Host range of enterococcal vanA plasmids among Gram-positive intestinal bacteria

Guido Werner1, Ana R. Freitas2,3, Teresa M. Coque3–5, Johanna E. Sollid6, Camilla Lester7, Anette M. Hammerum7, Lourdes Garcia-Migura8, Lars B. Jensen8, Maria V. Francia9, Wolfgang Witte1, R. J. Willems10 and Arnfinn Sundsfjord6

1Robert Koch-Institute, Wernigerode, Germany; 2REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; 3Fundación para la Investigación en Biomedicina del Hospital Ramón y Cajal (FIBioHRYC, IRYCIS), Madrid, Spain; 4Unidad de Resistencia a Antibióticos y Virulencia Bacteriana, Madrid, Spain; 5CIBER en Epidemiología y Salud Pública e Internacional, Madrid, Spain; 6University of Trondheim, Trondheim, Norway; 7Statens Serum Institute, Copenhagen, Denmark; 8Division of Microbiology and Risk Assessment, Danish Technical University, Copenhagen, Denmark; 9Servicio de Microbiología, Hospital Universitario Marques de Valdecilla-IFIMAV, Santander, Spain; 10University Medical Centre Utrecht, Utrecht, The Netherlands

*Corresponding author. Working group on enterococci, Unit FG13 Nosocomial Infections, Department of Infectious Diseases, Robert Koch Institute, Wernigerode Branch, Burgstr. 37, 38855 Wernigerode, Germany. Tel: +49-3943-679-210; Fax: +49-3943-679-207/317; E-mail: wernerg@rki.de

Received 4 August 2010; returned 16 September 2010; revised 24 September 2010; accepted 30 October 2010

Objectives: The most prevalent type of acquired glycopeptide resistance is encoded by the vanA transposon Tn1546 located mainly on transferable plasmids in Enterococcus faecium. The limited occurrence in other species could be due to the lack of inter-species transferability and/or stability of Tn1546-containing plasmids in other species. We investigated the in vitro transferability of 14 pre-characterized vanA-containing plasmids hosted by E. faecium (n=9), Enterococcus faecalis (n=4) and Enterococcus raffinosus (n=1) into several enterococcal, lactobacillar, lactococcal and bifidobacterial recipients.

Methods: A filter-mating protocol was harmonized using procedures of seven partner laboratories. Donor strains were mated with three E. faecium recipients, three E. faecalis recipients, a Lactococcus lactis recipient, a Lactococcus lactis recipient and two Bifidobacterium recipients. Transfer rates were calculated per donor and recipient. Transconjugants were confirmed by determining their phenotypic and genotypic properties. Stability of plasmids in the new host was assessed in long-term growth experiments.

Results: In total, 282 enterococcal matings and 73 inter-genus matings were performed and evaluated. In summary, intra-species transfer was far more frequent than inter-species transfer, if that was detectable at all. All recipients of the same species behaved similarly. Inter-genus transfer was shown for broad host range control plasmids (pIP501/pAMβ1) only. Acquired resistance plasmids remained stable in the new host.

Conclusions: Intra-species transfer of enterococcal vanA plasmids was far more frequent than transfer across species or genus barriers and may thus explain the preferred prevalence of vanA-containing plasmids among E. faecium. A reservoir of vanA plasmids in non-enterococcal intestinal colonizers does not seem to be reasonable.

Keywords: vancomycin resistance, repA, relaxases

Introduction

The rapid global emergence of transferable high-level glycopeptide resistance in enterococci in the late 1990s has challenged the already limited therapeutic options in the treatment of severe enterococcal infections. Different types of acquired vancomycin resistance have been described in enterococci based on phenotypic and genotypic characteristics.1,2 By far the most prevalent type is vanA followed by vanB. The vanA gene is contained in Tn1546 or its derivatives usually located on transferable plasmids. The vanB gene is part of Tn1547 or the conjugative transposon CTn1549/5382, mainly chromosomally located and less frequently found on plasmids.2,3 The main clonally relevant reservoir of vanA and vanB is in Enterococcus faecium, at least in Europe, Northern and Latin America and Southeast Asia.2,4 Nowadays, acquired vanA-type vancomycin-resistant E. faecium (VREF) are widely prevalent in hospitals around the world.5 A distinct vanA reservoir exists also in...
commensal E. faecium isolates from commercial animal husbandry, which differ from hospital strains.6–10 and in Paenibacillus strains from environmental samples.11–13 Intestinal commensal bacteria such as Ruminococcus, Rhodococcus and others could serve as a reservoir for vanB and other van gene clusters.11,14,15 It has also been discussed whether commensal intestinal colonizers such as bifidobacteria, lactobacilli and lactococci may participate in lateral gene transfer of vancomycin resistance plasmids.16

A few plasmids containing Tn1546 or derivatives have been fully sequenced or partially characterized,17–19 including transferable plasmids of various types, such as pHTb-plasmid variants carrying isolates carrying vanA since many investigated vanA plasmids remain non-typeable.22–25 The mosaïc-like composition of enterococcal plasmids leads to problems in typing them when typing is performed only on single plasmid marker genes.18,21,22,24–26 The putative plasmid/gene reservoir that exists in related non-enterococcal species could supply resistance genes for future spread.16,29

Transfer of vanA (and vanB) clusters has been demonstrated in several studies30–35 including broad host range plasmid-mediated inter-genus transfer of Tn1546 into methicillin-resistant Staphylococcus aureus (MRSA).13,36,37 Transfer of vanA plasmids into other non-enterococcal species has also been described previously.38,39 Many questions remain unanswered with regard to transferability and host reservoir of the mobile vanA resistance cluster, for instance why prevalence of Tn1546 is mostly limited to E. faecium. Enterococcus faecalis, for instance, possesses a very efficient pheromone response system allowing plasmid exchange at high rates.40,41 The mobility of Tn1546-like elements might be limited by a preference for specific attachment sites.52

In the present study we assessed the role of horizontal plasmid transfer for successful dissemination of vanA-type clusters and plasmids among various Gram-positive intestinal colonizers. Several representative VRE clinical (outbreak) and animal strains were selected originating from several countries. VanA transferability and maintenance were assessed within various enterococcal and other Gram-positive intestinal colonizers known to exchange resistance determinants with enterococci (lactobacilli, lactococci, bifidobacteria). Altogether 355 matings were performed and corresponding transconjugants were confirmed by phenotypic and molecular methods.

Materials and methods

Bacterial strains and plasmids

Lists of donor and recipient strains are given in Tables 1 and 2, respectively. A number of non-enterococcal recipient bacteria were considered and marker resistances were introduced as part of this study (for details see Table 2). Project partners were asked to send VRE donor isolates that were representative of regional or countrywide outbreak strains, historical VRE from human infections or from colonizations in animals from the early 1990s until now (Table 1). For reasons of comparison E. faecalis RE25:pRE25 harbouring a completely sequenced Inc18-prototype plasmid (non-vanA) was included. Donor strains had tested positive in previous intra-species filter matings.

Antibiotic susceptibility testing

Enterococcal strains were examined by microbroth dilution according to the German standard DIN 58940 on antibiotic susceptibility testing. MICs for lactococcal, lactobacillary and bifidobacterial isolates were determined as described previously.43,44

Introducing marker resistances into selected recipients

Marker resistances for new enterococcal and non-enterococcal recipients were introduced randomly by streaking out exponentially grown cells (≥10^{10} cfu) on agar plates with stepwise increasing concentrations of the selective antibiotics (not described in detail). MICs were determined as described above using microbroth dilution and Etest® (bioMérieux, Marcy L’Etoile, France). All recipient candidate strains were plasmid free, except E. faecium AK-EM40, which contained several plasmids (see the Results section).

Filter-mating protocol

Seven partners shared their filter-mating procedures on the basis of which a harmonized protocol was established. All experiments were performed on three filters in parallel. For details including concentrations of used antibiotics please see the Supplementary data (available at JAC Online).

Phenotypic characterization of transconjugants

One cfu per filter (n=3) was again separated on selective agar plates. Species and antibiotic susceptibilities were determined. Transconjugants of recipient 64/3 were analysed in follow-up experiments investigating the stability of acquired resistance plasmids by long-term growth experiments over 4 weeks in BHI. Samples were passageed daily (0.1 mL of overnight culture in 5 mL of BHI). At the end of each week samples were taken and stored at −70 °C. Colony counts were measured on selective (vancomycin/fusidic acid/rifampicin or erythromycin/fusidic acid/rifampicin) and non-selective agar plates without antibiotics after each week. Generation times were calculated based on Sandegren et al.53 Transfer of broad host range plasmids pIP501 (E. faecalis OG1X:pIP501) and pAMβ1 (OG1X:pAMβ1) was used as a control for combinations that revealed no detectable vanA plasmid transfer (see the Results section).

Bacteriocin assays

Susceptibility of recipients to bacteriocin(-like) or other inhibitory substances released from donor strains was assayed in BHI agar plate assays. Recipient cells were grown for 3–4 h in BHI broth to reach cell densities equivalent in turbidity to that of a 0.5 McFarland standard. They were diluted 1:100 in saline and streaked onto the entire agar plates expecting semi-confluent growth after overnight incubation at 37 °C. Colony counts were measured on selective (vancomycin/fusidic acid/rifampicin or erythromycin/fusidic acid/rifampicin) and non-selective agar plates without antibiotics after each week. Generation times were calculated based on Sandegren et al.53

274
Table 1. List of donor strains

<table>
<thead>
<tr>
<th>Study-strain no.</th>
<th>Species</th>
<th>Strain name</th>
<th>Origin</th>
<th>Year</th>
<th>Plasmid characteristics (van, rep, PSK, tra)</th>
<th>No. of plasmids (sizes in kb)</th>
<th>MLST</th>
<th>Selected donor resistance marker (other resistances)</th>
<th>Remarks/other features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. raffinosus</td>
<td>TUH 50-23 (UW7358)</td>
<td>faecal, Canada</td>
<td>2006</td>
<td>170 kb vanA plasmid; PSK: axe-txe, rep(pRUM)</td>
<td>1 (170)</td>
<td>—</td>
<td>VAN</td>
<td>plasmid transferred previously detected between various enterococcal species from France, similar to BM4147::pIP816, but with transferable vanA plasmid; PSK: axe-txe; tra(Tn925); p6A; + 6 kb pCIZ2-like bacteriocin plasmid; + 6 kb pRI1-like plasmid + 6 kb unknown plasmid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>this study (Sollid/Sundsfjord)</td>
</tr>
</tbody>
</table>
| 2                | E. faecium       | 70/90       | clinical, France | 1990  | 100 kb; vanA, PSK: axe-txe(+); (rep-Inc18)  | 2 (100, 45)                   | ST25         | V EY                                             | strain from France, similar to BM4147::pIP816, but with transferable vanA plasmid epidemically spread worldwide since 1995–99 in Germany | 68
| 3                | E. faecium       | UW931       | BAL, Germany     | 1996  | 100 kb; p671-like vanA-ter(M) plasmid; rep(pEF1), T4SS, tra(Tn925); p6A; + 6 kb pCIZ2-like bacteriocin plasmid; + 6 kb pRI1-like plasmid + 6 kb unknown plasmid<sup>a</sup> | 4 (100, 6, 6, 4)               | ST117        | VAN (EY)                                         | QID-resistant outbreak strain with vanA and vatD resistance cluster on a transferable plasmid: TC only acquired the 80 kb plasmid on a transferable plasmid; PSK: axe-txe (vanA and vatD resistance cluster); rep<sup>-Inc18</sup> | 52
| 4                | E. faecium       | UW3540      | urine, Germany   | 2002  | 80 kb vanA-erm(B)-vatD plasmid (rep-Inc18); axe-txe(+), but not on vanA plasmid | 3 (80, 30, 10)                | ST145        | V EY (QD)                                       | QID-resistant outbreak strain with vanA and vatD resistance cluster on a transferable plasmid: TC only acquired the 80 kb plasmid on a transferable plasmid; PSK: axe-txe (vanA and vatD resistance cluster); rep<sup>-Inc18</sup> | 69
| 5                | E. faecium       | H182 (UW7353) | hepatic fluid, Portugal | 2002  | 92 kb vanA plasmid (rep-pRUM)<sup>b</sup> | 5 (12, 40, 92, 140, 230)       | ST18         | V EY (KAN)                                      | epidemic plasmid spread from Portugal to the USA; widespread and persistent in Portugal and Italy; larger plasmid in TC | 70
| 6                | E. faecium       | H311 (UW7483) | urine, Portugal  | 2002  | 60 kb vanA plasmid (rep-Inc18, rep-pRUM, PSK: axe-txe) | 4 (10, 50, 60, 200)           | ST132        | V EY (GEN)                                      | epidemic plasmid from Portugal (2001-02); widespread plasmid type (2001-07) | 70
| 7                | E. faecium       | E0292 (UW7486) | urine, USA       | 1992  | 200 kb vanA plasmid (rep-Inc18) | 6 (5, 8, 10, 22, 60, 200)     | ST20         | V EY (GEN, STR)                                  | epidemic plasmid from Portugal (2001-02); widespread plasmid type (2001-07) | 70
| 8                | E. faecium       | H74 (UW7490) | hepatic fluid, Portugal | 2001  | 50 kb vanA plasmid (rep-Inc18, rep-pRUM, PSK: axe-txe) | 7 (12, 30, 50, 60, 170, 200, 350) | ST132        | V EY (GEN)                                      | epidemic plasmid from Portugal (2001-02); widespread plasmid type (2001-07) | 70
| 9                | E. faecium       | E0013 (UW7492) | urine, Portugal  | 1992  | 50 kb vanA plasmid, rep-pRI1, rep-Inc18, PSK: axe-txe | 2 (25, 50)                    | ST18         | V EY (TET, STR)                                  | epidemic plasmid from Portugal (2001-02); widespread plasmid type (2001-07) | 70
| 10               | E. faecium       | UW261       | food, Germany    | 1995  | 172 kbvanA plasmid; unknown repa type (rep-pAD1-Inc18/-RUM-negative);PSK: axe-txe | 1 (172)                      | ND           | V EY (TET)                                      | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 68
| 11               | E. faecalis      | Val-1 (UW7489) | blood, Spain     | 1999  | 100 kb vanA plasmid (rep-pAD1)<sup>d</sup> | 2 (75, 100)                   | ST6          | V EY (GEN)                                      | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 46
| 12               | E. faecalis      | 229710 (UW7770) | clinical, Portugal | 1996  | 85 kb vanA plasmid (rep-pAD1, rep-Inc18; PSK: par) | 1 (85)                      | ST6          | V GEN (TET, ERY)                                | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 46
| 13               | E. faecalis      | 536540 (UW7771) | clinical, Portugal | 1999  | 85 kb vanA plasmid (rep-pAD1; PSK: par) | 2 (85, 145)                   | ST21         | V GEN (EY, TET)                                 | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 46
| 14               | E. faecalis      | H143 (UW7766) | wound, Portugal   | 2001  | 85 kb vanA plasmid (rep-pAD1; PSK: par) | 2 (85, 145)                   | ST30         | V EY (TET), ERY                                  | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 46
| 15               | E. faecalis      | RE25:pRE25   | food, Switzerland | 1995  | 50 kb plasmid pRE25; rep(pRE25) = Inc18; tra(T4SS), PSK: axe-txe | 1 (100)                      | ND           | V EY (TET), ERY                                  | epidemic plasmid from Spain and Italy; larger plasmid in TC clone B (ST6/67); strain widely disseminated in all over Portugal and other EU countries (1996-2008); clone X (CC21)<sup>c</sup> | 46

BAL, bronchoalveolar lavage; urine, from urinary tract infection; TC, transconjugant; ND, not determined; CHL, chloramphenicol, ERY, erythromycin; GEN, gentamicin (high-level); KAN, kanamycin (high-level); QID, quinupristin/dalfopristin; STR, streptomycin (high-level); TET, tetracycline; VAN, vancomycin; tra(Tn925), cluster of conjugative genes similar to Tn916; prototype of conjugative transposons; tra(T4SS), transfer region for type 4 secretion systems involved in horizontal gene transfer.

<sup>a</sup>Plasmid classification according to Jensen et al.<sup>48</sup>
<sup>b</sup>PSK systems: w<sup>v</sup>-z, axe-txe and par.
<sup>c</sup>The vanA plasmid is shown in bold.
<sup>d</sup>UW931: plasmid data were obtained from Southern hybridizations and 454 plasmid sequencing (not described in detail; G. Werner, unpublished data). Isolate also contained the following plasmids: 6 kb pCIZ2-like bacteriocin plasmid; 4 kb pRI1-like plasmid; and 6 kb unknown cryptic plasmid.
<sup>e</sup>Negative in PCR screenings for PSK systems w<sup>v</sup>-z, axe-txe and par.
<sup>f</sup>Plasmids rearranged when initial mating experiments were performed in the primary laboratory; plasmid rearrangements were not confirmed in experiments performed as part of this study (see also Figure S3).
**Plasmid analysis**

Plasmids were isolated using a modified mini-preparation based on phenol/chloroform purification. Alternatively, a modification of the protocol by Barton et al. describing S1 nuclease treatment of genomic DNA and resolution of the linearized plasmids in PFGE, was performed (see below).

**DNA isolation, PCR, sequencing and multilocus sequence typing (MLST)**

Genomic DNA was prepared using a DNA extraction kit (DNeasy Tissue Kit; Qiagen, Hilden, Germany). PCR was performed with Illustra® puRe Taq Ready-To-Go PCR beads (GE Healthcare, Freiburg, Germany) as described previously. MLST was carried out according to the reference MLST database (http://efaecium.mlst.net/). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using DNASTAR's Lasergene 8.0 software, TraceEditPro v. 1.1.1 from Ridom (http://www.ridom.de) and via the official MLST web site (http://efaecium.mlst.net/).

**SmaI- and S1-PFGE**

Genomic DNA of transconjugants was isolated, digested with SmaI and resolved in PFGE using 1% agarose gels and a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA). For sizing large plasmids genomic DNA was treated with S1 nuclease and conditions and criteria set by a protocol described recently, including ramped pulse times of 5–35 s for 22 h at 14°C. SmaI-digested S. aureus NCTC 8325 was used as a size marker. Data were analysed and plasmid sizes were calculated using a Dice coefficient and UPGMA clustering (BioNumerics v. 5.1; Applied Maths, Sint-Martens-Latem, Belgium).

**Results**

**Introducing marker resistances into recipients**

Most enterococcal recipients were already high-level resistant to rifampicin and fusidic acid (Table 2). We introduced strain AK-EM40, representing an antibiotic-susceptible MLST sequence (Applied Biosystems, Darmstadt, Germany). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using DNASTAR's Lasergene 8.0 software, TraceEditPro v. 1.1.1 from Ridom (http://www.ridom.de) and via the official MLST web site (http://efaecium.mlst.net/).

**Table 2.** List of candidates considered as recipients

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no.</th>
<th>Wild-type MICs</th>
<th>Mutant MICs</th>
<th>MLST type/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RIF</td>
<td>FUS</td>
<td>RIF</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>JH2-2</td>
<td>128</td>
<td>128</td>
<td>ST8</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>OG1RF</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>ST1</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>UV202 (JH2-2::recA-)</td>
<td>64</td>
<td>128</td>
<td>ST8^72</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>64/3</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>ST21</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>GE-1</td>
<td>32</td>
<td>256</td>
<td>ST515</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>BM4105-RF</td>
<td>&gt;256</td>
<td>256</td>
<td>ST172</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>AK-EM40RF</td>
<td>256</td>
<td>256</td>
<td>ST18^a,b</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>UW3767RF</td>
<td>1</td>
<td>32–128^c</td>
<td>256</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>UW5688RF</td>
<td>≤0.008</td>
<td>32–128^c</td>
<td>256</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>UW4127RF</td>
<td>16</td>
<td>4/2</td>
<td>(&gt;-1024</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>UW4268R</td>
<td>≤0.008</td>
<td>4/1</td>
<td>256</td>
</tr>
<tr>
<td><em>B. breve</em></td>
<td>UW4272R</td>
<td>≤0.008</td>
<td>16^o</td>
<td>256</td>
</tr>
<tr>
<td><em>B. longum</em></td>
<td>UW4385R</td>
<td>≤0.008</td>
<td>8/2</td>
<td>256</td>
</tr>
</tbody>
</table>

FUS, fusidic acid; RIF, rifampicin; NS, not successful (several failed attempts were performed to select fusidic acid-resistant bifidobacterial mutants (concentrations of 20 and 30 mg/L)). Characteristics marked in bold represent features introduced during this study.

^ST18 is MLST clonal complex CC17.

^rep gene PCRs revealed pRE25-like and pVEF1-like plasmids in AK-EM40, but were negative for rep(pRUM) und rep(pAD1).^48

^Varying MICS in repeated tests.

^Not considered for mating experiments.

^Unstable fusidic acid resistance.
Transferability of vanA plasmids

Bacteriocin production in donor strains

It was known from previous analyses that donor strain E. faecium UW931 is a very potent bacteriocin producer at least when tested against E. faecium 64/3 and E. faecalis JH2-2.51,52 Preliminary plasmid sequencing (G. Werner, unpublished results) revealed nucleotide identity with plasmid fragments of pCIZ2 and pEF1 encoding enterocins EntQ and A5-48/EntIJ, respectively.27,54 Nevertheless, we were able in previous filter-mating experiments to transfer the vancomycin resistance determinant into E. faecium 64/3.51,55 However, we used shorter filter incubation times (4 h). Preliminary analysis revealed bacteriocin production in UW931 during the late exponential growth phase (I. Klare and G. Werner, unpublished results). When counting recipients, donors and transconjugants after filter incubation, we noticed drastically reduced numbers for recipient cell counts (see matings nos 65–67; Table S1). This putative influence of mating time and supposed bacteriocin production on the outcome of mating experiments prompted us to study susceptibilities of the recipients used and putative bacteriocin production in donor strains in agar plate assays (Table S2 (available as Supplementary data at JAC Online) and Figure S1 (available as Supplementary data at JAC Online)). Inhibition of growth of recipients appeared frequently and was dependent on the donor strain tested and the recipient used. The effect was specific since certain donor strains (UW261) inhibited growth of only a subset of tested recipients (Table S2). Recipient AK-EM40RF appeared to be more susceptible to inhibitory substances released from donor cells than others. UW931 was again confirmed as a potent bacteriocin producer capable of inhibiting all tested E. faecium and E. faecalis recipients; however, with variable activity. Inhibition of growth of recipient cells should be considered as a possible reason for undetectable or low resistance gene transfer, which could be missed if only transfer rates per donor are assessed and/or when cell counts are determined only before filter incubation.

Matings with non-enterococcal recipients

To assess the host range of enterococcal resistance plasmids among commensal intestinal colonizers, filter matings with lactobacillary, lactococcal and bifidobacterial recipients were performed using the harmonized protocol (with only one instead of three filters). No transconjugants were obtained with any of the lactobacillary or bifidobacterial recipients (Table 3). Recipient counts of the L. lactis recipient were drastically reduced after filter incubation (<500 cfu/mL) independent of aerobic or anaerobic test conditions (Table S3, available as Supplementary data at JAC Online). Counts of the Lactobacillus acidophilus recipient cells were not affected, but after mating with E. raffinosus TUH50-23 recipient counts dropped to undetectable numbers (<50 cfu/mL). Bifidobacterial recipient counts were not affected by filter incubation, but the E. raffinosus donor strain TUH50-23 did not survive filter mating on M144 medium under anaerobic growth conditions.
Table 3. Results of filter matings with enterococcal, lactococcal, lactobacterial and bifidobacterial recipients

<table>
<thead>
<tr>
<th>Recipients</th>
<th>E. raffinosus</th>
<th>E. faecium</th>
<th>E. faecalis</th>
<th>RE25::pRE25</th>
<th>Vat-1</th>
<th>229710</th>
<th>536540</th>
<th>H143</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TUH50-23</td>
<td>H182</td>
<td>70/90</td>
<td>UW3540</td>
<td>H311</td>
<td>E0292</td>
<td>E0013</td>
<td>UW931</td>
</tr>
<tr>
<td>Efm 64/3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Efm BM4105RF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Efm GE-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Efm AK-EM40RF</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Efs OG1RF</td>
<td>+</td>
<td>–</td>
<td>(+)a</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Efs JH2-2</td>
<td>(+)a</td>
<td>–</td>
<td>(+)a</td>
<td>–</td>
<td>(+)a</td>
<td>(+)a</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Efs UV202</td>
<td>–</td>
<td>–</td>
<td>(+)a</td>
<td>–</td>
<td>–</td>
<td>(+)a</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bb UW4268R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bl UW4365R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

AA, anaerob; A, aerob; Bb, B. bifidum; Bl, B. longum; Efm, E. faecium; Efs, E. faecalis; La, L. acidophilus; Ll, L. lactis; ND, not determined.

| Only with low efficacy (transconjugants only grew on large selective plates; average rates <10^{-7}/10^{-8}). |
| No or only weak growth of recipient cells on selective plates (<10 cfu/mL filter content). |
| Performed three times. |
| No or only weak growth of donor cells on selective plates (<10 cfu/mL filter content). |
We performed control experiments with broad host range plasmids pIP501 and pAMβ1 shown to be transferable into lactic acid bacteria and bifidobacteria.\(^{56,57}\) We successfully transferred pIP501 into L. lactis UW4127RF but were unable to select for pIP501/pAMβ1 transfer into bifidobacterial recipients (B. bifidum UW4268R/B. longum UW4385R).

## Analysis of selected transconjugants

For matings using recipient E. faecium 64/3 one transconjugant per mating was confirmed by Smal macrorestriction of genomic DNA in PFGE. PFGE of transconjugants displayed banding patterns nearly indistinguishable from that of the recipient (Figure S2A, available as Supplementary data at JAC Online). The few additional bands in transconjugants compared with the recipient most probably derive from Smal-digested plasmids. Plasmid acquisition in transconjugants of recipient 64/3 was confirmed by S1 nuclease PFGE (Figure S2B, available as Supplementary data at JAC Online). In some matings only the vanA-containing plasmid was transferred (H182, E0292, UW3540) while in other matings all plasmids of the donor strain were transferred (H311) (Table 1 and Figure S2). It is important to note that small, cryptic plasmids of ≤10 kb are not well visualized in standard S1 PFGE, so it cannot be excluded that some of the donor plasmids were also mobilized into the transconjugants, while unnoticed in S1 PFGE.

Efficiency of transfer and capability of replication in the new host are important prerequisites for maintenance of resistance plasmids in new hosts. For long-term persistence and stable maintenance on a population scale, stability of plasmid persistence in the absence of selective pressure by antibiotics is another important characteristic. We performed growth experiments on all 12 transconjugants of recipient E. faecium 64/3 in non-selective (antibiotic-free) liquid BHI broth for 4 weeks. We did not see substantial differences in the colony counts on selective or non-selective media (Table S4, available as Supplementary data at JAC Online). Minor variations, e.g. for H74×64/3 TC1 with a factor of +3 to +3.5 between non-selective and selective growth, were reproducible over weeks and thus reflect less efficient growth of transconjugant cells under stringent selective conditions rather than plasmid loss. S1 nuclease-digested plasmid DNA resolved in PFGE revealed stable plasmid patterns in all transconjugants investigated before and after the growth period (Figure S3, available as Supplementary data at JAC Online). In conclusion, newly acquired resistance plasmids were all stably maintained at the population level under non-selective conditions for ~100 generations.

## Discussion

In this study we assessed the role of horizontal plasmid transfer for successful dissemination of vanA-type resistance among enterococci and other Gram-positive, commensal, intestinal bacteria known to share a common gene pool and a similar ecological niche with enterococci.

In general, the harmonized protocol worked well and could be recommended, at least for mating experiments among enterococcal strains (see also comments below). Mating time on the filter appeared to be of critical importance. Overnight incubation either on filters or in liquid broth is accepted for conjugation experiments with bacteria in general. Prolonged incubation times may affect survival of recipients and donors on filters and thus reduce transconjugant counts in a qualitative and quantitative manner (Table S3).\(^{56}\) Transconjugants could also serve as additional donors, being capable of transferring resistance plasmids to further recipients. Due to high cell counts, bacterial growth is not expected to happen during filter incubation. Recipient counts might also drop dramatically due to a supposed production of bacteriocin-(like) substances released from some donor strains (Table S2 and Figure S1). According to all these arguments it may be advisable to reduce the mating time to 2–4 h. Also it appeared beneficial to determine all cell counts after filter incubation and calculate rates per donor and per recipient numbers to visualize and address any effect of cell survival during filter incubation.

Mating results demonstrated a rather limited host range of the vanA plasmids examined. As expected, intra-species entero-coccal transfer rates were highest, and transfer was less efficient (if demonstrable at all) between different enterococcal species (Table 3). Transfer across species barriers revealed higher rates from E. faecalis donors to E. faecium recipients than vice versa. This is in agreement with recent reports demonstrating E. faecalis pheromone-like plasmid transfer into or prevalence among E. faecalis isolates;\(^{58}\) also here all E. faecalis vanA plasmids were of the pAD1 or pCF10 type, at least as determined by their replicase genes. Very low transfer efficiencies, e.g. specific for certain E. faecium vanA plasmids transferred into E. faecalis recipients, were only detectable after a high inoculum on large selective agar plates (plasmid types: Inc18-, pEF1-, pH5B-like; Tables 1 and 3). Most common mating protocols do not consider using large selection plates to increase detection limit by a factor of 5–10-fold, which may change completely the overall outcome of the experiment in the case of very low transfer rates near the detection limit (<10\(^{-8}\) cfu/mL). Recipient strains of the same species revealed similar mating results; however, mating rates varied (Table S1). Plasmid transfer rates were independent of recA status since mating rates did not differ between isogenic H2-2 (recA+) and UV202 (recA–) recipients. Our results are in congruence with results of recent plasmid classification schemes suggesting a narrow host range of some plasmid housekeeping genes determined by replicases (repA_N) and relaxases (not documented here).\(^{56,58,48}\) Some investigated E. faecium vanA plasmids contained Inc18-family repA genes (70/90, E0292); however, mating results did not confirm a supposed broad host range. Locating these determinants to the investigated vanA plasmids does not reliably indicate their functional involvement in plasmid replication and for certain plasmids already several rep homologues have been detected by Southern hybridization (H311, H74). Due to the mosaic-like structure of enterococcal plasmids, indentifying rep classes like the Inc18-like does not unambiguously allow prediction of the plasmid type and host range since other features like conjugation modules and relaxases may be as important, and little is known to what extent these determinants or PSK systems determine and influence the host range and stable maintenance of their plasmids in various hosts.

The recipient AK-EMRF representing hospital-associated E. faecium (CC17) did not acquire vanA determinants. These findings contradict the current hypothesis that members of this clonal complex generally acquire resistance genes, plasmids or...
mobile resistance elements more easily than their colonizing counterparts and question the model that multiresistance genotypes among enterococcal clinical strains derive from an enhanced plasmid acquisition capability. Nevertheless, only a single CC17 recipient was examined and it remains to be elucidated whether these findings are generally applicable.

Recent reports demonstrated a considerable reservoir of vanB and other van determinants in non-enterococcal intestinal colonizers. In addition, it was speculated that lactobacilli may participate in lateral gene transfer of vancomycin resistance plasmids. Our results would not support this hypothesis. However, it cannot be excluded that results are biased by the corresponding in vitro filter-mating model and the experimental setting (conditions, strains, plasmids) used here. Parameters set for our protocol were in concurrence with a recent harmonized protocol to assess antibiotic resistance gene transfer among lactococcal species. In that study it was discussed that single parameters, such as donor to recipient ratio, the time of filter incubation and even the donor/recipient strains used influence transfer efficacies and even the overall outcome tremendously.

Using filter mating to determine transfer efficiencies of genetic traits such as resistance plasmids among bacteria generally possesses certain limitations. First, filter mating may not reflect in vivo situations. In natural ecosystems such as within the mammalian intestine various stimuli may influence and also trigger horizontal gene transfer. Transfer efficiencies of vanA/B gene clusters in mice intestines were higher compared with rates assessed in vitro. Biofilms may also play an important role in lateral gene transfer supporting very tight cell-to-cell contacts between unrelated bacteria also allowing transfer of non-conjugative plasmids.

Long-term in vitro growth experiments revealed stable maintenance of the acquired vanA plasmids in transconjugants of a single recipient. For some of the plasmids, specific maintenance and PSK systems were already determined (Table 1). It remains to be speculated whether acquisition of plasmids harbouring them reveals stable maintenance also in the new host or if this comparably stable persistence is also host dependent, since we tested only transconjugants of a single recipient strain. In this context it is of special interest that despite glycopeptides (avoparcin) having been banned for more than a decade as growth promotors, a reservoir of vanA-type resistance and corresponding plasmids in E. faecalis from commercial animal farming still exists. Data from the recent, present and previous unpublished (G. Werner) studies suggest a mosaic-like structure of enterococcal resistance plasmids also encoding many functions unrelated to antimicrobial resistance, but associated with virulence and/or primary or secondary metabolism. Most probably some of these additional features influence persistence and fitness in the plasmid-acquiring hosts; an argument that remains to be investigated in future analyses.

In conclusion, results of in vitro filter-mating experiments showed a considerably narrow host range of the investigated enterococcal vanA plasmids. Recipient strains of the same species behaved similarly thus allowing general conclusions to be drawn when using only a single recipient per species. Representatives of Gram-positive species like Lactococcus, Lactobacillus or Bifidobacterium known to share a common gene pool with Enterococcus do not seem to participate in the dissemination of commonly encountered enterococcal vanA plasmids. Acquisition of resistance plasmids in enterococcal recipients was stable over many generations even in the absence of selective antibiotic pressure.

Acknowledgements
Special thanks to Dr E. Sadowy (Warsaw, Poland), Dr P. Ruiz-Garbajosa (HRC, Spain) and Dr H. Leavis (UMCU, The Netherlands) for sharing their individual filter-mating protocols and experiences. We acknowledge excellent technical assistance by Mrs Christine Guenther, Mrs Carola Fleige and Mrs Christina Aaby Svendsen. Special thanks to Professor I. Nes for providing strain E. faecalis OG1X:pAMB1 and to Dr Vincent Perreten for the gift of strain E. faecalis RE25::pRE25.

Funding
This work was supported by a grant from the European Commission for an EU-funded project called ‘Approaches to Control multi-resistant Enterococci’ (ACE; grant number LSHE-CT-2007-037410).

Supplementary data
The harmonized filter-mating protocol, Tables S1–S4 and Figures S1–S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
Transferability of vanA plasmids


29 Jacobsen L, Wilcks A, Hammer K et al. Horizontal transfer of tet(M) and erm(B) resistance plasmids from food strains of Lactobacillus plantarum to Enterococcus faecalis HJ2-2 in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiol Ecol 2007; 59: 158–66.


44 Klare I, Konstabel C, Muller-Bertling S et al. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli,

278


