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Phage Conversion for β-Lactam Antibiotic Resistance of *Staphylococcus aureus* from Foods S

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Temperate phages have been suggested to carry virulence factors and other lysogenic conversion genes that play important roles in pathogenicity. In this study, phage TEM123 in wild-type *Staphylococcus aureus* from food sources was analyzed with respect to its morphology, genome sequence, and antibiotic resistance conversion ability. Phage TEM123 from a mitomycin C-induced lysate of *S. aureus* was isolated from foods. Morphological analysis under a transmission electron microscope revealed that it belonged to the family *Siphoviridae*. The genome of phage TEM123 consisted of a double-stranded DNA of 43,786 bp with a G+C content of 34.06%. A bioinformatics analysis of the phage genome identified 43 putative open reading frames (ORFs). ORF1 encoded a protein that was nearly identical to the metallo-β-lactamase enzymes that degrade β-lactam antibiotics. After transduction to *S. aureus* with phage TEM123, the metallo-β-lactamase gene was confirmed in the transductant by PCR and sequencing analyses. In a β-lactam antibiotic susceptibility test, the transductant was more highly resistant to β-lactam antibiotics than *S. aureus* S133. Phage TEM123 might play a role in the transfer of β-lactam antibiotic resistance determinants in *S. aureus*. Therefore, we suggest that the prophage of *S. aureus* with its exotoxin is a risk factor for food safety in the food chain through lateral gene transfer.

Keywords: Genome, metallo-β-lactamase, phage conversion, *Staphylococcus aureus*

Introduction

The emergence and spread of antimicrobial resistance has increased morbidity and mortality, and has become a global issue. Since the mid-twentieth century, genetic determinants of antibiotic resistance have spread in strongly selective environments, particularly among pathogenic bacteria in hospitals, in certain communities, and along the commercial food chain. Excessive usage of antibiotics, greater movement of people, and advanced industrialization might have contributed to the changing epidemiology of resistance via horizontal gene transfer [11]. Such genetic transfer and genetic exchange in communities explains the emergence of successive multidrug-resistant strains of *Staphylococcus aureus* [32]. Gene transfer that causes resistance to antibiotics, such as β-lactams, may occur through plasmids, transposons, or phages by transduction, transformation, and conjugation [17]. Phages are present in all habitats on earth; there are an estimated 10^{31} phages worldwide or approximately 10^{6} phages/cm^{3} across all environments. Phages usually persist in aquatic environments, where their higher survival rate makes them more suitable than free DNA for the transfer of genes among bacteria. In fact, many bacteria contain identifiable prophages that represent about 16% of their chromosomal DNA [5]. Therefore, the contribution of phages to gene transfer in natural extra-intestinal environments and in human-generated environments might be greater than that of plasmids and transposons [9]. However, the importance of phages in gene transfer is still underestimated, unlike that of other transfer routes, such as transformation and conjugation [28]. Phage-mediated transduction of antibiotic resistance was reported between species of enterococci [23], suggesting that phages participate in far more extensive genetic

exchange among species of bacteria than previously believed [8]. However, there is little information available on the direct contribution of phages, a type of mobile gene element. Only a few groups have analyzed antibiotic resistance genes in phage DNA isolated from wastewater environments. β-Lactamase genes have been detected in naturally occurring phage particles isolated from sewage, and it has been suggested that an isolated single phage encoding β-lactamase transduced the character to other host strains [25]. β-Lactamase genes from phage DNA obtained from sewage were transferred by electroporation to ampicillin-sensitive bacteria, which then became resistant to ampicillin [9]. β-Lactamase activity introduces resistance to β-lactam antibiotics in *S. aureus* via the hydrolysis of the β-lactam ring of penicillin-based antibiotics [21, 24, 30]. Phages that encode enterotoxin A, exfoliative toxin A, and Panton-Valentine leukocidin (PVL) also contribute to the evolution of virulent strains [3, 15, 29, 34]. Phages play a significant role in the rapid evolution of antibiotic resistance in *S. aureus* [19]. For phage DNA to serve as a reservoir of resistance genes and to act as an agent of transduction in the ecosystem, sensitive host strains and natural phage particles that support infection with these phages and subsequent antibiotic conversion are necessary. The objective of the present study was to show the direct conversion of antibiotic resistance in *S. aureus* from food samples by the β-lactamase gene from the phage TEM123.

Materials and Methods

Isolation of Wild-Type *S. aureus* from Foods

The type strain *S. aureus* KCCM12103 was purchased from the Korean Culture Center of Microorganisms. Wild-type *S. aureus* S123 and S133 were isolated from ready-to-eat kimbap (rice rolled in laver) purchased from a convenience chain store and identified by PCR and biochemical characteristics. Briefly, 25 g of sample was homogenized in a stomacher and the homogenate was inoculated in 10% NaCl Tryptic soy broth (Difco Laboratories Inc., Detroit, MI, USA) for enrichment. The enriched broth was incubated at 37°C for 24 h under aerobic conditions, diluted with 0.1% sterile peptone water, and spread onto Baird-Parker agar supplemented with egg-yolk tellurite (Difco Laboratories Inc.). Presumptive colonies were picked out, placed in new Baird-Parker agar, and further identified by Gram staining and catalase and coagulase tests. Then, PCR was carried out for further identification and primers targeting the staphylococcal *nuc* gene and 16S rRNA were synthesized commercially [18]. Primer sets for the identification of *S. aureus* are shown in Table 1. Additionally, the antibiotic resistance patterns of wild-type *S. aureus* S123 and S133 were confirmed. Wild-type *S. aureus* S123 showed resistance to ampicillin (Amp^R) whereas wild-type *S. aureus* S133 was susceptible to ampicillin (Amp^S).

Phage Induction and Morphology

To induce the temperate phage TEM123 from *S. aureus* S123, 0.1 ml of bacterial culture was transferred into 50 ml of fresh LBC broth. The induction of temperate phages was performed by the addition of mitomycin C (Merck Korea, Seoul, Korea) at a final concentration of 1 μg/ml for approximately 2 h after inoculation (OD_{660} = 0.2–0.3). After further incubation at 37°C for 5 h, the cell lysate was centrifuged for 15 min at 10,000 ×*g* and the supernatant was passed through a syringe filter with a 0.2 μm pore size (Millipore, Billerica, MA, USA). To determine their morphological characteristics, purified phage particles were negatively stained with 2% aqueous uranyl acetate (pH 4.5) on a carbon-coated grid and examined by transmission electron microscopy.

Genome Sequence Analysis

Phage TEM123 DNA was isolated from polyethylene-glycol-

Table 1. Primer sets for PCR.

Target region	Primer	Sequence (5′ → 3′)	Length
16S rRNA	16sF	CCGCCTGGGGAGTACG	240 bp
	16sR	AAGGGTTGCGCTCGTTGC	
nuc gene	Sa-1	GAAAGGGCAATACGCAAAGA	482 bp
	Sa-2	TAGCCAAGCCTTGACGAACT	
Metallo-β-lactamase	lactamase FOR	TCGCAGCTCTTGCTTTGCCT	159 bp
	lactamase REV	GCAGGGTGTCTTATCACACACGAA	
Front region	I-For	GCTCTAACGAGTCCGGTAGC	620 bp
	I-Rer	AAAACCTCGCCTTCGTCGAT	
Intermediate region	II-For	TTACGCTTCCGCTGGTTTCT	820 bp
	II-Rev	TGCAACACTACCTGAGGCTG	
End region	V-For	TGAGACGCAACTCTTCGCTT	500 bp
	V-Rev	GATCGTTGACGGGCGTAGAT	

precipitated phage particles using the method described by Manfioletti *et al.* [22], with some modifications. All chemical reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). Briefly, DNase I (10 μg/ml) and RNase A (20 μg/ml) were added to the phage lysate. After incubation at room temperature for 15 min, 0.5 M EDTA (pH 8) and proteinase K (1 mg/ml) were added, followed by incubation at 65°C for 30 min. After incubation, the nucleic acids were extracted using phenol-chloroform-isoamyl alcohol. They were precipitated with ethanol and dissolved in sterile distilled water. Phage DNA was stored at −80°C. The genomic sequence was determined using ultra-high-throughput sequencing on the Genome Sequencer FLX (GS-FLX) with 20-fold redundancy, on average. The nucleotide sequences were compared with those of other genes in GenBank using BLAST. Open reading frames (ORFs) were identified using the NCBI ORF Finder. The molecular weight and isoelectric point were calculated using the ExPASy Compute pI/Mw program. The tRNA sequences were analyzed using tRNAscan-SE. A conserved protein domain analysis was performed using BLASTP and the NCBI Conserved Domains Database.

Transduction of Resistance and PCR

S. aureus S133 as the recipient was incubated at 37°C for 18 h. High-titer phage TEM123 (~10^9 PFU/ml) was added to the bacterial recipient after overnight culture in Luria-Bertani (LB) broth (Difco Laboratories Inc.) and incubated at 37°C for 1 h. The bacterial cells were washed with TSB and then resuspended in 1 ml of TSB and incubated for 1 h at 37°C. Then, 100 μl of each mixture was spread onto LB agar containing ampicillin (100 μg/ml). These plates were incubated at 37°C for 24–48 h and a single colony was picked out. Transduction was confirmed using PCR. Genomic DNA from the transductant was isolated using DNA tissue kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for the metallo-β-lactamase gene in phage TEM123 were designed and synthesized commercially (Bioneer, Daejon, Korea). The presence of the phage TEM123 genome in the transductant of *S. aureus* S133 was confirmed with PCR; all primer sets are shown in Table 1.

Antibiotic Resistance of the Transductant

The growth properties of *S. aureus* S133 and its transductant were examined in LB broth supplemented with antibiotics at various concentrations and incubated at 37°C with constant agitation (150 rpm). Bacterial growth was monitored by measuring the absorbance at 660 nm and all experiments were conducted in triplicates. *S. aureus* S133 and its transductant were tested for resistance to β-lactam antibiotics, such as penicillin G, ampicillin, amoxicillin, cephalothin, cefoxitin, imipenem, and meropenem, and other antibiotic groups.

Nucleotide Sequence Accession Number

The complete genome sequence of temperate phage TEM123 is available in GenBank under the accession number JQ779024.

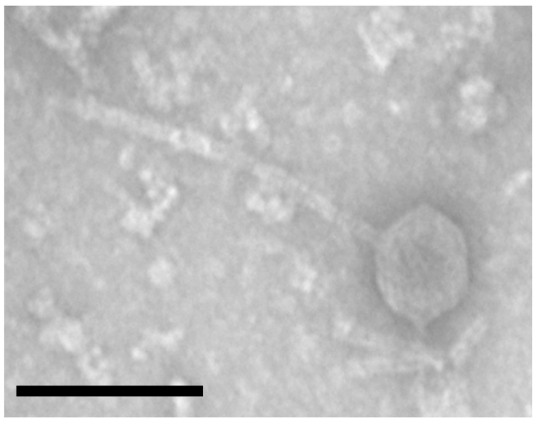

Fig. 1. Morphology of staphylococcal phage TEM123 under transmission electron microscopy (scale bar: 100 nm).

Results

Induction and Morphology of Phage TEM123

To isolate wild-type *S. aureus* S123 and S133 from ready-to-eat kimbap, suspicious colonies on Baird-Parker agar were analyzed on the basis of Gram stains as well as catalase and coagulase tests, and were confirmed by PCR. The isolates showed products of 482 bp (*nuc* gene) and 240 bp (16S rRNA gene) in the PCR analysis, which were the same as those of *S. aureus* KCCM12103 (data not shown). To induce the temperate phage, *S. aureus* S123 was treated with mitomycin C, a DNA-damaging agent that activates *recA*-dependent phage induction in gram-negative and -positive bacteria. Temperate phage induction was confirmed by growth curve analysis and electron microscopy (Fig. 1). The phage particles had long non-contractile tails and icosahedral heads. They belonged to *Siphoviridae* in *Caudovirales*.

Genomic Sequence Analysis

The phage TEM123 genome was 43,786 bp with a G+C content of 34.06%. A BLASTN search revealed that its nucleotide sequence shared a high degree of similarity with that of the phages phiNM1 (70%) and TEM126 (84%). A bioinformatics analysis of the phage genome identified 43 putative open reading frames (ORFs). These 43 ORFs were similar to those of genes in the GenBank database with annotated functions. Phage genomes consisted of various structural and functional genes for survival or propagation. The phage TEM123 genes were organized in the following functional modules: replication, DNA packaging, morphogenesis, lysis, and lysogeny (Fig. 2 and Table S1). ORF40 encoded a protein that was homologous

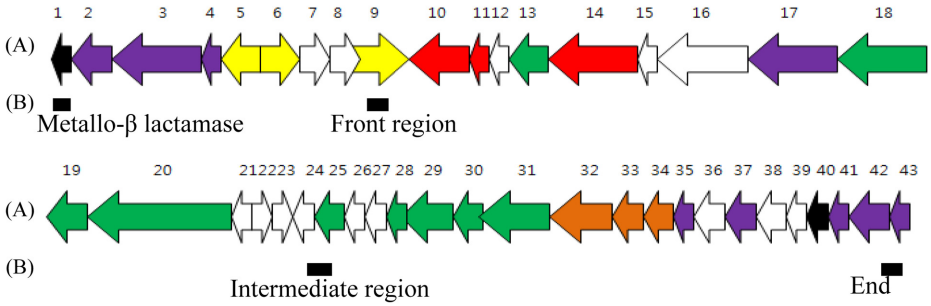

Fig. 2. Schematic representation of the phage TEM123 genome.
Putative open reading frames (ORFs) are represented as arrows with predicted functions, when available. Proposed modules are based on predicted functions. Black: virulence-related protein; Purple: regulation/replication/modification; Yellow: lysogenic module; Red: lysis; Green: morphogenesis; Orange: DNA packaging. (**B**) The target region for PCR in the *S. aureus* S133 transductant.

to the phiPVL-ORF50-like family from *S. aureus*. The phiPVL ORF050 protein was approximately 130 residues long and it was found to be a part of the PVL group of genes, which encode members of the leukocidin group of bacterial toxins that kill leukocytes by creating pores in the cell membrane. PVL is encoded by diverse prophages of *S. aureus*, and it appears to be a virulence factor associated with a number of human diseases. ORF1 was located in the putative metallo-β-lactamase conserved domain, based on a search using the NCBI Conserved Domain Database. Metallo-β-lactamase is an enzyme that confers bacterial resistance to a broad range of β-lactam antibiotics, such as penicillins and carbapenems. Many pathogenic bacteria become virulent after acquiring one or more prophages carrying toxin genes or other virulence factors. Prophages can also confer a diverse array of phenotypic traits to their hosts.

Transduction of Resistance and PCR

S. aureus S133 was used as a host for phage transduction. To isolate the transductant after the transduction assay, the colony was picked up and streaked onto LB agar containing ampicillin. The metallo-β-lactamase gene was detected in the transductant of *S. aureus* S133 by PCR (Fig. 3). The transductant showed a 159 bp product in the PCR analysis, which was the same for metallo-β-lactamase of phage TEM123 determined by sequencing the PCR product (data not shown). The presence of the phage TEM123 genome in the transductant was confirmed using PCR (Fig. 4). A PCR analysis using three primer sets confirmed the presence of an internal region in the genome of TEM123 in the transductant. However, wild-type *S. aureus* S133 was not detected in the internal region of phage TEM123.

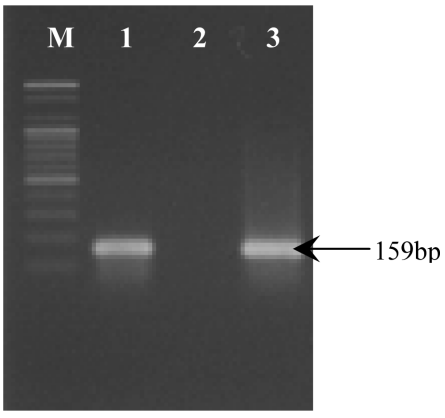

Fig. 3. PCR amplification of the metallo-β-lactamase gene in the wild-type *S. aureus* S133 transductant.
M: 100 bp DNA ladder; Lane 1: *S. aureus* S123; Lane 2: *S. aureus* S133; and Lane 3: *S. aureus* S133 transductant.

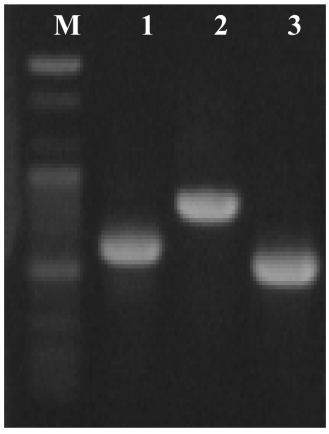

Fig. 4. PCR amplification of the phage TEM123 genome in *S. aureus* 133 transductant.
M: 100 bp DNA ladder; Lane 1: Front region; Lane 2: Intermediate region; and Lane 3: End region.

Antibiotic Resistance Property of the Transductant

The minimal inhibitory concentration (MIC) of a variety of β-lactam antibiotics tested against wild-type *S. aureus* S133, as shown in Table 2, ranged from 0.25 to 1 μg/ml. In the transductant *S. aureus* S133, the MICs of ampicillin and amoxicillin were approximately 128 μg/ml. The MIC of penicillin G was 16 μg/ml, and the MICs of cephalothin and cefoxitin were 2 and 1 μg/ml, respectively. The MICs of imipenem and meropenem were 4 and 0.5 μg/ml, respectively. Thus, the transductant *S. aureus* S133 was resistant to β-lactam antibiotics. However, the resistance of *S. aureus* S133 and its transductant to other antibiotics, such as streptomycin of the aminoglycoside group, vancomycin of the glycopeptide group, rifampicin of the rifamycin group, erythromycin of the macrolide group, tetracycline, and chloramphenicol, did not change.

Discussion

S. aureus produces various virulence factors, such as staphylococcal enterotoxin and toxic shock syndrome toxin 1, which induce superantigen activity [12]. Temperate phages of the pathogen could be carriers of virulence factors, thus playing an important role in pathogenicity [20]. When a temperate phage infects a bacterium, it can replicate via the lytic life cycle and cause lysis of the host bacterium, or it can incorporate its DNA into the bacterium's DNA and become a non-infectious prophage [2, 6]. In *S. aureus*, the number of prophages is generally high, and all *S. aureus* genomes sequenced to date contain at least one prophage. The number of known genome sequences of phages, including prophages of *S. aureus*, has increased, and most of the 76 staphylococcal phages registered in the genome database of EMBL-EBI (http://www.ebi.ac.uk/genomes/phage.html) belong to the *Caudovirales* order. In addition, genomes of more than 260 *S. aureus* strains have been reported, and the presence of prophages (or similar prophage sequences) has been confirmed in each. Through a bioinformatics analysis and an examination of molecular characteristics, the relationships between staphylococcal phages and *S. aureus* in antibiotic resistance, gene transfer, pathogenesis, and evolution have been discussed. Phage-encoded virulence factors provide *S. aureus* with various toxins, such as staphylococcal enterotoxin A, staphylococcal enterotoxin E, exfoliative toxin A, and PVL [27]. Enterotoxins cause food poisoning and toxic shock syndrome. Exfoliative toxin is involved in skin infections, and PVL forms pores in leukocytes. Phage-mediated mobile genetic elements are very important to *S. aureus* because they mediate virulence-related gene transfer. For example, genes encoding toxic shock syndrome toxin (TSST-1) and the enterotoxins SEB, SEC, SEK, SEL, and SEM are located on pathogenicity islands (SaPI1–SaPI4 and SaPIbov). SaPIs, which are typical pathogenicity islands, are replicated and mobilized either in response to SOS-induced excision of a helper prophage in the same strain, or following infection by the joint entry of SaPIs and a helper phage. The molecular mechanism for induction involves the specific interaction of a SaPI repressor and a derepressor encoded by the helper phage. Different proteins of a particular helper phage may be involved in the induction of different SaPIs. Hence, SaPI mobilization represents a remarkable example of evolutionary adaptation involving pathogenicity islands and phages [10, 26].

Table. 2. Antibiotic susceptibility patterns of β-lactam antibiotics against wild-type *S. aureus* S133 and its transductant.

Antimicrobial agents	MIC (μg/ml)		
	Wild-type *S. aureus* S123	Wild-type *S. aureus* S133	Transductant
Penicillin G	16	0.25>	16
Ampicillin	128	0.25>	128
Amoxicillin	>128	1	>128
Cephalothin	4	0.25>	2
Cefoxitin	2	0.25>	1
Imipenem	4	1	4
Meropenem	1	0.25	0.5

S. aureus has become resistant to various antibiotics, and resistant strains have become a serious problem because they cause hospital- and community-associated infections that are difficult to treat [12]. β-Lactam antibiotics are among the most widely used antibiotics for the treatment of staphylococcal infections [16]. Since penicillin was first introduced in the early 1940s, it improved the prognosis and therapy of patients with staphylococcal infection [7]. However, more than 80% of both community- and hospital-acquired staphylococcal isolates were resistant to penicillin by the late 1960s, and many hospitals had outbreaks of multidrug-resistant *S. aureus*. Methicillin was the first semi-synthetic penicillinase-resistant penicillin, but only 2 years later, the first case of methicillin-resistant *S. aureus* (MRSA) was reported in England [13]. MRSA has become the most problematic gram-positive bacterium in public health, and has recently acquired resistance to vancomycin [31]. Generally, *S. aureus* has two resistance mechanisms against β-lactam antibiotics [20]. First, β-lactamase, produced by *S. aureus*, destroys β-lactam

antibiotics by hydrolysis. The genes for the expression of staphylococcal β-lactamases are organized in a cluster of *blaZ* (β-lactamases)–*blaR1* (signal transducer)–*blaI* (repressor). Second, methicillin resistance requires the presence of the chromosomal *mecA* gene, which is responsible for the synthesis of penicillin-binding protein 2a (PBP2a) and is intrinsically insensitive to methicillin and to most β-lactam antibiotics. Resistance against methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome *mec* (SCC*mec*). The *mecA* gene codes for an altered form of PBP2a that has a lower binding affinity for β-lactam antibiotics. Generally, β-lactamase enzymes are divided into classes A, B, C, and D, based on their molecular characteristics. Classes A, C, and D enzymes utilize serine for the hydrolysis of β-lactam antibiotics, and class B (metallo-β-lactamase) enzymes require divalent ions for hydrolysis [4]. In *S. aureus*, classes A, C, and D are located in plasmids, whereas class B enzymes reside on the chromosome. Voladri and Kernodle [33] first reported the nucleotide and deduced amino acid sequences of type B β-lactamase in the chromosome of *S. aureus*. Kahlon *et al*. [14] demonstrated that metallo-β-lactamase and other drug-resistant genes are found in vancomycin-resistant *S. aureus* Mu50, based on in silico phylogenetic motif profiling. In a search for metallo-β-lactamase in prophages of *S. aureus* in the UniProtKB database (http://www.uniprot.org/), 18 metallo-β-lactamases were found to be encoded on prophages of *S. aureus* strains. In our study, we discovered that phage TEM123 was induced by mitomycin C from wild-type *S. aureus*, and we analyzed its morphological and genomic characteristics. Through bioinformatics analysis, we found that the genome of phage TEM123 from wild-type *S. aureus* S123 contains a gene for metallo-β-lactamase. Conversion of phage TEM123 might play a role in the transfer of β-lactam antibiotic-resistant determinants in *S. aureus*. Thus, we present evidence that food is a potential route for the transfer of antibiotic resistance in bacteria that cause foodborne outbreaks.

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