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Molecules and Cells

Powerful Usage of Phylogenetically Diverse *Staphylococcus aureus* Control Strains for Detecting Multidrug Resistance Genes in Transcriptomics Studies

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***Staphylococcus aureus* is an important human pathogen responsible for life-threatening septicemia, endocarditis, and toxic shock syndrome. Although positive (MRSA; ATCC 33591) and negative (MSSA; ATCC 25923) control strains have been used for various pathogenesis or assay studies, little is known about the genomic structure of the strains, and there has been little genome-wide expression analysis. Phylogenetic analyses revealed that ATCC 33591 and ATCC 25923 are the most genetically diverse strains of the 15 *S. aureus* genomes studied. Microarray analysis showed that the most significantly upregulated group of MRSA genes was the transport group, which includes ATP-binding cassette (ABC) transporters, the two-component system, and the phosphotransferase system. Analysis of the KEGG pathway showed that ABC transporters and the two-component system were the most significantly altered in MRSA. Transcriptional profiling showed a clear difference in gene expression between MRSA and MSSA due to the great genetic distance between the two control strains. Therefore, we suggest that use of the two control strains in comparative genomics or transcriptomics studies would facilitate the identification of major genes for drug resistance in *S. aureus*.**

INTRODUCTION

Staphylococcus aureus is an important human pathogen responsible for life-threatening septicemia, endocarditis, and toxic shock syndrome (Corey, 2009; Woo et al., 1997). It is the most common etiological agent of contagious bovine mastitis, which causes significant losses in the dairy industry (Sears and McCarthy, 2003). It is a persistent resident of the nose in 20% of the human population; another 60% of the population are intermittent carriers, whereas approximately 20% almost never carry it (Kluytmans et al., 1997). Most carriers harbor a single strain, whereas others carry several strains, suggesting that the nares are likely sites for horizontal genetic exchange among strains (Cespedes et al., 2005). Methicillin-resistant *S. aureus* (MRSA) is a multidrug-resistant bacterium whose increasing prevalence in hospitals is of concern worldwide (Gould, 2005). MRSA in animals, such as dogs (Loeffler et al., 2005), horses (Weese et al., 2005), and pigs (Huijsdens et al., 2006), is also a cause for concern, because these animals could act as reservoirs for human isolates or could lead to the evolution of more pathogenic and resistant strains. Resistance genes are variably present as a result of being carried on mobile genetic elements, such as the staphylococcal cassette chromosome *mec*, plasmids, and transposons (Lindsay and Holden, 2004; 2006). Many *S. aureus* pathogenesis studies have used transcription profiling using microarrays to monitor the change in expression in a non-hemolytic variant (Yarwood et al., 2007), to examine the expression profiles of two vancomycin intermediately resistant *S. aureus* strains (Jansen et al., 2007), and to determine the contribution of Panton-Valentine leukocidin in the pathogenesis of community-associated MRSA (Diep et al., 2008).

Nevertheless, little is known about the genomic structure of the *S. aureus* strains ATCC 33591 and ATCC 25923, and little genome-wide expression analysis has been conducted. These two strains are the positive (MRSA; ATCC 33591) and negative (MSSA; ATCC 25923) controls used most frequently in various pathogenesis or assay studies. They have been used as model strains for the restoration of susceptibility to beta-lactams (Lemaire et al., 2008a; 2008b), for the large-scale analysis of clinical isolates (Sergi et al., 2009), and for the development of rapid assays (Al-Talib et al., 2009; Saiful et al., 2006; 2009). Here we compare the genome-wide gene expression of two representative MRSA and MSSA strains.

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MATERIALS AND METHODS

Strains and susceptibility testing

Staphylococcus aureus ATCC 33591 (MRSA) and ATCC 25923 (MSSA) were used in this study. They were grown in tryptic soy broth. Susceptibility to vancomycin, kanamycin, chloramphenicol, tetracycline, penicillin, streptomycin, and erythromycin was determined by disk diffusion using antibiotics discs (Becton, Dickinson and Company, USA) in accordance with the guidelines of the Clinical Laboratory Standard Institute (CLSI 2006).

Phylogenetic analysis using three sas gene sequences

Chromosomal DNA was extracted from the two strains using the AquaPure genomics DNA extraction kit (Bio-Rad). The primers of Robinson and Enright (2003) were used for multilocus sequencing of three *sas* genes (*sasB*, 320 bp; *sasF*, 122 bp; and *sasH*, 211 bp) in the two *S. aureus* strains. PCR amplification was performed using a Thermal Cycler DICE Gradient (Takara-Bio, Japan), and then the PCR products were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Germany). The DNA of both strands was sequenced using an ABI 3730XL automated sequencer (PE Applied Biosystems).

Phylogenetic analysis was performed using our sequences and 13 published sequences: JH1 (NC_009632), JH9 (NV_009487), Mu3 (NC_009782), Mu50 (NC_002758), N315 (NC_002745), MRSA252 (NC_002952), MW2 (NC_003923), MSSA476 (NC_002953), NCTC8325 (NC_007795), Newman (NC_009641), COL (NC_002951), USA300 (NC_010079), and USA300_TCH1516 (NC_007793). The 15 *S. aureus* sequences were initially aligned using ClustalX 1.83 (Thompson et al., 1997) and then adjusted manually where necessary. Regions of uncertain alignment were eliminated from the final analyses, and alignment gaps were treated as missing data. Thus, the analyses were limited to reliably aligned regions comprising a total of 653 nucleotide positions. Phylogenetic reconstructions were conducted using the combined *sas*B + F + H dataset of the 15 strains using Bayesian inferences. Bayesian methods were executed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under the best fit models HKY, which were selected using hLRT in Modeltest 3.7 (Posada and Crandall, 1998). We set the parameters nst = 2 and rates = equal as the likelihood settings. Monte Carlo Markov chains were run for 2,000,000 generations and sampled every 100 generations: four chains were run and 20,000 initial trees were discarded (burn in). Bayesian posterior probabilities were estimated based on the 50% majority rule consensus of the trees. Tree graphic output was produced using TreeView 1.6.1 (Page, 1996).

Generation of microarray data

We performed global gene expression analyses using Affymetrix GeneChip® *S. aureus* Genome arrays. Samples were prepared according to the manufacturer's recommendations. The *S. aureus* strains were grown in tryptic soy broth at 37°C. Total RNA was isolated using a RiboPure Bacteria Kit (Ambion, UK) as described by the manufacturer. RNA quality was assessed with an Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, The Netherlands), and quantity was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, USA). For each RNA sample, 10 μg was used as input into the Affymetrix procedure, as recommended by protocol (http://www.affymetrix.com). Briefly, 10 μg total RNA from each sample was converted into double-strand cDNA using random primers. The double-stranded cDNA was purified with a MinElute PCR purification kit (Qiagen) and quantified using an ND-1000 spectrophotometer. The purified double-stranded cDNA was fragmented using 0.6 U/μg DNase I and end-labeled by a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. The fragmented end-labeled cDNA was hybridized to the GeneChip® *S. aureus* Genome arrays for 16 h at 45°C and 60 rpm, as described in the GeneChip Expression Analysis Technical Manual (Affymetrix). After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450 (Affymetrix) and scanned using a GeneChip Array scanner 3000 7G (Affymetrix).

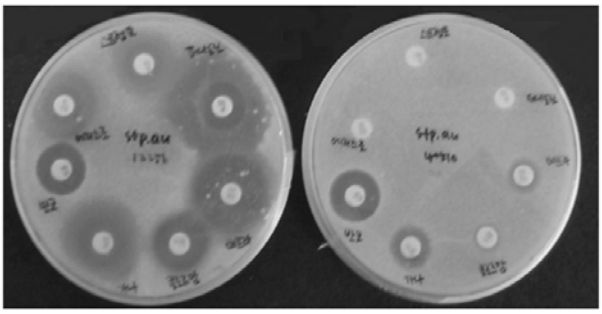

Fig. 1. Tests of the susceptibility of *S. aureus* ATCC 25923 (left) and ATCC 33591 (right) to antibiotics.

Analysis of microarray data

The quality of the array image was assessed as described in the GeneChip Expression Analysis Technical Manual (Affymetrix). All arrays were processed to determine the "robust multi-array average" (RMA) using the "affy" software package (Gautier et al., 2004). Expression values were computed in detail from raw CEL files by applying the RMA model of probe-specific correction for perfect-match probes. These corrected probe values were then subjected to quantile normalization, and a median polish was applied to compute one expression measure from all probe values. The resulting RMA expression values were ln-transformed. Individual gene expression levels were compared using an unpaired Welch's *t*-test. The Benjamini-Hochberg correction for the false discovery rate (FDR) was used for all probe-level normalized data. We selected genes as differentially expressed when the FDR-adjusted *P* value was less than 0.01 according to Welch's *t*-test. Hierarchical clustering was performed using the Hclust function and displayed using the Heatmap software package of the R statistical package (version 2.5). Enrichment analysis of the KEGG pathway and gene ontology were performed using DAVID bioinformatics resources (Huang et al., 2009).

RESULTS AND DISCUSSION

Antibiotic susceptibility testing shows full concordance with previous reports

The reference strains *S. aureus* ATCC 25923 and MRSA ATCC 33591 were tested for their susceptibility to antibiotics using the standardized agar disc diffusion method. These strains have been used as quality control organisms in many antimicrobial susceptibility tests of *S. aureus*. As expected, ATCC 25923 was susceptible to all of the antibiotics tested; MRSA ATCC 33591 was resistant to all of the antibiotics tested, with the exception of vancomycin, and had intermediate susceptibility to kanamycin (Fig. 1). With the increase in staphylococcal resistance to methicillin, vancomycin is often the treatment of choice for MRSA infection. ATCC 33591 is susceptible to vancomycin, and the methicillin control strain is also susceptible to

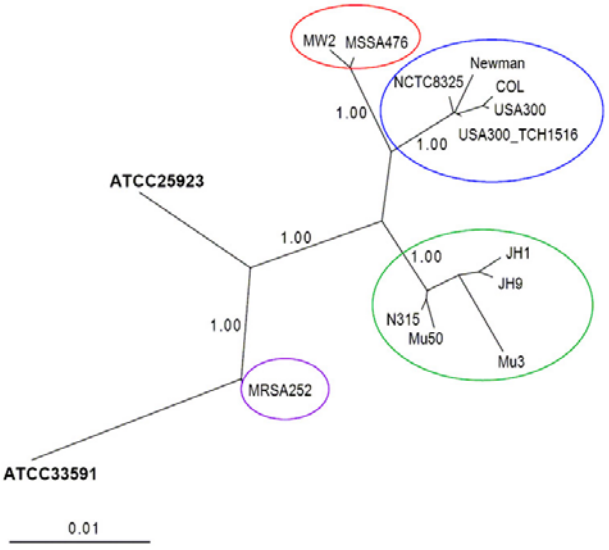

Fig. 2. Bayesian inference tree (-lnL = 1157.9191) showing the phylogenetic relationships of the 15 *S. aureus* strains obtained from the three *sas* gene sequences (*sasB*, *sasF*, and *sasH*) under the HKY model. Posterior probabilities (≥ 0.80) are indicated above the branches. The base frequencies were A = 0.2929, C = 0.2089, G = 0.1508, and T = 0.3473. The strains examined are in bold type.

Kanamycin-tobramycin and ofloxacin (Hamdad et al., 2006). The findings of our susceptibility testing were fully concordant with those of previous reports.

The two positive or negative control strains are genetically diverse

Three *sas* gene sequences of the two *S. aureus* strains were determined and then analyzed along with 13 published sequences. In concordance with previous results (Baba et al., 2008), the 13 published *S. aureus* sequences revealed three phylogenetic clusters with the genetically diverse MRSA252 (Fig. 2). MRSA252 is one of the most genetically diverse *S. aureus* strains–about 6% of the genome is novel compared with other published genomes (Holden et al., 2004). Our phylogenetic analysis revealed that of the strains analyzed, ATCC 33591 was the most genetically diverse and ATCC 25923 was the next most diverse, whereas MRSA252 fell between these two strains. MRSA252 is a clinically important, globally prevalent lineage that has been compared to MSSA476 (Holden et al., 2004). Because the two control strains are located the greatest genetic distance apart compared with published *S. aureus* sequences, they constitute a good control reference for pathogenesis studies. For example, the phylogenetic distance between MSSA476 and ATCC 25923 is almost as great as that between MSSA476 and MRSA252. The first pair of strains are MSSA, which is the most phylogenetic diverse. A three-way comparison between the two MSSA and the MRSA strains would be useful to screen out background genes and facilitate the identification of the major genes responsible for methicillin resistance. ATCC 33591 could also be used for the same purpose in both genomics or transcriptomics comparative analysis.

In hospitals where multiresistant MRSA is endemic, the appearance of vancomycin-resistant *S. aureus* (VRSA) would be a serious problem in terms of treating infected patients. The potential transfer of vancomycin resistance from vancomycin-resistant enterococci to human *S. aureus* could be the background for the emergence of vancomycin fully resistant *S. aureus*. This would be a major problem for human health care (Sung et al., 2008) and a great concern for agricultural settings,

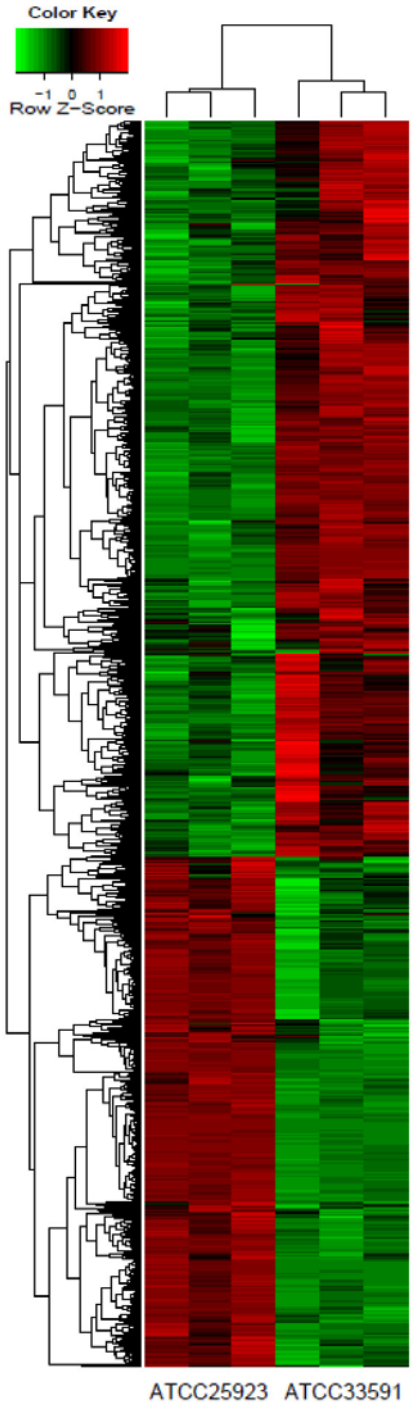

Fig. 3. The heatmap and dendrogram of differentially expressed genes. The heatmap shows the expression levels of the 3579 probes. A total of 1467 probes were upregulated (red) in ATCC 25923, and 2112 probes were upregulated (red) in ATCC 33591. Red represents high expression; green represents low expression. The heatmap and dendrogram were drawn using the Heatmap.2 R package using hierarchical clustering.

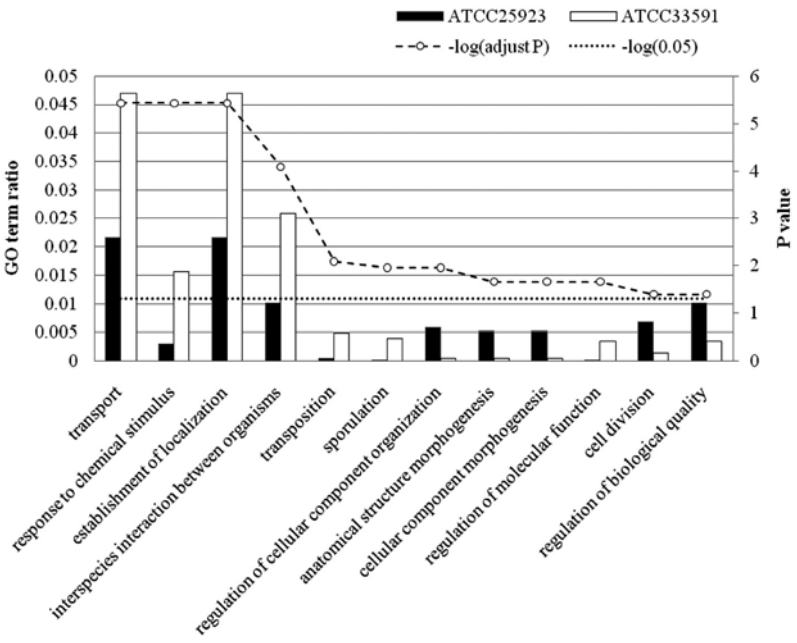

Fig. 4. Enriched biological process terms of differentially expressed genes. Differentially expressed genes of the third-level biological process term of gene ontology were counted using the Affymetrix annotated data. The enrichment test was performed using the two-sided Fisher's exact test to compare ATCC 25923 and ATCC 33591. *P* values were adjusted with the FDR correction. The vertical axis represents the ratio of BP terms to sum of BP terms, and the supporting vertical axis shows the adjusted *P* value (FDR) for each GO term. Only significantly enriched GO terms are shown, *i.e.*, those over the -log(0.05) dashed line in this figure. The sum of the BP terms in ATCC 2523 and ATCC 33591 was 4387 and 2044, respectively.

in which vancomycin-resistant enterococci are found (Witte, 2000). A comparative analysis using two diverse MRSA strains would help experts identify the major genes responsible for VRSA or vancomycin intermediately resistant *S. aureus*.

Microarray analysis reveals that genes highly upregulated in the MRSA strains are in transport group

Microarray analysis revealed that 3579 gene probes showed genes that were differentially expressed between the two control strains. Of these, 2112 gene probes were upregulated in ATCC 33591 and 1467 in ATCC 25923 (Fig. 3). The enrichment test of the GO biological process revealed that the most significantly enriched group in MRSA was the transport group (Fig. 4). Genes in the transport group upregulated in MRSA included ATP-binding cassette (ABC) transporters, the two-component system (TCS), and the phosphotransferase system. In concordance with the GO enrichment result, analysis of the KEGG pathway showed that ABC transporters and the TCS were the most significantly enriched in MRSA (Table 1).

Multidrug resistance is frequently associated with the overexpression of ABC transporters and the TCS, which is consistent with the result seen in the resistant *S. aureus*. ABC transporters are integral membrane proteins that actively transport chemically diverse substrates across the lipid bilayers of cellular membranes. In bacterial cells, ABC transporters contribute to multidrug and antibiotic resistance by extruding drugs or antibiotics (Dawson and Locher, 2007). This is of clinical importance because multidrug resistance in human cancer cells is mostly the result of the overexpression of ABC transporters that catalyze the extrusion of the cytotoxic compounds used in cancer therapy (Gottesman and Ambudkar, 2001). Bacterial drug resis tance has become an increasing problem. In bacteria, ABC exporters extrude diverse substrates, including drugs and antibiotics, whereas ABC importers mediate the uptake of essential nutrients. The high-resolution structure of a homologous bacterial multidrug ABC transporter, Sav1866 from *S. aureus*, has been determined (Dawson and Locher, 2006).

The TCS serves as a basic stimulus-response coupling mechanism that allows organisms to sense and respond to changes in environmental conditions. The TCS typically consists of two types of signal transducers: a sensor kinase and its response regulator. The sensor kinase monitors a certain environmental condition and modulates the phosphorylation state of the response regulator that controls genes. One of the most attractive aspects of the TCS is its regulation of antimicrobial resistance factors. The TCSs of bacteria consist of two proteins, histidine kinase and response regulators, and have received increasing attention for their potential as novel antibacterial drug

Table 1. Enriched KEGG pathways of the differentially expressed genes

Strain	Term	Count	*P**
ATCC 25923	Ribosome	53	5.4E-10
	Pyruvate metabolism	21	9.7E-03
	Oxidative phosphorylation	19	4.3E-02
	Citrate cycle (TCA cycle)	18	2.3E-03
	Pentose phosphate pathway	14	3.7E-02
	Reductive carboxylate cycle (CO_2 fixation)	11	3.6E-02
	Valine, leucine, and isoleucine degradation	10	1.1E-02
	Photosynthesis	9	1.8E-02
	Lysine degradation	8	2.8E-02
	Fatty acid metabolism	7	4.3E-02
ATCC 33591	ABC transporters	23	4.6E-13
	TCS	14	1.2E-05
	Phenylalanine, tyrosine, and tryptophan biosynthesis	8	1.9E-04
	Glycine, serine, and threonine metabolism	6	5.2E-03
	Histidine metabolism	5	6.6E-03
	Beta-lactam resistance	2	2.1E-02

*The enrichment test of each KEGG pathway between whole genes and upregulated genes in a strain was performed using Fisher's exact test. Multiple testing corrections were performed with the FDR correction.

targets (Macielag and Goldschmidt, 2000; Matsushita and Janda, 2002). Some TCSs regulate the expression of antibiotic resistance determinants, including drug-efflux pumps (Hirakawa et al., 2003). The overexpression of response regulators of bacterial two-component signal transduction systems confers drug resistance as a result of controlling the expression of some drug transporter genes.

Various TCSs are ubiquitous in bacteria and regulate the transcription of different gene products. The regulation of osmolarity, nutrient uptake, redox potential, and sporulation and the expression of virulence factors are under the control of TCSs. Higher eukaryotes, including mammals, use a distinct signal transduction system that incorporates serine, threonine, and tyrosine phosphorylation. Although common in eukaryotes, the phosphorylation of serine, threonine, and tyrosine residues has only recently been identified in prokaryotes. Eukaryotic-type serine/threonine kinases (STKs) and phosphatases (STPs) are expressed in many prokaryotes, including *S. aureus*. Prokaryotic STKs regulate various cellular functions, such as stress responses, biofilm formation, cell wall biosynthesis, sporulation, and metabolic and developmental processes. The modulation of cellular processes by STKs and STPs is widespread in bacteria. PknB/Stk and Stp are modulators of cell wall structure and susceptibility to cell wall-acting antibiotics, such as certain beta-lactams and tunicamycin.

The Gram-positive bacteria cell envelope is essential for both survival and pathogenicity. The cell envelope is the target of numerous antibiotics. The enzymatic reactions in cell wall synthesis are performed by the penicillin-binding protein (PBP) and can be inhibited by the two major groups of cell wall active antibiotics: beta-lactams and glycopeptides. *S. aureus* generally becomes resistant to beta-lactams through the production of additional PBP and PBP2a encoded by *mecA*. PBP2a has a decreased affinity for beta-lactams due to its distorted active site. Therefore, in the presence of beta-lactams, PBP2a is able to maintain cell wall biosynthesis with the help of the beta-lactam-insensitive transglycosylase domain of PBP2.

In summary, based on phylogenetic analysis, we postulate that use of the two control strains studied here in comparative genomics or transcriptomics studies could facilitate the identification of major genes for drug resistance in *S. aureus*. We think that the result of transcriptional profiling implies a high statistical power resulting from the great genetic distance between the two control strains.

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