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Transfer of Antibiotic Resistance Genes in *Enterococcus faecium* of Animal and Human Origin and the Significance of Meat Products*

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Introduction

By the end of the 1960ties transferable antibiotic resistance had been detected and analyzed. When plasmid encoded oxytetracycline resistance was found in a zoonotic pathogen, namely *S. typhimurium*, a large reservoir of transferable antibiotic resistance had been identified in animal husbandry. This led to the recommendation of the Swann Committee to the British Government that antibiotics which are also used in human chemotherapy, or which select for cross resistance against them, should not be used as feed additives in animal husbandry (1). Later this criterion was also established for licensing antibacterials as growth promoters in EU countries (2). At least in these countries broad spectrum antibiotics were no longer used as growth promoters, the majority of substances lincensed for this purpose in the 1970ties have a Gram-positive activity spectrum (table 1). At this time acquired and transferable resistance against most of these substances was unknown. Although there was still an ungoing debate whether reservoirs of transferable resistance genes in food animals and in humans communicate, the use of the antibacterials listed in table 1 was not questioned.

This situation changed, however, with the emergence and spread of transferable glycopeptide resistance in enterococci (for summary see (3)).

The pioneering work was performed by *S. Levy* and coworkers on dissemination of plasmid encoded oxytetracycline resistance in *E. coli* demonstrating spread from farm animals to humans working and living on farms (4, 5). At this time, however, oxytetracycline resistance was already very frequent in coliforms in humans as well as other animals, and there was also a substantial use for human chemotherapy.

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Table 1
Antibacterials used as growth promoters in EU countries

Substance	*Enteric resorption*	*Type of anti-bacterial activity*	*Activity spectrum*	*Mechanism of activity*	*Cross resistance to other antibacterial substances*
Quinoxalines[1] (carbadox, olaquindox)	+	bactericidal	Gram+, gram–	inhibition of DNA synthesis	not demonstrated
Glycopeptides[2] (avoparcin)	–	bacteriostatic	Gram+	inhibition of peptidoglycan biosynthesis	Vancomycin, Teicoplanin
Ionophores (monensin, salinomycin)	±	bactericidal	Gram+	disorder of zytoplasmic membrane	not demonstrated
Macrolides[3] (tylosin, spiramycin)	±	bacteriostatic	Gram+	inhibition of protein synthesis	Macrolides/Lincosamidines
Phosphoglycolipids (flavomycin)	–	bacteriostatic	Gram+	inhibition of cell wall synthesis	not demonstrated
Streptogramins[3] (virginiamycin)	–	bacteriostatic	Gram+	inhibition of protein synthesis	other streptogramines, macrolides, lincosamidines
Polypeptides[3] (Zn-bacitracin)	–	bactericidal	Gram+	inhibition of peptidoglycan biosynthesis	not demonstrated
Oligosaccharides (avilamycin)	–	bactericidal	Gram+	inhibition of protein biosynthesis	Everninomycin

[1] No longer used by the summer of 1999 for toxicological reasons (workers health protection).
[2] Use discontinued in all EU-countries by April 1997.
[3] Use discontinued in all EU-countries by January 1999.

Once a resistance gene has already become widely disseminated among different ecosystems, it is always difficult to trace it back to its origin. This is only possible by genetic labelling of a particular resistance gene and following its further ways or by prospective studies after the introduction of an antibiotic into use in only one field of application as e. g. animal feeding.

In the following, studies on selection and spread of resistance genes from enterococci in the bacterial flora of meat animals selected by use of growth promoters to the bacterial flora of humans will be reviewed.

Glycopeptide resistance in enterococci

In enterococci there are four different genotypes of acquired glycopeptide resistance (6–8) from which the *vanA* genotype is most frequent in Central Europe (9).

The mechanism of glycopeptide resistance mediated by the *vanA* gene cluster is based on alteration of the target for glycopeptides (formation of a depsipeptide instead of the D-ala-D-ala group of N-acetylmuraminic acid) (7). The gene cluster is located on transposons of the Tn*1546* type (6), which are integrated into conjugative plasmids (fig. 1).

The detection of glycopeptide resistant *Enterococcus faecium* (GREF) possessing *vanA* in waste water treatment plants of small towns in Germany which had no hospital was the first indication to a reservoir outside hospitals (10). The assumption that the use of the glycopeptide avoparcin as feed additive had selected for GREF in animal husbandry was confirmed by demonstration of GREF in animal faeces (pigs and chicken) from farms using avoparcin, however not or only rarely in those which do not (11, 12).

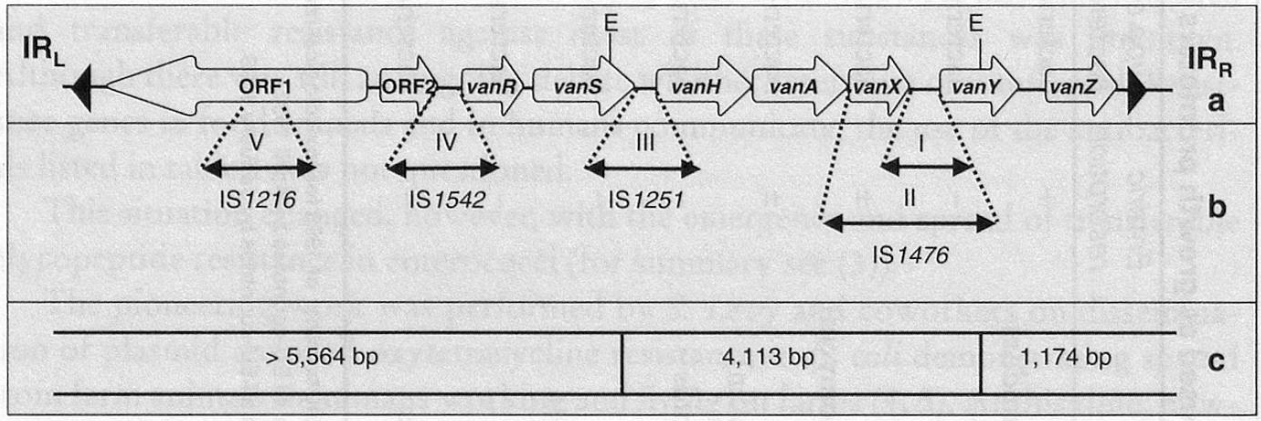

Figure 1 **Structure of transposon Tn 1546 carrying the *vanA* gene cluster**
a Gene cluster of Tn *1546* prototype
b Insertions in different polymorphes
c size measure

All GREF isolated in our studies were of the *vanA* genotype with the *vanA* gene cluster mostly located on conjugative plasmids (13). How GREF with their transferable resistance genes reach humans? Different ways are imaginable: direct contact with farm personnel (for a study on this issue see (14)), via waste and surface water and with meat products. Although the hygienic standards of meat production are fairly high in most developed countries, a faecal contamination of meat products can not completely be avoided.

GREF have been detected in the thawing liquid of poultry carcasses offered in supermarkets and also in samples of raw minced pork (15, 16). It is a custom in central Europe to eat raw minced pork spiced with pepper, salt and onion. Although their is no consumption of raw poultry, bacteria carried on these products can contaminate other food as we especially know from transmission of enteric *Salmonellae.*

If contaminated meat products are of significance for transfer of GREF to humans, these germs should be found as colonizers of the intestinal flora of non hospitalized humans. This assumption was confirmed by a study in an rural area of Germany where a hospital origin was rather unlikely (table 2). Carriage of GREF by humans in the community was further reported from other European countries (17, 18). However, GREF have not been detected in faecal samples from strong vegetarians (19).

Due to creation of a large reservoir of GREF in humans in the community they can reach hospitals by patient admission and also more directly with meat products.

The data reviewed indicate the way of dissemination from meat animals to humans in general. For tracing of spread of transferable resistance the following questions had to be answered:

- Is there a clonal spread of particular GREF strains among animals and humans?
- Does glycopeptide resistance spread by transfer of the *vanA* gene cluster among different *E. faecium* strains?
- Is the structural configuration of the *vanA* gene cluster uniform in GREF from human and from animal sources or are there different "types" in enterococcal populations of humans and of meat animals?

Results obtained until now have led to the following conclusions: a variety of different *Sma*I-macrorestriction as well as multilocus electrophoreses patterns demonstrate the polyclonal nature of GREF and suggest a frequent dissimination of the *vanA* gene cluster among different strains (11). According to these data there are probably no host specific ecovars in *E. faecium*. When a GREF strain of porcine origin was ingested by a human volunteer, it persisted over several months (20). Plasmids from GREF of different ecological origin exhibit rather different restriction endonuclease cleavage patterns; this suggests a frequent transposition of the *vanA* gene cluster among different plasmids.

Different structural types of the *vanA* gene cluster resulting from integration of insertion sequences into noncoding regions or from deletions concerning genes for

Table 2
Occurrence of glycopeptide-resistant *E. faecium* (*vanA* mediated) outside hospitals in animals, in meat products and in faecal samples of non-hospitalized humans in a provincial district of Germany

Hospitals	*Community*	*Food*	*Animal husbandry*
District's two hospitals (A, B) each with ~ 700 beds and 12000–15000 in-patients per year[a]	100 non-hospitalized humans (inhabitants)	Samples of raw minced meat	Large pig farm with ergotropic use of avoparcin
– no glycopeptide-resistant enterococci isolated from infections in humans from 1990 until now	– 12 faecal samples positive for glycopeptide resistant *E. faecium* (*vanA* mediated)	– 5 positive for glycopeptide-resistant *E. faecium* (*vanA* mediated)	– Glycopeptide-resistant *E. faecium* (*vanA* mediated) frequent in manure
~ 300 enterococcal strains assayed per year from hospitalized patients			11 small individual holdings of pigs in villages, no avoparcin use – No glycopeptide-resistant *E. faecium* in faecal samples

[a] Therapeutic use of glycopeptides in 1994: hospital A, 60 daily doses vancomycin (VM), 40 daily doses teicoplanin (TE), no oral applications; hospital B, 42 daily doses VM, 125 daily doses TE, no oral applications (for both hospitals this corresponds on average to 26 patients treated).
(For details, see 13)

transposition have been described (21). If the animal and the human reservoir of GREF communicate, the same structural types should be demonstrated. Until now different molecular methods have been applied to molecular typing of the *vanA* gene cluster in different countries (see table 3). Besides a wide spread of Tn*1546* like elements in their original configuration (13) there are different types which are characteristic for GREF from chicken and pigs. In humans, however, "animal specific" types of the *vanA* gene cluster have been found which furthermore demonstrates a dissemination from the animal reservoir (table 3).

Streptogramin resistance in *E. faecium*

The semisynthetic streptogramin combination quinupristin/dalfopristin (Q/D) is one of the few antibiotics of last resort for a treatment of infections due to multiply resistant Gram-positive bacteria, including multi- and vancomycin-resistant enterococci (25). Q/D is a derivative of pristinamycin which, as every streptogramin antibiotic, is a combination of two chemically unrelated compounds, B and A. The combination of both acts synergistically when given in the 30:70 ratio as produced by *Streptomyces pristinaespirales* (26). A combination of streptogramins A and B overcomes resistance to B compounds, but is inactive in case of resistance to A compounds (27). However, recent studies have indicated that a combination of resistance mechanisms to streptogramins A and B is essential for conferring resistance to Q/D in *Enterococcus faecium* (28). Q/D has been released recently for a clinical use in the USA and the UK and is prior to licensing in other EU countries.

Resistance to streptogramins in enterococci of human origin is still rare (29). It is mediated by two acetyltransferases SatA and SatG (27, 30). Resistance to B compounds is mediated via the *ermB* gene encoded 23S r-RNA methylase and in 2 isolates demonstrated to be *vgb* related (31).

Besides a very limited use of the older streptogramins in humans in France and of Q/D in a few clinical trials in Central Europe, virginiamycin has been used to a large extent for growth promotion in animal husbandry in Northern America and in Europe since the early 1970ties (32). Recent studies have indicated a considerable reservoir of streptogramin resistant enterococci resulting from an agricultural use of virginiamycin. Figure 2 shows structural formulas of virginiamycin and of quinupristin/dalfopristin. Further investigation performed in the US (33), in Denmark (32), and in Germany (34) have shown that the same resistance genes, namely *satA* and *satG* have been found in *E. faecium* of animal and human origin (35). The results of the German study are summarized in table 4, quinupristin/dalfopristin resistant *E. faecium* (QDRE) have not been detected in farm animals not fed with antibiotics (35).

QDRE quite frequently contaminated meat products and most probably as a consequence of this were also found in the intestinal tracts of non hospitalized humans in the community of a rural area in Germany. As it has already been reported for GREF, also QDRE were polyclonal as deduced from macrorestriction

Table 3
Molecular typing of the *vanA* gene cluster of *Enterococcus faecium* from human and animal sources

Origin	*Methodology applied*	*Results*	*References*
Germany	overlapping PCR	majority of isolates from humans, poultry and pig uniform	(13)
Denmark	overlapping PCR, sequencing of *vanX*	Type 1: humans, chicken, turkey Type 2: humans, pigs Type 3: humans Type 4: humans Types 5–16: rare types of various sources	(21)
Great Britain	overlapping PCR (ten primer pairs)	24 different types Type A: 34% of non human sources Type H: 32% of human, 5% non human Types T, U, W: 4–10% from human and non human sources	(22)
Norway	RFLP of long PCR amplimers	uniform for isolates from humans and from chickens	(23)
Netherlands	overlapping PCR	*vanX-vanY:* 1300 bp: poultry (42%) 543 bp: poultry (58%) 543 bp: humans (100%)	(24)

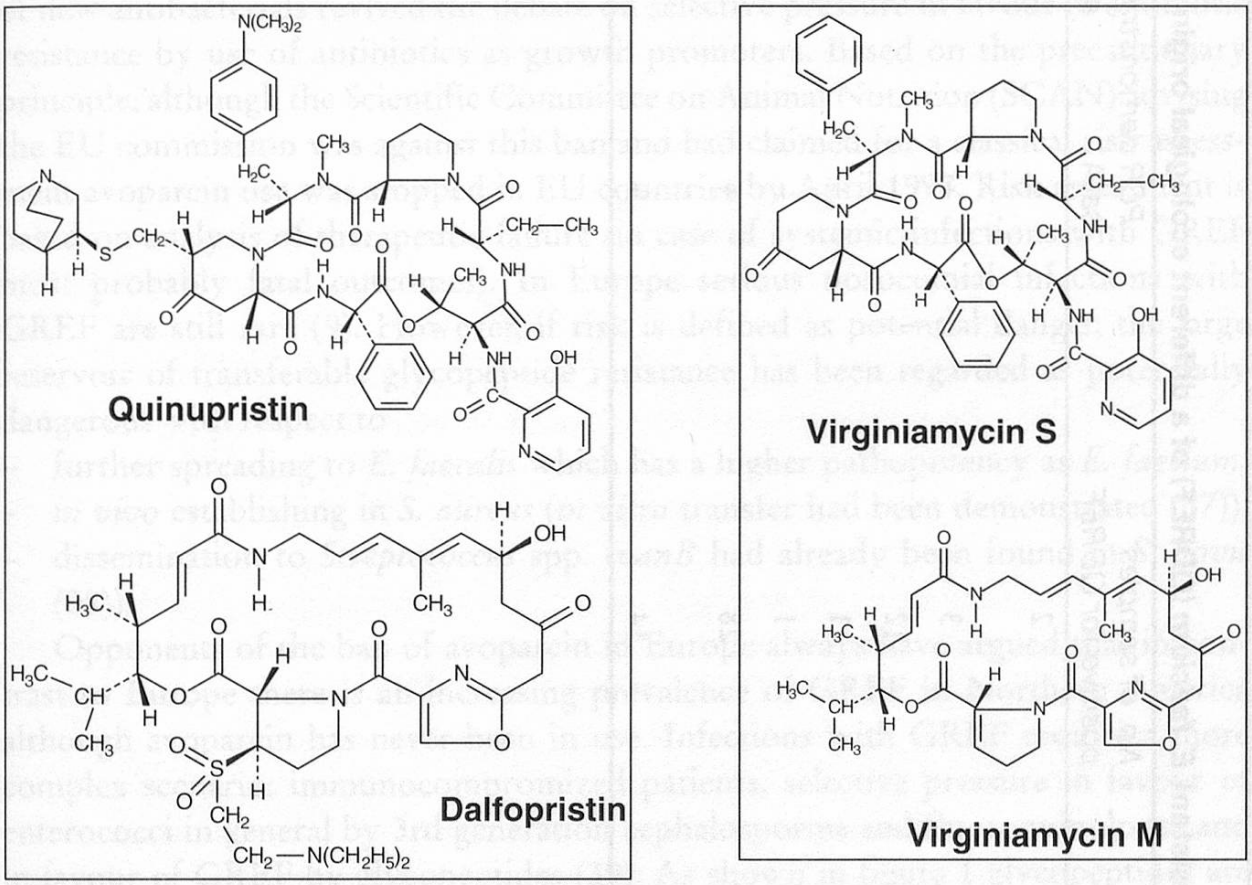

Figure 2 **Structure formulas of quinupristin/dalfopristin (S_B/S_A) and virginiamycin S and M**

analysis of genomic DNA. Also plasmids carrying *satG* exhibited different *EcoR*I cleavage pattern (35).

As already seen for the *vanA* gene cluster, spread of *satA* and *satG* is more likely due to the spread of the resistance genes than to clonal dissemination of particular resistant strains or of particular plasmids. The nature of transferable elements into which *satA* and *satG* are most probably integrated is subject to further investigation.

Conclusions

The studies performed on oxytetracycline and streptothricin resistance in *E. coli* and on glycopeptide and streptogramin resistance in enterococci clearly reveal that reservoirs of antibiotic resistance genes in bacterial flora of different ecosystems communicate. As commensals of the digestive tract both species obviously have a significance for acquisition and spread of resistance genes as probably other bacteria of this ecosystem like lactococci (36). The role *E. faecium* as a pathogen in immunocompromised patients, the resistance development in this species (especially

Table 4
Streptogramin resistance genes in Quinupristin/Dalfopristin resistant *E. faecium* (QDREF) of a different ecological origin (37)

Origin	*No. of samples*	*No. of samples positive for QDREF*	*PCR demonstration satA*	*satG*
sewage	from 2 municipal sewage treatment plants	2	+	+
chicken, manure	3 from 2 farms	3	+	+
pigs	12 from 6 farms	12	–	+
poultry carcasses	24	11	+	+
minced pork	10	1	–	+
stool samples, nonhospitalized humans	200	28	+	+
hospitalized patients	190	14	+	+

transferable glycopeptide resistance an antibiotic of last resort) and the shortcome of new antibacterials revived the debate on selective pressure in favour of antibiotic resistance by use of antibiotics as growth promoters. Based on the precautionary principle, although the Scientific Committee on Animal Nutrition (SCAN) advising the EU commission was against this ban and had claimed for a classical risk assessment avoparcin use was stopped in EU countries by April 1997. Risk assessment is based on analysis of therapeutic failure (in case of systemic infections with GREF most probably fatal outcomes). In Europe serious nosocomial infections with GREF are still rare (9). However, if risk is defined as potential danger, the large reservoir of transferable glycopeptide resistance has been regarded as potentially dangerous with respect to

- further spreading to *E. faecalis* which has a higher pathopotency as *E. faecium,*
- *in vivo* establishing in *S. aureus* (*in vitro* transfer had been demonstrated (37)),
- dissemination to *Streptococcus* spp. (*vanB* had already been found in *S. bovis* (38)).

Opponents of the ban of avoparcin in Europe always have argued that in contrast to Europe there is an increasing prevalence of GREF in Northern America although avoparcin has never been in use. Infections with GREF require a more complex scenario: immunocompromized patients, selective pressure in favour of enterococci in general by 3rd generation cephalosporins and fluoroquinolones and in favour of GREF by glycopeptides (39). As shown in figure 1 glycopeptides are used in human chemotherapy units to larger extent in the US than in Europe (40).

Streptogramin resistance in *E. faecium* and in particular in GREF of hospital origin observed in Germany and earlier described in the US (33, 34) has not been selected by streptogramin use in humans, the origin in animal husbandry is rather likely. As already seen with streptothricin resistance in *E. coli,* streptogramin resistance genes were obviously disseminated in the absence of direct selective pressure (food chain). The example of virginiamycin also shows that an antibacterial used for animal feeding becomes risky when related compounds are introduced into human chemotherapy.

The condensing evidence described above and the conclusions of a scientific committee of the EU council (41) have led to the stop of feeding virginiamycin, tylosin, spiramycin and Zn-bacitracin by January 1999. Will this reduction of selective pressure lead to a decreased reservoir of resistance genes? First observations are encouraging.

In Germany the carrier rate of GREF in the community declined after the ban of avoparcin in 1996 (1994: 12 %, by end of 1996: 6.0 %, 1998: 3,3 % (42)). Also the rate of contamination of poultry carcasses declined as observed in parallel in Italy 18 months after stop of avoparcin (43). In Denmark a considerable reduction of GREF in chicken broilers was observed, however, not in pigs (44). The latter had been explained by the continuing use of tylosin since *ermB* is nearly always located on the same conjugative plasmids as the *vanA* gene cluster. After stop of tylosin in

Denmark, also the frequency of GREF in pigs has been observed. The observations on avilamycin in Denmark have shown that it's use had increased from 10 kg in 1990 to 2,740 kg in 1996 and afterwards decreased to 7 kg in 1998. The frequencies of avilamycin resistance in *E. faecium* paralleled these figures: 64 % in 1995, 81 % in 1997 and 23.3 % by the end of 1998 (45).

The transfer of resistant bacteria is not restricted to a particular country or continental league with common regulations as EU. Trade of meat products and dissemination of bacteria with transferable resistance genes is world wide as recently reported from Japan. This country imports 600 000 tons of chickens a year, it was demonstrated that chicken produced in France and in Thailand were contaminated with GREF (46). Prevention of further spreading of antibiotic resistance from the bacterial flora of food animal via meat products requires global regulations.

Summary

Selective pressure by use of avoparcin and virginiamycin as growth promoters in animal husbandry has created a considerable reservoir of transferable, plasmid determined resistance against glycopeptides (*vanA* gene cluster) and to streptogramins (*vatD, vatE, ermB*) in *Enterococcus faecium* from meat animals. Molecular typing of resistant *E. faecium* isolates and characterization of the resistance genes have revealed that spread of these resistances is mainly due to dissemination of the resistance genes among a variety of different plasmids and different strains. Humans acquire resistant enterococci from meat animals by contamined meat products.

Zusammenfassung

Der Selektionsdruck durch den früher erfolgten Einsatz von Avoparcin und von Virginiamycin als Leistungsförderer in der Tiermast hat bei *Enterococcus faecium* von Masttieren ein beträchtliches Reservoir der übertragbaren, plasmiddeterminierten Resistenz gegen Glykopeptide (*vanA*-Gencluster) und gegen Streptogramine (*vatD, vatE, ermB*) geschaffen. Die molekulare Typisierung der resistenten Stämme und Charakterisierung der Resistenzgene haben gezeigt, dass die Verbreitung der Resistenzen hauptsächlich durch die Ausbreitung der Resistenzgene zwischen einer Vielzahl unterschiedlicher Plasmide und Stämme erfolgt. Zum Menschen gelangen die resistenten *E. faecium* über kontaminierte Fleischprodukte.

Résumé

L'avoparcine et la virginiamycine ont été largement utilisés par le passé dans l'engraissement des animaux de boucherie. Il en résulta une émergence chez *Enterococcus faecium* de souches résistantes aux glycopeptides (cluster des gènes *VanA*) et aux streptogramines (*VatD, VatE, ermB*). Ces résistances sont transférables et, comme l'a révélé la biologie moléculaire, codées dans une large variété de plasmides. La progression de ces résistances résulte principalement de la dissémination des gènes responsables dans les dits plasmides et partant, dans différentes souches de

l'espèce *E. faecium*. La transmission aux humains de ces entérocoques se fait par le biais de produits carnés contaminés.

Key words

Transferable resistance, Glycopeptides, Streptogramins, Enterococci

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