Use of an excision reporter plasmid to study the intracellular mobility of the conjugative transposon Tn916 in Gram-positive bacteria

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An excision reporter plasmid was constructed to characterize the intracellular mobility of Tn916 in various Gram-positive bacteria. The reporter component of this plasmid is a chloramphenicol-resistance gene which has been insertionally inactivated with the integrative vector pAT112 containing the attachment site of Tn916. Tn916-mediated excision of pAT112, to produce clones resistant to chloramphenicol, was detected in *Enterococcus faecalis* BM4110, *Listeria monocytogenes* L028-Str and *Streptococcus gordonii* BM120, but not in *Lactococcus lactis* MG1363-RF or in *Streptococcus pneumoniae* BM124, and always depended upon the ability of the bacterial host to generate circular forms of the transposon. The results suggest that (i) the excision event, although required, is not sufficient for conjugal transfer to occur and (ii) there is no linear relationship between the donor potential of *E. faecalis* strains and either the excision frequency of pAT112 or the copy number of Tn916 circular intermediates per cell in these hosts. Excision of pAT112 occurred mainly during the late exponential phase of growth of *E. faecalis* and *L. monocytogenes* and this recombination event was not stimulated by heat shock, salt and alcohol stresses or by the presence of tetracycline in the medium.

Keywords: conjugative transposons, Tn916, excision, conjugation

INTRODUCTION

The 18 kb transposon Tn916, first detected in the chromosome of *Enterococcus faecalis* strain DS16, is the prototype of a closely related family of conjugative transposons widespread in Gram-positive cocci (Clewett *et al.*, 1995; Scott & Churchward, 1995). Transposable elements which belong to this family are large DNA segments (≥ 18 kb) and contain the tetracycline-resistance determinant *tet(M)* either alone (e.g. Tn916) or associated with other resistance genes (e.g. Tn3703 and Tn1545). Transposition of these elements proceeds by excision to a free, non-replicative, covalently closed circular intermediate that is a substrate for integration. The integration–excision system of these elements is structurally and functionally related to that of lambdoid phages (Poyart-Salmeron *et al.*, 1989, 1990). Excision and integration occur by reciprocal site-specific recombination between non-homologous sequences of 6 bp. Excisive recombination requires two transposon-encoded proteins, an excisionase (Xis-Tn) and an integrase (Int-Tn), whereas integration requires only Int-Tn. However, as in the λ site-specific recombination

system, Int-Tn alone can catalyse a low level of excision but this activity is greatly stimulated by Xis-Tn. It is thus likely that the direction of recombination, integration versus excision, is determined by the relative amounts of the two proteins in a cell.

Conjugal transfer of Tn916 and related elements can be viewed as a transposition event involving donor and recipient replicons that happen to be present in different cells and is therefore referred to as conjugative transposition. This event involves circularization of the element in the donor, and transfer of the non-replicative intermediate by conjugation to the recipient where it integrates (Storrs *et al.*, 1991). Bacterial hosts such as *Lactococcus lactis* in which Tn916 cannot excise are unable to promote conjugative transposition (Bringel *et al.*, 1991). Thus, in addition to their transfer functions, transfer of this class of elements requires transposon-encoded excision and integration functions. The complete nucleotide sequence of Tn916 has been determined (Flannagan *et al.*, 1994), which enabled the identification of some putative polypeptides displaying significant homology with other known proteins involved in

conjugation (antirestriction protein Ard of plasmid ColIb-P9, MbeA mobilization protein of plasmid ColE1) and the location of a functional *oriT* (Jaworski & Clewell, 1995).

The frequency of conjugative transposition of Tn916 between isogenic strains of *E. faecalis* may range over more than four orders of magnitude between different Tn916-containing donors and is not related to the number of transposon insertions in the donor strain, although strains with a high donor potential generally contain multiple insertions. The host range of transfer of Tn916 might depend on the donor potential of the transposon-delivery strain: only strains having a high donor potential can undergo conjugative transposition from Gram-positive to Gram-negative bacteria (Poyart *et al.*, 1995). Tn916 insertions located at different sites of the bacterial genome may possess different donor potentials when considered independently and it has been suggested that the insertion with the greatest potential would be the most likely to excise initially and that this event could stimulate, by *trans*-activation, movements of all other transposons present in the cell (Flannagan & Clewell, 1991). The dosage of Tn916 circular intermediates in *E. faecalis* performed by quantitative PCR assay has revealed that the frequency of Tn916 conjugation is dependent on the copy number of circular intermediates present in the donor cell population (Manganelli *et al.*, 1995). Thus, excision appears to be the initial and rate-limiting step of both the intracellular and intercellular mobility of this class of elements. In this study, we have constructed an excision reporter plasmid to characterize, qualitatively and quantitatively, the intracellular mobility of Tn916 in various Gram-positive bacteria including isogenic strains with different donor potential. Our results are not fully consistent with the mechanism of *trans*-activation described above and do not support the proposal that there is a linear relationship between excision and transfer.

METHODS

Bacterial strains and growth conditions. The main characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. All the strains were grown at 37 °C in brain heart infusion (BHI) broth or on BHI agar plates (Difco). *Streptococcus gordonii* and *Streptococcus pneumoniae* were grown on BHI blood agar and incubated under a 5% CO₂ moist atmosphere. For Gram-positive bacteria, antibiotics were used at the following concentrations: streptomycin, 1000 µg ml⁻¹; rifampicin, 40 µg ml⁻¹; fusidic acid, 20 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹ (*E. faecalis*), 10 µg ml⁻¹ (*L. lactis*, *Listeria monocytogenes*) or 5 µg ml⁻¹ (*S. gordonii*, *S. pneumoniae*); erythromycin, 8 µg ml⁻¹; tetracycline, 8 µg ml⁻¹. For *Escherichia coli*, 20 µg chloramphenicol ml⁻¹, 60 µg spectinomycin ml⁻¹ and 8 µg tetracycline ml⁻¹ were used.

DNA manipulations. Plasmid DNA was extracted from *E. coli* as described by Sambrook *et al.* (1989). Total DNA from Gram-positive bacteria was purified as described previously by Storrs *et al.* (1991). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and were used according to the manufacturer's recommendations.

Restriction-endonuclease-generated DNA fragments were separated by electrophoresis on 0.8% (w/v) agarose gels and transferred to reinforced nitrocellulose BA-S 83 membranes (Schleicher & Schuell). Prehybridization and hybridization were performed under stringent conditions as described by Sambrook *et al.* (1989). DNA probes were labelled with [α -³²P]dCTP (Amersham) by using the Nick Translation kit (Amersham). Detection of the segments corresponding to the transposon attachment site (*att*-Tn) specific for Tn916 circular intermediates and to the transposon integrase gene *int*-Tn was carried out by PCR using the oligonucleotides CT31 plus CT32 (Storrs *et al.*, 1991) and O_{int1} (5'-GTCGTATCAAGGC-TCTTTCC-3') plus O_{int2} (5'-GTACTACTAAGCAACAAG-ACGC-3'), respectively. PCR reactions were performed in a final volume of 100 µl containing 50 ng genomic DNA, 0.1 µM each primer, 200 µM each deoxynucleoside triphosphate and 2 U Taq DNA polymerase in 1 × amplification buffer (10 mM Tris/HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂). The PCR mixture was submitted to a denaturation step (10 min at 95 °C), followed by 35 cycles of amplification (60 s of annealing at 55 °C, 90 s of elongation at 72 °C and 60 s of denaturation at 95 °C).

Mating experiments. Overnight cultures of donor and recipient cells were diluted 1:100 in fresh BHI broth without antibiotics. Cells were grown for 5 h (approximate OD₆₀₀ 0.7) and 100 µl each of donors and recipients was mixed and spread onto 0.45 µm pore-size nitrocellulose membrane filters (Millipore) placed on BHI agar plates. After 18 h incubation at 37 °C, cells were suspended in 1 ml 0.9% NaCl and serial dilutions of this mixture were plated on BHI agar containing appropriate antibiotics to select transconjugants and to enumerate donor cells. Transfer frequencies were expressed as the number of transconjugants per donor c.f.u. after the mating period.

Construction of the excision reporter plasmid pTCR9. The 901 bp *TaqI*-*Sau3A* DNA fragment containing the *cat* gene of the streptococcal plasmid pIP501 (Trieu-Cuot *et al.*, 1992) was cloned into pGB2 to produce pTCR7. Plasmid pAT112 is a mobilizable and integrative vector that utilizes the transposition properties of Tn1545/Tn916. Competent cells of *E. coli* SM10 harbouring pAT112 and pTCR7 were transformed (Sambrook *et al.*, 1989) with 1 µg pAT294 DNA, a pUC18-derivative directing synthesis of the transposon-encoded integrase Int-Tn. After heat shock, the cells were diluted 10-fold in BHI broth containing ampicillin and incubated for 5 h with gentle agitation. Serial dilutions of the culture were mixed with an equal volume of a late exponential phase culture of *E. coli* K802N::Tn10 and the resulting mixture was plated on BHI agar containing spectinomycin and tetracycline to select *E. coli* K802N::Tn10 transconjugants harbouring co-integrates of the integrative vector and of the target replicon. Analysis of the plasmid content of chloramphenicol-susceptible (Cm^s) transconjugants by agarose gel electrophoresis showed two classes of pTCR7::pAT112 co-integrates on the basis of their *Sau3A* restriction profiles. The insertion site of pAT112 was characterized on one plasmid representative of each class by double-stranded DNA template sequencing (Zhang *et al.*, 1988), using a Sequenase 2.0 kit (United States Biochemical) and the oligonucleotides CT31 and CT32. Insertion of pAT112 had occurred either in the *cat* promoter region or at position 333 of the 643-bp-long *cat* gene (Trieu-Cuot *et al.*, 1992). The plasmid of the latter class, designated pTCR8, was introduced by transformation (Trieu-Cuot *et al.*, 1988) into *S. gordonii* harbouring the low copy number plasmid pIL205, a pIP501 derivative conferring resistance to chloramphenicol but not to erythromycin, and transformants

Table 1. Bacterial strains and plasmids used in this study

Strain*/plasmid	Relevant properties†	Reference or origin
Strains		
<i>Enterococcus faecalis</i>		
BM4110	Str (spontaneous mutant of JH2)	Courvalin & Carlier (1986)
JH2-2	Rif, Fus (spontaneous mutant of JH2)	Jacobs & Hobbs (1974)
NEM429	BM4110::Tn916 (four copies), Str Tc	This study
NEM575	BM4110::Tn916 (two copies), Str Tc	This study
NEM574	BM4110::Tn916 (one copy), Str Tc	This study
<i>Listeria monocytogenes</i>		
L028-Str	Str (spontaneous mutant of L028)	This study
NEM766	L028-Str::Tn916 (one copy), Str Tc	This study
<i>Streptococcus gordonii</i>		
BM120	Str	Le Bouguénec <i>et al.</i> (1984)
NEM768	BM120::Tn916 (one copy), Str Tc	This study
<i>Streptococcus pneumoniae</i>		
BM124	Str	Le Bouguénec <i>et al.</i> (1984)
NEM751	BM124::Tn916 (two copies), Str Tc	This study
BM4200	Wild strain carrying Tn1545, Cm Km Em Tc	Courvalin & Carlier (1986)
<i>Lactococcus lactis</i>		
MG1363-RF	Rif Fus (spontaneous mutant of MG1363)	Poyart & Trieu-Cuot (1994)
NEM536	MG1363-RF::Tn916 (one copy), Rif Fus Tc	This study
<i>Escherichia coli</i>		
SM10	F ⁻ , RP4-2-Tc::Mu, Km, <i>thi-1 recA1 thr-1 leuB6 tonA21 supE44 λ⁻ lacY1</i> , Tra ⁺	Simon <i>et al.</i> (1983)
K802N::Tn10	<i>hsdR hsdM⁺ gal met supE</i> , Nal Rif Tc	Trieu-Cuot <i>et al.</i> (1988)
Plasmids		
pGB2	Sp/Sm, <i>oriR</i> pSC101, 4.0 kb	Churchward <i>et al.</i> (1984)
pAT112	Em, Tra ⁻ , Mob ⁺ , <i>oriR</i> pACYC184, <i>att</i> -Tn1545, 4.9 kb	Trieu-Cuot <i>et al.</i> (1991)
pIL205	Cm, Tra ⁺ , 27.3 kb	Langella <i>et al.</i> (1993)
pAT294	Ap, pUC18 with a 1.8 kb <i>SspI</i> - <i>Sau3A</i> fragment carrying <i>int</i> -Tn, 4.6 kb	Poyart-Salmeron <i>et al.</i> (1989)
pTCR7	pGB2 with a 0.9 kb <i>TaqI</i> - <i>Sau3A</i> fragment containing <i>cat</i> of pIP501, Sp/Sm Cm, 4.9 kb	Trieu-Cuot <i>et al.</i> (1992) and this study
pTCR8	pTCR7::pAT112, pAT112 integrated into <i>cat</i> , Sp/Sm Em, 9.8 kb	This study
pTCR9	pIL205::pAT112, Em, 32.2 kb, Tra ⁺	This study

* Bacteria were grown in BHI broth or agar.

† Ap, Cm, Em, Fus, Km, Nal, Rif, Sm (or Str), Sp and Tc indicate resistance to ampicillin, chloramphenicol, erythromycin, fusidic acid, kanamycin, nalidixic acid, rifampin, streptomycin, spectinomycin and tetracycline, respectively.

were selected on BHI agar containing 5% defibrinated horse serum and erythromycin. Six selected transformants were tested for antibiotic resistance by agar disk diffusion, and their plasmid contents were analysed by agarose gel electrophoresis. As expected, all the clones were erythromycin-resistant (Em^R) but were Cm^S and contained a single plasmid of 32.2 kb, i.e. the size of pIL205 (27.3 kb) plus that of pAT112 (4.9 kb). This plasmid, designated pTCR9 (Fig. 1), retained the relevant characteristics of the parental replicon pIP501: it transfers efficiently by filter mating, but not in liquid media, to a large variety of Gram-positive bacteria, where it replicates stably in the absence of selective pressure.

Excision assay. Tn916-mediated excision of pAT112 from pTCR9 was monitored as follows. Bacterial strains harbouring the excision reporter plasmid pTCR9 were grown overnight at 37 °C in BHI broth containing erythromycin, diluted 1:100 in preheated BHI broth and incubated with gentle agitation in a rollerdrum to prevent sedimentation of

cells. Aliquots of the cultures were removed at the desired period of growth, serially diluted and plated both on BHI agar containing chloramphenicol to enumerate clones in which pAT112 has excised from pTCR9, and hence designated excisants, and on BHI agar without antibiotics to enumerate the cells. In each experiment, we studied the ability of selected excisants to transfer by conjugation at a high frequency (i.e. that of pIL205) their chloramphenicol-resistant (Cm^R) determinant to an appropriate *E. faecalis* recipient. The excision frequency of pAT112 from pTCR9 was expressed as the number of Cm^R excisants/total c.f.u., after 48 h incubation at 37 °C. For salt, alcohol, antibiotic or heat stresses, cultures of *E. faecalis* NEM429 harbouring pTCR9 were grown at 37 °C to late exponential phase (10⁹ c.f.u. ml⁻¹). At this point, the cells were stressed for 30 min by addition (final concentration) of NaCl (2 M), ethanol (10%, v/v) or tetracycline (10 µg ml⁻¹), or were transferred to 45 °C. Each experiment was carried out in duplicate and the excision frequency of

pAT112 from pTCR9 was determined immediately after the stress treatment as described above.

RESULTS

Donor potential of Tn916 delivery strains

The conjugative transposon Tn916 was transferred by conjugation from a suitably marked *E. faecalis* donor to *E. faecalis* BM4110, *L. monocytogenes* L028-Str, *S. gordonii* BM120, *S. pneumoniae* BM124 and *L. lactis* MG1363-RF. In each experiment, the ability of 12 selected transconjugants to deliver Tn916 into the appropriate *E. faecalis* recipient, JH2-2 or BM4110, was evaluated by filter mating. Retransfer of Tn916 was obtained with *E. faecalis* or *L. monocytogenes* donors but was never observed when *S. gordonii*, *S. pneumoniae* and *L. lactis* were used as transposon delivery strains (Table 2 and data not shown). During the course of this work, we isolated three *E. faecalis* strains (NEM429, NEM574 and NEM575) that delivered Tn916 at significantly different frequencies (Table 2). One strain of *L.*

monocytogenes (NEM766), *S. gordonii* (NEM768), *S. pneumoniae* (NEM751) and *L. lactis* (NEM536) harbouring Tn916 was also selected for further studies. Southern analysis was used to determine the number of copies of Tn916 in these strains (Table 1), evaluated as the number of transposon-chromosome junction fragments containing *int*-Tn (Fig. 3, lanes 1, 7 and 13, shows part of this analysis).

Excision of pAT112 in bacterial hosts harbouring Tn916

The structure of plasmid pTCR9 is shown in Fig. 1. The reporter component of this plasmid is a *cat* gene which has been insertionally inactivated with an integrative vector containing the *att*-Tn site of Tn1545 which is identical to that of Tn916. The functioning of this reporter plasmid system is based on the fact that the two transposon-encoded proteins that are required for excision of this class of transposon will freely diffuse in the cytoplasm of the cells (Poyart-Salmeron *et al.*, 1989).

Table 2. Analysis of the excision and transfer events in different bacterial strains harbouring Tn916

Bacterial host harbouring pTCR9*	Trial	Excision frequency of pAT112†	Relevant resistance phenotype of Cm ^R excisants (%)				Transfer frequency of Tn916 to JH2-2‡
			Tc ^S Em ^S	Tc ^S Em ^R	Tc ^R Em ^S	Tc ^R Em ^R	
<i>E. faecalis</i>							
BM4110	1	< 10 ⁻⁹	—	—	—	—	—
NEM429 (four copies of Tn916)	1	2.6 × 10 ⁻⁷	0	5	90	5	6.7 × 10 ⁻⁶
	2	2.7 × 10 ⁻⁷	10	0	80	10	5.1 × 10 ⁻⁶
NEM575 (two copies of Tn916)	1	4.5 × 10 ⁻⁸	60	0	20	20	2.1 × 10 ⁻⁷
	2	5.0 × 10 ⁻⁸	69	6	25	0	3.6 × 10 ⁻⁷
NEM574 (one copy of Tn916)	1	8.7 × 10 ⁻⁸	89	11	0	0	1.1 × 10 ⁻⁸
	2	8.0 × 10 ⁻⁸	80	20	0	0	8.8 × 10 ⁻⁹
<i>L. monocytogenes</i>							
L028-Str	1	< 10 ⁻⁹	—	—	—	—	—
NEM766 (one copy of Tn916)	1	5.5 × 10 ⁻⁶	100	0	0	0	5.5 × 10 ⁻⁶
	2	4.2 × 10 ⁻⁶	90	10	0	0	6.3 × 10 ⁻⁶
<i>S. gordonii</i>							
BM120	1	< 10 ⁻⁹	—	—	—	—	—
NEM768 (one copy of Tn916)	1	2.7 × 10 ⁻⁶	60	40	0	0	< 10 ⁻⁹
	2	0.9 × 10 ⁻⁶	67	33	0	0	< 10 ⁻⁹
<i>S. pneumoniae</i>							
BM124	1	< 10 ⁻⁸	—	—	—	—	—
NEM751 (two copies of Tn916)	1	< 10 ⁻⁸	—	—	—	—	< 10 ⁻⁸
	2	< 10 ⁻⁸	—	—	—	—	< 10 ⁻⁸
<i>L. lactis</i>							
MG1363-RF	1	< 10 ⁻⁹	—	—	—	—	—
NEM536 (one copy of Tn916)	1	< 10 ⁻⁹	—	—	—	—	< 10 ⁻⁹ §
	2	< 10 ⁻⁹	—	—	—	—	< 10 ⁻⁹ §

*NEM429, NEM575 and NEM574 are BM4110::Tn916; NEM766 is L028-Str::Tn916; NEM768 is BM120::Tn916; NEM751 is BM124::Tn916; and NEM536 is MG1363-RF::Tn916.

†Number of Cm^R c.f.u./total c.f.u. in early stationary phase.

‡Number of transconjugants per donor c.f.u. after mating.

§*E. faecalis* BM4110 was used as a recipient in these matings.

Fig. 1. Structure of the excision reporter plasmid pTCR9. Fragments of pTCR9 corresponding to pAT112 and pIL205 are represented by black and open areas, respectively. The 6 bp overlap sequences (atttta, ataata) at the integration site of pAT112 in the *cat* gene of pIL205 to produce pTCR9 are indicated. Resolution of pTCR9 into its two components occurs by excision of pAT112 provided that the bacterial host synthesizes the appropriate amount of excisionase and integrase. The two alternative hexanucleotides in pAT112 and pIL205 after excision are shown. *aphA3*, 3'-aminoglycoside phosphotransferase gene; *attTn_L* and *attTn_R*, left and right attachment sites, respectively; *cat*, chloramphenicol acetyltransferase gene; Δcat and *cat* Δ , 5'- and 3'-truncated *cat* gene; *erm*, erythromycin-resistance methyltransferase gene; *Int-Tn*, transposon-encoded integrase; *mob*, mobilization functions; *oriR*, replication origin; *tra*, transfer functions; Xis-Tn, transposon-encoded excisionase. The plasmids are not drawn to scale.

Thus, provided that a co-resident transposon synthesizes the appropriate intracellular amount of Xis-Tn and Int-Tn, pTCR9 can be resolved into its two basic components, the Cm^R plasmid pIL205 and the Em^R plasmid pAT112 which does not replicate in a Gram-positive host (Fig. 1). Plasmid pTCR9 was introduced by conjugation into *E. faecalis* strains BM4110, NEM429, NEM574 and NEM575, *L. monocytogenes* strains L028-Str and NEM766, *S. gordonii* strains BM120 and NEM768, *S. pneumoniae* strains BM124 and NEM751, and *L. lactis* strains MG1363-RF and NEM536. For each of these strains, aliquots of early stationary phase cultures were spread onto BHI agar containing chloramphenicol. Excision of pAT112 from pTCR9 to produce Cm^R clones was detected in strains of *E. faecalis*, *L. monocytogenes* and *S. gordonii* and always depended upon the presence of Tn916 in the bacterial host (Table 2). The frequency of excision of pAT112 ranged from 5.5×10^{-6} to 4.5×10^{-8} , depending upon the

Fig. 2. Agarose gel electrophoresis of PCR products obtained by using primers enabling amplification of *int-Tn* (a) and of *att-Tn* (b). The substrates were as follows. Lanes: 1, 1 kb ladder (Gibco BRL); 2, *E. faecalis* NEM429; 3, *L. monocytogenes* NEM766; 4, *S. gordonii* NEM768; 5, *S. pneumoniae* NEM751; 6, *L. lactis* NEM536.

strains considered. Cm^R clones were never obtained in *S. pneumoniae* NEM751 and *L. lactis* NEM536 carrying Tn916, which suggests that, in these bacteria, the transposon is unable to promote the excision of pAT112 as well as its own excision. This hypothesis was further substantiated by using a PCR assay which allowed amplification of a 240 bp fragment corresponding to the attachment site of the circularized transposon. As expected, this specific DNA fragment was detected within the DNA templates of *E. faecalis* NEM429, *L. monocytogenes* NEM766 and *S. gordonii* NEM768, but not in those of *L. lactis* NEM536 and *S. pneumoniae* NEM751 (Fig. 2), or in that of *S. pneumoniae* BM4200, which harbours the closely related conjugative transposon Tn1545 (data not shown). In these experiments, the amplification of a 1215 bp DNA fragment corresponding to *int-Tn* served as a positive control (Fig. 2). This analysis confirmed that Tn916 is unable to excise in *L. lactis* NEM536 and in *S. pneumoniae* NEM751 and BM4200.

Phenotypic and genotypic analysis of pAT112 excisants

The resistance phenotype of a minimum of 20 Cm^R colonies of *E. faecalis*, *L. monocytogenes* and *S. gordonii* obtained on a single plate inoculated with the penultimate proficient dilution was studied by the agar disk diffusion technique (Table 2). The mean values obtained from two experiments revealed that, depending upon the strain considered, 5–36% of the selected excisants were also Em^R . As opposed to the Cm^R determinant, the gene conferring resistance to erythromycin in these strains was not transferable by conjugation (data not shown). These results indicated that the non-replicative plasmid pAT112, following excision from pTCR9, had inserted into the chromosome of the host. The presence of pAT112 sequences in the chromosome of Em^R excisants was consistently revealed by Southern blot

Fig. 3. Southern blot analysis of Cm^R excisants of strains of *E. faecalis* harbouring plasmid pTCR9. Bacterial DNA was purified, digested with *Hind*III, resolved by agarose gel (0.8%, w/v) electrophoresis, transferred onto a nitrocellulose membrane, and hybridized with ^{32}P -labelled *int*-Tn to detect sequences related to Tn916 and to characterize the transposon-chromosome junction fragment(s) carrying *int*-Tn. Lanes: 1, *E. faecalis* NEM429; 2, NEM429(pTCR9); 3–5, $\text{Tc}^R \text{Em}^S$ NEM429(pTCR9) excisants; 6, $\text{Tc}^R \text{Em}^R$ NEM429(pTCR9) excisants; 7, *E. faecalis* NEM575; 8, NEM575(pTCR9); 9 and 10, $\text{Tc}^R \text{Em}^S$ NEM575(pTCR9) excisants; 11, $\text{Tc}^S \text{Em}^R$ NEM575(pTCR9) excisants; 12, $\text{Tc}^S \text{Em}^S$ NEM575(pTCR9) excisants; 13, *E. faecalis* NEM574; 14, NEM574(pTCR9); 15 and 16, $\text{Tc}^S \text{Em}^R$ NEM574(pTCR9) excisants; 17 and 18, $\text{Tc}^S \text{Em}^S$ NEM574(pTCR9) excisants. Black dots show transposon-chromosome junction fragments containing *int*-Tn in the parental strains.

hybridization (data not shown). It is noteworthy that almost all Cm^R excisants originating from *E. faecalis* NEM574, *L. monocytogenes* NEM766 and *S. gordonii* NEM768 were susceptible to tetracycline. By contrast, the majority of pAT112 excisants in *E. faecalis* NEM429 and 25–40% of those originating in *E. faecalis* NEM575 were tetracycline-resistant (Tc^R). Southern analysis revealed that none of the genomic DNA extracted from *E. faecalis*, *L. monocytogenes* and *S. gordonii* excisants susceptible to tetracycline hybridized with a DNA probe specific to the transposon-encoded integrase gene *int*-Tn (Fig. 3, lanes 11, 12 and 15–18, shows part of this analysis). The intracellular mobility of Tn916 in Tc^R excisants derived from strains containing multiple copies of the transposon was studied as follows. The genomic DNA of 18 Tc^R excisants of *E. faecalis* NEM429 and corresponding to all Cm^R clones obtained on a single plate were purified and analysed by Southern blot hybridization using the *int*-Tn-specific DNA probe. All but one clone possessed a hybridization pattern indistinguishable from that of the parental strain (Fig. 3, lanes 3, 4 and 6, shows part of this analysis) and the movement of one copy of Tn916 from one chromosomal locus to another was observed in the remaining clone (Fig. 3, lane 5). A similar study revealed that all Cm^R excisants of *E. faecalis* NEM575 resistant to tetracycline possessed a hybridization pattern indistinguishable from that of the parental strain (Fig. 3, lanes 9 and 10, shows part of this analysis).

Excision of pAT112 during bacterial growth and under various stress conditions

The excision of pAT112 was studied during the growth of *E. faecalis* NEM429 and *L. monocytogenes* NEM766. The ratio of Cm^R excisants/total c.f.u. increased

Fig. 4. Excision of pAT112 from pTCR9 during the growth of (a) *E. faecalis* NEM429 and (b) *L. monocytogenes* NEM766. Experiments were performed in duplicate and the mean values $\pm$ standard deviations were plotted. $\square$, c.f.u. ml^{-1} ; $\bullet$, Cm^R c.f.u./total c.f.u.

biphasically during the exponential phase of growth of both bacteria and a maximum rate of increase was observed during the late exponential phase (Fig. 4). This ratio slightly decreased as the cells entered the stationary phase and then reached a plateau. These results indicate that excision of pAT112 occurred only during the exponential growth phase of *E. faecalis* and *L. monocytogenes* and mainly during their late exponential phase of growth.

To investigate if excision of pAT112 might be triggered by environmental stresses, cells of *E. faecalis* NEM429 in the late exponential growth phase were submitted for a short period to commonly used stress inducers (salt, ethanol, heat) at doses that severely reduced cell growth and Cm^R excisants were enumerated immediately after the treatment. None of these stresses was found to increase the frequency of excision of pAT112 in NEM429 (data not shown). Similarly, neither the addition of tetracycline during this phase of growth nor the presence of this antibiotic throughout the cultivation of NEM429 modified the excision frequency of pAT112 (data not shown).

DISCUSSION

This paper describes the use of the excision reporter plasmid pTCR9 to carry out a detailed analysis of the intracellular mobility of the conjugative transposon Tn916 in various Gram-positive bacteria. Resolution of this plasmid into its two replicon components to give

Cm^R clones might occur by *trans*-complementation in strains synthesizing the appropriate amount of excisionase and integrase (Fig. 1). To test the functionality of this approach, plasmid pTCR9 was introduced into *E. faecalis* BM4110, *L. monocytogenes* L028-Str, *S. gordonii* BM120, *L. lactis* MG1363-RF and *S. pneumoniae* BM124 harbouring or not harbouring Tn916. Cm^R excisants were only obtained in strains of *E. faecalis*, *L. monocytogenes* and *S. gordonii* containing Tn916 (Table 2) and, as expected, a PCR analysis revealed that the accomplishment of the recombination event responsible for the expression of this resistance phenotype always depends upon the ability of the bacterial host to generate circularized forms of the transposon (Fig. 2). These results demonstrate the reliability of the excision reporter system developed in this study. Excision of Tn916 and Tn916-mediated excision of pAT112 were not detected in *S. pneumoniae* BM120 whereas the *att*-Tn site of circular intermediates of the Tn916-like transposon Tn5251 has been amplified by PCR in *S. pneumoniae* strain DP1322 (Provvedi *et al.*, 1996). The ability of Tn916-like elements to excise could therefore differ between strains belonging to the same species. Bacterial strains in which Tn916 and pAT112 do not excise are unable to act as transposon donors in mating experiments (Table 2; Fig. 2). These results are consistent with the demonstration that excision is the initial step of both the intracellular and intercellular mobility of this class of elements (Storrs *et al.*, 1991). It should be mentioned, however, that the Tn916-related element Tn1545, which does not excise in *S. pneumoniae* BM4200, can be conjugatively transferred from this strain to *E. faecalis* recipients (Buu-Hoi & Horodniceanu, 1980; Courvalin & Carlier, 1987). Structural analysis of Tn1545 in *S. pneumoniae* BM4200 has revealed that this transposon constitutes the central part of a large conjugative element that possesses its own transposition and transfer functions (Le Bouguénec *et al.*, 1990). Conjugal transfer of Tn1545 from *S. pneumoniae* BM4200 to *E. faecalis* recipients might therefore simply result from its passive transfer in association with the external element. The fact that conjugal transfer of the Tn916-like element Tn5251, which constitutes the central part of the composite element Tn5253, could not be detected independently from that of the bearing conjugative element Tn5252 in matings between *S. pneumoniae* strains constitutes further support for this hypothesis (Provvedi *et al.*, 1996). Excision of Tn916 and Tn916-mediated excision of pAT112 were detected in *S. gordonii* BM120, whereas this strain did not transfer Tn916 at a detectable frequency. These results suggest that the transfer functions of Tn916 are not conveniently expressed in this strain and demonstrate that excision is not sufficient for conjugal transfer to occur. That Tn916 cannot undergo conjugative transposition from *S. gordonii* BM120 to *E. faecalis* JH2-2 opposes the previous report of conjugal transfer of the Tn916-related transposon Tn3703 between the same bacterial strains (*S. gordonii* BM124 was formerly designated *S. sanguis* Challis BM124) (Le Bouguénec *et al.*, 1984). These conflicting

results possibly reflect the fact that Tn3703 is part of a large conjugative element designated Tn3701 (Le Bouguénec *et al.*, 1990). Thus, the acquisition of a broader host range of transfer by Tn916-like elements such as Tn1545 or Tn3703 might constitute the selective pressure responsible for the genesis of the large composite conjugative transposons that are widespread in streptococci (Clermont & Horaud, 1994; David *et al.*, 1993; Le Bouguénec *et al.*, 1990).

It is noteworthy that, whatever the bacterial species considered, all Cm^R excisants derived from strains containing a single copy of Tn916 were cured of their element (Table 2; Fig. 3, lanes 11, 12 and 15–18, shows part of this analysis). This phenomenon is less pronounced in the case of *E. faecalis* NEM575, which carries two copies of Tn916, since only 60–75 % of its Cm^R excisants have lost the two elements present in the parental strain. By contrast, only 5–10 % of the Cm^R excisants derived from *E. faecalis* NEM429 have lost the four copies of Tn916 (Table 2). Southern blot analysis of Cm^R Tc^R excisants revealed that all clones derived from NEM575 (Fig. 3, lanes 9 and 10) and that all but one clone derived from NEM429 (Fig. 4) have retained all copies of Tn916 at the same locations as in the parental strains. Taken together, these results suggest that (i) an excision event in a strain carrying multiple Tn916 insertions may produce two major classes of excisants, those in which all copies of the element excised (typified by the excisants cured of Tn916) and those in which a single copy excised, and (ii) an inverse correlation between the copy number of chromosome-borne transposons and the relative proportions of these two classes of excisants may exist. It has been proposed that when there is more than one copy of Tn916 in the cell, the movement of one element activates the movement of all others (Clewell *et al.*, 1995; Flannagan & Clewell, 1991). Our data are not fully consistent with this hypothesis since the mobility of all copies of Tn916 was found to accompany that of pAT112 in 67 % and only 7.5 % of the Cm^R excisants derived from NEM575 and NEM429, respectively. Moreover, the detection of the movement of a single copy of Tn916 in a Cm^R excisant derived from *E. faecalis* NEM429 (Fig. 3, lane 5) demonstrated the ability of a copy of Tn916 to excise alone in a high donor potential strain carrying multiple Tn916 insertions.

Determination of the dosage of Tn916 circular intermediates in various *E. faecalis* strains by using a quantitative PCR assay has led to the conclusion that Tn916 conjugation frequency is directly proportional to the copy number of circular intermediates present in the donor cell population (Manganelli *et al.*, 1995). However, data obtained by using non-quantitative PCR assays have led to a similar (Jaworski & Clewell, 1994) or to the opposite (Rice *et al.*, 1992) conclusion. The excision frequency of pAT112, defined as the number of Cm^R excisants/total c.f.u., reflects the number of cells within a given bacterial population in which the amount of Xis-Tn and Int-Tn enabled an excision event to occur. The excision frequency of pAT112 obtained with

the three isogenic strains *E. faecalis* NEM429, NEM574 and NEM575 is within the same order of magnitude whereas their transposon donor potential varies by more than two orders of magnitude (Table 2). Phenotypic (Table 2) and genotypic (Fig. 3, lanes 3–6, shows part of this analysis) characterization of Cm^R excisants of *E. faecalis* NEM429 harbouring pTCR9 revealed that the four copies of Tn916 plus pAT112 had excised in 7.5% of the clones studied, and one copy of Tn916 plus pAT112 had excised in 5% of the clones studied, whereas only pAT112 had excised in 87.5% of the clones studied. These data suggest that, once an excision event had occurred in NEM429, 87.5%, 5% and 7.5% of the excisants produced one copy, two copies and four copies of Tn916 circular intermediates, respectively. A similar analysis led us to conclude that, in the case of NEM575, 32.5% and 67.5% of the excisants produced one copy and two copies of Tn916 circular intermediates, respectively. One can therefore assume that, on average, every excisant of NEM429, NEM575 and NEM574 produces 1.28 copies (7.5% × four copies + 5% × two copies + 87.5% × one copy), 1.67 copies (67.5% × two copies + 32.5% × one copy) and one copy of Tn916 circular intermediates, respectively. The copy number of Tn916 circular intermediates per cell can then be estimated for NEM429, NEM575 and NEM574 as the mean number of Tn916 circular intermediates produced by the corresponding excisants multiplied by the frequency of pAT112 excision in these strains. These values were 3.4×10^{-7} , 7.9×10^{-8} and 8.35×10^{-8} for NEM429, NEM575 and NEM574, respectively. Thus, there is apparently no linear relationship between the donor potential of *E. faecalis* NEM429, NEM574 and NEM575 and either the excision frequency of pAT112 or the copy number of Tn916 circular intermediates per cell in these strains. Taken together, these results indicate that, although excision might contribute to defining the donor potential of the host, other factors are likely to be involved in the conjugation process. It has been proposed that the differences in Tn916 transfer potential are related to differences in excision frequencies influenced by the nucleotide sequence of the 6 bp junction fragments (Jaworski & Clewell, 1994). An alternative possibility could be that the sequence of the 6 bp junction fragments influences the binding and/or activity of proteins that modulate the expression of the transfer functions. Analysis of excision of pAT112 during the growth of *E. faecalis* NEM429 and *L. monocytogenes* NEM766 revealed an increase in the rate of excision during the late exponential phase (Fig. 4). A similar observation was made when the copy number of Tn916 circular intermediates was quantified by PCR (Manganelli *et al.*, 1995). These results suggested that the excision of Tn916 may be regulated by some host factors, as previously suggested by Bringel *et al.* (1991). It is conceivable that these factors are missing in bacterial hosts such as *L. lactis* MG1363 and *S. pneumoniae* BM124 and BM4200 where Tn916 is unable to excise. Finally, the role of environmental stresses in the excision of Tn916 was studied and our results indicate that this recombination event is ap-

parently not stimulated by heat shock, salt and alcohol stresses.

We demonstrated that the presence of tetracycline in the mating medium increased the transfer frequency of Tn1545 (Doucet-Populaire *et al.*, 1991). This observation was confirmed for several Tn916-like elements (Rice *et al.*, 1995; Torres *et al.*, 1991), including the prototype element Tn916 (Showsh & Andrew, 1992), and it has been proposed that tetracycline-stimulated excision of the element would result in an increase in excision frequency and, in turn, in an increase in conjugation frequency (Manganelli *et al.*, 1995). The molecular basis for this hypothesis would be that the tetracycline-inducible *tetM* transcript might activate the transcription of the downstream genes *xis*-Tn and *int*-Tn (Rice *et al.*, 1992). We do not agree with the hypothesis that tetracycline induces the excision of Tn916-like elements for the following reasons: (i) increased transcription of *xis*-Tn and *int*-Tn is not supposed to result in an increase of excision since this would not modify the ratio of the corresponding proteins; (ii) excision of Tn916 from the insertionally inactivated haemolytic gene carried by the IncHly plasmid pIP964, as measured by the reversion towards the haemolytic phenotype, did not increase when an *E. faecalis* host was cultivated in the presence of tetracycline (data not shown); (iii) the frequency of excision of pAT112 from pTCR9 was not increased when *E. faecalis* NEM429 was cultivated in the presence of tetracycline. We propose rather that, following excision, a tetracycline-inducible transcript reads through the newly formed transposon attachment site to activate the *tra* determinants carried by the other arm of the transposon. Further work is required to substantiate this hypothesis.

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