Molecular Microbiology (2002) **45**(2), 375–395

Transfer origins in the conjugative *Enterococcus faecalis* plasmids pAD1 and pAM373: identification of the pAD1 *nic* site, a specific relaxase and a possible TraG-like protein

M. Victoria Francia[1] and Don B. Clewell[1,2]*
[1]*Department of Biologic and Materials Sciences, School of Dentistry, and* [2]*Department of Microbiology and Immunology, School of Medicine, The University of Michigan, Ann Arbor, MI 48109, USA.*

Summary

The *Enterococcus faecalis* conjugative plasmids pAD1 and pAM373 encode a mating response to the peptide sex pheromones cAD1 and cAM373 respectively. Sequence determination of both plasmids has recently been completed with strong similarity evident over many of the structural genes related to conjugation. pAD1 has two origins of transfer, with *oriT1* being located within the *repA* determinant, whereas the more efficiently utilized *oriT2* is located between *orf53* and *orf57*, two genes found in the present study to be essential for conjugation. We have found a similarly located *oriT* to be present in pAM373. *oriT2* corresponds to about 285 bp based on its ability to facilitate mobilization by pAD1 when ligated to the shuttle vector pAM401; however, it was not mobilized by pAM373. In contrast, a similarly ligated fragment containing the *oriT* of pAM373 did not facilitate mobilization by pAD1 but was efficiently mobilized by pAM373. The *oriT* sites of the two plasmids each contained a homologous large inverted repeat (spanning about 140 bp) adjacent to a series of non-homologous short (6 bp) direct repeats. A hybrid construction containing the inverted repeat of pAM373 and direct repeats of pAD1 was mobilized efficiently by pAD1 but not by pAM373, indicating a significantly greater degree of specificity is associated with the direct repeats. Mutational (deletion) analyses of the pAD1 *oriT2* inverted repeat structure suggested its importance in facilitating transfer or perhaps ligation of the ends of the newly transferred DNA strand. Analyses showed that Orf57 (to be called TraX) is the relaxase, which was found to induce a specific nick in the large inverted repeat inside *oriT*; the protein also facilitated site-specific recombination between two *oriT2* sites. Orf53 (to be called TraW) exhibits certain structural similarities to TraG-like proteins, although there is little overall homology.

Accepted 2 April, 2002. *For correspondence. E-mail dclewell@umich.edu; Tel. (+1) 734 763 0117; Fax (+1) 734 763 9905.

Introduction

pAD1 (60 kb) is representative of a widely disseminated family of conjugative plasmids commonly found in clinical isolates of *Enterococcus faecalis*. Bacterial virulence in animal models is associated with a pAD1-encoded cytolysin (Gilmore *et al*., 1994) and a surface protein known as aggregation substance (AS) is associated with the size of vegetations appearing in rabbit endocarditis models (Chow *et al*., 1993), as well as binding to mammalian cells in culture (Kreft *et al*., 1992; Sussmuth *et al*., 2000). AS also plays a significant role in the formation of mating aggregates resulting from a response induced by a peptide sex pheromone, cAD1, produced by recipient (plasmid-free) enterococci (Dunny *et al*., 1979; Galli *et al*., 1989). Regulation of the pAD1 pheromone response has been investigated in some detail (for recent reviews, see Clewell, 1999; Clewell and Dunny, 2002), and the nucleotide sequence has recently been completed (Francia *et al*., 2001).

pAD1 has two origins of transfer (*oriT*) that are separated by about 180° on the circular element (Francia *et al*., 2001). *oriT1* is located within *repA*, which is close to or may overlap the vegetative origin of replication (Weaver *et al*., 1993; An and Clewell, 1997), whereas *oriT2* is in a region of the plasmid containing genes related to conjugation (Francia *et al*., 2001). When a segment containing either of the transfer origins is cloned on the vector plasmid, pAM401, the latter can be mobilized *in trans* by pAD1. A chimera containing *oriT2* is mobilized with an efficiency several orders of magnitude greater than one containing *oriT1* (Francia *et al*., 2001) and is probably the preferred transfer origin on pAD1, at least in intraspecies matings.

Transfer origins (*oriT*'s) and their role in the initiation of conjugative DNA transfer have been investigated in a number of laboratories, with the focus being mainly on Gram-negative systems (for reviews, see Lanka and

Wilkins, 1995; Firth *et al*., 1996; Zechner *et al*., 2000). Initiation of plasmid transfer generally requires the assembly at the *oriT* site of a protein complex containing a relaxase (nickase) and accessory DNA binding proteins. The relaxase catalyses the cleavage of a specific phosphodiester bond at the *nic* site, within the related *oriT,* during which it becomes covalently linked to the 5′-end of the cleaved strand through a tyrosine residue. Single-stranded DNA is transferred to the recipient cell, and the ends are subsequently re-ligated through the cleaving–joining activity of the relaxase. The process has features of the first round of DNA synthesis during rolling circle replication (Erickson and Meyer, 1993; Pansegrau *et al*., 1993; Waters and Guiney, 1993; Lanka and Wilkins, 1995). 'Relaxosomes' consisting of plasmid DNA–protein complexes have been isolated directly from bacteria or reconstructed *in vitro* whereby nicking may subsequently be activated causing a relaxation of the supercoiled plasmid. 'Relaxation complexes' of this nature were originally identified in *Escherichia coli* in the case of the mobilizable plasmid *Col*E1 (Clewell and Helinski, 1969) and the conjugative element *Col*Ib-P9 (Clewell and Helinski, 1970), although it was several years later that a relationship to conjugative transfer was established (Inselburg, 1977; Warren *et al*., 1978; Willetts and Wilkins, 1984; Pansegrau *et al*., 1990; Wilkins and Lanka, 1993; Lanka and Wilkins, 1995).

Analyses of plasmids from Gram-positive systems have been more recent and have begun to define transfer origins and related relaxosome components. Specific *nic* sites have been identified on the conjugative plasmids pIP501 from *Streptococcus agalactiae* (Wang and Macrina, 1995) and pGO1 from *Staphylococcus aureus* (Climo *et al*., 1996), as well as several mobilizable plasmids such as the streptococcal plasmid pMV158 (Guzman and Espinosa, 1997). Surprisingly, the sequences of the *nic* regions of both pGO1 and pIP501 were shown to be quite similar to *nic* region sequences of a family of IncQ type elements from Gram-negative bacteria (Lanka and Wilkins, 1995; Wang and Macrina, 1995; Climo *et al*., 1996). pMV158 is representative of a new *oriT* family, mainly made up of Gram-positive mobilizable plasmids that replicate by rolling circle mechanisms (Grohmann *et al*., 1997; Guzman and Espinosa, 1997). Conservation of DNA sequences around the *nic* is in general also reflected in homologies of their relaxase genes. To date, all known relaxases can be grouped in four families and all have several common motifs (Balzer *et al*., 1994; Pansegrau *et al*., 1994; Zechner *et al*., 2000), with just one exception, the recently published relaxase of CloDF13 (Nunez and de la Cruz, 2001).

The recent completion of the nucleotide sequences of both pAD1 (Francia *et al*., 2001) and another sex pheromone-responding plasmid pAM373 (De Boever *et al*., 2000) did not reveal any determinants bearing significant homology with known relaxases, although these two plasmids resembled each other over many of the structural genes related to conjugation. In this communication, we report on an investigation of the *oriT2* site of pAD1 including identification of the specific nick site and show that a similar site is present in pAM373. We also show in the case of pAD1 that determinants on either side of the *nic* site are necessary for conjugation, and one, *orf57*, encodes the specific nicking enzyme. In addition, Orf57 is shown to facilitate a site-specific recombinational event between *oriT2* sites on two replicons, resulting in a cointegrate structure.

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1.

Results

Characterization of oriT2 *in pAD1 and a similar sequence in pAM373*

The identification of *oriT2* (Francia *et al*., 2001) was based on the mobilization of a pAM401 chimera (pAM8100) carrying a cloned 0.7 kb segment of pAD1 by a pAD1::Tn*917* derivative (pAM714) with wild-type conjugation properties. To determine the minimal size of the mobilization-enabling region, we constructed and examined deletions of pAM8100 (Figs 1 and 2). Constructs were tested for their ability to facilitate mobilization of the vector-encoded *cat* determinant (Cm-resistance) from RecA-negative donors (UV202) in short (10 min) matings in broth after a 90 min exposure to sex pheromone cAD1. The smallest mobilizable fragment was that of pAM8103, which corresponded to a 285 bp segment providing a high efficiency of transfer. As seen in Fig. 2, pAM8103 contains a group of five direct repeats of 6 bp each with 5 bp spacing, followed by a large inverted repeat over a 141 bp span. (Other repeats are seen outside the critical region as shown in Figs 1 and 2.) Figure 3A illustrates a 'folded', single-strand view of the inverted repeat. Both the direct-repeat and the inverted repeat regions are essential as deletion of either of these (e.g. pAM8104 or pAM8105) results in loss of transfer activity (Fig. 1).

Interestingly, a site with sequences resembling the large inverted repeat is also present in the *Enterococcus faecalis* pheromone-responding plasmid pAM373 (De Boever *et al*., 2000) (see Fig. 2 (pAM8300) and Fig. 3B). To determine if the region in pAM373 also functions as an origin of transfer, a representative segment (447 bp) from this plasmid was generated by polymerase chain reaction (PCR) and cloned in pAM401 giving rise to pAM8301. The latter was readily mobilized by a pAM373::Tn*917lac* derivative, pAM4020, with wild-type transfer properties as shown in Fig. 1. Mobilization was specific, as a clone containing the pAM373-related *oriT* was not mobilized by the

Table 1. Bacterial strains, plasmids and oligonucleotides used.

	Relevant features	Reference
Strains:		
E. faecalis		
JH2-2	*rif*, *fus*	Jacob and Hobbs (1974)
FA2-2	*rif*, *fus*	Franke and Clewell (1981)
UV202	*rif*, *fus*, *recA*$^-$	Yagi and Clewell (1980)
S. aureus		
879R4S	*str*, cryptic plasmid, 12.5 kb	Schaberg *et al*. (1982)
E. coli		
DH5α	*end*A1 *rec*A1 *gyr*A96 *thi*-1*hsd*R17 *sup*E44 *rel*A1 Δ(argF-lacZYA)U169	
ϕ80lacZΔM15	BRL	
BL21(DE3)	F$^-$ ompT r_b^- m_b^- DE3	Invitrogen
BL21pLysS	BL21(DE3) derivative	Invitrogen
Plasmids:		
pAM714	pAD1::Tn*917*, Hly-Bac *erm*; wild-type mating properties	Ike and Clewell (1984)
pAM307	pAD1::Tn*917* in *Eco*RI H fragment, *erm*, non-haemolytic; wild-type mating properties	Clewell *et al*. (1982)
pAM373	Encodes response to cAM373 pheromone	Clewell *et al*. (1985)
pAM4020	pAM373::Tn917, *erm*; wild-type mating properties	De Boever *et al*. (2000)
pAM401	*E. coli-E. faecalis* shuttle; *cat tet*	Wirth *et al*. (1986)
pMGS100	*E. coli-E. faecalis* shuttle; *cat tet*; Bac promoter	Fujimoto and Ike (2001)
pMSP3535VA	*E. coli-E. faecalis* shuttle; Km^r; Nis promoter	Bryan *et al*. (2000)
pMSP3545	*E. coli-E. faecalis* shuttle; *erm*; Nis promoter	Bryan *et al*. (2000)
pDL278	*E. coli-E. faecalis* shuttle; *spc*	Tenover *et al*. (992)
pET30b	*E. coli* expression vector	Novagen
pASK60	*E. coli* expression vector	Biometra
pSU18	*E. coli* cloning vector, *cat*, p15A	Bartolome *et al*. (1991)
pTAd	*E. coli* cloning vector, Amp^r, Km^r, colE1	Clontech
pAM3314	pAM401 with cloned 504 bp PCR fragment carrying *oriT1*	An and Clewell (1997)
pAM8100	pAM401 with cloned 779 bp PCR fragment carrying *oriT2*	Francia *et al*. (2001)
pAM8101	pAM401 with cloned 573 bp PCR fragment carrying *oriT2*	This study
pAM8102	pAM401 with cloned 425 bp PCR fragment carrying *oriT2*	This study
pAM8103	pAM401 with cloned 285 bp PCR fragment carrying *oriT2*	This study
pAM8104	pAM401 with cloned 153 bp PCR fragment carrying the direct repeats from *oriT2*	This study
pAM8105	pAM401 with cloned 198 bp PCR fragment carrying the invert repeat from *oriT*2	This study
pAM8106	pAM401 with cloned 334 bp PCR fragment carrying the direct repeats from *oriT2* and the inverted repeat from *oriT373*	This study
pAM8107	pAM401 with cloned 283 bp PCR fragment carrying *oriT2*, CG deletion	This study
pAM8108	pAM401 with cloned 283 bp PCR fragment carrying *oriT*2, GC deletion	This study
pAM8110	pAM401 with cloned 285 bp PCR fragment carrying *oriT2*, 4 bp substitutions	This study
pAM8111	pAM401 with cloned 233 bp PCR fragment carrying *oriT2*, 52 bp deletion	This study
pAM8112	pAM8111 plus additional 14 bp deletion	This study
pAM8113	pAM401 with cloned 198 bp PCR fragment carrying *oriT2*, 87 bp deletion	This study
pAM8114	pAM8111 plus additional 22 bp deletion	This study
pAM88	Suicide vector, pSU18 with cat (Gram+) cloned as PCR fragment/*Eco*RI	This study
pAM8121	pTAd with cloned 705 bp from the 5′-end of *orf53* (1–705 bp) (PCR fragment)	This study
pAM8122A	pTAd with cloned internal fragment of *orf53* (742–1516 bp) (PCR fragment)	This study
pAM8122B	pTAd with cloned 1516 bp fragment from the 5′-end of *orf53*, deletion 36 bp, +*Sal*I new restriction site	This study
pAM8123	pAM88 with cloned 1516 bp fragment from the 5′-end of *orf53*, deletion 36 bp, +*Sal*I new restriction site	This study
pAM8124	pTAd with cloned 307 bp from the 5′-end of *orf57* (194–501 bp) (PCR fragment)	This study
pAM8125A	pTAd with cloned 141 bp from the 3′-end of *orf57* (547–687 bp) (PCR fragment)	This study
pAM8125B	pTAd with cloned 448 bp fragment from *orf57* (194–687 bp), deletion 45 bp, +*Sal*I new restriction site	This study
pAM8126	pAM88 with cloned 448 bp fragment from orf57 (194–687 bp), deletion 45 bp, +*Sal*I new restriction site	This study
pAM8128	pAD1 non-haemolytic, recombinant *orf53* insertion mutant	This study
pAM8129	pAD1 non-haemolytic, recombinant *orf57* insertion mutant	This study
pAM8130	pAD1 non-haemolytic, Orf57 15aa in-frame deletion mutant	This study
pAM8131	pAD1 non-haemolytic, Orf53 12aa in-frame deletion mutant (Walker motif)	This study
pAM8132	pET30b with cloned *Bgl*II/*Nco*I fragment carrying Nis promoter	This study
pAM8133	pAM8132 with cloned *Nco*I/*Xho*I fragment carrying *orf57*	This study
pAM8134	pMSP3535VA with cloned *Bgl*II/*Bse*A1 fragment carrying Nis promoter + *orf57*-His tag	This study

Table 1. *cont.*

	Relevant features	Reference
pAM8135	pASK60 with cloned *Not*I/*Sal*I fragment carrying *orf53*	This study
pAM8136	pSU18 with cloned *Eco*RI fragment carrying Bac promoter from pMGS100	This study
pAM8137	pAM8136 with cloned *Not*I/*Hin*dIII fragment carrying *orf53*-Strep tag	This study
pAM8138	pDL278 with cloned *Eco*RI/*Hin*dIII fragment carrying Bac promoter + *orf53*-Strep tag	This study
pAM8150	pTAd with cloned 325 bp PCR fragment containing *oriT2*	This study
pAM8151	pSU18 with cloned 350 bp *Eco*RI fragment (from pAM8150) containing *oriT2*	This study
pAM8152	pSU18 with cloned 350 bp *Eco*RI fragment (from pAM8150) containing *oriT2*, inverse orientation	This study
pAM8155	pET30b with cloned 781 bp PCR fragment carrying *orf57*	This study
pAM8300	pAM401 with cloned 1022 bp PCR fragment carrying *oriT373*	This study
pAM8301	pAM401 with cloned 447 bp PCR fragment carrying *oriT373*	This study

Oligonucleotides:

Name	Sequence (5′-3′)	Plasmid generated using this primer/use
8100/5	GAAACGCACTCGAATGGT	pAM8100, 1, 2, 3, 4, 6, 7, 8, 10, 11, 12 13, 14, run-off assays
8100/3	CAATTTGGGAACATTCCAC	pAM8100
8101/3	TAGGTTTCAAAGTATCACC	pAM8101
8102/3	TTGCTTTGGGCGATAGTCCC	pAM8102
8103/3	TACTCTATTCTGTTTGTCCT	pAM8103, 4, 7, 8, 10, run-off assays
8105/5	AAATTGTAGTGGCGTGTCC	pAM8105
8105/3	CTTCTATCACCTTACTCATTC	pAM8105
8106/B	GGTTCCACCTTTCTTAATTCCACACACTTTCCAGTGGT	pAM8016
8106/C	GGAAAGTGTGTGGAATTAAGAAAGGTGGAACC	pAM8106
8111/3	CCTGACCATTTTCGCTTAATGG	pAM8111, 12, 13, 14
88.5	GGTGCATCTTCCAAGTAAAGT	pAM88
88.3	AATATTATCGACTACATGGAAG	pAM88
8121/5	TTGTTTAACAATGGCTTGCTACAAC	pAM8121
8121/3	GGTCGACTGCGGGAACAAGAACATGCTG	pAM8121
8122/5	GGTCGACCTTGTCCAACACGCAGTC	pAM8122
8122/3	TGGCATACAAATAAAACGGTG	pAM8122
8124/5	GGAATGTTCCCAAATTGGA	pAM8124
8124/3	GGTCGACCCACTCTACTTCTCGTCC	pAM8124
8125/5	GGTCGACCAGGGTGTGTTTCCAAAATAC	pAM8125
8125/3	GTACTCGTCCCCTTCTTTGTC	pAM8125
8133/5	CCCCATGGAGGAGGTGATACTATGAAACCATTAC	pAM8133
8133/3	CCCCTCGAGCTCCCAATTAATGAATTTATC	pAM8133
8135/5	TTGCGGCCGCCATGTTTAACAATGGCTTGCTACAAC	pAM8135
8135/3	GGTCGACTTTTTGAATTACTTTTTTGTAACC	pAM8135
8136/5	AGAGCGTCGACTGATTGAA	pAM8136
8136/3	GGGGTACCGTCGATCTTATCGCGATT	pAM8136
8155/5	CCCCATATGAAACCATTACTTTACAATTAC	pAM8155
8155/3	CCCAAGCTTCTCCCAATTAATGAATTTATC	pAM8155
8300/5	GAAAGTTTATGTGCGAAATG	pAM8300, 1
8300/3	TTGTGAACGAGAAATGATTC	pAM8300
8301/3	GGTTGAAAGAGAACGGAAAGAG	pAM8301
#401/A	GAGCAAGAGATTACGCGCAG	Sequencing junctions transconjugants *S. aureus*
#401/B	TGCCGGCCACGATGCGTCC	“
#15	TTGGAGTGAAACAATGGG	“
#21	TGGTTATTACAAAGTTCCA	“
P.E./5.2	GTGTGGAATAATAAATTGTAGTGG	Run-off assays

Hly, haemolysin; Bac, bacteriocin; Nis, nisin; His, histidine; Strep, streptavidin.

pAD1 system nor was the pAD1-related *oriT* mobilized by the pAM373 system. It is noteworthy that the large inverted repeats of the two systems bear significant sequence identity and, whereas the pAM373-sequence corresponding to the direct repeats of pAD1 is totally different, it does contain direct repeats (i.e. six hexanucleotide repeats with 6 bp spacings).

oriT2 *facilitates transfer into* Staphylococcus aureus

Although we have not been able to establish pAD1 in *S. aureus* (unpublished data), an earlier report (Jones *et al*., 1987) suggested transfer occurred at extremely low frequencies (10^{-10}–10^{-9} per recipient). It was not certain whether pAD1 (in our hands) was not able to

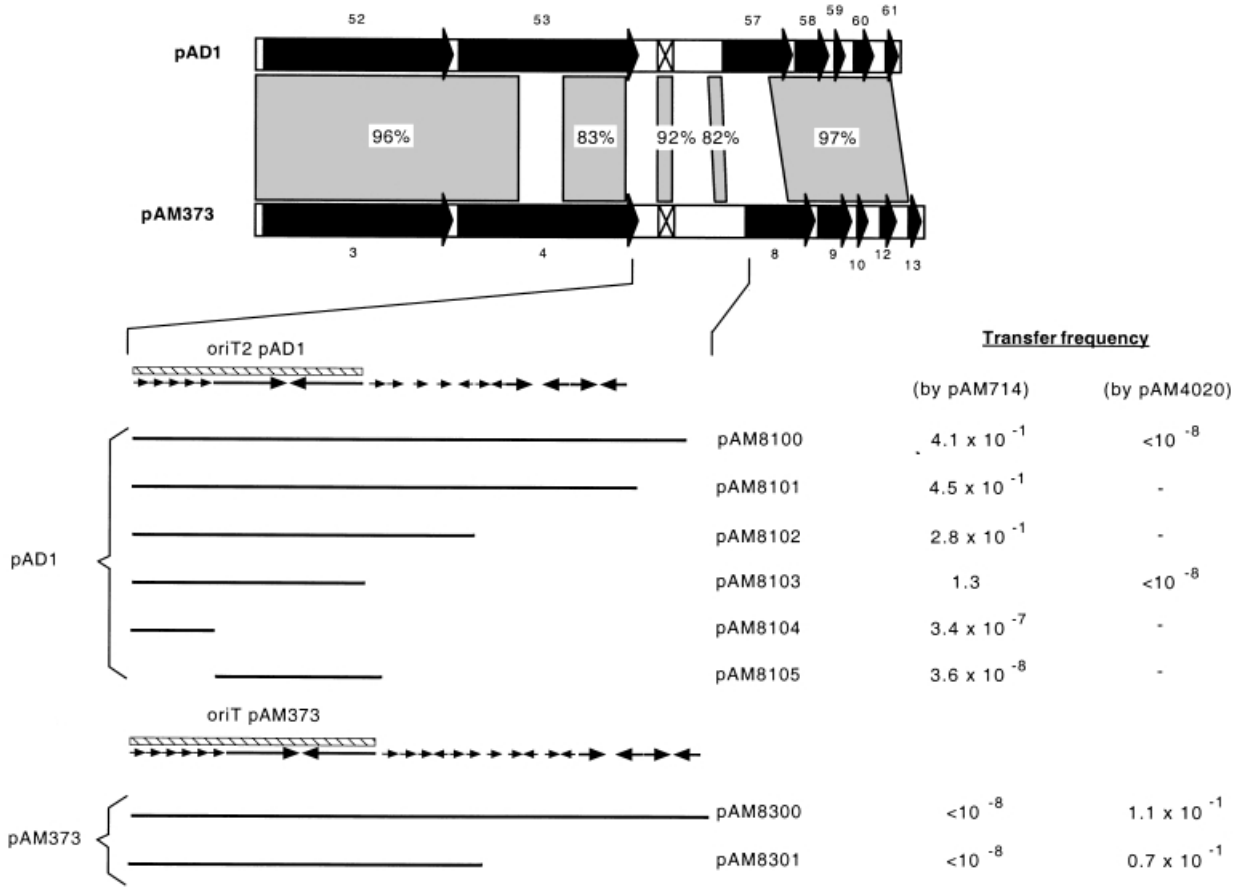

Fig. 1. Comparison of pAD1 and pAM373 *oriT* regions and transfer specificity. The numbers above and below the plasmid maps refer to the ORFs encoded by that region for pAD1 and pAM373 respectively. The degree of identity at the DNA level is shown in the shaded regions. The regions in between (white) showed no significant homology. The linear segments below the map represent the region within pAD1 or pAM373 that was tested in the mobilization experiments. The minimum *oriT* regions are indicated by hatched bars. The small arrows represent direct and inverted repeats present in the intergenic regions. The indicated segments were amplified and cloned in the non-mobilizable shuttle vector pAM401 and placed in *Enterococcus faecalis* UV202 (deficient in homologous recombination) together with pAM714 (pAD1::Tn*917* with wild-type conjugation properties) or pAM4020 (pAM373::Tn*917lac* with wild-type conjugation properties). Matings were conducted using *E. faecalis* OG1SS as the recipient with selection for transconjugants on plates containing spectinomycin and chloramphenicol. The transfer frequencies are indicated as the number of colonies resistant to spectinomycin and chloramphenicol divided by the number of donors (resistance to rifampicin and erythromycin) at the end of the 10 min mating period. The frequencies indicated represent in each case the average of three independent matings.

replicate in *S. aureus* or simply could not transfer into this species. Insofar as the vector pAM401 does replicate in *S. aureus*, we examined the ability of pAM307 (pAD1::Tn*917* (non-haemolytic) with wild-type transfer properties) to mobilize pAM401 chimeras carrying *oriT1 (*pAM3314) or *oriT2 (*pAM8103). As shown in Table 2, only pAM8103 could be mobilized from *E. faecalis* to *S. aureus* in overnight filter matings, and this occurred only when synthetic pheromone (cAD1) was present. Cm-resistant transconjugants appeared at approximately 10^{-3} per donor, and restriction analyses of three isolates using the restriction enzyme *Eco*R1 showed that the pAM8103 was intact in the staphylococcal host (not shown). Er-resistant transconjugants appeared at a frequency two orders of magnitude lower, and these were also Cm-resistant. Analysis of the plasmid content in these cases showed a pattern consistent with a pAM8103::pAM307 cointegrate structure (not shown). The data are consistent with the notion that pAM307 can transfer into *S. aureus* but is not able to replicate; that is, maintenance requires a covalent association (cointegration) with a plasmid (pAM8103 in this case) able to replicate in this host.

Table 2 shows similar results relating to the pAM373 system. Here pAM4020 is able to mobilize the vector chimera pAM8301 carrying the *oriT* of pAM373. Addition of the synthetic pheromone peptide cAM373 significantly enhanced transfer. The fact that some transfer was also observed in the absence of the added peptide is probably due to the fact that *S. aureus* itself produces a

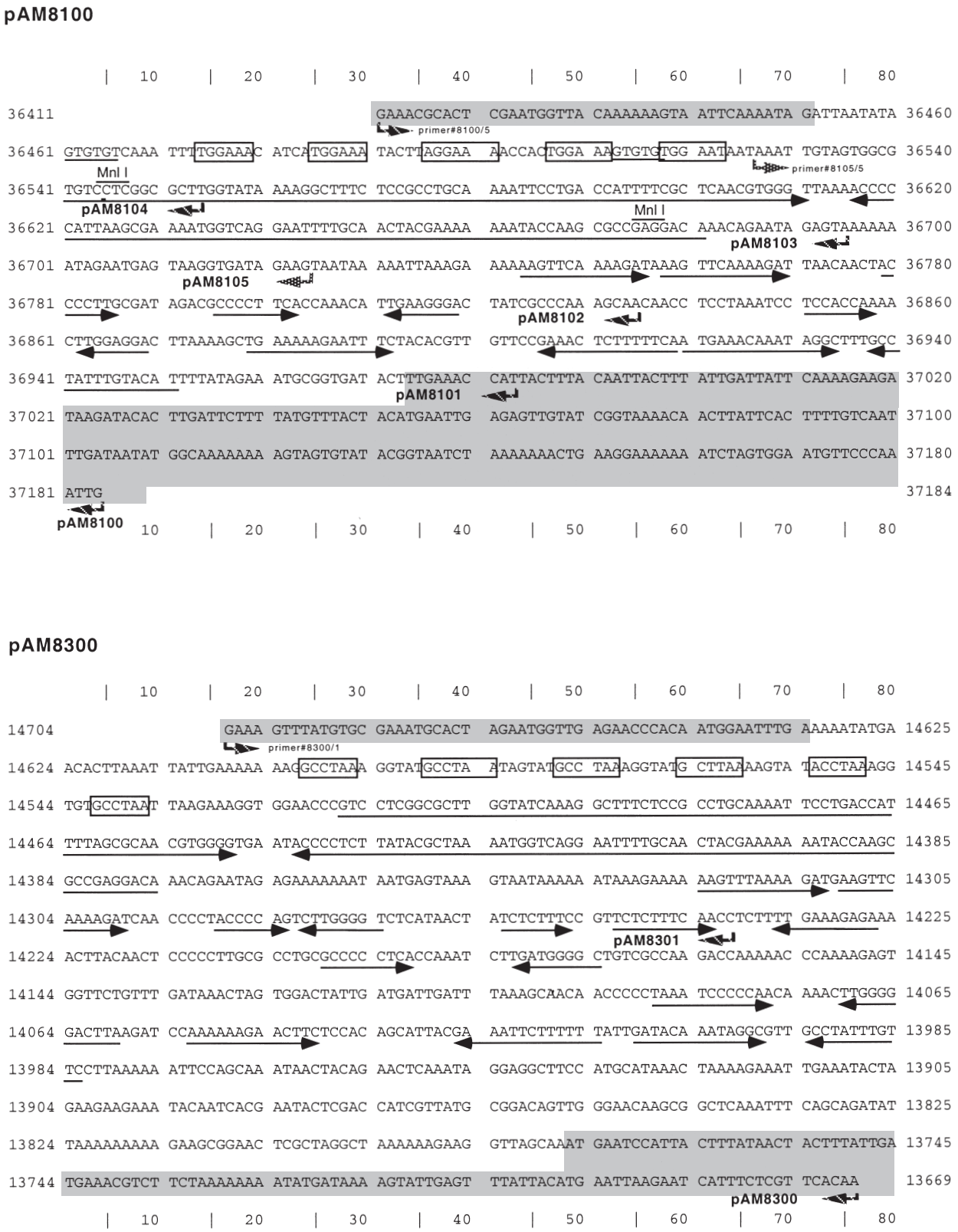

Fig. 2. Nucleotide sequences of *oriT*-containing regions of pAD1 and pAM373 tested in the mobilization experiments. The nucleotide numbers correspond to those published by Francia and colleagues (Francia *et al.*, 2001 and de Boever *et al.*, 2000 for pAM373). Sequences boxed in or underlined with arrows represent direct or inverted repeats. The shaded regions correspond to the 3′-end of *orf53* and the 5′-end of *orf57* in the case of pAM8100, and the 3′-end of *orf4* and the 5′-end of *orf8* in pAM8300.

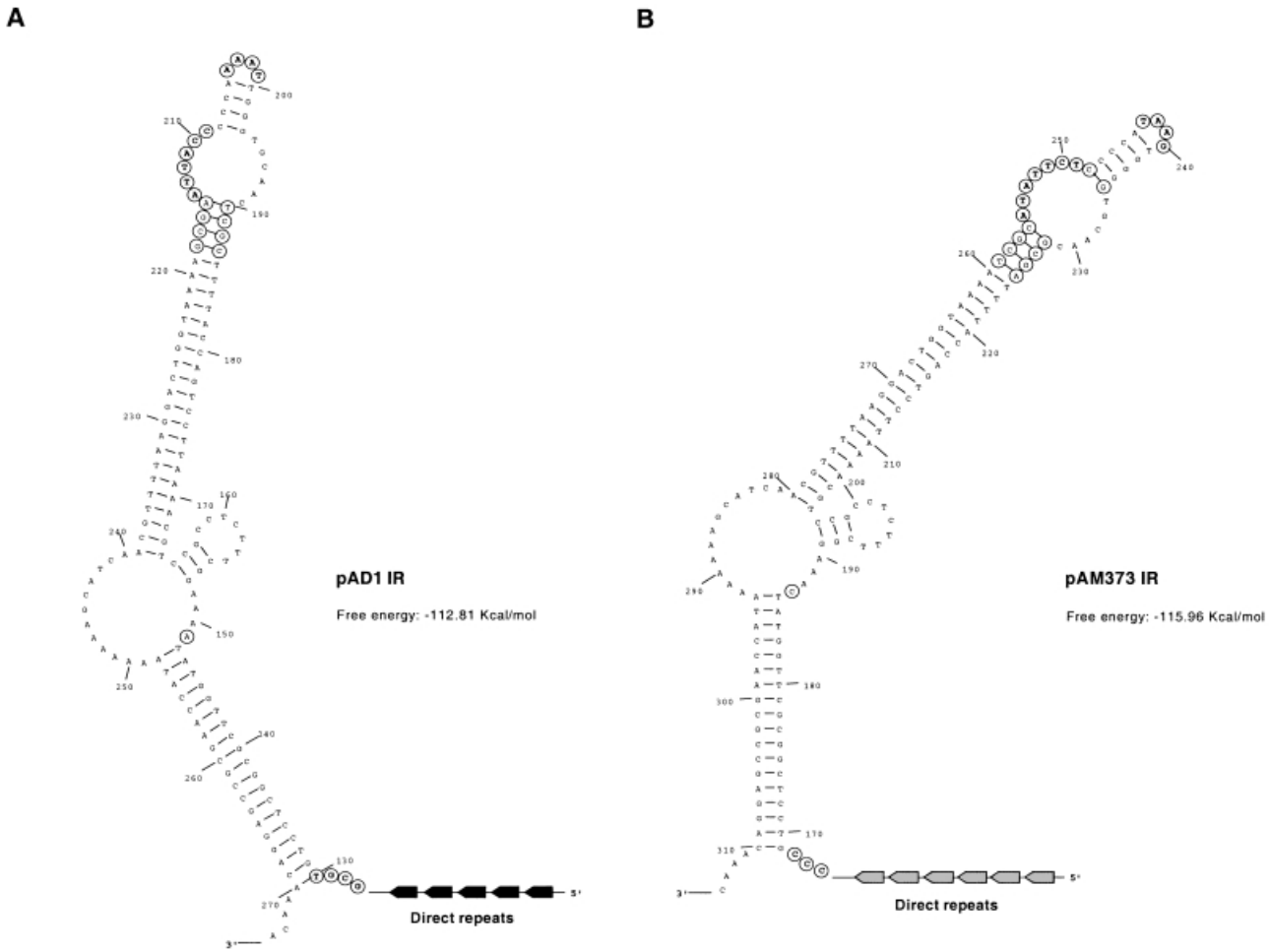

Fig. 3. Folded DNA from the corresponding *oriT* regions of pAD1 and pAM373. The circled nucleotides indicate the differences between the inverted repeat of the two plasmids.

cAM373 activity (Clewell *et al*., 1985). Er-resistant transconjugants, reflecting transfer of pAM4020, occurred at a much lower frequency and was observed only when cAM373 was provided; and these were also Cm-resistant. Analysis of plasmid DNA from three independently isolated transconjugants also revealed the presence of cointegrate structures (not shown).

The data indicate that *oriT2* of pAD1 and the *oriT* of pAM373 are utilized in the movement of these plasmids from enterococci to staphylococci and that a *trans*-acting

Table 2. Transfer frequency *E. faecalis* × *S. aureus*, involving *oriT1* or *oriT2* of pAD1 and *oriT* of pAM373, in the presence or absence of pheromone.

Donor	cAD1/cAM373[a]	Frequency Cm^r/donor[b]	Frequency Er^r/donor[b]
UV202/pAM307, pAM401	–	$<10^{-7}$	$<10^{-8}$
UV202/pAM307, pAM3314	–	$<10^{-7}$	$<10^{-8}$
UV202/pAM307, pAM8103	–	$<10^{-7}$	$<10^{-8}$
UV202/pAM307, pAM401	+	$<10^{-7}$	$<10^{-8}$
UV202/pAM307, pAM3314	+	$<10^{-7}$	$<10^{-8}$
UV202/pAM307, pAM8103	+	1.0×10^{-3}	1.5×10^{-5}
UV202/pAM4020, pAM401	–	$<10^{-7}$	$<10^{-8}$
UV202/pAM4020, pAM8301	–	2.9×10^{-6}	$<10^{-8}$
UV202/pAM4020, pAM401	+	$<10^{-7}$	$<10^{-8}$
UV202/pAM4020, pAM8301	+	8.3×10^{-4}	7.0×10^{-7}

a. +, Pheromone present; –, pheromone absent.
b. The mobilization frequencies indicated for each derivative represent the average of three independent experiments and are expressed as the number of Cm-resistant or Er-resistant transconjugants per donor cell.

product(s) from the parent system is necessary. The inability to observe *oriT1* of pAD1 to facilitate mobilization of the vector chimera implies that this origin at least does not play a selective role in transfer of DNA to the different genus. The possibility that it could facilitate transfer at a frequency below that detectable here cannot be ruled out. (Recall that even in enterococcal matings *oriT1* operates at frequencies several orders of magnitude lower than that of *oriT2*.)

Cointegrate formation involves recombination between oriT *regions*

The above results dealing with transfer of pAD1 DNA (pAM307) from *E. faecalis* to *S. aureus* also implied that cointegrate formation was dependent on *oriT2*, as neither the vector alone nor the *oriT1* clone (pAM3314) resulted in Er-resistant transconjugants. This raised the question of whether cointegrate formation involves a site-specific recombination between the *oriT2* sites of the chimera (pAM8103) and pAM307 that was consistent with the restriction analyses of the transconjugants (above). To further examine this notion we attempted to generate PCR products using primers flanking each of the two expected junctions. An illustrative scheme is shown in Fig. 4A. Figure 4B shows products corresponding to 'left' and 'right' junctions of DNA obtained from three independent Er-resistant transconjugants. Such products were not observed in the case where pAM8103 or pAM307 alone were used as template DNA, and sequence analysis (not shown) confirmed the presence of *oriT2* in each junction (PCR product). Similar PCR products were also obtained from various enterococcal donors (no recipients present) harbouring pAM8103 and a pAD1 derivative and grown in the presence or absence of pheromone. An example is shown in Fig. 4C. Furthermore, the amount of product reflecting each cointegrate junction was significantly greater after exposure of the cells to synthetic cAD1 (compare lane 1 with lane 2, and lane 3 with lane 4). The control amplification products corresponding to the *oriT2* region of the pAD1 derivative (i.e. using primers #15 and #21) were similar regardless of whether or not the cells were exposed to cAD1 (lanes 5 and 6). This indicates that the pheromone-related differences seen for the cointegrate junctions (lanes 1 through 4) were not due to differences in plasmid recovery under the two conditions. Insofar as pheromone is known to induce the synthesis of a number of proteins necessary for conjugation (Clewell, 1993a; b), it is likely that this includes production of one or more products necessary for recognition of *oriT2*. The low level observed in the non-induced donors may reflect the previously identified phase variation phenomena, which reversibly switches on constitutive expression of conjugation functions at a frequency of 10^{-4}–10^{-3} per cell per generation (Pontius and Clewell, 1991; Heath *et al*., 1995). In the case of the pAM373 system, cointegrate formation probably arose by a similar recombination, as no transfer was observed (Table 2) without the presence of the *oriT* segment within the vector and as suggested by the restriction analyses of DNA from Er-resistant transconjugants; however, we did not further address that system.

Transfer of pAD1 requires genes flanking oriT2

Open reading frames (ORFs) previously noted as *orf53* and *orf57* (Francia *et al*., 2001) are located on either side of *oriT2* as indicated in Fig. 1. Interestingly, both are among the few ORFs of the pAD1 conjugation-related proteins that show significant differences when compared with their counterparts in pAM373. Although most of the conjugation genes show >95% identity compared with the pAM373 counterpart, Orf53 and Orf57 are 82% and 59% identical to Orf4 and Orf8 of pAM373 respectively. This and the results presented above suggest a possible relationship of these products to transfer specificity. The *orf53* gene encodes a protein of 747 amino acids (MW 85 562) with a pI of 6.1; the SMART program (Schultz *et al*., 2000) predicts it to have three transmembrane segments in its amino-terminal region, the first of which corresponds to a signal sequence. The predicted Orf53 also has motifs corresponding to ATP-binding sites (Walker *et al*., 1982) and a putative *Fts*K–*Spo*IIE domain (BLAST program). These characteristics are common to 'TraG-like' proteins that are essential for conjugal transfer (Cabezon *et al*., 1997; Gomis-Ruth *et al*., 2001). Database searches revealed homologous ORFs from plasmids in *Streptococcus thermophilus*, *Streptococcus mutans*, *Pseudomonas putida*

Table 3. Conjugation and mobilization frequencies of pAM307 and its *orf53* and *orf57* mutants in the presence or absence of the complementing proteins.

	Transfer frequency[a] (UV202 × OG1SS)	
Plasmid in donors	pAD1 derivatives (Er^r/donor)	pAM8103 (Cm^r/donor)
pAM307 (parent plasmid)	9.0×10^{-2}	NA
pAM8130 (*orf57* mutant)	$<10^{-8}$	NA
pAM8131 (*orf53* mutant)	$<10^{-8}$	NA
pAM307, pAM8103	ND	6.0×10^{-1}
pAM8130, pAM8103	ND	$<10^{-7}$
pAM8131, pAM8103	ND	$<10^{-7}$
pAM8130, pMSP3535VA	$<10^{-8}$	NA
pAM8130, pAM8134	4.1×10^{-2}	NA
pAM8131, pDL278	$<10^{-8}$	NA
pAM8131, pAM8138	6.0×10^{-4}	NA

a. The transfer frequencies indicated for each derivative represent the average of at least two independent experiments and are expressed as the number of Cm-resistant or Er-resistant transconjugants per donor cell.

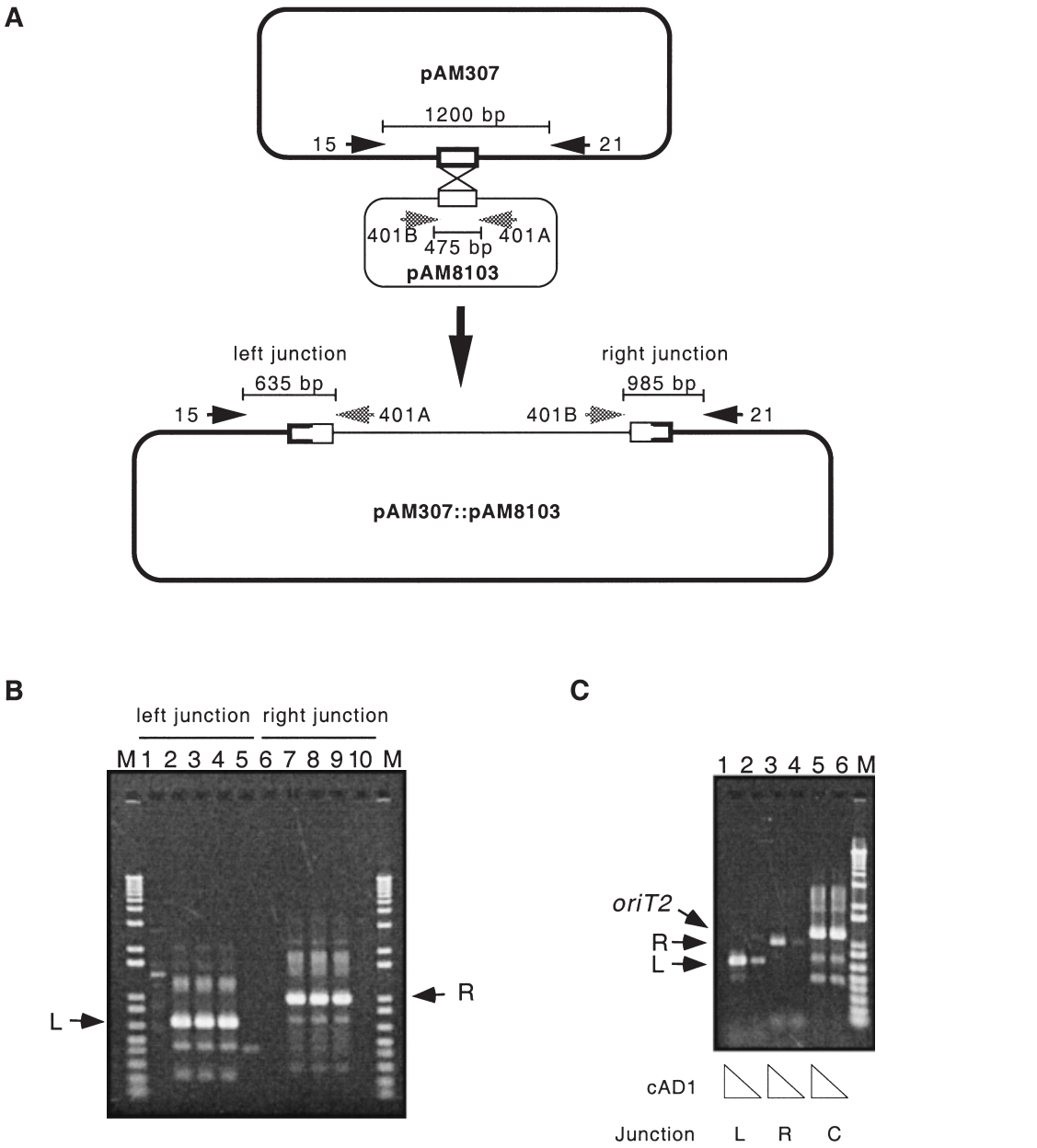

Fig. 4. Site-specific recombination between two *oriT2* sites.
A. Diagram of the process envisioned for cointegrate formation. The related primers used to generate PCR products from the cointegrate structure recovered from Er-resistant transconjugants of *Staphylococcus aureus* are indicated.
B. PCR products representing the left (lanes 2–4) and right (lanes 7–9) junctions of the pAM8103::pAM307 cointegrates recovered from three independent Er-resistant staphylococcal transconjugants. pAM8103 and pAM307 were used as templates for the PCR negative controls for the left and right junctions (lanes 1 and 5, and lanes 6 and 10 respectively). In all cases, 1:100 dilutions of alkaline lysis preparations were used as template DNA for the PCR reaction.
C. PCR products representing the left (lanes 1 and 2) and right (lanes 3 and 4) junctions of pAM8103::pAM714 cointegrates recovered directly from *E. faecalis* UV202 cells grown in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of pheromone (cAD1). DNA from alkaline lysis preparations (dilution 1:1) were used as template for the PCR reaction. Lanes 5 and 6 represent the products from the PCR control in which donor (±cAD1) DNA preparations (dilution 1:100) and oligonucleotides #15 and #21 (Table 1) were used.

and *Salmonella enterica*; and, interestingly, several of them are cited as hypothetical TraG proteins. To determine if Orf53 plays a role in transfer, we generated an in-frame deletion removing the complete Walker motif A (Fig. 5). The mutation was placed in pAM307, a pAD1::Tn*917* derivative defective in production of cytolysin but with wild-type transfer properties. As shown in Table 3, the mutated plasmid pAM8131 completely abolished the ability to transfer and was not able to mobilize pAM8103 (pAM401 vector bearing *oriT2*). Only when

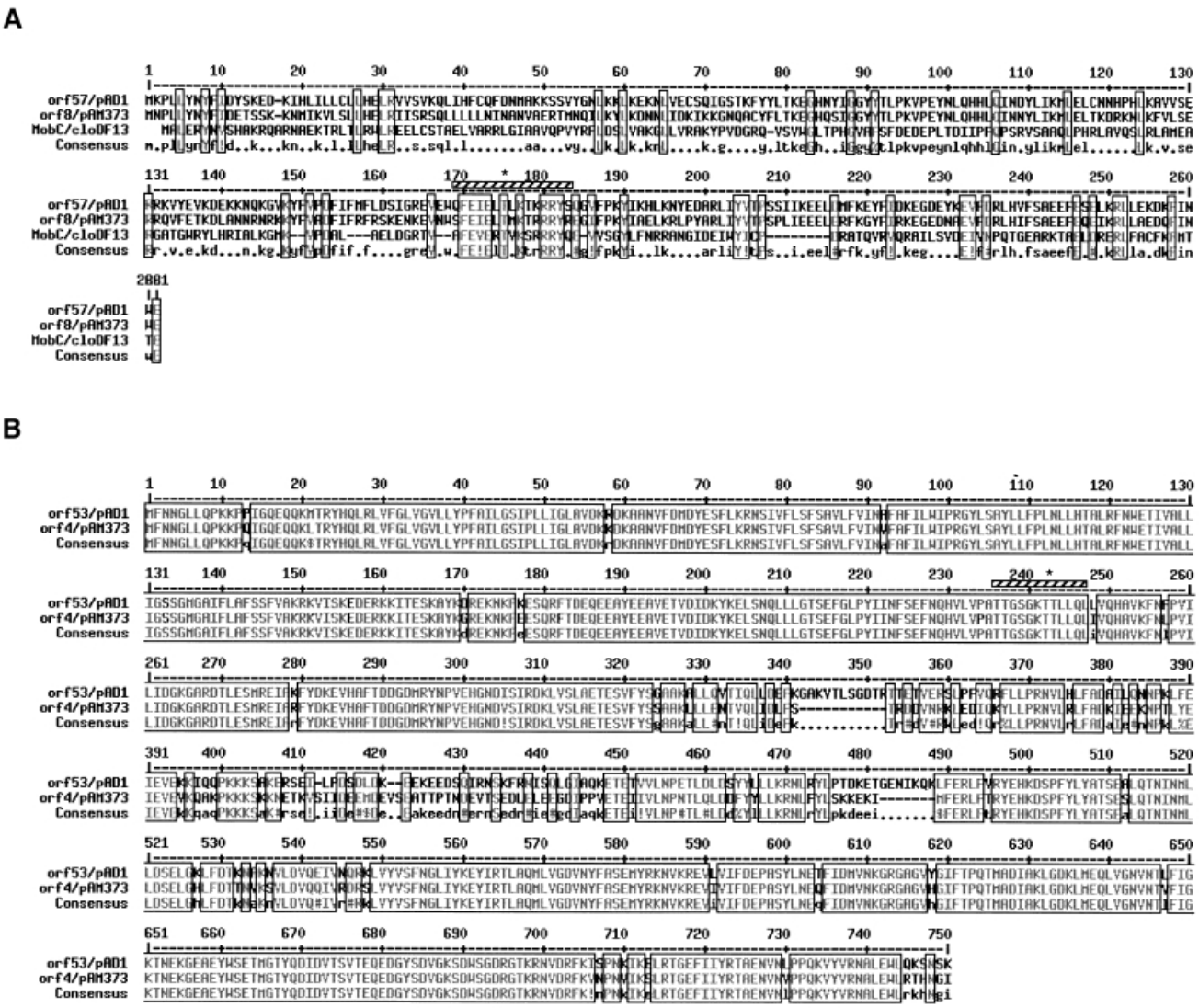

Fig. 5. Sequence comparisons of pAD1 and pAM373.
A. Predicted amino acid sequences of the putative relaxases of pAD1 (Orf57) and pAM373 (Orf8) with comparisons to each other and to MobC of CloDF13. The hatched bar with the asterisk over it indicates the region deleted in the case of the in-frame *orf57* deletion mutant.
B. Comparison of the predicted amino acid sequences for the putative TraG-like proteins of pAD1 (Orf53) and pAM373 (Orf4). The hatched bar with the asterisk over it indicates the region deleted in the case of the in-frame *orf53* deletion mutant.

the complete Orf53 was provided *in trans via* pAM8138 could transfer be partially restored, indicating that this product is essential for pAD1 conjugation.

The product of *orf57 has* a predicted size of 262 amino acids (MW 31 517) and a pI of 8.12. Database searches revealed no homology to known proteins. Interestingly, an internal 21-amino-acid segment is very similar to a portion of the CloDF13 protein MobC (Nunez and de la Cruz, 2001); however, when the total lengths of the proteins are compared there is not a strong resemblance (Fig. 5). MobC has been reported to be a relaxase without significant similarity to other known proteins including previously published relaxases (Nunez and de la Cruz, 2001). An in-frame deletion was generated in pAM307 which eliminated 15 amino acids of the segment of Orf57 common to MobC (Fig. 5) giving rise to pAM8130. The latter was unable to transfer nor could it mobilize pAM8103 (Table 3); however, transfer was almost fully restored when Orf57 was supplied via complementation (pAM8134). Thus Orf57 appears to be essential for pAD1 conjugation.

Genetic analyses of oriT2

As noted above, the large inverted repeat in *oriT2* of pAD1 and the *oriT* of pAM373 exhibit interesting similarities. As seen in Fig. 3, single-stranded forms could take on similar folded structures; however, differences mainly situated in

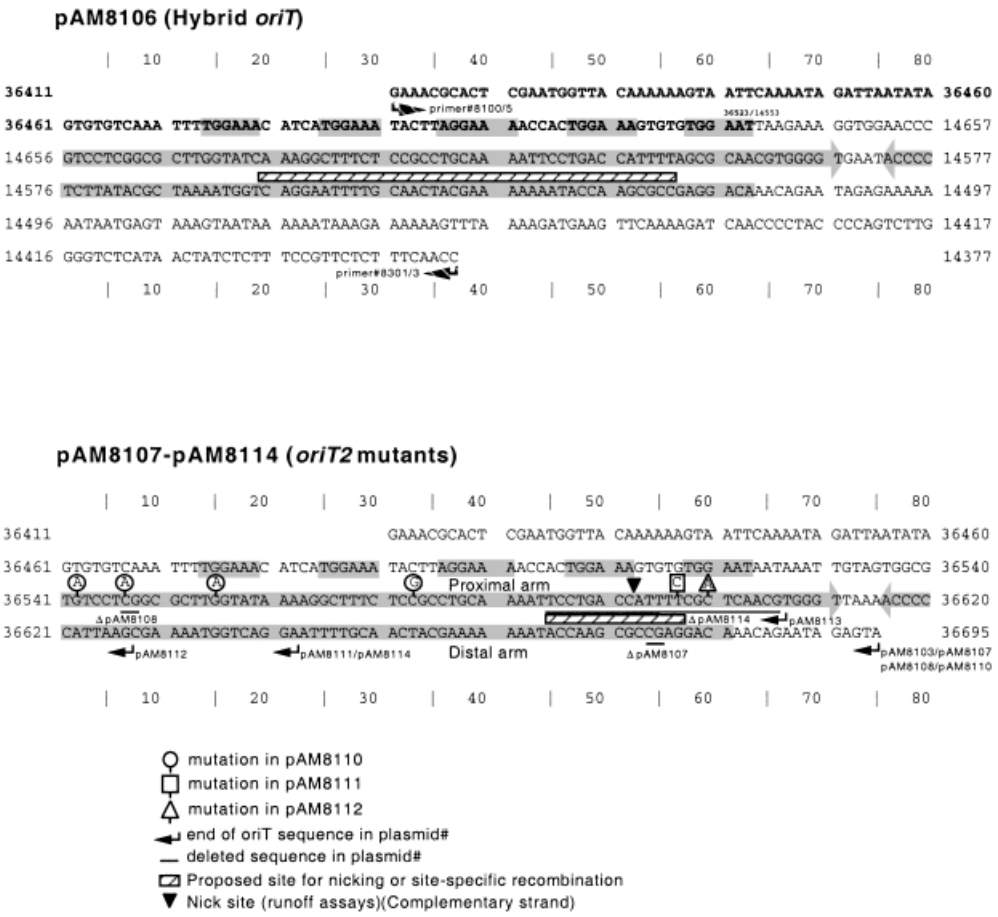

Fig. 6. Nucleotide sequences of the *oriT2/oriT (*pAD1/pAM373) hybrid construct (pAM8106) and the mutations involved in the case of the *oriT2* derivatives assayed in Table 4. The shaded regions represent the segments containing the direct and inverted repeat sequences. The small arrows refer to the location of specific primers used. In the case of pAM8106, the large inverted repeat of *oriT2* was replaced by the corresponding inverted repeat of pAM373. The above hatched bar represents the region where recombination appears to have taken place in the generation of cointegrates similar to that shown in Fig. 4A, but involving pAM8106. The below hatched bar indicates the overlapping region where the *nic* site is proposed to be according to the genetic experiments. The black triangle indicates the *nic* site obtained in the run-off DNA synthesis assays. (Note that the nicked strand is actually the one complementary to the one shown.)

the loops are clearly evident. To determine if these differences could have a role in transfer specificity, a DNA segment representing a hybrid containing the inverted repeat of the pAM373 origin and the direct repeats of the pAD1 *oriT2* was generated (see *Experimental procedures*) and placed in the pAM401 vector, resulting in pAM8106 (Fig. 6). As indicated in Table 4, pAM8106 was mobilized by pAM714 almost as well as (within an order of magnitude) the control with *oriT2 (*pAM8103). However, when the pAM373 system was used (pAM4020), transfer of the hybrid origin was very low (reduced by five orders of magnitude). The data imply that transfer specificity mainly resides in the region containing the direct repeats and that the inverted repeats may be interchangeable with only minor loss of efficiency.

Several vector-*oriT2* derivatives with deletions or base substitutions in the inverted repeat sequences (pAM8107–pAM8114; see Fig. 6 and Table 1) were then examined with respect to their mobilization in the presence of pAM714. The derivatives pAM8107–pAM8110 involved changes in only 2–4 bp, presumably affecting folded conformations and, as seen in Table 4, the slight reductions in transfer frequency do not appear significant. Even in the case of pAM8111, which contains a 52 nt deletion in the arm of the inverted repeat distal to the direct repeats, mobilization was still relatively efficient (Table 4). When larger deletions of the distal arm of this repeat were introduced (pAM8112, 66 nt deletion; and pAM8113, 87 nt deletion), a much greater reduction, about three to four orders of magnitude, was evident; although this level (on the order of 10^{-4} per donor) still represents significant transfer (e.g. compared with non-pheromone-responding plasmids). In the case of pAM8114, however, which contained an additional 22 nt deletion (compared with pAM8111) affecting the proximal arm of the inverted repeat (see Fig. 6), transfer was nearly abolished. The

Table 4. Mobilization frequencies for the derivatives containing base substitutions or deletions in the inverted repeat of the minimal pAD1 *oriT* site along with the hybrid *oriT* site (represented in Fig. 6), into *E. faecalis* OG1SS recipients.

	Transfer frequency (UV202 × OG1SS)[a]	
Plasmid in donors	Cm^r/donor	Er^r/donor
pAM714, pAM8103	1.06	9.5×10^{-2}
pAM714, pAM8106	1.3×10^{-1}	3.0×10^{-1}
pAM714, pAM8107	6.9×10^{-1}	2.5×10^{-1}
pAM714, pAM8108	1.3×10^{-1}	1.4×10^{-1}
pAM714, pAM8110	2.3×10^{-1}	2.0×10^{-1}
pAM714, pAM8111	2.8×10^{-2}	ND
pAM714, pAM8112	3.9×10^{-4}	ND
pAM714, pAM8113	1.2×10^{-4}	ND
pAM714, pAM8114	1.0×10^{-7}	ND
pAM4020, pAM8301	2.2×10^{-1}	0.7×10^{-1}
pAM4020, pAM8106	4.0×10^{-6}	2.2×10^{-1}
pAM4020, pAM8103	$<10^{-8}$	1.2×10^{-1}

a. The mobilization frequencies indicated for each derivative represent the average of at least two independent experiments and are expressed as the number of Cm-resistant or Er-resistant transconjugants per donor cell.

data suggest that the ability to form a hairpin structure involving the two repeats contributes significantly to the ability to transfer, and at least one specific segment of the inverted repeat proximal to the direct repeats is absolutely essential to the process.

In the cases where plasmid chimeras had structures that still allowed for folding identical to that shown in Fig. 3A, mobilization was functionally 'wild-type' (e.g. pAM8103 and pAM8107–pAM8110), and 100% of the transconjugants analysed contained an intact version of the donor plasmid, including the *oriT* site. (Thirty transconjugants of each were examined by restriction analysis, and PCR and sequencing was conducted on several representatives.) However, in cases where the distal arm of the inverted repeat was partially or totally missing (i.e. pAM1112 and pAM1113), 90% of the transconjugants appeared devoid of *oriT2*, judging by the negative PCR results obtained with specific primers. Also, different restriction patterns could be observed suggesting that some reorganization was occurring in the recipient, probably related to difficulty in aligning the 3′- and 5′-ends of the transferred strand for ligation. This percentage was reduced to just 10% in the case of pAM8111. The data are consistent with a role for the inverted repeat in assuring the recircularization of the transferred strand in the recipient.

Identification of nic *site within the* oriT2 *and the involvement of Orf57*

In an effort to determine the location of the transfer initiation site (or *nic* site) of pAD1, we first took advantage of the selection for cointegrate plasmids appearing in *S. aureus* and the site-specific recombination at *oriT* that produced such structures. In addition, we made use of the *oriT* hybrid (pAM8106) (see Fig. 6) which contained the large inverted repeat of the pAM373 origin and the direct repeats of the pAD1 *oriT2* (the differences between the inverted repeat of pAD1 and the one of pAM373 are noted in Fig. 3). Er-resistant transconjugants appearing in *S. aureus* using *E. faecalis* UV202 donors contained cointegrates with distinguishable *oriT* sequences at the plasmid junctions (not shown). Importantly, the sequences of both junctions implied that site-specific recombination occurred inside the proximal arm of the inverted repeat, in the 37 nt region indicated in Fig. 6. This also overlaps partially with the 22 nt segment that was deleted in the non-mobilizable pAM8114 (see above section). The inability of pAM714 to mobilize pAM8114 is consistent with the possibility that a *nic* site necessary for transfer is located within the region that was deleted. And, assuming that the site-specific recombination occurs in the *nic* site as known to be the case for other conjugative systems, it should be located in the 13 nt overlap between positions 36585 and 36597 in Fig. 6.

To determine if a *nic* site is indeed located in this region, we performed 'run-off' DNA synthesis analyses, a sensitive technique detecting DNA strand discontinuities generated *in vivo*. *E. faecalis* cells carrying pAM307 (pAD1::Tn*917* with wild-type mating properties) that had been induced or not with cAD1 and mixed with recipient bacteria were used. (The utilized primer was P.E./5.2.) As shown in Fig. 7A (lane 6), a band corresponding to 78 nt, which terminates at a specific site within the inverted repeat, is clearly evident in the case for the cAD1-induced cells. No band is present when the cells were not exposed to cAD1 (lane 2). The *Taq* DNA polymerase used is known to display terminal transferase activity, adding an additional nucleotide once it reaches the end of the template; thus the cleavage should be located between the T and G as indicated in Fig. 7A. The same site of interruption was observed when a different primer (8100/5) was used (not shown). A primer (8103/3) designed to detect a terminus on the opposite strand did not reveal a run-off product. Parallel experiments were conducted examining cells harbouring pAM8130 (mutated *orf57*) or pAM8131 (mutated *orf53*); and as shown in Fig. 7A, a similar termination site was observed in the case of pAM8131 (*orf53* mutant) but not for pAM8130 (*orf57* mutant) (lanes 7 and 8 respectively). The data imply that Orf57 has specific nicking activity and that the strand cleaved is on the strand complementary to the reading frames of *orf53* and *orf57*.

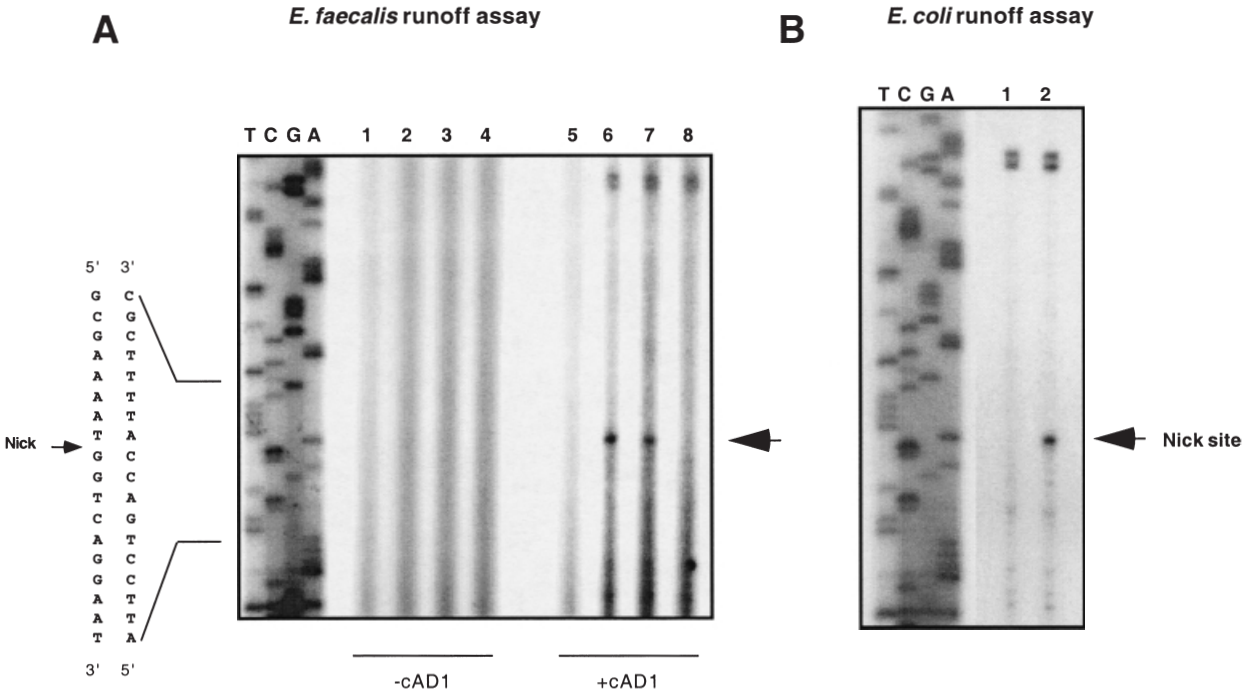

Fig. 7. Location of the specific nick site in *oriT2* of pAD1.
A. *E. faecalis* run-off DNA synthesis assays using the ^{32}P-labelled 'run-off primer' (P.E./5.2). The T, C, G, and A lanes indicate the corresponding sequencing reactions using the fmol DNA sequencing kit. Template DNA was pAM8103 for the sequencing reaction. Cells containing no plasmid (lanes 1 and 5), pAM307 (lanes 2 and 6), pAM8131 (lanes 3 and 7) or pAM8130 (lanes 4 and 8), preinduced with cAD1 (lanes 5–8) or not (lanes 1–4) were processed as described for the run-off assays. The arrow on the right indicates the run-off band whereas the arrow on the left notes the corresponding site within the sequence. [An essentially identical result was obtained using the primer 8100/5 (not shown).]
B. *E. coli* run-off assays. BL21 cells containing pAM8151 and pET30b (lane 1) or pAM8151 and pAM8155 (lane 2) were processed as described in *Experimental procedures*. In both cases, the cells were preinduced with 1 mM IPTG. The arrow indicates the band representing the run-off point.

Orf57 appears sufficient to generate cleavage in vivo *in* E. coli

To determine if Orf57 was sufficient for generating the nick within *oriT2*, an examination using *E. coli* was conducted. pAM8155 represents the His-tag expression vector pET30b carrying *orf57*. It was placed together with the pAM8151 plasmid that carries *oriT2* in *E. coli* BL21. Exponentially growing cells preinduced with IPTG were then examined using a run-off DNA synthesis assay in the same manner as described above for *E. faecalis*. Figure 7B, lane 2, shows a band representing the same interruption that was noted for the fully conjugative pAD1 derivative (pAM307) in *E. faecalis*; the control experiment performed with the empty vector plus pAM8151 did not exhibit such a band (lane 1). Western blotting assays (anti His-tag) carried out in parallel showed that only in the case where pAM8155 was used, was Orf57 indeed present (not shown). The data imply that the pAD1 Orf57 protein on its own was able to specifically nick within the *oriT2* site. It should be kept in mind that the excess of Orf57 in the *E. coli* system may suppress the need for accessory proteins that could be necessary for nicking to occur in the original *E. faecalis* system under normal physiological conditions.

Discussion

A characteristic known thus far to occur exclusively in enterococci is the involvement of sex pheromones in the transfer of certain conjugative plasmids (Dunny *et al*., 1978; 1979). Such plasmids, which can transfer at frequencies approaching 100% under optimal conditions, are ubiquitous in *E. faecalis*; and there is evidence that they facilitate the mobilization of resident non-conjugative plasmids as well as enhance the transfer of other self-mobilizing elements such as conjugative transposons (Franke and Clewell, 1981; Clewell and Gawron-Burke, 1986; Clewell, 1990; 1999). pAD1 has been a useful model system for studying the pheromone response and is now yielding significant information directly relating to plasmid DNA transfer. In the work presented here, we

have localized the second *oriT* identified in pAD1 (*oriT2*) to a 285 bp segment between *orf53* and *orf57*, two genes required for plasmid transfer and located near the downstream end of a large group of similarly oriented structural genes relating to conjugation. We identified a structurally similar *oriT* site in pAM373 between *orf4* and *orf8*, homologues of *orf53* and *orf57* respectively. Both sites were shown to facilitate transfer to *S. aureus*, and analyses of the pAD1 *oriT2* sequence revealed the precise nicking site. Orf57 was found to be the relaxase that cleaves within the *nic* site, whereas Orf53 was found to be absolutely required for transfer.

The *oriT* sites in both pAD1 and pAM373 possessed multiple repeats (Fig. 2), a characteristic also found in other bacterial *oriT* regions (Lanka and Wilkins, 1995). A large inverted repeat sequence as well as nearby direct repeats within *oriT2* were shown to be important for conjugation and constituted the minimal *oriT* site; and a similar organization was evident in pAM373. Indeed, the sequences of the two systems are closely related in the inverted repeats, but quite different in the direct repeats. Interestingly, examination of an *oriT2/oriT* (pAD1/pAM373) hybrid construct suggested that plasmid transfer specificity resides mainly in the direct repeats. A schematic representation of the minimal *oriT* is shown in Fig. 8A. A very similar sequence containing a highly homologous inverted repeat near a series of direct repeats (non-homologous) is present in a cryptic plasmid, pER371, in *S. thermophilus* (Solaiman and Somkuti, 1998), although its function as an origin of transfer has not been reported. Similarly, there are sequences present in two plasmid systems in *E. faecalis* V583 (complete genome sequenced by TIGR) that are highly homologous and are likely to serve as *oriT* sites. (Based on extensive homology outside these sites, the V583 plasmids appear closely related to pheromone-responding elements.) Although these five systems exhibit strong similarity in their large inverted repeat, particularly around the *nic* site (*oriT2*), significant homology with other known *oriT* sites from both Gram-positive and -negative systems is not evident. However, a similar arrangement to the one shown in Fig. 8A has been recently reported for the non-conjugative, but mobilizable, Gram-negative plasmid CloDF13 *oriT* (Nunez and de la Cruz, 2001). Only a small region close to the *nic* site of CloDF13 exhibits some sequence similarity to the *nic* sequence of interest in the present study (Fig. 8B).

It is interesting that a sequence organization very similar to the *oriT* structures identified in the present study is present in the double-stranded replication origins (*dso*) of the pMV158 family of rolling circle replication plasmids (del Solar *et al*., 1998). The *dso* of these elements consists of two or three direct repeats (called the *bind* region), a spacer region, and an inverted repeat called the *nic* region or hairpin I. The distance between the conserved *nic* site and the non-conserved *bind* locus ranges between 14 and 95 nt among the different replicons of the family. In the case of pMV158, RepB protein binds *in vitro* to a dsDNA fragment containing the direct repeats. These sequences appear to be essential for plasmid replication *in vivo*, but not for *in vitro* relaxation of supercoiled DNA. Hairpin I of pMV158 is sufficient for the nicking-closing reaction mediated by RepB, which is also able to recognize *in vitro* the *nic* regions of other plasmids of the family (del Solar *et al*., 1998).

A reasonable hypothesis consistent with the data from the pAD1 and pAM373 systems is that the direct repeats serve as specific binding sites for the protein involved in the nicking reaction providing the observed specificity, whereas the nick site would be located in the inverted

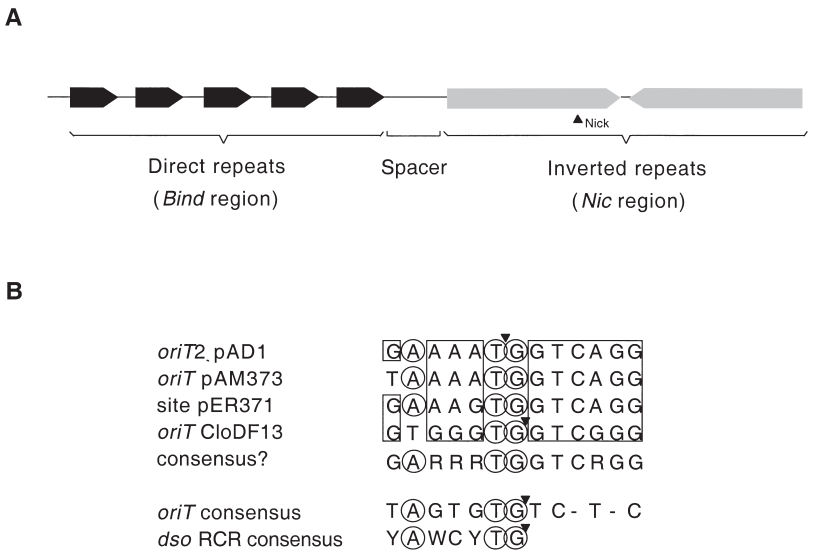

Fig. 8. Map of the minimum *oriT* region and location of *nic* site.
A. Schematic representation of regions in both pAD1 and pAM373 with the key sequences indicated.
B. Comparison of the nucleotide sequences of the *nic* site of pAD1 (*oriT2*), pAM373 (*oriT*), and CloDF13 and a similar region in pER371. A comparison is also made with the consensus *oriT* representative of a variety of other plasmids as well as the consensus site for *dso* of RCR plasmids (Zechner *et al*., 2000). Nick sites are indicated by the black triangles. Circled or boxed nucleotides represent identity or similarity, respectively, with other plasmids. The nick site of pAD1 *oriT2* is on the strand complementary to that containing the sense reading frames of *orf53* and *orf57*.

repeat. Also, it is possible that the inverted repeat could assume a hairpin structure that may be essential to the initiation of DNA transfer. This would be consistent with the 1000-fold drop in the transfer frequency when the distal arm of the inverted repeat was deleted. However, the frequent appearance of aberrant structures among the reduced number of transconjugants that do arise may relate to a role for the inverted repeat in termination of DNA transfer by recircularizing the newly transferred DNA strand. Extensive studies of the *oriT* sites of other plasmids have shown that initiation and termination of transfer are processes requiring different sequence characteristics (Bhattacharjee *et al*., 1992; Zhang and Meyer, 1995; Becker and Meyer, 2000; Furuya and Komano, 2000), reflecting the generally accepted model maintaining that initiation requires supercoiled dsDNA whereas termination involves ssDNA (Lanka and Wilkins, 1995;de la Cruz and Lanka, 1998). Indeed, termination usually requires an inverted repeat commonly found in the *oriT* in the last portion of the plasmid to be transferred (Bhattacharjee *et al*., 1992; Furuya and Komano, 2000). Thus, the 1000-fold reduction we observed in transfer when the distal arm of the inverted repeat was missing may relate to the inability to terminate efficiently in the recipient.

Five families of *oriT* sites have been noted (Guzman and Espinosa, 1997; Zechner *et al*., 2000) upon comparison of a large variety of origins. Previously studied Gram-positive (conjugative and mobilizable) plasmids can be included in one or another of these five families by sequence similarities close to the nick site. Thus, pIP501 (Wang and Macrina, 1995), pGO1 (Climo *et al*., 1996), pSK41 (Berg *et al*., 1998) and pMRC101 (Dougherty *et al*., 1998) *oriT* sites exhibit similarities to IncQ *oriT*´s, whereas pNZ4000 *oriT* (van Kranenburg and de Vos, 1998) and *oriT1* of pAD1 (An and Clewell, 1997) are highly homologous to the IncP type. The pMV158 *oriT* gave rise to the formation of a new family, that now includes mainly Gram-positive mobilizable RCR plasmids (Guzman and Espinosa, 1997); and several recently identified Gram-negative members (Smith and Parker, 1998; Szpirer *et al*., 2001) are also associated with this family. *oriT2* of pAD1 and *oriT* of pAM373 are not similar to any of these groups. Figure 8B shows a comparison to the *oriT* consensus sequence for all five *oriT* families and to the consensus sequence of the *dso* of RCR plasmids (Zechner *et al*., 2000). The only similarities relate to the centrally located TG site and an A, located four residues away, that interestingly, represent the highest conserved nucleotides when compared with the *nic* sites in both *oriT*´s and *dso*'s (Fig. 8B). It would appear that the group consisting of pAD1, pAM373, pER371 and CloDF13 represents a new family of *oriT* sites, also reflecting the existence of specific relaxases.

To date, conjugative relaxases have been classified in four groups, according to conservation of their amino acid sequences and of their target *nic* sites (Zechner *et al*., 2000). Based on the high degree of homology with Orf57 of pAD1, Orf8 of pAM373 is also likely to represent the specific relaxase for that plasmid. Orf57 and Orf8 have no significant homologues in the database other than with respective determinants on plasmids in the recently sequenced *E. faecalis* V583 genome. They do not have the '3-histidine motif' characteristic of other relaxases and apparently essential for relaxase activity, nor does MobC, the functional relaxase of CloDF13 (Nunez and de la Cruz, 2001). Interestingly, when they are compared with MobC, there is a 21-amino-acid segment that is conserved but, whereas the sizes of Orf57 and MobC are very similar, significant homology over the entire reading frames is not evident. In addition, their hydrophilicity profiles do not closely resemble each other outside the 21-residue segment they have in common. The present study showed that an in-frame deletion (15 amino acids, including a tyrosine residue) in this region eliminated the nicking function of Orf57. This possibly suggests an important function for these amino acids at or near an active site of the protein, although we cannot rule out that the mutation inactivated the protein via a major conformational change. The ability of Orf57 to facilitate site-specific recombination between *oriT* sites, as revealed in the present work by the cointegration phenomena associated with transfer from *E. faecalis* to *S. aureus*, is not unique in that other relaxases are known to exhibit such an activity (Broome-Smith, 1980; Llosa *et al*., 1994).

Although Orf53 was found to be required for pAD1 transfer, it was not necessary for nicking; and it is likely that this is the case for Orf4 of pAM373. The TraG-like characteristics present in these proteins suggest they could be the 'coupling proteins' that interact with both DNA processing (relaxosome) and mating-pair-formation functions specific for each system, similar to the situation observed for other well characterized conjugative plasmids (Balzer *et al*., 1994; Cabezon *et al*., 1997; Errington *et al*., 2001; Gomis-Ruth *et al*., 2001). It is conceivable therefore that Orf53 interacts with Orf57 or accessory proteins forming the pAD1 relaxosome at some point during DNA transfer. It is interesting that MobB of CloDF13, the proposed TraG-like protein in that system, is required for nicking at *oriT*, suggesting that the nature of such interactions in the two systems may be different (Nunez and de la Cruz, 2001). The fact that the Orf53 in-frame deletion mutant was not fully complemented *in trans* may imply that the protein functions as a multimer and that the mutant protein had a dominant-negative effect. A similar behaviour was observed in the complementation studies of TrwB, the TraG-like protein of the Gram-negative plasmid R388 (Moncalian *et al*., 1999). According to its

recently solved crystal structure (Gomis-Ruth *et al*., 2001), six equivalent protein monomers would associate to form an almost spherical quaternary structure, with a central channel that would be able to accommodate a single DNA strand.

Our studies provide evidence that when *oriT2* is utilized, pAD1 transfers from donor to recipient as a single strand of DNA conforming to the model established for the conjugative transfer of other Gram-negative and -positive plasmids (Lanka and Wilkins, 1995; Zechner *et al*., 2000), except for *Streptomyces* in which double-strand DNA was recently shown to transfer (Possoz *et al*., 2001). Assuming that the direction of transfer is 5′ to 3′, as in the other systems described so far (Zechner *et al*., 2000), then the *orf53* determinant would enter the recipient first, whereas the relaxase determinant would enter last. Transfer specificity appears to relate to the cognate relaxase as well as its specific recognition site, which is consistent with our data involving the direct repeats in the transfer specificity; however, the studies with the hybrid origin suggest that an additional component is also involved. This relates to the finding that the transferable pAM373 derivative (pAM4020) was able to mobilize the hybrid *oriT (*pAM8106), albeit at a greatly reduced efficiency compared with pAD1, but not the *oriT2 (*pAM8103) of pAD1 (Table 4). Preliminary negative results (not shown) from complementation experiments in which Orf57 was supplied *in trans* to attempt to rescue the mobilization of pAM8103 by pAM4020, are also consistent with the involvement of an additional specificity factor(s). Conceivably Orf53 and/or maybe another protein forming part of the pAD1 relaxosome, will contribute in this regard, as it is the case for other well studied conjugation systems (Cabezon *et al*., 1997; Fekete and Frost, 2000; Hamilton *et al*., 2000). In the future, *orf53* and *orf57* will be referred to as *traW* and *traX* respectively.

Finally, the fact that the transfer origins of both pAD1 and pAM373 facilitate transfer from *E. faecalis* to *S. aureus* is significant from the following perspective. Essentially all *S. aureus* strains produce a cAM373 activity (Clewell *et al*., 1985; Muscholl-Silberhorn *et al*., 1997), and strains of *S. aureus* carrying the plasmid pSK41 produce a cAD1 activity (Firth *et al*., 1994). These peptides represent signal sequence components of specific lipoprotein precursors (Firth *et al*., 1994; Flannagan and Clewell, 2002), similar to the case for the pheromone determinants in *E. faecalis* (Clewell *et al*., 2000; An and Clewell, 2002; Antiporta and Dunny, 2002; Flannagan and Clewell, 2002) and although pAD1 and pAM373 do not appear to replicate in *S. aureus*, their ability to transfer into these organisms may have bearing on future acquisition of vancomycin resistance by staphylococci. Although some vancomycin-resistant staphylococcal isolates have appeared in recent years (Tenover *et al*., 2001), the related determinants are not of the highly evolved *vanA*, *vanB*, etc. variety that have become common in enterococci (Evers *et al*., 1996) and are frequently associated with transposable elements (Arthur *et al*., 1993; Jensen *et al*., 1999; Willems *et al*., 1999). Interestingly, a highly conjugative, cAM373-responding plasmid (pAM368) carrying a *vanA* determinant has recently been identified in a multiply resistant strain of *E. faecalis* (Showsh *et al*., 2001). The passage of such an element, or at least mobilization of a *vanA* determinant, to *S. aureus,* an important human pathogen, may therefore be imminent.

Experimental procedures

Bacterial strains, plasmids, oligonucleotides and reagents

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *E. faecalis* strains were grown in Todd–Hewitt broth (THB) (Difco Laboratories) at 37°C, unless otherwise noted. *E. coli* strains were grown in Luria–Bertani (LB) broth (Sambrook *et al*., 1989). Plating was on THB agar. The following antibiotics were used at the indicated concentrations when using *E. faecalis*: erythromycin, 20 μg ml^{-1}; streptomycin, 500 μg ml^{-1}; kanamycin, 500 μg ml^{-1}; spectinomycin, 500 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; rifampin, 25 μg ml^{-1}; and fusidic acid, 25 μg ml^{-1}. When using *E. coli,* concentrations were: ampicillin 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; erythromycin, 200 μg ml^{-1}; and nalidixic acid, 20 μg ml^{-1}. All antibiotics were obtained from Sigma Chemical Co. Xgal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and IPTG were from Invitrogen and were used at concentrations of 40 μg ml^{-1} and 1 mM respectively. Synthetic cAD1 peptide was prepared at the University of Michigan peptide synthesis core facility.

Plasmid/DNA methodology

Recombinant plasmids were generated in *E. coli* DH5α. Introduction of plasmid DNA into bacterial cells was by transformation as described previously (Hanahan, 1983; Dower *et al*., 1988). Electrotransformation of *E. faecalis* was as described by Flannagan and Clewell (Flannagan and Clewell, 1991). Plasmid DNA was purified from *E. coli* using established techniques described elsewhere (Sambrook *et al*., 1989). Isolation of plasmid DNA from *E. faecalis* was also as previously described (Weaver and Clewell, 1988). When necessary, DNA fragments were purified with silica gel as described by Boyle and Lew (Boyle and Lew, 1995). Recombinant DNA methodology as well as analyses of plasmid DNA using restriction enzymes and agarose gel electrophoresis involved procedures described by Sambrook and colleagues (Sambrook *et al*., 1989). Restriction enzymes were purchased from Invitrogen, and reactions were carried out under the conditions recommended. Polymerase chain reaction (PCR) was performed with a Perkin-Elmer Cetus apparatus under conditions recommended by the manufacturer. Spe-

cific primers were purchased from Invitrogen and *Taq* DNA polymerase from Roche. PCR-generated fragments were purified by using QIAquick-spin columns (Qiagen). Ligations made use of T4 DNA ligase from New England Biolabs. Nucleotide sequence analyses were carried at the University of Michigan sequencing core facility or using the fmol DNA cycle sequencing system as specified by the manufacturer (Promega).

Conjugation experiments

Filter matings were performed as previously described (Clewell *et al*., 1985). Broth matings (in THB) were for 20 min, unless otherwise indicated, after induction of donors for 90 min with synthetic pheromone (5 ng ml^{-1} to 5 μg ml^{-1}, depending on the experiment). Transfer frequencies are expressed as the number of transconjugants per donor cell (at the end of the mating). The plasmid content of transconjugants was verified electrophoretically when appropriate.

Genetic analyses of oriT

Segments of pAD1 containing *oriT2* with various amounts of flanking DNA were amplified by PCR using the oligonucleotides indicated in Table 1 and cloned into pTAd *via* TA cloning. The same strategy was used to construct the deletion derivatives and clones containing the pAM373 *oriT* (see Table 1 for related primers used for each construction). From here *Xba*I–*Bam*HI fragments were then subcloned into the shuttle plasmid pAM401. The derivatives with point mutations were picked up as 'unexpected' variants noticed upon sequencing PCR products as was the deletion relating to pAM8114.

The clone representing a hybrid of the *oriT*´s of pAD1 and pAM373 was constructed as follows. The 128 nt PCR fragment containing the direct repeats of pAD1 was generated using primers 8100/5 and 8106/B and pAM714 as template DNA. The 240 nt fragment containing the inverted repeat of pAM373 was generated using 8106/C and 8301/3 as primers and pAM373 as template. Both fragments were purified using QIAquick-spin columns (Qiagen), diluted 1:1000, mixed and used as a template for a new PCR reaction using 8100/5 and 8301/3 primers; the resulting PCR product (334 bp) was purified and ligated to pTAd. And, as before, a *Xba*I–*Bam*HI fragment was cloned into pAM401 obtaining pAM8106.

All clones were confirmed by sequencing.

Generation of orf53 *and* orf57 *mutants and complementation experiments*

The plasmid pAM88 was used as a suicide plasmid in *E. faecalis* to generate the *orf53* and *orf57* pAD1 mutants. It was constructed as follows. A 1590 bp fragment containing the *cat* determinant of pAM401, was amplified by PCR (primers used shown in Table 1) and cloned into the pTAd vector, via TA cloning. The 1.6 kb *Eco*RI fragment from this plasmid was gel-extracted and ligated into the *Eco*RI site of pSU18 (Bartolome *et al*., 1991), resulting in pAM88.

A mutant of *orf53* containing a 36 bp in-frame deletion substituted with a *Sal*I restriction site was generated as follows. A PCR fragment of 705 bp was generated using primers 8121/5 and 8121/3 (contains added *Sal*I site) and cloned into pTAd (no *Sal*I site in vector) obtaining pAM8121. Another PCR fragment, of 774 bp, was generated using primers 8122/5 (contains added *Sal*I site) and 8122/3 and cloned into pTAd, obtaining pAM8122A. This plasmid was digested with *Sal*I–*Xba*I restriction enzymes and the 0.8 kb segment was cloned into the same sites of pAM8121, obtaining pAM8122B. The 1.5 kb *Xba*I–*Hin*dIII fragment derived from the digestion of this plasmid was then cloned into the vector pAM88 resulting in pAM8123 with a 36-bp deletion in *orf53*.

A similar approach was used to construct an *orf57* mutation with a 45 bp in-frame deletion and again containing a new *Sal*I restriction site. A PCR fragment of 307 bp was generated using primers 8124/5 and 8124/3 (contains added *Sal*I site) and cloned into pTAd obtaining pAM8124. The other PCR fragment of 141 bp used primers 8125/5 (contains added *Sal*I site) and 8125/3 and was cloned in pTAd resulting in pAM8125A. This was then digested with *Sal*I–*Xba*I enzymes and the resulting fragment (≈ 0.2 kb) was cloned in pAM8124 resulting in pAM8125B, which contained the deletion. The 448 bp *Xba*I–*Hin*dIII fragment of pAM8125B was then cloned in pAM88 resulting in pAM8126.

The plasmids used for the complementation experiments were generated as follows. The 0.3 kb *Bgl*II–*Nco*I fragment of pMSP3545 (contains nisin promoter) was cloned into the same sites of pET30b, obtaining pAM8132. A PCR fragment of 0.8 kb containing *orf57* was generated using primers 8133/5 and 8133/3, purified, digested with *Nco*I–*Xho*I, and cloned into the same sites of pAM8132, obtaining pAM8133. This plasmid was digested with *Bgl*II–*Bse*A1, and the resulting 1.2 kb fragment (containing the nisin promoter, the *orf57* gene and the His-tag) was cloned into the *Bgl*II–*Xma*I sites of the pMSP3535VA *E. coli-E. faecalis* shuttle vector, resulting in pAM8134.

The construction of pAM8138 was as follows. A PCR fragment of 2.25 kb containing *orf53* was generated, purified, digested with *Not*I–*Sal*I restriction enzymes and cloned into the same sites of pASK60, generating pAM8135. The 0.2 kb fragment carrying the Bacteriocin 21 promoter was generated by PCR using pMGS100 as template, purified, digested with *Eco*RI, and cloned into the *Eco*RI site of pSU18, obtaining pAM8136. A 2.5 kb *Not*I–*Hin*dIII fragment carrying *orf53*-streptavidin tag (from pAM8135) was cloned into the *Eag*I–*Hin*dIII sites of pAM8136, resulting in pAM8137. This plasmid was digested with *Eco*RI–*Hin*dIII and the 2.7 kb fragment (containing the bac promoter and *orf53*-streptavidin tag) was gel-extracted and cloned into the same sites of the *E. coli-E. faecalis* shuttle vector pDL278, obtaining pAM8138.

The plasmid constructs carrying the mutated *orf53* and *orf57* segments (pAM8123 and pAM8126 respectively) were introduced into *E. faecalis* JH2-2/pAM307 by electrotransformation, and integrants occurring via homologous recombination were selected using chloramphenicol. A representative transformant from each (pAM8128 and pAM8129) was subcultured for several passages in THB without drug and plated on medium containing erythromycin (to maintain pAM307 recombinants). Colonies were then replica-plated on medium containing chloramphenicol, and candidates (erythromycin-resistant but chloramphenicol-sensitive) were screened using PCR amplification (primers 8121/5 and 8122/3 for the *orf53*

mutant, and 8155/5 and 8155/3 for the *orf57* mutant) to obtain DNA to be examined for the presence of the *Sal*I cleavage site. Representative recombinants of pAM307 were designated pAM8130 (*orf57* mutation) and pAM8131 (*orf53* mutation) and confirmed by sequencing.

For the related complementation studies, chimeras representing *orf53* (pAM8138) and *orf57* (pAM8134) were introduced into the strains containing pAM8131 and pAM8130 respectively. The empty vectors, pDL278 and pMSP3535VA, respectively, were used as negative controls.

Determination of the nic *location*

The location of the *nic* site was determined using a run-off DNA synthesis assay (primer extension using Taq polymerase (Roche) coupled with amplification using a Perkin Elmer thermocycler) (Zechner *et al*., 1997). Parallel reactions providing sequence data (used as size marker) were conducted using the fmol DNA cycle sequencing system (Promega) and a DNA template containing *oriT2 (*pAM8103). Bacterial strains cultured overnight were diluted 1:20 for *E. coli* (BL21 derivatives) or 1 : 50 for *E. faecalis* (UV202 derivatives) in fresh media (5 ml) and grown to an optical density of 0.3–0.5 (600 μm). When *E. faecalis* was used, cAD1 (5 μg ml^{-1}) was added, if indicated, during last 90 min of growth. *E. coli* was induced with IPTG (1 mM). A 10-fold excess of *E. faecalis* 'recipients' (OG1SS) was added to culture, and after 5 min the cells were rapidly cooled to 0°C. Aliquots of cultures containing 10–30 × 10^6 colony-forming units (cfu) were collected and added to reaction mixtures as described by Zechner and colleagues (Zechner *et al*., 1997). The conditions for the cycle programme were chosen empirically by first optimizing the yield and specificity of DNA amplification from plasmid-carrying bacteria with the appropriate oligonucleotides. 8100/5 or P.E/5.2 primers were used for the strand that is nicked, and 8103/3 for the complementary strand (see Table 1). The cycle programme was set for 35 cycles as follows: (i) for *E. faecalis*/pAD1, 95°C × 40 s, 55°C × 1 min, and 72°C × 1 min; (ii) for *E. coli*/pAM8151, 95°C × 30 s, 60°C × 30 s, and 72°C × 30 s. The reaction products were treated as described and analysed on 6% polyacrylamide gels containing 6 M urea. Gels were visualized by autoradiography at –70°C for 1–5 d with Kodak X-Omat film and intensifying screens.

Protein analysis

Cells were disrupted with SDS and β-mercaptoethanol and subjected to SDS–PAGE (12%), stained with Coomassie brilliant blue R-250, according to the method of Laemmli (Laemmli 1970). Western blotting was performed as described elsewhere (Sambrook *et al*., 1989), and the His-tag fusion protein (Orf57) detected using polyclonal anti-His antibody (#sc803, Santa Cruz Biotechnology) and the ECL Western Blotting analysis system (Amersham Pharmacia Biotech).

Acknowledgements

This work was supported by National Institutes of Health grant GM33956. M.V.F. was supported in part by a grant from NATO. We thank Gary Dunny for providing the vectors pMSP3535VA and pMSP3545, and Shuhei Fujimoto for the vector pMGS100. We thank all members of our laboratory for helpful discussions.

References

An, F.Y., and Clewell, D.B. (1997) The origin of transfer (*oriT*) of the Enterococcal, pheromone-responding, cytolysin plasmid pAD1 is located within the *repA* determinant. *Plasmid* **37:** 87–94.

An, F.Y., and Clewell, D.B. (2002) Identification of the cAD1 sex pheromone precursor in *Enterococcus faecalis*. *J Bacteriol* **184:** 1880–1887.

Antiporta, M.H., and Dunny, G.M. (2002) *ccfA*, the genetic determinant for the cCF10 peptide pheromone in *Enterococcus faecalis* OG1RF. *J Bacteriol* **184:** 1155–1162.

Arthur, M., Molinas, C., Depardieu, F., and Courvalin, P. (1993) Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* **175:** 117–127.

Balzer, D., Pansegrau, W., and Lanka, E. (1994) Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer of plasmid RP4. *J Bacteriol* **176:** 4285–4295.

Bartolome, B., Jubete, Y., Martinez, E., and de la Cruz, F. (1991) Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* **102:** 75–78.

Becker, E.C., and Meyer, R.J. (2000) Recognition of oriT for DNA processing at termination of a round of conjugal transfer. *J Mol Biol* **300:** 1067–1077.

Berg, T., Firth, N., Apisiridej, S., Hettiaratchi, A., Leelaporn, A., and Skurray, R.A. (1998) Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multiresistance plasmids. *J Bacteriol* **180:** 4350–4359.

Bhattacharjee, M., Rao, X.M., and Meyer, R.J. (1992) Role of the origin of transfer in termination of strand transfer during bacterial conjugation. *J Bacteriol* **174:** 6659–6665.

Boyle, J.S., and Lew, A.M. (1995) An inexpensive alternative to glassmilk for DNA purification. *Trends Genet* **11:** 8.

Broome-Smith, J. (1980) RecA independent, site-specific recombination between ColE1 or ColK and a miniplasmid they complement for mobilization and relaxation: implications for the mechanism of DNA transfer during mobilization. *Plasmid* **4:** 51–63.

Bryan, E.M., Bae, T., Kleerebezem, M., and Dunny, G.M. (2000) Improved vectors for nisin-controlled expression in Gram-positive bacteria. *Plasmid* **44:** 183–190.

Cabezon, E., Sastre, J.I., and de la Cruz, F. (1997) Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. *Mol Gen Genet* **254:** 400–406.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., and Zervos, M.J. (1993) Plasmid-associated hemolysin and aggregation substance production contributes to virulence in experimental enterococcal endocarditis. *Antimicrob Agents Chemother* **37:** 2474–2477.

Clewell, D.B. (1990) Movable genetic elements and antibiotic resistance in enterococci. *Eur J Clin Microbiol Infect Dis* **9:** 90–102.

Clewell, D.B. (1993a) Bacterial sex pheromone-induced plasmid transfer. *Cell* **73:** 9–12.

Clewell, D.B. (1993b) Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*. In: *Bacterial Conjugation.* Clewell, D.B., (ed.). New York: Plenum Press, pp. 349–367.

Clewell, D.B. (1999) Sex pheromone systems in enterococci. In: *Cell-Cell Signaling in Bacteria*. Dunny G.M., and Winans, S.C. (eds). Washington, DC: American Society for Microbiology Press, pp. 47–65.

Clewell, D.B., and Dunny, G.M. (2002) Conjugation and genetic exchange in enterococci. In: *The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance*. Gilmore M.S., *et al*. (eds). Washington, DC: American Soc. Microbiology, pp. 265–300.

Clewell, D.B., and Gawron-Burke, C. (1986) Conjugative transposons and the dissemination of antibiotic resistance in streptococci. *Ann Rev Microbiol* **40:** 635–659.

Clewell, D.B., and Helinski, D.R. (1969) Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc Nat Acad Sci USA* **62:** 1159–1166.

Clewell, D.B., and Helinski, D.R. (1970) Existence of the colicinogenic factor-sex factor *Col*Ib-P9 as a supercoiled circular DNA-protein relaxation complex. *Biochem Biophys Res Comm* **41:** 150–156.

Clewell, D.B., Tomich, P.K., Gawron-Burke, M.C., Franke, A.E., Yagi, Y., and An, F.Y. (1982) Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917. J Bacteriol* **152:** 1220–1230.

Clewell, D.B., An, F.Y., White, B.A., and Gawron-Burke, C. (1985) *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn*918*) *J Bacteriol* **162:** 1212–1220.

Clewell, D.B., An, F.Y., Flannagan, S.F., Antiporta, M., and Dunny, G.M. (2000) Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. *Mol Microbiol* **35:** 246–247.

Climo, M.W., Sharma, V.K., and Archer, G.L. (1996) Identification and characterization of the origin of conjugative transfer (*oriT*) and a gene (*nes*) encoding a single-stranded endonuclease on the staphylococcal plasmid pGO1. *J Bacteriol* **178:** 4975–4983.

de la Cruz, F., and Lanka, E. (1998) Function of the Ti-plasmid Vir proteins: T-complex formation and transfer to the plant cell. In: *Rhizobiaceae.* Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J. (eds). Hingham, MA: Kluwer Academic Publishers, pp. 281–301.

De Boever, E.H., Clewell, D.B., and Fraser, C.M. (2000) *Enterococcus faecalis* conjugative plasmid pAM373: complete nucleotide sequence and genetic analyses of sex pheromone response. *Mol Microbiol* **37:** 1327–1341.

Dougherty, B.A., Hill, C., Weidman, J.F., Richardson, D.R., Venter, J.C., and Ross, R.P. (1998) Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147. *Mol Microbiol* **29:** 1029–1038.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16:** 6127–6145.

Dunny, G.M., Brown, B.L., and Clewell, D.B. (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci USA* **75:** 3479–3483.

Dunny, G.M., Craig, R.A., Carron, R.L., and Clewell, D.B. (1979) Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. *Plasmid* **2:** 454–465.

Erickson, M.J., and Meyer, R.J. (1993) The origin of greater-than-unit-length plasmids generated during bacterial conjugation. *Mol Microbiol* **7:** 289–298.

Errington, J., Bath, J., and Wu, L.J. (2001) DNA transport in bacteria. *Nat Rev Mol Cell Biol* **2:** 538–545.

Evers, S., Quintiliani, R., Jr, and Courvalin, P. (1996) Genetics of glycopeptide resistance in enterococci. *Microb Drug Resist* **2:** 219–223.

Fekete, R.A., and Frost, L.S. (2000) Mobilization of chimeric *oriT* plasmids by F and R100–1: role of relaxosome formation in defining plasmid specificity. *J Bacteriol* **182:** 4022–4027.

Firth, N., Fink, P.D., Johnson, L., and Skurray, R.A. (1994) A lipoprotein signal peptide encoded by the staphylococcal conjugative plasmid pSK41 exhibits an activity resembling that of *Enterococcus faecalis* pheromone cAD1. *J Bacteriol* **176:** 5871–5873.

Firth, N., Ippen-Ihler, K., and Skurray, R.A. (1996) Structure and function of the F factor and mechanism of conjugation. In: *Escherichia Coli and Salmonella*. Neidhard, F.C., Curtis, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Schaechter, M., and Umbarger, H.E. (eds). Washington DC: American Society for Microbiology Press, pp. 2377–2401.

Flannagan, S.E., and Clewell, D.B. (1991) Conjugative transfer of Tn*916. Enterococcus faecalis*: *trans* activation of homologous transposons. *J Bacteriol* **173:** 7136–7141.

Flannagan, S.E., and Clewell, D.B. (2002) Identification and characterization of genes encoding sex pheromone cAM373 activity in *Enterococcus faecalis* and *Staphylococcus aureus*. *Mol Microbiol* **44:** 803–81.

Francia, M.V., Haas, W., Wirth, R., Samberger, E., Muscholl-Silberhorn, A., Gilmore, M.S., *et al.* (2001) Completion of the nucleotide sequence of the *Enterococcus faecalis* conjugative virulence plasmid pAD1 and identification of a second transfer origin. *Plasmid* **46:** 117–127.

Franke, A.E., and Clewell, D.B. (1981) Evidence for a chromosome-borne resistance transposon (Tn*916)* in *Streptococcus faecalis* that is capable of 'conjugal' transfer in the absence of a conjugative plasmid. *J Bacteriol* **145:** 494–502.

Fujimoto, S., and Ike, Y. (2001) pAM401-based shuttle vectors that enable overexpression of promoterless genes and one-step purification of tag fusion proteins directly from *Enterococcus faecalis*. *Appl Environ Microbiol* **67:** 1262–1267.

Furuya, N., and Komano, T. (2000) Initiation and termination of DNA transfer during conjugation of *IncI*1 plasmid R64: roles of two sets of inverted repeat sequences within *oriT* in termination of R64 transfer. *J Bacteriol* **182:** 3191–3196.

Galli, D., Wirth, R., and Wanner, G. (1989) Identification of aggregation substances of *Enterococcus faecalis* after induction by sex pheromones. *Arch Microbiol* **151:** 486–490.

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., and Clewell, D.B. (1994) Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J Bacteriol* **176:** 7335–7344.

Gomis-Ruth, F.X., Moncalian, G., Perez-Luque, R., Gonzalez, A., Cabezon, E., de la Cruz, F., and Coll, M. (2001) The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase. *Nature* **409:** 637–641.

Grohmann, E., Zechner, E.L., and Espinosa, M. (1997) Determination of specific DNA strand discontinuities with nucleotide resolution in exponentionally growing bacteria harboring rolling circle-replicating plasmids. *FEMS Microbiol Lett* **152:** 363–369.

Guzman, L.M., and Espinosa, M. (1997) The mobilization protein, MobM, of the streptococcal plasmid pMV158 specifically cleaves supercoiled DNA at the plasmid oriT. *J Mol Biol* **266:** 688–702.

Hamilton, C.M., Lee, H., Li, P.L., Cook, D.M., Piper, K.R., von Bodman, S.B., *et al.* (2000) TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. *J Bacteriol* **182:** 1541–1548.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580.

Heath, D.G., An, F.Y., Weaver, K.E., and Clewell, D.B. (1995) Phase variation of *Enterococcus faecalis* pAD1 conjugation functions relates to changes in iteron sequence region. *J Bacteriol* **177:** 5453–5459.

Ike, Y., and Clewell, D.B. (1984) Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. *J Bacteriol* **158:** 777–783.

Inselburg, J. (1977) Studies of colicin E1 plasmid functions by analysis of deletions and TnA insertions of the plasmid. *J Bacteriol* **132:** 332–340.

Jacob, A.E., and Hobbs, S. (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes. J Bacteriol* **117:** 360–372.

Jensen, L.B., Hammerum, A.M., Poulsen, R.L., and Westh, H. (1999) Vancomycin-resistant *Enterococcus faecium* strains with highly similar pulsed-field gel electrophoresis patterns containing similar Tn*1546*-like elements isolated from a hospitalized patient and pigs in Denmark. *Antimicrob Agents Chemother* **43:** 724–725.

Jones, J.M., Yost, S.C., and Pattee, P.A. (1987) Transfer of the conjugal tetracycline resistance transposon Tn*916* from *Streptococcus faecalis* to *Staphylococcus aureus* and identification of some insertion sites in the staphylococcal chromosome. *J Bacteriol* **169:** 2121–2131.

van Kranenburg, R., and de Vos, W.M. (1998) Characterization of multiple regions involved in replication and mobilization of plasmid pNZ4000 coding for exopolysaccharide production in *Lactococcus lactis*. *J Bacteriol* **180:** 5285–5290.

Kreft, B., Marre, R., Schramm, U., and Wirth, R. (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infect Immun* **60:** 25–30.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lanka, E., and Wilkins, B.M. (1995) DNA processing reactions in bacterial conjugation. *Annu Rev Biochem* **64:** 141–169.

Llosa, M., Bolland, S., Grandoso, G., and de la Cruz, F. (1994) Conjugation-independent, site-specific recombination at the oriT of the IncW plasmid R388 mediated by TrwC. *J Bacteriol* **176:** 3210–3217.

Moncalian, G., Cabezon, E., Alkorta, I., Valle, M., Moro, F., Valpuesta, J.M., *et al.* (1999) Characterization of ATP and DNA binding activities of TrwB, the coupling protein essential in plasmid R388 conjugation. *J Biol Chem* **274:** 36117–36124.

Muscholl-Silberhorn, A., Samberger, E., and Wirth, R. (1997) Why does *Staphylococcus aureus* secrete an *Enterococcus faecalis*-specific pheromone? *FEMS Microbiol Lett* **157:** 261–266.

Nunez, B., and de la Cruz, F. (2001) Two atypical mobilization proteins are involved in plasmid CloDF13 relaxation. *Mol Microbiol* **39:** 1088–1099.

Pansegrau, W., Balzer, D., Kruft, V., Lurz, R., and Lanka, E. (1990) In vitro assembly of relaxosomes at the transfer origin of plasmid RP4. *Proc Natl Acad Sci USA* **87:** 6555–6559.

Pansegrau, W., Schroder, W., and Lanka, E. (1993) Relaxase (TraI) of IncP alpha plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. *Proc Natl Acad Sci USA* **90:** 2925–2929.

Pansegrau, W., Schroder, W., and Lanka, E. (1994) Concerted action of three distinct domains in the DNA cleaving-joining reaction catalyzed by relaxase (TraI) of conjugative plasmid RP4. *J Biol Chem* **269:** 2782–2789.

Pontius, L.T., and Clewell, D.B. (1991) A phase variation event that activates conjugation functions encoded by the *Enterococcus faecalis* plasmid pAD1. *Plasmid* **26:** 172–185.

Possoz, C., Ribard, C., Gagnat, J., Pernodet, J.L., and Guerineau, M. (2001) The integrative element pSAM2 from *Streptomyces*: kinetics and mode of conjugal transfer. *Mol Microbiol* **42:** 159–166.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaberg, D.R., Clewell, D.B., and Glatzer, L. (1982) Conjugative transfer of R-plasmids from *Streptococcus faecalis* to *Staphylococcus aureus*. *Antimicrob Agents Chemother* **22:** 204–207.

Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P., and Bork, P. (2000) SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res* **28:** 231–234.

Showsh, S.A., De Boever, E.H., and Clewell, D.B. (2001) Vancomycin resistance plasmid in *Enterococcus faecalis* that encodes sensitivity to a sex pheromone also produced by *Staphylococcus aureus*. *Antimicrob Agents Chemother* **45:** 2177–2178.

Smith, C.J., and Parker, A.C. (1998) The transfer origin for *Bacteroides* mobilizable transposon Tn*4555* is related to

a plasmid family from gram-positive bacteria. *J Bacteriol* **180:** 435–439.

Solaiman, D.K., and Somkuti, G.A. (1998) Characterization of a novel *Streptococcus thermophilus* rolling-circle plasmid used for vector construction. *Appl Microbiol Biotechnol* **50:** 174–180.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., and Diaz-Orejas, R. (1998) Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62:** 434–464.

Sussmuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., and Rozdzinski, E. (2000) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect Immun* **68:** 4900–4906.

Szpirer, C.Y., Faelen, M., and Couturier, M. (2001) Mobilization function of the pBHR1 plasmid, a derivative of the broad-host-range plasmid pBBR1. *J Bacteriol* **183:** 2101–2110.

Tenover, F.C., Fennell, C.L., Lee, L., and LeBlanc, D.J. (1992) Characterization of two plasmids from *Campylobacter jejuni* isolates that carry the *aph*A-7 kanamycin resistance determinant. *Antimicrob Agents Chemother* **36:** 712–716.

Tenover, F.C., Biddle, J.W., and Lancaster, M.V. (2001) Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. *Emerg Infect Dis* **7:** 327–332.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1:** 945–951.

Wang, A., and Macrina, F.L. (1995) Streptococcal plasmid pIP501 has a functional *oriT* site. *J Bacteriol* **177:** 4199–4206.

Warren, G.J., Twigg, A.J., and Sherratt, D.J. (1978) *Col*E1 plasmid mobility and relaxation complex. *Nature* **274:** 259–261.

Waters, V.L., and Guiney, D.G. (1993) Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. *Mol Microbiol* **9:** 1123–1130.

Weaver, K.E., and Clewell, D.B. (1988) Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: construction and characterization of *lacZ* transcriptional fusions in a key control region of the plasmid. *J Bacteriol* **170:** 4343–4352.

Weaver, K.E., Clewell, D.B., and An, F.Y. (1993) Identification, characterization, and nucleotide sequence of a region of *Enterococcus faecalis* pheromone-responsive plasmid pAD1 capable of autonomous replication. *J Bacteriol* **175:** 1900–1909.

Wilkins, B.M., and Lanka, E. (1993) DNA processing and replication during plasmid transfer between Gram-negative bacteria. In: *Bacterial Conjugation*. Clewell, D.B. (ed.). New York: Plenum Press, pp. 105–136.

Willems, R.J., Top, J., van den Braak, N., van Belkum, A., Mevius, D.J., Hendriks, G., *et al.* (1999) Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. *Antimicrob Agents Chemother* **43:** 483–491.

Willetts, N., and Wilkins, B. (1984) Processing of plasmid DNA during bacterial conjugation. *Microbiol Rev* **48:** 24–41.

Wirth, R., An, F.Y., and Clewell, D.B. (1986) Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. *J Bacteriol* **165:** 831–836.

Yagi, Y., and Clewell, D.B. (1980) Recombination-deficient mutant of *Streptococcus faecalis*. *J Bacteriol* **143:** 966–970.

Zechner, E.L., Pruger, H., Grohmann, E., Espinosa, M., and Hogenauer, G. (1997) Specific cleavage of chromosomal and plasmid DNA strands in Gram-positive and Gram-negative bacteria can be detected with nucleotide resolution. *Proc Natl Acad Sci USA* **94:** 7435–7440.

Zechner, E.L., de la Cruz, F., Eisenbrandt, R., Grahn, A.M., Koraimann, G., Lanka, E., *et al.* (2000) Conjugative-DNA transfer processes. In: *The Horizontal Gene Pool. Bacterial Plasmids and Gene Spread.* Thomas, C.M. (ed.). The Netherlands: Harwood Academic Publishers, pp. 87–174.

Zhang, S., and Meyer, R.J. (1995) Localized denaturation of *oriT* DNA within relaxosomes of the broad-host-range plasmid R1162. *Mol Microbiol* **17:** 727–735.