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Transcriptomic analysis of *Staphylococcus aureus* under the stress condition of antibacterial erythorbyl laurate by RNA sequencing

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ABSTRACT

Erythorbyl laurate (EL) is a novel multi-functional emulsifier with antibacterial activity against Gram-positive bacteria. The objective of this study was to find genetic evidences of the antibacterial mechanism of EL against food-borne pathogenic *Staphylococcus aureus* Newman by transcriptomic analysis. Total RNA samples were extracted from non-treated and 0.1 mM (sublethal concentration) EL-treated *S. aureus* Newman, and then transcriptional profiling was performed by RNA sequencing (RNA-Seq). In EL-treated *S. aureus* Newman, 242 and 225 genes out of the 2687 genes, were up-regulated and down-regulated greater than 2-fold change, respectively. The majority of up-regulated genes in EL-treated *S. aureus* Newman were cell wall stress stimulon including genes related to VraSR two-component regulatory system (*vra*S, *vra*R, and *vra*T), cell envelopment (*mur*, *sgt*, *fmt*, and etc.) and L-lysine biosynthesis (*lys*, *dap*, and etc.), and there were significant differences in the regulation of peptidoglycan biosynthesis between EL-treated and control samples ($p < 0.01$). On the other hand, genes involved in energy metabolism, nucleic acid metabolism, translation, cell division, and transporter were down-regulated. Finally, these results of the transcriptional profiling from RNA-Seq were validated by quantitative real-time PCR. This transcriptomic study could provide the genetic evidence of the EL stress response, which suggests that EL act as a cell wall-active (cell membrane targeting) agent.

1. Introduction

The majority of emulsion-based products contain many different additives for specific purposes such as emulsification, antioxidation, and preservation (Branen, Davidson, Salminen, & Thorngate, 2001). Lipid oxidation and microbial contamination of emulsion-based products have still been considered as the major hazards, although emulsion-based products are extensively used in the food, cosmetic, and pharmaceutical industries (Park et al., 2017). Lipid peroxides formed by lipid oxidation can decrease quality, nutritional value, and safety of emulsion-based products (Luther et al., 2007). Especially, microbial contamination by pathogens should be paid attention as it can cause food poisoning or skin disease. In a previous study where we developed a strategy for simultaneously controlling these hazards, we successfully performed lipase-catalyzed esterification, which resulted in the synthesis of a multi-functional emulsifier named erythorbyl laurate (EL, 6-*O*-lauroyl-erythorbic acid) (Park, Lee, Sung, Lee, & Chang, 2011).

EL is a novel multi-functional emulsifier that is enzymatically esterified by immobilized lipase between erythorbic acid (D-isoascorbic acid) and lauric acid, which are strong antioxidant and antibacterial agent, respectively. The utilization of EL as an effective multi-functional food additive was firstly proposed by investigating its interfacial characteristics and antioxidant activity (Park et al., 2017). In a recent study on the antibacterial activity of EL, consistent with previous reports of lauric acid (Lieberman, Enig & Preuss, 2006), EL showed growth-inhibitory effects on a range of pathogens (Park et al., 2018). In particular, Gram-positive pathogens such as *Staphylococcus aureus, Listeria monocytogenes*, and *Bacillus cereus* were very susceptible to EL, whereas Gram-negative bacteria were not significantly affected.

Understanding how pathogens respond to antibacterial agents and the mechanism of action of antibacterial agents are crucial for optimizing the treatment conditions of novel antibacterial agents. In an

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aforementioned study, we found physiological evidence (alteration in the permeability and integrity of the cell membrane) that provided insight on the mechanism of antibacterial activity of EL using Live/Dead BacLight assay based on propidium iodide dye and energy filtered transmission electron microscopy (Park et al., 2018). Thus, to further understand the mechanism at the transcriptome level, we performed transcriptomic analysis of changes in gene expression caused by EL. Recently, thanks to advances in proteomics and transcriptomics based on next-generation sequencing (NGS), quantitative analyses of gene or protein expression and consequential comparative analyses under various conditions have become possible. The most powerful approach for transcriptomics is RNA sequencing (RNA-Seq), which is based on deep-sequencing technologies and allows for more precise measurements of transcript levels and their isoforms than other methods (Wang, Gerstein, & Snyder, 2009).

The objective of this study was to elucidate the antibacterial mechanism of EL against a model Gram-positive bacterium at the transcriptome level using RNA-Seq. *S. aureus* Newman, a severe human pathogen and common cause of food poisoning, was used as a model strain. *S. aureus* Newman is a suitable model strain for transcriptomic analysis because it displays robust virulence properties in models of disease and its complete genome sequence was reported (Baba, Bae, Schneewind, Takeuchi, & Hiramatsu, 2008). In this study, we determined whether there were strong EL-specific or general stress responses triggered by EL and compared these results with our hypothesis about the antibacterial mechanism of EL.

2. Materials and methods

2.1. Materials

Novozym® 435 (*i.e.* immobilized lipase from *Candida antarctica* with a catalytic activity of 10,000 PLU/g) was kindly supplied by Novozymes (Bagsværd, Denmark). Erythorbic acid (≥99.0%) and lauric acid (≥99.0%) were purchased from Fluka Co. (Buchs, Switzerland) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. High-performance liquid chromatography (HPLC)-grade acetonitrile, purchased from J. T. Baker Co. (Phillipsburg, NJ, USA) was dehydrated using 4 Å molecular sieves (8–12 mesh; Sigma-Aldrich Co.) and filtered through a membrane filter (0.45 μm) before use. All of the other chemicals were of analytical grade and used without further purification.

2.2. Preparation and quantitative analysis of EL

EL was prepared as previously described (Park et al., 2018). In brief, erythorbic acid (0.12 mmol) and lauric acid (0.60 mmol) were mixed in a screw-capped glass vial with 20 mL acetonitrile and incubated at 50 °C for 30 min in an orbital shaking water bath at 200 rpm. The reaction was initiated by adding 200 mg immobilized lipase to the reactor maintaining temperature at 50 ± 1 °C and the synthesized EL was purified by solvent-separation. And then quantitative analysis was performed using a LC-2002 HPLC apparatus (JASCO Inc., Tokyo, Japan) equipped with a Spherisorb-ODS column (5 μm, 100 Å, I.D. 4.6 × 250 mm; Waters Corp., Milford, MA, USA) and Auto Flex II mass spectrometer (Bruker Daltonics, Bremen, Germany) for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS). The mobile phase used in the analysis was acetonitrile/water/acetic acid (90:5:5, v/v/v) at a flow rate of 1.0 mL/min for 15 min.

2.3. Extraction and purification of RNA from S. aureus Newman

S. aureus Newman strain was selected for experiments and cultured overnight at 37 °C in tryptic soy broth (TSB). An Erlenmeyer flask (100 mL) containing TSB (40 mL) was inoculated with the overnight-grown culture (500 μL) and incubated at 37 °C with shaking at 220 rpm. When the optical density (OD_{600}) reached 0.8 (*i.e.* at late logarithmic phase), 250 μL of the sample was taken for RNA extraction. Samples were treated with 0.1 mM of EL for 15 min. Control cultures without EL treatment were also incubated in the same conditions for 15 min. Total RNAs were extracted from each sample using an easy-RED™ BYF total RNA extraction kit (iNtRON Biotechnology, Inc., Seongnam, Gyeonggi-do, Korea). And then DNase digestion and RNA cleanup were performed with an RNase-Free DNase set (QIAGEN, Hilden, Germany) and RNeasy MinElute cleanup kit (QIAGEN), respectively. All processes were conducted according to the manufacturer's instructions. The purified total RNAs were subjected to construct cDNA library for RNA-Seq.

2.4. Construction of cDNA library for RNA-Seq

The mRNA in the total RNA was converted into a library of complementary DNA (cDNA) suitable for subsequent cluster generation using reagents provided in the Illumina® TruSeq™ RNA Sample Preparation Kit (San Diego, CA, USA), in accordance with the TruSeq™ RNA Sample Preparation Guide. The first step in the workflow involved removing the rRNA from the total RNA using a Ribo-Zero rRNA Removal kit (epicentre®, Madison, WI, USA). Following this step, the remaining mRNA was fragmented into small pieces and primed for cDNA synthesis using divalent cations under elevated temperature. The cleaved RNA fragments primed with random hexamers were copied into first-strand cDNA using reverse transcriptase and random primers, followed by synthesis of second-strand cDNA and removal of the RNA template using DNA polymerase I and RNase H. Next, Ampure XP beads (Beckman Coulter, Inc., Brea, CA, USA) were used to separate the double-stranded cDNA from the second-strand reaction mix. Finally, a single adenine (A) nucleotide was added to the 3′ ends of cDNA fragments using a 3′ to 5′ exonuclease to prevent them from ligating to one another (*i.e.* end repair process) during adapter ligation reaction, and these adenylated cDNA fragments were ligated to multiple indexing adapters. The cDNA products were selectively purified and enriched with PCR to create the final cDNA library for RNA-Seq.

2.5. RNA-seq and data analysis

The resulting cDNA libraries were sequenced on an Illumina® HiSeq™ 2000 (RNA-Seq). Sequencing of each sample was duplicated and raw RNA-Seq reads were subjected to a quality check using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) (Andrews, 2014). Subsequent analysis of the RNA-Seq data was performed by TopHat and Cufflinks software. RNA-Seq reads were mapped to the reference genome of *S. aureus* Newman (NC_009641) using TopHat software (http://ccb.jhu.edu/software/tophat), which aligns RNA-Seq reads to the genome using the high-throughput short read aligner software Bowtie 2 (Trapnell, Pachter, & Salzberg, 2009). Cufflinks software (http://cole-trapnell-lab.github.io/cufflinks) was used to assemble the alignments into a parsimonious set of transcripts and estimate their relative abundances (Trapnell et al., 2012). The expression level of each gene was normalized by calculating the reads per kilobase per million mapped reads (RPKM) (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008) as follows.

$$RPKM = \frac{gene\ mapped\ reads}{total\ mapped\ reads(millions) \times gene\ length(kb)}$$

Differentially expressed genes (DEGs) between EL-treated and control samples were identified by calculating the fold-change in gene expression. Fold-changes (*i.e.* expression changes) were calculated from normalized values of RPKM and transformed to represent plus/minus direction for convenience.

$$\log_2(fold\ change) = \log_2\frac{(EL\ treated\ sample)}{(control\ sample)}$$

Gene enrichment and functional annotation of significantly

Table 1
Summary of genes up-regulated by 0.1 mM erythorbyl laurate.

Gene ID	Gene name[a]	Annotation	Fold-change[b]	Category
Cell envelopment				
5331053	***sgt*B**	Glycosyltransferase	3.8	Peptidoglycan biosynthesis
5331236	***murZ***	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	2.9	Peptidoglycan biosynthesis
5330559	***fmt*A**	Autolysis and methicillin resistant-related protein FmtA	2.1	Peptidoglycan, biofilm biosynthesis
5331477	*fnb*A	Fibronectin binding protein A precursor	2.7	Peptidoglycan-anchor
5331791	*cap*B	Capsular polysaccharide synthesis enzyme CapB	2.7	External encapsulating structure organization
5331797	*cap*E	Capsular polysaccharide biosynthesis protein CapE	2.3	External encapsulating structure organization
5331802	*cap*I	Capsular polysaccharide biosynthesis protein CapI	2.1	External encapsulating structure organization
5331985	*eng*B	Ribosome biogenesis GTP-binding Protein YsxC	2.6	Cell cycle and division
5330218	lpl1nm	Tandem lipoprotein	4.6	Cell membrane proteins
5332092	lpl4nm	Tandem lipoprotein	4.3	Cell membrane proteins
5332094	lpl6nm	Tandem lipoprotein	3.3	Cell membrane proteins
5330219	lpl3nm	Tandem lipoprotein	3.0	Cell membrane proteins
5332093	lpl5nm	Tandem lipoprotein	2.6	Cell membrane proteins
5330179	*tat*A	Twin-arginine translocation protein TatA	2.8	Cell membrane proteins
5332033	*ica*A	*N*-glycosyltransferase	2.5	Cell membrane proteins
Amino acid biosynthesis				
5331999	*his*C	Histidinol-phosphate aminotransferase	6.9	Nitrogen compound biosynthetic process
5332564	*trp*C	Indole-3-glycerol-phosphate synthase	4.8	Nitrogen compound biosynthetic process
5332003	*his*A	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase	4.8	Nitrogen compound biosynthetic process
5331741	*arg*R	Arginine repressor	4.3	Nitrogen compound biosynthetic process
5330228	*cys*M	Cysteine synthase	4.0	Nitrogen compound biosynthetic process
5330774	*lys*C	Aspartate kinase	15.2	Lysine biosynthesis
5332108	*asd*	Aspartate semialdehyde dehydrogenase	10.6	Lysine biosynthesis
5331853	*dap*B	Dihydrodipicolinate reductase	8.8	Lysine biosynthesis
5331759	*dap*A	Dihydrodipicolinate synthase	7.8	Lysine biosynthesis
5331854	*dap*D	Tetrahydrodipicolinate acetyltransferase	6.1	Lysine biosynthesis
5332450	*sbn*H	Putative diaminopimelate decarboxylase (= lysA)	2.5	Lysine biosynthesis
5331732	*lys*A	Diaminopimelate decarboxylase	3.7	Lysine biosynthesis
5332539	*met*L	Homoserine dehydrogenase	4.5	Lysine, branched-chain amino acid biosynthesis
5332039	*leu*A	2-Isopropylmalate synthase	3.2	Branched-chain amino acid biosynthesis
5332038	*ilv*C	Ketol-acid reductoisomerase	2.8	Branched-chain amino acid biosynthesis
5331962	*glt*D	Glutamate synthase subunit beta	3.5	Alanine, aspartate and glutamate metabolism
5332079	*leu*D	Isopropylmalate isomerase small subunit	2.7	Branched-chain amino acid biosynthesis
5332078	*leu*C	Isopropylmalate isomerase large subunit	2.6	Branched-chain amino acid biosynthesis
5332077	*leu*B	3-Isopropylmalate dehydrogenase	2.5	Branched-chain amino acid biosynthesis
5332541	*thr*B	Homoserine kinase	2.7	Glycine, serine and threonine metabolism
5332129	*thr*C	Threonine synthase	2.5	Glycine, serine and threonine metabolism
Protein function				
5331192		Iron compound ABC transporter iron compound-binding protein	6.3	Metal ion transmembrane transporter activity
5332448	*sbn*F	Siderophore biosynthesis IucC family protein	4.4	Metal ion transmembrane transporter activity
5331103	*pep*S	Aminopeptidase PepS	5.6	Peptidase activity
5331103	*pep*S	Aminopeptidase PepS	5.6	Peptidase activity
5331855	*hip*O	Hippurate hydrolase	3.8	Peptidase activity
5332332		Type III leader peptidase family protein	3.0	Peptidase activity
5331023	***prs*A**	Peptidyl-prolyl cis/trans-isomerase	3.2	Protein folding & chaperone
5331929	*hsl*O	Hsp33-like chaperonin	2.7	Protein folding & chaperone
5330268	*hch*A	Chaperone protein HchA	2.4	Protein folding & chaperone
5331387		Acetyltransferase, GNAT family protein	3.1	N-acetyltransferase activity
5330314		Acetyltransferase, GNAT family protein	2.9	N-acetyltransferase activity
5331371		Drug resistance transporter EmrB/QacA subfamily protein	2.7	Drug transporter activity
5332032	*ica*B	Intercellular adhesion protein B	6.1	Signal peptide
5331477	*fnb*A	Fibronectin binding protein A precursor	2.7	Signal peptide
Cellular process				
5332008	*hlg*C	Gamma-hemolysin component C	10.4	Cytolysis of cells of another organism
5332104	*luk*S	Leukocidin/hemolysin toxin subunit S	8.6	Cytolysis of cells of another organism
5331190	*luk*F	Leukocidin/hemolysin toxin subunit F	7.8	Cytolysis of cells of another organism
5332443	*hlg*A	Gamma-hemolysin component A	7.1	Cytolysis of cells of another organism
5332010	*hlg*B	Gamma hemolysin, component B	6.9	Cytolysis of cells of another organism
5331610	***drp*35**	Drp35	8.6	Metal ion binding, response to antibiotic
5332445	*sbn*C	Siderophore biosynthesis IucC family protein	4.8	Metal ion binding, response to antibiotic
5331606		ABC transporter ATP-binding protein	4.3	ATP, adenyl nucleotide binding
5330486		NADH-dependent flavin oxidoreductase	4.3	Nuleotide, cofactor binding
5330536		Lipoate-protein ligase A	2.7	Nucleotide, ATP-binding
5331248		Zinc and cobalt transport repressor protein	2.6	Winged helix repressor DNA-binding
5331888	*ipd*C	Indole-3-pyruvate decarboxylase	2.6	Cofactor binding
5331959	*glp*D	Aerobic glycerol-3-phosphate dehydrogenase	3.5	Nucleotide phosphate-binding region:FAD
5330424	*trx*B	Thioredoxin reductase	2.8	Nucleotide phosphate-binding region:FAD, electron carrier activity
5331258		Arginase	2.6	Arginine and proline metabolism, metal ion binding
5331746	*arg*D	Ornithine aminotransferase	4.1	Arginine and proline metabolism
5329983	*ald*A	Aldehyde dehydrogenase-like protein	4.3	Glycerolipid metabolism, oxidoreductase activity
5332031	*lip*	Lipase precursor	3.1	Glycerolipid metabolism

(*continued on next page*)

Table 1 (*continued*)

Gene ID	Gene name[a]	Annotation	Fold-change[b]	Category
5332483	set9nm	Superantigen-like protein	3.7	Staphylococcal toxin
5332480	set6nm	Superantigen-like protein	2.5	Staphylococcal toxin
5331488	*fbp*	Fructose-1,6-bisphosphatase	3.6	Carbohydrate biosynthetic process
5331907	*acs*	Acetyl-CoA synthetase	3.5	Glycolysis/Gluconeogenesis
5331997	*alr*2	Alanine racemase 2	3.5	Pyridoxal phosphate
5330989		Putative translaldolase	2.9	Pentose phosphate pathway
5331971	*gnt*K	Gluconate kinase	2.7	Pentose phosphate pathway
5331430		Amino acid permease	2.7	Amino acid transporter
5331898		Acetyl-CoA/acetoacetyl-CoA transferase	7.1	
5330680		Putative transposase	5.6	
5331783	*bsa*A2	Lantibiotic precursor	4.7	
5331829	*fad*A	Acetyl-CoA acetyltransferase-like protein	4.5	
5331489		Phospholipase/carboxylesterase family protein	3.5	
5330230		ABC transporter substrate-binding protein	2.7	
5329997	*ent*B	Isochorismatase	2.7	
5330500		Truncated MHC class II analog protein	2.5	
Regulatory function				
5331535		Transcriptional regulator TetR family protein	3.4	Regulation of transcription, DNA-dependent
5331549		Transcriptional regulator TetR family protein	3.0	Regulation of transcription, DNA-dependent
5331404		MerR family regulatory protein	3.0	Regulation of transcription, DNA-dependent, negative regulation of macromolecule biosynthetic process
5331741	*arg*R	Arginine repressor	4.3	Winged helix repressor DNA-binding
5330495		LysR family regulatory protein	3.0	Winged helix repressor DNA-binding
Signal transduction				
5332564	*trp*C	Indole-3-glycerol-phosphate synthase	4.8	Two-component systems
5332603	***vra*T (*yvq*F)**	Hypothetical protein (positive modulator of VraSR)	2.7	Two-component systems
5332602	***vra*S**	Sensor histidine kinase VraS	2.1	Two-component systems
5331107	***vra*R**	DNA-binding response regulator VraR	3.5	Two-component systems

[a] Bold genes are related to the *vra*SR two-component regulatory system.
[b] Fold-changes (*i.e.* expression changes) were calculated from normalized values of RPKM.

expressed genes or gene clusters were analyzed using DAVID 6.8 tools (https://david.ncifcrf.gov/home.jsp) with Fisher exact test for statistical analysis (Huang, Sherman, & Lempicki, 2008, 2009).

2.6. Quantitative real-time PCR

For validation of transcriptional profiling from RNA-Seq, quantitative real-time PCR (qRT-PCR) was performed. The *vra*SR genes and randomly selected up-regulated genes related to peptidoglycan biosynthesis were subjected to qRT-PCR. The cDNA for qRT-PCR was synthesized using a QuantiTect reverse transcription kit (QIAGEN). qRT-PCR was performed using specific primer pairs and iQTM SYBR Green supermix (Bio-Rad, Hercules, CA, USA). The primers were designed with the Primer-BLAST (NCBI) and purchased from Macrogen, Inc. (Seoul, Korea). The amplification and detection of PCR products were performed using CFX Connect™ Real-Time System (Bio-Rad). The thermal cycling conditions were as follows. After activation of the polymerase and a DNA denaturation step at 95 °C, 40 amplification cycles were performed with a denaturation step at 95 °C followed by an annealing and extension step at 55 °C. The cDNA values were normalized with the value of 16s rRNA, which was constant in different conditions (data not shown).

3. Results and discussion

3.1. Growth of S. aureus Newman

EL has bacteriostatic and bactericidal activities simultaneously on Gram-positive bacteria, beyond 0.1 mM concentration (Park et al., 2018). To identify changes in gene expression caused by treatment of EL properly, *S. aureus* Newman was treated with a sublethal concentration (0.1 mM) of EL, which can cause stress but not any reduction (death) of cells (*i.e.* a dose of a potentially lethal substance that is no large enough to cause death). Consequentially, 0.1 mM EL inhibited the growth of cells (extension of lag time) but did not cause any death of cells (reduction of cells); in other words, EL did not show bactericidal activity but inhibited bacterial growth as expected (data not shown).

3.2. Mapping and quantification of reads

S. aureus Newman has a total of 2687 genes with 2614 coding DNA sequences (CDS), 56 tRNA genes, 16 rRNA genes, and 1 tmRNA gene (Baba et al., 2008). RNA-Seq generated about 2.8 million and 11 million reads with 101 bp length from the EL-treated and control cDNA libraries, respectively (Table S1). For quality control, raw sequence data from RNA-Seq was checked using FastQC, and no problem was identified that required further analysis (data not shown). A total of 8.3% reads from EL-treated sample and 26.6% reads from control sample were successfully aligned to the reference genome of *S. aureus* Newman (NC_009641). Full information about the quantification of reads (RPKM values) and the annotations corresponding to each CDS are represented in Table S2.

3.3. Differentially expressed genes in EL-treated S. aureus Newman

Gene expression levels were compared in EL-treated and control sample. Of the 2614 CDS regions in *S. aureus* Newman, 198 regions with at least one zero RPKM value in each sample (118 regions in the EL-treated sample and 80 regions in the control sample) were excluded, leaving 2416 regions to be analyzed. A total of 467 genes/regions were differentially expressed in EL-treated *S. aureus* Newman (Fig. S3), and of these, 242 were up-regulated and 225 were down-regulated ($|\text{fold change}| \geq 2$). We found that there were significantly up-regulated or down-regulated genes when *S. aureus* Newman was treated with a sublethal concentration of EL. These genes were classified according to their functionality in the cell to investigate how EL affects *S. aureus* Newman. As shown in Table 1, genes involved in cell envelopment (*mur*, *sgt*, *fmt*, *vra*, etc.) and amino acid biosynthesis (*lys*, *dap*, *his*, etc.) were distinctively up-regulated, as well as genes encoding chaperones (*drp*, *prs*, *hsl*, *hch*) and virulence factors such as toxins (*hlg*, *luk*) and siderophores (*sbn*). In the case of down-regulated genes (Table 2), genes related to energy metabolism, nucleic acid metabolism, cell division,

Table 2
Summary of genes down-regulated by 0.1 mM erythorbyl laurate.

Gene ID	Gene name	Annotation	Fold-change[a]	Category
Cell envelopment				
5331038		Extracellular glutamine-binding protein (putative polar amino acid transport system)	−4.2	Peptidoglycan-based cell wall
5330047	*scd*A	Cell wall biosynthesis protein ScdA	−2.8	Cell division
5331856		Cell division protein	−2.6	Cell division
5332180	*mur*E	UDP-N-acetylmuramoylalanyl-D-glutamate–L-lysine ligase	−2.3	Peptidoglycan biosynthesis
5331865	*dlt*D	D-alanine-activating DltD protein	−2.1	D-alanylation
Amino acid biosynthesis				
5331750	*arg*G	Argininosuccinate synthase	−19.5	Nitrogen compound biosynthetic process
5331961	*arg*H	Argininosuccinate lyase	−16.5	Nitrogen compound biosynthetic process
5330838		Pyrroline-5-carboxylate reductase	−5.7	Nitrogen compound biosynthetic process
5332191		Nitric oxide synthase oxygenase	−3.6	Nitrogen compound biosynthetic process
5329996	*arg*J	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	−2.6	Nitrogen compound biosynthetic process
5331561		Glycine betaine aldehyde dehydrogenase	−4.1	Nitrogen compound biosynthetic process
5332342	*ahr*C	Arginine repressor	−2.5	Arginine biosynthesis
Protein function				
5331527		Immunodominant antigen A	−10.9	Extracellular region signal peptide
5332586	*ure*G	Urease accessory protein UreG	−4.7	Nucleotide-binding
5331747		Branched-chain amino acid transport system II carrier protein	−4.2	Amino acid transmembrane transporter activity
5330693	*rpm*B	50S ribosomal protein L28	−3.6	Translation
5331492		Acetyltransferase, GNAT family protein	−3.2	N-acetyltransferase activity
5331526		Acetyltransferase, GNAT family protein	−2.6	N-acetyltransferase activity
5330883	*rps*U	30S ribosomal protein S21	−3.2	Translation
5332532	*rpm*E2	50S ribosomal protein L31 type B	−2.6	Translation
5331300	*rps*L	30S ribosomal protein S9	−2.5	Translation
5330887	*rps*T	30S ribosomal protein S20	−2.5	Translation
5331354		Peptidase M20/M25/M40 family protein	−3.0	Peptidase activity
5332558	*rpl*S	50S ribosomal protein L19	−2.5	Structural constituent of ribosome
Cellular process				
5330168		Glycerol-3-phosphate transporter	−4.8	Carbohydrate transport
5330048		PTS system, IIA component	−4.7	Carbohydrate transport
5332334	*rbs*U	Ribose transporter RbsU	−3.1	Carbohydrate transport
5332223		Oligopeptide transport system permease	−4.7	Cell membrane
5330797		Cell wall associated fibronectin-binding protein	−2.6	Cell membrane
5332479	set5nm	Superantigen-like protein 5	−4.5	*Staphylococcus aureus* exotoxin
5330660	*hla*	Alpha-hemolysin precursor	−2.6	Cytolysis of cells of another organism
5329981	*adh*E	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	−4.5	Oxidoreductase activity
5331561		Glycine betaine aldehyde dehydrogenase	−4.1	Oxidoreductase activity
5330560	*qox*D	Quinol oxidase polypeptide IV	−3.9	Cytochrome-c oxidase activity
5332329	*qox*C	Quinol oxidase polypeptide III	−3.3	Cytochrome-c oxidase activity
5332221	*opp*1C	Oligopeptide transporter putative membrane permease	−3.3	ABC transporters
5332173	*fmt*B	Methicillin resistance determinant FmtB protein	−3.1	Gram-positive signal peptide
5330374		Anion transporter family protein	−3.2	Monovalent inorganic cation transport
5331356	*fof*B	Fosfomycin resistance protein FosB	−3.2	Response to antibiotic
5329920	*hut*H	Histidine ammonia-lyase	−4.0	Histidine metabolism
5331808	*cap*O	Capsular polysaccharide biosynthesis protein CapO	−3.1	Amino sugar and nucleotide sugar metabolism
5330023	*pfl*B	Formate acetyltransferase	−3.0	Pyruvate, Propanoate, Butanoate, propanoate metabolism
5332182	*fba*A	Fructose-bisphosphate aldolase	−2.7	Fructose and mannose metabolism
5330952	*pls*C	1-acyl-sn-glycerol-3-phosphate acyltransferase	−2.7	Glycerolipid metabolism
5329990		Formate dehydrogenase	−2.9	Cofactor binding
5331795	*cap*B	Polysaccharide biosynthesis protein CapD	−2.5	Cofactor binding
5332135	*mnh*B	Putative monovalent cation/H+ antiporter subunit B	−2.9	Metal ion binding protein
5331565		Anaerobic ribonucleotide reductase, small subunit	−2.7	Metal cluster binding
5331439		General substrate transporter involved in chloramphenicol resistance	−2.6	Major facilitator superfamily MFS-1
Regulatory function				
5331138		ABC transporter ATP-binding protein	−10.7	Nucleotide-binding
5331217	*kdp*C	Potassium-transporting ATPase subunit C	−5.3	Nucleotide-binding
5331139		GntR family regulatory protein	−5.0	Winged helix repressor DNA-binding
5331485		Transcriptional regulator MarR family protein	−4.2	Winged helix repressor DNA-binding
5331029		DNA-binding response regulator	−2.9	Winged helix repressor DNA-binding
5331400		MarR family regulatory protein	−2.9	Winged helix repressor DNA-binding
5332100		Transcriptional regulator, GntR family protein	−2.8	Winged helix repressor DNA-binding
5329969	*pnp*	Purine nucleoside phosphorylase	−3.2	RNA-binding
5331037		Glutamine transport ATP-binding protein	−2.7	Nucleoside binding
5331621		Integrase	−7.7	DNA metabolic process
5331277		Putative transposase for IS1272	−3.9	DNA metabolic process
5331047		A/G-specific adenine glycosylase	−3.2	DNA metabolic process
Signal transduction				
5331402		Nitrite transport protein	−3.0	Two-component system

[a] Fold-changes (*i.e.* expression changes) were calculated from normalized values of RPKM.

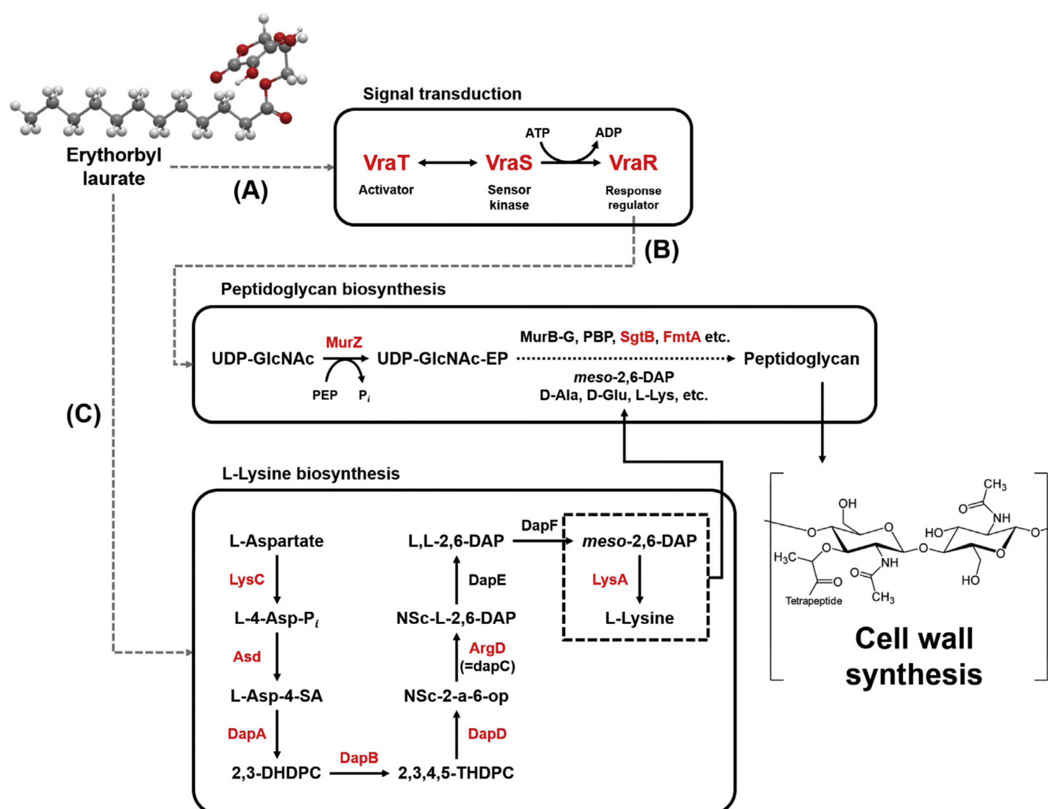

Fig. 1. Proposed mechanism of cell wall induction by erythorbyl laurate in *S. aureus* Newman. When *S. aureus* Newman is exposed to EL, it perceives cell wall stress through VraSR TCRS. VraR subsequently activates genes related to peptidoglycan biosynthesis to construct cell wall to endure stress. Simultaneously, genes related to the biosynthesis of L-lysine biosynthesis, a component of peptidoglycan, are up-regulated. The proteins in the diagram are crucial factors for each metabolic pathway and the proteins that are up-regulated in the presence of EL are shown in red color (greater than 2-fold change). EL probably triggered cell wall stress because the majority of the proteins related to cell wall biosynthesis were up-regulated, however, the signal is still unknown (gray dashed arrow). The detail pathway of peptidoglycan biosynthesis was abbreviated (black dashed arrow) and other pathways related to these metabolic process were not included for clarity. GlcNac, *N*-acetylglucosamine; PEP, phosphoenolpyruvate; EP, enolpyruvate; DAP, diaminopimelate; SA, semialdehyde; DHDPC, dihydrodipicolinate; THDPC, tertrahydrodipicolinate; NSc-2-a-6-op, *N*-succinyl-2-amino-6-oxopimelate.

and translation were identified. An analysis of DEGs suggested that *S. aureus* Newman was stressed by EL, which consequently induced the expression of specific genes for adaptation and survival, while simultaneously repressing genes related to cell growth.

3.3.1. Up-regulation of vraSR signal transduction

In our previous study, we revealed that the primary activity of EL was disintegration of the cell membrane and cell wall (Park et al., 2018). Consistent with our findings, many transcriptomic studies of *S. aureus* revealed cell wall stress stimulon induced by cell wall-active agents (Kuroda et al., 2003; Muthaiyan, Silverman, Jayaswal, & Wilkinson, 2008; Utaida et al., 2003). Exploiting this fact, DEGs related to this mode of action were investigated in detail, and up-regulated genes encoding the two-component regulatory system (TCRS), *vra*S and *vra*R (*vra*SR), were identified in EL-treated *S. aureus* Newman (2.1- and 3.5-fold change, respectively) (Fig. 1A). VraS (sensor kinase) and VraR (response regulator) construct a VraSR TCRS which is activated by cell wall-targeting antibiotics or the deletion of genes encoding enzymes for cell wall synthesis. This system induces the expression of genes involved in peptidoglycan biosynthesis resulting in reducing the damage to the cell wall or membrane (McCallum, Stutzmann Meier, Heusser, & Berger-Bächi, 2011). *yvq*F (also called *vra*T), which is encoded in upstream of *vra*SR (*vra*TSR operon, *orf*1-*var*T-*vra*S-*vra*R) and known as a positive modulator of VraSR (Boyle-Vavra, Yin, Jo, Montgomery, & Daum, 2013), was also up-regulated by EL (2.7-fold change). In addition, the majority of up-regulated genes (*mur*, *sgt*, *fmt*, *prs*, and etc.) were confirmed to be related to *vra*SR signal transduction (VraSR-dependent cell wall stress stimulon) (Muthaiyan et al., 2008). These up-regulations of *vra*SR and *vra*SR-related genes can be genetic evidences that EL stresses *S. aureus* Newman in the aforementioned manner because VraSR responds exclusively to cell wall-active agents.

3.3.2. Peptidoglycan biosynthesis

On closer inspection of the genes controlled by VraSR, those involved in peptidoglycan biosynthesis, *mur*Z (encoding a redundant MurA isozyme, 2.8-fold change), *sgt*B (3.8-fold change), and *fmt*A (2.1-fold change) were up-regulated more than 2-fold in EL-treated *S. aureus* Newman (Fig. 1B). In *S. aureus*, the *mur*A and *mur*Z both encode UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA and MurZ), which catalyzes the first committed step in peptidoglycan biosynthesis and therefore plays an important role in cell wall growth (Du et al., 2000). According to the previous research on these two genes, only the expression of *mur*Z was strongly induced when exposed to cell wall-active agents (Blake et al., 2009). This report corresponds to the significant changes in expression of the *mur*Z (not *mur*A) in our transcriptional profiling and provides another strong evidence that EL acted as a cell wall-active agent.

Furthermore, the up-regulated genes known as the core cell wall stress stimulon, *sgt*B and *fmt*A, encode monofunctional glycosyltransferase and β-lactamase in *S. aureus*, respectively, and are also regulated by VraSR. These two enzymes participate in enhancing peptidoglycan biosynthesis in different ways. In Gram-positive bacteria such as *S. aureus* Newman, SgtB might catalyze the incorporation of UDP-*N*-acetylglucosamine into peptidoglycan (elongation of the cell

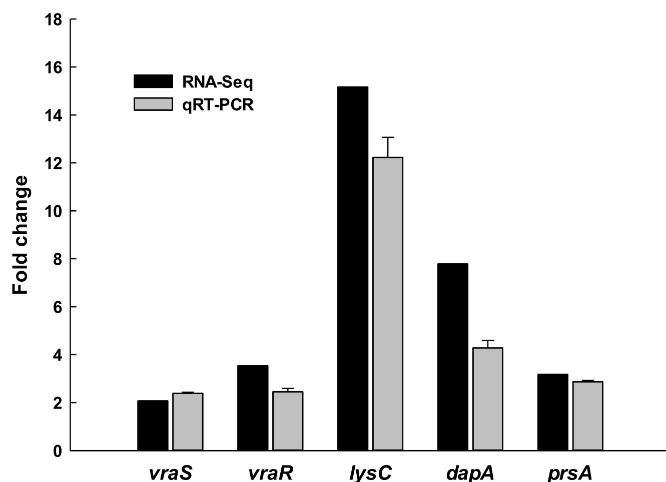

Fig. 2. Validation of RNA sequencing data by quantitative real-time PCR. The *vra*SR genes and randomly selected up-regulated genes related to peptidoglycan biosynthesis were selected for validation. Data are expressed as fold change in gene expression between control and EL-treated samples.

wall), and FmtA might be involved in peptidoglycan synthesis for teichoic acid attachment or biofilm formation (Qamar & Golemi-Kotra, 2012; Wang et al., 2001). These results suggest that *S. aureus* Newman increased cell wall synthesis to avoid excessive spreading of cytoplasmic materials caused by disintegration of the cell membrane or cell wall in an EL-existing environment. DAVID analysis in accordance with functionality, verified that there were significant differences in the frequency of clusters related to peptidoglycan biosynthesis between EL-treated and control samples ($p < 0.01$).

3.3.3. L-Lysine biosynthesis

The expression of various genes involved in amino acid biosynthesis also increased in EL-treated *S. aureus* Newman. Among them, genes in *dap* operon (L-lysine biosynthesis) were highly up-regulated (2.5–15.2-fold change, Fig. 1C). The gene *lys*C, which was up-regulated fully 15.2-fold, encodes aspartate kinase that catalyzes the first step in the L-lysine biosynthesis. Together with glutamine and alanine, L-Lysine is utilized for not only protein synthesis but also cell wall synthesis in bacteria. *Meso*-2,6-Diaminopimelate, which is synthesized during L-lysine biosynthesis, is likewise utilized as a component of peptidoglycan (Grundy, Lehman, & Henkin, 2003). Thus, it was shown that the synthesis of L-lysine and *meso*-2,6-diaminopimelate, which are the major components of peptidoglycan, was promoted as cell wall synthesis was increased by EL. The relationship between VraSR TCRS and L-lysine biosynthesis was not elucidated, however, several studies have found that cell wall-active agents affect these genes (Hessling et al., 2013; Kuroda et al., 2003). For this reason, the up-regulation of these genes shown in transcriptional profiling could be genetic evidence for the antibacterial mechanism of cell wall-active agents. DAVID analysis verified that there were significant differences in the frequency of clusters related to L-lysine biosynthesis between EL-treated and control samples ($p < 0.01$).

3.3.4. Other genes

There were other up-regulated genes related to the VraSR TCRS or the cell wall stress stimulon. The genes *prs*A (directly controlled by VraSR, 3.2-fold change) and *drp*35 (8.6-fold change) respond to cell wall-active agents and encode a chaperone, which assists the proper folding or unfolding of macromolecular structures (Jousselin et al., 2012). The cytoplasmic environment can be seriously affected by damage to the cell membrane and is directly connected to the life of the cell. Because EL can alter the permeability and integrity of the cell membrane and cause disorder of the cytoplasm, a recovery system involving chaperones, which target unfolded or misfolded proteins, needs to be activated in an EL-existing environment. Hence, the up-regulation of these genes (*prs*A, *drp*35, and etc.) in EL-treated *S. aureus* Newman was understandable. In addition, several genes encoding virulence factors including two toxins (hemolysin and leukotoxin, above 7-fold change) and a siderophore (above 4-fold change), were up-regulated by EL. These results show that *S. aureus* Newman manifested aggression and maintained high expression of these genes for survival against harsh conditions.

On the other hand, 225 genes in various categories were significantly down-regulated by EL and these genes are related to energy metabolism, nucleic acid metabolism, translation, cell division, transporter, and binding proteins. To focus on adaptation and survival in an EL-existing environment, *S. aureus* Newman inhibited cell growth (highly energy-consuming process) itself through the down-regulation of these genes. Furthermore, these results were consistent with the previous transcriptional profiling of nisin-treated *S. aureus* (McCallum et al., 2011). Nisin, an antibacterial peptide used as a food preservative, inserts into the membrane of bacteria and forms pores causing membrane depolarization and the rapid efflux of cytoplasmic materials. EL, which is expected to have a similar mode of antibacterial activity, showed comparable transcriptional profiles from RNA-Seq in this study.

3.4. Validation of RNA-Seq data by qRT-PCR

qRT-PCR was used to validate the transcriptional profiles from RNA-Seq. The *vra*SR genes and randomly selected up-regulated genes related to peptidoglycan biosynthesis were subjected to qRT-PCR, and the results of qRT-PCR were compared to the transcriptional profiles from RNA-Seq. As shown in Fig. 2, the fold changes in each gene (*vra*S, *vra*R, *lys*C, *dap*A, and *prs*A) were similar between qRT-PCR and RNA-Seq. This showed that RNA-Seq was properly performed and validated the genetic evidences from transcriptional profiling that EL is a cell wall-active agent against *S. aureus* Newman affecting the cell membrane.

4. Conclusions

In this study, we found genetic evidences for the antibacterial mechanism of EL against food-borne pathogenic *S. aureus* Newman by transcriptomic analysis using RNA-Seq. In EL-treated *S. aureus* Newman, 242 and 225 genes were significantly up-regulated and down-regulated, respectively. Up-regulated genes were largely related to cell wall stress stimulon and down-regulated genes were related to cell growth, which indicates that there was cell wall stress to *S. aureus* Newman in an EL-existing environment. Collectively, this transcriptional profiling proposed that EL acted as a cell wall-active (cell membrane targeting) agent, consistent with physiological evidence previously reported. This evidence enables to facilitate further studies on the synergistic effect of cell wall-active preservatives with EL and ligand-receptor interactions using mutants deficient in cell wall synthesis for an in-depth understanding of the antibacterial mechanism of EL. These researches would lead to broad utilization of EL as a multi-functional emulsifier with antibacterial activity to various emulsion-based products, particularly in the food industry.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2018.08.021.

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