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Regulation of Transfer of the *Enterococcus faecalis* Pheromone-Responding Plasmid pAD1: Temperature-Sensitive Transfer Mutants and Identification of a New Regulatory Determinant, *traD*

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The enterococcal, conjugative, cytolysin plasmid pAD1 confers a mating response to the peptide sex pheromone cAD1 secreted by plasmid-free strains of *Enterococcus faecalis*. Cells carrying pAM714, a pAD1::Tn*917* derivative with wild-type conjugation properties, were mutagenized with ethyl methanesulfonate to obtain variants that were induced (in the absence of pheromone) to transfer plasmid DNA upon shifting from 32 to 42°C. Of 31 such mutants generated, the results of analyses of 7 are presented in detail. All seven strains were thermosensitive in the *E. faecalis* host FA2-2; colony morphology, clumping, and DNA transfer correlated well with each other at the two temperatures. In the nonisogenic host *E. faecalis* OG1X, however, only one derivative (pAM2725) exhibited correlation of all three traits at both temperatures. Three (pAM2700, pAM2703, and pAM2717) clumped and had colonies characteristic of pheromone-induced cells at 32°C but transferred plasmid DNA at a higher frequency only at the elevated temperature. The other three (pAM2708, pAM2709, and pAM2712) were derepressed at both temperatures for all three characteristics. Four of the mutations, including that of pAM2725, mapped within the *traA* determinant, whereas two mapped identically in a previously unnoted open reading frame (designated *traD*) putatively encoding a short (23-amino-acid) peptide downstream of the inhibitor peptide determinant *iad* and in the opposite orientation. One mutant could not be located in the regions sequenced. Studies showed that the *traA* and *traD* mutations could be complemented in *trans* with a DNA fragment carrying the corresponding regions.

Certain conjugative plasmids in *Enterococcus faecalis* encode a mating response to specific peptide sex pheromones secreted by potential recipient (plasmid-free) cells. (See references 4, 5, 11, and 40 for recent reviews.) One such element is pAD1, a 60-kb hemolysin-bacteriocin (cytolysin) plasmid originally identified in the *E. faecalis* clinical isolate DS16 (9, 35). Transfer functions of pAD1 and a number of closely related cytolysin plasmids (19), as well as the antibiotic resistance plasmid pBEM10 (25), are specifically induced by the octapeptide cAD1 (24).

The pAD1 mating response is characterized by the synthesis of a proteinaceous surface aggregation substance (Asa1) which binds to the surface of recipients as well as other donors (16). Donors exposed to culture filtrates of recipients undergo a clumping response, which is easily visualized. Plasmid-containing cells that have been plated on solid medium give rise to colonies with a watery (soft) morphology when teased with a toothpick; however, if synthetic cAD1 is present in the plates, the colonies exhibit a distinguishable dry (fracturable) morphology (39). The latter characteristic, which relates to induced aggregation occurring within the colony, has been useful in screening for pAD1 mutations that affect regulation of the mating response (13, 38). In addition to inducing Asa1 expression, pheromone also activates expression of genes related to DNA transfer (7, 13). Once a copy of the plasmid is received by the recipient, the resulting transconjugant shuts down the production of pheromone; however, different pheromones specific for other conjugative plasmids continue to be secreted.

The regulatory region for the pAD1 pheromone response is organized as illustrated in Fig. 1. Previous studies have shown that transposon insertions (Tn*917* or Tn*917lac*) into *traA* or *traB* resulted in constitutive clumping and the ability to transfer plasmid DNA at a relatively high frequency in short (10-min) matings (18). Most *traA* mutants exhibit a dry colony morphology in the absence of cAD1, and the TraA protein has been shown to be a key negative regulator of the expression of *traE1*. In turn, TraE1 positively regulates most if not all of the structural genes relating to conjugation (28, 29, 38). Regulation of *traE1* by TraA relates to the control of transcription from the *iad* promoter through two terminators (TTS1 and TTS2) downstream of *iad* and into *traE1* (17, 26, 30, 33).

The product of *traB* is involved in reducing the production of endogenous cAD1 (1); *traB* mutants release a small but not wild-type amount of cAD1 that results in a self-induction. Colonies of *traB* mutants appear ringed due to being dry in the central part of the colony and watery on the perimeter (39). The ring phenomenon is related to the production of higher levels of cAD1 under an anaerobic, compared to an aerobic, environment (39); the central part of the colony is more anaerobic.

iad encodes iAD1, which is a peptide competitive inhibitor of cAD1 (8, 23), whereas *traC* encodes a surface protein involved in binding to cAD1 and iAD1 (32). The region between the *iad* promoter and TTS1 is quite complex in that it predicts significant secondary structure in potential transcripts (reference 30 and unpublished data), and there is evidence for at least three RNA species being involved (33). Whereas regulation of the pheromone response relates to a control of tran-

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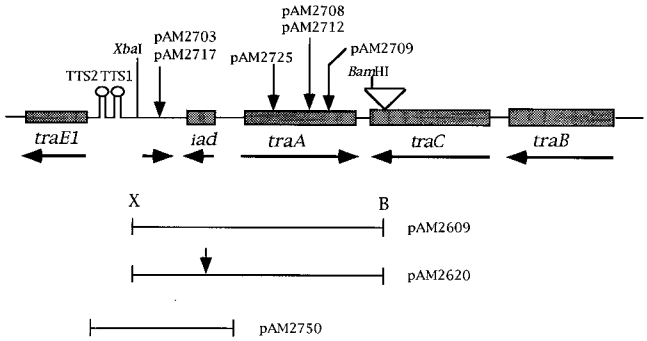

FIG. 1. Map of pAD1 region involved in regulation of the pheromone response. The horizontal arrows below the map indicate the orientation of the corresponding genes. TTS1 and TTS2 are sites involved in termination of transcription from the *iad* promoter. The vertical arrows above the map indicate the positions of specific point mutations that give rise to temperature sensitivity. The inverted triangle above *traC* represents a Tn*917lac* insert in pAM2180 with a *Bam*HI site near one end. This *Bam*HI site (B) was used together with the *Xba*I site (X) just to the right of TTS2 in the construction of two of the plasmid chimeras (pAM2609 and pAM2620) that were used in complementation studies. The arrow over pAM2620 indicates the position of a mutation that eliminates production of iAD1. The short rightward-pointing arrow just downstream of *iad* corresponds to the ORF designated *traD*.

scriptional readthrough of TTS1-TTS2, details of the mechanism by which this occurs are not clear. The TraA product is known to bind at the *iad* promoter (33), and induction appears to involve up-regulation of an already elevated basal level of expression from this site. Transcriptional analysis of *traA* mutants supports this notion (33); however, while the data would suggest that detectable extracellular iAD1 levels should be increased significantly, it rises by only a factor of two at most. From these considerations, as well as the notion that the TTS1-TTS2 termination must be unusually efficient due to the apparent ability of TraE1, once expressed, to up-regulate itself at its own promoter, it would appear that regulation involves factors additional to the action of TraA at the *iad* promoter and that identification of these controlling processes requires a more detailed analysis of the region between the *iad* promoter and TTS1.

We have previously utilized transposon mutagenesis (Tn*917* and Tn*917lac*) to examine regulation (13, 18, 38); however, the potential polarity effects of this approach could mask the importance of key regulatory features in a region of high structural complexity. As an alternative approach, we considered the derivation of point mutations using chemical mutagenesis and a search for variants that were temperature inducible with respect to conjugation functions. Here we present the results of such a study. Thermosensitive (TS) mutations were generated, and while some were found to occur within *traA*, as expected, others were found in the region between *iad* and *traE1*. The latter were found in a short open reading frame (ORF) that appears to play a role in regulation. Interestingly, some of the mutants, including mutants for *traA*, behaved differently in two different (nonisogenic) *E. faecalis* host strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Plasmid pAM714 is a derivative of pAD1 that carries an insertion of the erythromycin resistance transposon Tn*917* in a region that is not involved in transfer. This plasmid was used to represent the wild type in several experiments.

Media and reagents. Media used for *E. faecalis* were Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) and N2GT (nutrient broth no. 2 [Oxoid Ltd., London, United Kingdom] supplemented with 0.2% glucose and 0.1

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Strains		
E. faecalis		
OG1X	*str gel*	20
FA2-2	*rif fus*	9
JH2SS	*str spc*	34
DS16	Clinical isolate; carries pAD1 and pAD2	35
E. coli DH5α	F^- ϕ80 *lacZ* Δ*M15 endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* Δ*gyrA96* Δ*(lacZYA-argF) U169*	Promega Research Laboratories
Plasmids		
pBluescript KS^+	*E. coli* plasmid vector; *amp*	Stratagene
pAM401	*E. coli-E. faecalis* shuttle: *cat tet*	
pAM714	pAD1::Tn*917*; Hly/Bac, *erm*	
pAM2011	pAD1::Tn*917lac* with insert 4 bp downstream from TTS2	30
pAM2011E	Miniplasmid derivative of pAM2011	37
pAM2100	pAD1 with Tn*917lac* insert in *traA*	29
pAM2120	pAD1 with Tn*917lac* insert in *traA*	29
pAM2180	pAD1 with Tn*917lac* insert in *traC*	29
pAM2609	*Xba*I-*Bam*HI fragment of pAM2180 cloned in pAM401	This study
pAM2614	*Xba*I-*Bam*HI fragment of pAM2609 cloned in *Xba*I-*Bam*HI sites in pBluescript	This study
pAM2620	pAM2609 with the *iad19* mutation	This study
pAM2700	pAM714 with EMS-generated mutation	This study
pAM2703	pAM714 with point mutation 119 bp downstream of *iad*	This study
pAM2708	pAM714 with point mutation affecting amino acid residue 181 of TraA	This study
pAM2709	pAM714 with point mutation affecting amino acid residue 229 of TraA	This study
pAM2712	pAM714 with point mutation affecting amino acid residue 181 of TraA	This study
pAM2717	pAM714 with point mutation 119 bp downstream of *iad*	This study
pAM2725	pAM714 with point mutation affecting amino acid residue 71 of TraA	This study
pAM2750	809-bp PCR product containing *iad*, TTS1, and TTS2 cloned in pAM401	This study
pAM2756	pAM2011E with mutation of pAM2725	This study

M Tris-HCl [pH 7.5]). N2GT medium was used in all experiments dealing with the pheromone response. Luria-Bertani broth (21) was used for culturing *Escherichia coli*. Solid media were prepared by including 1.5% agar. Cultures were incubated at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: tetracycline, 10 μg/ml; chloramphenicol, 20 μg/ml; ampicillin, 100 μg/ml; erythromycin, 20 μg/ml; streptomycin, 1 mg/ml; spectinomycin, 500 μg/ml; rifampin, 25 μg/ml; and fusidic acid, 25 μg/ml. Hemolysis was detected, when necessary, on THB plates containing 4% horse blood (Colorado Serum Co., Denver, Colo.). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GIBCO/BRL, Inc., Gaithersburg, Md.) was used at a concentration of 100 μg/ml. Synthetic cAD1, prepared at the University of Michigan core peptide synthesis facility, was used at a concentration of either 40 or 80 ng/ml.

Mutagenesis and selection. TS mutants affected in regulation of the mating response were isolated after mutagenesis with ethyl methanesulfonate (EMS; Sigma Chemical Co., St. Louis, Mo.) as described below. Cultures of strain OG1X(pAM714) or FA2-2(pAM714) were grown overnight in the presence of erythromycin. A cell suspension containing approximately 10^9 cells/ml was prepared in antibiotic-free N2GT medium. A 1.0-ml aliquot of the suspension was removed and used as a control. One-milliliter aliquots of the same suspension were treated with EMS (282 to 940 μM) for 2 h. The cells were washed twice in phosphate-buffered saline (2) and grown at 37°C for 4 h, followed by incubation at 42°C for 1 h, after which 100 μl of the mutagenized cells was mated in 10-min broth matings at 42°C with 500 μl of either FA2-2 or OG1X cells in a total of 5 ml. The mixtures were then plated on N2GT at 42°C for selection of transconjugants. After 72 h, transconjugants exhibiting a dry colony morphology were screened for their characteristics at 32°C. Those whose colonies were dry at 42°C and watery at 32°C were extensively purified and subjected to further characterization.

To determine the percent survival after treatment with different concentrations of EMS, aliquots were diluted in phosphate-buffered saline and plated onto THB. After 48 h, colony counts were performed. Percent survival was estimated in relation to the number of colonies obtained for the control, which was taken to represent 100% survival.

DNA techniques and strain constructions. Routine screening of plasmid DNA was carried out by a small-scale alkaline lysis procedure previously described (36). Purified plasmid DNA from *E. coli* was prepared with the Plasmid Midi kit (Qiagen, Inc., Chatsworth, Calif.) as recommended by the manufacturer. Plasmid DNA was analyzed by digestion with restriction enzymes and separation of restriction fragments in 0.8% agarose gels. All restriction enzymes were purchased from GIBCO/BRL, Inc., and reactions were carried out under the conditions recommended. Standard recombinant DNA techniques were used in the construction of plasmids (2). PCR was performed with a Perkin-Elmer Cetus apparatus, under conditions recommended by the manufacturer. Specific primers were synthesized at the Biomedical Research DNA Core Facility of the University of Michigan. When appropriate, restriction sites for *Bam*HI were incorporated at the 5′ ends of each PCR product for subsequent cloning. PCR-generated fragments were purified by using QIAquick-spin columns (Qiagen Inc.), cleaved with the appropriate restriction enzyme and ligated to the vector plasmid. pBluescript KS^+ (Stratagene) was used for cloning DNA into *E. coli* strictly for sequencing purposes, whereas the shuttle vector pAM401 (41) was used for cloning DNA in *E. coli* and subsequent introduction into *E. faecalis*. In the case of the PCR procedure used to construct pAM2750, the primers used were 5′-GCGCGGATCCAGTGCATTCGTGGAATGATT-3′, whose 3′ end is 133 bp upstream of the initiation codon of *iad*, and 5′-GGCCGGATCCAATACTG AAAAGCAACTCAT-3′, whose 3′ end is 35 bp downstream of TTS2.

Transformation of *E. coli* and *E. faecalis* with the constructed plasmids was done by electroporation as described elsewhere (10, 14).

DNA sequencing. PCRs (at least two independent reactions per mutant) were used to amplify from each mutant a 1,593-bp fragment that included the region encoding *iad* and *traA*. The PCR products were then cloned into pBluescript KS^+. Dideoxy chain termination sequencing directly from double-stranded plasmids (3) was performed by the method described by Redston and Kern (31) with custom primers. Both DNA strands were sequenced. DNA sequences were analyzed with a MacVector software package from Eastman Kodak.

Characterization of colony morphology. Isolates were streaked on N2GT plates with or without cAD1, depending on the experiment. After incubation at the appropriate temperature for at least 72 h, colonies were viewed by oblique light microscopy to determine whether aggregation factors were expressed. A soft (watery [W]) colony appearance was previously correlated with lack of expression of aggregation substance, whereas a dry (D) colony appearance was correlated with expression (18). The latter phenotype is easily detected because the colonies fracture when touched with a toothpick (39).

Conjugative transfer experiments. Ten-minute broth matings were done as previously described (9, 18) at either 32 or 42°C. Before the matings, the donor cells were incubated for 1 h at either 32 or 42°C. Transconjugants were always checked for hemolysin production and for the absence of donor chromosomal markers. Transfer frequencies were calculated as the number of transconjugants per donor cell.

Pheromone and inhibitor assays. The concentrations of cAD1 and iAD1 were determined by the microtiter assay method described previously (8, 12). The cAD1 titer was defined as the reciprocal of the highest dilution of culture filtrate which induced aggregate formation in responder cells. The inhibitor titer was represented as the cAD1 titer in the absence of inhibitor divided by the titer in its presence. Strain DS16 was used as a responder in both cAD1 and iAD1 determinations.

Clumping assays. Cultures were diluted 1:100 in N2GT broth (total volume of 5 ml) containing chloramphenicol and/or erythromycin and were incubated statically at the desired temperature for at least 18 h. Aggregate formation was indicative of constitutive clumping.

Construction of *iad19*. Site-directed mutagenesis of *iad* was conducted, resulting in the changing of the amino-terminal leucine codon (TTA) of iAD1 to the nonsense codon TAA. This was carried out with a mutagenesis kit purchased from United States Biochemical (Cleveland, Ohio) and a synthetic oligonucleotide with the structure 5′-GACAACAAATT*AAGTTATCAA-3′ (the T with the asterisk occupies the position that is an A in the wild-type determinant). For single-strand DNA template preparation, *E. coli* NM522 (supplied in kit) carrying pAM2614 was infected with helper phage M13KO7 (supplied in kit) at a multiplicity of infection of 10 and grown overnight with vigorous shaking at 37°C. Preparation of phage particles and their DNA was done by standard protocols (2). Phage particles were recovered from supernatant with polyethylene glycol 8000. Single-strand DNA was prepared by phenol-chloroform treatment followed by ethanol precipitation. According to the method recommended by the manufacturers of the kit, the synthetic primer was treated with T4 polynucleotide kinase (Bethesda Research Laboratories) and annealed with the template. The complementary strand was synthesized with T7 DNA polymerase in the presence of 5-methyl-dCTP, and the newly synthesized strand was ligated with T4 ligase. The heteroduplex DNA was digested by *Msp*I (5′. . .CCGG. . .3′), which nicks only the nonmethylated parental strand. The DNA was then digested with exonuclease III. After inactivation of the enzyme, the DNA was used for transformation of the nonrestrictive host *E. coli* SDM supplied in the kit. Transformants which did not produce iAD1 were screened by the microtiter assay. One of two resulting iAD1-negative derivatives was designated pAM2616. An *Xba*I/*Bam*HI fragment was then subcloned from pAM2616 to pAM401, resulting in pAM2620. An *Xba*I/*Bam*HI fragment containing the wild-type equivalent is present in pAM2609 (33).

Complementation-suppression studies. In experiments involving the complementation of TS mutations, heteroplasmid strains were constructed. OG1X or FA2-2 cells containing the "helper" plasmids (Fig. 1) were used as recipients in matings in which strains harboring pAM714 derivatives containing a TS mutation were used as donors. All helper plasmids were derivatives of the shuttle vector pAM401. Transconjugants were selected on N2GT plates containing erythromycin, chloramphenicol, and either streptomycin or rifampin and fusidic acid. This procedure ensured the maintenance of both the helper plasmid and the test plasmid in the cells. Complementation of the TS phenotype was determined by examination of colony morphology and clumping of the heteroplasmid strains at 32 and 42°C and by measuring the transfer frequencies of the mutant plasmid at 42°C during short (10-min) broth matings. Recipient strains were FA2-2 when the donor was an OG1X host or JH2SS when the donor was an FA2-2 host.

Allelic exchange experiments. Allelic exchange (recombination) of wild-type sequences with homologous segments containing point mutations was performed as follows. Overnight filter matings were conducted with FA2-2(pAM2725) as a donor and OG1X(pAM2011E) as a recipient. Both donor and recipient plasmids carry *erm*, but pAM2725 also encodes production of hemolysin. Transconjugants were selected at 42°C on THB plates containing streptomycin (which selects for OG1X), erythromycin, and X-Gal (100 μg/ml). Blue colonies were then tested for β-galactosidase production at 32°C on the same medium. Colonies that were Hly^- and that consistently appeared blue at 42°C and white at 32°C were selected for further study. This involved plasmid DNA isolation and digestion with *Eco*RI, testing for plasmid transfer to FA2-2, and DNA sequencing in order to identify the presence of the point mutation on the recombinant plasmid.

Kinetics of β-galactosidase production. To analyze the kinetics of β-galactosidase production in the FA2-2 strain carrying pAM2756 or the parental plasmid pAM2011E, 1:10 dilutions were made from overnight cultures and grown at 32°C to mid-exponential phase (40 to 50 Klett units; Klett-Summerson colorimeter with no. 54 filter). Cultures were then subjected to growth under different conditions (i.e., maintained at 32°C, maintained at 32°C and exposed to cAD1, transferred to 42°C, or transferred to 42°C and exposed to cAD1), during which 2.5-ml aliquots were removed at 30-min intervals and assayed for enzyme activity as described by Miller (22). Activities were expressed in Miller units.

RESULTS

Generation of TS conjugation control mutants. Mutants exhibiting a colony morphology that was watery (W) at 32°C and dry (D) at 42°C were selected after mutagenesis with EMS, as described in Materials and Methods. Eleven independent mutagenesis experiments with either *E. faecalis* OG1X(pAM714) or FA2-2(pAM714) gave rise to a number of TS mutants identified after transfer of the plasmid to FA2-2 or OG1X, respectively, in short (10-min) broth matings at 42°C. From 6,145 transconjugant FA2-2 colonies, 24 (0.39%) showed a colony

TABLE 2. Colony morphology and constitutive clumping of pAD1 derivatives[a]

Plasmid	FA2-2 background				OG1X background			
	32°C		42°C		32°C		42°C	
	Colony morphology	Clumping[b]	Colony morphology	Clumping	Colony morphology	Clumping	Colony morphology	Clumping
pAM2700	W	−	WF/SD	++	SD	++	SD	++
pAM2703	W	−	WF/SD	++	D	++	D	++
pAM2708	W	−	SD/D	++	SD	++	D	++
pAM2709	W	−	WF/SD	++	D	++	D	++
pAM2712	W	−	WF/SD	++	D	++	D	++
pAM2717	W	−	WF/SD	++	D	++	D	++
pAM2725	W	−	D	++	W	−	D	++
pAM2120	D	++	D	++	D	++	D	++
pAM714	W	−	W	−	W	−	W	−

[a] Plasmids pAM714 and pAM2120 represent controls: the former plasmid is the "wild type," and the latter one is a *traA* mutant generated upon insertion of the transposon Tn*917lac* into the 5′ region of *traA*.
[b] −, no clumping observed; ++, clumping observed.

morphology expected for TS mutants, whereas from 6,325 OG1X colonies tested, 7 (0.11%) represented presumptive TS mutants. The variants were isolated in experiments which resulted in 4 to 6% survival of mutagenized cells with EMS concentrations of 470 to 564 μM. When survival was lower (0.1 to 1%) or higher (20 to 30%), no TS mutants were detected.

Characterization of the presumptive mutants. All 31 presumptive TS mutants were subjected to phenotypic characterization; in all cases, the mutation was linked to the plasmid. The results from seven representative mutants are described here. Plasmids pAM714 (typifies the wild type) and pAM2120 were included as controls in most experiments. pAM2120 is a previously derived *traA* mutant containing the transposon Tn*917lac* inserted into the 5′ region of *traA* (29); it is fully derepressed for clumping and plasmid transfer. Plasmid DNA from all TS mutants exhibited the same *Eco*RI restriction profile (eight bands) as pAM714, and the cells produced hemolysin.

By oblique light microscopy, colony morphologies could be distinguished, not all of which were of the W or D type. Some had intermediate morphologies and were designated as watery-fracturable (WF) or semidry (SD). Colony phenotypes and clumping in broth were examined in both OG1X and FA2-2 background. The results are summarized in Table 2. Interestingly, there were differences observed in the two hosts. In the FA2-2 background, the mutants had a W colony morphology at 32°C and a WF, SD, or D morphology at 42°C. In the OG1X background, however, most exhibited a D morphology at 42°C but also a D or SD morphology at 32°C. Thus, most of the plasmid mutations appeared as TS mutations only in the FA2-2 background. pAM2725 was the only derivative that exhibited a clear-cut temperature-sensitive behavior in both hosts (i.e., W colonies at 32°C and D colonies at 42°C); we refer to this plasmid as our prototype TS variant. In OG1X, the ability to clump in broth correlated with the D (or SD) colony morphology. In FA2-2, clumping was observed in all cases at 42°C and in no case at 32°C.

Transfer frequencies. Transfer potential of the TS mutants was determined by matings at both 32 and 42°C. Each mating was for 10 min; however, in each case the donors, pregrown at 32°C, were preincubated at either 32 or 42°C for 1 h prior to the matings. The matings were done at the same temperature as the specific preincubation. When the donors represented FA2-2 hosts, the recipients were JH2SS, and when the donors involved OG1X, the recipients were FA2-2. Ten-minute matings generally do not result in much transfer of wild-type plasmid, because the response to pheromone being produced by recipients usually takes 30 to 40 min. Thus, significant transfer observed in 10-min matings means that conjugation functions in the donors are already active. The results are shown in Table 3.

When present in FA2-2, the mutant derivatives of pAM714 showed little or no difference in transfer at 32°C compared to the parental plasmid. However, at 42°C, the mutant plasmids transferred at frequencies on the order of 100-fold higher. Prior incubation at 42°C of donors with the wild-type plasmid

TABLE 3. Transfer frequencies during 10-min matings at different temperatures[a]

Plasmid	From FA2-2 host to JH2SS		From OG1X host to FA2-2	
	32°C	42°C	32°C	42°C
pAM2700	$3.1 (\pm 4.2) \times 10^{-6}$	$7.2 (\pm 4.8) \times 10^{-5}$	$1.8 (\pm 3.1) \times 10^{-7}$	$5.1 (\pm 2.1) \times 10^{-5}$
pAM2703	$1.8 (\pm 1.4) \times 10^{-6}$	$1.6 (\pm 2.2) \times 10^{-4}$	$1.2 (\pm 1.2) \times 10^{-6}$	$6.0 (\pm 1.9) \times 10^{-5}$
pAM2708	$1.9 (\pm 2.6) \times 10^{-6}$	$7.8 (\pm 7.0) \times 10^{-5}$	$1.2 (\pm 0.6) \times 10^{-4}$	$3.7 (\pm 4.1) \times 10^{-3}$
pAM2709	$2.4 (\pm 5.4) \times 10^{-7}$	$7.9 (\pm 7.3) \times 10^{-5}$	$6.0 (\pm 6.1) \times 10^{-5}$	$2.3 (\pm 1.6) \times 10^{-3}$
pAM2712	$5.6 (\pm 3.4) \times 10^{-7}$	$3.8 (\pm 1.2) \times 10^{-5}$	$1.2 (\pm 2.0) \times 10^{-4}$	$2.4 (\pm 2.5) \times 10^{-3}$
pAM2717	$4.6 (\pm 5.5) \times 10^{-7}$	$4.3 (\pm 3.6) \times 10^{-5}$	$6.3 (\pm 3.0) \times 10^{-7}$	$3.2 (\pm 2.5) \times 10^{-4}$
pAM2725	$1.2 (\pm 0.2) \times 10^{-8}$	$3.5 (\pm 2.3) \times 10^{-4}$	$2.8 (\pm 1.2) \times 10^{-6}$	$1.6 (\pm 0.5) \times 10^{-3}$
pAM2120	$5.6 (\pm 3.1) \times 10^{-4}$	$2.4 (\pm 1.7) \times 10^{-3}$	ND[b]	ND[b]
pAM714	$<2.8 (\pm 4.5) \times 10^{-7}$	$6.5 (\pm 4.4) \times 10^{-6}$	$1.4 (\pm 1.0) \times 10^{-6}$	$9.1 (\pm 9.5) \times 10^{-6}$

[a] Prior to the matings, the donor cells were preincubated for 1 h at either 32 or 42°C. The conjugation frequencies represent the numbers of transconjugants per donor cell and were calculated as means (± standard deviations) of at least three independent experiments.
[b] ND, not done.

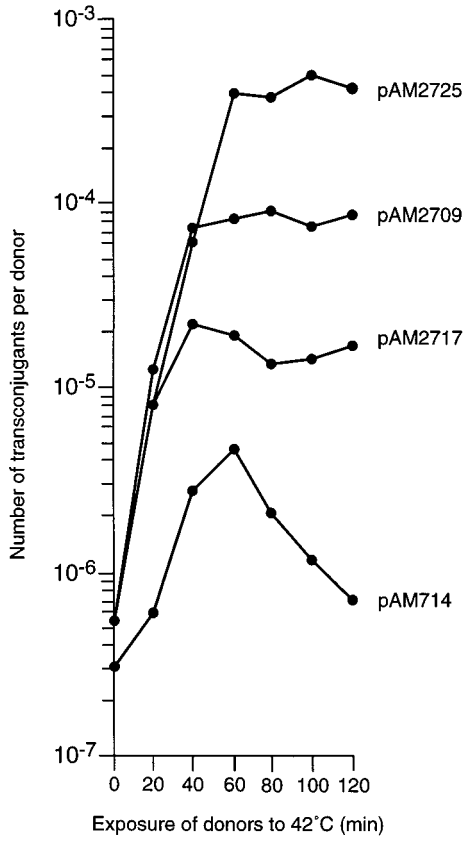

FIG. 2. Plasmid transfer in 10-min broth matings after prior exposure of donors to 42°C for increasing lengths of time. The recipient strain was JH2SS; the donors were derivatives of strain FA2-2 carrying plasmid pAM2725, pAM2709, pAM2717, or pAM714. The number of transconjugants per donor at each point represents the mean of at least three independent experiments.

pAM714 and the *traA* mutant pAM2120 seemed to increase transfer some, but this was small in comparison to the TS mutants.

When present in OG1X (Table 3), the mutants fell into two groups. Some (pAM2700, pAM2703, pAM2717, and pAM2725) did not exhibit a significant transfer potential at 32°C but transferred at relatively high frequencies at 42°C. While this was expected for pAM2725, it had been anticipated that the other three, because of their D or SD morphology on plates, would show elevated frequencies at 32°C. The second group, exemplified by plasmids pAM2708, pAM2709, and pAM2712, transferred at relatively high frequencies at 32°C, which correlated well with the D colony morphology and self-clumping exhibited at this temperature.

Temperature induction kinetics. Figure 2 shows the 10-min transfer frequencies of representative FA2-2 donors after prior incubation at 42°C for increasing lengths of time. All mutants tested were found to require 40 to 60 min at 42°C to fully express the derepressed phenotype, at which point the transfer frequencies leveled off. The final levels of transfer potential differed, depending on the particular mutant tested. pAM2725 was observed to reach the highest potential, whereas pAM2709 and pAM2717 reached levels that were 1 and 2 orders of magnitude lower, respectively. Interestingly, the wild-type pAM714 showed an increase in plasmid transfer during the first 60 min at 42°C, after which the frequency dropped back to a level similar to that of cells not exposed to the higher temperature.

TABLE 4. Extent of plasmid transfer in short broth matings after preincubation of the donor cells for 1 h at 42°C in the presence of cAD1 (40 ng/ml)[a]

Plasmid	Transfer frequency	
	− cAD1	+ cAD1
pAM2700	$5.1 (\pm 2.6) \times 10^{-5}$	$8.7 (\pm 4.1) \times 10^{-4}$
pAM2703	$3.2 (\pm 2.0) \times 10^{-5}$	$1.4 (\pm 1.0) \times 10^{-3}$
pAM2708	$2.2 (\pm 2.0) \times 10^{-4}$	$3.7 (\pm 2.0) \times 10^{-3}$
pAM2709	$3.3 (\pm 2.5) \times 10^{-5}$	$3.8 (\pm 2.1) \times 10^{-3}$
pAM2712	$5.1 (\pm 0.5) \times 10^{-5}$	$3.8 (\pm 4.0) \times 10^{-3}$
pAM2717	$7.9 (\pm 4.4) \times 10^{-5}$	$2.4 (\pm 2.2) \times 10^{-3}$
pAM2725	$4.0 (\pm 1.8) \times 10^{-4}$	$5.0 (\pm 2.9) \times 10^{-4}$

[a] The conjugation frequencies represent the numbers of transconjugants per donor cell and were calculated as means of three independent experiments. The donor strains were derivatives of FA2-2 carrying different mutations, and the recipient strain was JH2SS.

Sensitivity of mutants to pheromone. To test if the mutants could still respond to pheromone, colony morphologies were examined with the FA2-2 host on plates at 32°C containing synthetic cAD1 (40 ng/ml). With the exception of pAM2725, the colonies were of the D type at the low temperature, indicating a pheromone response. Cells harboring pAM2725 remained type W, implying an absence of a pheromone response. Table 4 shows results in which pheromone (40 ng/ml) was present for 1 h at 42°C prior to 10-min matings. All mutants exhibited at least a 17-fold enhancement of transfer after exposure to cAD1 compared to temperature-induced cells not exposed to pheromone. However, this was not the case for pAM2725, which was not affected.

When cells harboring pAM2725 were exposed to cAD1 at 32°C for 1 h prior to matings at 32°C, a relatively small (about sevenfold) increase in transfer was observed from the FA2-2 background (Table 5). A similar experiment in the OG1X background showed a 250-fold enhancement of transfer. In this background, some stimulation (27-fold) was observed at 42°C as well. In these experiments, one of our strains harbored the plasmid pAM2100, which contains a Tn*917lac* insertion near the 3′ end of *traA* (29). This plasmid had been previously reported (36) as not responding to cAD1, and as shown here (Table 5), it did not respond at 32°C.

Production of cAD1 and iAD1. The level of production of endogenous cAD1 and the pAD1-encoded inhibitor (iAD1) was analyzed for the strains carrying mutant plasmids and

TABLE 5. Transfer frequencies of plasmid pAM2725 in short broth matings compared with plasmid pAM714[a]

Origin	Temp (°C)	Plasmid	− cAD1	+ cAD1
FA2-2	32	pAM2725	$3.9 (\pm 3.1) \times 10^{-6}$	$2.2 (\pm 2.3) \times 10^{-5}$
		pAM714	$<1.7 (\pm 0.5) \times 10^{-8}$	$3.7 (\pm 1.5) \times 10^{-4}$
		pAM2100	$3.2 (\pm 2.6) \times 10^{-5}$	$3.0 (\pm 3.0) \times 10^{-5}$
	42	pAM2725	$4.0 (\pm 1.8) \times 10^{-4}$	$5.0 (\pm 2.9) \times 10^{-4}$
OG1X	32	pAM2725	$2.2 (\pm 1.0) \times 10^{-6}$	$5.5 (\pm 2.1) \times 10^{-4}$
		pAM714	$1.4 (\pm 1.0) \times 10^{-6}$	$5.2 (\pm 1.3) \times 10^{-4}$
		pAM2100	$5.2 (\pm 2.2) \times 10^{-5}$	$5.2 (\pm 1.6) \times 10^{-5}$
	42	pAM2725	$3.1 (\pm 0.5) \times 10^{-3}$	$8.3 (\pm 1.9) \times 10^{-2}$

[a] Prior to the matings, the donor cells were incubated at either 32 or 42°C for 1 h in either the presence or the absence of synthetic cAD1 (40 ng/ml). The conjugation frequencies represent the numbers of transconjugants per donor cell and were calculated from three independent experiments.

TABLE 6. Complementation analyses at 42°C in the genetic background of OG1X[a]

Tested plasmid	Value for helper plasmid:											
	pAM401			pAM2609 (*iad* plus *traA*)			pAM2620 (*iad19* plus *traA*)			pAM2750 (*iad*)		
	M	C	Transfer	M	C	Transfer	M	C	Transfer	M	C	Transfer
pAM2700	SD	+	1.2×10^{-4} 2.2×10^{-4}	W	−	1.5×10^{-5} 4.2×10^{-6}	D	++	1.5×10^{-5} 4.2×10^{-5}	W	−	6.2×10^{-5} 3.1×10^{-5}
pAM2703	D	+	8.8×10^{-5} 5.2×10^{-5}	W	−	4.2×10^{-6} 2.3×10^{-6}	SD	++	7.6×10^{-5} 2.8×10^{-5}	W	−	1.6×10^{-5} 3.2×10^{-5}
pAM2709	SD	+	3.6×10^{-4} 2.2×10^{-4}	W	−	2.5×10^{-6} 5.5×10^{-6}	WF/SD	++	1.1×10^{-4} 8.8×10^{-5}	SD	++	5.3×10^{-4} 5.1×10^{-4}
pAM2712	D	++	3.5×10^{-4} 5.8×10^{-4}	W	−	3.2×10^{-6} 7.2×10^{-6}	WF/SD	++	5.3×10^{-5} 4.8×10^{-3}	D	++	3.1×10^{-4} 2.4×10^{-4}
pAM2717	D	+	4.8×10^{-5} 3.8×10^{-5}	W	−	9.3×10^{-6} 1.7×10^{-6}	SD/D	++	1.5×10^{-5} 2.2×10^{-5}	W	−	3.9×10^{-5} 3.4×10^{-5}
pAM2725	SD	++	3.3×10^{-3} 2.2×10^{-3}	W	−	3.4×10^{-5} 3.5×10^{-5}	WF	++	1.8×10^{-4} 3.0×10^{-4}	D	++	4.2×10^{-3} 8.3×10^{-4}
pAM2120	D	++	4.8×10^{-3} 2.6×10^{-3}	W	−	1.1×10^{-6} 1.8×10^{-5}	SD/D	++	8.4×10^{-5} 8.7×10^{-4}	D	++	1.0×10^{-2} 3.2×10^{-3}
pAM714	WF	−	4.8×10^{-6} 2.6×10^{-6}	W	−	1.3×10^{-6} 2.1×10^{-6}	WF/SD	++	6.5×10^{-5} 5.1×10^{-5}	WF	+	2.5×10^{-4} 8.1×10^{-5}

[a] M, colony morphology; C, clumping; −, no clumping; +, weak clumping; ++, strong clumping. The transfer frequencies represent the numbers of transconjugants per donor cell.

grown at either 32 or 42°C. cAD1 and iAD1 production was assayed in OG1X and FA2-2 host backgrounds, respectively. None of the strains examined produced detectable levels of cAD1 (i.e., titers of <2) and thus resembled the wild-type plasmid in this regard. Levels of iAD1 detected in the supernatants of all mutants were similar to those for the wild type at both temperatures.

Complementation studies. As a first step in locating the specific mutational lesions on the plasmid, complementation analyses were performed. Due to host-related differences already noted, the studies were conducted in both OG1X and FA2-2. Since regulatory determinants seemed most likely to be involved, constructs carrying the *iad-traA* region were examined for the ability to complement. Figure 1 illustrates the segments tested; these were present in the shuttle vector pAM401.

pAM2609 contains a segment extending from an *Xba*I site upstream of TTS1 (a transcription termination site) to the *Bam*HI site of a transposon (Tn*917lac*) that had been inserted downstream of *traA* in *traC*. pAM2620 is a similar clone, except that *iad* carries a mutation that converts the codon for the first amino acid of iAD1 to a stop codon (see Materials and Methods; note that iAD1 corresponds to the last 8 amino acid residues in a 22-amino-acid precursor peptide [8]). pAM2750 carries a PCR product containing *iad* as well as the two transcription termination sites, TTS1 and TTS2.

Experiments with the OG1X background. When OG1X cells contained any of the mutant plasmids along with pAM2609, they no longer gave rise to dry (type D) colonies at 42°C, nor did they give the appearance of clumping at the high temperature in broth. In addition, as shown in Table 6, transfer during short matings at 42°C was reduced (at least 20-fold) compared to the case when the empty shuttle vector was present. Thus, for all the mutants tested an apparent complementation was provided by pAM2609.

When the pAM2620 construct was used, most strains showed only a small effect on colony morphology at 42°C. The colonies never became as dry as the case for the controls (i.e., when pAM401 was present); however, they were not watery. They all clumped in broth at 42°C and transferred the plasmid DNA at relatively high frequencies at that temperature. Interestingly, the strain carrying the wild-type plasmid pAM714 together with pAM2620 gave rise to colonies that were WF and/or SD at 42°C; this strain also clumped at 42°C and transferred plasmid DNA at higher frequencies than expected. This was in contrast to the results for cells carrying pAM714 along with pAM401 or pAM2609.

In another set of experiments, pAM2750 containing an intact *iad* determinant but no *traA* was examined (Table 6). In this case, the mutants fell into two groups. The first included plasmids pAM2709, pAM2712, and pAM2725, whereby complementation was not observed. The second group, which included pAM2700, pAM2703, and pAM2717, behaved in an unexpected way; they showed complementation for colony morphology and clumping but not for transfer. Unexpected also were the results observed with the wild-type plasmid (pAM714), which were similar to those described when the pAM2620 construct was used (see above); that is, there was increased expression of conjugation functions.

Experiments with the FA2-2 background. All mutations could be complemented by plasmid pAM2620 (Table 7); at 42°C, the strains did not exhibit the dry colony morphology, self-clumping, or transfer of plasmid DNA at high frequencies. Therefore, complementation by pAM2609 was not tested, since it had already been shown to occur in OG1X. These results ruled out the possibility that any of the mutations could

TABLE 7. Complementation analyses at 42°C in the genetic background of FA2-2[a]

Tested plasmid	Value for helper plasmid:								
	pAM401			pAM2620 (*traA* plus *iad19*)			pAM2750 (*iad*)		
	M	C	Transfer	M	C	Transfer	M	C	Transfer
pAM2700	D	++	2.3×10^{-5} 3.2×10^{-5}	W	−	$<1.8 \times 10^{-8}$ $<1.1 \times 10^{-8}$	W	−	6.4×10^{-5} 5.2×10^{-5}
pAM2703	SD/D	++	2.0×10^{-5} 2.9×10^{-5}	W	−	5.3×10^{-7} 2.4×10^{-7}	W	−	5.7×10^{-5} 3.9×10^{-5}
pAM2709	WF	++	1.7×10^{-4} 1.0×10^{-4}	W	−	5.9×10^{-7} 1.3×10^{-6}	WF	++	5.7×10^{-4} 2.9×10^{-4}
pAM2712	D	++	3.3×10^{-4} 1.6×10^{-4}	W	−	1.3×10^{-6} 1.5×10^{-6}	D	++	4.2×10^{-4} 2.6×10^{-4}
pAM2717	SD/D	++	2.6×10^{-5} 3.1×10^{-5}	W	−	$<1.1 \times 10^{-8}$ 7.7×10^{-7}	W	−	6.5×10^{-5} 4.6×10^{-5}
pAM2725	D	++	6.5×10^{-4} 3.1×10^{-4}	W	−	5.7×10^{-6} 1.2×10^{-6}	SD	++	3.1×10^{-4} 3.6×10^{-4}
pAM2120	D	++	4.0×10^{-4} 6.9×10^{-4}	W	−	4.0×10^{-6} 3.5×10^{-6}	D	++	5.5×10^{-4} 4.0×10^{-4}
pAM714	W	−	5.7×10^{-6} 2.6×10^{-6}	W	−	2.1×10^{-6} 4.0×10^{-7}	W	−	7.3×10^{-5} 4.8×10^{-5}

[a] M, colony morphology; C, clumping; −, no clumping; +, weak clumping; ++, strong clumping. The transfer frequencies represent the numbers of transconjugants per donor cell.

be located within *iad*. In relation to the helper plasmid pAM2750, the results were similar to those observed in OG1X. There was no complementation of the mutations found in pAM2709, pAM2712, and pAM2725. Mutants pAM2700, pAM2703, and pAM2717 could be complemented only in relation to colony morphology and clumping; conjugal transfer remained high. In the case of the wild-type pAM714, a higher (10-fold) transfer frequency was observed at 42°C in the presence of pAM2750; however, unlike the case for OG1X, neither dry colonies nor clumping was detected at this temperature.

Since the complementation studies did not make use of a Rec^- host, it was conceivable that a low level of recombination might have taken place. To test this possibility, the heteroplasmid strains were grown for about 30 generations in the absence of chloramphenicol to allow segregation of the helper plasmids. (pAM401 derivatives are relatively unstable in *E. faecalis* in the absence of selective pressure [41].) Cm^s colonies were checked for colony morphology at 42°C and were shown to have reverted to the colony morphology exhibited in the absence of the helper plasmids. This implies that recombinants were not significantly present in the heteroplasmid strains.

DNA sequence analysis of TS mutants. Since the evidence showed or implied that all mutations could be complemented by pAM2609 in both hosts and by pAM2620 in at least one host (FA2-2), the region from the *Xba*I site left of *iad* through *traA* of each mutant plasmid was sequenced. The positions of each mutation found are shown in Fig. 1 and 3. All corresponded to transitions in which a GC pair was replaced by an AT pair; in four cases, they were located within *traA*. pAM2708 and pAM2712 carry the same mutation, which affects amino acid residue 181 of TraA (CCA→TCA). Since these were derived from the same mutagenesis experiment, they are probably siblings. These mutants, and that represented in pAM2709 (CCG→TCG), replace a proline residue with a serine residue; the latter was at amino acid position 229. pAM2725 showed the replacement of a serine residue at position 71 with a phenylalanine (TCT→TTT).

pAM2703 and pAM2717 had the same mutation, although these were derived from independent mutagen exposures. The lesion is located 119 bp downstream of the TAA stop codon of the *iad* gene, in a region that had not been previously noted as bearing a specific determinant (Fig. 3). However, the mutation appears within a small ORF that would encode a highly hydrophobic peptide of 23 amino acid residues (2,522 Da). The ORF is in the opposite orientation from *iad*; a potential ribosome binding site (AGGA) and a characteristic promoter are found upstream of the putative ATG initiation codon. The mutation replaces a glycine residue (fourth amino acid residue from the N terminus) with an aspartate (GGT→GAT).

The mutation carried by mutant pAM2700 could not be found within either *traA* or *iad* and has thus far not been identified.

Introduction and analysis of a TS mutation introduced onto a miniplasmid with a Tn*917lac* transcriptional fusion downstream from TTS2. pAM2011E is a plasmid that corresponds to the *Eco*RI-B fragment of pAD1 carrying a Tn*917lac* insertion 3 bp downstream of TTS2, making LacZ expression dependent upon induction by cAD1 (30, 37). The plasmid carries all of the regulatory determinants of pAD1 as well as those necessary for autonomous replication and maintains the low copy number of pAD1; it is devoid of essentially all structural genes related to conjugation and hemolysin production. The TS mutation of the prototype plasmid pAM2725 was introduced into pAM2011E by allelic exchange as described in Materials and Methods so that the effect of temperature shifts could be quantitated by monitoring β-galactosidase expression. The resulting construction was designated pAM2756.

Kinetic studies were done comparing pAM2756 and its pa-

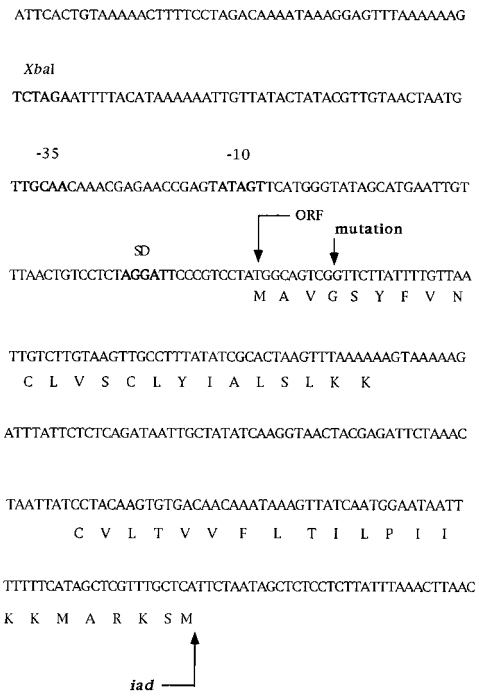

FIG. 3. DNA sequence of the region comprising a small ORF (*traD*) located downstream of the *iad* gene. Putative Shine-Dalgarno (SD) and promoter sequences are also indicated. The mutation found in plasmids pAM2703 and pAM2717 is indicated by an arrow. (The previously published [30] GenBank accession number that includes this sequence is M87836.)

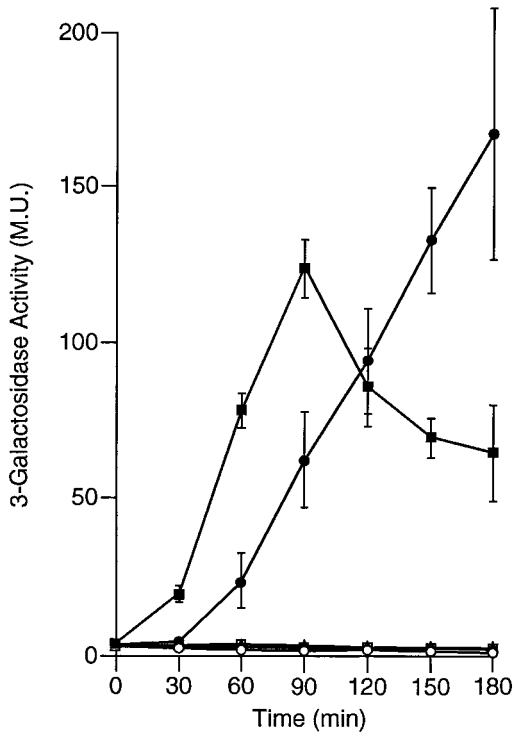

FIG. 4. Kinetics of induction of β-galactosidase activity in strain FA2-2(pAM2756) compared to strain FA2-2(pAM2011E). The strains were grown at 32°C in THB until growth reached 40 to 50 Klett units, at which point they were subjected to different growth conditions. At the indicated times, samples were taken, and enzyme activity was measured. Each curve represents the mean of three independent experiments. ■, FA2-2(pAM2756) at 42°C; □, FA2-2(pAM2756) at 32°C; △, FA2-2(pAM2756) at 32°C plus cAD1; ●, FA2-2(pAM2011E) at 32°C plus cAD1; ○, FA2-2(pAM2011E) at 42°C. M.U., Miller units.

rental pAM2011E, and the results are seen in Fig. 4. FA2-2(pAM2756) failed to express β-galactosidase at 32°C in the presence of cAD1, a phenotype consistent with data above showing that this *traA* mutation rendered the plasmid insensitive to cAD1 when present in the FA2-2 background. In contrast, enzyme activity was fully inducible by cAD1 in strain FA2-2(pAM2011E). Incubation of strain FA2-2(pAM2756) at 42°C caused a sharp increase in LacZ activity detected in culture lysates, with the highest activity being detected after 90 min. This differed sharply with the behavior of strain FA2-2(pAM2011E), which produced the same basal level of enzyme throughout the entire experiment.

Kinetic experiments were not performed with the OG1X host; however, experiments performed on solid medium containing X-Gal, and either in the presence or in the absence of synthetic cAD1 (80 ng/ml), showed that strain OG1X(pAM2756) produced enzyme activity (blue colonies) at 32°C only in the presence of cAD1. When incubated at 42°C, this strain was able to produce significant LacZ activity. The addition of cAD1 to the medium seemed to increase the level of the enzyme further, since the bacterial growth developed a slightly darker blue color.

Thus, LacZ activity in cells carrying pAM2756 is constitutively expressed only at 42°C, as expected for a plasmid carrying a TS mutation in *traA*. Moreover, like the case for pAM2725, pAM2756 is defective in responding to cAD1 in the FA2-2 background.

DISCUSSION

An important consideration regarding the control of transcriptional readthrough of TTS1-TTS2 relates to previously reported evidence that the downstream *traE1* product is able to up-regulate itself from its own (*traE1*) promoter located within TTS2 (15, 33). Because read-through may trigger a burst of *traE1* expression, the prevention of even small amounts of transcription through TTS1-TTS2 in the absence of pheromone would seem important. Indeed, the complexity of the region upstream of the terminators may relate in some way to the need for highly efficient termination. In this view, TraE1 itself or some step in its expression is believed to be relatively unstable or inefficient, and therefore, maintaining TraE1 expression at a level needed for activating conjugation functions may require at least some degree of continued transcriptional read-through of TTS1-TTS2. The data presented here show that in addition to TraA and its action at the *iad* promoter there is indeed at least one other control factor, and it involves genetic information located between the *iad* promoter and TTS1.

Of particular interest, it was found that two of our mutations (those of pAM2703 and pAM2717, which were identical) mapped within a small putative ORF that would encode a 23-amino-acid peptide. This ORF had not been given serious attention previously because of its small size; however, it does appear to have putative promoter and ribosome binding sites. It will subsequently be referred to as *traD*; it is in the opposite orientation from *iad*, which encodes the 22-amino-acid precursor of the pheromone inhibitor peptide iAD1. As noted earlier, a transcript encompassing the entire region between the 5′ end of *iad* and TTS1 has the potential for significant secondary structure. A *traD* transcript would also exhibit secondary struc-

ture and of course would be a countertranscript to at least a portion of RNA transcribed from the *iad* promoter to TTS1-TTS2. The potential interactions of such transcripts and the role of corresponding mutational lesions in *traD* in conferring temperature sensitivity are difficult to assess at this time.

A significant feature of the data presented here is that a number of the TS mutants behaved differently in the two *E. faecalis* host strains, FA2-2 and OG1X. The one exception was the pAM2725-related mutation, which was similar in both hosts with regard to temperature inducibility. The lesion in pAM2725 mapped about 25% in from the 5′ end of *traA*. Interestingly, when in the FA2-2 host at 32°C, pAM2725-carrying cells were relatively insensitive to cAD1, whereas in the OG1X host significant cAD1 induction occurred at this temperature. This was consistent with observations of LacZ expression from cells harboring the pAM2756 miniplasmid (which harbors the same TS mutation as pAM2725) carrying a transcriptional fusion just downstream from TTS2. Essentially, no LacZ induction by cAD1 could be detected in FA2-2 at 32°C, whereas enzyme activity was expressed readily in the pheromone-exposed OG1X host. Kinetic studies of temperature-induced conjugation found that pAM2725 exhibited the greatest increase in transfer frequency among the mutant strains examined. Maximum transfer frequencies in 10-min matings were generally attained within 60 min of exposure to 42°C.

Of the seven temperature-sensitive lesions studied, four (pAM2708, pAM2709, pAM2712, and pAM2725) mapped within *traA*, whereas three (pAM2703, pAM2717, and pAM2700) were located outside of *traA*. A recurring theme in several aspects of our investigation was an absence of correlation between aggregation and transfer functions in the OG1X host. In FA2-2, all the mutants showed good correlation among clumping, colony morphology, and plasmid transfer; all characteristics were temperature sensitive. However, in the OG1X host only plasmid transfer was temperature sensitive; except for pAM2725, we observed dry colony morphology and clumping at 32°C as well as 42°C. Of these, only pAM2708, pAM2709, and pAM2712 (mutations in *traA*) exhibited an elevated transfer frequency at 32°C (around 10^{-4}) compared to parental pAM714 (around 10^{-6}). The other mutants exhibiting dry colonies at 32°C, which mapped outside of *traA*, had transfer frequencies closer to that of pAM714 at the low temperature, and at 42°C, the mating frequencies were lower (around 10^{-4}) than for those mapping within *traA* (around 10^{-3}). Thus, it appears that in the OG1X host those mutants with lesions outside of *traA* have functions relating to aggregation and presumably the initiation of mating pair formation being expressed at 32°C without significant transfer of plasmid DNA.

Complementation studies showed that in *trans* a region containing *traA* along with *iad* and *traD* (pAM2609) was able to complement at 42°C the mutations located in *traA* or *traD* in OG1X cells. Interestingly, when the same region of DNA contained a point mutation eliminating iAD1 expression (pAM2620), the ability to complement was lost in the OG1X host but not in FA2-2. Thus, it would appear that, in the OG1X host, production of iAD1 is an important cofactor in the complementation (or suppression) of both the *traA* and *traD* mutations. Apparently, one or more host functions are involved differently in recognizing *iad* expression or interacting with iAD1 in the two backgrounds. It is intriguing that, when the DNA defective in iAD1 production (pAM2620) was present with the wild-type pAM714, the latter exhibited an induced state at 42°C in OG1X. In FA2-2, this was not the case.

A region containing *iad* and *traD*, but not *traA*, was, as expected, not able to complement the *traA* mutations but did appear to affect the *traD* mutation and the outside mutation of pAM2700 with respect to colony morphology and the clumping phenomenon in both the OG1X and FA2-2 hosts. Interestingly, the plasmid transfer phenotype was not changed; that is, transfer still occurred at an elevated frequency at 42°C. However, because the *trans*-acting chimera (pAM2750) had the effect of elevating transfer of the parental pAM714 in OG1X, as well as facilitating clumping and a slight change in colony morphology (from W to WF), it is likely that the apparent absence of DNA transfer-related complementation actually relates to a stimulation or activation of the conjugation system. A similar but lesser effect on DNA transfer was observed in the FA2-2 host, but in this case, noticeable clumping or a change in colony morphology was not detected. One reasonable explanation for enhancement of transfer functions would be that TraA of pAM714 is being titrated by the multicopy plasmid chimera (pAM2750). (TraA is known to bind to a site within the *iad* promoter [33].) However, it cannot be ruled out that pAM2750 may be providing a positively acting regulatory product.

It is noteworthy that differences between OG1X and FA2-2 in connection with the pAD1 system have been reported previously (38, 39). For example, it is known that plasmid-free OG1X cells secrete about eightfold more cAD1 than do FA2-2 cells. And when pAD1 is present, OG1X host cells are significantly more sensitive to exogenous cAD1 than FA2-2 cells carrying the plasmid; this appears in part to be related to the pAD1-borne *traC*, whose product is a pheromone-binding protein (TraC) located on the cell surface (32). Published data (38) imply that, while TraC enhances cAD1 sensitivity in OG1X, it is nonfunctional or not expressed in FA2-2. It is not clear whether these host differences relate to the differences observed in the present study, although it is tempting to speculate that the different levels of host-encoded cAD1 expression are involved. It is known that expression of endogenous cAD1 in donor (plasmid-containing) cells is not shut down completely (27) and that the plasmid-encoded inhibitor peptide iAD1 probably plays an important role in preventing self-induction by small amounts of endogenous pheromone. Thus, since OG1X cells produce a lot more (eightfold) cAD1 than do FA2-2 cells, iAD1 may play a more significant role in preventing self-induction. In the case when pAM2609 was present together with the mutant plasmids, or even the wild-type pAM714, the cells may be more sensitive to cAD1 (or bind more cAD1) because of larger amounts of TraA present. (As noted below, there is evidence that TraA binds directly to cAD1.) In this case, however, the additional iAD1 that is also present may compensate for the increased sensitivity and might even enhance TraA affinity for DNA. Since iAD1 is not produced in the case of pAM2620, the excess TraA would be available to bind to cAD1, resulting in self-induction. In FA2-2, the absence of *iad* expression (by pAM2620) may not be critical because the host produces much lower levels of endogenous cAD1. This would appear to be a reasonable explanation of the differences in the complementation data involving pAM2620 in the two hosts.

It is clear that there are multiple loci in the pheromone response regulatory region of pAD1 that play key roles in governing the pheromone response and that mutations can exhibit different phenotypes in different hosts. We have also seen that, within *traA*, TS lesions in different sites can result in different phenotypes, an observation that is consistent with differences in the behavior of various transposon insertions in *traA* (i.e., certain Tn*917* insertions in *traA* near the 3′ end do

not result in a full derepression of conjugation genes and are insensitive to cAD1 exposure [29].) The newly identified *traD* determinant in which two similar TS lesions were found is particularly interesting and is currently the subject of further study. Recent data indicate that it is transcribed readily in the absence of exposure to pheromone but becomes shut down during induction (2a); conceivably, *traD* expression somehow enhances the efficiency of termination at TTS1-TTS2.

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