

Regulation of the pAD1-Encoded Sex Pheromone Response in *Enterococcus faecalis*: Expression of the Positive Regulator TraE1

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Received 28 August 1992/Accepted 30 November 1992

pAD1 is a conjugative, 60-kb, hemolysin-bacteriocin plasmid in *Enterococcus faecalis* that encodes a mating response to a small peptide sex pheromone, cAD1, secreted by potential recipient bacteria. The response is regulated by a cluster of genes that includes a positive regulatory determinant, *traE1*, able to activate key structural genes involved in the conjugative process. A negative regulatory determinant, *traA*, affects the expression of *traE1* and is sensitive to the pheromone signal. Between the two determinants is a gene, *iad*, which encodes a small peptide, iAD1, a competitive inhibitor of cAD1. The determinants (*traE1-iad-traA*) are oriented such that *iad* and *traE1* are transcribed in the same direction, opposite that of *traA*. Transcription of *iad* and *traA* starts between these determinants and moves outward in each case. A recent report from our laboratory, dealing with transcriptional fusions in the *traE1-iad* region (L. T. Pontius and D. B. Clewell, J. Bacteriol. 174:3152–3160, 1992), indicated that *traE1* expression may be dependent on transcriptional read-through of a terminator(s) between *iad* and *traE1*. The present report provides direct analyses of relevant RNA species before and during induction and shows that indeed transcriptional read-through from *iad* is important in the initial expression of *traE1*. However, the data show that once *traE1* is activated, it can then be expressed independently, probably because of TraE1 activating its own promoter. This view is also supported by genetic complementation studies. In addition, DNA binding studies with TraA showed that the protein binds to the promoter of *iad*. Binding of TraA to the region between *iad* and *traE1* could not be detected; however, the involvement of TraA in influencing transcription termination in this region is still not ruled out, since additional factors could be involved. A model for the regulation of the pheromone response is presented.

pAD1 is a conjugative, 60-kb, hemolysin-bacteriocin plasmid believed to contribute to virulence in the opportunistic pathogen *Enterococcus faecalis* (for recent reviews, see references 4 and 9). Like a number of other *E. faecalis* plasmids that are transferred at a high frequency in broth matings, pAD1 encodes a response to a specific peptide sex pheromone (cAD1 in this case [21]) secreted by potential recipients. Induction results in the synthesis of a surface “aggregation substance” that facilitates the formation of mating aggregates (13, 16). cAD1 also activates genes required for DNA transfer and surface exclusion (6, 12). Donor cells secrete a peptide, iAD1, which acts as a competitive inhibitor of the pheromone; however, inhibition is significant only when cAD1 concentrations are relatively low (5, 18, 20).

Genetic analyses with Tn917 and Tn917lac have found that all the determinants relating to conjugation are contiguously located on a region occupying at least half of the plasmid (approximately 30 kb), with the genes responsible for regulation of the process being clustered in a 7-kb segment at one end (12, 17, 22, 28–30). Key regulatory genes that have been identified include (i) *traE1*, which encodes a positive regulator necessary for the expression of all conjugation functions (24); (ii) *traA*, which is involved in the negative control of *traE1* expression (23, 30); (iii) *traB*, which is involved in the shutdown of endogenous cAD1

production (30, 31); and (iv) *traC*, which encodes a product involved in binding to and/or sensing of exogenous pheromone (4, 30). In addition, the determinant for iAD1, *iad*, is located between *traE1* and *traA* and encodes a 22-amino-acid precursor, with the last 8 residues corresponding to the active product (7). The determinants are arranged as *traE1-iad-traA-traC-traB*, with all but *traA* being in a right-to-left (5'-to-3') orientation. Analyses of the nucleotide sequence and transcriptional fusions relating to the *traE1-iad-traA* region (7, 23, 24) suggested that the TraA protein, which shows limited homology to certain DNA binding proteins (23), controls *traE1* expression by regulating transcriptional read-through from the promoter that drives *iad*. The sequence data identified two apparent factor-independent transcription termination sites located close together and between *iad* and *traE1*. *lacZ* fusions on the downstream side (*traE1* side) of the first terminator (TTS1) were inducible, while upstream derivatives (*iad* side) were expressed at a relatively high level in the absence of the pheromone (24). It was suggested that TraA may exert its effect via an interaction with DNA somewhere within the few hundred nucleotides separating the end of the *iad* reading frame and TTS1, a region that appears devoid of any other obvious terminators but that could possibly give rise to some complex RNA secondary structure (24). Induction could be viewed as facilitating an increased level of transcription through TTS1 (as well as the closely located TTS2), leading to expression of downstream *traE1*.

Here we present results from both genetic and biochemical analyses further detailing the regulation of *traE1* tran-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Strains		
<i>E. faecalis</i>		
OG1X	<i>str gel</i>	18
FA2-2	<i>rif fus</i>	8
UV202	<i>rif fus Rec⁻</i> (derived from JH2-2)	34
<i>E. coli</i>		
XL1-Blue	F ⁻ :Tn10 <i>proA⁺ B⁺ lacI^q Δ(lacZ)M15/recA endA gyrA thi hsdR supE relA lac</i>	Stratagene
TH688	CSH57b <i>thr::Tn5</i>	26
DH5α	F ⁻ φ80 <i>lacZ</i> ΔM15 <i>endA1 recA1 hsdR1</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 d⁻ gyrA96 Δ(lacZYA-argF)U169</i>	Promega Research Laboratories; 23
Plasmids		
pBluescriptSK	<i>E. coli</i> vector plasmid; <i>amp</i>	Stratagene
pAD1	<i>hly-bac</i> ; pheromone response	27
pAM1.3SK	Fragment containing <i>traE1</i> and <i>iad</i> (no promoter) cloned in pBluescriptSK	7
pAM1.3SKΔXbaI	Deletion derivative of pAM13SK; lacks <i>traE1</i> , TTS1, and TTS2	This study
pAM1.6SK	Fragment containing <i>traE1</i> and <i>iad</i> cloned in pBluescriptSK	7
pAM2011	pAD1 with a Tn917 <i>lac</i> insertion affecting <i>traE1</i>	28
pAM2120	pAD1 containing <i>traA/A120 Tn917lac</i>	28
pAM2145	pAD1 containing NR5 and M14 Tn917 <i>lac</i> insertions	30
pAM2180	pAD1 containing a PR18 Tn917 <i>lac</i> insertion	28
pAM2604	<i>Bam</i> HI fragment of pAM2145 cloned in pAM401	This study
pAM2609	<i>Xba</i> I- <i>Bam</i> HI fragment of pAM2180 cloned in pAM401	This study
pAM2610	<i>Alu</i> I fragment (134 to 395 bp; see Fig. 3) cloned in the <i>Sma</i> I site of pBluescriptSK	This study
pAM2611	pAM2604 <i>traA::Tn5</i>	This study
pAM2622	Cointegrate of pAM401 and pAM1.3SK at <i>Bam</i> HI sites	This study
pAM2623	Cointegrate of pAM401 and pAM1.6SK at <i>Bam</i> HI sites	This study
pAM401	Shuttle vector for <i>E. coli</i> and <i>E. faecalis</i>	33
pAM7500	<i>Hind</i> III fragment of pAD1 cloned in pBluescriptSK	23
pAM7520	Nested deletion derivative of pAM7500 containing part of the <i>traE1</i> gene; lacks the 5' end of <i>traE1</i>	This study
pAM7557	Nested deletion derivative of pAM7500; <i>traA</i> is downstream of the <i>lacZ</i> promoter of pBluescriptSK	This study

scription by TraA. The data support the view that induction indeed results in a necessary extension of transcription through the above-noted termination sites but that this process subsequently yields to the expression of *traE1* from its own promoter located within TTS2. TraA was found to bind to DNA in the promoter region of *iad*, but an association downstream, closer to TTS1, could not be detected. The results of complementation studies involving both *traA* and *traE1* are also presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. Media used for *E. faecalis* were Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and N2GT (nutrient broth no. 2 [Oxoid Ltd., London, England] supplemented with 0.2% glucose and 0.1 M Tris-HCl [pH 7.5]). N2GT medium was used in all experiments involved in a pheromone response. Luria broth (10) was used for culturing *Escherichia coli*. Solid media were prepared by including 1.5% agar. Growth was always accomplished at 37°C. Antibiotics were used at the following concentrations: ampicillin, 60 μg/ml; erythromycin, 10 μg/ml; chloramphenicol, 20 μg/ml; streptomycin, 1,000 μg/ml; and kanamycin, 40 μg/ml.

Analysis of transcripts. RNA was prepared in a manner similar to that previously described (25). Overnight cultures of *E. faecalis* OG1X carrying the plasmid of interest were diluted to 20% in 25 ml of N2GT medium, and the cells were

grown for 2 h. Synthetic cAD1 was then added to the cultures at a concentration of 40 ng/ml. After a specified time, chloramphenicol (200 μg/ml) and sodium azide (20 mM) were added; the cells were chilled on ice, harvested, and suspended in 2 ml of 25% sucrose in Tris-HCl (pH 8.0). After the addition of 2 μl of 250 mM EDTA (pH 8.0) and 50 μl of 20-μg/ml lysozyme, the cells were maintained on ice for 30 min, pelleted, and suspended in 300 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 3 mM EDTA, 200 mM NaCl). Three hundred microliters of lysis solution (1% SDS [sodium dodecyl sulfate] in lysis buffer) was added, and the mixture was maintained at 100°C for 2 min, with occasional mixing. Six hundred microliters of hot phenol saturated with lysis buffer (65°C) was added, and the mixture was kept at 65°C for 5 min, with occasional inversion of the tube. The phases were then separated by centrifugation, and the aqueous phase was extracted once with phenol-chloroform and then twice with ether. A 1/10 volume of 3 M sodium acetate was added, after which nucleic acid was recovered by ethanol precipitation. The pellet was suspended in 100 μl of DNase I buffer (100 mM sodium acetate, 5 mM MgCl₂ [pH 5.0]), and DNA was digested with DNase I (RNase free; Boehringer Mannheim Biochemicals) for 2 h at 37°C. RNA was recovered by ethanol precipitation. RNA species were then separated by electrophoresis in a 1.2% agarose-MOPS (3-[N-morpholino]propanesulfonic acid)-formaldehyde system (1). Northern (RNA) hybridization was carried out at 42°C in the presence of 50% formamide (1). The two probes consisted of

pAM1.3SK Δ XbaI (probe 1) and pAM7520 (probe 2); these probes were labeled by nick translation with a kit from Bethesda Research Laboratories. RNA marker molecules were from Bethesda Research Laboratories.

Determination of the initiation site of transcription. Primer extension was conducted essentially as described elsewhere (1). The synthetic primer was made by the University of Michigan DNA Core Facility and corresponded to the structure 5'-CCTACAAGTGTGACAACAAATAAAGTTATC-3', which is located in the *iad* determinant (see Fig. 3). Primer DNA (3.2 μ g) was treated with 10 U of T4 polynucleotide kinase (Bethesda Research Laboratories) in the presence of 200 μ Ci of [γ - 32 P]ATP (10 mCi/ml; Amersham). Forty two micrograms of RNA prepared from OG1X(pAD1) induced with 40 ng of cAD1 per ml for 20 min was hybridized with 30 ng of kinase-treated primer overnight at 30°C in 30 μ l of hybridization solution (166 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 1.0 M NaCl, 0.33 mM EDTA). Primer extension was conducted with 37.5 U of avian myeloblastosis virus reverse transcriptase (Boehringer-1.6 mM each deoxynucleoside triphosphate-50 mM Tris-HCl (pH 8.0)-5 mM MgCl₂-50 mM KCl-5 mM dithiothreitol-50 μ g of gelatin per ml. The reaction mixture was incubated at 42°C for 90 min. Samples were run on a sequencing gel after pancreatic RNase I treatment, and the gel was autoradiographed after being dried. Related sequencing reactions were carried out as described elsewhere (2).

iAD1 production after induction. An overnight culture of FA2-2(pAD1) was diluted to 2% in 10 ml of N2GT broth. After growth for 4 h at 37°C, synthetic cAD1 was added at a concentration of 10 ng/ml. At 0, 10, 20, and 40 min after the addition of cAD1, 1.5-ml samples were removed; culture supernatants were quickly prepared and boiled for 10 min. The iAD1 titer was determined as previously described (18) by a microtiter assay.

Complementation studies. For studies involving the complementation of *traA*, plasmid pAM2604 was constructed by cloning the *Bam*HI fragment of pAM2145 in shuttle plasmid pAM401, whereas plasmid pAM2609 was prepared by cloning the *Xba*I-*Bam*HI fragment of pAM2180 in pAM401 (see Fig. 5). Tn5 mutagenesis of pAM2604 was performed as follows. The plasmid was introduced into *E. coli* TH688 (with Tn5 in the *thr* locus) by transformation; transformants were streaked on selective plates and left at room temperature for 2 weeks. Plasmid DNA was prepared from a mixture of colonies and used to transform plasmid-free *E. coli* DH5 α , with selection for kanamycin resistance (for Tn5) and vector-borne chloramphenicol resistance. Resulting colonies were purified and examined for the specific location of Tn5 in the plasmid. One derivative, found to have an insert in *traA*, was designated pAM2611 (see Fig. 5) and tested for complementation in *E. faecalis*. Plasmid DNA was introduced into *E. faecalis* by electroporation (22) with a Bio-Rad Gene Pulser. Complementation of the *traA* phenotype was determined by use of short broth matings as well as examination of colony morphology in the *E. faecalis* UV202 (recombination-deficient) background.

Complementation of the *traE1* phenotype was determined with plasmids pAM2622 and pAM2623, which were generated by ligation of pAM1.3SK and pAM1.6SK, respectively, to pAM401 via the *Bam*HI sites in each. The plasmids were then introduced into *E. faecalis* OG1X(pAM2011) by electroporation. Transformants were plated on N2GT agar containing cAD1 (40 ng/ml), erythromycin (10 μ g/ml), and chloramphenicol (20 μ g/ml). This procedure assured the maintenance of both pAM2011 and the test plasmid during

determination of whether the colony morphology exhibited the "dry" phenotype indicative of pheromone induction. For the clumping assays, 0.5-ml portions of overnight cultures were diluted in 4.5 ml of fresh N2GT medium to which cAD1 was then added to a final concentration of 100 ng/ml. Examination for clumping was done after 2 h.

Overproduction and partial purification of the TraA protein. Overproduction of TraA in *E. coli* was accomplished with pAM7557, a derivative carrying *traA* downstream of the *lacZ* promoter of pBluescriptSK. The construct was generated originally as a nested deletion of pAM7500 (see reference 23 and Fig. 5). *traA* lacked its own promoter but maintained its ribosome binding site.

Five milliliters of an overnight culture of *E. coli* XL1-Blue(pAM7557) was added to 500 ml of Luria broth, and cells were grown for 3 h. IPTG (isopropyl- β -D-thiogalactoside) was added to a final concentration of 1 mM to induce transcription from the *lacZ* promoter, and the culture was incubated for another 5 h. The cells were then harvested, frozen (-70°C), and thawed. The pellet was suspended in 2 ml of A0 buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) and sonicated for 3 min at 60 to 80 W. After the removal of particulate material by centrifugation, ammonium sulfate was added to a final concentration of 60%; precipitated protein was collected by centrifugation. The protein was then dissolved in 20 ml of PBS (140 mM NaCl, 1.5 mM KH₂PO₄, 15 mM Na₂HPO₄, 2.7 mM KCl [pH 7.4]), and the solution was dialyzed overnight against PBS. The sample was applied to a heparin-Sepharose column (10 ml; Pharmacia LKB Biotechnology) at a flow rate of 100 μ l/min, and the column was washed with 50 ml of PBS at a flow rate of 200 μ l/min. Proteins were eluted with an NaCl gradient (140 mM to 2 M in a total of 200 ml) in PBS and collected as 1-ml fractions. Fractions were examined for protein content by SDS-polyacrylamide gel (10%) electrophoresis and staining with Coomassie brilliant blue. Size markers were a "rainbow set" of proteins from Amersham.

Gel retardation experiments. The DNA substrate (pAM7500) was digested with a restriction enzyme(s), extracted with phenol-chloroform, and precipitated with ethanol. DNA binding was carried out essentially as described elsewhere (1). The binding mixture contained the DNA restriction digest, 5 to 10 μ l of the TraA fraction, 10% glycerol, 1 mM dithiothreitol, 200 μ g of bovine serum albumin per ml, and 2 μ g of salmon sperm DNA. The mixture was incubated at 30°C for 30 min and then examined by gel electrophoresis with 3% NuSieve (FMC BioProducts) in Tris-glycine buffer (50 mM Tris-HCl [pH 8.5], 380 mM glycine, 2 mM EDTA). DNA bands were visualized under UV light after staining was done with ethidium bromide.

DNA footprinting. The *Alu*I DNA fragment (134 to 395 bp) containing the *traA* binding site was cloned in the *Sma*I site of pBluescriptSK, located between the *Bam*HI and *Kpn*I sites. The resulting plasmid was designated pAM2610. Target DNA was prepared by digestion of pAM2610 with *Kpn*I and *Bam*HI, agarose gel electrophoresis, and elution of DNA from an agarose block. The *Bam*HI cohesive end was filled in with polymerase (Klenow) in the presence of [α - 32 P]dATP. Protein-DNA binding was allowed to occur as described above for the gel retardation experiments. Magnesium and calcium ion concentrations were adjusted to 5 mM each. Two microliters of diluted DNase I (50 ng/ml) (Bethesda Research Laboratories) was added to 50 μ l of the reaction mixture, and the mixture was incubated at 25°C for 2 min. The reaction was stopped by the addition of 50 μ l of phenol-chloroform. DNA was recovered by ethanol precipitation, suspended in 4 μ l of DNA sequencing buffer, and run

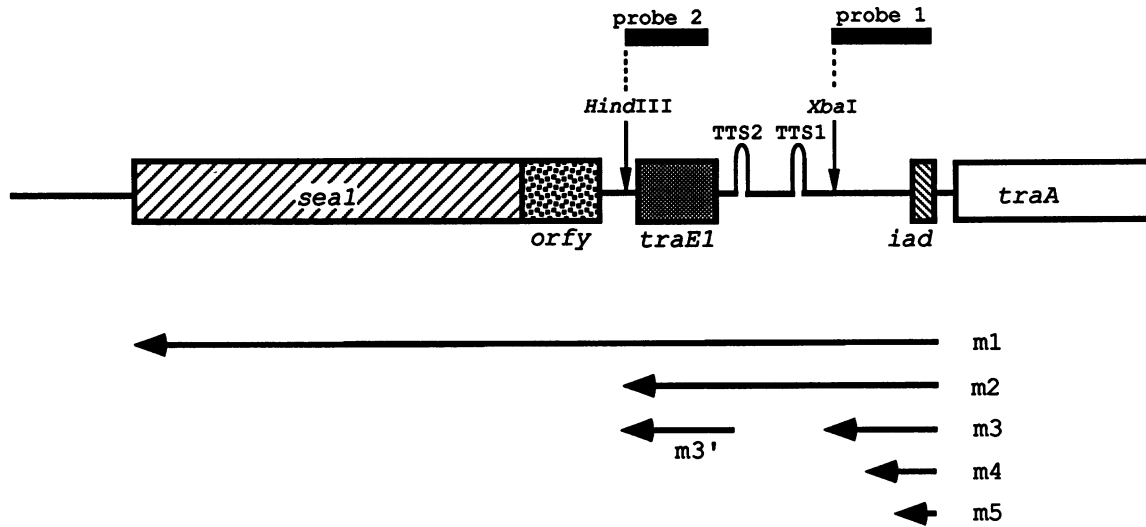


FIG. 1. Map of the key regulatory region of the pheromone response of pAD1 along with notations relevant to transcription analyses. The locations of probes 1 and 2, used in the experiments shown in Fig. 2, are indicated, and the arrows represent interpretations of the various transcripts (m1 to m5 and m3') detected. TTS1 and TTS2 are previously identified (24) putative transcription termination sites.

alongside a DNA sequence analysis preparation. After drying was complete, autoradiography was done at -70°C , usually overnight.

RESULTS

Analysis of pheromone-induced transcripts. Figure 1 shows the region that was analyzed for RNA products during induction; probes 1 and 2 cover the locations indicated. The results of the Northern analyses are shown in Fig. 2A. When probe 1 (Fig. 2A), which contained *iad* and flanking regions, was used, three transcripts were clearly evident in uninduced cells (lane 1). These were designated m3 (~400 bases),

m4 (~170 bases), and m5 (~110 bases). After 20 min of exposure to cAD1 (40 ng/ml), two additional transcripts, designated m1 (~4,400 bases) and m2 (~1,050 bases), were detected (Fig. 2A, lane 2). When probe 2 (Fig. 2B) was used, no RNA was detected in uninduced cells (lane 1); however, m1 and m2, as well as a band similar in size to m3, here designated m3', were evident after 20 min of induction (lane 2). The m3' transcript must be different from m3, since it was barely detectable in uninduced cells. The data are consistent with the arrows in Fig. 1 indicating m1 through m5 (including m3'). m3 corresponds well in size to a transcript beginning at the *iad* promoter and terminating at TTS1, whereas m4 and

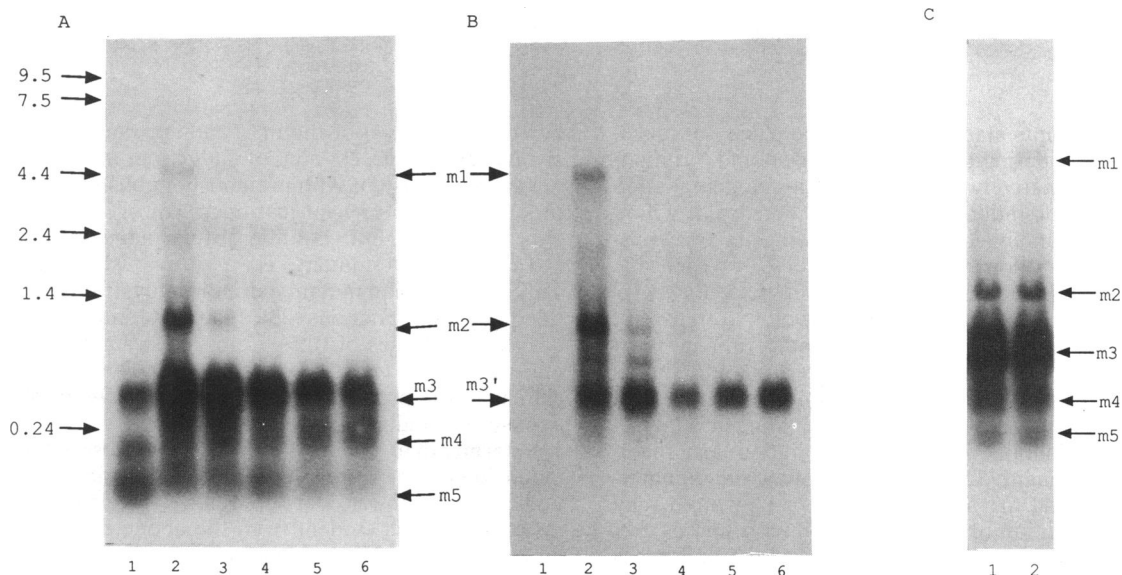


FIG. 2. Northern blot analyses of the regulatory region depicted in Fig. 1. (A and B) Wild-type pAD1 tested with probe 1 (A) and probe 2 (B). (C) *traA* mutant pAM2120 tested with probe 1. In all three panels, lane 1 represents cells without the addition of cAD1, and lane 2 represents exposure to cAD1 for 20 min. In panels A and B, lanes 3 to 6 correspond to cAD1 exposure times of 40, 80, 120, and 160 min, respectively. Arrows indicate transcripts m1 to m5 and m3'. The positions of RNA markers are noted on the left (in kilobases).

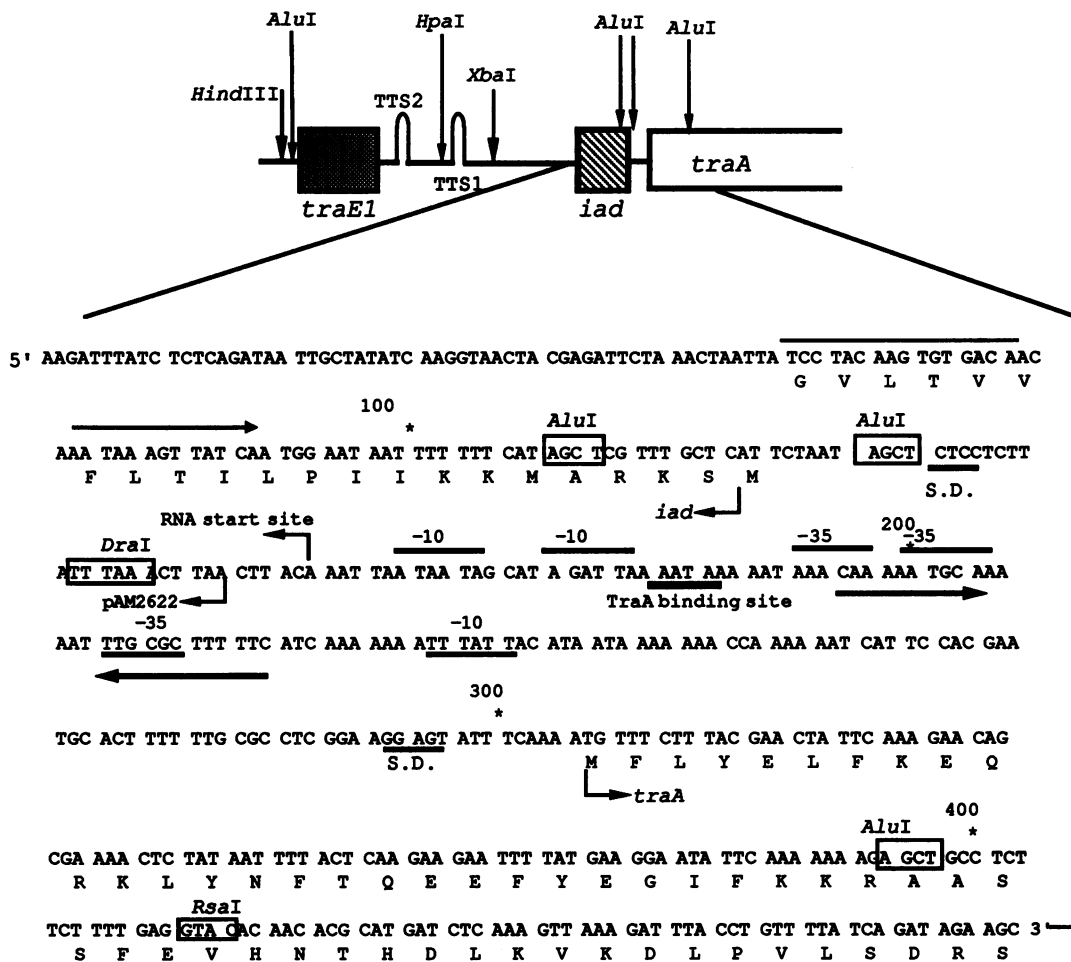


FIG. 3. Restriction map of the regulatory region of pAD1 along with the DNA sequence of the segment containing *iad* and a portion of *traA*. Putative -10 and -35 boxes and Shine-Dalgarno (S.D.) sites are noted for both *traA* and *iad*. A region of dyad symmetry between *iad* and *traA* is noted by inverted arrows. The designation pAM2622 indicates one end of the pAD1 fragment contained in pAM2622, pAM1.3SK, and pAM1.3SK Δ XbaI. The arrow above the *iad* sequence represents the oligomer used in the primer extension study (see Fig. 4); the deduced RNA start site is also noted. The TraA binding site relating to the data shown in Fig. 8 is shown between the -10 and -35 boxes of *iad*.

m5 may be transcripts starting at the same place but terminating earlier or initiating further downstream and terminating at TTS1. Alternatively, m4 and m5 may be degradation products of m3. The induced m3' band is consistent with a transcript corresponding to *traE1* and using its own promoter and transcription termination site. (Two possible transcription termination sites are evident between *traE1* and *orfy* [32]). The m2 band corresponds well to a transcript that extends through TTS1 and TTS2 and terminates just beyond *traE1*, presumably at the same termination site as that used by m3'. The m1 band corresponds to a transcript extending into the *sea-1* determinant and ending at a factor-independent termination site that was identified near the 3' end of that determinant (32). The nature of the band migrating between m2 and m3' in Fig. 2B is not known. It may represent a degradation or processed product of m1 or m2.

Figure 2 also shows that the induction of m1 and m2 is maximal within 20 min; their presence is greatly reduced after 40 min (lane 3) and barely detectable after 80 min (lane 4). The m3' transcript, however, remains at a high level throughout the entire 160-min time period. The m4 and m5 bands do not appear to change much during induction;

however, the level of the m3 band appears to be increased significantly after 20 min of induction. An inducible m3 would be consistent with previously published analyses (24) of a *lacZ* fusion present just upstream of TTS1; this fusion expressed *LacZ* but could be further induced with cAD1.

The *traA120* mutant is a previously characterized Tn917*lac* insertion mutant exhibiting constitutive expression of conjugation functions (28). Figure 2C shows the results of Northern analyses of RNA from this strain both before and after exposure to cAD1 for 20 min. The profiles appear similar to the profile corresponding to a 20-min pheromone exposure of inducible pAD1.

Determination of the initiation site of transcription. Primer extension can be used to determine transcription start sites, with the assumption that the transcript being primed is not processed. As is evident from the above-described results, all transcripts, with the exception of m3', can be viewed as initiating from a promoter upstream of *iad* (Fig. 3). Figure 4 shows the results of a primer extension analysis performed on RNA prepared after a 20-min exposure of cells to the pheromone. Transcription was found to start with a G located downstream of two previously apparent -10 boxes,

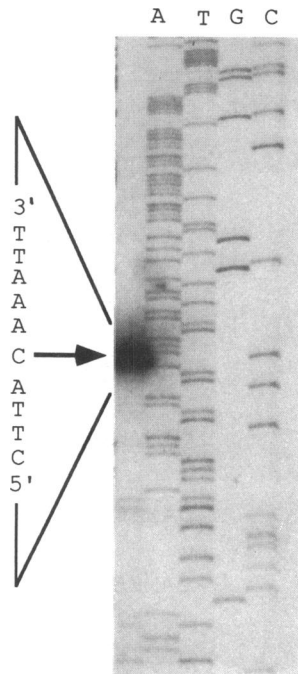


FIG. 4. Primer extension analysis. The primer used is within the *iad* determinant (Fig. 3). The results are shown in the leftmost lane alongside a DNA sequence determination performed with the same primer and a DNA template (pAM7500) representing this region. The specific location of the first nucleotide is noted by the arrow and is also noted in Fig. 3.

6 and 17 bases away. As noted above, there is a possibility that m4 and/or m5 initiated further downstream; however, if either species still overlapped the primer, there should have been an indication in the data in Fig. 4. Initiation further downstream of the primer would not be detected here.

Expression of *iad* during induction. While the transcription data imply that induction is associated with increased read-through of TTS1, the very high level of m3 present after 20 min suggests that transcription from the *iad* promoter is also initiated at an increased rate. Although the levels of m4 and m5 do not appear to differ dramatically from those observed for uninduced cells, the fact that all three transcripts represent *iad* raises the question of whether induction is associated with an increased production of iAD1. For examination of this point, FA2-2(pAD1) cells were induced for 10, 20, and 40 min with 10 ng of cAD1 per ml and then removed by a short centrifugation step. The cAD1 titers in supernatants were then determined to ascertain whether they were decreased because of an increase in the production of iAD1. We observed a consistent decrease of about 50% (one dilution); thus, it is conceivable that there is some increase in iAD1 secretion, but it is not dramatic (data not shown). (It is important to note that this type of experiment must be done with the FA2-2 host rather than the isogenically different OG1-related hosts. cAD1 and iAD1 both bind to the surface of OG1(pAD1) cells because of a specific pheromone binding factor; however, this factor is not evident in the FA2-2 host [30]).

***traA* acts in *trans*.** Although it has been assumed that the *traA* product is a repressor that acts in *trans*, this assumption has not been directly confirmed by complementation experiments. Here we make use of the Rec⁻ strain of UV202 to

demonstrate that this is indeed the case. Figure 5 shows a series of chimeric plasmids containing various segments of the regulatory region. Plasmids pAM2604 and pAM2609, both of which contain *traA*, were introduced into strain UV202 (by electroporation) containing pAD1 *traA120*, a constitutively aggregating high-frequency transfer derivative. (*traA120* is a Tn917*lac* insertion about 25% of the way into the 5' end of the gene.) Colonies of cells containing pAD1 *traA120* are characteristically dry (able to be easily fractured with a toothpick) and easily distinguished from the wild type (17, 31). They also transfer plasmid DNA at a relatively high frequency (e.g., 10⁻⁴) in short (10-min) broth matings. When the cells also contained pAM2604 or pAM2609, they no longer had the dry colony appearance or transferred plasmid DNA at a high frequency in broth. However, when pAD1 *traA120* was used together with pAM2611, which had a Tn5 insertion in *traA*, no complementation was observed (data not shown). (We were not able to test for inducibility in the UV202 strain because of a host-related trait that prevents the inducibility of pAD1 [17].) The observations clearly support the view that TraA acts in *trans* to regulate transcription into *traE1* and beyond.

Partial purification of TraA. pAM7557 is a pBluescriptSK vector carrying *traA* on the segment indicated in Fig. 5. With *traA* under the control of the *lacZ* promoter of the vector, expression could be enhanced by exposure of the *E. coli* XL1-Blue host to IPTG. After exposure to IPTG, a 40-kDa band was detectable by SDS-polyacrylamide gel electrophoresis (PAGE); this band was not seen in a similar preparation from cells containing only the vector (data not shown) and is presumed to represent TraA. Bacterial sonicates were precipitated with ammonium sulfate (60%) and fractionated on a heparin-Sepharose column as described in Materials and Methods. As shown in Fig. 6, a protein of the expected size was detected in fractions 24 to 26. This band was not present in a similarly fractionated preparation from cells harboring only pBluescriptSK. Fractions 24 to 26 were combined and dialyzed against PBS, as were corresponding fractions from a control (vector only) preparation. These were used in the examination of TraA binding to DNA as described below.

DNA binding of TraA. Gel retardation studies were performed with restriction digests of pAM7500 (Fig. 5). Partially purified TraA was mixed with plasmid DNA digested with *DraI* or *RsaI*, after which the restriction fragments were separated by agarose gel electrophoresis (Fig. 7). The shifting of a *DraI* fragment (Fig. 7A) and an *RsaI* fragment (Fig. 7B) suggested that TraA bound between these two sites in the region shown in Fig. 3. For further examination of this region, DNA was cleaved with a mixture of *AluI* and *HpaI* prior to being mixed with TraA. Figure 7C shows that an *AluI* fragment corresponding in size to that bounded by positions 134 and 395 in Fig. 3 was missing (lanes 3 and 4) when TraA was present. We believe that the band was shifted as a smear (i.e., because of the dissociation of protein during migration), making it difficult to resolve on the gel. It is also important to note that the band bounded by the *HpaI* site just downstream of TTS1 and the *AluI* site at position 113 did not shift; thus, binding in the region between *iad* and TTS1 was not evident.

A footprinting analysis was performed as follows. The above-noted *AluI* fragment exhibiting binding to TraA was eluted after agarose gel electrophoresis and cloned in the *SmaI* site in pBluescriptSK; the resulting chimera was designated pAM2610. The cloned fragment was separated by cleavage with *BamHI* and *KpnI* (which target sites flanking

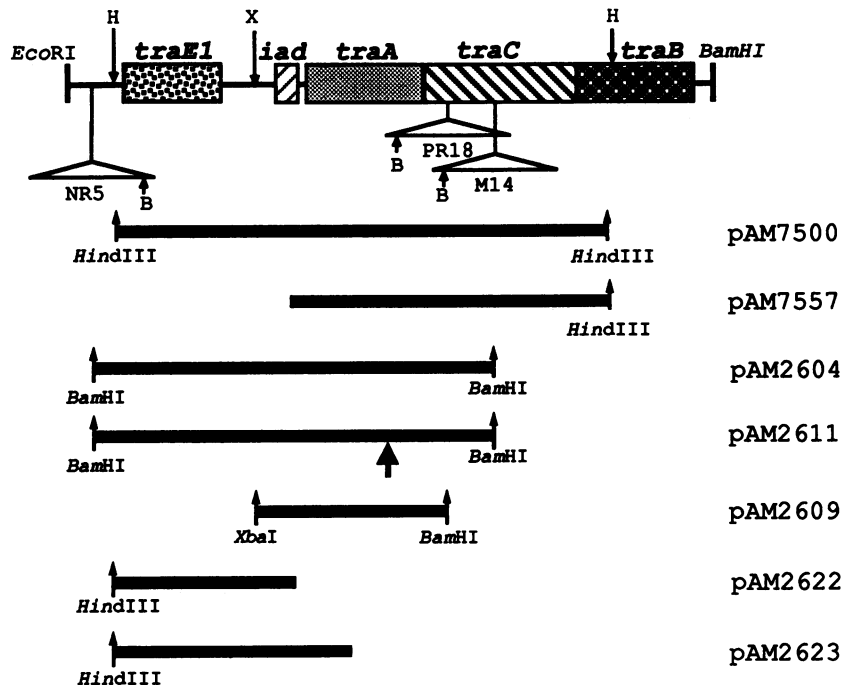


FIG. 5. pAD1 regulatory region and plasmids used for complementation and DNA binding studies. The triangles represent Tn917lac insertions that have been generated and characterized elsewhere (28); they are present in plasmids pAM2145 (NR5 and M14) and pAM2180 (PR18). The *Bam*HI site in these insertions was used in the construction of certain plasmid derivatives. The notations for restriction sites are as follows: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; and X, *Xba*I. The vertical arrows on the ends of the designated plasmid segments indicate the restriction sites used for construction; the absence of an arrow indicates that the end was generated in a nested deletion process. A larger arrow for pAM2611 indicates the location of a Tn5 insertion.

the *Alu*I fragment) and purified by gel electrophoresis. This fragment was then end labeled by filling in of the *Bam*HI cohesive end with polymerase and [α - 32 P]dATP. The *Alu*I 134-base site (Fig. 3) was near the labeled end; the *Alu*I 395-base site was near the *Kpn*I site. The labeled fragment was mixed with TraA, digested with DNase I, and analyzed by gel electrophoresis (Fig. 8). As shown in lane 1, some regions appeared protected from digestion with DNase I, even in the absence of TraA. However, a 5-base region was specifically protected in the presence of the protein (lane 2). Although faint bands were sometimes observed in this region, they were always much weaker in intensity than those in the control. The corresponding region within the sequence is indicated in both Fig. 8 and 3 and is located within the *iad* promoter. It was clear that the region nearer the *Bam*HI site (or *iad*) was not protected. It is conceivable that there was some TraA binding nearer the *Kpn*I site (toward the *traA* determinant). The presence of a segment with dyad symmetry (hairpinning potential) close to the *traA* promoter may protect this region, even in the absence of TraA; however, bands in this region tended to be slightly fainter when TraA was present.

Complementation of *traE1*. It was of interest to determine whether a mutation in *traE1* could be complemented in *trans*. In addition, since the transcriptional data and DNA binding studies implied that TraA may affect *traE1* expression via influence at the *iad* promoter, it was important to determine genetically whether the presence of the *iad* promoter on the complementing DNA segment was necessary.

pAM2623 contains a segment extending from the *Hind*III site downstream of *traE1* into *traA* (Fig. 5). pAM2622 is a similar clone, except that the region upstream of *iad*, includ-

ing the *iad* promoter, is absent. Each of these plasmids was introduced by electroporation into an OG1X host already carrying the pAD1 derivative pAM2011, which contains a Tn917lac insertion between the -10 and -35 boxes of *traE1*. Normally, OG1X(pAD1) cells give rise to dry colonies on plates containing cAD1, whereas cells carrying pAM2011 exhibit a typical *traE1* mutant phenotype and therefore do not exhibit dry colonies on pheromone-containing plates. OG1X(pAM2011) cells also do not undergo the characteristic clumping when exposed to the pheromone for a few hours in broth. OG1X(pAM2011, pAM2623) exhibited a dry colony morphology in the presence of cAD1 (40 ng/ml); however, the colonies did not appear quite as dry as for OG1X(pAD1). Similarly, exposure to cAD1 in broth resulted in clumping, but it was clearly less dramatic than for OG1X(pAD1). In contrast, OG1X(pAM2011, pAM2622) cells were no different from OG1X(pAM2011) cells with respect to their appearance in the presence of the pheromone on plates or in broth.

The data imply that when *traE1* is provided in *trans* it does indeed complement the mutation in pAM2011, but only if the *iad* promoter is also present. It would have been more desirable to perform the experiment with a Rec⁻ host such as UV202 but, as noted earlier, the pAD1 system is not inducible in that host. Unfortunately, there is currently no Rec⁻ host available that is isogenic with the OG1X host. Analysis of plasmid DNA from OG1X(pAM2011, pAM2623) cells provided no detectable evidence that recombination had occurred between the two plasmids (data not shown), although it is conceivable that a low level of recombination might have taken place and was responsible for the complementation observed at a lower level than might have been expected. However, this idea is not relevant to the fact that

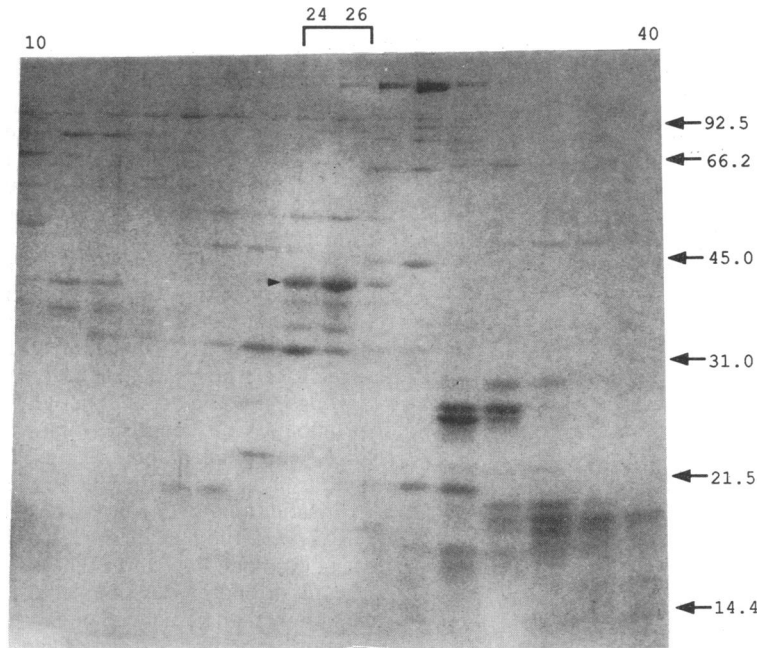


FIG. 6. SDS-PAGE of fractions from a heparin-Sepharose chromatography column used to partially purify TraA. An extract containing TraA was prepared and applied to the column as described in Materials and Methods. Aliquots (60 μ l) from every second fraction between fractions 10 and 40 were analyzed. (Fraction numbers are indicated on the top.) The arrowhead points to the TraA protein, which was absent in similarly prepared fractions from a control strain containing only vector DNA. The arrows on the right indicate the molecular masses (in kilodaltons) of marker proteins.

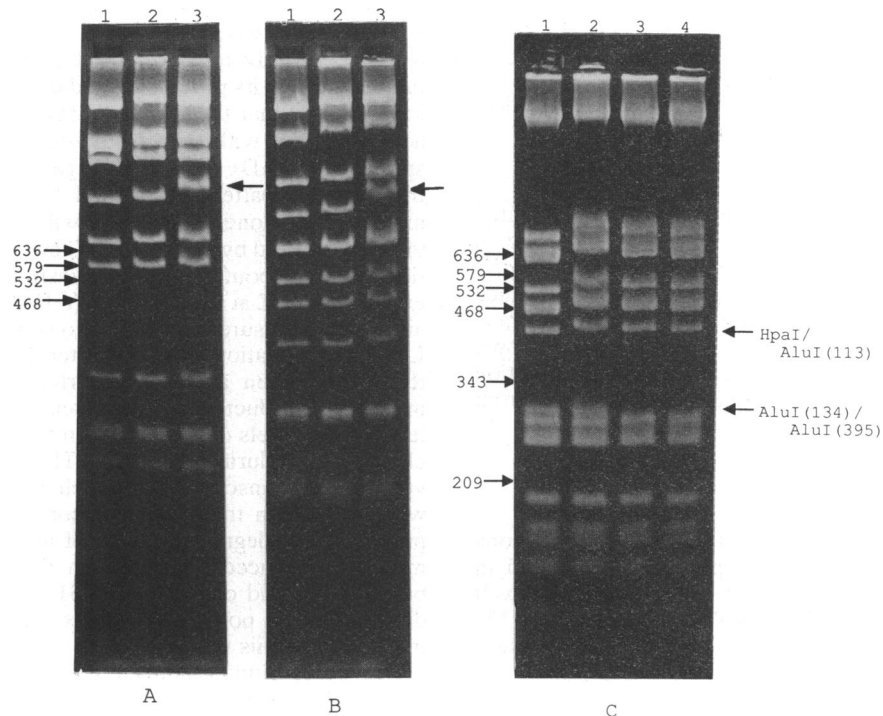


FIG. 7. Gel retardation as a result of TraA binding to DNA. Plasmid pAM7500 was digested with a restriction enzyme(s) and analyzed by gel electrophoresis as described in Materials and Methods. (A) *DraI* digest. Lanes: 1, control (no protein added); 2, protein fraction from control strain added; 3, TraA fraction added. (B) *RsaI* digest. Lanes: 1, control (no protein added); 2, protein fraction from control strain added; 3, TraA fraction added. Arrows on the right sides of panels A and B indicate bands that shifted. (C) *HpaI-AluI* digest. Lanes: 1, control (no protein added); 2, protein fraction from control strain added; 3 and 4, TraA fraction added. Equivalent amounts of protein were added in each case. The numbers in parentheses after the names of the restriction enzymes indicate the positions of the corresponding restriction sites in Fig. 3. Molecular size markers (in base pairs) are shown on the left sides of the gels.

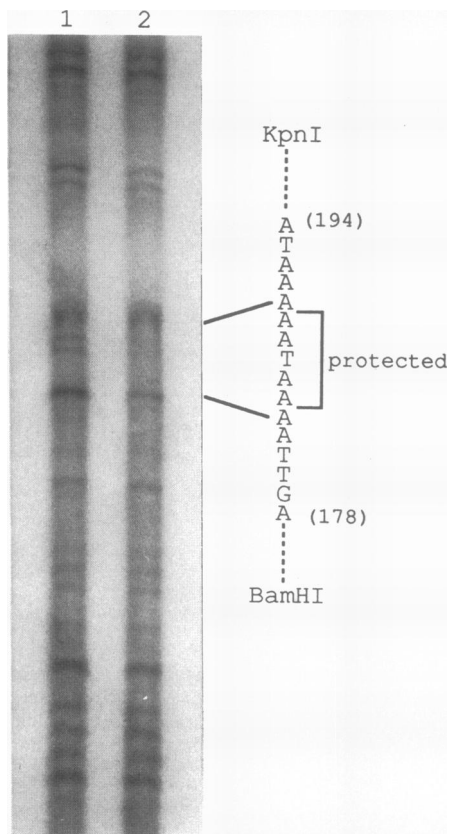


FIG. 8. DNA footprinting with TraA. The *KpnI*-*BamHI* fragment of pAM2610 was mixed with (lane 2) or without (lane 1) TraA, digested with DNase I, and run on a sequencing gel as described in Materials and Methods. A sequencing reaction (not shown) was run in parallel to position the region of protection. The nucleotides protected are indicated on the right.

the absence of the *iad* promoter in pAM2622 eliminated complementation. The data are reasonably conclusive in revealing the requirement for the *iad* promoter for *traE1* expression, at least for the initial stages of induction. Since the transcription data identified an RNA species (m3') corresponding to *traE1* and being expressed from its own promoter, it is evident that at some stage in the induction process, expression no longer requires transcription from the *iad* promoter.

DISCUSSION

The previously reported analysis of transcriptional fusions (24) implied that induction by the pheromone resulted in significant read-through of putative terminator TTS1 as well as a second possible terminator (TTS2) located about 125 bases further downstream. Our RNA analyses in the present study yielded data consistent with those observations and provided additional insight into these events. Read-through from the *iad* promoter to the end of *traE1* (transcript m2) appeared maximal at 20 min of induction, after which it became significantly reduced so that it was barely detectable at 80 min. A separate transcript, m3', appearing to be expressed under the control of the *traE1* promoter, was also induced; this transcript, however, continued to be expressed well beyond the time at which the larger read-through

transcript (m2) became undetectable. The m3' signal was strong even after 160 min of pheromone exposure.

The transcription data suggest that the expression of *traE1* initially requires read-through of TTS1 and TTS2 and that this may in turn trigger the expression of the gene from its own promoter. In this regard, it is noteworthy that Galli et al. (14) recently identified a transcriptional start site upstream of *traE1* in the right repeat of TTS2. One interpretation is that activation of the *traE1* promoter is somehow coupled to transcription from upstream through TTS2. However, considering the fact that TraE1 is a positively acting regulator at one or more sites downstream, it is very possible that it activates its own promoter as well. Thus, once enough TraE1 can be made as a result of transcriptional read-through, it could begin to activate transcription from its own promoter. Consistent with this view were the results of the *traE1* complementation studies, indicating again that TraE1 synthesis must initially utilize the *iad* promoter.

In addition to the increased read-through of TTS1 and TTS2, our studies indicated that during the first 20 to 40 min of pheromone exposure, the initiation of transcription from the *iad* promoter was significantly increased over its already relatively high basal level of expression. The precise location of this promoter was identified, and the highly dominant transcript, m3, corresponded to an RNA molecule terminating in the TTS1-TTS2 region. The data suggested that iAD1 should be detected in culture supernatants at a much higher level for induced cells compared with that normally observed for uninduced cells; however, we detected only about a twofold increase. It is not clear why the levels of iAD1 were not higher; nonetheless, the data were consistent with those observed for *traA* mutants, which also exhibited only a twofold increase (28, 30). Conceivably, iAD1 can be modified (inactivated) posttranslationally, or there may be factors limiting its processing and/or secretion. (It should be kept in mind that the peptide is synthesized as a 22-amino-acid precursor with the carboxy-terminal 8 residues corresponding to iAD1 [7].) With regard to *traA* mutants, the transcription pattern resembled that observed for a 20-minute pheromone exposure of wild-type cells. It was previously reported by Pontius and Clewell (24) that a transcriptional fusion about 40 bases downstream of *iad* (pAM7304) expressed LacZ at similar levels (relatively high) both before and after exposure to cAD1. However, the postinduction LacZ determination was done after 90 min, a time at which the transcription analyses reported in the present study indicated a reduction in m3 levels to nearly preinduction levels. The levels of the m4 and m5 RNAs detected did not change much during induction. These RNAs were shorter versions of transcripts for which no obvious terminators were evident in the sequence; conceivably they represent processed (or degraded) forms of m3, and their generation may be influenced by signals in the nucleotide sequence between the end of *iad* and TTS1. (See reference 24 for a discussion of a potential complex secondary structure that may occur in this region.)

Our DNA binding studies showed that the *trans*-acting TraA protein was associated with the promoter region of *iad*, an observation consistent with the notion that TraA negatively regulates transcription from this region. It had been anticipated that TraA might act between *iad* and TTS1 to prevent transcriptional read-through; however, our inability to detect binding within this region implies that if TraA does indeed affect read-through, it is weak or requires additional factors. The possible involvement of a short (14-amino-acid-

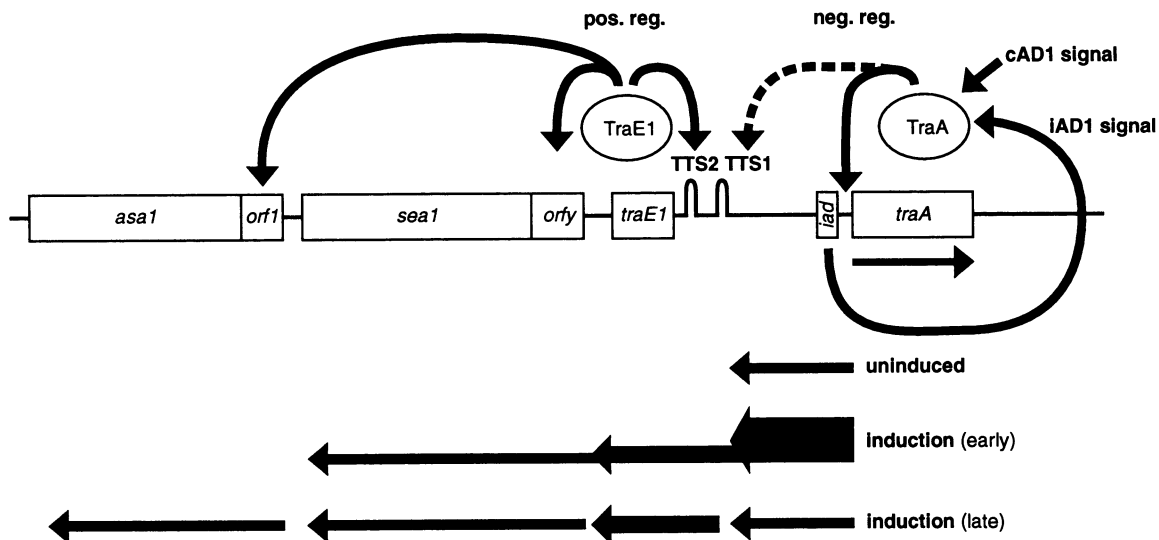


FIG. 9. Model for the regulation of the pAD1 pheromone response. Induction is viewed as the pheromone signal initially causing the release of the negative effect of TraA on the *iad* promoter as well as possibly effecting a transcription antitermination event at TTS1 and TTS2, allowing increased transcription and read-through into *traE1*. Once the TraE1 product is made, it positively self-regulates at the *traE1* promoter as well as at the downstream promoters of *sea1* and *asa1*. TraA repression resumes quickly, in part influenced by the burst of iAD1 expression that then competes with the cAD1 signal and also possibly due to derepression of *traA* (i.e., a transient increase in TraA then negatively regulates itself). pos. reg., positive regulation; neg. reg., negative regulation.

residue) open reading frame starting within TTS1 (7, 29) should be kept in mind.

We could not detect TraA binding closer to or within the *traA* promoter. However, the fact that footprinting experiments revealed a DNase insensitivity of this region in the absence of TraA made it difficult to rule out binding. A self-regulation of *traA* by its product would seem a reasonable possibility. However, Tn917lac insertions in *traA*, generating transcriptional fusions, result in very low levels of LacZ synthesis (28), implying that if derepression occurs, the increased level of TraA synthesis still involves a relatively low level of transcription. This result would not be surprising for a negatively acting regulatory protein.

Weidlich et al. (32) recently identified the determinant for surface exclusion protein Seal1, which is a 98-kDa protein encoded downstream from *traE1* (Fig. 1). Immediately preceding *sea1* is an open reading frame, *orfy*, which would encode a 12-kDa protein. Our Northern blot analyses identified an inducible RNA species, m1, that would easily correspond to a transcript extending to the 3' end of *sea1*. The data are consistent with the notion that the surface exclusion property of pAD1 is indeed inducible (6). Larger RNA species that might reflect extension into the determinant for the aggregation substance, *asa1* (15), were not observed. Galli et al. (14) reported that both *sea1* and *asa1* can be transcribed independently from promoters upstream of *orfy* and *orf1* (small reading frames just upstream of *sea1* and *asa1*), respectively.

The model shown in Fig. 9 illustrates our current working hypothesis for how the various elements of regulation interact. It differs from a model recently proposed by Galli et al. (14) that does not view *traE1* as able to be transcribed independently. These workers suggested that TraE1 activates the *iad* promoter to enhance the synthesis of transcripts reading through TTS1 TTS2. Their primer extension study revealed a transcriptional start site that was close to the *traE1* translational start site and that was interpreted as

an artifact relating to a secondary structure (TTS2) present in an RNA molecule extending further upstream (i.e., to the *iad* promoter). We believe that their inability to detect the *traE1* transcript (our m3' transcript) was because of the electrophoresis conditions that they used, which would have allowed this small RNA species to migrate off the end of the gel. We believe that their data are actually consistent with our model.

Finally, it is worth noting that conjugative *E. faecalis* plasmid pCF10 appears in some ways to be organized in a manner similar to pAD1 (11, 19). Both plasmids have a regulatory region adjacent to a surface exclusion determinant, which is in turn next to the gene for the aggregation substance. Like pAD1, pCF10 contains both positively and negatively acting regulatory products. The pheromones, however, are different, and there are recent indications that there are significant differences in the mechanisms of regulation (3).

ACKNOWLEDGMENTS

We thank Y. Su, D. Heath, F. An, and R. Wirth for helpful discussions relating to different aspects of this work.

This study was supported by Public Health Service grants GM33956 and AI10318 from the National Institutes of Health.

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