Movable Genetic Elements and Antibiotic Resistance in Enterococci

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The enterococci possess genetic elements able to move from one strain to another via conjugation. Certain enterococcal plasmids exhibit a broad host range among gram-positive bacteria, but only when matings are performed on solid surfaces. Other plasmids are more specific to enterococci, transfer efficiently in broth, and encode a response to recipient-produced sex pheromones. Transmissible non-plasmid elements, the conjugative transposons, are widespread among the enterococci and determine their own fertility properties. Drug resistance, hemolysin, and bacteriocin determinants are commonly found on the various transmissible enterococcal elements. Examples of the different systems are discussed in this review.

The association of antibiotic resistance with plasmid content in enterococci was first noted by Courvalin et al. (1) in 1972 in a strain of *Enterococcus* (formerly *Streptococcus*) *faecalis* (BM6201), and reports showing that erythromycin (Em) and tetracycline (Tc) resistance were encoded by two different plasmids (designated pIP613 and pIP614, respectively) in this strain soon followed (2). In 1974 Jacob and Hobbs (3) reported that plasmid-encoded resistance determinants in *Enterococcus faecalis* JH1 could be transferred by conjugation in broth matings. In this case, resistance to erythromycin, tetracycline, kanamycin (Km) and neomycin was encoded on a plasmid designated pJH1, and a co-resident plasmid, pJH2, was found to determine hemolysin and bacteriocin activity. About the same time, Tomura et al. (4) reported a relatively high rate of conjugal transfer of an *Enterococcus faecalis* hemolysin/bacteriocin trait; however, a physical demonstration of plasmid involvement was not conducted. At the time, conjugative transfer was a rather novel phenomenon in gram-positive bacteria; with the exception of a single unconfirmed report nine years earlier in *Enterococcus faecalis* (5), the only gram-positive organisms in which conjugation was known to occur were the strepto-

mycetes. Today, of course, numerous plasmids, both conjugative and nonconjugative, have been identified in enterococci and characterized to various degrees (6, 7, 8). Some nonconjugative plasmids such as pAMα1 can be mobilized at high frequency by certain conjugative plasmids (9, 10), whereas other nonconjugative plasmids such as pAD2 are mobilized very inefficiently (11, 12, 13). Chromosomal determinants were also found to be mobilized at low frequencies (14, 15).

Enterococci also commonly harbor conjugative transposons, elements able to transfer in the absence of a mobilizing plasmid (14, 16). The prototype of such an element is the Tc-resistance transposon Tn*916*, originally identified in *Enterococcus faecalis* DS16 (14). Its discovery paralleled studies of a transferable, nonplasmid, multiple-resistance element in *Streptococcus pneumoniae* (17).

Conjugative Plasmids

There are two basic types of conjugative transfer in enterococci: i) that which occurs in broth and at relatively high frequencies, and ii) that which occurs only on solid surfaces (e. g. a filter membrane) and at frequencies usually less than 10^{-3}. Thus far, plasmids which transfer in broth have been found to encode a response to recipient-produced sex pheromones, whereas surface-requiring matings do not appear to involve these compounds (6, 8). In the case of conjugative transposition, transfer generally occurs only on surfaces.

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Multiple antibiotic resistance is common among the enterococci, especially among *Enterococcus faecalis*, and plasmids are ubiquitous in these organisms. After Jacob and Hobbs (3) reported conjugative resistance transfer, additional reports by others soon followed (3, 9, 11–26). Many examples have involved donor strains also containing a highly transferable hemolysin plasmid (3, 9, 11, 20, 26–33), and the extent to which the latter facilitated the mobilization of unlinked resistance determinants has not always been clear. There has been some effort to place resistance plasmids into different incompatibility groups (24, 34).

Clinical isolates shown to produce or respond to sex pheromones are much more likely to be resistant to antibiotics than strains that do not exhibit pheromone phenomena (35). Strains bearing hemolysin plasmids, which usually encode a pheromone response (see below), also tend to be resistant to multiple antibiotics, but it is rare for the hemolysin and resistance traits to be linked (26, 36). Hemolysin plasmids have probably played a significant role in the mobilization of resistance determinants located on other elements.

The plasmid pAMβ1, originally identified in *Enterococcus faecalis* DS5, has a very broad host range and transfers on solid surfaces to most gram-positive species that have been tested (6, 7). This 26 kb plasmid encodes resistance to erythromycin, although the single *erm* determinant encodes the so-called MLS phenotype, indicating resistance to macrolides, lincosamides and streptogramin B. The MLS phenotype is common for many *erm* determinants among gram-positive cocci (7, 37). The plasmid has been characterized by LeBlanc and Lee (38), who have mapped positions of *erm*, a transfer-related region, and a replication region. It was also found that certain regions tend to delete specifically, when the plasmid is introduced into *Streptococcus sanguis* (39) or *Bacillus subtilis* (40).

The *erm* determinant, which has recently been sequenced (41) and which expresses in *Escherichia coli*, has been utilized in the construction of various *Escherichia coli*-streptococcal/enterococcal shuttle vectors such as pVA838 (42, 43). Recently, Trieu-Cuot et al. (44, 45) have constructed shuttle plasmids utilizing components of pAMβ1, pBR322 and RK2, and have observed conjugative transfer from *Escherichia coli* to several species of gram-positive bacteria and from *Enterococcus faecalis* to *Escherichia coli*.

Other *Enterococcus faecalis* plasmids, such as pAM81 and pAM490 (46), also exhibit a broad host range among gram-positive organisms and probably have much in common with a number of plasmids that have been identified in various streptococcal species (7). These plasmids tend to be similar in size and usually determine erythromycin resistance; they sometimes encode additional resistances. One such plasmid, pIP-501 (47), originally from *Streptococcus agalactiae*, is easily acquired by enterococci and has been significantly characterized (48, 49, 50).

Plasmids that Respond to Sex Pheromones

Over one-third of clinical isolates of *Enterococcus faecalis* exhibit a clumping response when exposed to culture filtrates of plasmid-free strains (35, 36). The clumping-inducing agent was shown to correspond to a sex pheromone that induced a mating response by cells harboring certain conjugative plasmids (35, 51). Thus, when a plasmid-bearing donor culture is mixed in broth with potential recipient cells, the "clumping" that results represents the formation of large mating aggregates. The induction requires 30 to 40 minutes, and results in the synthesis of several surface proteins collectively referred to as "aggregation substance" (30, 52, 53). A wall component referred to as "binding substance", to which aggregation substance is believed to bind, is present on the surface of both donors and recipients (35).

Once the recipient acquires a copy of the plasmid it shuts down the synthesis of the related sex pheromone but continues to synthesize other pheromones that are specific for donors with different plasmids (35). A plasmid-free strain produces at least five different sex pheromones, and it is likely that many more are actually excreted (8). It is not surprising, then, that single strains have been found to harbor multiple pheromone-responding plasmids (10, 54). If a strain harboring more than one plasmid is exposed to a pheromone specific to only one of the plasmids, then only the related plasmid is induced to transfer (53). Table 1 lists the known pheromone-responding plasmids along with some of their properties. It is noted that *Enterococcus faecalis* strains DS5 and HH22 each harbor three different pheromone-responding plasmids.

Induction results in the appearance of immunologically unique surface proteins with sizes ranging from 52,000 to 190,000 daltons (Da) (52–55; K. E. Weaver and D. B. Clewell, unpublished data). In the case of pAD1, these proteins are referred to as AD52, AD74, AD130, AD153 and AD157; the dominant protein is AD74. Although the designations were originally intended to reflect the relative sizes, recent data have

Table 1: Plasmids known to encode a pheromone response.

Plasmid	Size (kb)	Original host	Phenotype encoded[a]	Related pheromone	Related reference(s)
pAD1	60	DS16	Hly/Bac, Uv	cAD1	(11, 13, 35)
pPD1	56	39-5	Bac	cPD1	(30, 51)
pAM373	36	RC73	?	cAM373	(73)
pCF10	54	SF-7	Tc^r(Tn925)	cCF10	(71)
pAMγ1	60	DS5	Hly/Bac, Uv	cAD1	(10)
pAMγ2	~60	DS5	Bac	cAMγ2	(10)
pAMγ3	~60	DS5	?	cAMγ3	(10)
pOB1	71	5952	Hly/Bac	cOB1	(6, 28)
pJH2	59	JH1	Hly/Bac	cAD1	(6, 27)
pBEM10	70	HH22	Pen^r, Gm^r, Km^r, Tm^r	cAD1	(54)
pAM323	66	HH22	Em^r	cAM323	(54)
pAM324	53	HH22	?	cAM324	(54)

[a]Hly, hemolysin; Bac, bacteriocin; Uv, ultraviolet light resistance; Tc^r, tetracycline resistance; Pen^r, penicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tm^r, tobramycin resistance; Em^r, erythromycin resistance.

Table 2: Structures of sex pheromones and some related inhibitors.

Pheromone or inhibitor (molecular weight)	Peptide structure	Reference
cPD1 (912)	H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH	(59)
cAD1 (818)	H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH	(60)
cAM373 (733)	H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OH	(61)
cCF10 (789)	H-Leu-Val-Thr-Leu-Val-Phe-Val-OH	(62)
iPD1 (828)	H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser-OH	(63)
iAD1 (846)	H-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-OH	(64)

shown that AD153 and AD157 are larger than originally thought; they are closer to 170,000 and 190,000 Da, respectively (K. E. Weaver and D. B. Clewell, unpublished data). In the case of pPD1, the dominant protein is designated PD78, and is probably analogous to pAD1's AD74. A similar analogy may occur with the SA73 protein of pCF10 (52).

Wirth and colleagues (56, 57) isolated AD74 and raised polyclonal antibodies against it. The antiserum was used in immunogold labeling experiments to observe by electron microscopy a dense layer of "hairs" on the surface of induced cells. The distribution of hairs, after a relatively short induction time (< 40 min), was uneven and appeared to occur only on "old wall". That is, wall synthesized during exposure to pheromone (cAD1) appeared not to give rise to aggregation substance. The purpose of the uneven distribution is not clear, but it might serve as a basis for dissociation of donor and recipient cells after mating. That is, absence of hairs on newly synthesized wall would mean that cell division within a mating aggregate could lead to cells that could escape.

I. Nakayama et al. (to be published) have also purified and characterized AD74 as well as PD-78. Antiserum raised against PD78 was able to block aggregation of induced pPD1-containing cells. They also performed amino-terminal analyses of both proteins and found them to be significantly different. This is consistent with observations showing that the two proteins were immunologically distinct (53).

Aggregation of induced cells requires the presence of divalent cations such as Mg^{++}, as well as phosphate ions (30). There is evidence that binding substance probably has a lipoteichoic acid component, since low concentrations (0.1 to 1.0 μg/ml) of lipoteichoic acid were able to block aggregation (53). In this regard it is noteworthy that lipoteichoic acid corresponds to the Lancefield group D antigen of *Enterococcus faecalis* (58).

Table 2 lists the structures of four sex pheromones (cPD1, cAD1, cAM373 and cCF10) that have been characterized by Suzuki and collaborators (59–62). They are all very hydrophobic octa- or hepta-peptides, and each contains one

hydroxyamino acid residue, which conceivably could be modified as part of the plasmid-determined shutdown process. In addition, the structure of two inhibitor peptides, iPD1 and iAD1 (63, 64), is shown. The latter are competitive inhibitors that are excreted by donors containing pPD1 or pAD1, respectively. The role of the inhibitors is not clear; however, they probably serve to prevent self-induction by low levels of endogenous pheromone or by different pheromones with low levels of cross-reacting activity for the plasmid-specific pheromone receptor site. The iAD1 peptide is 50 % homologous with its corresponding pheromone, cAD1, whereas iPD1 and cPD1 are only 25 % homologous. Interestingly, iPD1 and iAD1 have three adjacent residues in common, and the heptapeptide cCF10 has four adjacent residues in common with iAD1.

Synthetic sex pheromone and inhibitor peptides were found to have full activity and exhibited strong specificity for the related plasmid system. The pheromones are active at concentrations as low as 5×10^{-11} M (59–62), and studies with c-CF10 (62) showed that a donor cell may sense as few as one or two molecules. Synthetic hybrids corresponding to cPD1 on the amino-terminal half and cAD1 on the carboxy-terminal half exhibited 10 % of the activity of cPD1 but had no cAD1 activity (65). The reverse was true for the converse experiment, with 1 % of the activity of cAD1 and no cPD1 activity. The specificity of the peptide is therefore determined by the amino-terminal sequences.

There is evidence that the structural determinant for iAD1 is located on pAD1, in that when the plasmid was introduced into a strain not exhibiting a cAD1 pheromone system (e. g. *Enterococcus faecium* 9790), iAD1 was excreted (66). In addition, recent studies (D. B. Clewell, F. Y. An and L. Pontius; to be published) have revealed the nucleotide sequence of iAD1 on pAD1. The genomic sequence shows what is probably a 22 amino acid precursor with the final eight residues at the carboxy-terminus corresponding to iAD1. Efforts to clone pheromone genes from the *Enterococcus faecalis* chromosome are ongoing but have thus far been difficult due to hybridization of probes to multiple regions.

Genetic analyses have been conducted on pAD1 and pCF10 using transposon mutagenesis. Youngman (67) has developed several temperature-sensitive plasmid-transposon (Tn*917*) delivery systems which have been particularly useful. It has been possible to generate mutants defective in various aspects of the mating process (55, 68, 69). In the case of pAD1 (Figure 1), mutants have been obtained that are defective in the ability to aggregate (region F), and in these

cases production of certain surface proteins is usually affected. Variants of this type usually transfer plasmid DNA if matings are performed on filter membranes. Other mutants are defective in the ability to transfer DNA (region G and H), but are normal with regard to aggregation. In addition, there are mutants that neither aggregate nor transfer (region E); these appear to involve a positive regulator necessary for expression of multiple transfer determinants. Tn*917-lac* transcriptional fusions within this area of pAD1 result in derivatives that can be induced to produce beta galactosidase upon exposure to cAD1 (55, 70).

There are also at least two determinants involved in negative regulation; insertions into these regions, *traA* and *traB*, result in constitutive expression of transfer functions and constitutive clumping (68). There is evidence that the *traA* product negatively regulates expression of region E, and recent data relating to *traB* suggest its product is involved in the shutdown of endogenous cAD1 production (K. E. Weaver and D. B. Clewell, to be published). Region C, as well as *traA*, may be involved in sensing or transducing the pheromone signal (55, 70; K. E. Weaver and D. B. Clewell, to be published). A more in-depth account of the related genetic analyses is given in a recent review by Clewell and Weaver (8) as well as in References 55 and 70.
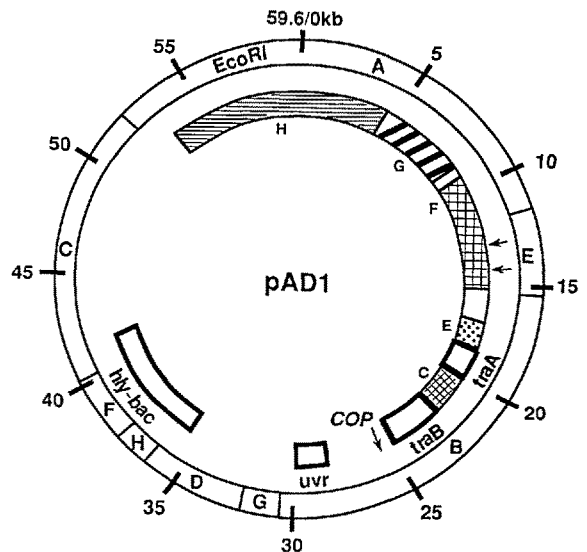

Figure 1: Physical and functional map of pAD1. Regions important for transfer are indicated as shaded or open boxes on the interior of the map and labeled *traA*, *traB*, and C through H. Regions *hly-bac*, *uvr* and *cop* represent genes for hemolysin-bacteriocin, ultraviolet light resistance, and copy number (plasmid), respectively. *Eco*R 1 fragments are labeled within the outer circle.

Analyses of pCF10 (71) have revealed some similarities to pAD1 in that there is evidence for both negative and positive regulation (72). In this case, two positive regulator loci designated R130 and R150 have been reported to independently affect surface exclusion and aggregation, respectively. It will be interesting to compare pCF10 and pAD1 as more information becomes available.

It is surprising that in the 11 years since sex pheromone-induced plasmid transfer was discovered in *Enterococcus faecalis*, this phenomenon has still not been identified in other species of bacteria. Interestingly, a substance similar to the pheromone cAM373 is produced by practically all strains of *Staphylococcus aureus* and about 15 % of *Streptococcus sanguis* strains (73); however, there is no evidence that the substance serves as a pheromone in these species.

It has been speculated (74) that the pheromone peptides excreted by plasmid-free strains of *Enterococcus faecalis* may actually have other functions. It is conceivable that the peptides preceeded the plasmids, and the latter evolved in such a way as to take advantage of these substances as mating signals. If this is true it raises the question of what other roles these compounds might play. Recently, it has been reported that some of these peptides exhibit potent neutrophil chemotaxis activity (75, 76). The phenomenon relates, at least in part, to receptors that also recognize f-Met-Leu-Phe. The fact that certain mammalian leukocytes appear to recognize such compounds as cPD1 and cAM373 raises the possibility that these substances could influence the course of a related bacterial infection by enhancing or perhaps modifying the host response. Conceivably, some peptides could act to competitively inhibit the activity of certain chemotactic factors; such compounds might then be considered virulence factors.

An Amplifiable *tet* Nonconjugative R-Plasmid

The nonconjugative 9.3 kb plasmid pAMα1 bearing *tet* was originally identified in *Enterococcus faecalis* strain DS5 and was co-resident with the four conjugative plasmids pAMβ1, pAM∂1, pAM∂2 and pAM∂3 (9, 10, 77, 78). It could be mobilized readily by any of the four conjugative plasmids, and it was possible to generate transconjugants that harbored only pAMα1 (9). When cells harboring pAMα1 were grown in the presence of tetracycline for a number of generations, an increased level of resistance could be detected, and this could be cor-

related with a reversible generation of tandem repeats of a 4.1 kb segment containing *tet* (78, 79, 80). In addition, cells grown in the absence of tetracycline gave rise to tetracycline-sensitive derivatives with a 4.1 kb deletion. The 4.1 kb segment is flanked by two directly repeated 380 base pair sequences designated RS1 (recombination sequence), which are presumed to facilitate the phenomenon (80). Both amplification and deletion failed to occur in a recombination deficient (Rec⁻) enterococcal host, and models based on homologous recombination between the direct repeats have been suggested (80, 81). This type of resistance amplification is also known to occur with certain R-plasmids in gram-negative bacteria (82). In the case of pAMα1, it is interesting that with increased amplification, the mass of plasmid DNA calculated as a percentage of the chromosomal mass does not change (79); this suggests the involvement of a copy number control mechanism sensitive to total plasmid mass.

Perkins and Youngman (83) have reported that pAMα1 is actually a composite of two separable replicons and that the amplifiable segment is actually a replicon, designated pAMα1Δ1, capable of autonomous replication in *Bacillus subtilis*. The other component, designated pAMα1Δ2, corresponds to that of tetracycline-sensitive deletion variants in *Enterococcus faecalis*. When *Bacillus subtilis* is transformed with pAMα1, tetracycline-resistant transformants appear to have only pAMα1Δ1 (83, 84). It is possible that pAMα1Δ1 is not able to replicate independently in *Enterococcus faecalis* and that pAMα1Δ2 is not able to replicate in *Bacillus subtilis*. Interestingly, pAMα1Δ1 has restriction sites showing it to be virtually identical to the similarly sized, *tet*-containing *Bacillus cereus* plasmid pBC16 (85). Plasmids with high similarity have been found in other species of *Bacillus* (86), and there is a close relationship with the *Staphylococcus aureus* plasmid pUB110 (87), which differs only in having a Km-resistance determinant rather than *tet*. In addition, the *Enterococcus faecalis* plasmid pJH1 (88) and the *Bacillus subtilis* Marburg 168 chromosome (89) have been shown to contain integrated pAMα1Δ1-like elements. The *tet* of pAMα1 has been sequenced and found to encode a 458 amino acid protein (90). Burdett et al. (91) have categorized this particular determinant as *tetL*.

Conjugative Transposons

The initial identification of a conjugative transposon occurred in *Enterococcus faecalis* strain DS16 (14). The latter is a multiply antibiotic resistant strain originally found to contain two

plasmids, pAD1 (see above) and pAD2 (11). The latter determines resistance to erythromycin, kanamycin and streptomycin (Sm). The *erm* determinant is located on what was eventually found to represent Tn917 (12). A *tet* determinant was found to be located on the chromosome. When *tet* was mobilized in mating experiments, most transconjugants were hemolytic, and some were found to be hyperhemolytic. The latter contained a 16 kb insertion in pAD1 close to the hemolysin determinant. Insertions of a similar size into different sites on pAD1 could be found in other derivatives; some completely inactivated hemolysin expression. It was apparent that *tet* was located on a 16 kb transposon that could insert at multiple sites on pAD1; it was designated Tn916.

It was surprising at the time to find that plasmid-free derivatives of DS16 were able to transfer tetracycline resistance in filter matings (14, 92). Transfer required cell-to-cell contact, and insertions occurred at different locations in the recipient chromosome. This type of movement can be viewed as an intercellular transposition event – thus, the term conjugative transposon. Both intracellular and intercellular transposition are Rec-independent.

Similar elements, designated Tn918 (73), Tn920 (54) and Tn925 (93), all encoding tetracycline resistance, have been identified in different strains of *Enterococcus faecalis*; and additional elements, also carrying *tet* (Tn5031, Tn5032 and Tn5033), have been revealed in strains of *Enterococcus faecium* (H. Fletcher et al., to be published). Conjugative transposons have been found primarily on the bacterial chromosome, although Tn925 was originally found on pCF10.

Tn916 has a very broad host range and will transfer into numerous gram-positive genera (16). Chromosome-borne conjugative transposons have also been found in gram-positive species such as *Streptococcus sanguis* (94), *Streptococcus pneumoniae* (95, 96), *Streptococcus agalactiae* (97, 98), *Streptococcus pyogenes* (99) and *Clostridium difficile* (100). In some cases these nonplasmid transferable elements are much larger (more than 60 kb) and carry multiple resistances. All include a Tc-resistance determinant of the *tetM* variety (91), and, in all cases tested, there appears to be strong homology with Tn916 (16). Like Tn916, the transposon Tn1545 (determines resistance to tetracycline, erythromycin and kanamycin) from *Streptococcus pneumoniae* has been the subject of detailed analyses (95, 101, 102). The *tet* determinant of Tn1545 has been sequenced (103), and recent analysis of the *tet* of Tn916 show it to be extremely similar (Y. Su and D. B. Clewell, unpublished data). The *tetM* protein is estimated to have a size of 72,500 daltons.

Enterococcus faecalis transconjugants with Tn916 inserted at different sites on the chromosome donated the element at frequencies of 10^{-9} to 10^{-5} per donor, depending on the strain (92).

The differences in frequency are most likely influenced by sequences outside the transposon. There is a positive correlation between conjugative donor potential and transposition to a subsequently introduced plasmid. For example, two strains whose transfer potential differed by a factor of 100 also exhibited a 100-fold difference in the ability to generate inserts into pAD1 (92).

Tn916 has been cloned in *Escherichia coli* on plasmid vectors where it has been mapped and studied genetically. In the *Escherichia coli* background there is a strong tendency for the transposon to excise, giving rise to tetracycline-sensitive variants at a relatively high frequency under nonselective conditions (104). It was proposed (92, 104) that an excision giving rise to a nonreplicating circular intermediate is the first and rate-limiting step in a transposition event, and that its high frequency in the *Escherichia coli* background reflects an aberrant expression of this step. Indeed, circular intermediates have been isolated in *Escherichia coli* and found to transform *Bacillus subtilis*, giving rise to normal insertions in the chromosome (105).

Tn916 can be easily reintroduced into *Enterococcus faecalis* via protoplast transformation (106) with *Escherichia coli* plasmid chimeras (107). This leads to an insertion into the enterococcal chromosome. Using Tn5 as an insertional mutagen (107, 108) in *Escherichia coli*, it was possible to generate insertions into numerous sites in Tn916. Some insertions (close to the left end of the transposon) resulted in an inability to excise in *Escherichia coli*. Such derivatives could not give rise to insertions into the enterococcal chromosome upon protoplast transformation. The ability to excise in *Escherichia coli* could be complemented in trans when the corresponding DNA was made available on a co-resident plasmid. Other Tn5 insertions specifically affected the ability to conjugate (108).

The ends of Tn916 have been sequenced (109, 110) and have been found to contain imperfect inverted repeat sequences with identity at 20 of 26 nucleotides. Farther in from the ends, imperfect directly repeated sequences are present, with 24 of 27 nucleotides matching. Within the right terminus is a potential outwardly reading promoter site. The transposon junction regions contain homologous segments, but of a nature not consistent with a direct duplication of the target sequence. Essentially identical sequence termini have been found in the case of Tn1545 (102). On the basis of combined junction data from Tn916 and Tn1545, a novel mechanism has been proposed whereby a transposon-specific

excisase generates staggered nicks of 5 or 6 base pair (111; M. Caparon et al., to be published) at each nonidentical junction, leading to a circular molecule with heteroduplexes at the recombination sites. It was proposed that resulting mismatches might be rectified by the mismatch repair system of the host or by replication.

Other Transposons

More conventional (i. e. nonconjugative) transposons have also been found in enterococci. The above-mentioned Tn917 (5.3 kb; carrying *erm*) was originally identified on the nonconjugative plasmid pAD2 (12, 13) and is a member of the Tn3 family of transposons (112). It was discovered on the basis of its ability to transpose to the highly conjugative pAD1. The general structure of the transposon, based on a sequence analysis (113, 114), is shown in Figure 2. All open reading frames (ORFs) are such that transcription is from left to right. The *erm* (MLS) determinant is essentially identical to that of the plasmid pAM77 that originally came from *Streptococcus sanguis* A1 (115, 116). Horinouchi et al. (116) had previously sequenced the latter and showed that it contained a complex translational attenuation control region for *erm* expression. This region is also present in Tn917 and corresponds to ORF1. The region corresponding to ORF3 is also present in pAM77, but homology terminates two base pairs beyond the ORF3 stop codon (113); the function of ORF3 is not known. ORF4 corresponds to a resolvase determinant (*tnpR*) (112) and exhibits homology with other *tnpR* determinants. The remainder of the transposon was originally reported to consist of two additional ORFs, ORF5 and ORF6 (113); a recent

sequence correction, however, has shown that the two are actually joined to make a single ORF (F. Y. An and D. B. Clewell, unpublished data). The latter exhibits some homology to *tnpA* (transposase) of other Tn3-family transposons (117).

Tn917 generates a 5 base pair duplication of target DNA and contains 38 base pair repeats at the termini, typical of Tn3-family elements. It also contains an internal 38 base pair repeat that is inverted with respect to the right end terminus (113). The segment defined by the latter two sequences, which does not include *erm*, may be capable of transposing on its own, although this has not been directly demonstrated. An analogous situation is known to occur in the case of Tn1721, in which an internal minor transposon designated Tn1722 is present (118, 119). Tn4430, from *Bacillus thuringiensis*, is an element with no determinants other than those necessary for transposition (120); interestingly, it has significant homology with the corresponding region of Tn917 (117, 121, 122).

The internal 38 base pair sequence in Tn917 is in direct orientation with respect to the left end terminus; and extensions to the right of these two sequences, giving rise to direct repeats of 73 rather than 38 base pairs, provide additional homology (113). Since they flank *erm*, recombination between the two might lead to deletion or amplification of the resistance determinant. Although *erm* is inducible to a high level of resistance with erythromycin, it is important to note that some macrolides and lincosamides do not induce (37); in the latter cases, amplification might offer another means of elevating expression.

An especially interesting property of Tn917 is its ability to undergo enhanced transposition on exposure to erythromycin (12). Both resistance and transposition were inducible at erythromycin concentrations as low as 0.001 µg/ml but not 0.0001 µg/ml, suggesting a common or similar regulatory mechanism. Studies showed that this is probably related to transcriptional readthrough from the *erm* determinant into the remainder of the transposon (113). The Tn3-family transposon Tn501, encoding mercury resistance, is another example of an element that can be induced to transpose by the compound to which it encodes resistance (112, 123).

Tn917 is extremely similar to Tn551, an element originally identified on a staphylococcal plasmid (114). Tn551, however, expresses *erm* constitutively, whereas the *erm* of Tn917 is inducible. A Tn917-like transposon (Tn3871) occurs on the enterococcal multiple resistance plasmid pJH1 (124), and very similar elements have been identified in isolates from various humans, chickens and pigs (125, 126). These
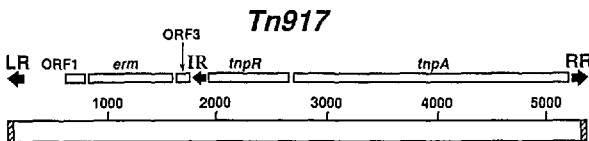

Figure 2: Map of transposon Tn917. The five open reading frames (ORFs) are all on the same strand and are transcribed from left to right. ORF2 is indicated as *erm* (the erythromycin-resistance determinant), whereas ORF4 and ORF5 are indicated as *tnpR* (resolvase determinant) and *tnpA* (transposase determinant), respectively. The location and orientation of the left, internal and right repeats are indicated as LR, IR and RR, respectively. Although not apparent from the figure, the IR is actually almost completely within the 3' end of ORF3. The map represents a revision of that shown in Reference 113; ORF5 and what had been viewed as a separate ORF (ORF6) are now joined together to represent *tnpA*.

data, and the fact that Tn917, Tn 3871 and Tn551 came from isolates in Michigan, London and Japan, respectively, imply that this element is widely disseminated. It is also interesting that a number of *Enterococcus faecalis* plasmids, including pJH1 and pAD2, have been found to contain Km- and Sm-resistance determinants adjacent to Tn917-like elements and on a 9–11 kb DNA segment (126).

As noted earlier, Youngman (67) has developed a number of very useful transposon delivery vectors based on plasmid temperature sensitivity (67); these include various Tn917 derivatives that can be used for the generation of transcriptional fusions and for cloning purposes. Although developed primarily for *Bacillus*, the system has been found to work nicely in enterococci, where it can be used to generate both plasmid and chromosomal mutations (127).

It is likely that other resistance transposons will soon be identified in enterococci. The fact that gentamicin-resistance determinants have recently been found on a variety of apparently unrelated plasmids (128, 129) strongly suggests the involvement of one or more transpositional elements.

Hemolysin/Bacteriocin

A significant percentage of clinical isolates of *Enterococcus faecalis* produce a cytolytic protein that lyses human, rabbit and horse erythrocytes (6, 130, and see above). A recent study in Japan found that 60 % of *Enterococcus faecalis* strains associated with parenteral infections were hemolytic, in contrast to 17 % of strains isolated from faecal specimens (36); and hemolysin activity has been shown to contribute to virulence in a mouse model (131).

The *Enterococcus faecalis* hemolysin also possesses a bacteriocin activity which inhibits a broad range of gram-positive species (132, 133). The hemolysin/bacteriocin phenotype is usually encoded by a plasmid, which in the majority of cases is conjugative and determines a pheromone response (6, 36). Plasmids such as pAD1 and pAM∂1 as well as a number of hemolysin/bacteriocin plasmids from Japanese isolates exhibit almost identical *Eco*R1 restriction profiles (134). Other studies indicate most belong to the same incompatibility group (32), and many encode a response to the sex pheromone cAD1 (8, 36).

The hemolysin/bacteriocin activity is a two-component system where one component serves to activate lytic activity possessed by the other (135). The two components are referred to as A (activator) and L (lytic). When mutants defec-

tive in A grow as colonies on blood agar near mutants defective in L, a zone of hemolysis is generated on blood plates in a region between the two strains. The activation process therefore can occur extracellularly. The two proteins have been purified from *Enterococcus faecalis* strain X-14 and partially characterized (136, 137).

The hemolysin/bacteriocin determinant (*hly/bac*) of pAD1 has recently been analyzed by insertional mutagenesis and cloning approaches, and the regions determining the A and L components have been further defined (138). Approximately 8.4 kb of DNA was necessary for *hly/bac* expression. In addition, the A component was found to be associated with host immunity to the related bacteriocin activity.

It is interesting that hemolysin/bacteriocin plasmids such as pAD1 possess a site (or small region) in or close to the *hem/bac* determinant which behaves as a hot spot for many conjugative transposons (16). This frequently results in inactivation or hyperexpression of the hemolysin/bacteriocin. In the case of hyperexpression, colonies give rise to zones of hemolysis three to four times the normal diameter on blood agar. In some cases, hyperhemolysis is stimulated by the presence of tetracycline in the blood plates. If tetracycline is not present in these cases, colonies initially appear nonhemolytic; but if the plates are allowed to incubate further for a few days at room temperature, a "wave" of hemolysis may be seen to spread through the plate. Hemolysis around one colony appears to trigger hemolysis of nearby colonies, until all have a hyperhemolytic appearance. The effect of tetracycline may be nonspecific, since a pAD1::Tn916 derivative of this type which also had a Tn917 insertion could be similarly stimulated with either tetracycline or erythromycin (Y. Yagi and C. Gawron-Burke, unpublished data). It is therefore likely that the enhancement of hyperhemolytic expression in these cases arises from a drug-related "stress effect." The basis for hyperhemolytic expression relating to the insertion of conjugative transposons remains unknown. The possible role of an outwardly reading promoter on the transposon has been considered (110). It is important to note that such a phenomenon has never been observed to occur with insertions of the more conventional Tn917, despite the fact that numerous insertions into the pAD1 *hem/bac* area have been generated.

Concluding Remarks

Like many other bacteria, the enterococci have a variety of elements that facilitate the intra-

cellular and intercellular movement of genetic information. The intestinal location of these organisms and their abundance of plasmids and transposons suggests they may serve as a significant reservoir of genetic information available to other gram-positive bacteria in the gut – much the way *Escherichia coli* is sometimes viewed as a reservoir of information for gram-negative bacteria.

The increasing clinical significance of enterococcal infections and related problems of multiple antibiotic resistance emphasizes the importance of conducting further research aimed at elucidating factors associated with colonization and survival of enterococci. The relatively recent acquisition of plasmid-borne resistance to gentamicin (20, 23, 128, 129) and a β-lactamase-associated penicillin resistance (128, 139, 140) has left vancomycin as one of the few drugs remaining for treating serious infections such as subacute bacterial endocarditis, and the very recently detected plasmid-borne vancomycin resistance in a few clinical isolates is particularly disquieting (141, 142).

Acknowledgements

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