

SHORT REPORT

Nucleotide substitutions in *vanC-2* gene of *Enterococcus casseliflavus* isolates obtained from chickens

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SUMMARY

DNA sequencing of the *vanC-2* gene was partially carried out on 10 isolates of *Enterococcus casseliflavus* obtained from 8 samples of imported chickens in Japan between July 1999 and June 2001 to evaluate the variation in the gene. Forty nucleotide substitutions in 36 codons were identified within 345 base pairs when compared with the *vanC-2* sequence of the reference strain *E. casseliflavus* ATCC25788. Identical nucleotide substitutions were commonly found in the isolates recovered from chickens imported from both Brazil and China. Pulsed-field gel electrophoresis (PFGE) patterns of *NotI*-digested chromosomal DNA of these strains were distinguished by two, or more than six, band differences. These observations suggest that sequencing of the *vanC-2* gene may be helpful for epidemiological investigation in combination with the PFGE analyses of the isolates, although particular genotypes are unlikely to be restricted to each of the countries that exported chickens.

The *vanC-2* ligase gene has been known to be harboured in *Enterococcus casseliflavus* and can confer low to moderate levels of resistance to vancomycin [1]. It is also demonstrated that clinical enterococcus isolates carrying the *vanC-2* gene exhibited up to 7% variation within 346 base pairs when the *vanC-2* gene sequences were compared to the reference strain *E. casseliflavus* ATCC25788 [2]. Because occurrence of the *vanC-2* genes in enterococcus strains isolated from poultry has been reported [3–5], they have been suspected as a possible route of colonization of *E. casseliflavus* to humans. It is of interest that the identical nucleotide substitutions with those in the human isolates can be found in the isolates from poultry origin, although there is no sequencing analyses of the *vanC-2* genes in these strains. Approximately one third of the chicken meat consumed in Japan is imported from various countries [6]. We thus analysed DNA sequences of *vanC-2* genes in entero-

coccus isolates obtained from imported and domestic chicken meat in Japan. Additionally, pulsed-field gel electrophoresis (PFGE) patterns were compared among the isolates to determine whether the specific variation of the *vanC-2* gene in the isolates is associated with a certain PFGE pattern.

A total of 34 pieces of chicken meats were investigated. Of the 34 chickens, 4 were imported from China in July 1999, and 7 and 8 were imported from China and Brazil, respectively, between April 2000 and June 2001. Chickens were packed and frozen in the country of origin and shipped to Japan. Fifteen chicken meats produced in Japan were collected during April 2000 and June 2001. The chicken meat was swabbed on the surface with sterilized cotton swab and incubated in a heart-infusion broth for 20 h at 37 °C. The cultures were streaked on to EF agar (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) or bile-esculin-azide agar (Becton Dickinson Microbiology Systems Sparks, MD, USA) containing 6 µg/

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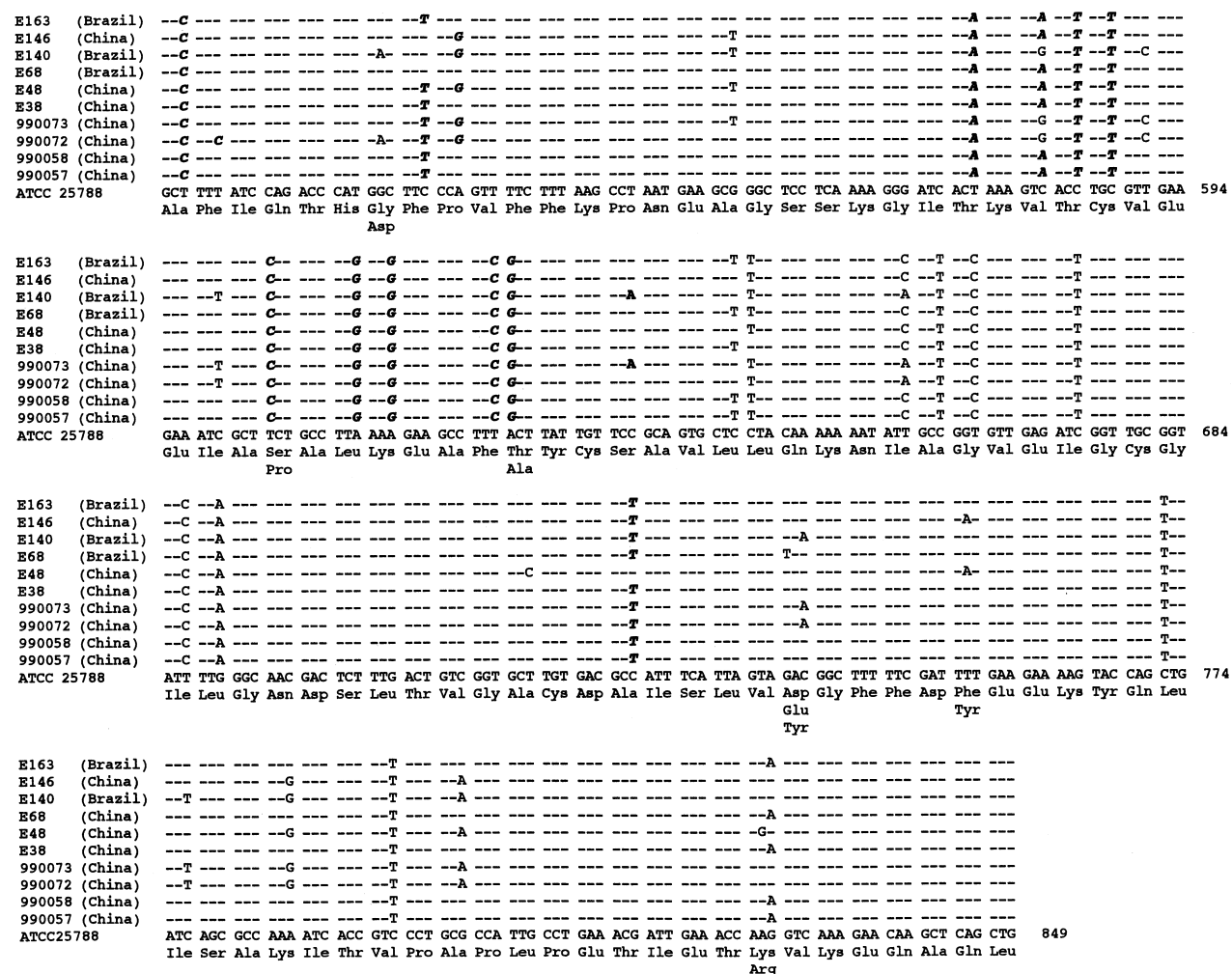


Fig. 1. Comparison of DNA sequences of *vanC-2* genes from chicken isolates with the reference strain ATCC25788. The numbers on the right refer to the nucleotide position described in a reference [2]. The nucleotide substitutions that were previously reported [2] appear in italics.

ml of vancomycin (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 48 and 24 h, respectively. Suspected colonies were identified as *Enterococcus* sp. by positive Gram stain, absence of catalase, growth on bile-esculine agar with esculin hydrolysis, and 6.5% NaCl tolerance. Susceptibilities to vancomycin of the enterococcus isolates were determined by the agar dilution method [7]. Vancomycin resistance genes were detected by a multiplex PCR assay with previously published primer pairs [8]. DNA sequencing analysis of the *vanC-2* genes was partially performed with the primers described above by using a DNA sequencer (GeneRapid, Amersham Pharmacia Biotech, Upssala, Sweden). The reference strain *E. casseliflavus* ATCC25788 was also used. 16S ribosomal RNA (rRNA) in the *vanC-2*-containing strains was amplified with primers complementary to nucleotides 149–174 and 550–524 of the 16S rRNA sequence

of *E. casseliflavus* [9]. DNA sequencing analysis of the 16S rRNA genes was performed with the same primers. For PFGE analysis of the strains with the *vanC-2*, chromosomal DNA was prepared in agar plugs as described elsewhere [10]. The *Sma*I- and *Not*I-digested patterns of the strains were analysed using a CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA, USA).

A total of 29 enterococcus isolates were obtained from 7 of the 11 chicken samples from China, 7 of the 8 samples from Brazil, and 11 of the 15 chicken meat samples produced in Japan. These isolates showed MICs of 4–8 µg/ml to vancomycin. Of the 29, 10 isolates (7 isolates obtained from 5 samples from China during 1999 and 2001 and 3 isolates from 3 samples from Brazil during 2000 and 2001) had *vanC-2* genes and they were all identified as *E. casseliflavus* by sequencing of the 16S rRNA genes. The others (3 isolates

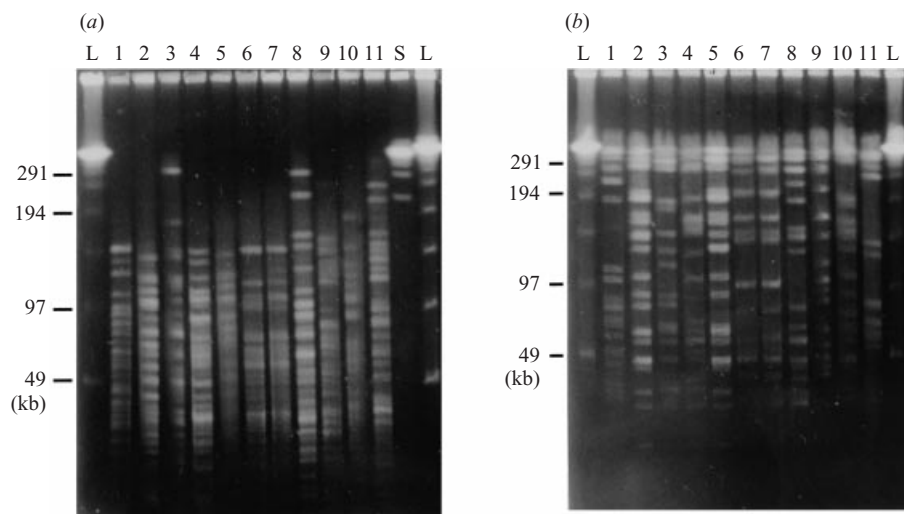


Fig. 2. *SmaI*- (a) and *NotI*-digested (b) PFGE patterns of *E. casseliflavus* isolates. Lanes: 1, E38; 2, E48; 3, E68; 4, E140; 5, E146; 6, E163; 7, 990057; 8, 990058; 9, 990072; 10, 990073; 11, ATCC25788; L, lambda 48.5-kb ladder; S, *Saccharomyces cerevisiae* size standards.

from 3 chicken samples from China, 5 isolates from 5 chicken samples from Brazil, and 11 isolates from 11 chicken samples produced in Japan during 2000 and 2001) carried *vanC-1* genes.

We sequenced 345-bp DNA sequences for the 10 *vanC-2*-containing isolates from the chickens and for the reference strain *E. casseliflavus* ATCC25788 (Fig. 1). In the 10 chicken isolates, 40 nucleotide substitutions in 36 codons were identified when compared with the *vanC-2* sequence of *E. casseliflavus* ATCC25788. Fifteen of the 40 sites found in this study have previously been reported [2]. We found 15 substitutions within nucleotides 505–726 of the *vanC-2* sequence that were not reported by Patel et al. [2]. In addition, 10 substitutions within nucleotides 727–849 were identified in this study. Six substitutions resulted in amino acid changes in the inferred VanC-2 protein. All the isolates had the identical nucleic substitution in each of 16 nucleotide positions. Of these, two substitutions at positions 604 and 625 were accompanied by amino acid changes [2]. No insertions or deletions were found. The numbers of band difference in their *SmaI*- and *NotI*-digested PFGE patterns between isolates E163 and 990057 were one and two, respectively, and the results were interpreted to be closely related according to the criteria for bacterial strain typing by the PFGE methodology [11]. The other strains were distinguished by more than six band differences (Fig. 2).

The number of nucleotide substitutions within nucleotides 505–726 identified in the present study was greater than that in the previous report [2]. Two

possible reasons can be suggested. One possibility is that the sources of enterococcus isolates are different in each study. Patel et al. [2] analysed human clinical isolates collected from 1992–5 in the United States whereas our collections were obtained from chicken meat between January 1999 and June 2001. Alternatively, substitutions may have accumulated from year to year, because 15 of the 19 changes that were observed within nucleotides 505–726 by Patel et al. [2] were also found in our isolates. Further analyses of the *vanC-2* genes of recent isolates from human clinical cases are needed to clarify these questions. Most nucleotide substitutions in the present study did not result in amino acid changes. We have reported variations in the *sic* gene of *Streptococcus pyogenes*, in which almost all the nucleotide changes were accompanied by amino acid replacement [12]. It has been considered that a complement-inhibitory protein of *S. pyogenes*, encoded by the *sic* gene, is one of the virulence factors for this organism [13] and that the novel variations in the *sic* gene gave an aetiological advantage to the novel strains having these variations [12]. On this point, variations observed in the *vanC-2* genes are likely to have occurred as a result of mechanisms different from those in the case of the *sic* gene in *S. pyogenes*.

An identical sequence was found in one isolate (E163) obtained from a sample from Brazil in June 2001 and two isolates (990057 and 990058) obtained from a sample from China in January 1999. In another instance (E140) obtained from a sample from Brazil in March 2001 and two isolates (990072 and

990073) obtained from a sample from China in January 1999, each of six nucleotide positions 582, 591, 600, 660, 741, and 777, respectively, had the identical nucleic substitution, although these were not found in the other strains. These observations suggest that the specific variation of the *vanC-2* genes is unlikely to be associated with the country that exported the chicken from which *E. casseliflavus* isolates were obtained. The isolates having the identical nucleic substitutions in the *vanC-2* sequence were distinguished by their PFGE patterns in the present study, suggesting that PFGE are more discriminative than sequencing of the *vanC-2*. Sequence variation in the *vanC-2*, however, is so notable that sequencing of the gene is likely to be helpful for epidemiological purpose. Although *E. casseliflavus* is not frequently recovered from clinical specimens, they may cause serious invasive infections [14, 15]. PFGE analyses as well as sequencing of the *vanC-2* genes of isolates may be necessary for epidemiological investigation [2].

Nucleotide sequence accession number. The nucleotide sequences of isolates 990057, 990058, 990072, 990073, E38, E48, E68, E140, E146, and E163 have been submitted to DNA Data Bank of Japan (DDBJ) and have been given accession no. AB070696, AB070697, AB070698, AB070699, AB070700, AB070701, AB070702, AB070703, AB070704, and AB070705, respectively.

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