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Screening of Essential Genes in *Staphylococcus aureus* N315 Using Comparative Genomics and Allelic Replacement Mutagenesis

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Abstract To find potential targets of novel antimicrobial agents, we identified essential genes of *Staphylococcus aureus* N315 by using comparative genomics and allele replacement mutagenesis. By comparing the genome of *S. aureus* N315 with those of *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae*, a total of 481 candidate target genes with similar amino acid sequences with at least three other species by >40% sequence identity were selected. Of 481 disrupted candidate genes, 122 genes were identified as essential genes for growth of *S. aureus* N315. Of these, 51 essential genes were those not identified in any bacterial species, and 24 genes encode proteins of unknown function. Seventeen genes were determined as non-essential although they were identified as essential genes in other strain of *S. aureus* and other species. We found no significant difference among essential genes between *Streptococcus pneumoniae* and *S. aureus* with regard to cellular function.

Key words: *Staphylococcus aureus*, essential genes, genomics, allelic replacement mutagenesis, new antimicrobial agent

Although the emergence and spread of antibiotic resistance in major bacterial pathogens for the past decades pose a growing challenge to public health, discovery of novel antimicrobial agents from natural products or modification of existing antibiotics cannot circumvent the problem of antimicrobial resistance. The recent development of bacterial genomics and availability of genomic sequences have allowed the identification of potentially novel antibacterial targets [1]. Although the identification of new drug targets does not guarantee the development of new chemical compound, it could be the first step towards the discovery of novel antibiotics to combat such resistant pathogens. The global effort to completely sequence bacterial genomes has generated a large amount of raw material for further analyses. Specifically, genomics can be applied to evaluate the suitability of potential targets for new antimicrobial drugs, based on the criteria of "essentiality" or "selectivity" [2]. The target for new antimicrobial drugs must be essential for the growth, replication, or survival of the bacterium. Genes that are conserved in different bacterial genomes often turn out to be essential [3–5]. Thus, the combination of comparative genomics and the gene knock-out system provides effective ways to identify the essential genes of bacterial pathogens [6]. Identification of essential genes in bacteria may be utilized for the development of new antimicrobial agents, because common essential genes in diverse pathogens could be novel targets for broad-spectrum antimicrobial agents.

Recently, compilation of essential genes identified in several bacteria, including *Mycoplasma genitalium*, *Haemophilus influenzae*, *Vibrio cholerae*, *Bacillus subtilis*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*, has been available [7]. Previously, using the inducible expression method of antisense RNA, two independent study groups identified 186 conserved essential genes in *S. aureus*, which is the most frequent causative agent of nosocomial infections [8–10]. However, their studies randomly screened essential or non-essential genes, and thus many essential genes of *S. aureus* may not have been included in their list of essential genes. In the present study, we found new essential genes in *S. aureus* by using comparative genomics, followed by allele replacement mutagenesis.

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Bacterial Strains and Selection of Target Genes

S. aureus N315 strain, the genome sequence of which has been completely determined [11], was used in this study. The strain was subcultured and maintained routinely on tryptic soy broth (TSB) or agar (TSA) (Difco, Becton-Dickinso, Sparks, MD, U.S.A.) supplemented with 2% lysed sheep blood. Genome sequence data of *S. aureus* N315 were obtained from a TIGR (The Institute for Genomic Research) database (http://www.tigr.org). Target genes were selected using Microbial Concordance tool as follows: (i) a total of 2,592 ORFs were compared, (ii) amino acid sequences of *S. aureus* N315 were compared with those of *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*, and (iii) genes that have similar amino acid sequences with at least three other species by >40% sequence identity were selected.

Allelic Replacement Mutagenesis

The scheme for allelic replacement mutagenesis for the generation of the *S. aureus* knock-out mutant is presented in Fig. 1, which is a modification of the method of Song *et al.* [12]. An erythromycin resistance cassette (958 bp) containing the Em^R gene of plasmid pE194 was amplified

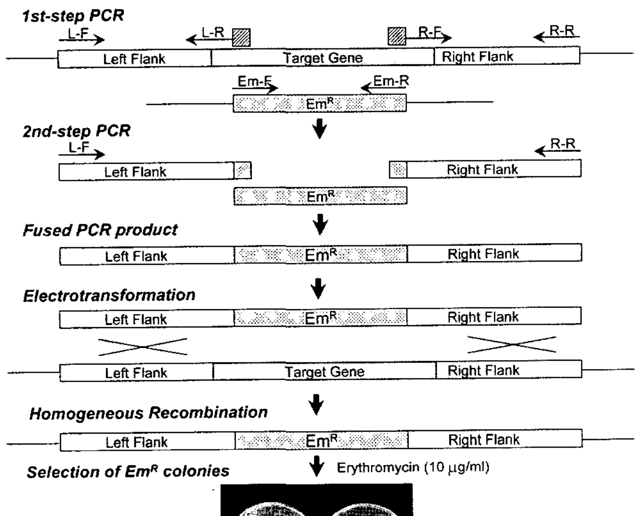

Fig. 1. Overall scheme of allelic replacement mutagenesis and gene knock-out by homologous recombination used in this study. In the first PCR reaction, an erythromycin resistance cassette (958 bp) containing the Em^R gene of plasmid pE194 and up- and downstream regions of target genes were amplified. In the second PCR reaction, the up- and downstream fragments were fused to the amplified Em^R gene by using primers L-F and R-R. The fused PCR product was then introduced into the *S. aureus* strain by electrotransformation. By homologous recombination, the target gene can be replaced with the Em^R gene. If the target gene is essential, no Em^R colony is obtained. Otherwise, the target gene is considered as non-essential.

with a primer set, Em-F (5'-CAA TAA TCG CAT CCG ATT GCA -3') and Em-R (5'-TTA CTT ATT AAA TAA TTT ATA GCT -3'). Two pairs of gene-specific primers, L-F/L-R and R-F/R-R, were used to amplify the left and right flanking regions of each target gene, generating PCR products of 500 to 800 bp in length. Primers L-R and R-F consisted of 21 nucleotides (5'-TGC AAT CGG ATG CGA TTA TTG-3' and 5'-TAT AAA TTA TTT AAT AAG TAA-3', respectively), which are identical to the promoter region, the 3'-end of the Em^R gene, and 23 nucleotides of a target gene-specific sequence. In order to minimize the potential polar effect in mutagenesis, primers were designed so that flanking genes and intergenic regions including potential promoters would remain intact in the mutants. In addition, transcriptional termination signals were removed from the erythromycin resistance gene marker (Em^R), and the cassettes were designed to integrate in the same orientation as the target genes to ensure transcription of the downstream region. PCR amplifications were run in a 96-well plate format under the following conditions: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec, and final extension of 72°C for 10 min. Each PCR product was purified using the Core-One PCR purification kit (Corebio system Co., Seoul, Korea) [13]. A template mixture of the amplified Em^R gene and two PCR products flanking the target gene were then subjected to PCR amplification to produce a linear fused product using primers L-F and R-R. The PCR condition of this step was carried out in a volume of 50 µl containing 2 µl each of the left and right flanking PCR products and the Em^R gene cassette, 5 µl of 10× buffer, 1 µl each of primers (L-F and R-R) (25 pmol/µl), 5 µl of dNTP mix (25 mM each), and 1 unit of *Taq* polymerase. The PCR condition used was as follows: 30 cycles of 94°C for 40 sec, 50°C for 40 sec, and 72°C for 2 min 30 sec, and the final extension of 72°C for 10 min.

To prepare *S. aureus* N315 competent cells, an overnight culture of *S. aureus* N315 was diluted 1 to 50 into TSB (100ml) and shaken at 37°C until OD_{660} of 0.3–0.8. The cells were collected by centrifugation at 8,000 ×g for 10 min and washed once with an equal volume of 10% glycerol. The cells were re-centrifuged and resuspended in 0.1 volume (approximately 10× concentration) of 10% glycerol. Aliquots (ca. 0.2 ml) of this suspension were used directly or stored at −80°C. The linear fused product was introduced into the chromosomal genome of *S. aureus* N315 by electrotransformation and homologous recombination.

As a result of introduction of the fused product into the genome of *S. aureus*, the Em^R gene cassette replaces the chromosomal copy of the target gene, thereby creating a gene knock-out. Electrotransformation was executed under the following conditions [14]. The 50 µl of *S. aureus* N315 competent cell was mixed with 1 µg of DNA samples in a 0.2-cm BioRad Gene Pulser cuvette and incubated on ice for 1 min. The cuvette was then placed in the sample chamber and was electroporated by a single pulse with the apparatus (BioRad Gene Pulser) set at 25 μF, 2.5 kV, and 100 Ω. Immediately after the pulse, 1.0 ml of SMMP medium [15] was added to the cuvette and mixed by inversion. The suspension was incubated at 42°C for 5 h in the presence of 10 μg/ml of erythromycin. The cells were then plated on blood agar plate containing 50 μg/ml of erythromycin, and then were grown at 37°C for 24 h in a CO_2 incubator. If no Em^R colony was obtained, the transformation was repeated at least twice more. Genes were regarded as essential if no colony was shown in all three transformations. If one or more Em^R colonies were obtained, the target genes were considered to be non-essential after the performance of transformation.

In a previous study, we developed a convenient and efficient method for identification of essential genes in bacteria and identified 133 essential gene sets in *S. pneumoniae* R6 [12]. Comparing other methods such as random insertional mutagenesis by transposon or plasmid, antisense RNA method, site-directed mutagenesis, and systematic gene inactivation [5, 8, 9, 16–21], this method has some advantages: First, based on simple criteria as indicated previously [16, 22], we could reduce the number of genes to be tested by stepwise filtering of ORFs through

Table 1. List of newly found essential genes of *S. aureus* N315.

N315 gene No.	Gene name	Gene description	Spn†	Bsu†	Eco†	Hin†	Mge†
I. Information storage and processing							
I-1. Translation, ribosomal structure, and biogenesis (J)							
SA0460	pth	Peptidyl-tRNA hydrolase	E		E	E	
SA0486	gltX	Glutamyl-tRNA synthetase (glutamate--tRNA ligase)	E	E	E	E	
SA0709	prfB	Peptide chain release factor 2, authentic frameshift	E	E	E		
SA0877	prfC	Peptide chain release factor 3				E	
SA1067	rpmB	Ribosomal protein L28		E	E		
SA1076	rnc	RNase III		E	E	E	
SA1113	rbfA	Ribosome-binding factor A					
SA1287	asnS	Asparaginyl-tRNA synthetase		E	E		
SA1359		Translation elongation factor P					
SA1404	rpsU	30S ribosomal protein S21		E	E		
SA1414	rpsT	30S ribosomal protein S20		E			
SA1704	map	Methionine aminopeptidase	E	E	E	E	
SA1713		RNA methyltransferase homolog		E	E		
SA1922	rpmE	Ribosomal protein L31		E	E		
SA2030	rpmD	50S ribosomal protein L30		E	E		
SA2039	rpmC	50S ribosomal protein L29		E	E		
SA2502	rnpA*	Ribonuclease P - protein component	E	E	E	E	
SAS033	rpmF	Ribosomal protein L32		E	E		
SAS047	rpmG	50S ribosomal protein L33		E	E		
I-2. Transcription (K)							
SA1109	nusA	Transcription termination	E	E	E		
SA1390	sigA	RNA polymerase major rho factor		E			
SA1438	greA	Transcription elongation factor GreA	E				
SA1923	rho	Transcription termination factor Rho			E		
I-3. DNA replication, recombination, and repair (L)							
SA0004	recF	Recombinational DNA repair ATPase (RecF pathway)					
SA0353	ssb	Single-strand DNA-binding protein		E	E		
SA0713	uvrB	Exonuclease ABC subunit B		NE		E	
SA0993	uvrC*	Exonuclease ABC subunit C					
SA1055	priA	Primosomal replication factor Y		E			
SA1093	topA	DNA topoisomerase I	E	E	E	E	
SA1128	recA	RecA/RadA recombinase					E
SA1391	dnaG	DNA primase	E	E	E	E	
SA1513	polA	DNA polymerase I			E		
SA1720	lig*	DNA ligase	E		E		
SA1792		Single-strand DNA-binding protein					

genome comparison with other species. Second, it was unnecessary to sequence target genes a posteriori for gene identification because of *a priori* knowledge of target genes. Third, it did not require a vector for recombination because allelic replacement mutagenesis could be completed only by two-step PCR. Fourth, this method could minimize the polar effect and be applied to both monocistronic and polycistronic genes [12].

Identification of essential genes by allelic replacement mutagenesis used in this study has been proven as accurate and useful in the previous studies [12, 23]. To confirm the method, we evaluated essential and non-essential genes in *S. aureus* N315, based on a previous report [8]. The mutant with knock-out non-essential genes, SA1525 (*dnaE*) and SA2434 (*fruA*), typically produced many colonies, whereas the mutants with knock-out of essential genes, SA0198 (*oppF*) and SA2442 (*secA*), produced no colonies. Mutant strains with successful recombination showed larger or smaller fragments on PCR than wild-type strain, similar to the result by Song *et al.* [12] (data not shown). Data from previous studies and this study suggested that identification of essential genes by allelic replacement mutagenesis is accurate and useful in *S. aureus*. Based on this confirmation, *S. aureus* genes that had already been reported as essential in previous studies [8, 9] were excluded in further selection and analysis.

The gene replacement of mutant clone was confirmed by PCR assay. Genomic DNAs of mutant and wild-type strains were used as templates in PCR amplification with primers L-F and R-R to verify the correct incorporation of the fused construct into the mutant genome. Thus, PCR reaction was carried out under the same condition as the step for fusion of three PCR products (30 cycles of 95°C for 40 sec, 50°C for 40 sec, and 72°C for 2 min 30 sec). Depending on target genes, the correct incorporation of the fused construct results in a larger or smaller PCR product in mutant than that in wild-type strain. By comparing the genome of *S. aureus* N315 with those of *B. subtilis*, *E. faecalis*, *E. coli*, *S. pneumoniae*, and *P. aeruginosa*, a total of 481 candidate target genes were selected by the criterion described above.

The essential genes of *S. aureus* that were identified in the present study are listed in Table 1. In this study, we identified 122 genes in *S. aureus* N315 that are essential for its growth. Of 122 essential genes, 51 were identified

Table 1. Continued.

N315 gene No.	Gene name	Gene description	Spn†	Bsu†	Eco†	Hin†	Mge†
II. Cellular processes							
II-1. Cell division and chromosome partitioning (D)							
SA0616	vraF	ABC transporter ATP-binding protein					
SA1028	ftsA	Cell division protein FtsA	E	E	E	E	
SA1852	vga	Hypothetical ABC transporter ATP-binding protein					
II-2. Post-translational modification, protein turnover, chaperones (O)							
SA1408	dnaJ	Molecular chaperones (contain C-terminal Zn finger domain)					
SA1409	dnaK	Molecular chaperone					
SA1837	groES	Protein fate: Protein folding and stabilization		E			
SA2082	ureA	Urease gamma subunit					
SA2083	ureB	Urease beta subunit					
SA2084	ureC	Urease alpha subunit					
II-3. Cell envelope biogenesis, outer membrane (M)							
SA0457	gcaD	UDP-N-acetylglucosamine pyrophosphorylase		E			
SA0997	murI	Glutamate racemase	E		E		
SA1025	mraY	Phospho-N-muramic acid-pentapeptide translocase	E	E	E		
SA1026	murD	UDP-N-acetylmuramoyl-L-alanine--D-glutamate ligase	E	E	E	E	
SA1251	murG	Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc GlcNAc transferase	E	E	E	E	
SA1561	murC	UDP-N-acetylmuramate-alanine ligase	E	E	E	E	
SA1886	murF	UDP-N-acetylmuramoylalanine-D-glutamyl-lysine-D-alanyl-D-alanine ligase	E	E	E		
SA1887	ddlA	D-Alanine-D-alanine ligase	E	E			
SA1959	glmS	Glucosamine-fructose-6-phosphate aminotransferase	E	E			
II-4. Cell motility and secretion (N)							
SA0206	msmX	Multiple sugar-binding transport ATP-binding protein					
II-5. Inorganic ion transport and metabolism (P)/Signal transduction (T)							
SA1054		Pantothenate metabolism flavoprotein homolog					
SA1557	ccpA	Catabolite control protein A					

Table 1. Continued.

N315 gene No.	Gene name	Gene description	**Spn†**	**Bsu†**	**Eco†**	**Hin†**	**Mge†**
III. Metabolism							
III-1. Energy production and conversion (C)							
SA0947	pdhD*	Dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3					
SA1244	odhB	Dihydrolipoamide succinyltransferase		E			
SA1524		Malate dehydrogenase homolog				E	
SA1533	ackA	Acetate kinase homolog					
SA1554	acsA	Acetyl-CoA synthetase					
SA2156		L-Lactate permease lctP homolog					
SA2185	narG	Respiratory nitrate reductase alpha chain					
SA2395	ldh	L-Lactate dehydrogenase				E	
	fer	Ferredoxin				E	
SA2406	gbsA	Glycine betaine aldehyde dehydrogenase gbsA					
III-2. Carbohydrate transport and metabolism (G)							
SA0134	drm	Phosphopentomutase					
SA0728	pgk	Phosphoglycerate kinase	E	E	E	E	
SA0729	tpi	Triose phosphate isomerase	E	E		E	
SA1510	gapB	Glyceraldehyde-3-phosphate dehydrogenase 2					
SA1962	mtlA	Mannitol PTS EII	E				
SA2326	ptsG	PTS system, glucose-specific IIABC component					
SA2435	pmi	Mannose-6-phosphate isomerase					
III-3. Amino acid transport and metabolism (E)							
SA0776	nifS	Pyridoxal-phosphate-dependent aminotransferase	E				
SA0847	oppD	Oligopeptide transport system ATP-binding protein OppD homolog					
SA0950	potA	Spermidine/putrescine ABC transporter, ATP-binding protein homolog					
SA1204	trpB	Tryptophan synthase beta chain	E			E	
SA1545	serA	Phosphoglycerate dehydrogenase	E			E	
SA0179		Ornithine aminotransferase					
III-4. Nucleotide transport and metabolism							
SA0374	pubX	Xanthine permease					
SA0375	guaB	Inositol-monophosphate dehydrogenase		E			
SA0376	guaA	GMP synthase				E	
SA0440	tmk	Thymidylate kinase homolog		E	E		
SA0458	prs	Ribose-phosphate pyrophosphokinase		E	E	E	
SA0686	nrdE	Ribonucleoside-diphosphate reductase (major subunit)	E	E			E
SA0924	purN	5-Phosphoribosylglycinamide transformylase 1	E			E	
SA1117	pnpA	Polyribonucleotide nucleotidyltransferase					
SA1260	thyA	Thymidylate synthase	E	E	E		E
SA1309	cmk	Cytidylate kinase		E	E	E	
III-5. Coenzyme metabolism (F)							
SA0785	lipA	Lipoic acid synthetase					
SA0898	menB	Naphthoate synthase		E		E	
SA1303	gerCD	Menaquinone biosynthesis methyltransferase					
SA1492	hemB	Delta-aminolevulinic acid dehydratase					
SA1728	nadE	NAD synthetase, prefers NH3 over glutamine		E	E		
III-6. Lipid metabolism (H)							
SA0869	fabI	Trans-2-enoyl-ACP reductase			E	E	
SA1072	plsX	Fatty acid/phospholipid synthesis protein		E			
SA1073	fabD	Malonyl CoA-acyl carrier protein transacylase	E	E	E	E	
SA1074	fabG	3-Oxoacyl-[acyl-carrier protein] reductase	E	E	E	E	
SA1126	pgsA	Phosphotidylglycerophosphate synthase	E	E	E	E	
SA1522	accA	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit	E	E	E	E	

Table 1. Continued.

N315 gene No.	Gene name	Gene description	**Spn**†	**Bsu**†	**Eco**†	**Hin**†	**Mge**†
IV. Poorly characterized							
IV-1. General function prediction only (R)							
SA0638	bacA	Bacitracin resistance protein	E				
SA1413	lepA*	GTP-binding protein					
SA1450		Iron-sulfur cofactor synthesis protein homolog					
SA1094	gid*	Glucose inhibited division protein gid					E
SA2344	copA	Copper-transporting ATPase copA					
SA2499	gidB	Glucose inhibited division protein B					E
IV-2. Function unknown (S)							
SA0021		Conserved hypothetical protein					
SA0085		Conserved hypothetical protein					
SA0181		Hypothetical protein					
SA0230		Conserved hypothetical protein					
SA0348		Hypothetical protein					
SA0422		Hypothetical protein					
SA0446		Conserved hypothetical protein					
SA0560		Conserved hypothetical protein					
SA0703		Conserved hypothetical protein					
SA0771		Conserved hypothetical protein					
SA0956		Hypothetical protein					
SA0998		Conserved hypothetical protein					
SA1147		Hypothetical protein					
SA1176		Conserved hypothetical protein					
SA1277		Conserved hypothetical protein					
SA1509		Conserved hypothetical protein					
SA2019		Conserved hypothetical protein					
SA2020		Hypothetical protein					

*Asterisks indicate genes that have been identified as essential in *S. aureus* in previous studies [8, 9].
†Essentiality in other species: Spn, *Streptococcus pneumoniae* [12, 16]; Bsu, *Bacillus subtilis* [5]; Eco, *Escherichia coli* (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp); Hin, *Haemophilus influenzae* [17]; Mge, *Mycoplasma genitalium* [18].

for the first time as essential genes, which had not been identified in any bacteria, judged by comparing the "Database of Essential Genes (DEG)" (http://tubic.tju.edu.cn/deg) [7]. Excluding poorly characterized genes, 31 genes were newly identified as essential in this study. These genes are classified on the basis of their clusters-of-orthologous-groups (COGs) functional categories [24], which are shown in Table 2. Whereas no essential genes were found to be related to post-translational modification, protein turnover, and chaperones (O) in previous studies [8, 9], we identified 6 essential genes with such cellular function in this study. In addition, we identified 3 essential genes related to cell division and chromosome partitioning (M), whereas only one essential gene (*ftsZ*) has been identified in the previous studies [8, 9]. We also identified 24 genes with unknown or poorly characterized function as essential. Most essential genes related to information storage and processing and cellular processes had been identified in other species. In addition, most essential genes related to nucleotide and lipid metabolism were also previously identified as essential in other species (Table 1). Newly identified essential genes in this study are concentrated in the categories of post-translational modification, protein turnover, chaperones (O), energy production and conversion (C), carbohydrate transport and metabolism (G), amino acid transport and metabolism (E), and coenzyme metabolism (H). However, this finding does not imply that the distribution of essential genes in *S. aureus* differs from other bacterial species, because of no significant difference of essential genes with regard to cellular function between *S. pneumoniae* and *S. aureus* [12]. Probably, our selection scheme for candidate genes to be mutated may select many conserved genes that have not been studied in the previous studies in some categories. This may indicate that screening of essential genes in bacteria may leave out many essential genes in most studies including our study. Genes that are essential in several species can be antimicrobial target and the essentiality of newly identified essential genes should be investigated in other bacterial species to explore more antimicrobial targets of broad spectrum.

Table 2. Functional classification of the 122 essential genes of *S. aureus*.

Cellular role*	Subtotal	Total
Information storage and processing		**34**
Translation, ribosomal structure, and biogenesis (J)	19	
Transcription (K)	4	
DNA replication, recombination, and repair (L)	11	
Cellular processes		**21**
Cell division and chromosome partitioning (D)	3	
Posttranslational modification, protein turnover, chaperones (O)	6	
Cell envelope biogenesis, outer membrane (M)	9	
Cell motility and secretion (N)	1	
Inorganic ion transport and metabolism (P)	1	
Signal transduction mechanisms (T)	1	
Metabolism		**43**
Energy production and conversion (C)	9	
Carbohydrate transport and metabolism (G)	7	
Amino acid transport and metabolism (E)	6	
Nucleotide transport and metabolism (F)	10	
Coenzyme metabolism (H)	5	
Lipid metabolism (I)	6	
Secondary metabolites biosynthesis, transport, and catabolism (Q)	-	
Poorly characterized		**24**
General function prediction only (R)	6	
Function unknown (S)	18	
Total		**122**

*Gene Classification was based on COG (Clusters of Orthologous Groups of proteins) functional categories of NCBI (http://www.ncbi.nlm.nih.gov/COG/).

We have found 17 genes, which are non-essential in *S. aureus* N315 but are essential in other strain of *S. aureus* and other species such as *S. pneumoniae*, *E. coli*, *B. subtilis*, *H. pylori*, and *H. influenzae* (Table 3). In particular, *purC* was identified as non-essential in *S. aureus* N315, although it had been identified as defective in another strain of *S. aureus*, WCHU29 [9]. In the screening of essential genes in *S. aureus* using the antisense RNA induction method, Ji *et al.* [9] identified two distinct phenotypes of essential genes; i.e., lethal (no-growth) and defective (giving rise to definitive small colonies). Several defective genes are known virulence factors. Although these defective genes were not lethal, and therefore were assumed to be non-essential, it did not rule out the possibility that some of the growth-defective genes may represent essential genes, because of suboptimal antisense effect or polycistronic operons [9]. In the Database of Essential Genes (DEG, http://tubic.tju.edu.cn/deg), these defective genes in *S. aureus* are considered as essential [7]. In this study, we included and investigated other two defective genes of Ji *et al.* [9], *rnpA* and *uvrC*. Unlike *purC*, the *rnpA* and *uvrC* were identified as essential in this study. Owing to an incongruent result on defective genes, a more detailed investigation of essentiality and cellular function of defective genes reported by Ji *et al.* [9] is needed.

One of the most unexpected results was that *trxA* and *trxB* were identified as non-essential. In a recent study using temperature-sensitive plasmid, *trxB* encoding thioredoxin reductase was shown to be essential [25]. Based on their finding, Uziel *et al.* [25] suggested that the thioredoxin system may provide a new target for the development of compounds against Gram-positive bacteria, because of structural differences between the bacterial and mammalian thioredoxin systems. Moreover, *trxA* has been identified as essential in *B. subtilis* [5], and *trxB* is essential in *S. pneumoniae* R6 [12], *B. subtilis* [5], and *H. pylori* [19]. In this study, however, more than 50 colonies with disruption of *trxA* or *trxB* genes were grown repeatedly in the plates containing 50 μg/ml of erythromycin, and therefore, they were evaluated as non-essential genes. This inconsistent result may be due to the method of gene knock-out or strain difference; however, no decisive speculation is now possible. Therefore, the essentiality of *trxA* and *trxB* in *S. aureus* and other bacterial pathogens should be further investigated.

One more interesting gene was *pgm*, which had been identified as essential in *B. subtilis* [5], but non-essential in this study. In *S. pneumoniae*, insertion mutants lacking the *pgm* gene were virulent in both immunologically normal and immunodeficient mice [26]. Namely, *pgm* is non-essential for growth, but essential for virulence in *S. pneumoniae*. In *S. aureus*, it was reported that *pgm* is essential for the

Table 3. Genes that are non-essential in *S. aureus* N315 but essential in other strains in *S. aureus* or other species [5, 9, 11, 15, 16, 19].*

Gene No.	Gene name	Gene description	*S. aureus*		*S. pneumoniae*		*B. subtilis*	*E. coli*	*H. pylori*	*H. influenzae*
			N315	WCHU29	R6	TIGR4		MG1655	26695	Rd
SA0366	ahpC	Peroxiredoxin	NE						E	
SA0474	folk	7,8-Dihydro-6-hydroxymethylpterin-pyrophosphokinase	NE					E		
SA0685	nrdI	Ribonucleotide reductase alpha subunit	NE					E		E
SA0719	trxB	Thioredoxin reductase	NE		E		E		E	
SA0918	purC	Phosphoribosylaminoimidazolesucci nocarboxamide synthase	NE	D						E
SA0992	trxA	Thiol-disulfide isomerase and thioredoxins	NE				E			
SA1082	rimM	16S rRNA processing protein RimM	NE			E		E		
SA1087	rnhB	Ribonuclease HII	NE					E		E
SA1226	Asd	Aspartate-semialdehyde dehydrogenase	NE				E	E		E
SA1410	grpE	Molecular chaperone GrpE (heat shock protein)	NE					E		
SA1498	clpX	ATP-dependent Clp protease ATP-binding subunit	NE		E					
SA1586	ribH	Riboflavin synthase γ-chain	NE				NE	E		
SA1905	atpD	Proton-translocating ATPase, F1 sector, γ-subunit	NE		E					
SA1906	atpG	Proton-translocating ATPase, F1 sector, γ-subunit	NE		E					
SA1907	atpA	Proton-translocating ATPase, F1 sector, α-subunit	NE		E					
SA2204	pgm	Phosphoglycerate mutase	NE				E			
SA2334	mvaS	3-Hydroxy-3-methylglutaryl coenzyme A synthase	NE		E					

*E, essential; NE, non-essential; D, defective.

optimal expression of methicillin resistance [27]. However, the role of the *pgm* gene in the virulence of *S. aureus* has not been investigated. Besides the genes described above, more rigorous investigation on the exact function of genes showing different essentiality among bacterial species should be performed.

Whereas *atpA*, *atpD*, and *atpG* are essential in *S. pneumoniae* R6 [12], they were evaluated as non-essential in *S. aureus* in this study (Table 3). The *atp* operon, including *atpA*, *atpD*, and *atpG* genes, encodes the F_1F_0-ATP synthase in bacteria, which play an important role in a number of vital cellular processes [28]. However, it has been shown to be dispensable for growth on fermentable carbon sources in several bacteria such as *E. coli* and *B. subtilis*, in which increased glycolytic flux can compensate for the loss of phosphorylation [29]. We speculate that the strategy for oxidative phosphorylation may be different between *S. aureus* and *S. pneumoniae*, although it needs to be further investigated.

In summary, we have identified 122 essential genes of *S. aures* N315 by using comparative genomics and allelic replacement mutagenesis through two-step PCR, which had been used in *S. pneumoniae*. Fifty-one genes were identified for the first time in this study as being essential genes, and 17 genes that had been evaluated as essential in other species were identified as non-essential.

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