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Co-transfer of *vanA* and aggregation substance genes from *Enterococcus faecalis* isolates in intra- and interspecies matings

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***Objectives*: The study was undertaken to investigate vancomycin-resistant (*vanA*) *Enterococcus faecalis* isolates carrying aggregation substance (AS) gene(s) for their ability to co-transfer *vanA* and AS genes.**

***Methods*: Six *vanA* clumping-positive *E. faecalis* isolates (five human and one food sample) carrying one or more AS genes (*prgB*, *asa1*, *asa373*) were analysed for co-transfer of *vanA* and AS genes to *E. faecalis* JH2-2 and *Enterococcus faecium* 64/3.**

***Results*: *E. faecalis* isolates harboured one or multiple plasmids carrying *vanA*, one or more AS gene(s) or both. *vanA* was transferred to JH2-2 (frequencies of 10^{-3}–10^{-6}) from all donors and to 64/3 (10^{-6}–10^{-8}) only from donors from humans. AS genes were detected in 51/60 (85%) of JH2-2 and in 20/50 (40%) of 64/3 *vanA* transconjugants (*prgB*, *asa1*, *asa373* or *prgB asa373*), of which a total of 53.6% were clumping-positive. The plasmid content of JH2-2 transconjugants from the same donor was either identical to that of the donor or it was completely different, suggesting different mechanisms for co-transfer (location on the same pheromone plasmid, mobilization of *vanA* plasmids by the pheromone-inducible conjugation system, rearrangement of plasmid content during matings). After induction with pheromones, a marked increase in adhesion to Caco-2 cells was observed in four isolates and in some JH2-2 transconjugants, all clumping-positive.**

***Conclusions*: Findings suggest that co-transfer of *vanA* and AS genes may be a common feature of *E. faecalis* isolates. Since AS is recognized as a virulence factor, this feature might contribute to the emergence of strains with enhanced ability to cause infection and disease in humans.**

Keywords: *E. faecalis*, *prgB*, *asa1*, *asa373*

Introduction

Over the last two decades, vancomycin-resistant enterococci (VRE) have emerged as major causes of nosocomial infections in clinical settings.[1] Vancomycin resistance is most commonly found in *Enterococcus faecium*, but is increasingly being seen in *Enterococcus faecalis*, the predominant enterococcal species in humans.[1] Clonal expansion has contributed significantly to the increasing incidence of VRE, e.g. the *E. faecium* clonal complex-17 (CC17) and the *E. faecalis* CC2 and CC9, which are associated with hospital outbreaks worldwide.[2] VanA (*vanA*) expresses inducible, high-level vancomycin resistance and cross-resistance to teicoplanin, and is prevalently detected in *E. faecium*.[1] VanA-type resistance in enterococcal clinical isolates is mediated by genetic elements, identical or closely related to Tn*1546*, that are generally carried by self-transferable plasmids.[3,4] Conjugal transfer of *vanA* plasmids appears to be responsible for the spread of *vanA* resistance in enterococci.[3,4]

Aggregation substance (AS) of *E. faecalis* is a pheromone-inducible surface protein encoded by pheromone plasmids that facilitates conjugative exchange, by mediating strong binding between cells and formation of cell aggregates (clumps).[1] AS also contributes to pathogenicity by enhancing cell adhesion and internalization as well as favouring intracellular survival within macrophages.[1] Although pheromone plasmids are frequently found in *E. faecalis*, the presence of AS genes in vancomycin-resistant isolates of this species has rarely been reported.[4–7]

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The present study was undertaken to investigate recently characterized *vanA E. faecalis* isolates carrying one or more AS genes (F. Biavasco, G. Foglia, C. Paoletti, G. Zandri, G. Magi, E. Guaglianone, A. Sundsfjord, C. Pruzzo, G. Donelli and B. Facinelli, unpublished data) for their ability to co-transfer *vanA* and AS genes in intra- and interspecies matings.

Materials and methods

Strains

Six *vanA E. faecalis* isolates (five human and one food sample) carrying one or more AS genes (*prgB*, *asa1*, *asa373*), all clumping-positive, were studied (Table 1). These strains have recently been detected among *vanA* enterococci collected in Italy and investigated for *vanA* location, Tn*1546* type, and presence of virulence determinants (F. Biavasco, G. Foglia, C. Paoletti, G. Zandri, G. Magi, E. Guaglianone, A. Sundsfjord, C. Pruzzo, G. Donelli and B. Facinelli, unpublished results); *prgB* with an additional *Eco*RI site (*prgB**) was detected in two isolates. Growth in the presence of pheromones gave rise to a strong clumping reaction rated as +++ (two strains) or ++ (four strains). Plasmid analysis and hybridization experiments demonstrated that all harboured plasmid DNA strongly hybridizing to a probe specific for the *vanA* resistance determinant; *Sma*I restriction analysis of total DNA resulted in different PFGE profiles.

E. faecalis OG1RF strains containing different pheromone plasmids (pCF10, *prgB*; pAD1, *asa1*; pAM373, *asa373*) were used to generate specific probes; fusidic acid- and rifampicin-resistant *E. faecalis* JH2-2, a pheromone-producer, and *E. faecium* 64/3 were used as recipients. Test strains also included four transconjugants obtained in another study using *E. faecalis* HC-VI4 as donor (G. Foglia, M. J. Flores, D. Mater, C. Paoletti, C. Vignaroli, E. Guaglianone, B. Facinelli, G. Cortier and F. Biavasco, unpublished results). Brain–heart infusion (BHI) broth and agar and blood agar base (BAB; Oxoid, Basingstoke, UK) supplemented with 5% defribinated sheep blood were used for routine growth. Isolates were maintained in glycerol at −70°C and subcultured twice in BHI agar before testing.

Conjugation experiments and clumping assay

Conjugal transfer and clumping assays were performed as described previously.[8]

Plasmid analysis and detection of vanA and AS genes

Plasmid analysis and PCR detection of *vanA* were done as described previously.[8] AS genes were detected by using primers internal to highly conserved regions in *asa1* and *prgB* (5′-AAGAAAAAGAAGTAGACCAAC; 3′-AAACGGCAAGAC AAGTAAATA), and to *asa373* (5′-GGACGCACGTACACAAAG CTAC; 3′-CTGGGTGTGATTCCGCTGTTA).[9,10] *Eco*RI digestion of amplicons was performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization experiments were performed using biotin-labelled *prgB*, *asa1* and *asa373* probes and the BrightStar BioDetect kit (Ambion, Huntingdon, UK).

Cell adhesion experiments

Adhesion was studied using Caco-2 cells, a human enterocyte-like cell line, as previously described.[8] Bacterial adhesion efficiency was quantified by determining the ratio of cell-associated cfu to the total cfu of the initial inoculum and was defined as high (>10%), intermediate (1–10%) or low (<1%). Each strain was tested in at least three separate assays on different days, each assay representing the average of triplicate wells.

Statistics

Fisher's exact test was applied with the Fisher test procedure of the S-Plus statistical package (S-PLUS 6.1 for Windows, Professional Edition, Release 1).

Results

Plasmid location of vanA and AS genes in E. faecalis isolates

Plasmid analysis and hybridization experiments performed on the donors demonstrated that they harboured 1–3 plasmids (27–62 kb) carrying *vanA*, AS gene(s) or both (Table 1). *vanA* and AS genes (*prgB*, *prgB**) were located on the same plasmid in HI-AN23, HC-AN21 and F-PM25, and on different plasmids in the remaining strains. In HC-VI4, *prgB* and *asa1* were found on one plasmid, which was different from both the *vanA* plasmid and the *asa373* plasmid. A *vanA* plasmid and two different *prgB* plasmids were detected in HC-AN22.

Co-transfer of vanA and AS genes in intra- and interspecies matings

vanA was successfully transferred from all *E. faecalis* isolates to *E. faecalis* JH2-2 (transfer frequencies of 10^{-3}–10^{-6}), whereas it was transferred to *E. faecium* 64/3 only when isolates from humans were used as donors (transfer frequencies of 10^{-6}–10^{-8}) (Table 1). To test co-transfer of *vanA* and AS genes, 10 *vanA* transconjugants from each mating experiment—for a total of 110 *vanA* transconjugants (60 from intra- and 50 from interspecific matings)—were randomly chosen and tested for the presence of AS genes by PCR and *Eco*RI restriction analysis of PCR products and for clumping activity (Table 1).

AS genes were detected in 71/110 (64.5%) *vanA* transconjugants [in 51/60 (85%) and in 20/50 (40%) of *E. faecalis* JH2-2 and *E. faecium* 64/3, respectively]. A weak (+) clumping reaction was detected in 59/110 (53.6%) *vanA* transconjugants [in 47/60 (78.3%) and in 12/50 (24%) of *E. faecalis* JH2-2 and *E. faecium* 64/3, respectively]. *Eco*RI restriction analysis made it possible to recognize different AS genes: *prgB* was the one most frequently detected in transconjugants, either alone or with *asa1* or *asa373*. *prgB* was detected in 40/51 *E. faecalis* JH2-2 and in 19/20 *E. faecium* 64/3 transconjugants, which were either clumping-positive (36/40 JH2-2 transconjugants and 11/19 *E. faecium* 64/3 transconjugants) or clumping-negative (4/40 *E. faecalis* JH2-2 transconjugants and 8/19 *E. faecium* 64/3 transconjugants). When using *vanA* donors carrying more than one AS gene (HC-VI4 and HC-AN21), transconjugants

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Table 1. Characteristics of *vanA E. faecalis* isolates carrying AS genes, transfer of *vanA* and co-transfer of *vanA* and AS genes in intra- and interspecies matings

Strains (source/year)	AS gene(s)/clumping activity	Plasmid content and location of *vanA* and AS genes	Transfer frequency[a] of *vanA* to: *E. faecalis* JH2-2	Transfer frequency[a] of *vanA* to: *E. faecium* 64/3	Co-transfer of *vanA* and AS genes (no. of transconjugants[b] carrying *vanA* and AS genes/no. of *vanA* transconjugants examined): *E. faecalis* JH2-2 (51/60)	Co-transfer of *vanA* and AS genes (no. of transconjugants[b] carrying *vanA* and AS genes/no. of *vanA* transconjugants examined): *E. faecium* 64/3 (20/50)
HI-AN23 (faeces/2002)	*prgB*/++	58 kb: *vanA* and *prgB* 45 kb	6×10^{-5}	1×10^{-8}	*vanA prgB* (10/10)[c]	0/10
HC-VI4 (blood/1997)	*prgB asa1 asa373*/+++	52 kb: *prgB* and *asa1* 45 kb: *vanA* 42 kb: *asa373*	6×10^{-4}	3×10^{-7}	*vanA prgB asa373* (5/10)[c] *vanA prgB* (4/10)[c,d] *vanA asa373* (1/10)[c]	*vanA prgB* (4/10)[c,d]
HC-AN21 (urine/1998)	*prgB**[e] *asa1*/++	56 kb: *vanA* and *prgB** 45 kb: *asa1*	1×10^{-5}	1×10^{-6}	*vanA asa1* (10/10)[c]	*vanA prgB* (4/10)[c,d] *vanA asa1* (1/10)[c]
HC-AN22 (urine/1998)	*prgB*/++	62 kb: *prgB* 56 kb: *prgB* 49 kb: *vanA*	2×10^{-5}	1×10^{-8}	*vanA prgB* (5/10)	*vanA prgB* (3/10)
HC-UD6 (bile/1997)	*prgB**[e]/++	61 kb 54 kb: *prgB** 27 kb: *vanA*	1×10^{-3}	5×10^{-6}	*vanA prgB** (6/10)	*vanA prgB** (8/10)
F-PM25 (poultry/2000)	*prgB*/+++	50 kb: *vanA* and *prgB*	1×10^{-6}	No *vanA* transconjugants	*vanA prgB* (10/10)[c]	—

[a]Transfer frequencies are expressed as the number of transconjugants per recipient cfu after mating.
[b]Ten transconjugants from each mating experiment were examined by PCR and clumping assays [*E. faecalis* JH2-2 and *E. faecium* 64/3 transconjugants with donor HC-VI4 were obtained in another study (G. Foglia, M. J. Flores, D. Mater, C. Paoletti, C. Vignaroli, E. Guaglianone, B. Facinelli, G. Cortier and F. Biavasco, unpublished results)].
[c]One transconjugant used in adhesion experiments.
[d]Clumping-negative.
[e]*prgB**: the *Eco*RI restriction profiles were similar to that derived from *E. faecalis* OG1RF (pCF10, *prgB*), except for the presence of an additional *Eco*RI site (F. Biavasco, G. Foglia, C. Paoletti, G. Zandri, G. Magi, E. Guaglianone, A. Sundsfjord, C. Pruzzo, G. Donelli and B. Facinelli, unpublished results).
—, no detectable transfer (i.e. a transfer frequency of $<1 \times 10^{-9}$).

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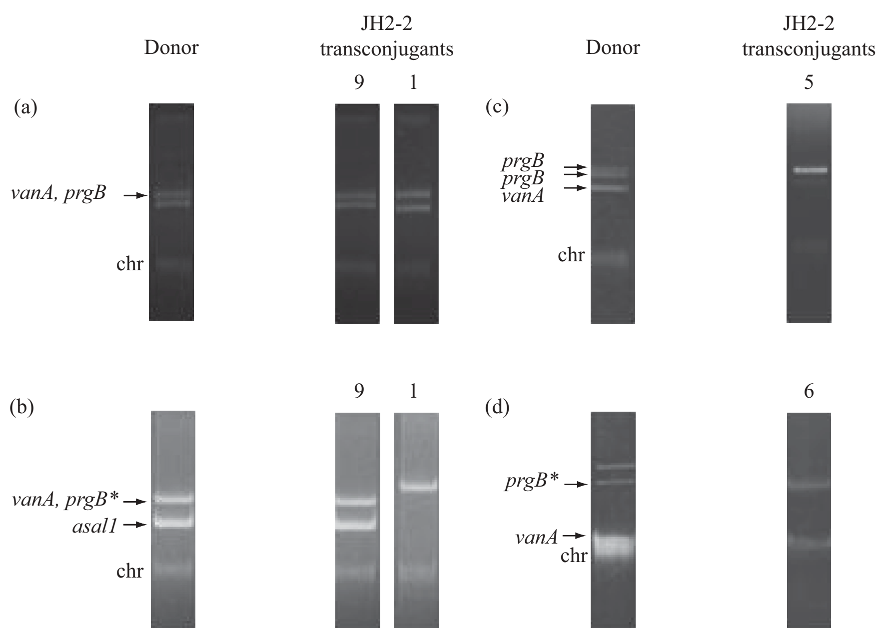

Figure 1. Plasmid profiles of donors and JH2-2 transconjugants carrying *vanA* and AS genes. (a) HI-AN23 donor: ten *vanA prgB* transconjugants; (b) HC-AN21 donor: ten *vanA asa1* transconjugants; (c) HC-AN22 donor: five *vanA prgB* transconjugants; (d) HC-UD6 donor: six *vanA prgB** transconjugants. Arrows indicate the plasmid location of *vanA* and AS genes in donors, as determined by hybridization experiments (not shown); chr, chromosome.

carrying *prgB* or *asa1* or *asa373* or *prgB asa373* were detected, all *prgB* transconjugants being clumping-negative. Moreover, transfer of *prgB** (bearing an additional *Eco*RI site) from HC-AN21 to *E. faecium* 64/3 resulted in transconjugants carrying *prgB* (Table 1).

Plasmid analysis performed on 31 *vanA* and AS gene-carrying JH2-2 transconjugants derived from four donors showed that the plasmid content of transconjugants, derived from the same donor and carrying the same AS gene, was either identical to that of the donor or it was completely different (Figure 1).

Induction with pheromones and adhesion to Caco-2 cells of E. faecalis isolates and transconjugants carrying vanA and AS genes

E. faecalis isolates and nine *vanA* transconjugants (six JH2-2 and three 64/3 carrying *asa1*, *prgB* and/or *asa373*) were tested for their ability to adhere to Caco-2 cells (Table 1).

In the absence of pheromones, adhesion efficiency was high in HI-AN23, low in HC-AN22 and HC-UD6, and intermediate in the remaining isolates, in the recipients and in the transconjugants (the only exception being the JH2-2 transconjugant, derived from HI-AN23, where adhesion efficiency was as high as in the donor).

After induction with JH2-2 pheromones, a significant association between the presence of AS genes and enhanced cell adhesion was noted ($P < 0.01$). Adhesion efficiency was increased in four (66.7%) isolates (HI-AN23, HC-VI4, HC-UD6 and F-PM25) and in three (50%) JH2-2 *prgB* or *asa1* transconjugants (from HI-AN23, HC-AN21 and F-PM25); these proportions are not significantly different ($P = 0.98$). The increase was 2.2- to 6.0-fold in isolates and 1.7- to 6.6-fold in transconjugants Adhesion efficiency remained unaffected in all *E. faecium* 64/3 (the recipient and its transconjugants).

Discussion

Co-transfer of pheromone response and antibiotic resistance has been reported in enterococci.[7,8,11,12] In *E. faecalis*, co-transfer of AS and VanA resistance has been described recently,[7] whereas in *E. faecium* it was demonstrated in 1996.[8,11] In the present study, six *vanA E. faecalis* isolates from human and food samples harbouring one or more AS genes were investigated for their ability to co-transfer *vanA* and AS genes to *E. faecalis* and *E. faecium* recipients. All isolates, independently of their source, were capable of co-transferring *vanA* and AS genes, suggesting that the ability to co-transfer these genes may be a common feature of *E. faecalis*. Since AS promotes colonic mucosal invasion by *E. faecalis*, this feature might contribute to the emergence of strains with enhanced ability to cause infection and disease in humans.

vanA was successfully transferred from all donors to *E. faecalis* JH2-2, albeit with different transfer frequencies, while it was transferred to *E. faecium* 64/3 only when isolates from humans were used as donors, and then at lower frequencies. About two-thirds of *vanA* transconjugants acquired one or two AS genes, co-transfer being more frequent in intra- than interspecies matings; when present, clumping was less evident in transconjugants. *prgB* was co-transferred more frequently in intraspecific than in interspecific matings and, when transferred, it was not always expressed, *asa1* was transferred alone in both intra- and interspecies matings and was always expressed, whereas *asa373* was co-transferred either alone or with *prgB* only in intraspecies matings. When using donor HC-AN21 carrying *prgB**, a reversion to *prgB* was detected in

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transconjugants, suggesting that the donor could have multiple *prgB* plasmids with polymorphic sequences.

After induction with pheromones, a marked increase in adhesion to Caco-2 cells was observed in intestinal, blood, bile and food *vanA* isolates and in half of *E. faecalis* transconjugants, all clumping-positive. Adhesion was not increased in *E. faecium* transconjugants, irrespective of clumping ability. These results demonstrated a significant association between the presence of AS genes and enhanced cell adhesion in *E. faecalis*; no significant differences were noted between donors and transconjugants.

Overall, our results demonstrated that co-transfer of *vanA* and AS genes is a fairly complicated process. In some *E. faecalis* isolates, *vanA* and AS genes were located on the same pheromone plasmid, whereas in others they were detected on different plasmids; moreover, two different AS genes could be located on the same plasmid, or the same AS gene could be found on two different plasmids. In addition, transconjugants derived from the same donor and carrying identical AS genes displayed different profiles that were also different from that of the donor. Data suggest that *vanA* and AS genes were co-transferred via different mechanisms: (i) location on the same pheromone plasmid; (ii) mobilization of *vanA* plasmids and (iii) rearrangement of the plasmid content during matings. In this regard, mobilization of plasmids by the pheromone-inducible conjugation system has recently been reported,[13] and there is strong evidence for the importance of co-integrate formation between multiple plasmids in the transfer of vancomycin resistance.[1]

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Transparency declarations

None to declare.

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