down-regulated by ethanol exposure.

Figure 3. Numbers of up- and down-regulated LP9 (open bars) and MM66 (solid bars) genes by gene functional category in response to ethanol exposure.

Figure 4. Numbers of up- and down-regulated LP9 (open bars) and MM66 (solid bars) genes by relevant gene functional sub-category in response to ethanol exposure.

Figure 5. Ethanol (9 % v/v) challenged growth assay.

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Figure 6. Numbers of genes by functional category for the 85 genes up-regulated in at least three transcriptional profiles among those for ethanol exposure and the four high-overlap SATMD environmental stress conditions.

Supplementary Material

Table S1. Ethanol MICs and MBCs.

Table S2. Ethanol exposure transcriptomic data for LP9 and MM66 as two-fold or greater expression changes by gene functional category; ND = Not Differentially regulated (< two-fold expression change).

Table S3. Numbers of differential gene expressions and genes involved by ethanol exposure by direction of expression change, strain, and gene functional category and sub-category.

Table S4. Differential regulation of 49 LP9 and MM66 ribosomal protein-encoding genes in response to ethanol exposure.

Table S5. Eighty-five genes up-regulated (✓) in at least three transcriptional profiles among those for ethanol exposure and the four high-overlap SATMD environmental stress conditions.

Figure S1. Growth response of MRSA LP9 (circles) and MM66 (squares) following 10% ethanol addition (open markers) vs. growth without ethanol (solid markers).

Table 1.

RT-qPCR primers	Gene	Sequence 5' → 3'
SA0481-F	<i>mcsA</i>	TGCACATGTTGGTAAATTTGG
SA0481-R		TTGTCCACCTTGAACCTCTGC
SA0835-F	<i>clpB</i>	CAAGCAGCACTTCAATCTCG
SA0835-R		TGTTTGTGCATCTTCCAACG
SA2307-F		GTTGCCATTTGAAGAAGTCG
SA2307-R		ATTTCAATGGGGCCTAATCC
<i>relP</i> inactivation and complementation primers		
attB1- <i>relP</i> -up:F		GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGATAGACTTACAATAAATGAACA
<i>relP</i> -up:R:SacII		AGGATTCCGCGGTTCGATCTACATACATTTTTATACTAA
<i>relP</i> -down:F:SacII		AGGATTCCGCGGGAGTAGATGTTTAGTAGGTGT
attB1- <i>relP</i> -down:R		GGGGACCACTTTGTACAAGAAAGCTGGGTTCGAGCACTAAAATAGTGAC
<i>relP</i> :F:KpnI		GGGGTACCGATTGGTATCGAGCGTTATCG
<i>relP</i> :R:XbaI		TGCTCTAGACACACCTACTAAACATCTACTC

*Nucleotide positions based on the published COL genome sequence.

Table 2.

Locus ID	Gene	Gene product	Fold-change in gene expression			
			LP9	(qRT-PCR)	MM66	(qRT-PCR)
SA0481	<i>mcsA</i>	protein-arginine kinase activator protein McsA	19.7	4.9	15.8	33.5
SA0835	<i>clpB</i>	ClpB chaperone homolog	52.2	2.6	67.1	31.3
SA2307		similar to chlorophenol degradation dioxygenase PcpA	28.4	2.4	8.1	3.2

Table 3.

Gene functional category	Locus ID	Gene	Gene product	LP9	MM66
Cell envelope metabolism and resistances	SA2308	NA	transcriptional regulator, MarR family	21.8	ND
Cell envelope metabolism and resistances	SA2343	<i>cwrA</i>	cell wall damage-inducible protein CwrA	ND	17.7
Central intermediary metabolism	SA0562	<i>adh1</i>	alcohol dehydrogenase	-8.2	-18.4
DNA replication, recombination and repair	SA1562	NA	putative cell division protein FtsK	16.9	3.9
Hypothetical proteins	SA0957	NA	hypothetical protein	11.7	34.1
Hypothetical proteins	SA1000	NA	hypothetical protein	12.7	16.7
Hypothetical proteins	SACOL0768	NA	hypothetical protein	16.2	17.5
Unknown function	SACOL1165	NA	hypothetical membrane protein	14.5	19.9
Hypothetical proteins	SAS037	NA	hypothetical protein	13.1	30.7
Nitrogen metabolism	SA2185	<i>narG</i>	respiratory nitrate reductase, alpha subunit	-3.2	-19.1
Nitrogen metabolism	SA2188	<i>nasD</i>	nitrite reductase, large subunit	-3.8	-15.5
Cofactors and secondary metabolites	SA2189	NA	similar to NirR protein	-3.3	-18.5
Nucleotide metabolism	SA1018	NA	noncanonical pyrimidine nucleotidase, YjjG family	12.2	24.9
Nucleotide metabolism	SA2438	NA	N-carbamoylsarcosine amidohydrolase	8.4	15.2
Protein fate	SA0803	<i>ampA</i>	probable cytosol aminopeptidase	13.2	19
Protein fate	SA1215	NA	hypothetical, similar to preprotein translocase	6.2	15.3
Protein fate	SA1216	NA	oligoendopeptidase F, putative	23.8	35
Protein fate	SA1343	NA	similar to tripeptidase PepT	12.8	18.8
Protein synthesis	SA2042	<i>rpIV</i>	ribosomal protein L22	-5.8	-19.1
Stress response - general	SA0480	<i>ctsR</i>	CtsR transcription repressor of class III stress genes	26.2	23.1
Stress response - general	SA0481	<i>mcsA</i>	protein-arginine kinase activator protein McsA	19.7	15.8

Stress response - general	SA0482	<i>mcsB</i>	protein-arginine kinase McsB	27.9	13.1
Stress response - general	SA0483	<i>clpC</i>	Clp protease, ATP-binding subunit ClpC, frameshift	17	14.1
Stress response - general	SA0835	<i>clpB</i>	ClpB chaperone homolog	52.2	67.1
Stress response - heat shock	SA1410	<i>grpE</i>	heat shock protein GrpE	13.5	15.6
Stress response - heat shock	SA1837	<i>groES</i>	heat shock protein GroES	18	10.3
Stress response - other	SA2307	NA	similar to chlorophenol degradation dioxygenase PcpA	28.4	8.1
Stringent response	SA2297	<i>relP</i>	GTP pyrophosphokinase	20.4	23.2
Transcription	SA2023	<i>rpoA</i>	DNA-directed RNA polymerase alpha subunit	-7.9	-17
Transcription	SAS010	NA	hypothetical XRE family transcriptional regulator	5.6	15.9
Transport	SA2227	NA	amino acid ABC transporter homolog	-10.1	-15.8
Virulence factor - secreted	SA0746	NA	staphylococcal thermonuclease homolog	8.8	24.8
Virulence factor - secreted	SA2207	<i>hlgA</i>	gamma-hemolysin chain II precursor	10.4	20.2

Table 4.

Gene functional category	Locus ID	Gene	Upregulated gene product	Ethanol 10% 15 min	Heat shock 43°C 10 min	Enduracidin 10xMIC 60 min	Vancomycin 10xMIC 60 min	Acid shock pH 4.5 5 min
Protein fate	SA1215	NA	hypothetical, similar to preprotein translocase	✓		✓	✓	
Stress response - general	SA0480	<i>ctsR</i>	CtsR class III stress gene transcription repressor	✓	✓	✓		✓
Stress response - general	SA0481	<i>mcsA</i>	Protein-arginine kinase activator protein McsA	✓	✓	✓		✓
Stress response - general	SA0482	<i>mcsB</i>	Protein-arginine kinase McsB	✓	✓	✓		✓
Stress response - general	SA0483	<i>clpC</i>	Clp protease ATP-binding subunit ClpC, frameshift	✓	✓	✓		✓
Stress response - general	SA0835	<i>clpB</i>	ClpB chaperone homolog	✓	✓	✓		✓
Stress Response - heat Shock	SA1410	<i>grpE</i>	heat shock protein GrpE	✓	✓	✓		
Stress Response - heat Shock	SA1837	<i>groES</i>	heat shock protein GroES	✓	✓	✓		
Stringent response	SA2297	<i>relP</i>	GTP pyrophosphokinase	✓		✓	✓	
Transcription	SAS010	NA	hypothetical XRE family transcriptional regulator	✓		✓	✓	
Virulence factor - secreted	SA2207	<i>hlgA</i>	gamma-hemolysin chain II precursor	✓		✓	✓

Figure 1.

Figure 2.

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Figure 3.

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Figure 4.

Figure 5.

Figure 6.