Veterinary Microbiology 184 (2016) 80–83

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Transferable genes putatively conferring elevated minimum inhibitory concentrations of narasin in *Enterococcus faecium* from Swedish broilers

Oskar Nilsson[a,*], Mattias Myrenås[a], Joakim Ågren[b]

[a] Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden
[b] Department of Microbiology, National Veterinary Institute (SVA), Uppsala, Sweden

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form 12 January 2016
Accepted 16 January 2016

Keywords:
Elevated MIC of narasin
Broilers
ABC-transporter

ABSTRACT

The minimum inhibitory concentration (MIC) of the polyether ionophore antibiotic narasin is elevated in a large proportion of *Enterococcus faecium* from Swedish broilers. The aim of this study was to identify gene(s) responsible for these elevated MICs.

Six plasmids, four conferring vancomycin resistance and elevated MIC of narasin and two only conferring resistance to vancomycin, were sequenced. The genes for a putative mechanism for elevated MIC of narasin was used to design a PCR assay which in turn was used to screen 100 isolates of *E. faecium* from Swedish broilers.

A 5.9 kb area was only found in the plasmids transferring elevated MIC of narasin. This area included two genes coding for an ABC-type transporter; an 'ABC transporter permease protein' and an 'ABC-type multidrug transport system, ATPase component'. These genes are known to confer resistance to the ionophore tetronasin. PCR investigation confirmed a correlation between the presence of the genes and a MIC of narasin $\geq$2 mg/L.

The results of this study indicate that the ABC permease together with the ABC ATPase are responsible for the elevated MIC of narasin present among *E. faecium* in Swedish broilers. To our knowledge, this is the first report of a putative transferable mechanism for elevated MIC of narasin.

1. Introduction

Polyether ionophore antibiotics are lipophilic molecules that can dissolve into cell membranes (Callaway et al., 2003). Once there, they cause cell death by disrupting the ion gradient across the cell membrane (Callaway et al., 2003). Today, ionophores are commonly used globally within the poultry industry for coccidiosis prophylaxis and the common regimen in Sweden is to include the ionophore narasin in broiler feed until 3–5 days before slaughter.

Resistance to narasin in enterococci was first suggested over 15 years ago but still the mechanisms behind the elevated minimum inhibitory concentration (MIC) is largely unknown (Butaye et al., 1999, 2000). This is generally also true for other ionophores, even if different mechanisms, for example alterations in the cell membrane have been described (Simjee et al., 2012).

Co-transfer of elevated MIC of narasin and resistance to vancomycin from vancomycin resistant enterococci (VRE) was coincidentally discovered by our group some years ago (Nilsson et al., 2012). The aim of this study was to identify the gene(s) responsible for these elevated MICs of narasin. Six plasmids from the earlier study (four conferring vancomycin resistance and elevated MIC of narasin and two only conferring resistance to vancomycin) were sequenced. A potential mechanism for elevated MIC of narasin was used to design a PCR assay and in turn used to screen a number of isolates.

2. Materials and methods

Seven isolates from a previous study were subjected to sequencing (Nilsson et al., 2012). The isolates were the *Enterococcus faecium* 64/3 recipient strain, without any plasmids, and six trans-conjugants, out of which in four co-transfer of vancomycin resistance and elevated MIC of narasin had occurred (isolates 05_028, 02_347, 02_304, 01_233). DNA was prepared from fresh colonies using an EZ1-robot (Qiagen, Hilden, Germany) and DNA-concentrations determined with the Qubit HS DNA-Kit (Life Technologies, Carlsbad, CA, USA). Sequencing libraries were prepared using the Nextera XT Kit (Illumina, San Diego, CA,

* Corresponding author. Fax: + 46 18 309162.
E-mail address: oskar.nilsson@sva.se (O. Nilsson).

http://dx.doi.org/10.1016/j.vetmic.2016.01.012
0378-1135/

USA) and 250 bp paired-end sequencing was performed on a MiSeq sequencer (Illumina). The reads were assembled using the SPAdes assembler with the '—careful' option (Bankevich et al., 2012).

By comparing assemblies in the genomic comparison software Gegenees (Agren et al., 2012) with the assembly from the recipient genome, contigs that only represented plasmid-DNA could be determined and extracted from the assemblies. The genome-finishing software Consed (Gordon et al., 1998) was used to elongate the plasmid contigs and for quality control of the assembled sequence. Open reading frames (ORF) of the plasmid from the trans-conjugant 2001_233, which showed elevated MIC of narasin, were determined using GeneMarkS (Besemer et al., 2001) and the amino acid sequences of the ORFs were then queried to the NCBI non-redundant protein database. The remaining five plasmid sequences were annotated using the myRAST software (Aziz et al., 2008; Overbeek et al., 2014). The sequence data have been deposited at The European Nucleotide Archive (ENA) under study accession number PRJEB8983.

Genes determined to be specific to the four plasmids that had elevated MIC of narasin and that were also suspected to be involved in conferring these elevated MICs (i.e., an 'ABC permease' and an 'ABC ATPase') were assessed using PCR assays on a larger set of strains ($n = 100$). The strains were selected at random from vancomycin resistant ($n = 15$) and vancomycin susceptible ($n = 85$) *E. faecium* from healthy broilers with MIC of narasin ranging from 0.25 to 8 mg/L (4–8 mg/L for the VRE) originally isolated and susceptibility tested within the Swedish veterinary resistance monitoring programme (Svarm). PCR-primers targeting conserved regions of the genes were designed using the NCBI Primer-BLAST tool and validated for specificity using the primer-alignment tool of Gegenees. For the ABC permease and the ABC ATPase, primers were designed (5′-AGCTGCGTATGGCTCCATTT-3′, 5′-GCTGATGC-TAAGCCAATGCC-3′ and 5′-TGTTCCTGGGGATGTTGCTC-3′, 5′-AGAGCGTCGCAAGTTTCTCA-3′ respectively). The primer pairs were used separately in two otherwise identical PCR assays. DNA was extracted by boiling or by using the EZ1 DNA Tissue kit (Qiagen). PCR was performed in a 25 μl PCR mixture using 2× HotStarTaq Master mix (Qiagen), 0.2 μM of each primer and 2 μl DNA template. PCR was run at 94 °C, 15 min +(94 °C, 30 s + 63 °C, 30 s + 72 °C, 50 s)× 35 cycles + 72 °C, 10 min in an Applied Biosystems 2720 Thermal Cycler and analysed by gel electrophoresis. The genomes of 16 of the 100 isolates assessed by PCR (10 PCR-positive and 6 PCR-negative) were also sequenced as above to verify the results from the PCR assays.

The correlation between the result from the PCR assays and the MIC of narasin for the 100 isolates was tested with Fisher's exact test using Stata software (release 13, StataCorp LP, College Station,

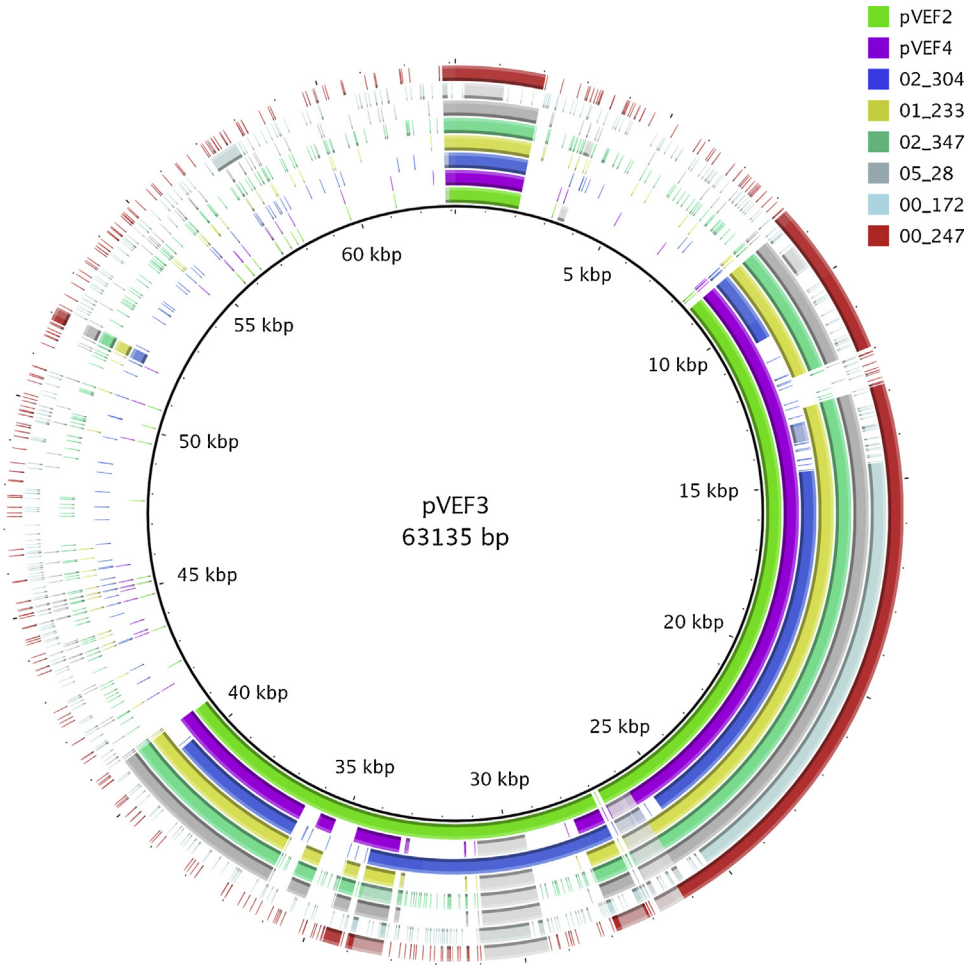

Fig. 1. Comparison of the six plasmids sequenced in this study and pVEF2, pVEF3 and PVEF4 from vancomycin resistant enterococci isolated from broilers in Norway (Sletvold et al., 2008, 2007, 2010). The genes coding for vancomycin resistance are situated between 15 and 22 kbp whereas the genes coding for the ABC-transporter putatively conferring elevated MIC of narasin are located between 36 and 40 kbp. Isolates 00_172 and 00_247 (the two outer circles) did not display elevated MIC of narasin.