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Enterococcus faecalis conjugative plasmid pAM373: complete nucleotide sequence and genetic analyses of sex pheromone response

Erika H. De Boever,[1] Don B. Clewell[1,2]* and Claire M. Fraser[3]

[1]*Department of Biologic and Materials Sciences, School of Dentistry, The University of Michigan, Ann Arbor, MI 48109, USA.*

[2]*Department of Microbiology and Immunology, School of Medicine, The University of Michigan, Ann Arbor, MI 48109, USA.*

[3]*The Institute for Genomic Research, Rockville, MD 20850, USA.*

Summary

pAM373 is a 36.7 kb conjugative plasmid in *Enterococcus faecalis* that encodes a response to a peptide sex pheromone, cAM373, secreted by plasmid-free (recipient) strains of enterococci. It was identified over 15 years ago as one of five plasmids in *E. faecalis* strain RC73 and was of interest because a related pheromone activity could be detected in culture supernatants of *Staphylococcus aureus* and *Streptococcus gordonii*. Because of increased clinical concern relating to the possibility of mobilizing vancomycin resistance determinants from enterococci, where they are becoming common, into pathogens such as *S. aureus*, efforts were initiated to characterize pAM373 further. The results of a complete nucleotide sequence determination of pAM373, as well as a genetic analysis of key genes related to regulation of the pheromone response, are reported here. With regard to determinants related to conjugation, the plasmid has a structural organization similar to other known pheromone-responsive plasmids such as pAD1, pCF10 and pPD1; however, there are several unique features. Although there are significant homologues relating to a pheromone-binding surface protein (TraC) and a negatively regulating protein (TraA), there is an absence of a determinant equivalent to *traB* of pAD1 (reduces endogenous pheromone) and a determinant for surface-exclusion protein. The precursor structure of the inhibitor peptide iAM373 was identified, and its determinant (*iam373*) was found to be about 500 nt upstream of an apparent transcription terminator t1. Tn*917–lac* insertion analyses provided interesting insights into aspects of control of the pheromone response and showed that, although the *traA* product is sensitive to pheromone, it appears to act differently from the *traA* homologue of pAD1.

Accepted 23 June, 2000. *For correspondence at Biologic and Materials Sciences, School of Dentistry, The University of Michigan, Ann Arbor, MI 48109-1078, USA. E-mail dclewell@umich.edu; Tel. (+1) 734 763 0117; Fax (+1) 734 763 9905.

Introduction

Enterococcus faecalis and *Enterococcus faecium* are normally harmless commensals that inhabit the human intestine, but they can act as opportunistic pathogens causing urinary tract infections, bacteraemia and infective endocarditis (Murray, 1990; Moellering, 1992; Jett *et al*., 1994). Enterococci are among the three most common types of bacteria involved in nosocomial infections in the USA (Lewis and Zervos, 1990). Multiple antibiotic resistance, including resistance to the 'last resort' antibiotic vancomycin, is widespread among the enterococci and represents a serious and growing clinical problem; endocarditis infections involving such organisms are associated with high mortality rates (Edmond *et al*., 1996; Huycke *et al*., 1998). The phenomenon of conjugative DNA transfer is particularly common among the enterococci and frequently involves highly transmissible plasmids and/or conjugative transposons, and antibiotic resistance determinants commonly reside on such elements (Clewell, 1990).

A group of highly conjugative plasmids found commonly in enterococci, especially in *E. faecalis*, are those which encode a mating response to small peptide sex pheromones secreted by plasmid-free (recipient) strains (for recent reviews, see Dunny and Leonard, 1997; Maqueda *et al*., 1997; Clewell, 1999). The response by donor bacteria involves synthesis of a plasmid-encoded protein surface adhesin referred to as 'aggregation substance' which facilitates the initial contact with recipient cells. Aggregation substance coats the donor cell surface and adheres to 'enterococcal-binding substance' (EBS) on the recipient surface. There is evidence that EBS corresponds at least in part to lipoteichoic acid (Ehrenfeld *et al*., 1986; Bensing and Dunny, 1993). As EBS is also located on the donor surface, donors exposed to a culture filtrate

of recipients undergo a clumping response, a phenomenon that serves as a convenient assay in the quantification of pheromone activity (Dunny *et al*., 1979).

Once a copy of the plasmid is acquired by the recipient, production of the related pheromone in the transconjugants is shut down or masked; however, unrelated pheromones that induce a mating response by donors bearing different families of plasmids continue to be secreted. Each plasmid encodes a peptide that acts as a competitive inhibitor of the corresponding pheromone and is believed to help prevent self-induction by endogenous pheromone that may be produced in plasmid-containing cells. Some of the best studied pheromone-responding plasmids are pAD1, pCF10 and pPD1, which confer responses to the octa- or heptapetides cAD1, cCF10 and cPD1 respectively; their corresponding inhibitor peptides are designated iAD1, iCF10 and iPD1. The chromosome-encoded pheromone determinants have recently been identified in *E. faecalis* genome databases and show that the peptides represent components of the signal sequences of lipoprotein precursors (Clewell *et al*., 2000).

pAM373 is a relatively small (36.7 kb) pheromone-responding plasmid that was identified as one of at least five plasmids present in the *E. faecalis* clinical isolate RC73 (Clewell *et al*., 1985). It was of interest at that time because, unlike the case for other plasmids, the related pheromone activity (cAM373) detectable in culture filtrates of plasmid-free *E. faecalis* was also produced by *Staphylococcus aureus* but not by coagulase-negative staphylococci. Activity was also produced by *Streptococcus gordonii* (formerly *Streptococcus sanguis*) (strains Challis and G9B) and *Enterococcus hirae* (strain 9790); however, no activity was detected in culture filtrates of a number of other species tested. Filter matings between *E. faecalis* cells carrying pAM373::Tn*917* (encodes erythromycin resistance) and recipient *S. aureus* or *S. gordonii* strains did not give rise to transconjugants, implying that the plasmid was unable to establish in these hosts; however, transfer to *E. hirae* was observed.

The cAM373 peptides from both *E. faecalis* and *S. aureus* have been characterized and found to differ only in their carboxyl-terminal amino acids; the structures were found to be AIFILAS and AIFILAA respectively (Mori *et al*., 1986; Nakayama *et al*., 1996). The inhibitor peptide encoded by pAM373 was determined to be SIFTLVA (Nakayama *et al*., 1995a).

pAM373 is also interesting in that although most other pheromone-responding plasmids have determinants for aggregation substance that are strongly homologous with each other based on hybridization data the determinant on pAM373 appears to be significantly different (Galli and Wirth, 1991; Hirt *et al*., 1996). This suggests possible differences in the mechanism of adherence to recipient cells. Indeed, Muscholl-Silberhorn (1999) has very recently reported that the pAM373 aggregation substance is not only structurally different but also binds to cells defective in EBS.

The initial report of pAM373 15 years ago (Clewell *et al*., 1985) preceded the rapid appearance of vancomycin resistance in enterococci that has occurred during the past decade. Now several classes of highly evolved vancomycin resistance gene clusters (e.g. *vanA*, *vanB*, *vanC*, etc.) can be found associated with clinical isolates of enterococci (Evers *et al*., 1996). Some are found on conjugative plasmids and reside on transposons such as Tn*1546* (Arthur *et al*., 1993; Jensen *et al*., 1999; Willems *et al*., 1999). Thus far, only a few vancomycin-resistant clinical isolates of *S. aureus* have been reported (Hiramatsu *et al*., 1997; Turco *et al*., 1998); these are relatively low levels of resistance and are unrelated to the resistances that have been appearing in the enterococci. Considering the ability of pAM373 to respond to a pheromone that is produced by *S. aureus*, it would seem that this plasmid, or closely related plasmids, could play a significant role in facilitating movement of vancomycin resistance determinants from enterococci into this important human pathogen. Although pAM373 itself may not be capable of replicating in *S. aureus*, an involvement in the mobilization or delivery of other resistance-bearing elements is a real possibility. For this reason and because, when compared with other pheromone-responding plasmids, pAM373 has been reported to have significantly different structural properties based on hybridization, further analyses of this interesting element were initiated. The complete sequence of the plasmid as well as genetic analyses which include identification of determinants relating to regulation of the pheromone response are presented.

Results and discussion

DNA sequence and gene organization of pAM373

Complete nucleotide sequencing of pAM373 revealed a circular molecule consisting of 36 679 bp with a G+C content of 34.7% – similar to that of enterococcal chromosomal DNA. Sequence analyses have identified 48 open reading frames (ORFs) with an average length of 688 bp. These are indicated on the map in Fig. 1; the first G of the unique *Sal*I site (GTCGAC) is designated as nucleotide number 1. Twenty per cent have GTG start sites and another 22% start with TTG. The ORFs are listed in Table 1 with annotations. Database comparisons indicate that there are 37 ORFs with sequences of unknown function; however, 16 of these correspond to homologues of genes on the enterococcal plasmids pAD1 and pPD1 or on the conjugative transposon Tn*916*. Two represent conserved hypothetical proteins in *Streptococcus thermophilis* or *Lactococcus lactis*.

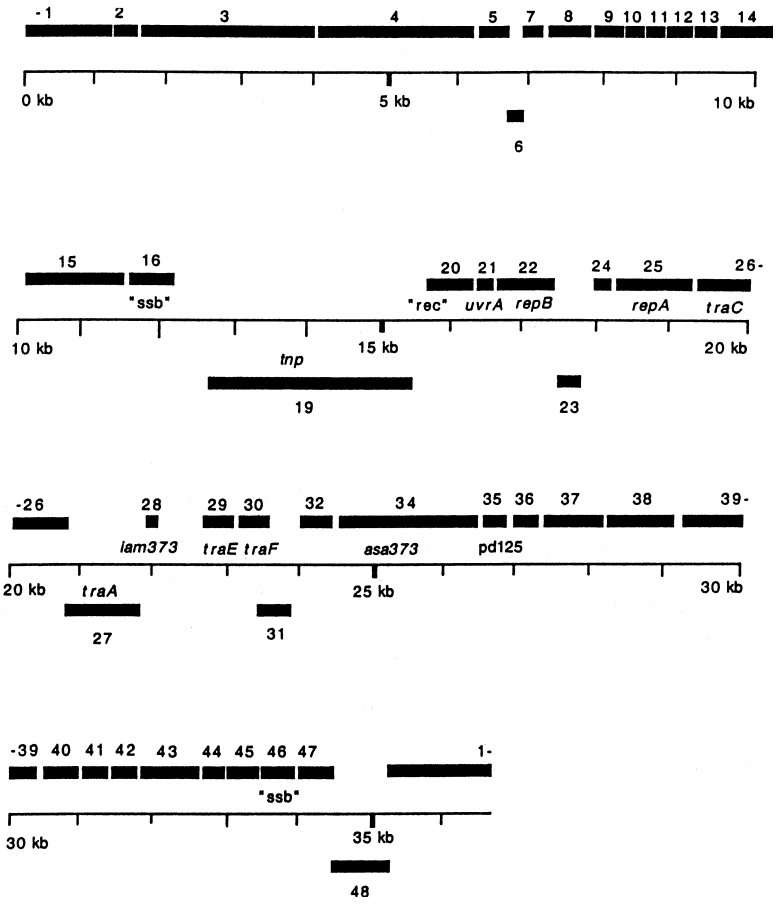

Fig. 1. Map of pAM373. The numbered open reading frames correspond to those listed in Table 1. ORFs shown above the scaled line are orientated 5′ to 3′ as left to right; whereas those below the line are in the opposite orientation. Nucleotide numbering starts with the unique *Sal*I site which is located within *orf1*.

orf26 is a significant homologue of *traC* of pAD1 and is addressed below along with *orf27*, a homologue of *traA* of pAD1, and *orf28* (*iam373*), which encodes the precursor of the pAM373 inhibitor peptide cAM373. Other relevant determinants to be discussed in more detail below in the context of the pheromone response are *orf34* (*asa373*), *orf30* and *orf29*.

orf25 and *orf22* are homologous with the replication/maintenance genes *repA* and *repB*, respectively, of pAD1. This is consistent with our ability to clone a 7.6 kb *Eco*RI fragment (fragment C; Clewell *et al*., 1985) of pAM373 containing these two determinants on the *E. coli* plasmid pVA891 (Macrina *et al*., 1983), giving rise to a chimera that will replicate in *E. faecalis* OG1X (F. An, personal communication). *orf21* bears some resemblance to the pAD1 *uvrA* determinant, and there are two ORFs (*orf16* and *orf46*) that resemble single-stranded DNA-binding proteins of *Bacillus subtilis*.

The pheromone response region

A cluster of regulatory and structural genes involved in the pheromone response is composed of eight putative ORFs which are organized as shown in Fig. 2. This figure also presents a comparison of the overall genetic organization of pAM373 with three other pheromone-responsive plasmids, pAD1, pCF10 and pPD1. Comparative Southern blot hybridization studies, using either whole pAD1 DNA or portions of the plasmid as probes, had previously shown that the general region involved in regulation of the pheromone response contained sequences that were significantly homologous with most pheromone response plasmids, except pAM373 (Hirt *et al*., 1996). The present study, however, demonstrates that this region in pAM373 has certain features that are similar. Several genes, with the exception of the gene encoding aggregation substance, are similar in size to the corresponding genes in pAD1, pCF10 and pPD1 and are in similar orientations. The degree of homology at the amino acid level is markedly weaker than that of genes from other pheromone systems, which explains the lack of hybridization signals reported in the previous studies.

Insertions of Tn*917–lac* into pAM373 were obtained as a result of transposition from the temperature-sensitive plasmid pTV32ts, as described in the *Experimental procedures*. One hundred and fifteen Tn*917–lac* insertions were mapped by comparing restriction enzyme profiles of plasmid DNA preparations with a physical map of wild-type

Table 1. Open reading frames identified in pAM373.

ORF	5′	3′	Amino acids	Identification (organism)	% Identity
1	35561	1371	830	ORF16 of Tn*916 (E. faecalis*)	24
2	1385	1723	113	Hypothetical protein	
3	1744	4086	781	ORF15 of Tn*916 (E. faecalis*)	21
4	4076	6286	737	Hypothetical protein	
5	6378	6653	92	[a]Cons. hypothetical protein (*S. thermophilus*)	71
6	6849	6733	39	Hypothetical protein	
7	6989	7147	53	Hypothetical protein	
8	7147	7929	261	Hypothetical protein	
9	7923	8423	167	Hypothetical protein	
10	8419	8619	67	Hypothetical protein	
11	8580	8750	57	Hypothetical protein	
12	8704	9024	107	Hypothetical protein	
13	9034	9297	88	[a]Cons. hypothetical protein (*L. lactis*)	57
14	9461	10234	258	Hypothetical protein	
15	10228	11565	446	Hypothetical protein	
16	11707	12180	158	SSB protein, putative (*B. subtilis*)	44
17	12272	12180	31	Hypothetical protein	
18	12512	12405	36	Hypothetical protein	
19	15576	12607	990	Transposase of Tn*5401 (B. thuringiensis*)	34
20	15710	16348	213	Recombinase of pSK1 (*S. aureus*)	43
21	16385	16585	67	UvrA of pAD1 (*E. faecalis*)	46
22	16664	17494	277	RepB of pAD1 (*E. faecalis*)	43
23	17469	17828	120	Hypothetical protein	
24	18042	18191	50	Hypothetical protein	
25	18198	19211	338	RepA of pAD1 (*E. faecalis*)	46
26	19277	20863	529	TraC of pAD1 (*E. faecalis*)	39
27	21918	20929	330	TraA of pAD1 (*E. faecalis*)	34
28	22100	22171	24	*iam373*	
29	22809	23144	112	TraE of pPD1 (*E. faecalis*)	62
30	23262	23570	103	TraF of pPD1 (*E. faecalis*)	91
31	23875	23597	93	Hypothetical protein	
32	24061	24381	107	ORF9 of pPD1 (*E. faecalis*)	84
33	24413	24511	33	Hypothetical protein	
34	24511	26475	655	[a]Agg. substance Asa1 of pAD1 (*E. faecalis*)	24
35	26548	26970	141	pd125 protein of pPD1 (*E. faecalis*)	100
36	27022	27279	86	ORF6 of pAD1 (*E. faecalis*)	97
37	27293	28168	292	ORF7 of pAD1 (*E. faecalis*)	86
38	28191	29048	286	ORF8 of pAD1 (anchor. protein) (*E. faecalis*)	96
39	29077	30345	423	ORF9 of pAD1 (*E. faecalis*); TraG (*S. aureus*)	85
40	30351	30965	205	ORF10/11 of pAD1 (*E. faecalis*)	65/78
41	31036	31365	110	ORF12 of pAD1 (*E. faecalis*)	72
42	31368	31679	104	ORF13 of pAD1 (*E. faecalis*)	87
43	31864	32706	281	ORF14 of pAD1(*E. faecalis*)	96
44	32699	32971	91	ORF15 of pAD1 (*E. faecalis*)	63
45	32974	33360	129	ORF16 of pAD1 (*E. faecalis*)	75
46	33527	34000	158	SSB protein (*B. subtilis*)	52
47	34035	34601	189	Hypothetical protein	
48	35516	34545	324	Hypothetical protein	

a. Cons, conserved; Agg, aggregated.

pAM373 (Clewell *et al*., 1985). In all cases, the transposon was linked to the plasmid and no chromosomal insertions were observed. The majority of the insertions occurred in one or the other of two *Eco*RI fragments (fragment A or C; see Clewell *et al*., 1985) with a significant clustering over a 2 kb region close to the junction of the two fragments (Fig. 3). Forty-eight independent insertion derivatives were mapped in detail by DNA sequencing outward from each end of the transposon and were categorized into groups on the basis of their phenotype. A list of representative derivatives along with their phenotypic properties is shown in Table 2. A number of derivatives displayed the wild-type phenotype with respect to mating, clumping behaviour and colony morphology, implying that the insertions were not in genes involved in the mating response. Some (e.g. pAM4020 and pAM4010) mapped outside the region expected to involve the pheromone response; and some (e.g. pAM4400) mapped outside a functional ORF (Fig. 3).

*A gene (*traC*) involved in pheromone sensing*

In pAD1, *traC* encodes a surface protein that binds exogenous pheromone and passes it to a host-encoded

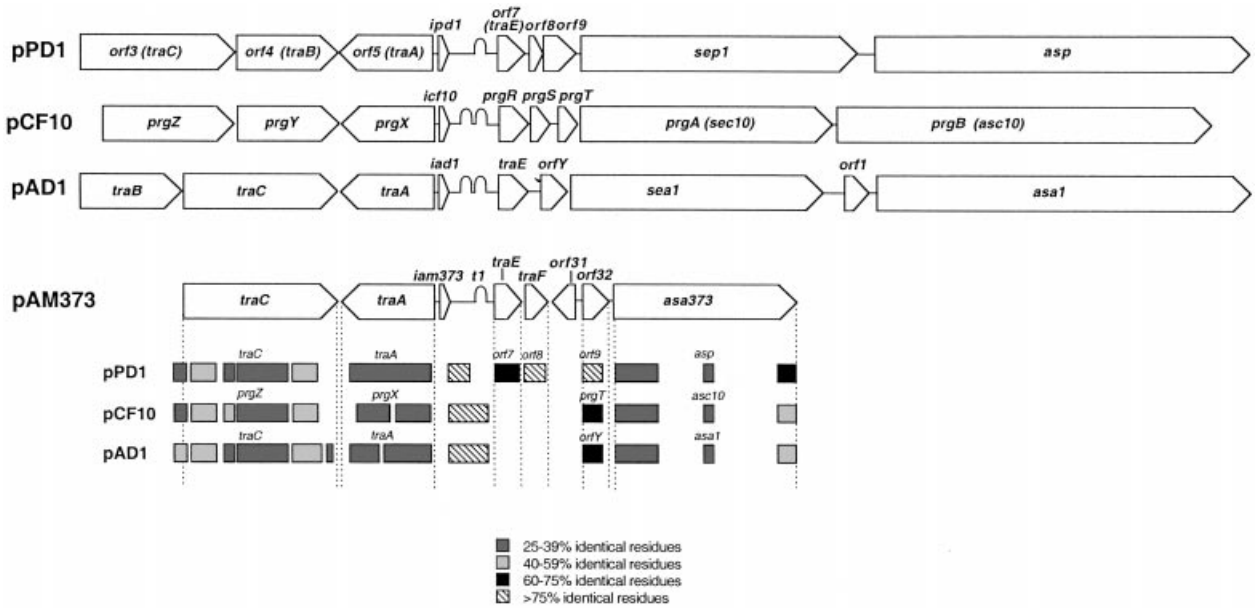

Fig. 2. Comparison of the pheromone-responsive region of pAM373 to the sex pheromone plasmids pAD1, pPD1 and pCF10. The boxes indicate regions of pAM373 showing homology to other pheromone-responsive plasmids. The values refer to identity at the amino acid level, with the exception of the region between *iam373* and *traE* for which homology refers to the extent of similarity at the nucleotide level.

oligopeptide uptake system; a characteristic of *traC* mutants is an increase in plasmid-encoded inhibitor peptide iAD1 in culture filtrates (Tanimoto *et al.*, 1993). A significant amount of iAD1 is believed to bind to TraC exposed on the cell surface; thus, the absence of the protein results in a four- to eightfold increase in inhibitor in culture filtrates (Weaver and Clewell, 1990). The pAD1 *traC* encodes a 543-amino-acid protein with a mass of 60.7 kDa and

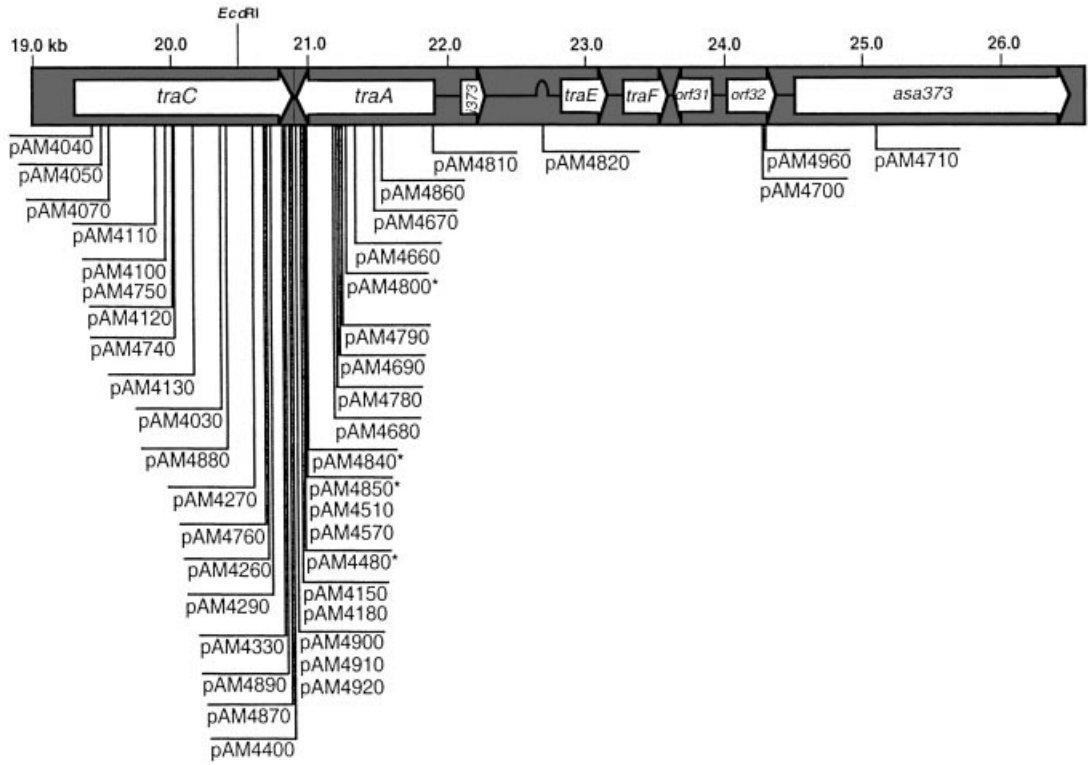

Fig. 3. Position of Tn*917–lac* insertions in pAM373. The asterisks indicate the members of the group D category in the case of the *traA* insertions.

Table 2. pAM373::Tn*917*–lac derivatives and their associated phenotypes.

Group	Phenotype	Plasmid derivatives	Colony morphology	Clumping		Inhibitor iAM373 titre[d]	Mating frequency[a]	
				Constit	cAM373 (ng ml^{-1})[b]		10 min mating	4 h mating
A	Wild type	pAM373, pAM4920 pAM4020, pAM4010 pAM4400, pAM4870 pAM4150, pAM4180 pAM4900, pAM4910	Normal	–	≤ 4	1	2×10^{-6} to 6×10^{-6}	2×10^{-3} to 6×10^{-3}
B	*traC* like (pAD1)	pAM4260, pAM4760 pAM4120, pAM4070 pAM4050, pAM4040 pAM4330, pAM4290 pAM4270, pAM4030 pAM4130, pAM4740 pAM4100, pAM4750 pAM4880, pAM4890 pAM4110, pAM4870	Normal	–	8–60	4–32	4×10^{-7} to 4×10^{-6}	3×10^{-7} to 8×10^{-6}
C	*traA* like (pAD1)	pAM4810, pAM4690 pAM4670, pAM4780 pAM4660, pAM4510 pAM4790, pAM4570 pAM4680, pAM4860	Dry	–	> 60[c]	1–2	8×10^{-5} to 2×10^{-5}	3×10^{-4} to 5×10^{-4}
D	*traA* like (pAD1)	pAM4800, pAM4480 pAM4840, pAM4850	Dry	+	n/a	1–2	2×10^{-4} to 3×10^{-3}	2×10^{-3} to 1×10^{-2}
E	Reduced transfer	pAM4710	Normal	–	> 5000	1–2	5×10^{-6}	6×10^{-5}
F	Reduced transfer	pAM4820	Normal	–	75	1	2×10^{-6}	5×10^{-6}

a. Transfer frequency, number of transconjugants per donor. Values are means of at least three mating experiments per strain.
b. Concentration of synthetic pheromone cAM373 needed to induce a clumping response.
c. Highest concentration of cAM373 used (60 ng ml^{-1}) did not result in a clumping response.
d. Inhibitor titre, pheromone titre without inhibitor present divided by the pheromone titre in the presence of inhibitor.

exhibits a very high homology with PrgZ (Ruhfel *et al.*, 1993), a protein encoded by the pheromone-responsive plasmid pCF10 (over 70% of aligned amino acids are identical). It is also similar to oligopeptide-binding proteins in *Escherichia coli, Salmonella typhimurium and B. subtilis* (see Tanimoto *et al.*, 1993).

In pAM373, *orf26*, now designated *traC*, encodes a 528-amino-acid protein that is 39% identical to the *traC* of pAD1; it appears to use the 'rare' start codon TTG. A conventional (ATG) start site 63 amino acids downstream is unlikely because it lacks a strong ribosomal binding site; and insertion mutations mapping to the region upstream of this ATG codon have a phenotype similar to that exhibited by insertions downstream of this codon (see below). In addition, the predicted size of the TTG-initiated protein is closer to that of the TraC protein of pAD1. The ORF has a strong Shine–Dalgarno sequence (GGAG) six bases upstream of the TTG start codon, and further upstream is a potential promoter region containing a −10 site (TATTAA) separated by 16 bases from a putative −35 site (ATGACT). Like its homologues, the putative TraC exhibits a sequence somewhat characteristic of a surface lipoprotein with amino acid positions 18–21 (FTGC) corresponding to the 'lipobox' (Wu, 1996), although the phenylalanine (F) is unusual for this position. Interestingly, *traC* appears to share a bidirectional transcription terminator (free energies are $\Delta G = -27.0$ kcal/mol and $\Delta G = -27.7$ kcal/mol) with the converging *traA* determinant positioned downstream and in the opposite orientation. It is interesting that all of the *traC*::Tn*917–lac* insertions were in the same orientation, with the *lacZ* determinant orientated the same as *traC*, suggesting insertions in the opposite orientation may be detrimental to the plasmid. In all cases, the colonies were deep blue on Xgal plates in the absence or presence of the inducing pheromone peptide. Conceivably, the relatively high transcription level compensates to some extent for inefficient translation due to the rare TTG start site; or perhaps the TraC product (defective in the insertion derivatives) down-regulates its own transcription. Alternatively, the *traC* transcript may play an additional role in the system and is required at levels above that needed for translation.

Mutations in *traC* gave rise to an elevated level of exogenous inhibitor peptide iAM373 detectable in culture filtrates, which would otherwise bind to the cell-surface *via* TraC (based on analogy with the pAD1 system). The inhibitor titres varied from mutant to mutant and there was some variability between independent experiments, but

the titres generally are four- to 32-fold higher than wild-type inhibitor levels (Table 2; group B).

A related characteristic of *traC* mutants is their reduced responsiveness to pheromone, as illustrated by the need for higher pheromone concentrations to induce clumping. These derivatives transferred poorly in 4 h broth matings (transfer frequencies were 1000- to 10 000-fold reduced compared with wild type). In contrast, overnight filter mating frequencies were of the order of 10^{-1} transconjugants per donor cell, which is similar to wild-type transfer frequencies under similar conditions. This implies that the cells can still transfer plasmid DNA, provided that the pheromone response impairment is circumvented by placing the donor and recipient cells in intimate contact with each other.

*Identification and analysis of a negative regulator determinant (*traA*)*

The following data revealed a determinant resembling *traA* of pAD1 but with interesting differences. The *traA* gene of pAD1 encodes a negative regulator in which mutations result in constitutive expression of conjugative functions (i.e. clumping, dry-colony morphology and high transfer frequencies in 10 min matings). However, insertions in the homologous sequence of pAM373 were associated with two distinct phenotypes (groups C and D) (see Table 2). As described in the *Experimental procedures* section, 36 independent mutagenesis experiments were carried out in which the mutagenized cells were mated for 10 or 20 min with plasmid-free OG1RF selecting for transconjugants on erythromycin (Em)-containing plates. The latter were screened for their ability to clump constitutively when grown in liquid medium, which would suggest that a key negative regulatory determinant may have been mutated. We expected these short matings to select for such derivatives; however, of 207 OG1RF pAM373::Tn*917–lac* transconjugants examined, only three (1.4%) exhibited a constitutive clumping phenotype (Table 2; group D). One additional mutant with this phenotype was identified without using the short-mating selection. Members of this subclass (pAM4800, pAM4480, pAM4840 and pAM4850) exhibited a phenotype very similar to those relating to most *traA* mutations in pAD1. These derivatives gave rise to large clumps in broth without addition of pheromone to the medium, and transferred plasmid DNA at frequencies of 2×10^{-4} to 3×10^{-3} and 2×10^{-3} to 1×10^{-2} in 10 min and 4 h broth matings respectively. The colonies were dry and fracturable (see *Experimental procedures*) when touched with a toothpick, implying that the cells are expressing aggregation proteins on the cell surface. (It is noted that wild-type cells do not result in a dry-colony morphology unless synthetic pheromone is included in the medium.)

The majority of insertion derivatives selected in the short matings were only partially derepressed in expression of plasmid transfer functions (Table 2; group C). Indeed, the constitutive clumping observed in the case of the group D derivatives (Table 2) was not exhibited by these variants; although they did form dry fracturable colonies on solid medium. Furthermore, exposure to 60 ng ml^{-1} of synthetic pheromone (cAM373) did not induce clumping by these derivatives. In addition, they transferred their corresponding plasmid with a lower efficiency in broth than the derivatives belonging to group D; although mating frequencies in 10 min and 4 h matings still were 10-fold higher than those of wild-type cells. Although cells previously unexposed to pheromone transferred plasmid DNA slightly better than wild-type cells, pre-exposure to pheromone did not significantly increase the mating potential.

Both types of mutations (i.e. groups C and D; Table 2) mapped to an open reading frame of 990 bases corresponding to the pAD1 homologue, which was also designated here as *traA*. This ORF would encode a 330-amino-acid protein with a mass of 38.1 kDa. It lacks a signal sequence typical for exported proteins, which is consistent with the cytoplasmic location of the TraA protein in pAD1 (Pontius and Clewell, 1992a; Fujimoto and Clewell, 1998). A strong ribosomal binding site (GGAGG) was found to precede the putative ATG start site by seven bases. An alternative upstream start site is unlikely because of the lack of a strong Shine–Dalgarno sequence. Examination of the region upstream of *traA* did not reveal any strong potential transcription initiation signals, based on comparison with the *E. coli* (sigma-70) consensus promoter. The deduced protein of *traA* exhibited moderate homology with the *traA* gene product of the pheromone-responsive plasmid pAD1 (31% identical amino acid residues), *orf5 (traA)* of pPD1 (33% identity), *prgX* of pCF10 (26% identical residues) and *orfX* of the *Enterococcus faecium* plasmid pHKK701 (24% identity).

Because there were only four insertions associated with the group D category, compared with 10 in group C, and because there were group C insertions between those of group D, the primary *traA* mutational phenotype may essentially correspond to that of group C. Conceivably, the group D representatives contain a second mutation outside *traA*, although sequence analysis of all four group D derivatives between the *iam373* promoter region to, and slightly beyond, t1 showed no difference from the wild type (see Fig. 3). It is interesting that both the C and D groups exhibit a similar dry-colony phenotype, whereas only the D group exhibits clumping in broth. This raises a question concerning the relationship between the two phenomena. In the case of pAD1, the dry-colony phenotype is generally correlated with clumping, but the absence of such a correlation in the case of the C group *traA*

insertion derivatives suggests that another factor could be contributing to the altered colony morphology. Conceivably, in the case of pAM373, the gene product(s) related to dry-colony morphology and regulated by TraA is different from the clumping-related product which is generally assumed to be aggregation substance. In this context, a second mutation may be responsible for derepression of aggregation substance. Efforts are currently under way to clarify this issue.

With respect to expression of β-galactosidase, colonies of cells containing *traA*::Tn*917–lac* insertions were moderately blue on Xgal plates regardless of the orientation and regardless of the presence or absence of cAM373 in the plates. For those derivatives with insertions in the same orientation as *traA*, some degree of expression is not surprising. However, expression in the case of oppositely orientated insertions suggests that there may be some degree of countertranscription from the direction of *traC*. Further data supporting this notion are addressed below in the section *Additional screening for mutants related to positive regulation*.

Interestingly, two transposon insertions near the 3′ end of *traA* (pAM4150 and pAM4180) exhibited the wild-type phenotype with respect to colony morphology, pheromone responsiveness and mating behaviour. The two insertions are located in the same site with the same orientation (*lacZ* orientated the same as *traA*), but as they were generated in different mutagenesis experiments they should not be siblings. The insertion site corresponds to 12 amino acid residues from the carboxyl terminus, suggesting that at least the terminal 12 residues are not essential for full TraA function.

Sequence analysis of iAM373

The inhibitor peptides that are encoded by all the pheromone-responsive plasmids studied to date are hydrophobic and correspond to the carboxyl-terminal seven or eight amino acids of precursor peptides seemingly designed to ensure export from the cell (see Clewell, 1999). They are believed to prevent the self-induction of donors by endogenous pheromone or other cross-reacting peptides and probably also serve to block induction by recipient-produced pheromone levels too low to result in the generation of mating aggregates (e.g. when recipients are too far away to be encountered by random collision).

Just upstream of *traA* is a small ORF that is transcribed in the opposite direction to *traA* and that encodes a 24-amino-acid peptide with its carboxy-terminal seven amino acids corresponding to the mature pheromone inhibitor iAM373 (MKKELILILKWLTPIGL**SIFTLVA**). As in the case of *traC*, the predicted ORF has an 'uncommon' initiation codon TTG, and there is an apparent ribosome binding site (AGAGG) 11 bases upstream. Further upstream, a potential promoter region consisting of −10 (TATACT) and −35 (TTTACT) hexamers separated by 16 bases is evident.

Over 500 bases downstream of the *iam373* determinant is a potential transcription termination site (t1) that consists of a 16 bp inverted repeat sequence (with two mismatches) ($\Delta G = -24.5$ kcal/mol) followed by a run of seven Ts. It is conceivable that this terminator has a similar function as in pAD1 and that the regulation of the pheromone response is mediated by transcription through this terminator. In pAM4820, the putative terminator is disrupted by a Tn*917–lac* transposon insertion, in which β-galactosidase would be expressed when transcription occurs upstream of the terminator. The *lacZ* fusion created by this Tn*917–lac* insertion is transcribed at an elevated basal level (i.e. dark blue colonies) in the absence of pheromone, which is consistent with the idea that transcription occurs constitutively from an upstream promoter into the terminator in the absence of cAM373. This derivative does not clump in response to 60 ng ml^{-1} of synthetic pheromone and does not transfer plasmid DNA efficiently in 10 min or 4 h broth matings, as illustrated by the low mating frequencies (2×10^{-6} and 5×10^{-6}) transconjugants per donor respectively).

It has been suggested in the case of pAD1 (Weaver and Clewell, 1990; Galli *et al*., 1992; Pontius and Clewell, 1992b; Tanimoto and Clewell, 1993; Bastos *et al*., 1997, 1998) that the region between the inhibitor determinant (*iad*) and the downstream *traE1* is of considerable regulatory importance. Southern blot analysis (Hirt *et al*., 1996) revealed that this region is conserved among most pheromone-responding plasmids. On pAM373, the region between the *iam373* and the *traE* determinant showed over 60%, 83% and 81% of the nucleotide residues being identical to the corresponding region on pPD1, pAD1 and pCF10 respectively.

The region downstream of t1

Downstream of the apparent transcription terminator is a 1.6 kb region with considerable homology to pPD1. It contains several determinants which were designated *traE, traF, orf31 and orf32* (see Fig. 3). *traE* (*orf29*), positioned just downstream of t1, has a good ribosome binding sequence (GGAGG) eight bases upstream of the ATG start codon; and further upstream were possible −10 (TATATT) and −35 (TTAAAA) hexamers, but with a spacing of 21 nucleotides that would seem to prevent efficient usage as a promoter. *traE* would encode a 105-amino-acid protein with a mass of 12.4 kDa. A sequence comparison showed significant homology at the amino acid level with the pPD1 product of *orf7 (traE)* (62% identity). Downstream of *traE* is another open reading frame *(traF)* that would encode a 103-amino-acid product

92% identical to the pPD1 TraF in its first 63 amino acids. Based on the similarities with other pheromone systems, we postulate that *traE* and perhaps *traF* may encode positive regulatory products, although the original screenings did not reveal any transposon insertions in these ORFs to provide direct support for this view. These data are consistent with those of Muscholl-Silberhorn (1999).

Additional screening for genes inducible in response to pheromone exposure

Pheromone-inducible genes may correspond to structural proteins related to the conjugation process or regulatory genes whose upregulation is necessary for a normal mating response. Appropriately orientated Tn*917–lacZ* insertions in these genes would be expected to give rise to white colonies on Xgal-supplemented medium and blue colonies if synthetic pheromone is also provided. The entire collection of Tn*917–lac* insertion derivatives was screened for inducible β-galactosidase expression. Of 425 isolates tested, five (1.2%) produced blue pigment on Xgal plates only in the presence of synthetic cAM373. These derivatives were subsequently sequenced to determine the site of the transposon insertion. None had insertions in *orf29 (traE)* or *orf30 (traF)*. One transposon insertion (pAM4710; Fig. 3) was found to be inside the aggregation substance gene (*asa373*; see below). A second derivative (pAM4960) mapped to a small 321-nt ORF (*orf32*) that was highly homologous to *orf9* of pPD1 (84% identity at the amino acid level); although it was slightly less responsive to pheromone, it exhibited the wild-type phenotype with respect to colony morphology and mating potential. A derivative with a similar phenotype (pAM4700), but with the transposon inserted in the opposite orientation, was white regardless of the presence of the pheromone (see Fig. 3).

The other three derivatives (pAM4900, pAM4910 and pAM4920) all mapped in an identical site with the same orientation immediately adjacent to the last codon (C-terminus) of *traA*. The orientation was such that β-galactosidase activity reflected transcription from the direction of *traC* (see Fig. 3). Although the three insertions were at identical sites, they appeared in independent mutagenesis experiments; thus, they should not represent siblings. They are located just a few base pairs from the apparent bidirectional terminator located between the converging *traA* and *traC* determinants and their phenotypes are identical to the wild type. The insertions within *traC* (see above) suggested that there is very strong transcription from the *traC* promoter (colonies are deep blue on Xgal plates). Assuming that this is not related to absence of a negative autoregulation, the terminator must be operating quite efficiently as the above-noted insertions just downstream give rise to white colonies in the absence of cAM373. The data would imply that pheromone exposure results in a significant decrease in termination strength. It is interesting that, in contrast to these insertions, similarly orientated insertions within *traA* exhibit a constitutive expression of β-galactosidase (see section above on *traA*). A reasonable interpretation would be that TraA itself is involved in minimizing transcriptional readthrough of the terminator from the *traC* promoter and that the absence of functional TraA (i.e. in the case of the *traA* insertions) results in a decrease in termination strength.

asa373*, a gene encoding aggregation substance*

It was previously shown that although pAM373 gives rise to the characteristic sex pheromone-mediated clumping response its aggregation substance is different from other known aggregation substances when compared by Southern blotting using pAD1-derived probes (Hirt *et al*., 1996). In the present study, *orf34* of pAM373 showed limited local homology with aggregation substance determinants of the other systems, i.e. pAD1, pCF10 and pPD1 (see Fig. 2); however, its putative product, Asa373, is only about half the size (655 amino acids). The C-terminal 57 amino acids of Asa373 contain a conserved cell wall anchoring motif LPXTG and a hydrophobic membrane anchor sequence that shows a relatively high degree of homology with *asp1* of pPD1 (82% identical amino acid residues), as well as *asa1* of pAD1, *asc10* of pCF10 and the conjugative *Enterococcus faecium* plasmid pHKK701 (56%, 57% and 61% identity respectively). Unlike the case for other characterized aggregation substance proteins, Asa373 does not have an RGD sequence (see Clewell, 1999). The data are consistent with those reported by Muscholl-Silberhorn (1999).

A Tn*917–lac* transposon insertion in this gene (see previous section and Table 2) resulted in a mutant (pAM4710) defective in several pheromone-mediated functions. For instance, this derivative failed to clump or to give rise to a dry-colony morphology when exposed to cAM373. Plasmid transfer was relatively poor in broth matings, although high transfer frequencies (4×10^{-1} transconjugants per donor) could be achieved when the donor and recipient cells were placed in intimate contact with each other (i.e. overnight filter matings). The same insertion mutant failed to hydrolyse Xgal in the absence of pheromone, but produced blue pigment in the presence of 30 ng ml^{-1} or more of synthetic pheromone.

Other sequences of note and possible relevance to conjugation control

Immediately downstream of the *asa373* gene is an open reading frame encoding a putative 141-amino-acid protein

that is 100% identical to the *pd125* determinant of pPD1. It also shows a high degree of homology to the pAD1 *orf3* and pCF10 *orf14* (> 90% identical residues). Further downstream are 10 open reading frames (*orf36–orf45*) which are all positioned in similar orientation (Fig. 1). These ORFs showed a high degree of sequence homology to genes identified in pAD1 (Table 1), but in most cases no significant similarities were found to other known proteins or genes in the database. An exception is *orf38*, which is 98% identical to *orf8* of pAD1 and also showed a high degree of sequence homology at the amino acid level to the pCF10 determinant *prgC* (61% identity). Hybridization studies of Hirt *et al*. (1996) showed that a pAD1 *orf8* probe generated a signal from all of 18 other plasmids tested. Searches also revealed a significant degree of similarity to *orfC* of the broad host range conjugative plasmid pAMβ1 and *orf6* of the broad host range, low copy number *Streptococcus pyogenes* plasmid pDB101 (31% and 41% identity at the amino acid level respectively). *orf38 has* some features typical of surface proteins of Gram-positive bacteria (Navarre and Schneewind, 1999), including a signal peptide sequence, a cell wall anchoring motif LPXTG and a C-terminal hydrophobic transmembrane helix. In the case of the pAD1 sequence *orf8,* it has been postulated that the gene product may enhance contact between mating pairs by providing additional cell–cell interactions independent of the aggregation substance, although this hypothesis was based on computer-aided analysis of the protein structure (Hirt *et al*., 1996).

The present study revealed a small (258 nucleotides) open reading frame, *orf36*, approximately 0.5 kb downstream of the *asa373* gene, which was 97% identical to *orf6* of pAD1. This is not consistent with the report that DNA prepared from pAM373 failed to hybridize to an *orf6* probe in Southern blotting experiments Hirt *et al*. (1996). Most of the remaining open reading frames showed significant homology only to determinants with unknown function identified on pAD1.

Concluding remarks

The data presented here show that whereas pAM373 exhibits some similarities to other pheromone-responding plasmids there are clearly significant differences. The determinant for aggregation substance (*asa373*) is only about half the size and contains unique regions; however, downstream (ORFs 36–45) there are significant similarities with the other plasmids (Table 1 and see Hirt *et al*., 1996). In the case of pAD1, genetic data have suggested that the latter region includes genes relating to the stabilization of donor–recipient aggregates and DNA transfer (Ehrenfeld and Clewell, 1987). A gene that most pheromone-responsive plasmids seem to have but that is lacking in the case of pAM373 relates to surface exclusion (Dunny *et al*., 1985; Weidlich *et al*., 1992). Normally, such determinants are located just upstream of the aggregation substance determinant. Recent biological data (E. H. De Boever and D. B. Clewell, in preparation) indicate that pAM373 actually does not utilize a surface exclusion system.

Another unique feature of pAM373 is the absence of a determinant resembling *traB* of pAD1 (An and Clewell, 1994) or its equivalent in pCF10 (*prgY*) (Ruhfel *et al*., 1993), or pPD1 (*orf4*) (Fujimoto *et al*., 1995; Nakayama *et al*., 1995b). These determinants encode apparent membrane proteins; and in the case of pAD1 and pPD1 the TraB product contributes to shut-down of endogenous pheromone production – at least in certain hosts (Nakayama *et al*., 1995c). It is known that cAM373 is indeed reduced in culture supernatants of cells carrying pAM373 (Nakayama *et al*., 1995c), so there may be another feature(s) of the system that is involved in this process.

TraC, and its apparent role in binding to exogenous pheromone (cAM373) and inhibitor peptide (iAM373), strongly resembles corresponding surface lipoproteins encoded by other pheromone-responsive plasmids. In the case of TraA, however, there are properties that distinguish it significantly from the homologue in pAD1. Although TraA appears to interact with the pheromone signal, the majority of *traA* insertions did not result in constitutive clumping, and donor potential in broth increased only slightly. The possibility that TraA might participate in controlling transcriptional readthrough from the *traC* promoter into its own determinant (*traA*) is an interesting one and is currently being further analysed.

The iAM373 precursor was found to be a 24-amino-acid peptide which, like those encoded by pAD1, pCF10 and pPD1, resembles a lone signal sequence, and the determinant *iam373* was located several hundred nucleotides upstream of a transcription terminator t1. An insertion in this terminator resulted in loss of mating potential, implying that a significant aspect of control may relate to transcriptional readthrough of this site – a feature that is common to some degree in the other systems (Clewell, 1999). Downstream of t1 is a region containing two ORFs resembling *traE* and *traF* of pPD1 (Fujimoto *et al*., 1995). Although nothing is known about the function of this region, an involvement with control of expression of the aggregation substance determinant *asa373* and perhaps other structural genes related to conjugation would appear likely.

Finally, this study represents significant ground work for further analyses and understanding of molecular control mechanisms involved in the transfer of pAM373 and related plasmids. Recently, our laboratory has identified an enterococcal plasmid encoding vancomycin resistance, which encodes a mating response to cAM373

(S. Showsh, personal communication). As, like pAM373, it also responds to the peptide in staphylococcal culture filtrates, this and related elements may be poised for entry into *S. aureus*.

Experimental procedures

Bacteria, media and reagents

E. faecalis host strains OG1X (Ike *et al*., 1983; chromosomal streptomycin resistance mutation) and OG1RF (Oliver *et al*., 1977; chromosomal rifampin and fusidic acid resistance mutations) were utilized in conducting both the genetic and DNA analyses reported in this study. The medium used for growth of *E. faecalis* strains was Todd Hewitt Broth (THB) (Difco Laboratories), and cultures were incubated at 37°C unless otherwise noted. Plating was on THB agar. The medium used in pheromone response or inhibitor production assays was N2GT (Oxoid Nutrient Broth no. 2 supplemented with 0.2% glucose and 0.1 M tris-HCl, pH 7.5) (Oxoid). Antibiotics were used in the following concentrations: 10 μg ml^{-1} erythromycin, 10 μg ml^{-1} tetracycline, 1 mg ml^{-1} streptomycin, 25 μg ml^{-1} rifampin, 25 μg ml^{-1} fusidic acid and 10 μg ml^{-1} chloramphenicol. Synthetic cAM373 was prepared by the University of Michigan Core Peptide Synthesis facility.

Transposon mutagenesis and isolation of pAM373 derivatives with Tn917–lac *insertions*

The transposon delivery vector pTV32ts (Youngman, 1987) was used for the mutagenesis procedure (Weaver and Clewell, 1988). This temperature-sensitive plasmid is replication deficient at 42°C but maintains itself normally at 30°C. It carries a plasmid-encoded chloramphenicol resistance gene (*cat*) and a derivative of Tn*917* with a promoterless copy of the *lacZ* gene near one end orientated such that transcriptional fusions can be easily monitored. Strain OG1X/pTV32ts was originally constructed by protoplast transformation (Wirth *et al*., 1986) and the plasmid pAM373 was introduced into this strain by conjugation. Although there is no selectable marker on pAM373, overnight filter matings are so efficient with this plasmid that transconjugants can be easily screened on the basis of their ability to respond (clump) when exposed to pheromone (cAM373). Cells carrying the two plasmids were maintained on THB agar plates supplemented with 10 μg ml^{-1} chloramphenicol (Cm) and 10 μg ml^{-1} erythromycin (Em). Individual cultures were grown overnight at 30°C in the presence of 10 μg ml^{-1} Cm to maintain the delivery plasmid and 0.05 μg ml^{-1} Em to induce transposition (Tomich *et al*., 1980). To allow segregation of pTV32ts, the cultures were washed and diluted 1:1000 in THB broth supplemented with 10 μg ml^{-1} Em and grown overnight at the non-permissive temperature (42°C). This was followed by a 1:200 dilution of the cultures into fresh THB-Em broth and overnight incubation at 42°C. An aliquot of each culture was then plated on 10 μg ml^{-1} Em to select for strains still harbouring the transposon. Em-resistant, Cm-sensitive (indicating loss of pTV32ts) isolates were selected for further study. In some cases, 10 min, 20 min or 4 h broth matings using OG1RF as the recipient strain were done before plating out the cells. The short matings (i.e. 10 and 20 min matings) would be expected to select for derivatives in which the pheromone response functions are constitutively expressed. These cells can easily be distinguished because they have a characteristic dry-colony morphology on agar plates and exhibit a constitutive clumping phenotype when grown in broth. Most other derivatives would still require the 30–40 min period necessary to respond to the pheromone of the recipients, and the mating time used would be too short for the pheromone response to be induced and for plasmid transfer to the recipient to occur.

Clumping assays

Pheromone response assays were performed by the microtitre serial twofold dilution method (Dunny *et al*., 1979) using an initial concentration of synthetic cAM373 peptide of 60 ng ml^{-1}. Pheromone inhibitor is quantified on the basis of its ability to reduce the sensitivity of a given responder strain to a given concentration of cAM373 pheromone. The assay to estimate inhibitor peptide levels was carried out as described by Ike *et al*. (1983) and using 60 ng ml^{-1} synthetic cAM373; the inhibitor titre was represented as the cAM373 titre in the absence of inhibitor divided by the titre in the presence of inhibitor (culture filtrate). Culture filtrates of test strains were prepared by inoculating 5 ml N2GT broth with 0.05 ml of an overnight culture and growing the cells to late log phase at 37°C with shaking. The cells were then pelleted by centrifugation and the supernatant boiled for 10 min.

Characterization of colony morphology

Colony morphology was determined by lightly touching isolates grown on N2GT for 48 h at 37°C with the end of a toothpick while viewed by oblique microscopy. Colonies were classified as 'normal' if they had a watery, translucent and soft appearance, which was previously correlated with the lack of expression of aggregation substance (Weaver and Clewell, 1991). Colonies that had an opaque appearance and fractured when touched with a toothpick were classified as 'dry'.

Mating procedures

When strains involving OG1X/pAM373::Tn*917–lac* were used as donors, the recipient was the plasmid-free OG1RF. When OG1RF/pAM373::Tn*917–lac* strains were used as donors, the recipient was the plasmid-free OG1X. Broth matings were performed using overnight cultures of donor and recipient bacteria and mixing 0.5 ml of the recipients with 0.05 ml of the donor culture in 4.5 ml fresh broth. This 1:10 mixture was incubated at 37°C with gentle agitation for either 10 min or 4 h and was then vortexed to obtain a uniform suspension. Tenfold serial dilutions were plated on THB medium with appropriate selective antibiotics. Separate platings, in which donors alone were selected, provided a basis for estimating the plasmid transfer frequency as transconjugants per donor.

Filter matings were essentially carried out as described by

Clewell *et al*. (1985) with the following modifications. Overnight 5 ml cultures of donors and recipients were mixed at a 1:10 donor–recipient ratio in a sterile microfuge tube (50 μl donor cells + 500 μl recipient cells) and pelleted by centrifugation. The supernatant was decanted, the cells washed in 0.5 ml fresh medium and the resulting pellet was resuspended in 25 μl fresh broth. The cell suspension was then dropped onto a sterile membrane filter (type HA, 0.45 μm pore size) that was placed on horse blood agar. The plate was incubated at 37°C, and the following day the cells were suspended in 1 ml THB medium; 10-fold serial dilutions were then spread on plates with appropriate selective antibiotics. Separate platings, in which donors alone were selected, provided a basis for estimating the plasmid transfer frequency.

Plasmid DNA isolation, restriction enzyme analysis and β-galactosidase activity

Small-scale plasmid preparations were obtained by a method described previously (Weaver and Clewell, 1988). Tn*917–lac* insertions were mapped by comparing plasmid restriction enzyme profiles with pAM373 wild-type profiles and relating them to a previously generated (Clewell *et al*., 1985) physical map. All restriction enzymes were purchased from Gibco BRL and all the reactions were carried out under the conditions recommended by the manufacturer.

β-Galactosidase activity of Tn*917–lac* derivatives was determined by streaking isolates on medium containing 100 μg ml^{-1} 5-bromo-2-chloro-3-indolyl-β-D-galactopyranoside (Xgal) with or without the addition of 30, 100 or 500 ng ml^{-1} of synthetic pheromone cAM373. Colony colour was evaluated after incubating for 48 h at 37°C.

DNA sequence analysis

Large-scale plasmid purifications from *E. faecalis* FA373 (Clewell *et al*., 1985) were performed by CsCl/ethidium bromide equilibrium density gradient ultracentrifugation, as previously described (Clewell *et al*., 1974). The latter strain is a derivative of FA2-2 (Clewell *et al*., 1982; chromosomal rifampin and fusidic acid resistance mutations) carrying pAM373 as well as a chromosome-borne Tn*918* (Clewell *et al*., 1985).

Small insert library construction made use of a nebulizer to shear purified pAM373 DNA by applying 82.8 kPa of nitrogen gas for 30 s. The sheared DNA was separated by preparative agarose gel electrophoresis, and DNA ranging from 1.5 to 2.5 kb was purified from gel slices by phenol extraction. A small insert library was made using the 'v + i' method of Fleischmann *et al*. (1995). The sheared insert DNA was treated with BAL 31 nuclease (New England Biolabs) and ligated to *Sma*I-digested pUC18 vector (Pharmacia) treated with alkaline phosphatase. A band corresponding to the vector plus single insert (v + i) DNA was gel purified, and the ends were polished with T4 polymerase (New England Biolabs). T4 DNA ligase (New England Biolabs) was then used to close the open v + i molecules.

Templates for DNA sequencing were prepared by electroporation of *E. coli* DH10B cells (Life Technologies) with aliquots of the small insert libraries. Cells were selected on SOB diffusion plates, as described elsewhere (Fleischmann *et al*., 1995). Colonies were picked and transferred into a 96-well growth plate and purified by an alkaline lysis procedure (Utterback *et al*., 1995). Purified pUC18 templates were sequenced (McDonald *et al*., 1995) using cycle-sequencing reactions with fluorescence-labelled forward and reverse primers (Applied Biosystems). For the project, a total of 791 out of 989 sequencing reactions were successful, with an average edited read length of 501 bp. The total sequence generated represented approximately 10-fold sequence redundancy. Random sequences were assembled using TIGR Assembler (Sutton *et al*., 1995) and gaps in the pAM373 molecule were closed by primer walking using oligonucleotides designed to contig ends and dye terminator chemistry. Final assembly and editing of the pAM373 plasmid resulted in a single molecule totalling 36 679 bp. The assembly was subsequently reoriented so that the first G of the unique *Sal*I site is designated as nucleotide 1.

The gene-finding algorithm GLIMMER (Salzberg *et al*., 1998) was used to identify ORFs. ORFs were searched against a non-redundant database of protein sequences (NRAA) developed at TIGR and curated from the public archives GenBank, Genpept and SWISSPROT. Alignments were evaluated and a database match was chosen for genes with significant similarity scores. The sequence was submitted to the GenBank database, where its accession number is AE002565.

Mapping of transposon insertions

Purified *E. faecalis* plasmid DNA used for mapping transposon insertions was prepared with the Plasmid Midi Kit (Qiagen) using a protocol for isolation of very low copy number, large size plasmid DNA as recommended by the manufacturer with a minor modification. Before treatment with NaOH, the cultures were incubated with 10 mg ml^{-1} lysozyme at 37°C for 45 min.

Dideoxynucleotide sequencing reactions were carried out directly on double-stranded DNA using either the 70770 Sequenase Version 2.0 Sequencing kit (Amersham) or the *fmol* DNA Cycle Sequencing System (Promega). Double-stranded supercoiled plasmid DNA was used as the template, and two primers (5′-CTATTCCTAAACACTTAAGAG-3′ and 5′-ACTGTACCACTAATAACTCAC-3′) reading outward from each end of Tn*917–lac* were used to determine the junction sequences of the transposon inserts in pAM373. The primers were prepared by the University of Michigan DNA Synthesis Core Facility. Both strands were sequenced and the obtained sequences were analysed using a MACVECTOR software package from Eastman Kodak.

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