Identification of Methicillin-Resistant Staphylococcus aureus (MRSA) Genetic Factors Involved in Human Endothelial Cells Damage, an Important Phenotype Correlated with Persistent Endovascular Infection

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Abstract: Methicillin-resistant Staphylococcus aureus (MRSA) is a leading cause of life-threatening endovascular infections. Endothelial cell (EC) damage is a key factor in the pathogenesis of these syndromes. However, genetic factors related to the EC damage have not been well studied. This study aims to identify genetic determinants that impact human EC damage by screening the genome-wide Nebraska Transposon Mutant Library (NTML). A well-established MTT assay was used to test the in vitro damage of human EC cell line (HMEC-1) caused by each mutant strain in the NTML. We first confirmed some global regulators and genes positively impact the EC damage, which is consistent with published results. These data support the utility of the high-throughput approach. Importantly, we demonstrated 317 mutants significantly decreased the EC damage, while only 6 mutants enhanced the EC damage vs. parental JE2 strain. The majority of these genes have not been previously defined to affect human EC damage. Interestingly, many of these newly identified genes are involved in metabolism, genetic and environmental information processing, and cellular processes. These results advance our knowledge of staphylococcal genetic factors related to human EC damage which may provide novel targets for the development of effective agents against MRSA endovascular infection.

Keywords: MRSA; human endothelial cell damage; virulence factors

1. Introduction

Staphylococcus aureus is the most common cause of endovascular infection, including infective endocarditis (IE). Despite the use of gold-standard antibiotics, morbidity and mortality associated with these syndromes remain unacceptably high [1]. In addition, the emergence of methicillin-resistant S. aureus (MRSA) further complicates the management of patients with these infections and emphasizes this public health threat [1]. Therefore, there is an urgent need to understand specific genetic factors involved in the pathogenesis and antibiotic treatment outcome of MRSA endovascular infection.

It is generally recognized that the pathogenesis of S. aureus is complex and probably involves the coordinate expression of multiple gene products, including a variety of surface adhesive proteins and exoproteins [2]. Once S. aureus enters into the bloodstream, it must avoid host innate defense killing to survive. When the organism has persisted in the bloodstream, it must then colonize and invade the endothelial cells (ECs) lining of the blood vessels, and, subsequently, damage the ECs to infect deeper tissues to cause organ dissemination [3]. It has been well demonstrated that EC damage plays a crucial role in the pathogenesis of many human diseases, including endovascular infections [4]. In addition,
we have recently demonstrated a positive correlation between in vitro human EC damage and virulence, as well as vancomycin treatment persistent outcome in an experimental endocarditis model caused by clinical MRSA isolates [5]. However, little is known about the genetic factors involved in the EC damage in \textit{S. aureus}.

The Nebraska Transposon Mutant Library (NTML) consists of 1920 sequence-defined transposon insertion mutants of non-essential genes in a community-associated (CA) MRSA USA300 strain, JE2 [6]. This library has been used for screening several biological phenotypes, including hemolysis, proteolysis, carotenoid pigment formation, antibiotic susceptibility, and biofilm formation [6–8]. These investigations demonstrate that the NTML may serve as a valuable genetic tool to study host-pathogen interaction.

Numerous investigations have used human umbilical vein EC (HUVECs) to study microbial–EC interactions. However, the use of HUVECs requires a constant supply of umbilical cords, and there are significant donor-to-donor variations in these ECs. To overcome these difficulties, immortalized ECs, including human microvascular EC (HMEC-1), have been developed. These cell lines have better availability and less variability [9]. In addition, we previously compared \textit{S. aureus} EC damage with HMEC-1 cell line and HUVECs, and found HMEC-1 cells were more susceptible to damage caused by \textit{S. aureus} vs. HUVECs [10]. In addition, the HMEC-1 cell line has been used to study the EC interactions with multiple microorganisms, including \textit{S. aureus} [10–12]. Thus, in the current investigation, the HMEC-1 cell line was employed to test the impact of all the mutant strains in the NTML on its damage.

In the current study, we aimed to identify staphylococcal genes associated with the EC damage by performing an unbiased genome-wide screening of all mutations in the NTML. This study will remarkably advance our understanding of staphylococcal genetic factors related to human EC damage which may provide novel targets for the development of effective compounds against MRSA endovascular infections.

2. Results

2.1. The MTT Assay Is Applicable to the High Throughput Screening of Genes Involved in HMEC-1 Damage

We confirmed some \textit{S. aureus} genetic factors which have previously been reported to affect EC damage. For instance, global regulator (e.g., \textit{agr}, \textit{saeSR}, and \textit{arlSR}) and structural genes related to gamma-hemolysin (e.g., \textit{hlg}) and serine-like protease (e.g., \textit{spl}) positively impact EC damage. In addition, the control \textit{arlR} mutant strain caused significantly less EC damage (<30%) vs. JE2 parental strain, which is in accordance with the previously reported results. These results proved the feasibility and reliability of this high throughput screening assay.

2.2. Identified Staphylococcal Genes Impacting HMEC-1 Damages

The mean HMEC-1 damage rate caused by the JE2 parental strain is 46.19 ± 2.97%. To focus on the genes which highly affect the EC damage, we set up the EC damage rates of ≤30% or ≥60% with \textit{p} values less than 0.05 as cutoffs for data analysis. Screening of the whole NTML displayed that 317 individual gene mutations led to significantly decreased HMEC-1 damage rates (≤30%; \textit{p} < 0.05; Figure 1, Table 1), suggesting these genes positively impact the EC damage. Only six mutant strains demonstrated significantly increased EC damage (≥60%, \textit{p} < 0.05; Figure 1, Table 2), including four genes with known functions (e.g., \textit{mepA}, \textit{azoR}, and \textit{moaD}, and SAUSA300_1197) and two hypothetical genes with unknown function. EC damage rates of the rest mutants from the NTML were presented in Supplementary Table S1. JE2 parental strain and randomly selected mutants showed similar EC damage rates between 24-well and 384-well plates assay (Table 3). Some of the mutants that caused significant changes to EC damage were successfully classified into KEGG categories, including metabolism, genetic information processing, environmental information processing, and cellular processes (Table 4). For the KEGG categories, ~65% of genes functioned in metabolism pathways, ~24% involved in environmental information
processing, ~11% acted in genetic information processes, and ~9% associated with cellular processes (Figure 2). In addition, some of these genes had multiple functions in the different KEGG pathways.

![Mean EC damage of JE2](image)

**Figure 1.** The global map of in vitro HMEC-1 damage rate caused by the mutant strains in the NTML. The vertical dashed line represents the mean of HMEC-1 damage rate of parental strain USA300 JE2 (46.19%); and the horizontal dashed line represents the p value of 0.05. The bright red dots represent ≤30% EC damage caused, while the bright blue dots represent ≥60% EC damage due to the study mutant strains in the NTML and p < 0.05 vs. JE2 WT strain. Damage rate below zero means the A_{560nm} of the test well is higher than the A_{560nm} of the negative damage control, which indicates that the mutant causes no damage to the EC.

**Table 1.** Mutants significantly decrease HMEC-1 damage vs. JE2 WT strain (EC damage rate ≤ 30%).

| Locus       | Gene Name | Description                                      | % EC Damage (Mean ± SD) |
|-------------|-----------|--------------------------------------------------|-------------------------|
| SAUSA300_0261 | hypothetical | conserved hypothetical protein                    | 29.83 ± 8.34            |
| SAUSA300_1172 | hypothetical | M16 family peptidase                             | 29.74 ± 4.80            |
| SAUSA300_0083 | hypothetical | hypothetical protein                             | 29.70 ± 10.14           |
| SAUSA300_1386 | hypothetical | phiETA ORF59-like protein                        | 29.57 ± 1.07            |
| SAUSA300_0076 | hypothetical | ABC transporter ATP-binding protein               | 29.57 ± 4.10            |
| SAUSA300_1712 | ribR      | 6,7-dimethyl-8-ribityllumazine synthase           | 29.49 ± 9.63            |
| SAUSA300_1457 | malR      | maltose operon transcriptional repressor         | 29.46 ± 2.79            |
| SAUSA300_1309 | hypothetical | IS2000 family transposase                        | 29.41 ± 8.13            |
| SAUSA300_1253 | gclT      | transcription antiterminator                     | 29.37 ± 4.04            |
| SAUSA300_1797 | hypothetical | conserved hypothetical protein                    | 29.37 ± 4.79            |
| SAUSA300_1759 | hypothetical | hypothetical protein                             | 29.13 ± 2.85            |
| SAUSA300_2386 | hypothetical | beta-lactamase                                   | 29.13 ± 1.62            |
| SAUSA300_2434 | hypothetical | transporter protein                              | 28.67 ± 5.28            |
| SAUSA300_2037 | hypothetical | ATP-dependent RNA helicase                       | 28.67 ± 8.90            |
| SAUSA300_1654 | hypothetical | proline dipeptidase                              | 28.46 ± 4.20            |
| SAUSA300_0615 | hypothetical | putative monovalent cation/H+ antiporter subunit F | 28.45 ± 4.24            |
| SAUSA300_1659 | tpx       | thiol peroxidase                                 | 28.44 ± 4.72            |
| SAUSA300_1478 | hypothetical | putative lipoprotein                             | 28.28 ± 4.37            |
| SAUSA300_2455 | hypothetical | putative fructose-1,6-bisphosphatase             | 28.27 ± 5.83            |
| SAUSA300_1297 | aqpP      | acylphosphatase                                  | 28.23 ± 4.50            |
| SAUSA300_2606 | hisF      | imidazole glycerol phosphate synthase subunit HisF | 27.62 ± 4.01            |
| SAUSA300_0795 | hypothetical | hypothetical protein                             | 27.38 ± 6.00            |
| SAUSA300_1683 | hypothetical | bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase | 27.26 ± 6.86 |
| SAUSA300_2618 | hypothetical | hypothetical protein                             | 27.23 ± 7.65            |
| SAUSA300_1398 | hypothetical | phiSLT ORF123-like protein                       | 27.16 ± 11.43           |
| Locus         | Gene Name       | Description                                      | % EC Damage (Mean ± SD) |
|--------------|-----------------|--------------------------------------------------|-------------------------|
| SAUSA300_0059| hypothetical    | conserved hypothetical protein                   | 27.07 ± 7.67            |
| SAUSA300_1764| epID            | lantibiotic epidermin biosynthesis protein EpID  | 26.84 ± 3.46            |
| SAUSA300_2332| hypothetical    | heat shock protein                               | 26.78 ± 8.46            |
| SAUSA300_1040| hypothetical    | hypothetical protein                            | 26.74 ± 8.21            |
| SAUSA300_2280| fosB            | fosfomycin resistance protein FosB               | 26.67 ± 8.68            |
| SAUSA300_1750| hypothetical    | conserved hypothetical protein                   | 26.62 ± 9.44            |
| SAUSA300_0883| hypothetical    | putative surface protein                        | 26.56 ± 12.91           |
| SAUSA300_1964| hypothetical    | hypothetical protein                            | 26.38 ± 7.19            |
| SAUSA300_0290| hypothetical    | putative lipoprotein                            | 26.29 ± 8.56            |
| SAUSA300_1672| nagE            | phosphotransferase system, N-acetylglycosamine-specific IIBC component | 26.21 ± 5.46            |
| SAUSA300_2023| rrbN            | anti-sigma-B factor, serine-protein kinase       | 26.01 ± 0.14            |
| SAUSA300_0190| gpcC            | indole-3-pyruvate decarboxylase                 | 25.81 ± 7.93            |
| SAUSA300_2413| hypothetical    | hypothetical protein                            | 25.79 ± 4.70            |
| SAUSA300_0798| hypothetical    | ABC transporter substrate-binding protein       | 25.59 ± 3.93            |
| SAUSA300_0489| ftsH            | putative cell division protein FtsH              | 25.55 ± 5.76            |
| SAUSA300_1093| pyrB            | aspartate carbamoyltransferase catalytic subunit | 25.49 ± 1.23            |
| SAUSA300_0517| hypothetical    | RNA methyltransferase                           | 25.39 ± 8.18            |
| SAUSA300_1740| hypothetical    | hypothetical protein                            | 25.37 ± 9.05            |
| SAUSA300_0540| hypothetical    | HAD family hydrolase                            | 25.26 ± 9.24            |
| SAUSA300_2272| hypothetical    | hypothetical protein                            | 25.25 ± 4.80            |
| SAUSA300_1968| hypothetical    | putative phage transcriptional regulator        | 25.23 ± 9.97            |
| SAUSA300_0642| hypothetical    | hypothetical protein                            | 25.21 ± 4.58            |
| SAUSA300_2358| hypothetical    | ABC transporter permease                        | 25.11 ± 6.08            |
| SAUSA300_1984| mntQ            | hypothetical protein                            | 25.07 ± 9.15            |
| SAUSA300_1266| trpE            | N-(5-phosphoribosyl)anthranilate isomerase      | 25.05 ± 7.12            |
| SAUSA300_2251| hypothetical    | dehydrogenase family protein                    | 25.00 ± 3.65            |
| SAUSA300_0706| hypothetical    | putative osmoprotectant ABC transporter ATP-binding protein | 24.95 ± 11.00           |
| SAUSA300_0941| hypothetical    | putative ferrichrome ABC transporter             | 24.69 ± 6.43            |
| SAUSA300_0951| sogA            | V8 protease                                     | 24.55 ± 8.41            |
| SAUSA300_1875| hypothetical    | exomuclease                                     | 24.52 ± 10.68           |
| SAUSA300_0566| hypothetical    | amino acid permease                             | 24.49 ± 5.06            |
| SAUSA300_0871| hypothetical    | hypothetical protein                            | 24.49 ± 12.19           |
| SAUSA300_0565| hypothetical    | conserved hypothetical protein                  | 24.43 ± 5.34            |
| SAUSA300_0391| hypothetical    | hypothetical protein                            | 24.38 ± 0.45            |
| SAUSA300_1328| hypothetical    | hypothetical protein                            | 24.10 ± 7.38            |
| SAUSA300_2279| hypothetical    | putative drug transporter                       | 23.92 ± 10.37           |
| SAUSA300_0505| hypothetical    | lysR family regulatory protein                  | 23.87 ± 10.37           |
| SAUSA300_0470| ksgA            | dimethyladenosine transferase                    | 23.86 ± 7.13            |
| SAUSA300_1106| hypothetical    | putative lipoprotein                            | 23.45 ± 8.92            |
| SAUSA300_1991| agrC            | accessory gene regulator protein C               | 23.44 ± 9.71            |
| SAUSA300_0108| hypothetical    | antigen, 67 kDa                                 | 23.31 ± 6.10            |
| SAUSA300_2326| agrC            | transcription regulatory protein                 | 23.30 ± 5.35            |
| SAUSA300_1399| hypothetical    | phoSLO ORF110-like protein                      | 23.29 ± 0.65            |
| SAUSA300_1942| hypothetical    | hypothetical protein                            | 23.29 ± 11.27           |
| SAUSA300_0079| hypothetical    | putative lipoprotein                            | 23.27 ± 6.02            |
| SAUSA300_1384| hypothetical    | phiSLO ORF100-like protein, holin                | 23.25 ± 6.98            |
| SAUSA300_1950| hypothetical    | hypothetical protein                            | 23.24 ± 9.64            |
| SAUSA300_0320| gdhB            | triacylglycerol lipase                           | 23.13 ± 9.02            |
| SAUSA300_0370| hypothetical    | putative enterotoxin                            | 23.06 ± 9.01            |
| SAUSA300_1224| hypothetical    | conserved hypothetical protein                  | 22.85 ± 4.12            |
| SAUSA300_1925| hypothetical    | phiPLV ORF17-like protein                       | 22.72 ± 9.85            |
| SAUSA300_1271| hypothetical    | hydrolase-like protein                          | 22.57 ± 5.67            |
| SAUSA300_0547| stdD            | stdD protein                                    | 22.52 ± 1.23            |
| SAUSA300_0561| hypothetical    | hypothetical protein                            | 22.57 ± 6.87            |
| SAUSA300_2367| hlgB            | gamma-hemolysin component B                     | 22.27 ± 7.70            |
| SAUSA300_1671| hypothetical    | hypothetical protein                            | 22.15 ± 10.08           |
| SAUSA300_2341| narJ            | respiratory nitrate reductase, subunit delta     | 22.11 ± 4.50            |
| SAUSA300_0420| hypothetical    | hypothetical protein                            | 22.10 ± 8.19            |
| SAUSA300_2281| hutG            | formimidoylglutamase                            | 22.09 ± 5.33            |
| SAUSA300_1427| hypothetical    | phiSLO ORF56-like protein                       | 21.94 ± 1.69            |
| SAUSA300_0691| saeR            | DNA-binding response regulator SaeR              | 21.93 ± 10.56           |
| SAUSA300_1519| hypothetical    | hypothetical protein                            | 21.86 ± 0.84            |
| SAUSA300_0253| stdA            | cell wall biosynthesis protein StdA              | 21.83 ± 12.24           |
| SAUSA300_2459| hypothetical    | MarR family transcriptional regulator           | 21.58 ± 6.37            |
| SAUSA300_2505| hypothetical    | acetyltransferase                               | 21.48 ± 5.28            |
| SAUSA300_0852| hypothetical    | hypothetical protein                            | 21.46 ± 9.86            |

Locus: Identifier of the locus on the SAUSA300 strain. Gene Name: Name of the gene. Description: Description of the gene function. % EC Damage (Mean ± SD): Percentage of EC Damage with mean and standard deviation.
| Locus       | Gene Name | Description                                      | % EC Damage (Mean ± SD) |
|------------|-----------|--------------------------------------------------|------------------------|
| SAUSA300_1213 | hypothetical | hypothetical protein                             | 21.42 ± 8.18           |
| SAUSA300_1216 | hypothetical | cardioplin synthetase                            | 21.40 ± 13.46          |
| SAUSA300_0395 | hypothetical | superantigen-like protein                        | 21.39 ± 9.28           |
| SAUSA300_1016 | cyoE      | protoheme IX farnesyltransferase                 | 21.38 ± 6.70           |
| SAUSA300_1126 | rnc       | ribonuclease III                                 | 21.34 ± 5.04           |
| SAUSA300_1437 | hypothetical | phiSLT ORF204-like protein                      | 21.26 ± 3.02           |
| SAUSA300_2145 | hypothetical | ABC transporter ATP-binding protein              | 21.19 ± 1.99           |
| SAUSA300_2288 | hypothetical | ABC transporter ATP-binding protein              | 21.10 ± 15.49          |
| SAUSA300_0698 | pabA      | para-aminobenzoate synthase, glutamine amidotransferase, component II | 21.05 ± 4.75           |
| SAUSA300_0519 | hypothetical | hypothetical protein                             | 20.86 ± 6.93           |
| SAUSA300_2330 | hypothetical | hypothetical protein                             | 20.82 ± 4.02           |
| SAUSA300_0141 | deoB      | phosphopentomutase                               | 20.69 ± 9.71           |
| SAUSA300_1684 | hypothetical | hypothetical protein                             | 20.53 ± 11.18          |
| SAUSA300_0456 | tgl       | queine tRNA-ribosyltransferase                   | 20.53 ± 9.07           |
| SAUSA300_0442 | hypothetical | hypothetical protein                             | 20.45 ± 3.70           |
| SAUSA300_0744 | lgt       | prolipoprotein dacylglyceryl transferase         | 20.44 ± 5.61           |
| SAUSA300_1576 | recD2     | helicase, RecD/TraA family                      | 20.41 ± 6.63           |
| SAUSA300_2088 | luxS      | S-ribosylhomocysteinate                          | 20.40 ± 7.33           |
| SAUSA300_0131 | hypothetical | putative Bacterial sugar transferase            | 20.28 ± 13.49          |
| SAUSA300_0649 | hypothetical | hypothetical protein                             | 20.23 ± 0.89           |
| SAUSA300_2550 | nrdG      | anaerobic ribonucleotide reductase, small subunit | 20.22 ± 10.12          |
| SAUSA300_2168 | hypothetical | hypothetical protein                             | 20.16 ± 4.12           |
| SAUSA300_2587 | hypothetical | accessoary secretory protein AspI                | 20.06 ± 9.42           |
| SAUSA300_2548 | hypothetical | hypothetical protein                             | 19.98 ± 7.37           |
| SAUSA300_1021 | hypothetical | hypothetical protein                             | 19.92 ± 15.09          |
| SAUSA300_0456 | rILA      | 23S ribosomal RNA                                | 19.91 ± 0.15           |
| SAUSA300_0431 | hypothetical | hypothetical protein                             | 19.86 ± 4.23           |
| SAUSA300_1247 | hypothetical | conserved hypothetical protein                   | 19.79 ± 10.23          |
| SAUSA300_2108 | milD      | mannitol-1-phosphate 5-dehydrogenase            | 19.74 ± 9.18           |
| SAUSA300_2516 | hypothetical | short chain dehydrogenase/reductase family oxidoeductase | 19.65 ± 10.14          |
| SAUSA300_0450 | tvrR      | trehalase operon repressor                       | 19.59 ± 13.38          |
| SAUSA300_0422 | hypothetical | hypothetical protein                             | 19.54 ± 2.66           |
| SAUSA300_1739 | hypothetical | hypothetical protein                             | 19.47 ± 8.56           |
| SAUSA300_0257 | lrgB      | antholin-like protein LrgB                       | 19.47 ± 17.61          |
| SAUSA300_0256 | hypothetical | hypothetical protein                             | 19.05 ± 4.22           |
| SAUSA300_2352 | hypothetical | hypothetical protein                             | 18.95 ± 11.52          |
| SAUSA300_2236 | hypothetical | hypothetical protein                             | 18.82 ± 4.26           |
| SAUSA300_1409 | hypothetical | hypothetical protein                             | 18.77 ± 11.78          |
| SAUSA300_1304 | hypothetical | hypothetical protein                             | 18.73 ± 5.92           |
| SAUSA300_1934 | hypothetical | phi77 ORF220-like protein, phage major tail protein | 18.68 ± 3.51           |
| SAUSA300_1279 | phoU      | phosphate transport system regulatory protein PhoU | 18.68 ± 4.74           |
| SAUSA300_1217 | hypothetical | ABC transporter ATP-binding protein              | 18.64 ± 8.42           |
| SAUSA300_0468 | hypothetical | TatD family hydrolase                           | 18.62 ± 0.90           |
| SAUSA300_2132 | hypothetical | hypothetical protein                             | 18.54 ± 17.28          |
| SAUSA300_0288 | essD/essD | hypothetical protein                             | 18.50 ± 12.03          |
| SAUSA300_2461 | hypothetical | glyoxalase family protein                        | 18.38 ± 6.48           |
| SAUSA300_1349 | bolA      | glycosyl transferase, group 1 family protein     | 18.26 ± 11.03          |
| SAUSA300_1009 | tycA      | GTP-binding protein                              | 18.22 ± 6.42           |
| SAUSA300_1755 | spID      | serine protease SpID                             | 18.20 ± 6.01           |
| SAUSA300_1966 | hypothetical | phi77 ORF14-like protein, phage anti-repressor protein | 18.04 ± 5.61           |
| SAUSA300_1307 | arlS      | sensor histidine kinase protein                  | 18.01 ± 7.14           |
| SAUSA300_1918 | lhb       | truncated beta-hemolysin                        | 17.91 ± 11.34          |
| SAUSA300_1569 | hypothetical | U32 family peptidase                            | 17.90 ± 6.37           |
| SAUSA300_1397 | hypothetical | phiSLT ORF213-like protein, major tail protein   | 17.88 ± 16.40          |
| SAUSA300_1032 | hypothetical | putative iron compound ABC transporter iron compound-binding protein | 17.87 ± 9.01           |
| SAUSA300_0259 | hypothetical | PTS system, IIA component                       | 17.72 ± 4.08           |
| SAUSA300_1070 | hypothetical | hypothetical protein                             | 17.66 ± 6.61           |
| SAUSA300_1474 | hypothetical | hypothetical protein                             | 17.57 ± 5.94           |
| SAUSA300_1451 | hypothetical | short chain dehydrogenase/reductase family oxidoeductase | 17.47 ± 4.46           |
| SAUSA300_0769 | hypothetical | hypothetical protein                             | 17.42 ± 7.43           |
| SAUSA300_2098 | arsR      | ArsR family transcriptional regulator           | 17.36 ± 8.42           |
| SAUSA300_0094 | hypothetical | hypothetical protein                             | 17.32 ± 9.77           |
| SAUSA300_1470 | ipaA      | geranyltransferase                               | 17.29 ± 13.19          |
| SAUSA300_1403 | hypothetical | hypothetical protein                             | 17.28 ± 10.80          |
| SAUSA300_2432 | hypothetical | MdtT/NeiDIX family hydrolase                    | 17.26 ± 15.62          |
| Locus       | Gene Name                  | Description                                      | % EC Damage (Mean ± SD) |
|-------------|----------------------------|-------------------------------------------------|------------------------|
| SAUSA300_0631 | hypothetical               | putative nucleoside transporter                 | 17.25 ± 11.20          |
| SAUSA300_1000 | potB                       | spermidine/putrescine ABC transporter permease   | 17.14 ± 5.86           |
| SAUSA300_2559 | hypothetical               | DNA-binding response regulator                   | 17.10 ± 8.85           |
| SAUSA300_2467 | srtA                       | sortase                                          | 17.01 ± 6.72           |
| SAUSA300_2300 | hypothetical               | transcriptional regulator, TetR family           | 16.92 ± 5.04           |
| SAUSA300_0916 | hypothetical               | hypothetical protein                            | 16.89 ± 2.85           |
| SAUSA300_1444 | srsB                      | segregation and condensation protein B           | 16.85 ± 6.40           |
| SAUSA300_0995 | hypothetical               | branched-chain alpha-keto acid dehydrogenase subunit E2 | 16.83 ± 18.68 |
| SAUSA300_0419 | hypothetical               | tandem lipoprotein                               | 16.78 ± 3.58           |
| SAUSA300_1563 | accC                       | acetyl-CoA carboxylase, biotin carboxylase       | 16.73 ± 11.04          |
| SAUSA300_2027 | alr                        | alanine racemase                                 | 16.70 ± 16.05          |
| SAUSA300_2607 | hisA                       | phoribosyl-5-((5-phosphoribosylamino)methylideneamino) imidazole-4-carboxamide | 16.70 ± 11.46 |
| SAUSA300_0023 | hypothetical               | hypothetical protein                            | 16.69 ± 16.09          |
| SAUSA300_1622 | tig                        | trigger factor                                   | 16.44 ± 5.67           |
| SAUSA300_0011 | hypothetical               | hypothetical protein                            | 16.37 ± 4.02           |
| SAUSA300_1097 | pyrF                      | orotidine 5'-phosphate decarboxylase             | 16.34 ± 8.94           |
| SAUSA300_1339 | hypothetical               | hypothetical protein                            | 16.25 ± 4.99           |
| SAUSA300_0585 | hypothetical               | hypothetical protein                            | 16.24 ± 13.38          |
| SAUSA300_0839 | nfu                        | hypothetical protein                            | 16.23 ± 12.30          |
| SAUSA300_0071 | hypothetical               | IS539-like transposase                           | 16.19 ± 3.17           |
| SAUSA300_0651 | hypothetical               | CHAP domain-contain protein                     | 16.09 ± 6.91           |
| SAUSA300_1599 | hypothetical               | hypothetical protein                            | 16.02 ± 7.75           |
| SAUSA300_1607 | hypothetical               | hypothetical protein                            | 16.02 ± 8.76           |
| SAUSA300_0588 | hypothetical               | hypothetical protein                            | 15.86 ± 15.72          |
| SAUSA300_2276 | hypothetical               | peptidase, M20/M25/M40 family                   | 15.84 ± 1.33           |
| SAUSA300_2055 | murA                       | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | 15.79 ± 10.49          |
| SAUSA300_0808 | hypothetical               | hypothetical protein                            | 15.69 ± 12.88          |
| SAUSA300_0759 | glmB                       | phosphoglyceromutase                            | 15.68 ± 9.84           |
| SAUSA300_0857 | rhpB                      | hypothetical protein                            | 15.66 ± 7.46           |
| SAUSA300_1051 | hypothetical               | hypothetical protein                            | 15.51 ± 14.05          |
| SAUSA300_1383 | hypothetical               | phiSLT ORF484-like protein, Lysin                | 15.46 ± 15.13          |
| SAUSA300_1566 | hypothetical               | hypothetical protein                            | 15.42 ± 14.25          |
| SAUSA300_2040 | hypothetical               | hypothetical protein                            | 15.42 ± 12.63          |
| SAUSA300_1145 | xrcC                       | tyrosine recombinase, xerC                       | 15.33 ± 4.57           |
| SAUSA300_0687 | hypothetical               | putative hemolysin                              | 15.14 ± 12.23          |
| SAUSA300_0630 | hypothetical               | ABC transporter ATP-binding protein              | 15.07 ± 10.45          |
| SAUSA300_1577 | hypothetical               | TPR domain-containing protein                   | 14.93 ± 1.75           |
| SAUSA300_1288 | dapA                       | dihydroidipicolinate synthase                    | 14.75 ± 7.53           |
| SAUSA300_1937 | hypothetical               | phi77 ORF045-like protein                       | 14.69 ± 8.83           |
| SAUSA300_1419 | hypothetical               | phiSLT ORF80-like protein                       | 14.65 ± 9.06           |
| SAUSA300_2245 | nirD                       | nitrite reductase (NAD(P)H), small subunit       | 14.54 ± 6.44           |
| SAUSA300_1365 | rpsA                       | 30S ribosomal protein S1                         | 14.48 ± 8.05           |
| SAUSA300_0029 | hypothetical               | hypothetical protein                            | 14.39 ± 3.30           |
| SAUSA300_2575 | hypothetical               | BgG family transcriptional antiterminator       | 14.12 ± 4.67           |
| SAUSA300_1497 | hypothetical               | glycine dehydrogenase subunit 1                 | 14.08 ± 4.09           |
| SAUSA300_1682 | cpvA                       | catabolite control protein A                     | 14.04 ± 8.43           |
| SAUSA300_0657 | hypothetical               | putative endodeoxyribonuclease RusA             | 13.92 ± 10.12          |
| SAUSA300_1955 | hypothetical               | sodium transport family protein                 | 13.85 ± 14.78          |
| SAUSA300_0924 | ktrD                       | ABC transporter ATP-binding protein              | 13.80 ± 6.67           |
| SAUSA300_0077 | hypothetical               | pyridoxal biosynthesis lyase, PdxS              | 13.58 ± 7.70           |
| SAUSA300_0504 | ptxS                       | transcriptional regulator                       | 13.06 ± 13.37          |
| SAUSA300_0195 | hypothetical               | DNA-binding response regulator                   | 13.05 ± 5.02           |
| SAUSA300_1308 | antR                      | NADH-dependent flavin oxidoreductase            | 12.99 ± 7.37           |
| SAUSA300_0859 | hypothetical               | hypothetical protein                            | 12.97 ± 3.93           |
| SAUSA300_1721 | argC                       | N-acetyl-gamma-glutamyl-phosphate reductase      | 12.92 ± 16.00          |
| SAUSA300_2641 | hypothetical               | hypothetical protein                            | 12.90 ± 8.36           |
| SAUSA300_0987 | hypothetical               | cytochrome D ubiquinol oxidase, subunit II       | 12.85 ± 10.22          |
| SAUSA300_1496 | dat                        | D-alanine aminotransferase                      | 12.71 ± 5.48           |
| SAUSA300_1283 | hypothetical               | phosphate ABC transporter, phosphate-binding protein PstS (dimethylallyl)adenosine RNA methylthiotransferase | 12.73 ± 9.23 |
| SAUSA300_1185 | mibB                       | gamma-hemolysin component A                     | 12.62 ± 10.40          |
| SAUSA300_2365 | hlgA                       | hypothetical protein                            | 12.56 ± 10.54          |
| SAUSA300_1394 | sirC                       | iron compound ABC transporter permease SirC     | 12.30 ± 6.17           |
| SAUSA300_2284 | hypothetical               | hypothetical protein                            | 12.20 ± 10.36          |
| SAUSA300_2225 | nypF                       | molybdenum cotator biosynthesis protein MoaC     | 12.05 ± 9.05           |
| Locus          | Gene Name          | Description                              | % EC Damage (Mean ± SD) |
|---------------|--------------------|------------------------------------------|-------------------------|
| SAUSA300_0244 | hypothetical       | zinc-binding dehydrogenase family oxidoreductase | 12.05 ± 9.79           |
| SAUSA300_2022 | rpoF               | RNA polymerase sigma factor SigB           | 12.05 ± 6.83            |
| SAUSA300_1089 | lppA               | lipoprotein signal peptidase               | 11.97 ± 6.81            |
| SAUSA300_1618 | hemX               | hemA concentration negative effector hemX | 11.88 ± 1.05            |
| SAUSA300_0117 | sirA               | iron compound ABC transporter iron compound-binding protein SirA | 11.83 ± 7.64 |
| SAUSA300_0899 | mscA               | adaptor protein                            | 11.58 ± 10.37           |
| SAUSA300_2492 | hypothetical       | acetyltransferase family protein           | 11.58 ± 9.50            |
| SAUSA300_1433 | hypothetical       | putative phage regulatory protein          | 11.41 ± 8.17            |
| SAUSA300_1244 | mscL               | large conductance mechanosensitive channel protein | 11.32 ± 7.21 |
| SAUSA300_0049 | hypothetical       | hypothetical protein                       | 11.30 ± 0.62            |
| SAUSA300_1667 | hypothetical       | putative glycophosphoryl diester phosphodiesterase | 11.30 ± 7.51 |
| SAUSA300_0994 | pdIB               | pyruvate dehydrogenase E1 component, beta subunit | 11.20 ± 8.12 |
| SAUSA300_0974 | putN               | phosphoribosylglycinamide formyltransferase | 11.07 ± 8.08            |
| SAUSA300_0067 | hypothetical       | universal stress protein                   | 11.02 ± 9.02            |
| SAUSA300_1590 | rsh (relA)         | GTP pyrophosphokinase                      | 10.95 ± 7.18            |
| SAUSA300_0526 | hypothetical       | methyltransferase small subunit            | 10.80 ± 10.78           |
| SAUSA300_0952 | hypothetical       | aminotransferase, class I                  | 10.57 ± 6.79            |
| SAUSA300_1694 | trnB               | tRNA (guanine-N(7)-)-methyltransferase     | 10.55 ± 16.08           |
| SAUSA300_0041 | hypothetical       | hypothetical protein                       | 10.41 ± 2.09            |
| SAUSA300_1449 | hypothetical       | MufT/nudix family protein                  | 10.11 ± 13.24           |
| SAUSA300_0724 | hypothetical       | hypothetical protein                       | 10.06 ± 2.60            |
| SAUSA300_1757 | sipB               | serine protease SpIB                       | 9.41 ± 4.17             |
| SAUSA300_0476 | hypothetical       | hypothetical protein                       | 9.18 ± 8.05             |
| SAUSA300_2052 | hypothetical       | single-stranded DNA-binding protein family | 9.11 ± 18.19            |
| SAUSA300_2176 | chiO               | cobalt transporter ATP-binding subunit      | 9.03 ± 9.11             |
| SAUSA300_1112 | stp1               | protein phosphatase 2C domain-containing protein | 8.98 ± 14.19 |
| SAUSA300_0789 | hypothetical       | putative thioredoxin                       | 8.89 ± 18.33            |
| SAUSA300_0379 | ahpF               | alkyl hydroperoxide reductase subunit F     | 8.46 ± 4.49             |
| SAUSA300_0548 | tatA               | twin arginine-targeting protein translocase | 8.36 ± 5.33             |
| SAUSA300_0469 | runMV              | hypothetical protein                       | 8.35 ± 0.35             |
| SAUSA300_1792 | hypothetical       | hypothetical protein                       | 8.20 ± 4.58             |
| SAUSA300_2061 | atpH               | F0F1 ATP synthase subunit delta            | 7.98 ± 1.29             |
| SAUSA300_1092 | pyrP               | uracil permease                            | 7.85 ± 2.60             |
| SAUSA300_0905 | hypothetical       | hypothetical protein                       | 7.61 ± 3.76             |
| SAUSA300_0444 | gflC                | LysR family regulatory protein             | 7.39 ± 2.70             |
| SAUSA300_2646 | trmE               | tRNA modification GTase TrmE               | 7.41 ± 8.81             |
| SAUSA300_2105 | wtE                 | PTS system, mannotol specific IIBC component | 6.95 ± 5.04 |
| SAUSA300_2486 | clpL                | putative ATP-dependent Clp protease        | 6.73 ± 0.02             |
| SAUSA300_1887 | pcrB               | geranylgeranylglycerol phosphate synthase-like protein | 6.58 ± 3.46 |
| SAUSA300_1653 | hypothetical       | metal-dependent hydrolase                 | 6.25 ± 8.63             |
| SAUSA300_2393 | opuCa              | glycine betaine/carnitine/choline ABC transporter ATP-binding protein | 6.25 ± 7.87 |
| SAUSA300_1183 | hypothetical       | 2-oxoglutarate ferredoxin oxidoreductase subunit beta | 6.19 ± 1.88 |
| SAUSA300_0393 | hypothetical       | hypothetical protein                       | 6.18 ± 2.30             |
| SAUSA300_0174 | hypothetical       | hypothetical protein                       | 6.15 ± 1.39             |
| SAUSA300_0841 | hypothetical       | hypothetical protein                       | 5.97 ± 2.99             |
| SAUSA300_1096 | carb                | carbamoyl phosphate synthase large subunit | 5.89 ± 2.89             |
| SAUSA300_2593 | hypothetical       | hypothetical protein                       | 5.84 ± 3.04             |
| SAUSA300_0221 | pflA                | pyruvate formate-lyase activating enzyme    | 5.68 ± 18.96            |
| SAUSA300_0996 | lydA                | dihydrolipoamide dehydrogenase             | 5.49 ± 2.87             |
| SAUSA300_1992 | agrA                | accessory gene regulator protein A          | 5.34 ± 14.81            |
| SAUSA300_1147 | hisU                | ATP-dependent protease ATP-binding subunit HslU | 4.99 ± 6.72 |
| SAUSA300_1120 | recG                | ATP-dependent DNA helicase RecG            | 4.60 ± 0.15             |
| SAUSA300_2078 | marA                | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | 3.18 ± 3.15 |
| SAUSA300_1583 | endA                | hypothetical protein                       | 2.48 ± 0.46             |
| SAUSA300_0992 | hypothetical       | hypothetical protein                       | 2.24 ± 20.30            |
| SAUSA300_0634 | flhB                | ferrichrome transport permease flhB        | 2.22 ± 4.57             |
| SAUSA300_0750 | whiA                | hypothetical protein                       | 1.88 ± 4.32             |
| SAUSA300_2485 | hypothetical       | methylated DNA-protein lysine methyltransferase | 1.78 ± 9.18 |
| SAUSA300_0426 | hypothetical       | hypothetical protein                       | 1.75 ± 5.09             |
| SAUSA300_2598 | capA                | capsular polysaccharide biosynthesis protein Cap1A | 0.85 ± 1.35 |
| SAUSA300_2246 | hypothetical       | hypothetical protein                       | 0.51 ± 16.59            |
| SAUSA300_2518 | hypothetical       | hydrolase family protein                   | 0.42 ± 7.59             |
| SAUSA300_0355 | hypothetical       | acetyl-CoA acetyltransferase               | 0.68 ± 4.44             |
| SAUSA300_0398 | hypothetical       | superantigen-like protein                  | 0.83 ± 2.42             |
| SAUSA300_2226 | moxB                | molybdenum cofactor biosynthesis protein B  | 1.15 ± 5.09             |
| SAUSA300_0945 | hypothetical       | isochorismate synthase family protein      | 1.17 ± 2.14             |
Table 1. Cont.

| Locus     | Gene Name | Description                                               | % EC Damage (Mean ± SD) |
|-----------|-----------|-----------------------------------------------------------|-------------------------|
| SAUSA300_0904 | gylL       | hypothetical protein                                       | −1.32 ± 0.61           |
| SAUSA300_0423 | hypothetical | hypothetical protein                                     | −2.20 ± 0.08           |
| SAUSA300_1422 | hypothetical | phISLT ORF65-like protein                                | −2.77 ± 6.35           |
| SAUSA300_0068 | hypothetical | cadmium-exporting ATPase, truncation                     | −2.79 ± 8.95           |
| SAUSA300_1870 | hypothetical | hypothetical protein                                     | −2.92 ± 15.58          |
| SAUSA300_1139 | sacD       | succinyl-CoA synthetase subunit alpha                     | −2.94 ± 8.32           |
| SAUSA300_0918 | ugdP       | diacylglycerol glucosyltransferase                        | −3.09 ± 8.63           |
| SAUSA300_0597 | hypothetical | putative endonuclease III                                | −3.15 ± 14.78          |
| SAUSA300_0326 | hypothetical | hypothetical protein                                     | −3.64 ± 2.40           |
| SAUSA300_0690 | sacS       | sensor histidine kinase SaeS                              | −4.88 ± 14.01          |
| SAUSA300_0560 | traB       | acetyl-CoA c-acetyltransferase                            | −5.06 ± 6.53           |
| SAUSA300_2334 | hypothetical | hypothetical protein                                     | −5.12 ± 7.55           |
| SAUSA300_2025 | rsbU       | sigma-B regulation protein                                | −5.19 ± 6.08           |
| SAUSA300_2152 | lacD       | tagatose 1,6-diphosphate aldolase                        | −5.59 ± 11.59          |
| SAUSA300_1680 | acuA       | acetoin utilization protein AcuA                           | −5.94 ± 10.87          |
| SAUSA300_2024 | rsbV       | anti-sigma-B factor, antagonist                           | −6.77 ± 14.71          |
| SAUSA300_0618 | mntC       | ABC transporter substrate-binding protein                 | −6.85 ± 4.61           |
| SAUSA300_1680 | hypothetical | DNA polymerase IV                                        | −6.91 ± 9.59           |
| SAUSA300_1465 | hypothetical | 2-oxoisovalerate dehydrogenase, E1 component, beta subunit | −7.15 ± 6.73           |
| SAUSA300_1573 | hypothetical | Holliday junction resolvase-like protein                  | −10.10 ± 6.68          |
| SAUSA300_1473 | musB       | transcription antitermination protein NusB                | −10.84 ± 10.00         |
| SAUSA300_1357 | aroC       | chorismate synthase                                       | −11.88 ± 0.89          |
| SAUSA300_1095 | carA       | carbamoyl phosphate synthase small subunit               | −14.12 ± 10.52         |
| SAUSA300_1469 | argR       | arginine repressor                                       | −14.16 ± 8.61          |
| SAUSA300_1615 | hcmB       | delta-aminolevulinic acid dehydratase                    | −14.95 ± 14.12         |
| SAUSA300_1467 | lpdA       | dihydrolipoamide dehydrogenase                           | −15.68 ± 14.07         |
| SAUSA300_0993 | pdhA       | pyruvate dehydrogenase E1 component, alpha subunit       | −17.05 ± 10.66         |
| SAUSA300_0752 | cipP       | ATP-dependent Clp protease proteolytic subunit            | −17.66 ± 11.34         |
| SAUSA300_1715 | ribD       | riboflavin biosynthesis protein                           | −23.78 ± 4.28          |

Note: EC damage below zero is due to the A560nm value of the mutant being higher than the A560nm of the negative control.

Table 2. Mutants significantly increase HMEC-1 damage vs. JE2 WT strain (EC damage rate ≥ 60%).

| Locus     | Gene Name | Description                                               | % EC Damage (Mean ± SD) |
|-----------|-----------|-----------------------------------------------------------|-------------------------|
| SAUSA300_1197 | ND ^a  | glutathione peroxidase                                     | 62.86 ± 5.67           |
| SAUSA300_1333 | hypothetical | conserved hypothetical protein                           | 62.17 ± 3.05           |
| SAUSA300_1485 | hypothetical | conserved hypothetical protein                           | 61.86 ± 6.12           |
| SAUSA300_2221 | moaD      | molybdopterin converting factor, subunit 1                | 61.64 ± 3.61           |
| SAUSA300_0206 | azoR      | flavodoxin family protein                                 | 60.82 ± 6.24           |
| SAUSA300_0335 | mecA      | MATE efflux family protein                                | 60.15 ± 8.31           |

Note: ^a ND: not determined.

Table 3. Verification of EC damage of JE WT strain and selected mutants using 24-well plates assay.

| Locus     | Group   | Gene Name | % EC Damage (Mean ± SD) |
|-----------|---------|-----------|-------------------------|
| SAUSA300_0904 | hypothetical | hypothetical | 62.86 ± 5.67 |
| SAUSA300_0423 | hypothetical | hypothetical | 62.17 ± 3.05 |
| SAUSA300_1485 | hypothetical | hypothetical | 61.86 ± 6.12 |
| SAUSA300_2221 | hypothetical | hypothetical | 61.64 ± 3.61 |
| SAUSA300_0206 | hypothetical | hypothetical | 60.82 ± 6.24 |
| SAUSA300_0335 | hypothetical | hypothetical | 60.15 ± 8.31 |
Table 3. Cont.

| Locus            | Group     | Gene Name | % EC Damage (Mean ± SD) | 384-Well Plates | 24-Well Plates |
|------------------|-----------|-----------|-------------------------|-----------------|----------------|
| SAUSA300_1040    | hypothetical |          | 26.74 ± 8.21            | 30.92<sup>a</sup> |
| SAUSA300_1875    | hypothetical |          | 24.52 ± 10.68           | 30.51<sup>a</sup> |
| SAUSA300_0871    | hypothetical |          | 24.49 ± 12.19           | 28.60<sup>a</sup> |
| SAUSA300_1950    | hypothetical |          | 23.24 ± 9.64            | 25.87<sup>a</sup> |
| SAUSA300_0253    | scdA       |          | 21.83 ± 12.24           | 22.52<sup>a</sup> |
| SAUSA300_0649    | hypothetical |          | 20.24 ± 0.89            | 22.65<sup>a</sup> |
| SAUSA300_2587    | hypothetical |          | 20.06 ± 9.42            | 26.45<sup>a</sup> |
| SAUSA300_0631    | hypothetical |          | 17.25 ± 11.20           | 23.00<sup>a</sup> |
| SAUSA300_2027    |            | air       | 16.70 ± 16.05           | 3.28 ± 1.38     |
| SAUSA300_2055    |            | murA      | 15.79 ± 10.49           | 7.62 ± 0.59     |
| SAUSA300_1682    |            | ccpA      | 14.04 ± 8.43            | 13.43<sup>a</sup> |
| SAUSA300_1696    |            | dat       | 12.74 ± 5.48            | 14.99 ± 1.34    |
| SAUSA300_0974    |            | purN      | 11.07 ± 8.08            | 20.58<sup>a</sup> |
| SAUSA300_1563    |            | accC      | 16.73 ± 11.04           | 11.82 ± 0.72    |
| SAUSA300_0041    |            | hypothetical | 10.41 ± 2.09           | 3.30<sup>a</sup> |
| SAUSA300_0994    |            | pdhB      | 11.20 ± 8.12            | 19.36<sup>a</sup> |
| SAUSA300_0186    |            | argC      | 12.92 ± 16.00           | 15.20 ± 2.13    |
| SAUSA300_1992    |            | agrA      | 5.34 ± 14.81            | −3.82 ± 1.77    |
| SAUSA300_0355    |            | hypothetical | −0.68 ± 4.44           | −1.20<sup>a</sup> |
| SAUSA300_0690    |            | sacS      | −4.89 ± 14.01           | −12.80 ± 1.77   |

<sup>a</sup> Verification of these mutants was performed once using the 24-well plates assay.

Table 4. Numbers of genes from different KEGG pathway categories.

| Categories               | Sub-Groups                  | No. of Mutants with Decreased HMEC-1 Damage | No. of Mutants with Increased HMEC-1 Damage |
|--------------------------|-----------------------------|---------------------------------------------|--------------------------------------------|
| Metabolism               | Carbohydrate metabolism     | 53                                          |                                            |
| Metabolism               | Amino acid metabolism       | 33                                          |                                            |
| Metabolism               | Metabolism of cofactors and vitamins | 11                                          |                                            |
| Metabolism               | Lipid metabolism            | 8                                           | 1                                          |
| Metabolism               | Nucleotide metabolism       | 8                                           |                                            |
| Metabolism               | Biosynthesis of other secondary metabolites | 7                                           |                                            |
| Metabolism               | Energy metabolism           | 7                                           |                                            |
| Metabolism               | Metabolism of other amino acids | 3                                           | 1                                          |
| Metabolism               | Metabolism of terpenoids and polyketides | 3                                           |                                            |
| Metabolism               | Glycan biosynthesis and metabolism | 2                                           |                                            |
| Metabolism               | Xenobiotics biodegradation and metabolism | 1                                           |                                            |
| Genetic information processing | Homologous recombination | 4                                           |                                            |
| Genetic information processing | DNA replication           | 2                                           |                                            |
| Genetic information processing | Mismatch repair             | 2                                           |                                            |
| Genetic information processing | Protein export             | 2                                           |                                            |
| Genetic information processing | Ribosome                   | 2                                           |                                            |
| Genetic information processing | Sulfur relay system        | 2                                           | 1                                          |
| Genetic information processing | RNA degradation           | 1                                           |                                            |
| Environmental information processing | Two-component system   | 13                                          |                                            |
| Environmental information processing | ABC transporters         | 9                                           |                                            |
| Environmental information processing | Other                     | 3                                           |                                            |
| Cellular processes       | Quorum sensing             | 9                                           |                                            |
| Total                    |                             | 185                                         | 3                                          |
In the current study, we first verified the reliability of our high-throughput screening system. Consistent with previous reports [13,16], we demonstrated that the inactivation of global regulators such as agr, arlRS, or saeRS significantly decreases EC damage. In addition, we also noticed that clinical MRSA strains collected from patients with persistent bacteremia cause significantly greater EC damage compared to clinical resolving MRSA isolates [15]. Moreover, the inactivation of agr, saeR, and arlSR has been proved significantly reduce EC damage as compared to their respective parental strains [13,16]. However, these studies only focused on a few virulence factors in S. aureus. Thus, the current study was designed to broadly define genetic determiners in S. aureus which involve in human EC damage using a high-throughput approach to screen a transposon mutant library containing 1920 non-essential gene mutants in MRSA USA300 JE2 background.

In the current study, we further verified the reliability of our high-throughput screening system. Consistent with previous reports [13,16], we demonstrated that the inactivation of global regulators such as agr, arlRS, or saeRS significantly decreases EC damage. In addition, we also noticed that clinical MRSA strains collected from patients with persistent bacteremia cause significantly greater EC damage compared to clinical resolving MRSA isolates [15]. Moreover, the inactivation of agr, saeR, and arlSR has been proved significantly reduce EC damage as compared to their respective parental strains [13,16]. However, these studies only focused on a few virulence factors in S. aureus. Thus, the current study was designed to broadly define genetic determiners in S. aureus which involve in human EC damage using a high-throughput approach to screen a transposon mutant library containing 1920 non-essential gene mutants in MRSA USA300 JE2 background.
addition, consistent results were obtained between 384-well and 24-well plates assays, which validated the improvement of testing significantly more samples each time.

Several interesting and important observations emerged from the present investigations. Overall, over 320 mutants had a significant impact on the EC damage. The majority of these mutants significantly reduced EC damage vs. JE2 parental strain. Using KEGG pathway analysis, mutant strains were classified into four categories, including metabolism, genetic information processing, environmental information processing, and cellular processes (Figure 3). Only six mutants were found with significantly increased EC damage vs. JE2 parental strain. Importantly, many of these genes are not previously defined to impact human EC damage in *S. aureus*.

**Figure 3.** Genetic factors in MRSA JE2 strain contribute to the HMEC-1 damage by KEGG analysis. These factors may ultimately impact the pathogenesis and treatment outcome in MRSA endovascular infection.

Many staphylococcal genetic factors related to metabolism were shown to intimately impact the EC damage. For instance, several gene mutants related to carbohydrate metabolism including tricarboxylic acid (TCA) cycle (e.g., *pdhA*, and *lpdA*) showed significantly decreased EC damage. Inactivation of *pdhA* or *lpdA* was reported to be associated with slower growth [17,18]. Since the TCA cycle processes produce the main energy resources for cellular activities [19], inactivation of corresponding TCA genes may result in lack of energy which may subsequently cause slower growth and decrease EC damage. In addition, mutants with genes related to energy metabolism (e.g., *cyoE*, and *atpH*) also displayed lower EC damage rates vs. parental strain JE2. It has been reported that *cyoE* encoding a protoheme IX farnesyltransferase is essential for processing heme into the electron transport chain and plays a critical role in cytolytic toxins production in *S. aureus*. Deletion of *cyoE* in *S. aureus* significantly decreases the expression of cytolytic toxins [20]. Turner et al. reported that mutation of *aptH* (associated with ATP synthase) had attenuated virulence and less invasiveness in vivo [21]. These results suggest that genetic factors associated with energy metabolism have activities on EC damage that may link to virulence.
Lipid metabolism genes (e.g., gehB, and ugpP) were reported to promote biofilm formation and host cell invasion [22]. We found that the mutation of these genes had significantly decreased EC damage vs. JE2 parental strain. These results may indicate a connection between lipid metabolism and EC damage. Genetic factors associated with nucleotides metabolism (e.g., purN) were also found to positively impact the EC damage. purN encodes the enzyme in de novo purine biosynthesis pathway which generates ATP and GTP that can be processed to stringent response alarmone, guanosine 3′-diphosphate-5-di(tri)phosphate ((p)ppGpp) [15]. Increased GTP and subsequent (p)ppGpp levels lead to enhanced persistent bacteremia (PB) phenotypes including a higher EC damage rate [15]. It is worthwhile to mention, genes related to staphylococcal cell-wall peptidoglycan biosynthesis (e.g., murA) and cell division (e.g., scdA) showed significant positive effects on EC damage. Cell-wall synthesis has long been considered an important target for novel anti-S. aureus agents [23,24], and our findings have implications for the approach.

In the genetic information processing pathways, genes involved in homologous recombination (e.g., recD, and recG), ribosome (e.g., rrlA, and rpsA), and protein export (e.g., lspA, and tatA) were identified to affect EC damage. For example, the signal peptidase encoded by lspA is required for biogenesis of bacterial lipoproteins, and failure to produce mature lipoproteins has previously been shown to impair pathogenicity and immune-modulating [25]. The results suggested that some genes related to genetic information processing also play a role in human EC damage.

The inactivation of genes involved in environmental information processing pathways such as ABC transporter (e.g., fluB, and mntC) and two-component system (e.g., saeSR, and arlSR) also decreased EC damage. These findings were in accordance with previous studies showing the presence of these gene products was associated with higher in vivo virulence potential vs. their respective WT strains [15,26–28].

Genes involved in cellular process, specifically quorum sensing (e.g., agr, and luxS), were identified to contribute to the EC damage. It is well known that quorum sensing via agr plays a central role in the pathogenesis of S. aureus. Under high cell density, agr is responsible for the increased expression of many toxins which may impact the EC damage [16], while the function of luxS in S. aureus has not been well investigated.

Genes unidentified in the KEGG pathways also showed a positive impact on the HMEC-1 damage in the current study. Some of these genes have been previously demonstrated to correlate with biofilm formation (e.g., xcrC), oxidative killing (e.g., nfu, and ybfI), hemolysis (e.g., hlb), and heat shock (e.g., hslU) [29–32]. In addition, few phage genes (SAUSA300_1433, SAUSA300_1934, SAUSA300_1936, SAUSA300_1968) were also shown impacts on the HMEC-1 damage.

Mutants of six genes had elevated EC damage indicating their negative impact on the EC damage. Among these genes, mepA encodes a multidrug efflux pump protein [33], azoR encodes quinone reductase [34], moaD encodes one of the subunits of molydopterin synthase involved in sulfur relay system pathway [35], gene SAUSA300_1197 encodes glutathione peroxidase. Further investigations related to the relationship between these genes and EC damage are needed.

4. Materials and Methods

4.1. Bacteria and Growth Conditions

The strains used in the current study include MRSA JE2 (a plasmid-cured derivative of LAC USA300) and 1920 transposon non-essential gene mutants within the NTML [6]. The NTML was kindly provided by the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA). The library was supplied in five 384-well microtiter plates. The plates containing MRSA mutant strains were duplicated and cultured in tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). On the experiment day, bacterial strains were freshly inoculated in TSB media and cultured at 37 °C for 3 h to obtain logarithmic phase cells [36], and adjusted to an OD$_{600nm}$ of 0.500 ($\sim$10$^8$ CFU/mL) and diluted accordingly. S. aureus inocula were confirmed by quantitative culture.
4.2. Endothelial Cell (HMEC-1) Culture

The HMEC-1 cell line was obtained from Kathryn Kellar, of the Centers for Disease Control (CDC), in the U.S., and maintained as recommended [10]. Primary cells were established from human dermal microvascular endothelial cells and immortalized by transfection with a Pbr322-based plasmid containing the coding region for the simian virus 40 large T-antigen [10].

4.3. HMEC-1 Damage Assay

The effect of MRSA strains on EC damage was determined using a well-established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [13,37,38]. Briefly, logarithmic phase MRSA cells (1 × 10^5 CFU/well) were added to HMEC-1 cells in 384-well plates with a density of ~5 × 10^3 EC/well in MCDB131 medium to reach a multiplicity of infection (MOI) of 20, which JE2 parental caused ~50% HMEC-1 damage as established in our pilot experiments. After 3 hr invasion, extracellular MRSA cells were killed by adding lysostaphin (10 μg/mL) in full medium MCDB131 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% bovine calf serum, 2 mM glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin [13,37]. At 18 hr incubation at 37 °C, MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) in Hank’s Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated for 2 h, then the medium was replaced with 0.04 M HCl in absolute isopropanol (Thermo Fisher Scientific, Waltham, MA, USA) to stop the reaction and lyse the cells. Absorbance was measured at 560 nm (A560nm) using a microplate reader Synergy 2 (BioTek, Winooski, VT, USA). Uninfected HMEC-1 served as a negative control, and wells containing medium alone were used for background correction in each round. In addition, EC infected with ∆arlR in JE2 was selected as an additional control group as it was reported that arlSR inactivation leads to >70% reduction in human EC damage vs. JE2 parental strain [13]. EC damage was calculated using the following formula: 1 − (A560nm of test well/A560nm of 0% − damage control well) as previously described [37]. Each experiment was performed three times in triplicate.

4.4. Verification of the HMEC-1 Damage Screening Results

After the screening of the whole library, JE2 WT strain and 20 randomly selected mutant strains with significantly decreased EC damage were confirmed again with the same MTT method using 24-well plates. In addition, the mutant strains with significantly increased EC damage were also tested in 24-well plates to confirm the damage results with the same method.

4.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). p-values were determined using the paired rank-sum test between mutant and JE2 wild-type strains. p < 0.05 was considered statistically significant.

4.6. KEGG Enrichment Analysis

The genes that caused a significant change in EC damage were classified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper tool with the mode of Staphylococcus aureus subsp. aureus USA300-FPR3757 (saa) [39]. The genes from different KEGG pathway categories were further analyzed.

5. Conclusions

To our knowledge, the present study provides the first whole-genome screen to identify genetic factors that impact human EC damage in S. aureus. Importantly, we defined a set of staphylococcal genes, which are not previously known to be associated with EC damage, significantly contribute to this phenotype. Although these findings need to be further verified using mutation strains generated by gene deletion and complementation
techniques, our results provide new insights into the relationship between genetic factors and EC damage in *S. aureus*. These genetic factors may be ideal targets for the development of effective therapeutic strategies to treat invasive MRSA endovascular infection.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antibiotics11030316/s1, Table S1: HMEC-1 damage caused by all the mutant strains, except mutants presented in Tables 1 and 2 in the NTML.

**Author Contributions:** Y.Q.X. designed the study. X.X. and L.L. performed the experiments. Y.L., X.X. and Y.Q.X. performed data analysis and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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