

A pAD1-Encoded Small RNA Molecule, mD, Negatively Regulates *Enterococcus faecalis* Pheromone Response by Enhancing Transcription Termination

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pAD1 is a 60-kb hemolysin-bacteriocin plasmid in *Enterococcus faecalis* that encodes a conjugative mating response to a peptide sex pheromone, cAD1, secreted by plasmid-free bacteria. The pheromone response is regulated by two proteins: TraE1, which positively regulates all or most conjugative structural genes, and TraA, which negatively regulates *traE1*. TraA binds to pAD1 DNA at the *iad* (encoding the inhibitor peptide iAD1) promoter but is released upon binding to imported pheromone. This leads to enhanced transcription through two closely spaced downstream terminators (t1 and t2) into *traE1*. TraE1 is believed to then upregulate itself from a site located within t2; thus, a small amount of transcription through t1-t2 could lead to overall induction. It is important therefore that the t1-t2 terminators be tightly controlled to keep the response shut down in the absence of pheromone. A small (200-nucleotide) RNA molecule designated mD is encoded just upstream of t1 by a determinant (*traD*) oriented in the direction opposite to that of transcripts utilizing t1. mD is expressed at high levels in the uninduced state, but it decreases significantly upon induction. Here we present results of genetic studies relating to the activity of t1-t2 and show that mD strongly enhances transcriptional termination at t1. The mD activity is shown to influence transcription well downstream and can affect the determinant for aggregation substance *asa1*. The phenomenon is specific in that there is no effect of mD on the unrelated pheromone-responding plasmids pPD1 and pCF10.

The conjugative plasmid pAD1 (60 kb), originally identified in the multiple-antibiotic-resistant *Enterococcus faecalis* DS16 (12, 40), encodes a hemolysin-bacteriocin (cytolysin) that contributes to virulence in animal models (6, 25, 27). Hemolytic clinical isolates from globally diverse geographical locations frequently harbor a highly similar, if not identical, plasmid (8, 9). pAD1 encodes a mating response to an octapeptide sex pheromone (cAD1) secreted by plasmid-free recipient cells (32), and investigations into the regulation of this response have been ongoing in our laboratory for a number of years. (For recent reviews of the enterococcal pheromone response phenomenon, see references 9, 15, and 29). The response leads to synthesis of a proteinaceous aggregation substance (21, 22) encoded by *asa1*; this protein binds to enterococcal binding substance which is present on the surface of both recipients and donors and may correspond in part to lipoteichoic acid (16). A second surface protein, Sea1, is expressed at a low level in the uninduced state but is upregulated upon exposure to pheromone and appears to affect surface exclusion (43). When donors are exposed to a culture filtrate of recipients (plasmid-free bacteria), they exhibit a clumping response which can even result in donor-donor mating, although at a reduced frequency compared to that involving transfer to plasmid-free cells (10). The clumping response provides the basis of a convenient assay for the quantitation of pheromone by serial dilution. The locations of *asa1* and *sea1*, as well as other determinants relating

to the regulation of the pheromone response, are shown in Fig. 1.

When pAD1 is acquired by a recipient, it shuts down or masks the production of cAD1; the extent of the shutdown depends on the bacterial host (34). The transconjugants continue to secrete different pheromones which are specific for donors carrying different families of responsive plasmids (8, 13). pAD1 itself encodes an octapeptide (iAD1) which is secreted and acts as a competitive inhibitor (antipheromone) of cAD1 (24, 31); its purpose is believed to be the prevention of self-induction of donors that may still be releasing some endogenous cAD1. The iAD1 peptide corresponds to the last 8 amino acid residues of a 22-amino-acid amphipathic precursor resembling a lone signal sequence (11); its determinant is designated *iad*.

pAD1 encodes two primary regulatory proteins: TraE1, which positively regulates structural genes required for conjugation as well as itself, and TraA, which negatively regulates expression of TraE1 (Fig. 1). TraA acts to downregulate transcription from the *iad* promoter which governs expression of a transcript extending about 450 nucleotides (nt) to a termination region (t1-t2). t1 and t2 are separated by 125 nt, and based on energy considerations, the stem of t1 is stronger than that of the downstream t2 (37). The initiation of *traE1* expression results from transcriptional readthrough of t1-t2 from the *iad* promoter (20, 37, 38). We have previously reported that TraA binds directly to imported cAD1, causing the protein to release its binding to DNA at the *iad* promoter, resulting in upregulation of transcription (18). This increase in transcription is assumed to contribute to readthrough into *traE1*.

Another factor has recently been identified which, like TraA, also negatively regulates *traE1*. This relates to a small open reading frame (*traD*), but there is evidence that it is the related transcript mD (200 nt) that is the active component (2,

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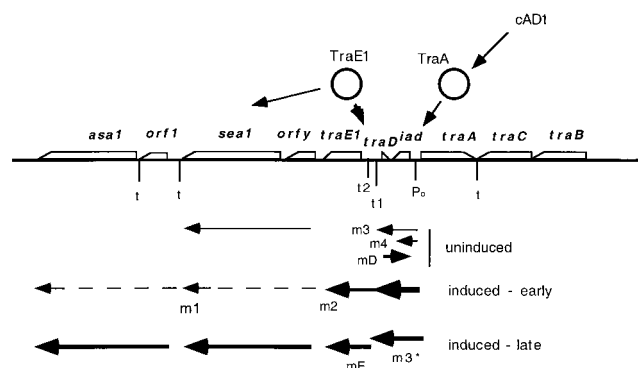


FIG. 1. pAD1 map showing regulatory determinants and transcripts observed in the uninduced, early-induced (e.g., 5 min), and late-induced (e.g., 30 min) states. The arrows below the map represent the various transcripts and their orientations. Induction is viewed as the pheromone (cAD1) binding to TraA, causing a release of its binding to the *iad* (which encodes inhibitor iAD1) promoter, leading to enhanced transcription from the promoter as well as transcription through *t1*. The "induced-early" transcripts reflect various extensions resulting from read-through of the *t1-t2* terminators giving rise to *m1* and *m2*. The dashed line indicates that, while transcription may continue in the indicated direction, the relative degrees have not been determined. The thickness of the arrows corresponds qualitatively with the relative levels of expression. *m3* terminates at *t1*, whereas *m3** is an extension of *m3* believed to terminate at *t2*. *mD* and *m4* overlap at their 3' ends by about 80 nt. *mE* is viewed as a transcript of *traE1* which has been activated at its own promoter within *t2* by TraE1 initially generated from the transient expression of *m2*. *mD* is generally expressed at a high level in the uninduced state but is downregulated by the pheromone-induced transcription from the opposing *iad* promoter.

3). *mD* is transcribed in the opposite direction from that of *iad*, from a promoter located downstream of *iad*. The *mD* transcript is present at a high level in uninduced cells; upon exposure of cells to cAD1, however, it becomes greatly reduced within 5 min and is almost undetectable after 10 min (3). It was suggested that *mD* acts negatively, along with TraA, to reduce transcription from the *iad* promoter and thus help prevent transcriptional readthrough into *traE1*. A temperature-sensitive mutant presumed to affect the conformation of *mD* was observed to cause induction at elevated temperature (32 to 42°C) as well as to enhance transcription from the *iad* promoter. Thermal induction of the conjugation response could be complemented in *trans* by plasmid clones carrying the *mD* (*traD*) determinant (2). It was hypothesized that reduction of *mD* synthesis upon pheromone exposure was related to the enhanced transcription from the *iad* promoter blocking transcriptional initiation from the downstream *mD* promoter on the opposite DNA strand. Based on structural considerations, both *mD* RNA and RNA transcribed from the *iad* promoter to *t1* exhibit extensive secondary structure, and it is thus reasonable that point mutations might significantly affect folding.

In the present communication, we focus on the function of *t1-t2* and show that, in addition to whatever influence *mD* may have at the *iad* promoter, it also has an effect independent of TraA on transcription through *t1*. We also show that, despite significant complementarity between the *mD* transcript and a potential transcript from a region just upstream of the *asa1* determinant (3), *mD* does not appear to affect transcription here.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. pAM714 (23) is a derivative of pAD1 carrying an insertion of the erythromycin resistance transposon Tn917 in a region (1) not involved in transfer. Its behavior with regard to transfer is therefore assumed to reflect the wild-type pAD1.

Media and reagents. Media used were Todd-Hewitt broth (Difco Laboratories) or N2GT (nutrient broth no. 2 [Oxoid Ltd., London, United Kingdom] supplemented with 0.2% glucose and 0.1 M Tris-HCl [pH 7.5]) for *E. faecalis* and Luria-Bertani broth (28) for *Escherichia coli*. Solid media were prepared by including 1.5% agar. Cultures were grown at 37°C unless otherwise noted. Cell density was determined using a Spectronic colorimeter at a wavelength of 600 nm. General reagents were essentially as previously described (2, 3). The following antibiotics were used at the indicated concentrations; ampicillin, 100 µg/ml; erythromycin, 20 µg/ml; streptomycin, 500 µg/ml; kanamycin, 500 µg/ml; spectinomycin, 500 µg/ml; chloramphenicol, 50 µg/ml in the case of *E. coli* and 20 µg/ml in the case of *E. faecalis*; rifampin, 25 µg/ml; and fusidic acid, 25 µg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Gibco BRL) was used at a concentration of 40 µg/ml for *E. coli* and 80 µg/ml for *E. faecalis*. Synthetic pheromone peptides were prepared at the University of Michigan Core Peptide Synthesis Facility.

Construction of plasmid chimeras for use in complementation studies. pAM-HTs (pAM1240) was constructed using the same PCR method and primers for construction of pAM-HT and pAM-HTd1 as described in the work of Bastos et al. (3). *Bam*HI sites were incorporated into the 5' termini of the primers, allowing for cloning into the *Bam*HI site of the shuttle vector pAM401. pAM-HTs has the same orientation in the vector as the corresponding segments in pAM-HT and pAM-HTd1.

Pheromone response and beta-galactosidase (LacZ) assays. The LacZ activities were determined as previously described (41), with the following minor modifications. One hundred microliters of an overnight culture of the appropriate strain was subcultured in 2.5 ml of fresh N2GT medium and grown for 30 min at appropriate temperatures. Cultures were then exposed for 90 min with synthetic cAD1 at concentrations of 0, 20, 40, 100, and 200 ng/ml. Cells were harvested from 2.1-ml aliquots, suspended in 200 µl of 0.05 M sodium phosphate buffer (pH 7.5), and permeabilized with 100 µl of toluene for 15 min at 37°C. Samples were incubated in 0.1 ml of 3.2 mM reduced glutathione–0.25 ml of 0.01 M *o*-nitrophenyl-β-D-galactopyranoside–2.0 ml of 0.05 M sodium phosphate buffer (pH 7.5) for 30 min at 37°C. The reaction was stopped with 0.5 ml of 1 M Na₂CO₃, and cell debris was removed by centrifugation. Absorbance was determined at 420 nm on a Spectronic colorimeter, and the results were expressed in Miller units (MU) (30).

Analysis of transcripts. RNA was prepared as described previously (3). The probe representing the region between *t1* and *t2* corresponded to 124 nt and was generated as follows. A PCR-amplified segment between *traD* and just upstream of *t2* was prepared using the upstream primer 5'-ATTTGGATCCATTGATAACTTTATTGTTGT-3' and the downstream primer 5'-AAATTGGATCCACGGCTCTACGAGTAGTTC-3' (both contain *Bam*HI sites near the 5' ends) and was cloned into the *Bam*HI site of pBlueScript II KS(+). Then an *Hpa*I/*Bam*HI fragment of the plasmid containing the region between *t1* and *t2* was subcloned into the *Eco*RV/*Bam*HI site of pBlueScript II KS(+) (designated pAM1235). A *Hind*III/*Bam*HI fragment of this construct was used as a probe in the Northern blot experiment. Northern blot analyses involved electrophoresis in a 1.2% agarose-MOPS (morpholinepropanesulfonic acid)-formaldehyde system. Hybridization was carried out at 42°C in the presence of 50% formamide. The probe was labeled by nick translation using a kit from Bethesda Research Laboratories.

Construction of the orfTTS1 mutation in pAM714. The orfTTS1 mutation was constructed as follows using the Promega Altered Site II in vitro mutagenesis kit. pAM1.6SK is a pBlueScript clone carrying a 1.6-kb pAD1 fragment extending from upstream of *iad* to downstream of *traE1* (11). The 1.6-kb pAD1 DNA fragment was removed using *Bam*HI and *Kpn*I and cloned into the *Bam*HI/*Kpn*I site of the pALTER-1 *E. coli* vector; the derivative was designated pALTER::1.6SK. Site-directed mutagenesis of pALTER::1.6SK was done using the kit protocol with some minor modifications. A synthetic 21-mer primer (5'-AGCGGGGAATATATACAGTTC-3') designed to make the desired orfTTS1 mutation (ATG to ATA; Met to Ile) was used. Mutated DNA was expected to be devoid of a *Bst*Z17I cleavage site (GTATAC) that could be screened easily. A 1.6-kb *Eco*RI fragment from a mutated plasmid was subcloned into the *Eco*RI site of the pSF141 (encoding Km^r and Cm^r determinants that are expressed in gram-positive bacteria [39]) vector that is suicidal in *E. faecalis*. The recombinant plasmid pSF141::1.6SK was introduced into *E. faecalis* OG1X/pAM714 by electroporation (17). Transformants (assumed to carry a cointegrate plasmid) were selected on plates containing erythromycin and kanamycin. A recombinant derivative was subcultured for 14 passages in Todd-Hewitt broth without drug and plated on medium containing erythromycin. Colonies were then replica plated on medium containing kanamycin, and candidates (Em^r Km^r) were screened using PCR amplification to obtain DNA to be examined for the absence of the *Bst*Z17I cleavage site. The two primers used for PCR and cloning here were 5'-TTACGGAATTCAGATCATGCGTGTGTGTACC-3' (within *traA*) and 5'-ATTGCGAATTCACAGCAAACATCCCCCTCAATT-3' (within *traE1*). One candidate containing the expected mutation was designated pAM1220; the mutation was confirmed by sequencing.

Construction of mutations within the *t1* region. The 1.6-kb *Eco*RI fragment of pALTER::1.6SK was cloned into the *Eco*RI site of pSF141 (designated pSF141::1.6SK). Four kinds of mutations were constructed using pSF141::1.6SK by utilizing the *Xba*I, *Acc*I, *Bst*Z17I, and *Hpa*I cleavage sites around the *t1* sequence. pAM1213 has a deletion of the segment between the *Bst*Z17I and

TABLE 1. Strains and plasmids used in this study^a

Strain or plasmid	Relevant features	Source or reference(s)
Strains		
<i>E. faecalis</i>		
OG1X	<i>str gel</i>	24
FA2-2	<i>rif fus</i>	12
JH2-2	<i>rif fus</i>	26
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1</i> Δ <i>gyrA96</i> Δ (<i>lacZYA-argF</i>)U169	Bethesda Research Laboratories
Plasmids		
pAD1	60-kb Hly-Bac	12, 40
pPD1	59-kb Bac	19, 45
pCF10	54-kb Tc(Tn925)	14
pAM714	pAD1::Tn917; Hly-Bac <i>erm</i> ; wild-type mating properties	1, 23
pAM714-d1HT	pAM714 derivative with point mutation (Met to Arg) in <i>traD</i>	3
pAM2703	pAM714 with ts mutation in <i>traD</i>	2
pAM2120	pAD1 with Tn917 insert in <i>traA</i>	34
pAM-HT	pAM401 with cloned 321-nt PCR fragment carrying <i>traD</i>	3
pAM-HTd1	Same as pAM-HT except with the d1 (<i>traD</i> Met-to-Arg) mutation	3
pAM-HTts	Same as pAM-HT except with the ts lesion of pAM2703	This study
pAM7304	pAD1 with Tn917 <i>lac</i> insertion between <i>traD</i> and <i>iad</i>	36
pAM7348	pAD1 with Tn917 <i>lac</i> insertion between <i>traD</i> and t1	36
pAM2019	pAD1 with Tn917 <i>lac</i> insertion within t1	36
pAM7343	pAD1 with Tn917 <i>lac</i> insertion between t1 and t2	36
pAM2011	pAD1 with Tn917 <i>lac</i> insertion between t2 and <i>traE1</i>	36
pAM7016	pAD1 with Tn917 <i>lac</i> insertion in <i>sea1</i>	35
pAM7025	pAD1 with Tn917 <i>lac</i> insertion in <i>asa1</i>	35
pAM2125	pAD1 with Tn917 <i>lac</i> insertion between <i>traE1</i> and <i>orfy</i> and a Tn917 <i>lac</i> insertion in <i>traA</i>	41
pBluescript KS(+)	<i>E. coli</i> plasmid vector; <i>amp</i>	Stratagene
pAM1.6SK	1.6-kb fragment (<i>iad</i> through <i>traE1</i>) cloned in pBluescript	11
pSF141	Cloning vector (Km ^r and Cm ^r); suicidal in <i>E. faecalis</i>	39
pAM401	<i>E. coli-E. faecalis</i> shuttle; <i>cat tet</i>	44
pALTER-1	Mutagenesis vector; Amp ^r	Promega
pALTER::1.6SK	1.6-kb <i>KpnI/BamHI</i> fragment from pAM1.6SK cloned in pALTER	This study
pSF141::1.6SK	1.6 kb <i>EcoRI</i> fragment from pALTER::1.6SK cloned in pSF141	This study
pAM1220	pAM714 with point mutation, ATG to ATA, at orfTTS1 start site	This study
pAM1221	pAM714 with AT insertion in t1 repeat	This study
pAM1222	pAM714 with AGCT insert in <i>XbaI</i> site in mD (<i>traD</i>) promoter	This study
pAM1224	pAM714 with <i>HpaI/BstZ171</i> deletion destroying t1	This study
pAM1231	pAM714 with <i>AccI/XbaI</i> deletion destroying t1	This study
pAM7016-dHB	pAM7016 also carrying the t1 deletion of pAM1224	This study
pAM7025-dHB	pAM7025 also carrying the t1 deletion of pAM1224	This study

^a Hly, hemolysin; Bac, bacteriocin; ts, temperature sensitivity.

HpaI cleavage sites. pAM1218 has a deletion of the segment between the *XbaI* and *AccI* cleavage sites. pAM1216 has an extra 4 bp generated by use of the Klenow enzyme to fill in the ends resulting from a cleaved *XbaI* site. pAM1217 has an additional 2 bp generated using the Klenow enzyme to fill in the ends resulting from a cleaved *AccI* site. The mutations in the four mutated plasmids were incorporated into pAM714 in the same manner as described above to generate pAM1220. The resulting plasmids were designated pAM1221, pAM1222, pAM1224, and pAM1231, respectively. In the cases where a t1-related mutation was incorporated into plasmid pAM7016 (Tn917*lac* inserted in *sea1*) and pAM7025 (Tn917*lac* inserted in *asa1*) to generate pAM7016dHB and pAM7025dHB, respectively, the pAM1213 plasmid (deletion between *BstZ171* and *HpaI*) was utilized for the allelic exchange.

RESULTS

pAM-HT corresponds to the *E. coli-E. faecalis* shuttle vector pAM401 carrying a 321-nt PCR fragment containing *traD*; when pAM-HT is present in *E. faecalis*, a high level of expression of mD RNA has been observed (3). pAM-HT and a derivative, pAM-HTd1, unable to translate *traD*, were previously shown to be capable of complementing in *trans* a temperature-sensitive pAD1 point mutation (pAM2703) located in

traD (3). mD RNA was therefore assumed to be the critical product in restoring the normal (temperature-stable) phenotype.

Table 2 shows that when *E. faecalis* cells carrying the wild-

TABLE 2. Effects of mD on the sensitivity of *E. faecalis* strains harboring pAD1, pPD1, or pCF10

Strain/plasmid	Pheromone	Pheromone concn (ng/ml) required to generate clumping response after addition of plasmid:		Difference (fold)
		pAM401 (vector)	pAM-HT (cloned <i>traD</i>)	
OG1X/pAD1	cAD1	16	125	8
FA2-2/pAD1	cAD1	64	500	8
JH2-2/pPD1	cPD1	250	250	0
FA2-2/pCF10	cCF10	0.5	1.0	2

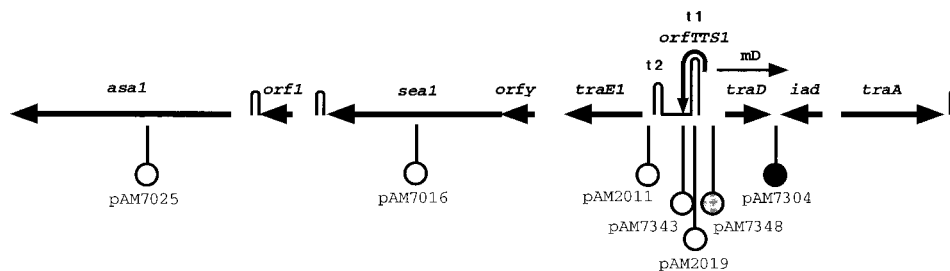


FIG. 2. Map showing the location of the Tn917lac insertions that are present in the indicated plasmid derivatives. All of the insertions are oriented such that the promoterless *lacZ* on the transposon will report transcription from right to left. The circles that are filled or contain shades of gray represent various levels of LacZ expression in the absence of pheromone. The unfilled circles mean that there is essentially no expression of LacZ in the uninduced state. The hairpin structures, in addition to t1 and t2, correspond to likely transcription terminators.

type pAD1 also carry pAM-HT, they become significantly less sensitive to cAD1; this was the case for two nonisogenic hosts, OG1X and FA2-2. To exhibit a clumping response, bacteria carrying pAM-HT required a pheromone concentration eight times higher than that when the cells were harboring the empty vector. The effect was specific for pAD1; no difference was observed when pAM-HT was present with plasmid pPD1 (which responds to cPD1), and only a twofold difference was noted for pCF10 (which responds to cCF10).

mD RNA influence at the transcription termination site t1. In an effort to determine how the mD product may affect transcription at different locations on pAD1, we examined a series of *lacZ* fusions corresponding to several pAD1::Tn917lac insertions. A map showing the location of these fusions, all of which are oriented to report transcription from right to left, is shown in Fig. 2. The data presented in Fig. 3 show that when excess mD is provided by pAM-HT or pAM-HTd1 there is a major effect on the expression of beta-galactosidase only on fusions that are located downstream of t1. For example, cells harboring pAM7343, containing the fusion just downstream of t1, were much less sensitive to cAD1, whereas there was no effect on pAM7348 with its insert just upstream of t1. With respect to pAM2019, where the insert is located within t1, presumably making the terminator nonfunctional, no effect was apparent. The effect of mD was observed well downstream as in the cases represented by the insertions in *asa1* (pAM7025) and *sea1* (pAM7016). Transcription in these determinants appeared lower than that for regions upstream. This is consistent with previous observations relating to Northern blot analyses (38) which showed that transcripts that extended to these genes were present at relatively low levels. The insertion between *iad* and *traD* (pAM7304) led to a full constitutive expression of LacZ, which is not complemented in *trans*—a result that we have noted previously (3).

pAM-HTts1 differs from pAM-HT in that the 321-nt segment that includes *traD* contains the temperature-sensitive lesion of pAM2703. As shown in Fig. 3, cells carrying the mutated chimera are still reduced in their sensitivity to pheromone but to a much lesser extent than that for cells carrying the corresponding wild-type DNA. We note that these experiments along with those utilizing pAM-HT and pAM-HTd1 were conducted at 37°C, a temperature midway between 32 and 42°C, the differential that served to define temperature sensitivity. The data presented in Fig. 4 are related to similar experiments using three of the insertion derivatives, but in this case, cells grown at 32 or 42°C were compared. It is apparent that the inhibitory effect of the wild-type product (from pAM-HT) was not affected by temperature in the case of pAM7343 (insertion downstream of t1) and pAM7025 (insert in *asa1*),

whereas when pAM-HTts1 was present, there was a much greater sensitivity to pheromone at the higher temperature. It is noteworthy that the cells in general appeared more sensitive to pheromone at the elevated temperature, even in the absence of additional *traD* product (i.e., see the case where the empty pAM401 was present). In the case of pAM7343, there was also an apparent temperature sensitivity related to the product of pAM-HTd1, which may be related to the nucleotide difference that resulted in altering the translational start codon, having an effect on RNA secondary structure. As anticipated, in the case of pAM7348 (insertion upstream of t1), none of the combinations significantly affected the sensitivity to pheromone at either temperature.

The effect of mD at t1 does not require functional TraA. In a previous communication (3), we discussed the possibility that mD might be able to interact with TraA, although this was in the context of events believed to be occurring at the *iad* promoter. To examine the possibility that an interaction might somehow influence transcriptional readthrough of t1, we conducted the following experiments.

Plasmid pAM2125 contains two oppositely oriented transposon insertions, as illustrated in Fig. 5A. One corresponds to Tn917lac inserted into *traA* (within the N-terminal 25% of the determinant); the other is a Tn917lac transposon inserted just downstream of *traE1*. The two insertions are in opposite orientations. The Tn917lac insertion downstream of *traE1* expresses beta-galactosidase at a high (constitutive) level because of the defective TraA, whereas the *lacZ* gene of the *traA* insertion is very low and not affected by pheromone (41). When pAM-HT or pAM-HTd1 was also present, there was a significant reduction (four- to fivefold) in enzyme expression (Fig. 5B), and this was not affected by exposure of the cells to cAD1. In the case where pAM-HTts1 was present with pAM2125, a less-than-twofold reduction was observed. These data imply that mD RNA effects a reduction of transcriptional readthrough at t1 without requiring an interaction with TraA. It is unlikely that the results could be related to an effect (upregulation) on expression from the *traA* promoter, since this would have been reflected in the data for pAM7348 in Fig. 3.

Analyses of transcription through t1 and effects of structural alterations in the terminator. In an earlier report (38), it was shown that in the uninduced state there was significant transcription from the *iad* promoter to the t1-t2 region, giving rise to a transcript that was designated m3. Upon induction, there was a major increase in this RNA; however, at that time the extent to which m3 termination was occurring at t1 or t2 was not distinguished. Northern blot hybridization data shown in Fig. 6, for which a probe corresponding to the region be-

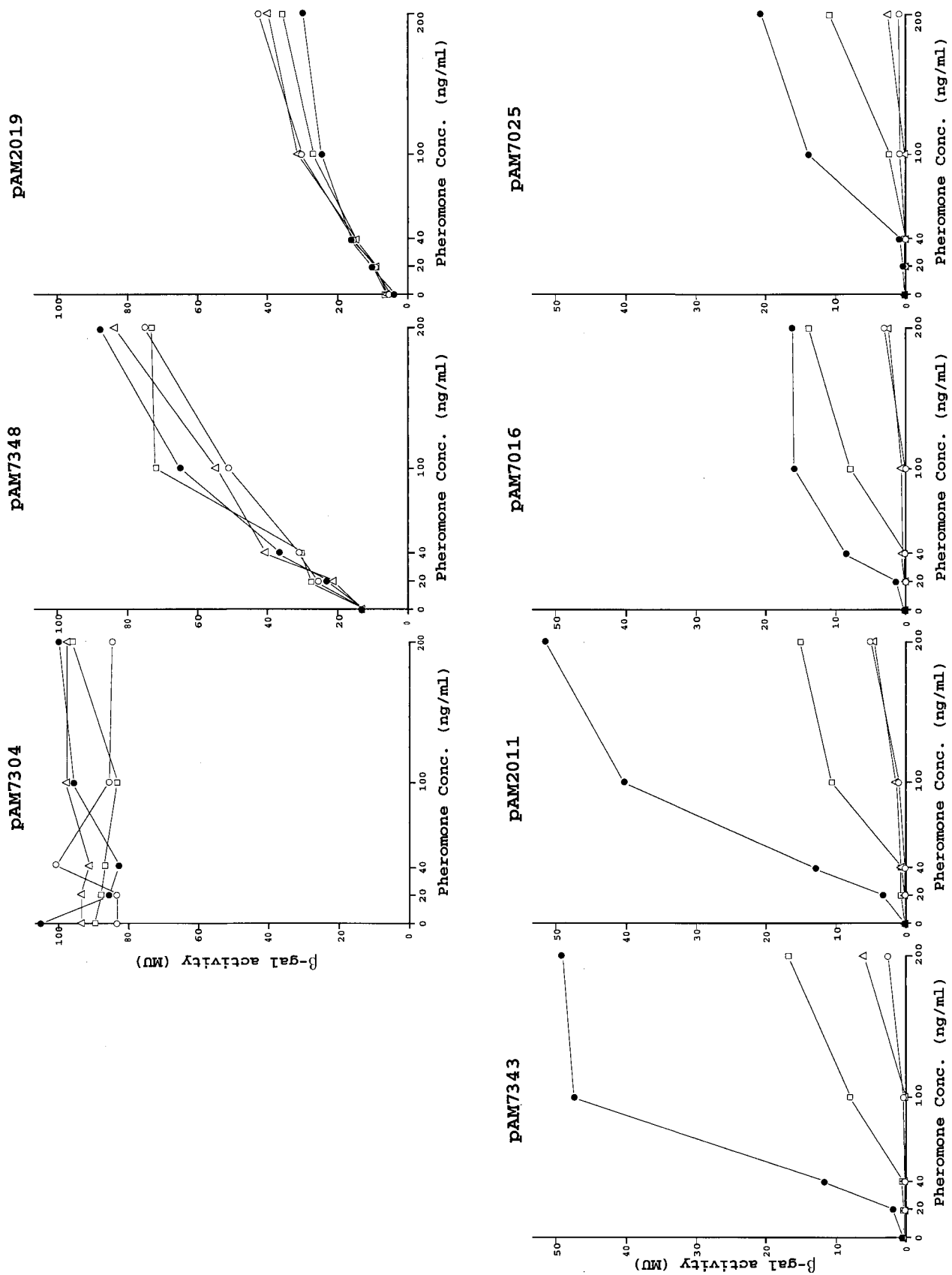
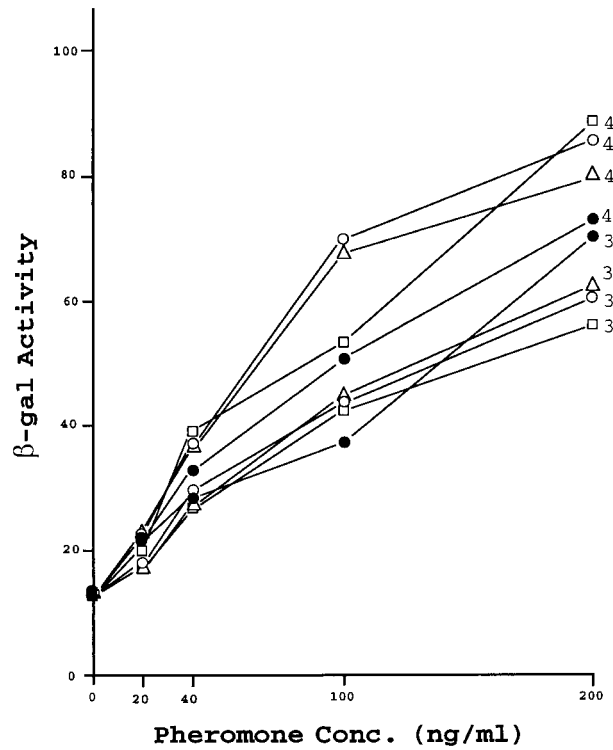


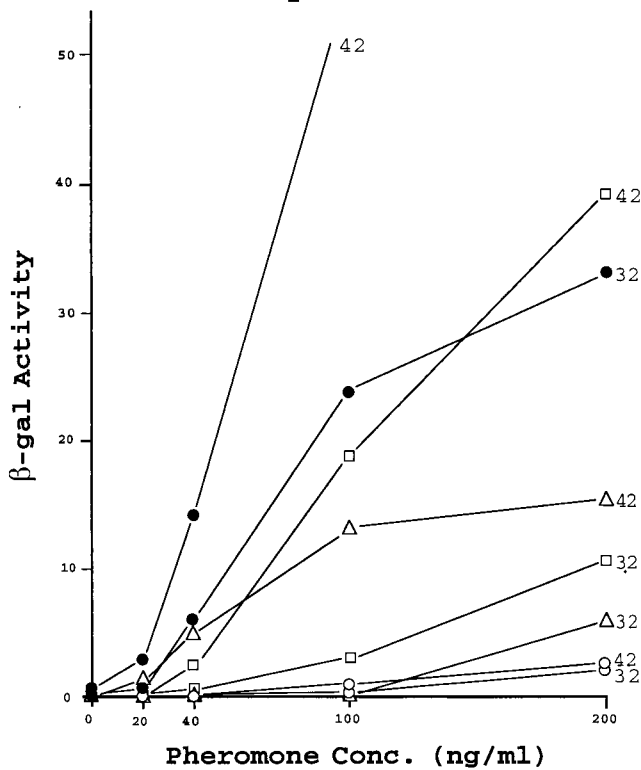
FIG. 3. Induced expression of β -galactosidase by various pAD1::Tn9/lac derivatives in response to increasing concentrations of cAD1 while in the presence of additionally provided mD in *trans*. The host strain was *E. faecalis* FA2-2 carrying the indicated plasmid. The plasmids provided in *trans* were pAM401 (●), pAM-HT (○), pAM-HTdI (△), and pAM-HTts1 (□). Exposure to cAD1 was for 1 h in each case.

FIG. 4. Effect of growth temperature on the cAD1 sensitivity of cells carrying various pAD1::Tn917lac derivatives in the presence of additionally provided mD in *trans*. The host strain was *E. faecalis* FA2-2 carrying the indicated plasmid. The plasmids provided in *trans* were pAM401, (●), pAM-HT (○), pAM-HTd1 (△), and pAM-HTts1 (□). Cells were grown at 32 or 42°C as indicated. Exposure to cAD1 was for 1 h in each case. The enzyme activity was expressed in MU.

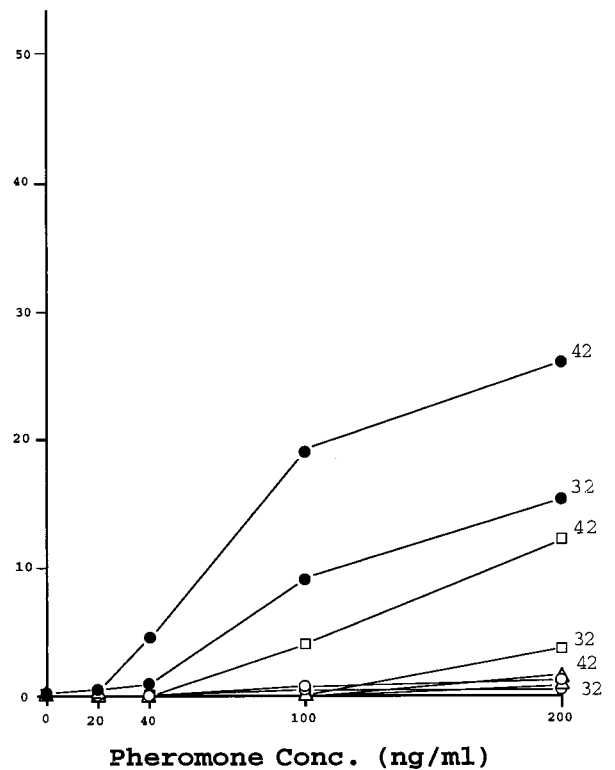
pAM7348



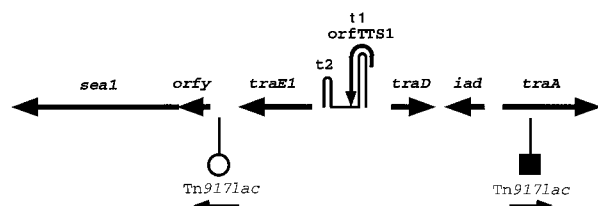
pAM7343



pAM7025



A.



B.

plasmid	β -gal activity (MU) (% of control)	
	-cAD1	+cAD1 (400ng/ml)
pAM401	77.0 (100%)	78.4 (102%)
pAM-HT	16.4 (21%)	19.0 (25%)
pAM-HTd1	20.8 (27%)	21.6 (28%)
pAM-HTts1	42.0 (55%)	49.6 (64%)

FIG. 5. Inhibition of t1 transcriptional readthrough by mD in the absence of TraA. (A) The host strain was *E. faecalis* FA2-2 carrying the plasmid pAM2125 with two transposon insertions as indicated. (B) Level of expression of β -galactosidase when the cells, also carrying the indicated additional plasmid, were exposed to cAD1 for 1 h or were not exposed. The percent activities were based on comparison with pAM401, set at 100%.

tween t1 and t2 was used, indicate the absence of detectable transcripts extending beyond t1 in the uninduced state (lane 1). This is supportive of the above data indicating that termination was occurring primarily at t1. Induction, however, resulted in detection of a transcript corresponding to a size in a range consistent with being about 150 nt longer than what would correspond to the \sim 500-nt m3 (Fig. 6, lane 2). Such a transcript is consistent with termination at t2, and we now designate this m3*. The probe also revealed a longer (\sim 1,050-nt) transcript which we had earlier noted (38) and called m2; this RNA appears to terminate downstream of *traE1*. The regions representing these transcripts are illustrated in Fig. 1. Thus,

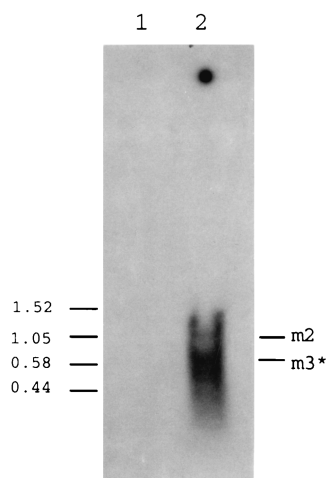


FIG. 6. Northern blot hybridization of RNA from induced and uninduced OG1X cells carrying pAM714. The probe corresponded to a 124-nt segment of DNA representing the region between t1 and t2. Lane 1, uninduced cells. Lane 2, cells induced with synthetic cAD1 for 20 min.

while extensive transcriptional readthrough occurs during induction, significant, but not highly efficient, termination occurs at t2.

A number of variants altered in the structure of t1 were constructed and introduced into pAM714 (a pAD1::Tn917 derivative with essentially wild-type properties) by allelic exchange (see Materials and Methods). Figure 7 illustrates the nature of the modifications (Fig. 7A) and associated phenotypes (Fig. 7B). Note in Fig. 7B that pAM714 transfers at a very low frequency during the relatively short (10-min) time period. Since significant induction of the mating response generally requires at least 30 min, the 10-min data reflect only the extent to which the donors are expressing mating functions in the absence of pheromone. In addition, cells carrying pAM714 give rise to colonies with a watery morphology, in contrast to those expressing aggregation-mating functions which exhibit a dry colony morphology (42).

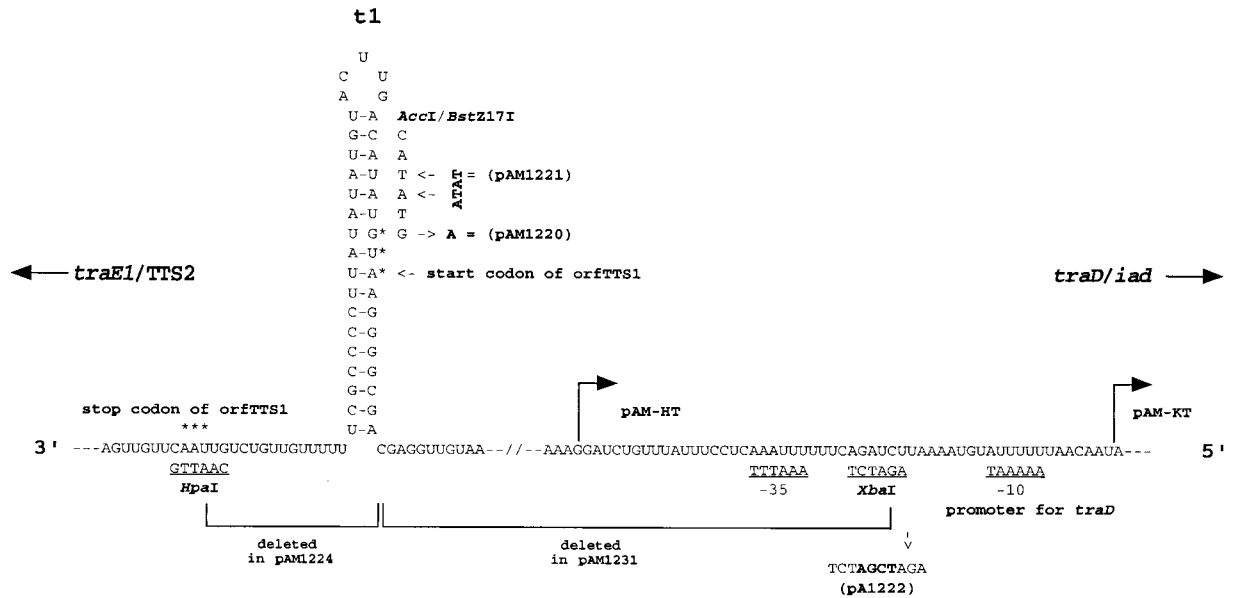
We had previously noted a possible short open reading frame (orfTTS1) corresponding to 15 amino acids with a start site within the upstream repeat of t1 (37). pAM1220 is a derivative in which the start codon was eliminated by changing ATG to ATA (i.e., a methionine to an isoleucine codon); the G-to-A substitution at the third position should not significantly affect the stem-loop structure of the related RNA. Although these cells exhibited a slight clumping, their colony morphology and mating properties were very similar to those of the wild type (pAM714); thus, orfTTS1 would not appear to be of major significance, at least under the conditions examined, if indeed it is expressed as a peptide at all.

pAM1224 contained a deletion between the *BstZ17I* site and the downstream *HpaI* site, essentially eliminating the termination structure and resulting in (i) a dry or semidry colony morphology (depending on the host), (ii) clumping, and (iii) a high frequency of transfer during a 10-min mating. A similar result was found for pAM1231, which contained a deletion between the *BstZ17I* and upstream *XbaI* sites. pAM1221 contains a disruption in t1 as a result of a 2-nt insertion (AT) into the *AccI* site located within one of the repeated sequences; this significantly weakened the terminator, since clumping was evident and plasmid transfer occurred at a greatly increased frequency during a 10-min mating.

Finally, a derivative, pAM1222, not involving t1 but affecting the promoter site of mD via the insertion of 4 nt between the -10 and -35 promoter hexamers resulted in cells exhibiting full expression of mating functions. The promoter modification is presumed to eliminate the expression of mD, which in turn results in a reduction in t1 termination strength, allowing enough readthrough of t1 (and also t2) to upregulate the mating response.

Influence of mD on *asaI* and *seaI* transcription appears related only to t1 readthrough. pAM7016-dHB and pAM7025-dHB correspond to pAD1::Tn917lac derivatives with insertions in *asaI* and *seaI*, respectively, which also carry a deletion of a major portion of t1 (deletion between the *HpaI* and *BstZ17I* sites). As shown in Fig. 8, in the uninduced state expression of LacZ occurs at a level significantly higher than that for the corresponding derivatives pAM7016 and pAM7025, which have a normal t1 (Fig. 3). The elimination of t1 resulted in an expression of about 6 MU, compared to values of below 1 MU when t1 was present. This would be anticipated since, as we observed above, transcriptional readthrough in the case of t1 mutants leads to at least some expression of mating functions. Enzyme activity increased upon exposure to increasing concentrations of pheromone, reaching a maximum value similar to those observed for the wild-type t1 shown in Fig. 3 (i.e., pAM7016 and pAM7025); however, when mD was pro-

A.



B.

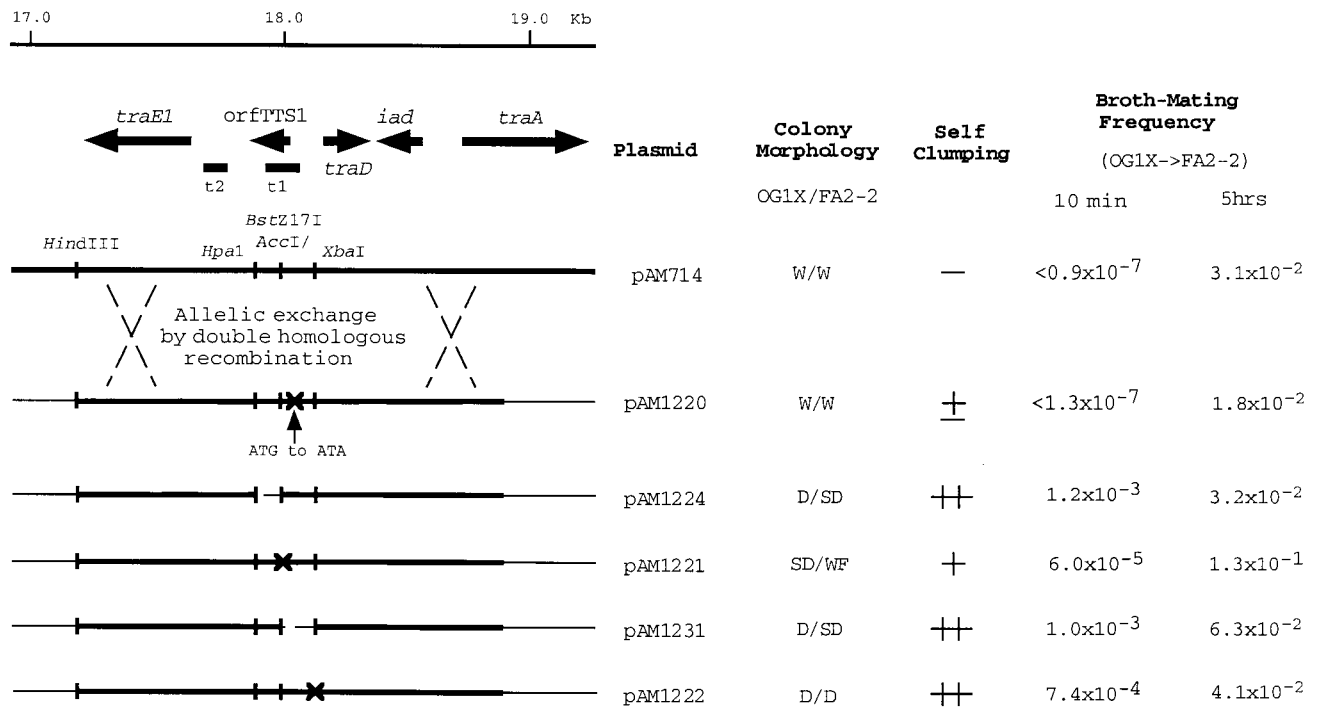


FIG. 7. Analyses of mutations in and around t1. (A) Specific genetic modifications that were generated. (B) Various structural changes and the resulting phenotypes. W, watery (typical of uninduced cells); D, dry (typical of cells fully induced); SD, semidry; WF, watery-fracturable. +, clumping; ++, extensive clumping; ±, slight clumping; —, no clumping. These are all in the absence of added cAD1. The mating frequencies are indicated as number of transconjugants per donor.

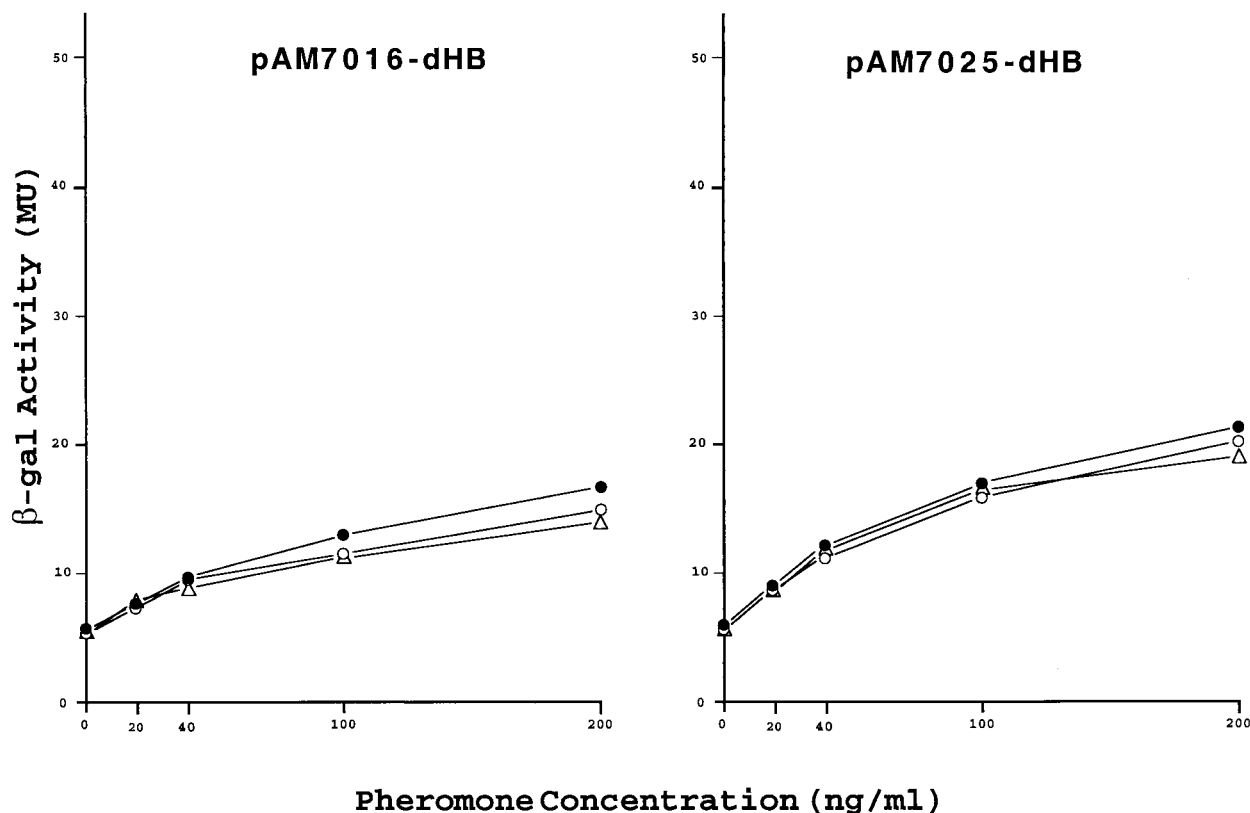


FIG. 8. Induced expression of β -galactosidase by pAD1::Tn917lac derivatives with a deleted t1 in response to increasing concentrations of cAD1 while in the presence of additionally provided mD in *trans*. The host strain was *E. faecalis* FA2-2 which carried the indicated plasmid with an insertion in *sea1* (pAM7016) or *asa1* (pAM7025). The plasmids provided in *trans* were pAM401 (●), pAM-HT (○), and pAM-HTd1 (△). Exposure to cAD1 was for 1 h in each case.

vided in *trans* (i.e., by pAM-HT or pAM-HTd1) there was no reduction in pheromone sensitivity. The data imply that, if mD RNA does target locations downstream of t1, it does not affect transcription. Like the case for the wild-type t1, the maximum levels of beta-galactosidase activities expressed are significantly less than those relating to Tn917lac insertions upstream of *sea1* (Fig. 3). In the case of pAM7016-dHB and pAM7025-dHB, the transcription observed would appear to reflect initiation from the *iad* promoter, and inducibility is interpreted to relate only to cAD1-induced expression (TraA related) from this site.

DISCUSSION

We have shown here that the small pAD1-encoded RNA molecule mD plays a significant role in transcription termination at t1. Excess mD provided in *trans* by a multicopy plasmid resulted in strong inhibition of pheromone-induced beta-galactosidase expression by pAD1::Tn917lac fusions only if the transposon insertions were located downstream of t1. Since data also revealed that mD did not target regions near the 5' end of *sea1* or *asa1*, the observed influence on the expression of these genes likely reflects its activity at t1. The effect of mD on t1 was also present in the case of a *traA* mutant normally fully derepressed for conjugation functions, indicating that interaction between mD and TraA was not required to enhance termination. Consistent with these data was our observation that the inducible clumping response of cells carrying wild-type pAD1 was significantly less sensitive to pheromone when excess mD was provided in *trans*.

Analyses of the behavior of several mutants with altered t1 structures showed the importance of this terminator in preventing transcription into *traE1*. When t1 was made dysfunctional, t2 was still able to terminate to some extent, giving rise to m3*, but significant readthrough leading to expression of conjugation functions was clearly evident. At least one function of t2 is presumably to stop any transcription that has escaped normal t1 termination in the absence of induction. In this regard, it is important to recall that, since TraE1 upregulates itself at a site within t2 (3), it is critical to block even small amounts of transcription from upstream to avoid spontaneous induction.

In a previous report (3), we hypothesized that mD associated with an opposing transcript (m3 or m4) by its complementarity to form a complex that in turn acted at the *iad* promoter to help maintain the basal level of transcription that occurs in cells not exposed to pheromone. The notion of interaction by complementarity was based on the overlap predicted to occur which was partial in the case of m4 (an 80-nt overlap) and complete in the case of m3 (see Fig. 9). A transposon insertion between mD and m4 would be expected to prevent such an overlap, and the inability to form a complex would be consistent with the finding that transcription from the *iad* promoter became constitutive and could not be complemented by mD in *trans*. The phenomenon was reflected in the present study in the case of pAM7304 (Tn917::lac insert between *traD* and *iad*) and the data of Fig. 3, where mD provided in *trans* had no effect. It was hypothesized that the activity of the mD::m4(m3) RNA complex involved interaction with either TraA or RNA polymerase. Our present data would suggest that, if indeed mD

acts at or near the site of the *iad* promoter, it is more likely to involve an association with RNA polymerase (see Fig. 9). A reasonable possibility is that mD, or mD complexed with m4 or m3, interacts with RNA polymerase in such a way as to enhance transcriptional termination upon its arrival at t1. A variation of this notion would be that mD anneals with nascent m3 in such a way as to cause premature release of RNA polymerase (close to t1) or to make the polymerase more sensitive to t1. In this regard, it is interesting that mD contains an 11-nt run, beginning at nt 50, that has complementarity to m3 in a region that would span the t1 loop region, a characteristic that brings to mind recent studies of Yarnell and Roberts (46) showing that oligonucleotides with complementarity to nascent RNA within its termination site can in some cases enhance termination.

An important feature of the pAD1 control system is that in the uninduced state there is already enough expression from the *iad* promoter to synthesize the iAD1 inhibitor peptide. The detection of transcripts such as m3 and m4 in the absence of induction is presumed to reflect, at least in part, the need to produce and secrete this peptide (38). At the same time there must be a way to ensure that transcription does not go beyond t1-t2, since as noted above even a small amount of synthesis of TraE1 may significantly upregulate, via subsequent autoregulation, the pheromone response. mD would appear to be a key factor in maintaining tight control of this process. The overall circuitry must be designed to allow RNA polymerase to initiate transcription to a limited degree in the absence of pheromone. This could be manifest as TraA acting as a relatively weak repressor, or RNA polymerase may be affected by another factor (e.g., mD alone or mD complexed with m4 or m3) in such a way as to more efficiently compete with TraA for access to the promoter.

The initial evidence suggesting that mD plays some role in affecting transcriptional initiation from the *iad* promoter was the earlier observation that the temperature-sensitive mD variant resulting from a point mutation within *traD* gave rise to enhanced transcription from this site upon shifting cells from 32 to 42°C (3). Since we have shown in the present study that this mutation relates to control of transcriptional readthrough of t1 but not transcription up to t1, the question of how mD affects initiation at the *iad* promoter is puzzling. Although it is conceivable that the previously observed thermally enhanced transcription was due to an unrelated temperature effect on the transcription machinery, another possibility could be related to an effect on stabilization of m3 or possible extensions of mD into *traA*. (We note that there is no apparent intrinsic terminator relating to mD transcription.)

Finally, the observation that transcription of *asaI* is regulated by mD activity at t1 and not at positions further downstream is interesting in connection with an earlier suggestion (3) that additional control may occur in closer proximity to *asaI*. The fact that the 5' end of mD exhibits significant complementarity over about 70 nt of a possible untranslated transcript 37 nt upstream of the *asaI* translational start site (3) is strongly suggestive of an interaction. If such an interaction indeed occurs, its main effect would now seem more likely to be on Asa1 translation rather than on transcription. And one might expect that the effect would be inhibitory, since mD is present at high levels in the uninduced state and at low levels after induction (Fig. 9). It is possible that mD prevents translation of small amounts of *asaI* transcript that may be expressed from a promoter other than that of *iad* in the uninduced state—perhaps the one reported to occur just upstream of *orf1* (33).

Muscholl et al. (33) reported that a segment of DNA con-

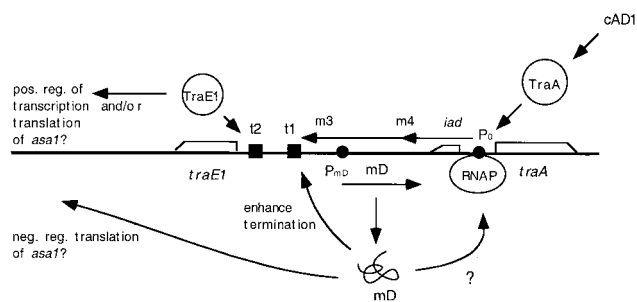


FIG. 9. Current view of the effect of mD on regulation of the pAD1 pheromone response. A primary function of mD is to enhance termination of transcription at t1. This may relate to an interaction between mD and RNA polymerase (RNAP). Such an interaction could occur near the *iad* promoter (P_0) or further downstream but prior to the RNA polymerase arrival at t1. P_{mD} refers to the promoter of mD (*traD*). pos. reg., positive regulation; neg. reg., negatively regulated.

taining only *orf1* and *asaI* cloned on the shuttle vector pWM401 was not able to express Asa1 in *E. faecalis* unless TraE1 was provided from a coresident plasmid clone in *trans*. The chimera that carried *traE1* also carried *iad* and its promoter but not an intact *traA*. Thus, it also encoded mD (unknown at the time), which was probably not expressed since TraA was absent and expression from the *iad* promoter would therefore be expected to be constitutive. Good expression of TraE1 and greatly reduced expression of mD would seem to have been likely in the reported experiments (33). Although it was assumed that transcription was being regulated by TraE1, expression was being monitored by immunoblotting to detect Asa1; it is possible, therefore, that translation rather than transcription was being influenced. We have previously speculated on the possible interplay between TraE1 and mD (3); conceivably, both factors relate to translation.

The Dunny group, working with the pheromone-responding plasmid pCF10, has reported that induction results in transcription from a promoter analogous to that of *iad* through the determinant for aggregation substance (*prgB*) located at a distance similar to that of *asaI* (7); indeed transcription appears to extend through several apparent terminators. (See reference 9 for a comparison of pAD1 and pCF10.) They also reported evidence that the large transcript is subsequently processed to give rise to smaller functional products (5), and there are data that support the view that PrgB is subsequently regulated at the translational level (4). A *traE1* homologue is not present in the pCF10 system (in its place are *prgR* and *prgS*), and an mD equivalent has not been reported. It is very possible, however, that an equivalent mD determinant is present, since there is near identity between pAD1 and pCF10 with respect to a point midway within *traD* (of pAD1), though through t2. A pCF10 RNA equivalent to mD with an identical promoter and with identity for over ~50% of the transcript can be easily envisioned. Such a transcript made by pCF10 would therefore be similar to the mD of pAD1 in only its 5' half. A similar case can be made for pPD1, based on examination of its sequence in the corresponding region (19). Perhaps relating to such a difference was our finding that when mD (of pAD1) was provided in *trans* it did not significantly affect pCF10 or pPD1 sensitivity to cCF10 or cPD1, respectively. It is interesting that the pCF10 system exhibits constitutive expression from its *icf* (which encodes inhibitor peptide iCF10) promoter (equivalent to that of *iad*) (4), since it would suggest that an mD equivalent might never be expressed because of the converging transcription. If this is the case, it would be consistent with the report that

transcription indeed occurs to a certain extent through the t1-t2 equivalent IRS1-IRS2 into *prgR* and *prgS* in the uninduced state (4). Induction of pCF10 is believed to involve a complex interaction of the internalized cCF10 with two regulatory factors (QL [an RNA equivalent to our m3*] and PrgS) and ribosomes. Such phenomena have not been identified in the pAD1 system, although they cannot be ruled out at this time.

Regulation of the pheromone response in *E. faecalis* is a complex process with both common and different features between various conjugative plasmids. The enhancement of transcription termination in *trans* by a small RNA molecule like mD is quite interesting and, to our knowledge, somewhat novel. Further characterization of this system and comparison to other systems such as pCF10 should provide new insights into the specific control mechanisms and their evolution.

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