Original Article

Inducible vancomycin resistance is common in porcine *Enterococcus gallinarum* and *E. casseliflavus* isolates

Wanida Tharvornvee[1,2] Chantima Pruksakorn[1,2*] Porntippa Lekcharoensuk[1,2]

Abstract

Enterococcus gallinarum and *E. casseliflavus* possess intrinsic vancomycin resistance, which is mediated by *vanC-1* and *vanC-2* gene clusters, respectively. The VanC resistance phenotype is expressed inducibly or constitutively. This study characterized the expression types for vancomycin resistance in 23 porcine *E. gallinarum* and 19 porcine *E. casseliflavus* isolates with vancomycin minimum inhibitory concentrations between 4 and 16 µg/ml. The $vanS_C$ and $vanS_{C-2}$ genes were sequenced and the deduced amino acid sequences were compared to those of the inducible and constitutive resistance reference strains. Analysis of growth curves revealed that the resistance phenotype in all isolates was induced by vancomycin. In the presence of vancomycin, lagging periods of these bacterial strains were 3 hours longer than when they were cultured in media without the drug. Transcriptional analysis using reverse transcription-polymerase chain reaction revealed that *vanC-1* and *vanC-2* genes were expressed by *E. gallinarum* and *E. casseliflavus*, respectively, when they were grown in the presence of vancomycin. Thus, the *vanC-1* and *vanC-2* mRNA expression were inducible. Alignment of the $VanS_C$ and $VanS_{C-2}$ protein sequences of the Van inducible and constitutive strains revealed conserved glycine residues at positions 320 and 296 in the inducible strains of *E. gallinarum* and *E. casseliflavus*, respectively. These results suggest that VanC enterococci from Thai swine may commonly express inducible vancomycin resistance phenotype and the inducible vancomycin resistance is associated with the conserved glycine residues of VanSc proteins.

Keywords: *Enterococcus*, inducible vancomycin resistance, *vanC-1*, *vanC-2*

[1]*Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand*
[2]*Center of Advanced Studies for Agriculture and Food, KU Institution for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand*
***Correspondence**: fvetctm@ku.ac.th*

Introduction

Enterococci are normally present in the intestine of humans and animals (Noble, 1978). These bacteria, previously considered as opportunistic pathogens, are now recognized as one of the leading causes of hospital-associated infections (Hidron et al., 2008). Much concern has been expressed regarding their resistance to several antimicrobials and particularly to glycopeptide antibiotics (Murray, 2000). Glycopeptide antibiotics such as vancomycin and teicoplanin are currently used for the treatment of severe Gram-positive bacterial infections (Arthur et al., 1996). Strains of enterococci resistant to vancomycin are typically multidrug-resistant (Cetinkaya et al., 2000) and therapeutic drugs that can cure the infection are rare.

Vancomycin resistance in enterococci is phenotypically and genotypically diverse. There are at least six distinct vancomycin resistance phenotypes: VanA, VanB, VanC, VanD, VanE and VanG, and these phenotypes are mediated by the *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG* genes, respectively (Courvalin, 2006). Among them, VanA, VanB and VanC are the major resistance phenotypes present in clinical specimens (Fisher and Philips, 2009). Most human vancomycin-resistant enterococcus (VRE) infections caused by *Enterococcus faecium* and *E. faecalis* commonly exhibit VanA and VanB phenotypes (Mazuski, 2008; Fisher and Philips, 2009). VanA VRE is inducibly resistant to high levels of vancomycin and teicoplanin (Courvalin, 2006). VanB phenotype is inducibly resistant to variable levels of vancomycin. VanC phenotype appears to be an intrinsic property of *E. gallinarum* (VanC-1) and *E. casseliflavus* (VanC-2) and confer a low level to vancomycin resistance (Courvalin, 2006). VanC species has been found to infect humans sporadically, but it causes serious invasive infections, including endocarditis and meningitis (Toye et al., 1997). In addition, failure of vancomycin treatment in an endocarditis model was associated with strains resistant to low level of vancomycin (Fantin et al., 1991).

Proteins expressed by the *van* gene clusters are involved in modification of the drug target, the peptidoglycan pentapeptide precursor (Reynolds et al., 1994; Arthur et al., 1996). The altered drug targets clearly reduce affinity for vancomycin and prevent the inhibitory effect of the drug on cell wall biosynthesis. *vanC* gene variants specific to *E. gallinarum* and *E. casseliflavus* are designated *vanC-1* and *vanC-2* subtypes, respectively (Navarro and Courvalin, 1994; Courvalin, 2006). The *vanC-1* cluster comprises *vanC-1*, $vanXY_C$, *VanT*, $vanR_C$ and $vanS_C$ genes (Arias et al., 2000). The encoded VanT protein produces D-Serine (Ser) from L-Ser (Arias et al., 2000). VanC has D-Alanine (Ala):D-Ser ligase activity, which is involved in the synthesis of modified peptidoglycan precursors ending with D-Ala-D-Ser. $VanXY_C$ is responsible for the elimination of normal precursors ending with D-Ala-D-Ala (Reynolds et al., 1999). $VanR_C$ and $VanS_C$ proteins are likely responsible for the regulation of gene expression (Arias et al., 2000). The *vanC-2* cluster of *E. casseliflavus*, which is composed of *vanC*-2, $vanXY_{C\text{-}2}$, $VanT_{C\text{-}2}$, $vanR_{C\text{-}2}$ and $vanS_{C\text{-}2}$ genes, shows similar organization to those of the *vanC-1* gene cluster of *E. gallinarum* (Dutta and Reynolds, 2002).

In a study of VanB strains, deletion of amino acids in the $VanS_B$ protein was found to be associated with the conversion of an inducible to a constitutive phenotype (Depardieu et al., 2003). The expression for VanC-type resistance can be either inducible or constitutive (Sahm et al., 1995; Panesso et al., 2005). It is likely that VanC enterococci commonly express inducible vancomycin resistance. However, there is no report regarding the characteristics of VanC enterococci from Thai swine. Therefore, this study aimed to determine the vancomycin resistance characteristics of *E. gallinarum* and *E. casseliflavus* isolates from pigs in various age ranges and from different locations to reveal whether they are inducible or constitutive. In addition, $vanS_C$ and $vanS_{C\text{-}2}$ genes were sequenced and deduced amino acid was compared with $VanS_C$ of the reference strains.

Materials and Methods

Bacterial strains and growth conditions: The bacterial strains and their minimum inhibitory concentration (MIC) of vancomycin are described in the Table 1. Of the total of 42 porcine enterococcus isolates (Pruksakorn et al., 2016), 23 were *E. gallinarum* (*vanC-1*; vancomycin MIC, 4-16 µg/ml) and 19 were *E. casseliflavus* (*vanC*-2; vancomycin MIC, 4-16 µg/ml). *E. casseliflavus* ATCC 25788 (*vanC*-2; vancomycin MIC 8 µg/ml) has been characterized as inducible vancomycin resistance as described elsewhere (Dutta and Reynolds, 2002). Cells were grown in brain heart infusion (BHI) medium (Becton, Dickinson and Company, USA) at 37°C.

Table 1 Strains of VanC enterococci and their vancomycin MICs (n = 42)

Strains	Number of isolates MIC of vancomycin (µg/ml)			Total isolates
	4	8	16	
E. gallinarum (*vanC-1*)	3	15	5	23
E. casseliflavus (*vanC-2*)	8	10	1	19

Growth curve analysis: Growth curve analysis was performed to detect constitutive or inducible vancomycin resistance phenotypes. Analysis of the growth curves for *E. gallinarum* and *E. casseliflavus* was modified from the protocols previously described (Sahm et al., 1995; Panesso et al., 2005). A test strain of *E. gallinarum* was grown in BHI broth. After incubation for 18 h, 1.5 ml aliquots of the culture were inoculated into two flasks, each containing 30 ml of fresh BHI broth. When the growth of bacterial culture reached an

optical density at 620 nm (OD620) of 0.05, subinhibitory vancomycin at concentration of 2 or 4 µg/ml (2 µg/ml for isolates with vancomycin MIC of 4 µg/ml, and 4 µg/ml for isolates with vancomycin MIC of 8 µg/ml and 16 µg/ml) was added into one of the flasks. The other flask was used as the control. OD620 of the supplemented and unsupplemented cultures was determined hourly for up to 8 h. Vancomycin (2 µg/ml) was used to analyze the growth curves of *E. casseliflavus*. Duplicate experiments were performed for each isolate. To measure lag time, growth curve fitting of OD data was performed using the USDA Integrated Pathogen Modeling Program (IPMP) 2013 (Huang, 2014).

RNA extraction and reverse transcription-polymerase chain reaction of vanC-1 and vanC-2 genes: mRNA expression of *vanC* was studied in *E. gallinarum* strains MA061022 (vancomycin MIC of 16 µg/ml), MB151020 (8 µg/ml) and MC131022 (4 µg/ml), and *E. casseliflavus* strains SB111012 (vancomycin MIC of 16 µg/ml), RA091020 (8 µg/ml) and SC161032 (4 µg/ml) using reverse transcription-polymerase chain reaction (RT-PCR) analysis. Bacterial cells grown to an OD620 of 1.0 with the absence (non-inducing) or presence (inducing) of vancomycin were collected for total RNA extraction. Total RNA was extracted with the RiboPure-Bacteria kit (Ambion®, Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Concentration and purity of the total RNA were measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

Specific primers for the RT-PCR analysis of *vanC-1* and *vanC-2* mRNA were vanC-1F, 5′-GGTATCAAGGAAACCTC-3′; vanC-1R, 5′-CTTCCG CCATCATAGCT-3′; vanC-2F, 5′-CTCCTACGATTCT CTTG-3′; vanC-2R, 5′-CGAGCAAGACCTTTAAG-3′ (Dutka-Malen et al., 1995). One-step RT-PCR was performed using the purified total RNA (100-150 ng) as a template in a 50 µl reaction containing 200 nM of each forward and reverse primer specific to *vanC-1* or *vanC-2*, SuperScript® III RT/Platinum® *Taq* mix (Invitrogen™, Thermo Fisher Scientific, USA) and the corresponding reaction buffer, according to the manufacturer's instructions. To verify the absence of genomic DNA, PCR amplification of the *vanC-1* or *vanC-2* genes was performed by excluding the reverse transcription step. 16S rRNA RT-PCR was included and served as an internal control using a primer pair RB1-RB2 (Panesso et al., 2005) and RNA samples extracted from *Enterococcus* spp. grown in the absence and presence of vancomycin. The reaction mixtures were subjected to thermal cycling amplification according to protocols described by the manufacturer.

PCR amplification of $vanS_C$ genes: Total DNA from *Enterococcus* spp. was prepared using the boiling lysis method (Yang et al., 2008) with modification. Briefly, five bacterial colonies of each isolate were taken from the BHI agar culture and washed with 1.0 ml of molecular biological-grade water. The resulting bacterial cell pellet was resuspended with 200 µl of TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). Then, the suspension was subjected to heating at 60°C for 30 min and boiling at 100°C for 10 min. After centrifugation at 13,000×*g* for 10 min, DNA extracts were retrieved from collection of the supernatant and kept at -20°C until subsequent PCR amplification.

PCR was conducted to obtain amplified $vanS_C$ and $vanS_{C-2}$ gene products using total DNA from each strain of *E. gallinarum* or *E. casseliflavus* as the template and primer pairs D1-E1 (Panesso et al., 2005) and vanSC2F-vanSC2R, respectively. The forward, vanSC2F (5′-AGTGATGGCACATATCG-3′), and reverse primers, vanSC2R (5′-TTAAGCGGTTGGTTCAG-3′), were designed from the DNA sequence of *E. casseliflavus* ATCC 25788 *vanC-2* cluster (Dutta and Reynolds, 2002). A 50 µl reaction mixture containing 1X-Phusion HF buffer (Thermo Fisher Scientific, USA), 200 µM of each dNTPs, 500 nM of each forward and reverse primer, 1 unit of Phusion DNA polymerase (Thermo Fisher Scientific, USA) and 4 µl of the DNA sample was subjected to PCR amplification on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA) with initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 54°C ($vanS_C$) or at 56°C ($vanS_{C-2}$) for 30 s, extension at 72°C for 45 s and final extension at 72°C for 5 min.

Nucleotide sequencing and sequence analysis: Nucleotide sequences of $vanS_C$ and $vanS_{C-2}$ were determined by sequencing of their PCR products. Briefly, $vanS_C$ and $vanS_{C-2}$ were amplified from 23 *E. gallinarum* and 19 *E. casseliflavus* isolates, respectively. The 1.8 kilobase (kb) $vanS_C$ and 1.2 kb $vanS_{C-2}$ PCR products were purified using a QIAquick® gel extraction kit (Qiagen, USA) according to the manufacturer's instructions and DNA sequencing was performed on both strands at Macrogen, Seoul, Korea. Nucleotide sequence data were analyzed using the Laser Gene software package (DNASTAR®, Inc., USA). Sequence trimming and assembly were carried out using the EditSeq and SeqMan programs. Multiple sequence alignment was performed using the CLUSTAL W method (Thompson et al., 1994) in the MegAlign program. *E. gallinarum* BM 4174 resistant to vancomycin constitutively (Panesso et al., 2005) was included in the analysis. Sequences of $vanS_C$ of *E. gallinarum* BM 4174 (Arias et al., 2000) and $vanS_{C-2}$ of *E. casseliflavus* ATCC 25788 (Dutta and Reynolds, 2002) were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Results

Growth curve analysis of VanC isolates from pigs: The expression of vancomycin-resistant characteristics of VanC isolates from pigs was determined using growth curve analysis. Induction growth curves by the presence of vancomycin were identified in all porcine isolates (23 *E. gallinarum* and 19 *E. casseliflavus*) (Fig. 1). For both *E. gallinarum* and *E. casseliflavus*, growth in the control cultures unsupplemented with vancomycin started after 2 h of lagging period. However, when they were in the cultures supplemented with a subinhibitory concentration of vancomycin, the normal growth was inhibited with an extended lag time to 5 h, resulting in distinctive growth patterns. Thus, the growth patterns of the porcine *E. gallinarum*

and *E. casseliflavus* populations were typical of inducible vancomycin resistance. Similarly, induction growth curves by the presence of vancomycin were identified in *E. casseliflavus* ATCC 25788 (data not shown), which normally expresses the inducible resistance phenotype.

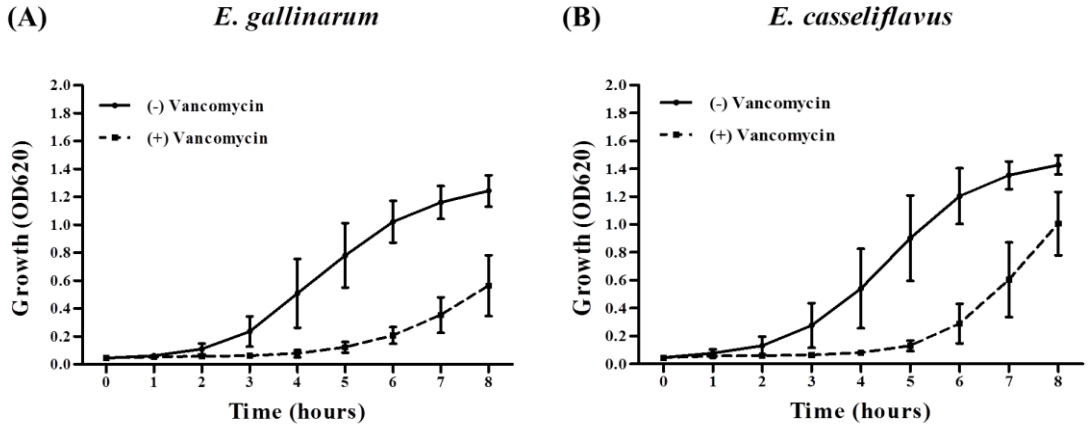

Figure 1 **Induction growth curves of 23 porcine *E. gallinarum* and 19 porcine *E. casseliflavus* isolates.** Each point represents the mean ± standard deviation of the optical density. *E. gallinarum* (A) and *E. casseliflavus* (B) were grown in BHI broth unsupplemented (-) and supplemented (+) with subinhibitory concentration of vancomycin.

Analysis of the vanC gene expressed by porcine E. gallinarum and E. casseliflavus: Vancomycin resistance phenotype in *E. gallinarum* and *E. casseliflavus* isolates from pigs was studied at the transcriptional level. The expression of *vanC-1* and *vanC-2* genes by *E. gallinarum* strains MA061022, MB151020 and MC131022, and *E. casseliflavus* strains SB111012, RA091020 and SC161032 was determined by RT-PCR analysis. Results showed that the *vanC-1*-specific RT-PCR products (822 base pairs, bp) were detected in RNA extracted from the *E. gallinarum* strains grown in the BHI broth supplemented with vancomycin (Fig. 2A, lanes 2, 5 and 8). However, no amplification product was observed in the unsupplemented cultures (Fig 2A, lanes 1, 4 and 7). To show that no products derived from residual genomic DNA contamination, the reactions without the reverse transcription step were also included as controls (lanes 3, 6 and 9). In parallel, the same RNA extracts were reverse transcribed and amplified with 16S rRNA specific primers served as internal controls. Results showed that an expected band of 222 bp was obtained in the RNA sample from enterococci grown under non-inducing and inducing conditions, but not in the controls without reverse transcription (Fig. 2B).

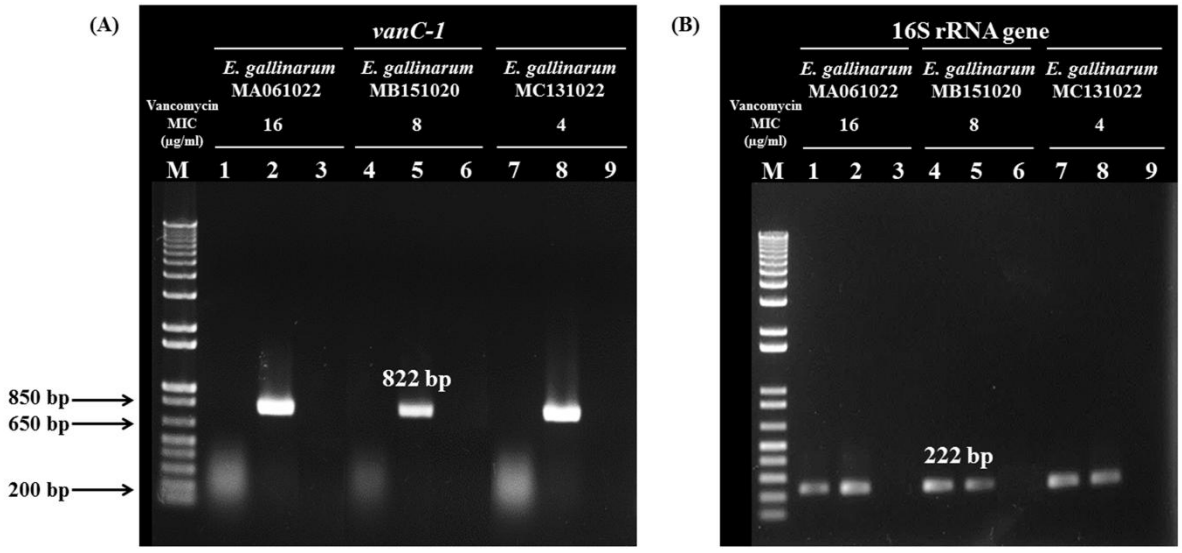

Figure 2 **RT-PCR analysis of vancomycin-induced *vanC-1* mRNA expression.** RT-PCR was performed using total RNA from *E. gallinarum* strains MA061022, MB151020 and MC131022, and primers specific to *vanC-1* (A) or 16S rRNA (B) genes. Lanes 1, 4 and 7 are cells grown in BHI broth without vancomycin; lanes 2-3, 5-6 and 8-9 are cells grown with vancomycin. Lanes 3, 6 and 9 are controls without reverse transcription.

Analysis of the *vanC-2* mRNA expression in the porcine *E. casseliflavus* revealed results similar to those for the *E. gallinarum* strains (Fig. 3). Transcription of the *vanC-2* gene was detected from the transcript derived from the *E. casseliflavus* strains cultured under inducing conditions (Fig. 3A, lanes 2, 5 and 8), but not

under non-inducing conditions (lanes 1, 4 and 7). In addition, no products derived from genomic DNA were identified in any of the negative control reactions (lanes 3, 6 and 9). RT-PCR products specific for 16S rRNA were obtained under the non-inducing (Fig. 3B, lanes 1, 4 and 7) and inducing (lanes 2, 5 and 8) conditions, but not in the controls without reverse transcription. Thus, inducible *vanC-1* and *vanC-2* mRNA expressions were demonstrated in the porcine *E. gallinarum* and *E. casseliflavus* isolates, respectively. These results confirmed that the expression of vancomycin resistance in the VanC isolates was inducible and the expression of *vanC-1* and *vanC-2* genes could be regulated at transcription level.

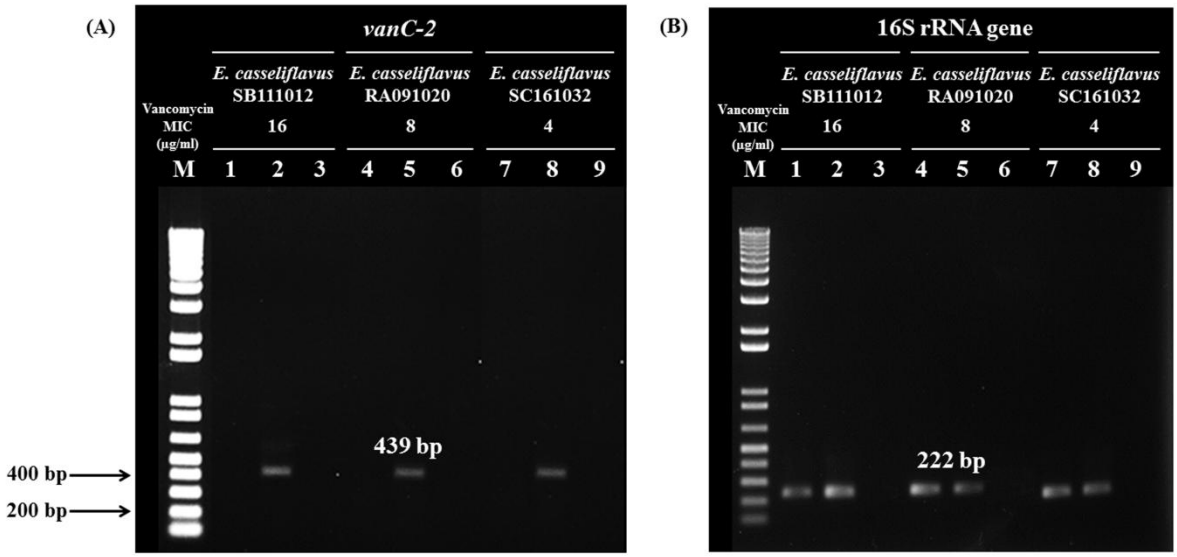

Figure 3 **RT-PCR analysis of vancomycin-induced *vanC-2* mRNA expression.** RT-PCR was performed using total RNA from *E. casseliflavus* strains SB111012, RA091020 and SC161032, and primers specific to *vanC-2* (A) or 16S rRNA (B) genes. Lanes 1, 4 and 7 are cells grown in BHI broth without vancomycin; lanes 2-3, 5-6 and 8-9 are cells grown with vancomycin. Lanes 3, 6 and 9 are controls without reverse transcription.

Comparison of VanS$_C$ amino acid sequences from porcine isolates E. gallinarum and E. casseliflavus: The deduced amino acid sequences of VanS$_C$ and VanS$_{C-2}$ from pig isolates were aligned with VanS$_C$ of constitutive *E. gallinarum* BM4174 and with VanS$_{C-2}$ of inducible *E. casseliflavus* ATCC 25788 (Fig. 4). Comparison of putative VanS$_C$ and VanS$_{C-2}$ protein sequences of the porcine isolates with the corresponding protein from a constitutive reference strain revealed conserved amino acid residues crucial for normal protein functions. The VanS$_C$ and VanS$_{C-2}$ amino acid sequences contained 340 and 329 residues. The VanS$_C$ sequences of *E. gallinarum* strain BM4174 and that of porcine isolates were almost identical and displayed 99.4-99.7% amino acid sequence identity. The VanS$_{C-2}$ sequences of porcine *E. casseliflavus* isolates and that of strain ATCC 25788 displayed 98.8-99.1% amino acid sequence identity. The 329 amino acid residues of the VanS$_{C-2}$ protein sequences of *E. casseliflavus* also exhibited a high degree of identity (81%) with VanS$_C$ from *E. gallinarum* BM4174, as previously reported (Dutta and Reynolds, 2002).

Alignment of the putative VanS$_C$ and VanS$_{C-2}$ protein sequences revealed conserved glycine (Gly)-320 and Gly-296 residues adjacent to the G2 domain in all inducible *E. gallinarum* and *E. casseliflavus* strains, respectively. These conserved residues were found in all inducible strains, but not in the constitutive strain, which had a Gly320Ser change. Several amino acid substitutions were found among the VanS$_C$ protein sequences of porcine *E. gallinarum* isolates including Ala37Ser (TYPE2: 1 isolate), Asn171Thr (TYPE3: 1 isolate), Arg176His (TYPE4: 4 isolates) and Asn244Lys (TYPE5: 1 isolate). Amino acid substitutions were found among the VanS$_{C-2}$ protein sequences obtained from porcine *E. casseliflavus* including Thr77Met (TYPE2: 3 isolates), Gly278Val (TYPE3: 2 isolates) and Ala289Val (TYPE4: 3 isolates). However, no correlation between the amino acid substitutions and vancomycin MICs was found.

Discussion

E. gallinarum and *E. casseliflavus* exhibit vancomycin resistance phenotype either inducibly or constitutively (Sahm et al., 1995; Panesso et al., 2005). However, the presence of two ligases in constitutively resistant strains is uncommon (Sahm et al., 1995). In the inducible VanC strains, the production of D-Ala-terminating precursors was shown to switch to D-Ser-terminating precursors in the presence of vancomycin (Billot-Klein et al., 1994; Sahm et al., 1995; Dutta and Reynolds, 2002; Panesso et al., 2005), while in the constitutive strains, D-Ser-terminating precursors were always detected in conditions with or without vancomycin (Reynolds et al., 1994; Sahm et al., 1995; Panesso et al., 2005). Therefore, in the inducible VanC strains, vancomycin resistance is modulated in response to vancomycin. An intriguing question is whether or not inducible or constitutive VanC-type resistance is a natural phenotype. Although the inducible VanC seems to be the wild type, one or more pieces of epidemiological evidence are required to support this hypothesis.

In this study, the occurrence of inducible or constitutive vancomycin resistance in a VanC population was examined. The results of the growth curve analysis and the RT-PCR results of porcine *E. gallinarum* and *E. casseliflavus* isolates indicated that the expression of the intrinsic vancomycin resistance phenotype by enterococci might be naturally inducible. Since the constitutive VRE strains were commonly obtained from clinical settings and *in vitro* selection on glycopeptides (Dutka-Malen et al., 1992; Rosato et al., 1995; Baptista et al., 1997; Depardieu et al., 2003; Panesso et al., 2005), they might have been regularly under selective pressure (Depardieu et al., 2003). Glycopeptides are prohibited in farm animals in Thailand. Thus, the absence of constitutive VRE found in this study may be in part due to the discontinued use of glycopeptides. In a report by Sahm et al. (1995), approximately half of the 26 *E. gallinarum* and 10 of the 11 *E. casseliflavus* isolates inducibly expressed vancomycin resistance. It is difficult to compare their detection rate with this study since details of the above bacterial strains were not disclosed. However, the high incidence of the inducible vancomycin resistance in *E. casseliflavus* is consistent with the current findings.

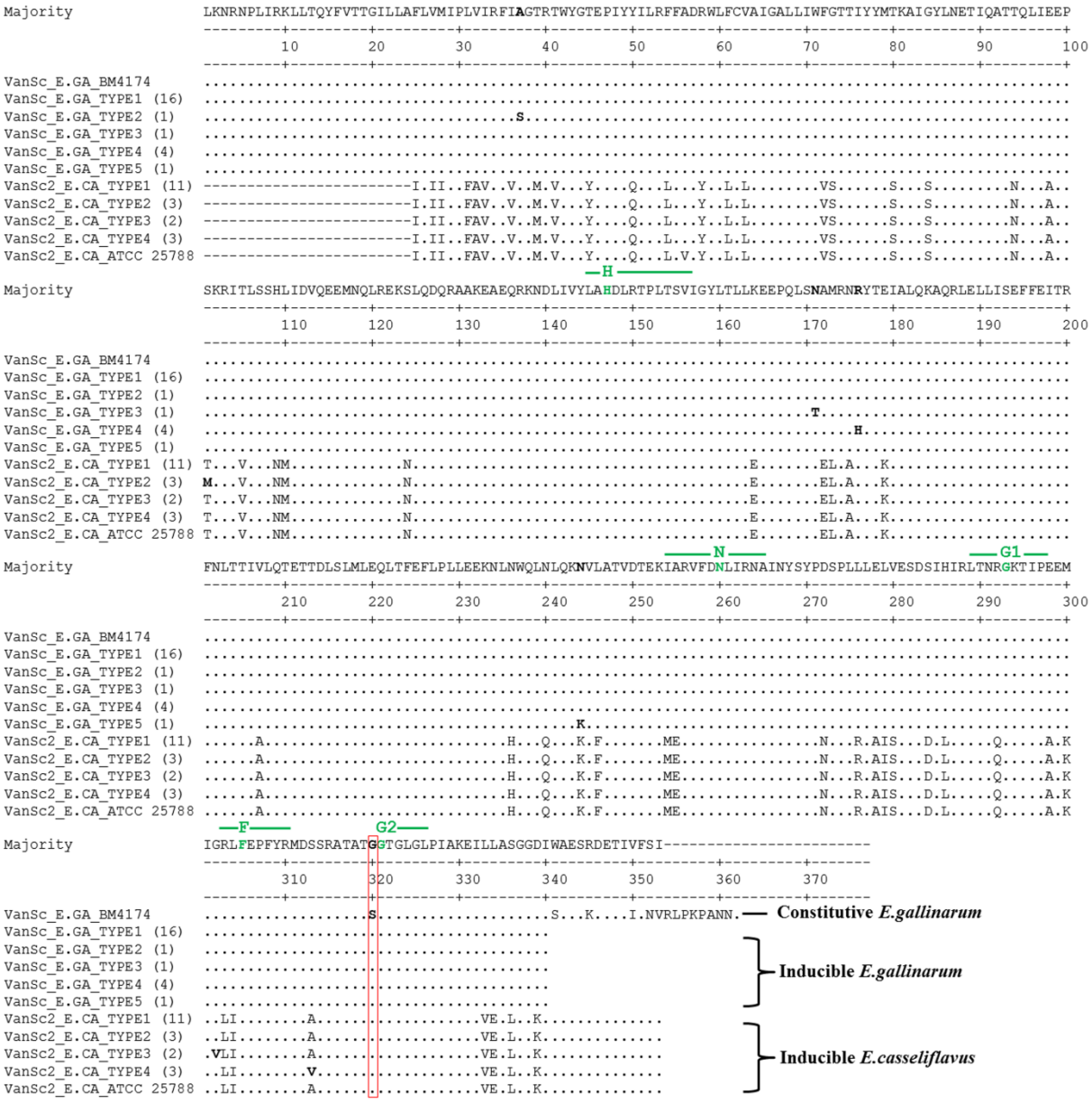

Figure 4 **Deduced amino acid alignment of VanSc sequences.** VanSc amino acid sequences of porcine *E. gallinarum* and *E. casseliflavus* from this study, *E. casseliflavus* ATCC 25788 and *E. gallinarum* BM4174, were aligned using MegAlign (DNASTAR®). The dots represent amino acids similar to the consensus. Numbers of isolates are in the parenthesis and H, N, G1, F, and G2 above the alignment indicate the conserved motifs (Arias et al., 2000). The conserved Gly-320 and Gly-296 residues found in the inducible *E. gallinarum* and *E. casseliflavus*, respectively, are in the open box.

Inducible expression, which is under regulation, is a common characteristic of acquired vancomycin resistance in enterococci (Courvalin, 2006). Our study also indicated that the regulation of gene expression was also common in intrinsic vancomycin resistance, apparently in both the VanC-1 and VanC-2 phenotypes. Although the expression of vancomycin resistance confers a high fitness cost to

enterococci, as demonstrated in the model of VanB strains, the tight regulation of gene expression dramatically reduces the biological cost associated with vancomycin resistance, resulting in widespread dissemination of the inducible vancomycin resistance phenotype (Foucault et al., 2010). Whether or not the wide spread of the inducible VanC phenotype in the current study is associated with the regulation of the *van* gene expression remains to be elucidated.

Growth curve analysis is a method used to determine constitutive or inducible expression of vancomycin resistance in enterococci (Sahm et al., 1995; Rosato et al., 1995; Panesso et al., 2005). In this study, the results obtained by growth curve and RT-PCR experiments correlated. Expression of the *vanC-1* gene cluster was found to be cotranscribed from a single promoter and controlled at a transcriptional level (Panesso et al., 2005). RT-PCR analysis was, therefore, used to confirm the expression type of VanC isolates primarily identified by growth curve analysis in which the detected *vanC-1* or *vanC-2* mRNAs represented the expression of genes in the operon. In addition, the RT-PCR analysis showing only *vanC-1* mRNA expression in the presence of vancomycin is consistent with previous findings (Panesso et al., 2005). Since the *vanC-2* gene cluster of *E. casseliflavus* is organized in the same manner as the *vanC-1* cluster of *E. gallinarum* (Dutta and Reynolds, 2002), expression of the *vanC-2* cluster could be controlled at the transcriptional level and result in a single transcript similar to the *vanC-1* cluster (Panesso et al., 2005). In the current study, the RT-PCR results of *vanC-2* isolates indicate that the regulation of gene expression at transcriptional level also occurs with the *vanC-2* gene cluster.

The prototype VanS/VanR found in VanA enterococci is a two-component regulatory system (Arthur et al., 1992). N-terminus of VanS contains a sensor domain with two transmembrane domains while C-terminus contains a cytoplasmic kinase domain with the characteristically conserved H, N, G1, F and G2 motifs (Dutta et al., 1999). In response to the presence of a glycopeptide, VanS and $VanS_B$ undergo autophosphorylation, in which the phosphate group is then transferred to VanR and $VanR_B$ (Arthur et al., 1992; Wright et al., 1993). The phosphorylated VanR then activates transcription of the vancomycin resistance genes. The VanS and $VanS_B$ also have a phosphatase activity which is presumably required for the negative regulation in the absence of glycopeptides (Depardieu et al., 2007). An 18 bp deletion mutation on $VanS_B$ that overlaps the G2 motif causes loss of phosphatase activity (Depardieu et al., 2003). $VanS_C$ and $VanS_{C-2}$ could function similar to VanS (Arias et al., 2000; Dutta and Reynolds, 2002). A Gly320Ser change of $VanS_C$ was associated with the constitutive vancomycin resistance of *E. gallinarum* isolates (Panesso et al., 2005). Our study found that there was no change in the Gly-320 residue in the inducible Van resistant protein of *E. gallinarum*, which is consistent with the previous findings. Our study of *E. casseliflavus* also supports the important role of $VanS_{C-2}$ in the inducible expression of the intrinsic vancomycin resistance.

The results of this study revealed that the vancomycin resistance phenotype of porcine *E. gallinarum* and *E. casseliflavus* was commonly inducible. Furthermore, the study of VanSc protein sequences suggested that the inducible expression was associated with the conserved Gly residues adjacent to the G2 domain.

Acknowledgements

The authors gratefully acknowledge the Kasetsart University Research and Development Institute (KURDI) for financial support. A part of this research was funded by the Center for Advanced Studies in Agriculture and Food, Institute for Advances Studies, Kasetsart University, Bangkok, Thailand.

References

Arias CA, Courvalin P and Reynolds PE 2000. *vanC* cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob Agents Chemother. 44(6): 1660-1666.

Arthur M, Molinas C and Courvalin P 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol. 174(8): 2582-2591.

Arthur M, Reynolds P and Courvalin P 1996. Glycopeptide resistance in enterococci. Trends Microbiol. 4(10): 401-407.

Baptista M, Depardieu F, Reynolds P, Courvalin P and Arthur M 1997. Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. Mol Microbiol. 25(1): 93-105.

Billot-Klein D, Gutmann L, Sablé S, Guittet E and Heijenoort VJ 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J Bacteriol. 176(8): 2398-2405.

Cetinkaya Y, Falk P and Mayhall CG 2000. Vancomycin-resistant enterococci. Clin Microbiol Rev. 13(4): 686-707.

Courvalin P 2006. Vancomycin resistance in gram-positive cocci. Clin Infect Dis.42 (Suppl 1): S25-34.

Depardieu F, Courvalin P and Msadek T 2003. A six amino acid deletion, partially overlapping the $VanS_B$ G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanB-type *Enterococcus faecium*. Mol Microbiol. 50(3): 1069-1083.

Depardieu F, Podglajen I, Leclercq R, Collatz E and Courvalin P 2007. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev. 20(1): 79-114.

Dutka-Malen S, Molinas C, Arthur M and Courvalin P 1992. Sequence of the vanC gene of *Enterococcus gallinarum* BM4174 encoding a D-alanine:D-alanine ligase-related protein necessary for vancomycin resistance. Gene. 112(1): 53-58.

Dutka-Malen S, Evers S and Courvalin P 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 33(1): 24-27.

Dutta R, Qin L and Inouye M 1999. Histidine kinases: diversity of domain organization. Mol Microbiol. 34(4): 633-640.

Dutta I and Reynolds PE 2002. Biochemical and genetic characterization of the *vanC-2* vancomycin resistance gene cluster of *Enterococcus casseliflavus* ATCC 25788. Antimicrob Agents Chemother. 46(10): 3125-3132.

Fantin B, Leclercq R, Arthur M, Duval J and Carbon C 1991. Influence of low-level resistance to vancomycin on efficacy of teicoplanin and vancomycin for treatment of experimental endocarditis due to *Enterococcus faecium*. Antimicrob Agents Chemother. 35(8): 1570-1575.

Fisher K and Phillips C 2009. The ecology, epidemiology and virulence of *Enterococcus*. Microbiology. 155(pt6): 1749-1757.

Foucault ML, Depardieu F, Courvalin P and Courvalin GC 2010. Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. Proc Natl Acad Sci USA. 107(39): 16964-16969.

Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA and Fridkin, SK 2008. Antimicrobial-resistant pathogens associated with healthcare-associated infections: Annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol. 29(11): 996-1011.

Huang L 2014. IPMP 2013—a comprehensive data analysis tool for predictive microbiology. Int J Food Microbiol. 171: 100-107.

Mazuski JE 2008. Vancomycin-resistant enterococcus: risk factors, surveillance, infections, and treatment. Surg. Infect (Larchmt). 9(6): 567-571.

Murray BE 2000. Vancomycin-resistant enterococcal infections. N Engl J Med. 342(10): 710-721.

Navarro F and Courvalin P 1994. Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens*. Antimicrob Agents Chemother. 38(8): 1788-1793.

Noble CJ 1978. Carriage of group D streptococci in the human bowel. J Clin Pathol. 31(12): 1182-1186.

Panesso D, Abadia-Patino L, Vanegas N, Reynolds PE, Courvalin P and Arias CA 2005. Transcriptional analysis of the *vanC* cluster from *Enterococcus gallinarum* strains with constitutive and inducible vancomycin resistance. Antimicrob Agents Chemother. 49(3): 1060-1066.

Pruksakorn C, Pimarn C, Boonsoongnern A and Narongsak W 2016. Detection and phenotypic characterization of vancomycin-resistant enterococci in pigs in Thailand. Agriculture and Natural Resources. 50(3): 199-200.

Reynolds PE, Snaith HA, Maguire AJ, Dutka-Malen S and Courvalin P 1994. Analysis of peptidoglycan precursors in vancomycin resistant *Enterococcus gallinarum* BM4174. Biochem J. 301(pt1): 5-8.

Reynolds PE, Arias CA and Courvalin P 1999. Gene $vanXY_C$ encodes D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in vancomycin-resistant *Enterococcus gallinarum* BM4174. Mol Microbiol. 34(2): 341-349.

Rosato A, Pierre J, Billot-Klein D, Buu-Hoi A and Gutmann L 1995. Inducible and constitutive expression of resistance to glycopeptides and vancomycin dependence in glycopeptide-resistant *Enterococcus avium*. Antimicrob Agents Chemother. 39(4): 830-833.

Sahm DF, Free L and Handwerger S 1995. Inducible and constitutive expression of *vanC-1*-encoded resistance to vancomycin in *Enterococcus gallinarum*. Antimicrob Agents Chemother. 39(7): 1480-1484.

Thompson JD, Higgins DG and Gibson TJ 1994. CLUSTAL W:improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22): 4673-4680.

Toye B, Shymanski J, Bobrowska M, Woods W and Ramotar KJ 1997. Clinical and epidemiological significance of enterococci intrinsically resistant to vancomycin (possessing the *vanC* genotype). J Clin Microbiol. 35(12): 3166-3170.

Wright GD, Holman TR and Walsh CT 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry. 32 (19): 5057-5063.

Yang JL, Wang MS, Cheng AC, Pan KC, Li CF and Deng SX 2008. A simple and rapid method for extracting bacterial DNA from intestinal microflora for ERIC- PCR detection. World J Gastroenterol. 14(18): 2872-2876.

บทคัดย่อ

ลักษณะการดื้อยาแวนโคมัยซินแบบเหนี่ยวนำได้พบบ่อย ในเชื้อ *Enterococcus gallinarum* และ *E. casseliflavus* จากสุกร

วนิดา ถาวรวีย์[1,2] จันทิมา พฤกษากร[1,2*] พรทิพภา เล็กเจริญสุข[1,2]

แบคทีเรีย *Enterococcus gallinarum* และ *E. casseliflavus* มีการดื้อยาแวนโคมัยซินตามธรรมชาติ ซึ่งเกิดจากการแสดงออกของกลุ่มยีน *vanC-1* และ *vanC-2* ตามลำดับ ลักษณะการดื้อยาแบบ VanC นี้มีการแสดงออกทั้งแบบเหนี่ยวนำได้หรือแบบตลอดเวลา การศึกษานี้จำแนกรูปแบบการแสดงออกของลักษณะการดื้อยาแวนโคมัยซินในเชื้อ *E. gallinarum* ที่แยกได้จากสุกรจำนวน 23 เชื้อ และเชื้อ *E. casseliflavus* จากสุกรจำนวน 19 เชื้อ ซึ่งมีค่าความเข้มข้นต่ำสุดของยาแวนโคมัยซินที่สามารถยับยั้งการเจริญเติบโตของแบคทีเรียได้ตั้งแต่ 4 ถึง 16 ไมโครกรัมต่อมิลลิลิตร ทำการหาลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนที่ทำนายได้ของยีน $vanS_C$ และ $vanS_{C-2}$ และเปรียบเทียบกับของสายพันธุ์มาตรฐานที่มีการดื้อยาแบบเหนี่ยวนำได้และแบบตลอดเวลา การวิเคราะห์กราฟการเจริญเติบโตของเชื้อพบว่าลักษณะการดื้อยาในทุกตัวอย่างเชื้อเป็นแบบเหนี่ยวนำได้ด้วยยาแวนโคมัยซิน ในสภาวะที่มียาแวนโคมัยซินระยะพักของเชื้อแบคทีเรียสายพันธุ์เหล่านี้ยาวนานขึ้น 3 ชั่วโมงเปรียบเทียบกับเมื่อถูกเพาะเลี้ยงในอาหารเลี้ยงเชื้อที่ไม่มียา การวิเคราะห์การแสดงออกของยีนในระดับ transcription ด้วยปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับพบว่ายีน *vanC-1* และ *vanC-2* มีการแสดงออกโดยเชื้อ *E. gallinarum* และ *E. casseliflavus* ตามลำดับ เมื่อเชื้อโตในสภาวะที่มียาแวนโคมัยซิน ดังนั้น การแสดงออกของ mRNA ของ *vanC-1* และ *vanC-2* เป็นแบบเหนี่ยวนำได้ จากการเปรียบเทียบลำดับของโปรตีน $VanS_C$ และ $VanS_{C-2}$ ของสายพันธุ์แบบเหนี่ยวนำได้และแบบตลอดเวลา พบไกลซีนตำแหน่งที่ 320 และ 296 จำเพาะในสายพันธุ์แบบเหนี่ยวนำได้ของเชื้อ *E. gallinarum* และ *E. casseliflavus* ตามลำดับ ผลการศึกษานี้ชี้แนะว่า VanC enterococci จากสุกรในประเทศไทยอาจมีการแสดงลักษณะการดื้อยาแวนโคมัยซินเป็นแบบเหนี่ยวนำได้ตามปกติ และลักษณะการดื้อยาแวนโคมัยซินแบบเหนี่ยวนำได้นี้สัมพันธ์กับไกลซีนตำแหน่งที่จำเพาะของโปรตีน $VanS_C$

คำสำคัญ: เอนเทอโรคอคคัส การดื้อยาแวนโคมัยซินแบบเหนี่ยวนำได้ ยีน *vanC-1* ยีน *vanC-2*

[1]ภาควิชาจุลชีววิทยาและวิทยาภูมิคุ้มกัน คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900
[2]ศูนย์วิทยาการขั้นสูงเพื่อเกษตรและอาหาร มหาวิทยาลัยเกษตรศาสตร์ แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900
****ผู้รับผิดชอบบทความ** E-mail: fvetctm@ku.ac.th*