Microbiology (2007), **153**, 1609–1618 DOI 10.1099/mic.0.2006/004812-0

Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin

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Received 1 December 2006
Revised 15 January 2007
Accepted 19 January 2007

It has been suggested that resistance to class IIa bacteriocins occurs at either a low or a high level. In listerial strains, low-level resistance (2–4-fold) to class IIa bacteriocins is attributed to alterations in membrane lipid composition. In *Listeria monocytogenes* and *Enterococcus faecalis*, high-level resistance (1000-fold) correlates with inactivation of the *mptACD* operon, which encodes the EII_t^{Man} mannose permease of the phosphotransferase system (PTS). Previous studies reported that in *L. monocytogenes*, high-level resistance involved the σ^{54} factor and the ManR activator. In this investigation, three genes associated with the resistance of *Ent. faecalis* JH2-2 to divercin V41, a pediocin-like bacteriocin from *Carnobacterium divergens* V41, were clearly identified by screening an insertional mutant library of *Ent. faecalis* JH2-2. These genes correspond to the well-known *rpoN* gene, which encodes σ^{54} factor, and to genes encoding a glycerophosphoryl diester phosphodiesterase (GlpQ) and a protein with a putative phosphodiesterase function (PDE). Resistance of the three mutants defective in the aforementioned genes appeared to be graduated: the *rpoN* mutant was more resistant than the *glpQ* mutant, which was more resistant than the *pde* mutant. Moreover, this resistance was specific to class IIa bacteriocins.

INTRODUCTION

The past few years have seen the emergence of class IIa bacteriocins produced by lactic acid bacteria as one of the most interesting groups of antimicrobial peptides for food preservation (Cleveland *et al.*, 2001) and in medicine as antiviral agents (Wachsman *et al.*, 1999, 2003). Class IIa bacteriocins, referred to as pediocin-like bacteriocins, are listericidal, small (<10 kDa), heat stable, non-lanthionine-containing peptides of 37 to 48 amino acids with a consensus sequence $YGNGVXCX_4(C)XVX_4A$ (X denotes any amino acid) in their charged N-terminal region (Drider *et al.*, 2006). These bacteriocins also have a similar tertiary structure (Frégeau-Gallagher *et al.*, 1997; Uteng *et al.*, 2003) and mode of action (Ennahar *et al.*, 2000). A promising method of food protection by using class IIa bacteriocins could be compromised by the development of resistant strains. This drawback could be overcome by using combinations of bacteriocins (Bouttefroy & Millière, 2000), or bacteriocins combined with other sublethal treatments such as sodium diacetate and sodium lactate (Uhart *et al.*, 2004). These combinations appear to be more efficient in controlling the growth of the foodborne pathogen *Listeria monocytogenes*. Resistance to class IIa bacteriocins has been studied but remains poorly understood overall. Studies aimed at characterizing the resistance mechanisms of bacterial targets have revealed the stability of this phenomenon (Rekhif *et al.*, 1994; Dykes & Hastings, 1998), which occurs at either a low or a high level. In *L. monocytogenes* and *Enterococcus faecalis*, low-level resistance is attributed to alterations in membrane lipid composition (Vadyvaloo *et al.*, 2002, 2004; Naghmouchi *et al.*, 2006) and high-level resistance results from the inactivation of the *mptACD* operon, which encodes the EII_t^{Man} mannose permease of the phosphotransferase system (PTS) (Dalet *et al.*, 2001; Héchard *et al.*, 2001). The aim of this study was to gain more insights into the molecular resistance mechanism developed by *Ent. faecalis* JH2-2 in response to pediocin-like bacteriocins. In the food quality area, *Ent. faecalis* is used as an indicator of faecal contamination and has been shown to be implicated in outbreaks of foodborne illness, while in the clinical area this bacterium has become a serious nosocomially transmitted pathogen. Our data

Abbreviations: ADT, agar diffusion test; AU, arbitrary units; DvnV41, divercin V41; DvnRV41, recombinant divercin V41; MesY105, mesentericin Y105; Ped PA-1/AcH, pediocin PA-1/AcH; PDE, phosphodiesterase; PTS, phosphotransfersase system; RAPD, random amplified polymorphic DNA.

strongly suggest that inactivation of genes encoding a glycerophosphoryl diester phosphodiesterase (GlpQ) and a protein with a putative phosphodiesterase function (PDE) confer a resistant character to *Ent. faecalis* JH2-2 towards divercin V41.

METHODS

Bacterial strains and growth conditions. *Ent. faecalis* JH2-2 (Yagi & Clewell, 1980) and its mutant derivatives were grown without shaking in M17 medium (Biokar) supplemented with glucose at 0.5 % (w/v) (Terzaghi & Sandine, 1975). When necessary, erythromycin (150 μg ml^{-1}) or tetracycline (15 μg ml^{-1}) was added to the growth medium. *Carnobacterium divergens* V41 (Pilet *et al.*, 1995), *Leuconostoc mesenteroides* Y105 (Héchard *et al.*, 1992) and *Pediococcus acidilactici* B5627 (University of Poitiers, France) were grown in MRS (de Man-Rogosa-Sharpe) medium (Biokar). *Escherichia coli* Origami, harbouring plasmid pCR03, which confers the ability to synthesize recombinant divercin V41 (Richard *et al.*, 2004), was grown at 37 °C with shaking in LB medium containing ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) (Richard *et al.*, 2004). *Listeria innocua* F (ENITIAA) was grown in Elliker medium (Biokar) at 30 °C, without shaking.

Production of pediocin-like bacteriocins. Divercin V41 (DvnV41), mesentericin Y105 (MesY105) and pediocin PA-1/AcH (Ped PA-1/AcH) were prepared from *C. divergens* V41, *Leuconostoc mesenteroides* Y105 and *P. acidilactici* B5627, respectively. Samples (1 ml) of cell cultures of each strain were centrifuged (6 min, 5000 ***g***, 4 °C), then the recovered supernatant was heated for 10 min at 100 °C and kept at −20 °C until use. Recombinant divercin V41 (DvnRV41) was obtained from the heterologous host *E. coli* Origami/pCR03 and purified as described previously (Richard *et al.*, 2004). Recombinant divercin V41 differs from natural divercin V41 by one amino acid in the N-terminal sequence, P-dvnV41 (Richard *et al.*, 2004). DvnV41 supernatant was used for established screening conditions and for screening the mutant library, then purified DvnRV41 was used for determining the MIC.

The anti-*Listeria* activity of DvnV41, DvnRV41, MesY105 and Ped PA-1/AcH against *L. innocua* F was verified as previously described (Pilet *et al.*, 1995). Briefly, 10 μl samples of a twofold serial dilution in Elliker broth for each bacteriocin studied were spotted on an Elliker agar (1 %) plate inoculated with the indicator strain, *L. innocua* F, at 10^7 c.f.u. ml^{-1}. Plates were incubated at 30 °C for 16 h. The bacteriocin activity was expressed in arbitrary units per ml (AU ml^{-1}) and was defined as the reciprocal of the lowest dilution that did not show growth inhibition of *L. innocua* F. Bacteriocin activity was found to be 820 000 AU ml^{-1} for DvnV41, 3 300 000 AU ml^{-1} for DvnRV41, and 256 000 AU ml^{-1} for MesY105 and Ped PA-1/AcH.

Isolation of *Ent. faecalis* JH2-2 divercin V41-resistant mutants. The library of insertional mutants of *Ent. faecalis* JH2-2 used in this study was constructed by Le Breton *et al.* (2002) as follows. DNA fragments of 200 bp to 1.5 kb were generated by partial digestion of chromosomal DNA from *Ent. faecalis* JH2-2 with *Alu*I then cloned into pORI19 (pWV01-derived Ori^+ $RepA^-$) using the $RepA^+$ helper *E. coli* strain EC101 (Law *et al.*, 1995) to obtain a bank of approximately 37 200 recombinant plasmids. A mixture of these recombinant plasmids was then transferred into *Ent. faecalis* JH2-2 that had previously received the pWV01-derived Ori^+ $RepA^{TS}$ pG^+host3 plasmid (pVE6007) (Maguin *et al.*, 1992). Clones were grown at 30 °C in GM17 medium containing erythromycin and chloramphenicol (the thermosensitive $RepA^{TS}$ protein is active at 30 °C and allowed replication of pG^+host3 and pORI19 recombinant plasmids). Cells were transferred into GM17 containing erythromycin (150 μg ml^{-1}) and the incubation temperature was shifted to 42 °C to inactivate the $RepA^{TS}$ protein and consequently to provoke the loss of pG^+host3 and the integration of the pORI19 recombinant plasmid by homologous recombination. Mutants (9600) were then screened for resistance to DvnV41 as follows: individual clones were grown at 37 °C in 96-well microtitre plates containing GM17 broth with 150 μg erythromycin ml^{-1} then replica plated in GM17 broth containing 5 % (v/v) DvnV41 (820 000 AU ml^{-1}). Integrated plasmids from all selected DvnV41-resistant mutants were excised as previously described by Le Breton *et al.* (2002) and used to identify the insertion loci. After sequencing the DNA insert carried by the pORI19 recombinant plasmid from each mutant, the nucleotide sequence was compared to that of the *Ent. faecalis* (V583) genomic sequence available at The Institute for Genomic Research (http://www.tigr.org/).

Analysis of mRNAs by RT-PCR. Total RNA of *Ent. faecalis* JH2-2 was isolated from exponentially growing cells in GM17 broth using the RNeasy Midi kit (Qiagen). For reverse transcriptions 10 μg of the RNA preparation was treated for 30 min at 37 °C with 20 U RNase-free DNase I (Roche Molecular Biochemicals) followed by a phenol-acid extraction at 65 °C. Reverse transcriptase reactions were performed on 2 μg of the RNA at 37 °C using the Omniscript RT kit (Qiagen) with 20 pmol random hexamer primers. cDNA was then purified using the QIAquick kit (Qiagen) and PCRs were performed with purified cDNA as template and 20 pmol primers listed in Table 1 using 2.5 U *Taq* DNA polymerase (Q-BIOgene). Amplifications were carried out for 30 cycles consisting of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, then the samples were analysed on a 1 % agarose gel in TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) buffer. The absence of contaminating genomic DNA was checked by regular PCR performed under the same conditions, except that the reverse transcriptase was replaced by H_2O. A positive control was also performed by replacing cDNA with genomic DNA (data not shown).

Complementation test. The EF0011 (*pde* gene) and EF2163 (*glpQ* gene) were amplified by PCR from chromosomal DNA of *Ent. faecalis* JH2-2 using specifically designed pairs of primers. The *pde* gene was amplified by primers *pde*F 5′-GACAATTTCGTCGACCAAAGAGC-3′ and *pde*R 5′-CTTTGGATCCCTTCACTCCTGTTC-3′, containing *Sal*I and *Bam*HI restriction sites (underlined) respectively; the *glpQ* gene was amplified with primers *glpQ*F5′-GTTGGATCCTAAAAAGAAGCCC-3′ and *glpQ*R 5′-CCTACGATATCTAATACTTTCTCC-3′, containing *Bam*HI and *Eco*RV restriction sites (underlined) respectively. Each PCR product was digested with appropriate restriction enzymes and cloned into pAM401 vector (Wirth *et al.*, 1986), itself cut with the same restriction enzymes. The recombinant plasmids were directly transferred into the *Ent. faecalis* mutant strain by electroporation using the following conditions: 25 μF, 400 Ω and 2500 V in a 0.2 cm cuvette (Pulse Controller Plus; Bio-Rad). The resulting cells were plated onto GM17 plates containing chloramphenicol (10 μg ml^{-1}) and erythromycin (10 μg ml^{-1}). Plates were incubated at 37 °C for 48 h. As a negative control, we used cells harbouring only plasmid pAM401.

Susceptibility/resistance test to divercin V41. Susceptibilities of *Ent. faecalis* JH2-2 wild-type, mutant strains 35A1 (*pde*), 36H4 (*glpxQ*) and transformants 35A1 + *pde*, 36H4 + *glpQ* toward DvnV41 were tested. Cultures were diluted (1/100) and plated onto GM17 medium containing 1 % agar; 10 μl samples of DvnV41 were spotted and the plates were incubated at 37 °C for 16 h. Inhibition haloes were observed and compared between the different strains.

Identification of *Ent. faecalis* JH2-2 divercin V41-resistant mutants. Identification of the mutants was performed by phenotypic and molecular methods. After Gram staining, each mutant strain was checked for its catalase and oxidase activities. Identification was completed by PCR-RAPD (random amplified

Table 1. Primers used in RT-PCR experiments

Locations of these primers are indicated in Fig. 1.

Primer set	Sequence (5′–3′)	Size of amplimer (bp)	Amplified gene
EF2163F	CACATCGAGGAAGTAAAGGC	508	*glpQ*
EF2163R	CCCGCTTGACTTAAATCCTC		
EF2162F	ACTCCATGCAAGTTTATCGC	617	*miaA*
EF2162R	TAGCCAATACCTTGAGCTGC		
EF2161F	GCATCAATGGAAGAACTGGC	694	Gene encoding putative
EF2161R	AGCGTAACTTCCATGCCTTG		GTP-binding protein
EF2160F	GAGTTACGCCGTTCCATGTC	304	*glnR*
EF2160R	CTGAGTAAGTCCTCCTTGCG		
J2163F	ATTCGCGCTTGTCGATAGTT	420	*glpQ–miaA*
J2162R	CTGACTTCACGGCAATCAAT		
J2162F	GCTATAAAGAATTTTTCCCG	436	*miaA*–gene encoding
J2161R	TCATAGGCATCTGTTAATTG		GTP-binding protein
J2161F	GTGAACTTCGCCGGATGACA	420	Gene encoding
J2160R	ATAGTAGCGAATTTGTCGAG		GTP-binding protein–*glnR*
EF0011F	TGTTGTGGGTCTTCTCTTAG	504	*pde*
EF0011R	TTATCGACAGACACAATGCC		
EF0012F	GTGCCAACTGGTTACGCCCA	349	*rpII*
EF0012R	GATGTAGTTTGACTGGTACG		
EF0013F	GCGATGGAATATGTGGAACC	603	*dnaB*
EF0013R	AGCTTTATCTGTCTTGGTGC		
J0011F	TCATTACCAAGCGGACGGAT	426	*pde* + *rpII*
J0012R	AAGGCTTGCATTTCTTTTGC		
J0012F	AGGTTACACTAACGTACCAG	439	*rpII* + *dnaB*
J0013R	GCACATCTGCATCTAAGAAA		
EF0783F	GATTTTATGTGCGAAGGATG	404	Gene encoding putative
EF0783R	GTCTGTTCCATAATAGACAC		acetyl transferase
EF0782F	AGAAGTTGGCCATGACTCAA	501	*rpoN*
EF0782R	TATACGCTAAATTAGGCGCG		
J0782F	TTCAGGCATGGAAATATCCC	403	*rpoN* + gene encoding
J0783R	GCATGACTTGGGGAAAAAGG		putative acetyl transferase

polymorphic DNA) using the following conditions: 1 µl (50 ng) chromosomal DNA, 5 µl 10× reaction buffer (New England Biolabs), 5 mM $MgCl_2$ (Sigma), 1 mM dNTPs (New England Biolabs), 0.5 µM primer M13V (5′-GTTTTCCCAGTCACGAC-3′) and 0.75 U *Taq* DNA polymerase (New England Biolabs). The PCR reactions were carried out on a PTC-100 thermocycler (MJ Research). The cycling programme was: 3 min at 94 °C, 5 min at 40 °C, 5 min at 72 °C for 3 cycles; 1 min at 94 °C, 2 min at 60 °C, 3 min at 72 °C for 32 cycles. Thereafter, 5 µl of each PCR product was separated on a 1.5 % agarose gel run at 100 V for 20 min and visualized by UV after ethidium bromide staining. A 100 bp ladder (New England Biolabs) was used as a reference for molecular size.

MIC determination. Twofold serial dilutions of DvnRV41 in GM17 medium were placed in microplate wells (Nunc). Each well was inoculated with 50 µl of *Ent. faecalis* JH2-2 wild-type or mutant cultures at 10^6 c.f.u. ml^{-1}. The microplate was incubated aerobically for 18 h at 30 °C then OD_{600} was measured hourly using an UltraMicroplate Reader (Bio-Tek Instruments). Sterile medium incubated under the same conditions was used as a blank. The MIC was calculated from the highest dilution showing complete inhibition of the tested strain (OD_{600} equals OD_{600} of the blank). The MIC determinations were repeated independently three times and the mean value is presented in the Results.

Cross-resistance to bacteriocins and antibiotics. The resistance level of *Ent. faecalis* JH2-2 and mutants resistant to DvnV41 was tested against class IIa bacteriocins (DvnV41, MesY105 and Ped PA-1/AcH), the class I bacteriocin nisin (Sigma) and the following antibiotics: cephalothin (0.01 mg ml^{-1}) (Sigma), penicillin G (10 IU) (Biomérieux), and streptomycin (10 IU) (Bio-Rad). Bacteriocin activity was assessed by the agar diffusion test (ADT) against *L. innocua* F as indicator strain (Pilet *et al.*, 1995). Briefly, brain heart infusion (BHI; Biokar) agar plates were inoculated with 1 ml of *Ent. faecalis* JH2-2 wild-type or mutant strains at 10^7 c.f.u. ml^{-1}. Next, 10 µl samples of a supernatant culture containing any of the class IIa bacteriocins cited above or nisin at 10 mg ml^{-1} were spotted onto the agar plate and incubated at 30 °C for 16 h. Cephalothin, or a disk of penicillin G or streptomycin, was applied in a similar way and plates were incubated at 30 °C for 16 h. After this, the plates were inspected for the formation of inhibition zones by measuring the diameter of each halo.

Database searches and sequence analyses. Searches for promoter locations and prediction of the *pde* and *glpQ* transcription start point were performed with the neural network program (http://www.fruitfly.org/seq_tools/promoter.html). *Ent. faecalis* genome analyses were performed using the *Ent. faecalis* (V583) genomic sequence available at The Institute for Genomic Research (http://www.tigr.org/).

RESULTS

Isolation of *Ent. faecalis* JH2-2 DvnV41-resistant mutants

The 9600 insertional mutant library of *Ent. faecalis* JH2-2 was screened for DvnV41 (820 000 AU ml^{-1}) resistant mutants. After 9 h of incubation at 37 °C, growth was observed for only six clones, named 35A1, 35H1, 35H6, 36H4, 36H7 and 36H9. The sequences of DNA inserts excised from the six DvnV41-resistant clones were compared to the DNA genome sequence of *Ent. faecalis* V583. Results revealed that the plasmid excised from mutant 35A1 contained a 385 bp DNA insert that corresponds to an internal region (nucleotides 854–1238) of a 1977 bp ORF named EF0011. The plasmid from mutant 36H4 contained a 431 bp DNA insert that corresponds to an internal region (nucleotides 234–664) of a 747 bp ORF (EF2163), while those of mutants 35H1, 35H6, 36H7 and 36H9 were identical and harboured a 412 bp insert corresponding to nucleotides 49–460 of the 1314 bp EF0782 ORF.

DNA sequence analysis

The *Ent. faecalis* (V583) genomic sequence in the region corresponding to the insertion locus in mutant 35A1 provided evidence for the presence of a ribosome-binding site sequence (GGAGG) located 6 nt upstream of the EF0011 ORF initiation codon (ATG). Translation of this ORF should allow synthesis of a 658 amino acid protein sharing homology with a putative phosphoesterase of the desert hedgehog (DHH) protein family, enzymes that hydrolyse polyphosphates into P_i. A prediction of transmembrane topology with the TMpred program (Hofmann & Stoffel, 1993) revealed that amino acid regions 10–28 and 34–53 could be membrane-spanning segments. In *Ent. faecalis* (V583), the EF0011 ORF is flanked by two genes, *rpsR* and *rpII*, encoding ribosomal protein S18 and ribosomal protein L9, respectively (Fig. 1). In this study, we refer to EF0011 ORF as the *pde* gene.

The EF0782 ORF (insertion locus 35H1, 35H6, 36H7 and 36H9) is preceded by a ribosome-binding site sequence (GGAGG) located 8 nt upstream of the initiation codon (ATG). This ORF, which corresponds to the *rpoN* gene, encodes the 443 amino acid RNA polymerase σ^{54} factor (Dalet *et al.*, 2000). It is preceded by a gene encoding a tRNA (tRNA-Arg2) oriented in the opposite direction and followed by the EF0783 ORF, which encodes a 625 amino acid putative acetyl transferase (Fig. 1). Immediately downstream of the last codon of the *rpoN* gene, there is a 201 bp intergenic region, which contains an inverted repeat (5′-AAACAACCAAAGCTTATGA(N_7)TCATAAGCTTTGGT-TGTTT-3′; $\Delta G = -30.2$ kcal mol^{-1}; -126.4 kJ mol^{-1}), which could act as a *rho*-independent terminator (Fig. 1).

The EF2163 ORF (insertion site in mutant 36H4) encodes a 248 amino acid protein (28.4 kDa) sharing high similarity with putative glycerophosphoryl diester phosphodiesterase enzymes, which are known to hydrolyse deacylated phospholipids into glycerol 3-phosphate and the corresponding alcohols. In this study, this ORF is named the *glpQ* gene. The entire amino acid sequence, deduced from the *glpQ* gene, was highly hydrophobic. Moreover, the *glpQ* gene is preceded by the EF2164 ORF, which encodes a putative membrane protein of 603 amino acids with an unknown function. Immediately downstream of the termination codon of the *glpQ* gene, the EF2162 ORF corresponds to the *miaA* gene, which encodes a 309 amino acid protein sharing high similarity with tRNA Δ^2-isopentenylpyrophosphate transferase (tRNA and rRNA base modification). Four base pairs downstream of the *miaA* gene, the EF2161 ORF encodes a 413 amino-acid putative GTP-binding protein. This latter is followed by an inverted repeat (5′-TAGAAATGATTTGT(N_4)ACAAATCATTTC-TA-3′; $\Delta G = -16.4$ kcal mol^{-1}; -68.6 kJ mol^{-1}), which could act as a *rho*-independent terminator. Further experiments were performed only with mutants 35A1 (*pde* inactivated), 36H4 (*glpQ* inactivated) and 35H1 (*rpoN* inactivated).

mRNA transcriptional analysis and complementation test

To identify accurately genes associated with resistance of *Ent. faecalis* JH2-2 to DvnV41, RT-PCR analyses were performed in order to study the mode of transcription of the inactivated genes as well as those located downstream of them. Thus, in mutant 36H4, defective in the putative GlpQ protein, the *glpQ* gene (EF2163 ORF) was shown to be cotranscribed with the *miaA* gene (EF2162 ORF), and the gene encoding a putative GTP-binding protein (EF2161 ORF) was itself cotranscribed with the *glnR* gene (EF2160 ORF) (Figs 1a and 2a). Regarding these RT-PCR results, we cannot discard a polar mutational effect resulting from the inactivation of the target *glpQ* gene. This inactivation could lead to either down- or up-regulation of *miaA* (EF2162 ORF), the gene encoding a putative GTP-binding protein (EF2161 ORF), and even *glnR* (EF2160 ORF) located downstream of the putative *rho*-independent terminator, as a band corresponding to a read-through transcript was obtained with primers J2161F and J2160R (Fig. 1). The same is true for the gene encoding a putative phosphoesterase (EF0011 ORF), which was found to be cotranscribed with the *rpII* gene (EF0012 ORF), which was itself cotranscribed with the *dnaB* gene (EF0013 ORF) (Figs 1b and 2b). However, as no RT-PCR product was detected with primers J0782F and J0783R (Fig. 2c), the *rpoN* gene (EF0782 ORF) appeared to be expressed as a monocistronic mRNA. To clearly ascribe resistance of *Ent. faecalis* 35A1 and 36H4

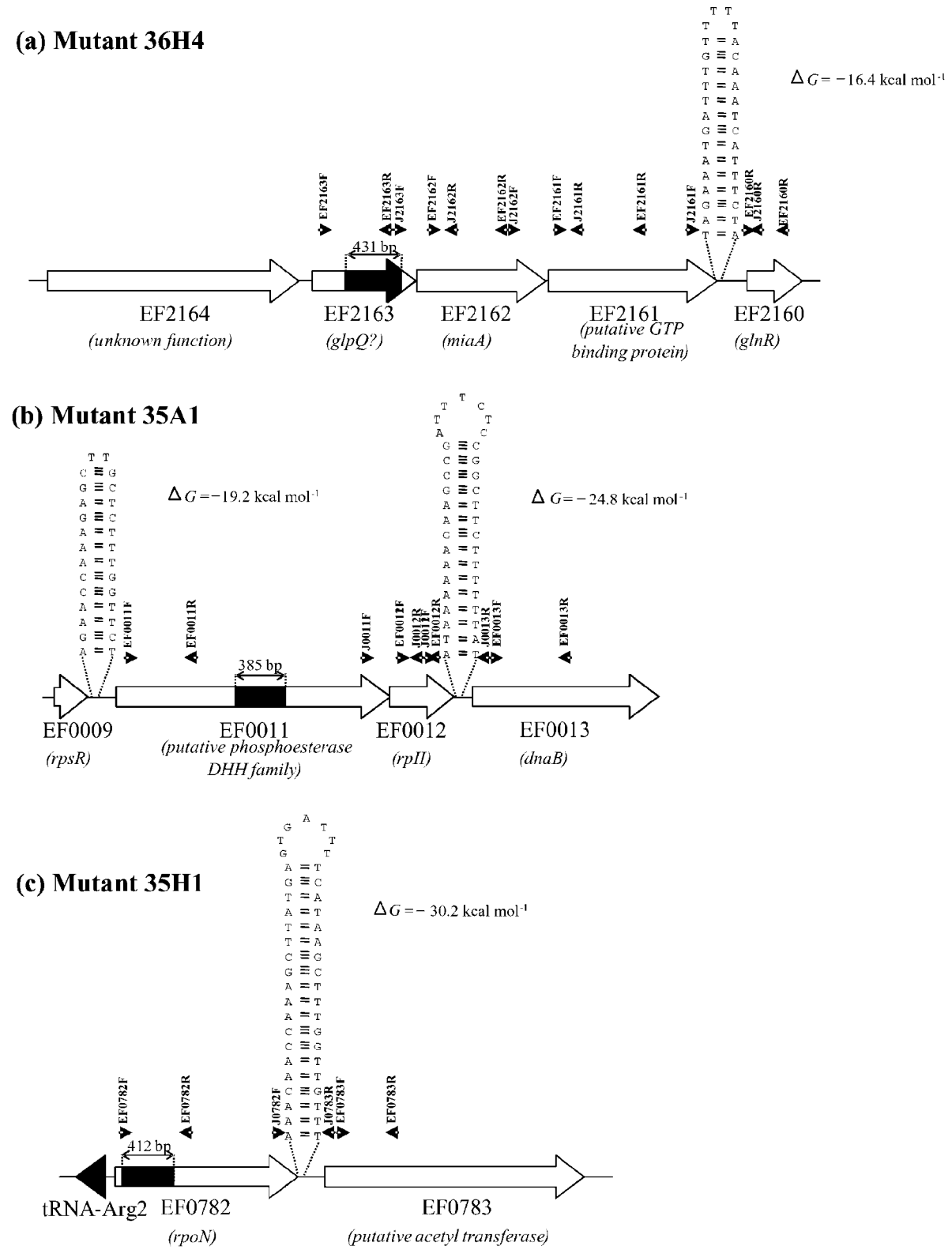

Fig. 1. Genetic organization of the *Ent. faecalis* chromosomal region surrounding integration loci in DvnV41-resistant mutants. Large arrows represent the ORFs and their orientation shows the transcriptional direction; black boxes represent the DNA regions harbouring plasmids integrated into the genomes of mutants; the black triangle represents a tRNA gene. The nucleotide sequences of the putative *rho*-independent terminators are shown. Primers used in RT-PCR experiments are indicated by black arrows.

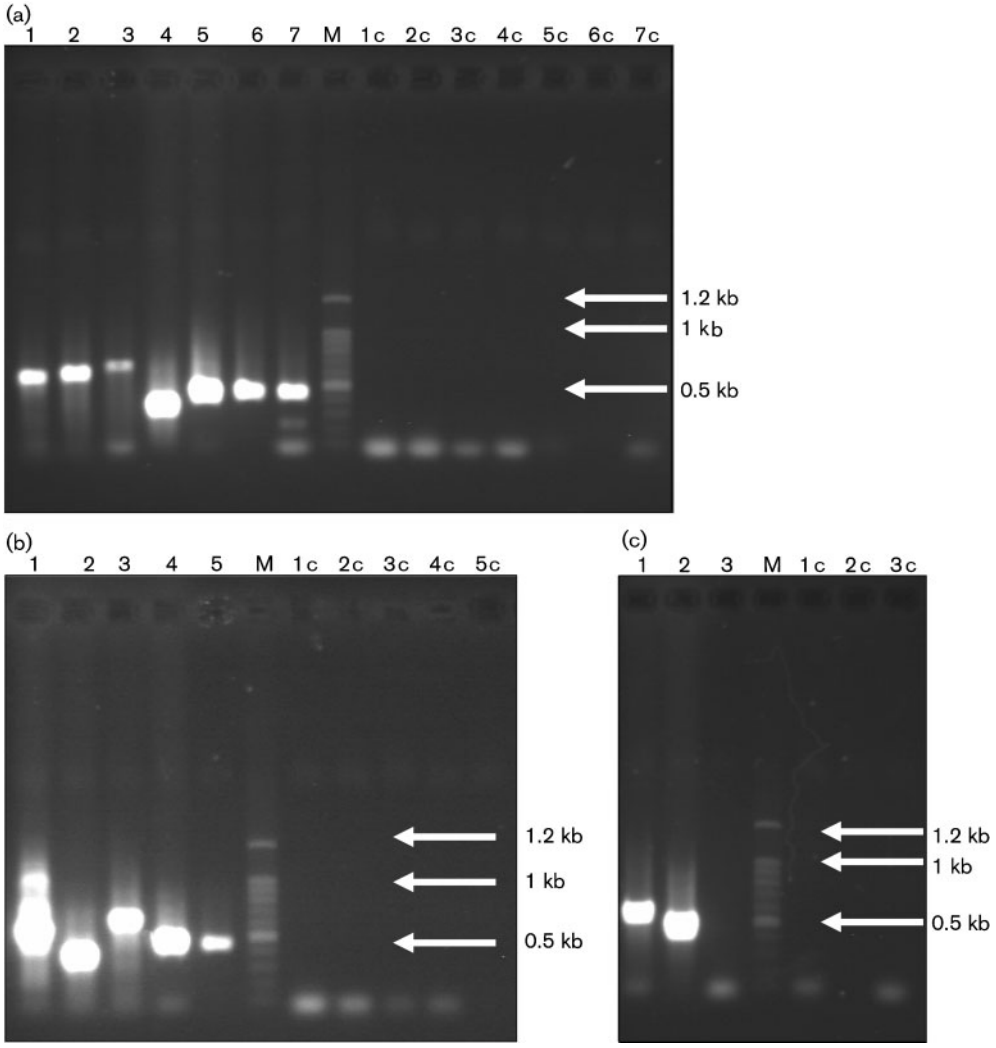

Fig. 2. RT-PCR assays conducted on mRNAs isolated from exponentially growing *Ent. faecalis* JH2-2 cells in GM17 broth. Reverse transcription was performed with random hexamer primers. In (a) PCR was performed with primers EF2163F and EF2163R (lanes 1 and 1c), EF2162F and EF2162R (lanes 2 and 2c), EF2161F and EF2161R (lanes 3 and 3c), EF2160F and EF2160R (lanes 4 and 4c), J2163F and J2162R (lanes 5 and 5c), J2162F and J2161R (lanes 6 and 6c) and J2161F and J2160R (lanes 7 and 7c). To ensure the absence of genomic DNA, negative controls were performed without reverse transcriptase (lanes 1c–7c). In (b) PCR was performed with primers EF0011F and EF0011R (lanes 1 and 1c), EF0012F and EF0012R (lanes 2 and 2c), EF0013F and EF0013R (lanes 3 and 3c), J0011F and J0012R (lanes 4 and 4c) and J0012F and J0013R (lanes 5 and 5c). Lanes 1c–5c correspond to the negative controls. In (c) PCR was performed with primers EF0783F and EF0783R (lanes 1 and 1c), EF0782F and EF0782R (lanes 2 and 2c) and J0782F and J0783R (lanes 3 and 3c). Lanes 1c–3c contain the negative controls. Lane M contains DNA fragments of the '100 bp DNA ladder' (Promega) molecular mass marker.

to inactivation of *pde* and *glpQ* genes rather than a polar effect of genes located in the neighbourhood, we performed a complementation test by incorporation *in trans* in each mutant pAM401 plasmids containing either the *pde* or *glpQ* gene. The new strains (transformants) 35A1 + *pde* and 36H4 + *glpQ* exhibited similar phenotypes to wild-type *Ent. faecalis* JH2-2 (Fig. 3a), whilst transformants harbouring only pAM401 vector remained resistant to DvnV41 (data not shown).

Characterization of *Ent. faecalis* JH2-2 mutants resistant to DvnV41

To examine if the DvnV41-resistant phenotype could be attributed to plasmid insertion, and not to an independent genomic mutation, the *Ent. faecalis* JH2-2 mutants 35A1, 36H4 and 35H1 were cured of their pORI19 recombinant plasmid as described previously (Connil *et al.*, 2002). This resulted in the restoration of the DvnV41-sensitive

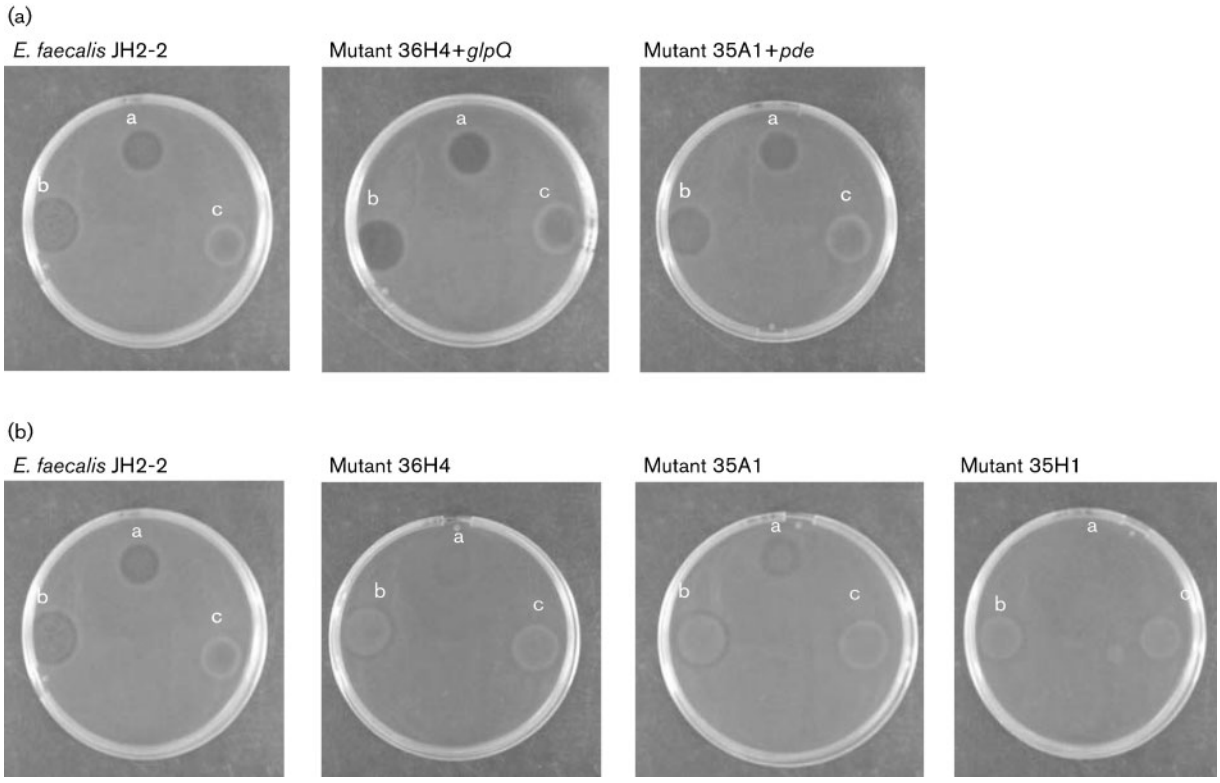

Fig. 3. Agar diffusion test (ADT) showing (a) complementation of mutants 36H4 + *glpQ* and 35A1 + *pde* and (b) resistance of *Ent. faecalis* JH2-2 and mutants 35H1, 36H4, 35A1 to (a) DvnV41, (b) PedA-1/AcH (c) MesY105.

phenotype, proving that plasmid insertion was responsible for this resistant phenotype (data not shown). Further experiments, based on phenotypic and molecular tests, were performed to confirm the enterococcal identity of each strain. As a result, all the strains were shown to be Gram-positive bacteria, cocci associated in pairs, and devoid of both catalase and oxidase activities. The identification procedure was completed by PCR-RAPD using the oligonucleotide primer M13V (Muller *et al.*, 2001). The PCR-RAPD profiles of the wild-type and mutant strains were identical (data not shown).

Growth characteristics of *Ent. faecalis* JH2-2 and its mutant derivatives were determined and compared. In the absence of DvnV41, the growth rates of wild-type and mutant strains were not significantly different. Addition of 5 µl of DvnV41, at 820 000 AU ml^{-1}, to 150 µl cultures, did not affect the growth rate of the mutant strains but extended the lag period of the wild-type strain by 4 h, or even 8 h when the amount of DvnV41 was doubled (data not shown). Characterization of the sensitivity of wild-type and mutant strains was carried out by measuring the MIC of DvnRV41, which ranged between 0.22 and 112 µg ml^{-1} (Table 2). The lowest value was obtained for the wild-type strain, which was 100 times more sensitive than mutants 35A1 (defective phenotype in putative PDE) and 36H4 (defective phenotype in putative GlpQ) and 500 times more sensitive than mutant 35H1 (defective phenotype in σ^{54} factor RNA polymerase).

Cross-resistance

Table 3 summarizes the related cross-resistance obtained with class IIa bacteriocins (DvnV41, Ped PA-1/AcH and MesY105), class I bacteriocin (nisin), and antibiotics. In direct comparison, the cross-resistance exhibited by the mutant strains to class IIa bacteriocins (Table 3; Fig. 3b) is in agreement with that formerly reported (Dalet *et al.*, 2000; Ramnath *et al.*, 2000; Gravesen *et al.*, 2002). Notably, the resistance was evaluated by two independent experiments including ADT and MIC (Tables 2 and 3). Both tests indicated that the resistance to DvnV41 developed by the wild-type and mutant strains was graduated since mutant 35H1 (defective in σ^{54} factor) was more resistant than mutant 36H4 (defective in putative GlpQ), which was itself slightly more resistant than the mutant 35A1 (defective in putative PDE) (Table 3). In contrast, no difference was revealed between the wild-type and mutant strains in terms of resistance to nisin (Table 3). The partial cross-resistance to class IIa bacteriocins, but not to the class I bacteriocin nisin, is consistent with the results of Dalet *et al.* (2000). Concerning resistance to antibiotics, there was no difference between the phenotype of *Ent. faecalis* JH2-2 wild-type and the three mutants. In summary, we suggest

Table 2. MIC for *Ent. faecalis* JH2-2 wild-type and DvnV41-resistant mutant strains towards DvnRV41

Values are means of triplicate independent experiments.

Strain	Gene knock-out	Putative function	MIC (μmol ml^{-1})
Ent. faecalis JH2-2 wild-type	–	–	0.22
Mutant 35A1 (*pde*)	EF0011	Phosphoesterase	28
Mutant 36H4 (*glpQ*)	EF2163	Glycerophosphodiester phosphodiesterase	28
Mutant 35H1 (*rpoN*)	EF0782	σ^{54} factor RNA polymerase	112

that a cross-resistance exists to class IIa bacteriocins but not to nisin and antibiotics.

DISCUSSION

Resistance to class IIa bacteriocins

High-level resistance of *Ent. faecalis*, *L. monocytogenes* and some other Gram-positive bacteria to class IIa bacteriocins results from the loss of *mpt* expression, either in defined mutants or in spontaneous resistant strains (Gravesen *et al.*, 2002). The *mpt* operon encodes a mannose permease (EII_t^{Man}) which belongs to the (PTS. The level of *mpt* expression correlates with the level of sensitivity (Dalet *et al.*, 2001; Héchard *et al.*, 2001), which implies that EII_t^{Man} permease might be a target molecule for class IIa bacteriocins. Given that the IIC and IID subunits are probably present in the membrane, they are potential targets of class IIa bacteriocins. The *mptACD* operon of *L. monocytogenes* heterologously expressed in an insensitive species, such as *Lactococcus lactis* (Ramnath *et al.*, 2004), renders this strain sensitive to various class IIa bacteriocins. Each gene of the *mptACD* operon was expressed independently in *Lac. lactis* and the expression of *mptC* alone was found to be sufficient to confer sensitivity. The IIC subunit has therefore been proposed as the target molecule of the class IIa bacteriocins (Ramnath *et al.*, 2004). Spontaneous resistance of *L. monocytogenes* is accompanied by a respective increase and decrease of two phosphoenolpyruvate-dependent PTSs, which are responsible for sugar uptake and phosphorylation in Gram-negative and Gram-positive bacteria (Postma *et al.*, 1993).

How is resistance to DvnV41 mediated in *L. monocytogenes* and *Ent. faecalis* JH2-2?

Earlier attempts to unravel the resistance of *L. monocytogenes* to DvnV41 involved a proteomic approach, comparing protein profiles of *L. monocytogenes* wild-type and a mutant resistant to DvnV41. *L. monocytogenes* with a DvnV41-resistant phenotype displayed differential protein synthesis (Duffes *et al.*, 2000). This mutant strain lacked at least nine protein spots, two of which have, interestingly, a molecular mass and pI matching those of the *mptA* cluster, which is controlled by the σ^{54} transcription factor in coordination with the ManR regulator (Dalet *et al.*, 2001). Undoubtedly, the identification of the proteins present within the two spots will be of major interest in understanding the resistance of *L. monocytogenes* to DvnV41. In the present study, we have identified three genes associated with the resistance of *Ent. faecalis* JH2-2 to DvnV41. The first one is the *rpoN* gene, which encodes the σ^{54} factor, an alternative subunit of RNA polymerase responsible for the transcription of a specific set of genes. The *rpoN* gene may or may not be involved directly in a general mechanism of sensitivity to class IIa bacteriocins. It has been postulated

Table 3. Resistance of *Ent. faecalis* JH2-2 wild-type and DvnV41-mutant strains to antibiotics and bacteriocins

Strain	Diameter (mm) of inhibition zone for antibiotic or bacteriocin*						
	Penicillin G†	Streptomycin†	Cephalosporin‡	Nisin‡	Divercin V41‡ (DvnV41)	Pediocin PA-1/AcH‡	Mesentericin Y105‡
Ent. faecalis JH2-2 wild-type	23	10	25	11	15	14	11
Mutant 35A1 (*pde*)	24	10	25	11	15§	14§	11§
Mutant 36H4 (*glpQ*)	24	9	25	11	15‖	14‖	0
Mutant 35H1 (*rpoN*)	24	10	26	11	0	0	0

*Values are the means of three experiments.

†Determined by disk diffusion method.

‡Determined by agar diffusion test.

§Limits of inhibition halo can be measured but halo is blurred.

‖Halo is more blurred than §.

that *rpoN* is involved in the expression of a target molecule for class IIa bacteriocins, loss of whose expression leads to resistance (Drider *et al.*, 2006). The second gene identified encodes a putative glycerophosphoryl diester phosphoesterase (GlpQ). GlpQ is an exoprotein found to participate in fatty acid and phospholipid degradation in many bacteria (Antelmann *et al.*, 2000). A hypothesis that could be drawn from our data is that the absence of GlpQ activity in mutant 36H4 leads to an intact fatty acid and phospholipid composition of the cell membrane, which should contribute to the resistant phenotype of this mutant. Moreover, the GlpQ protein was originally suggested to belong to the Pho regulon in *Bacillus subtilis* (Antelmann *et al.*, 2000), and to be governed by a pleiotropic two-component regulatory system PhoP-PhoR (Groisman, 2001). Interestingly, PhoP and Mg^{2+} also control the resistance of many Gram-negative bacteria to antimicrobial peptides, as supported by a number of lines of evidence (Groisman *et al.*, 1992; Moss *et al.*, 2001). Similarly, in Gram-positive bacteria, different studies have highlighted the role of a two-component regulatory system in the resistance of *L. monocytogenes* (Cotter *et al.*, 2002), *Ent. faecalis* (Comenge *et al.*, 2003) and *Staphylococcus aureus* (Kuroda *et al.*, 2003) to inhibiting substances such as antimicrobial peptides and antibiotics. At present, it is unclear what contribution a two-component regulatory system makes to *Ent. faecalis* JH2-2 under harsh environmental conditions with a significant amount of DvnV41. To answer this question, and in order to gain a more comprehensive view of the role of a two-component signal transduction system pathway in resistance to pediocin-like bacteriocins, we examined the resistance of each insertional mutant characterized so far in *Ent. faecalis* JH2-2 (Le Breton *et al.*, 2003) and *Ent. faecalis* V583 (Hancock & Perego, 2004). The results obtained reject any relationship between a two-component regulatory system and resistance to DvnV41. Finally, the third gene identified encodes a putative phosphoesterase (PDE), which belongs to the DHH family. At this stage of investigation, it is quite difficult to unravel the role of phosphoesterase in resistance mechanism of *Ent. faecalis* JH2-2 to divercin V41. The resistance measured by the ADT (Table 3) showed that the size of the inhibition halo is pediocin-like bacteriocin dependent. Interestingly, the resistance of mutant 36H4 to MesY105 was higher than that observed to DvnV41; this could be explained by the activity and/or potency of each bacteriocin.

Overall, it is clear that the *rpoN* gene is associated with the high level resistance and the newly identified genes with the intermediate resistance of *Ent. faecalis* JH2-2 to DvnV41, MesY105 and Ped PA-1/Ach.

ACKNOWLEDGEMENTS

The authors would like to thank Manolo Espinosa and Gisèle LaPointe for critical reading of the manuscript, Carol Robins for improvement of the English, Marta Perego and Pascale Serror for providing us with *Ent. faecalis* V583 mutant strains, and Kurt W. Miller for kindly providing the pAM401 vector. Ségolène Calvez received a PhD grant awarded by 'La Région des Pays de la Loire'. This work was partly supported by 'La Région des Pays de la Loire' and the European Union through VANAM II and the 6th PCRD, SEAFOODplus project, respectively.

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Edited by: G. M. Dunny