Vancomycin-Resistant *Lactococcus lactis* 1A-1 Isolated from a Competitive Exclusion Product Transfers Vancomycin Resistance Genes to *Staphylococcus aureus*

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Abstract: A vancomycin-resistant *Lactococcus lactis* isolate 1A-1 from a competitive exclusion (CE) product contained plasmid-encoded *vanA, B, C*1, and *C*2/3 genes. The *L. lactis* 1A-1 conjugatively transferred the genes to *Staphylococcus aureus in vitro*. CE product bacteria may be reservoirs for dissemination of *vanA, B,* and *C* genes to the human gastrointestinal microbiota.

INTRODUCTION

Vancomycin is a drug of last resort against antibiotic-resistant enterococci and methicillin-resistant staphylococci. Unfortunately, resistance to vancomycin is increasingly being reported [1, 2]. Studies on the dissemination and selection of antimicrobial drug resistance have focused mainly on clinically relevant bacterial species, but commensal bacteria may act as reservoirs of antimicrobial drug resistance genes similar to those found in human pathogens [3]. The commensal reservoir bacteria can be present in the intestines of farm animals exposed to antimicrobial drugs [4], and subsequently may contaminate the raw meat produced from these animals. Therefore, antimicrobial drug resistance genes from non-pathogenic as well as from pathogenic organisms are medically important.

Competitive exclusion (CE) products are mixtures of bacteria that are applied to newly hatched poultry chicks for rapid establishment of a protective gastrointestinal (GI) tract microbiota, which can impart colonization resistance against the human food-borne pathogens *Salmonella enterica* and *Campylobacter jejuni* [5]. A CE product may be manufactured by anaerobic culture of the bacterial mixture in large fermentation batches. During the growth phase of the bacteria, they can come into contact with other species in the mixture. If conjugation occurs between these bacterial species, genes could be transferred from one to another. A *Lactococcus lactis* strain 1A-1 was isolated from a CE product that was resistant to vancomycin [6], which is atypical of the species [7]. It is possible that the *L. lactis* isolate acquired a vancomycin resistance gene from a vancomycin-resistant *Enterococcus* sp. that was present in the CE product [8]. In the present study, we hypothesize that the vancomycin-resistant *L. lactis* isolate may have the potential to transfer resistance determinants to a *S. aureus* strain similar to those that normally transit the human GI tract [9]. The present study was conducted to identify the vancomycin resistance genes present in the *L. lactis* isolate. The ability of the *L. lactis* isolate to transfer vancomycin resistance genes by conjugation to *S. aureus* was also evaluated.

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MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The vancomycin-resistant *L. lactis* gene donor and *S. aureus* transconjugants were grown at 30ºC in MRS broth (Thermo Fisher Scientific, Houston, TX) supplemented with 30 μg/ml vancomycin (Sigma Chemical Co., St. Louis, MO) or in Elliker broth (Thermo Fisher) containing various concentrations of vancomycin at 30°C. Chemically competent *E. coli* (Invitrogen, Grand Island, NY) was grown at 37ºC in Luria Bertani (LB) broth (Invitrogen). *S. aureus* ATCC 25923 was grown at 37ºC in MRS broth.

Phenotypic Vancomycin Susceptibility Test

A disk diffusion method was used for antibiotic susceptibility testing [10]. Trypticase soy agar (TSA) plates (REMEL Laboratories, Lenexa, KS) were inoculated over the entire surface with a cotton-tipped applicator moistened in a MRS broth (Thermo Fisher) suspension of *L. lactis* 1A-1at a density of 10^7 CFU/ml (a 1:10 dilution of a 0.5 McFarland standard). The disks of vancomycin (30 μg) were applied to inoculated TSA plates, which were incubated overnight at 30ºC. Inhibition zone sizes of suspected vancomycin resistance gene-containing isolates were compared with zone sizes of bacteria that were susceptible to 30 μg/ml vancomycin in broth culture and *E. faecium* ATCC 51559, which contains the *vanA* gene and is resistant to 30 μg/ml vancomycin [11]. Control organisms were as follows: *Enterococcus faecalis* ATCC 51299 contained the *vanB* gene, *Enterococcus gallinarum* ATCC 49573 was used for the $vanC_1$ gene, the $vanC_{2/3}$ genes were present in *Enterococcus casseliflavus* ATCC 25788, and no vancomycin resistance gene was present in *Enterococcus faecalis* ATCC 29212 [11].

Curing of Episomally Encoded Antimicrobial Resistance

To determine if the resistance genes were carried by a plasmid, *L. lactis* 1A-1 was grown in MRS broth for 15 days

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in the presence of 10 μg/ml acridine orange [12]. Following repeated exposure to the curing agent, the bacteria were diluted in MRS broth and spread on MRS agar plates. The isolated colonies were replica-plated with nitrocellulose filters (Schleicher and Schuell, Keene, NH) to MRS agar plates without and with 30 μg/ml vancomycin. Susceptible colonies were tested by PCR to verify loss of the *vanA* gene. The isolate sensitivity to antibiotics was examined by the disk diffusion method.

Mating Experiments

A filter mating technique [13] was used to assess whether *L. lactis* 1A-1 could transfer vancomycin resistance by conjugation to *S. aureus* ATCC 25923. Bacteria were grown overnight in brain-heart infusion (BHI) broth (REMEL Laboratories). Donor (*L. lactis* 1A-1) and recipient (*S. aureus*) cells were mixed at a one to one ratio and incubated at 30°C for four hours in BHI broth. The cells were collected by suction filtration onto 0.45 μm cellulose acetate filters (Millipore Corp., Bedford, MA) and the filters were placed onto trypticase soy agar plates containing 5% sheep blood and 6 μg/ml vancomycin. The plates were incubated overnight at 37°C. Bacteria were recovered from the plates and filters by rinsing with BHI broth. Vancomycin-resistant colonies of the recipient *S. aureus* were subcultured on BHI agar containing 30 μg/ml of vancomycin. DNA was isolated from resistant recipients and tested by PCR for the presence of vancomycin resistance genes for verification of resistance mechanism transfer.

Screening, Isolation, Cloning and Sequencing Analysis of Vancomycin Resistance Genes

Existing PCR methods were used for detection of genes encoding vancomycin resistance [11, 14]. Genomic DNA from the *L. lactis* and *S. aureus* isolates was isolated using the Wizard Genomic DNA isolation kit (Promega, Madison, WI) and plasmid DNA was isolated using the technique described by Anderson and McKay [15]. The *van*A gene sequences on the plasmid DNA were isolated by PCR (forward primer: 5' -GGG AAA ACG ACA ATT GC-3', reverse primer: 5' -GTA CAA TGC GGC CGT TA-3') and the products were inserted into the *Eco* RI polylinker site of a pCR2.1-TOPO cloning vector, as described in the manufacturer's instructions (Invitrogen). *E. coli* DH5α cells (Invitrogen) were transformed with the recombinant plasmids. The inserted resistance genes were sequenced and gene sequences were submitted to GenBank BLAST searches [16] to determine if they were homologous to known resistance genes (i.e., *van*A). The PCR analyses [11] were used to detect *vanA*, *vanB*, $vanC_1$, and $vanC_{2/3}$, and the fragments lengths of the PCR product bands on 2% agarose electrophoresis gels were compared with a 100 bp ladder (Invitrogen).

RESULTS

Antimicrobial Susceptibility

The *L. lactis* 1A-1 isolated from the competitive exclusion product was resistant to vancomycin (30 μg/ml), when examined by the disk diffusion assay and compared to susceptible bacteria (Table **1**). The isolate was also able to grow in Elliker broth containing 1500 μg/ml vancomycin. The *S. aureus* transconjugants also exhibited smaller zones of inhibition than the original recipient strain (Table **1**).

Table 1. Phenotypic Detection of Vancomycin Resistance by *L. lactis* Donor and *S. aureus* Transconjugants

Isolate	Growth inhibition zone diameter (mm)
L. lactis 1A-1	0
L. lactis 1A-1 after curing	22
S. aureus wt*	20
S. aureus tc	15
S. aureus tc after curing	21
E. faecalis ATCC 51299	9
E. faecalis ATCC 29212	18

*wt = wild type, tc = transconjugant.

Mating Experiments

Conjugative transfer of high level vancomycin resistance from *L. lactis* 1A-1 to *S. aureus* ATCC 25923 (sensitive parent strain) was demonstrated *in vitro*. Five transconjugant clones were recovered that were resistant to 30 μg/ml of vancomycin, having inhibition disk zone diameters of 15 mm, compared with a 20 mm zone diameter of the wild type *S. aureus*.

Screening, Isolation, Cloning and Sequencing Analysis for Vancomycin Resistance Genes

The presence of the *vanA* vancomycin resistance gene was detected in *L. lactis* 1A-1 and the *S. aureus* transconjugant, but not the wild type *S. aureus* recipient strain by PCR (Fig. **1**). The nucleotide sequences of the *van*A gene of *L. lactis* 1A-1 were 97% identical to those of the *E. faecium*

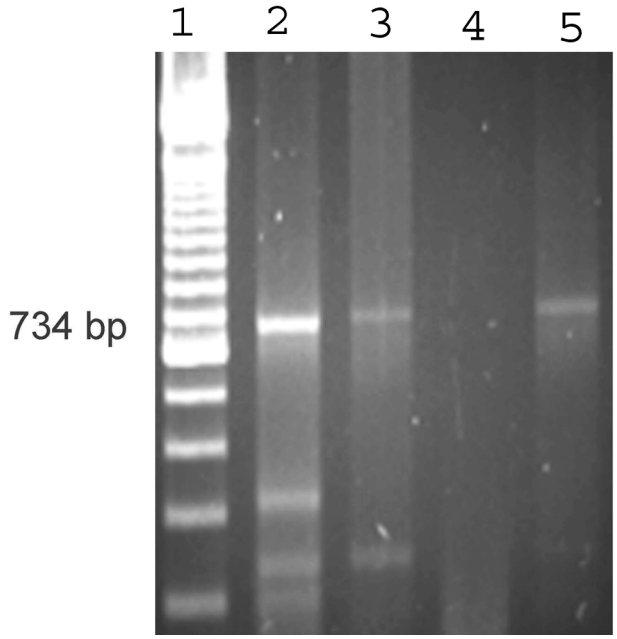

Fig. (1). Detection of the *vanA* gene in bacteria isolates by PCR. The gel lanes are: 1 = molecular weight ladder, 2 = *L. lactis* 1A-1, 3 = *S. aureus* transconjugant, 4 = *S. aureus* 25923, 5 = *E. faecium* 51559 positive control.

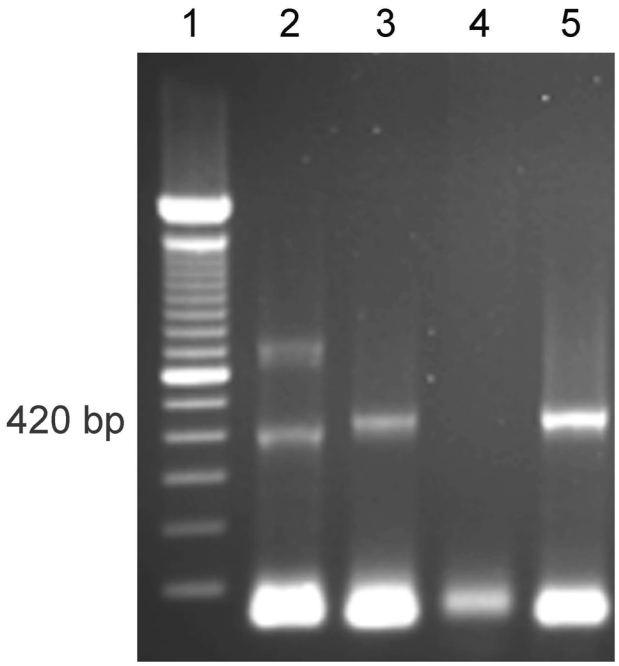

Fig. (2). The *vanB* gene was detected in the *L. lactis* donor and the *S. aureus* transconjugant by PCR. The gel lanes are: 1 = molecular weight ladder, 2 = *E. faecalis* 51299 positive control, 3 = *L. lactis* 1A-1, 4 = *S. aureus* 25923, 5 = *S. aureus* transconjugant.

strain F4/13 (54) in GenBank, according to a BLAST search (Accession no. EF206285.1). The *L. lactis* 1A-1 isolate and the *S. aureus* transconjugants also had *vanB* gene sequences detectable by PCR (Fig. **2**). The $vanC_1$ (Fig. **3**) and $vanC_{2/3}$ (Fig. **4**) genes were also detected in *L. lactis* 1A-1 and the *S. aureus* transconjugant, but not in the recipient wild type *S. aureus*. The $vanC_1$ PCR product had 97% sequence identity with the $vanC_1$ gene from *Enterococcus gallinarum* (Accession no. AY786180.1).

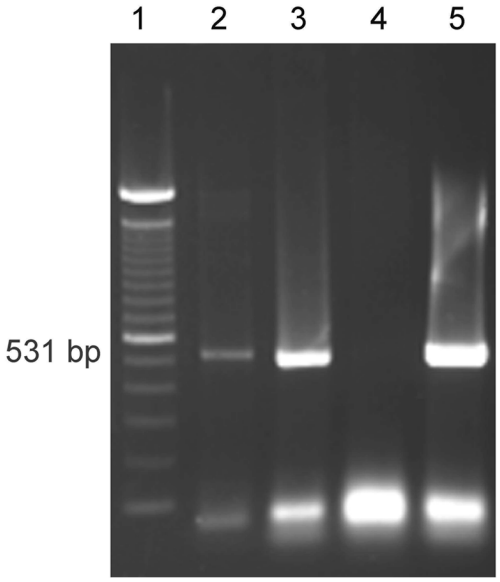

Fig. (3). The $vanC_1$ gene was detected in the *L. lactis* donor and the *S. aureus* transconjugant by PCR. The gel lanes are: 1 = molecular weight ladder, 2 = *L. lactis* 1A-1, 3 = *S. aureus* transconjugant, 4 = *S. aureus* 25923, 5 = *E. gallinarum* 49573 positive control.

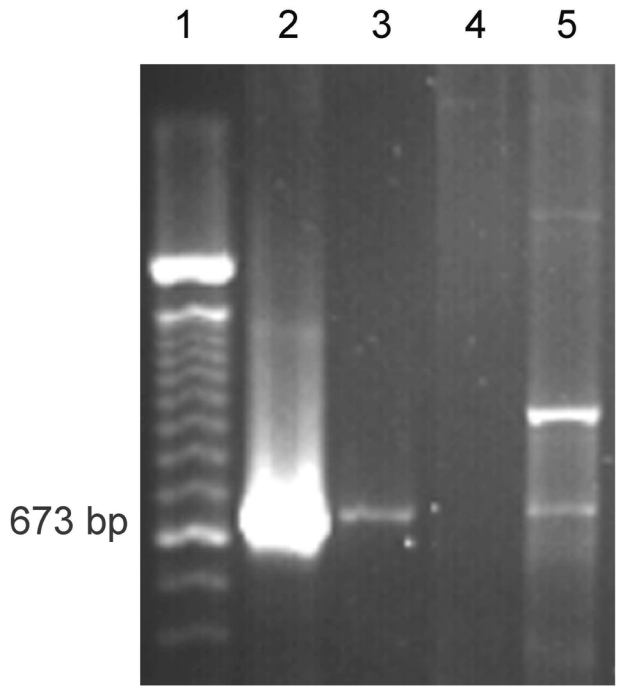

Fig. (4). The $vanC_{2/3}$ genes were detected in the *L. lactis* donor and the *S. aureus* transconjugant by PCR. The gel lanes are: 1 = molecular weight ladder, 2 = *L. lactis* 1A-1, 3 = *S. aureus* transconjugant, 4 = *S. aureus* 25923, 5 = *Enterococcus casseliflavus* 25788 positive control.

Curing of Episomally Encoded Antimicrobial Resistance

The acridine orange treatment yielded revertant *S. aureus* transconjugants at a rate of 86%. The reversion rate of *L. lactis* 1A-1 was 36%. As a control, *E. faecium* 51559 was also treated with acridine orange. It had a reversion rate of 93%. The ability to make the bacteria susceptible to vancomycin by acridine orange treatment suggests that *vanA* was present on episomal DNA in *L. lactis* 1A-1 and the *S. aureus* transconjugant. Plasmid-cured *L. lactis* 1A-1 and the plasmid-cured *S. aureus* transconjugant were more susceptible than the uncured clones, as shown with the disk diffusion assay (Table **1**).

DISCUSSION

The purpose of using CE products in poultry agriculture is to increase food safety by decreasing the incidence of adult animals reaching the human food supply contaminated with food-borne pathogens, such as *Salmonella enterica* and *Campylobacter jejuni*. The documented ability of these products to competitively exclude food-borne pathogens is encouraging, but not if the CE bacteria themselves pose a food safety risk [5]. The scientific community is aware that antimicrobial drug resistance genes can be mobilized between bacterial species in the gastrointestinal tract [17]. The vancomycin-resistant enterococci (VRE), once thought to be exclusively a problem associated with the medical use of glycopeptide drugs [18], are now being found in environmental sources that may contribute to the growing threat of VRE in and outside of the clinical setting [19]. CE products could contain VRE that transfer vancomycin resistance to other bacteria in the products during manufacture and to bacteria in the GI tracts of human poultry consumers. VRE have

the ability to conjugatively transfer vancomycin resistance genes to *Staphylococcus aureus in vitro* [13]. Since *S. aureus* is a normal constituent of human microbiota, the presence of VRE in poultry may have the potential to transform *S. aureus* in the microbiota of consumers, essentially infecting them with this troublesome opportunistic pathogen.

In the present study, we found a vancomycin-resistant *L. lactis* in a commercial CE product that may have acquired the *vanA* gene from a VRE reported to be present in the CE product [20]. We confirmed that *L. lactis* 1A-1 contained a *vanA* gene on a high molecular weight plasmid. The *van*A gene was conjugatively transferred to *S. aureus in vitro*, suggesting that this activity could occur *in vivo* in a human poultry consumer. We also observed PCR products of other vancomycin resistance genes that may have been transferred from *L. lactis* 1A-1 to *S. aureus*, including *vanB*, *vanC*$_1$, and *vanC*$_{2/3}$. These latter genes are associated with intermediate levels of resistance to vancomycin, while *vanA* is usually required for high resistance levels greater than 30 μg/ml [21]. In fact, *L. lactis* 1A-1 was resistant to extremely high concentrations, up to 1500 μg/ml. The *vanA* [22], *vanB*$_{1/2}$ [23, 24], *vanC*$_1$, and *van*C$_{2/3}$ [11] genes have been reported on different conjugative DNA elements from different species of bacteria. Detection of multiple vancomycin resistance genes on the same conjugatively transferred element is uncommon. The co-culture methods used to produce some CE products may promote higher rates of gene transfer than the GI tract environment. Several reports indicate that factors normally present in the diet and the GI tract environment may inhibit horizontal gene transfer events, making the risk of this phenomenon less than might be otherwise expected [18, 25, 26].

The nature of lactococci and enterococci appears to be that they can pass genes to each other. Horizontal gene transfer rates of mobilizable plasmids from *L. lactis* to *E. faecalis* have been measured in the digestive tracts of gnotobiotic mice at 3-4 $\log_{10}$ CFU/g feces [27]. Evidence also exists for the ability of lactococci to transfer genes to Enterobacteriaceae [28], so it is unclear how widely *L. lactis*-contaminated poultry meat could spread vancomycin resistance genes in the GI tracts of human consumers.

CONCLUSION

In the present study, multiple vancomycin resistance genes were transmissible by a *L. lactis* isolate from a CE product to a typical *S. aureus* that could be present in the GI tract of a human consumer. From the results presented in this study, it seems prudent to be concerned about the presence of transferable vancomycin resistance genes in live microbial animal feedstuffs and human food additives that contain enterococci and lactococci.

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