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Conjugative Transfer of *Enterococcus faecalis* Plasmid pAD1: Nucleotide Sequence and Transcriptional Fusion Analysis of a Region Involved in Positive Regulation

LINDA T. PONTIUS[1] AND DON B. CLEWELL[2]*

Department of Microbiology and Immunology, School of Medicine,[1] and Department of Biologic and Materials Sciences, School of Dentistry,[2] The University of Michigan, Ann Arbor, Michigan, 48109-0402

Received 30 December 1991/Accepted 10 March 1992

The *Enterococcus faecalis* plasmid pAD1 undergoes conjugative transfer in response to cAD1, a peptide sex pheromone emitted by potential bacterial recipients. Regulation of pAD1 transfer involves a number of plasmid-encoded determinants: *iad*, which determines a peptide-competitive inhibitor iAD1; signal sensing and transducing elements; and negative and positive regulators. The key positive regulator(s) of the pheromone response is believed to be encoded within a segment designated the E region of the plasmid. In this study, we analyzed the nucleotide sequence and transcription within the E region. An open reading frame designated *traE1* was identified; its inferred protein consists of 118 amino acids. Insertional mutagenesis of *traE1* resulted in a complete loss in plasmid transfer capability. Analysis of Tn*917-lac* insertions giving rise to transcriptional *lacZ* fusions showed that *traE1* is transcribed only under cAD1 inducing conditions. Analysis of additional *lacZ* fusions within the region provided some insight into the roles of potential regulatory signals within and around the nucleotide sequences reported here. A regulatory role appearing to involve read-through of certain key transcription termination sequences seemed evident.

Certain self-transmissible *Enterococcus faecalis* plasmids transfer in response to specific peptide pheromones secreted by potential recipients (recently reviewed in reference 9). The regulation of this process has been of continuing interest because many pheromone-responsive plasmids encode virulence factors (e.g., hemolysins) or antibiotic resistances (3, 4, 24).

One such plasmid is pAD1, a 60-kb conjugative plasmid which encodes a hemolysin-bacteriocin protein (8, 36). Transfer functions of this plasmid are specifically induced by the pheromone cAD1 (27). When induced, donors containing pAD1 express several novel surface proteins whose appearance has been correlated with the formation of mating aggregates (15, 37). In the absence of recipients, induced cells undergo a self clumping (12). On solid media containing pheromone, this self clumping causes colonies to appear dry and fracturable (37). The latter characteristic has proven useful in screening for pAD1 mutants defective in production of aggregation factors (37). In addition to inducing a surface protein(s) called aggregation substance, cAD1 also causes expression of genes required for plasmid transfer and surface exclusion (6, 13, 14, 29). After receiving a copy of pAD1, cAD1 production by the recipient (transconjugant) is shut down, and iAD1, a plasmid-encoded peptide serving as a competitive inhibitor of cAD1, is produced (26).

Genetic determinants related to pAD1 conjugation appear to be contiguously located within a segment of DNA spanning at least half (30 kb) of the plasmid. The structural genes are located in regions designated F, G, and H (14). The determinant encoding an aggregation substance factor has been localized within the F region (17), whereas the G and H regions relate to stabilization of mating aggregates in broth (broth matings) and plasmid transfer, respectively (9, 14). Tn*917-lac* mutagenesis of the F, G, and H regions revealed that transcription occurs in the same direction and occurs only under cAD1-inducing conditions (29).

Previous studies localized pAD1 determinants responsible for regulation of the mating response to a 6-kb region of the plasmid (Fig. 1). Negative regulators of the pheromone response are encoded within *traB* and *traA* and possibly within the C region of the plasmid (14, 22, 37). *traB* plays a role in cAD1 shutdown (38, 39), whereas *traA* encodes a repressor of the pheromone response which also appears to function in pheromone sensing or signal transduction (22, 37). The C region is believed to encode product(s) involved in sensing cAD1 (37). Insertional mutagenesis of the C region resulted in increased levels of iAD1 in the medium and a concurrently reduced sensitivity to cAD1 (14, 37). In addition, insertional mutagenesis of the *traA* proximal portion of the C region resulted in partial derepression of the mating response (37).

The regulatory region also contains the *iad* determinant which encodes iAD1 (7, 23). Exogenous cAD1 must be able to outcompete the donor-produced iAD1 for induction of the mating response to occur; for the case in which equal numbers of donors and recipients are present, cAD1 activity generally prevails. iAD1 is able to mask low levels of cAD1 (i.e., which might escape endogenous shutdown in the donor or be present when a potential recipient is far away), thereby preventing nonproductive activation of the transfer apparatus. The recently reported nucleotide sequence of the *iad* determinant revealed that iAD1 production involves a 22-amino-acid precursor, with the last eight (carboxyl-terminal) residues representing the mature peptide (7).

Tn*917* and Tn*917-lac* mutagenesis of the E region resulted in a nonresponder phenotype—the reduction or loss of ability to express both cellular aggregation and plasmid transfer functions (14, 37). Studies of *lacZ* fusions in the E region showed that transcription was cAD1 inducible (37) but occurred under noninducing conditions when *traA* was dysfunctional (38). Data generated in previous studies sug-

* Corresponding author.

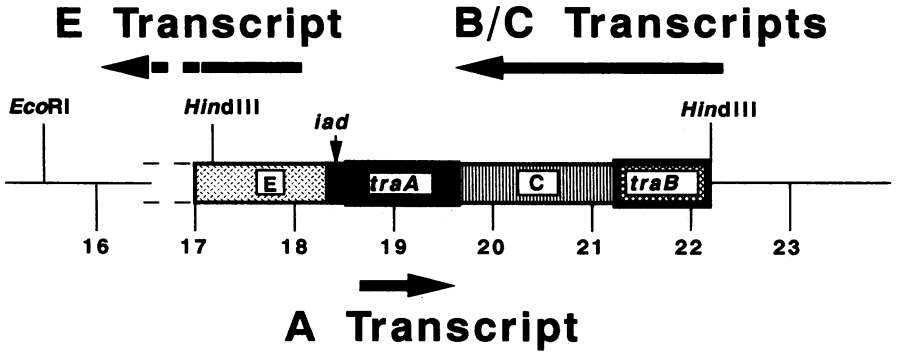

FIG. 1. The regulatory region of pAD1. Boldface arrows indicate the directions of transcription through the region, as determined in a previous study (37). The dashed lines around and over the E region indicate uncertainty as to the extent of sequences contained within the E region. The numbers refer to kilobases on the pAD1 map (34b, 37).

gested that the loss of aggregation and plasmid transfer function of E region mutants was not due to polarity effects (i.e., interruption of a transcript encoding the structural genes for the pheromone response) (14, 29). For example, a Tn*917-lac* insert mapping at 15.0 kb on the pAD1 map (between the E and F regions) transfers in broth matings and aggregates normally in response to pheromone (29). Taken together, this evidence suggested that the E region encodes a positive regulator(s) that is expressed in response to pheromone and may act at one or more downstream sites.

In order to gain further insight into this aspect of pAD1 regulation, we undertook a detailed analysis of the E region. Here we report nucleotide sequence data and identify a sequence (*traE1*) which appears to be essential for plasmid transfer. We also describe transcriptional analyses, making use of Tn*917-lac* fusions.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. pAD1::Tn*917* and Tn*917-lac* derivatives were generally characterized in an *E. faecalis* OG1X host background. *E. faecalis* FA2-2 was used as a recipient in filter mating experiments.

The construction of pAM7500 has been previously described (30). The recombinant consists of pBluescript with a segment of pAD1 DNA inserted into the *Hin*dIII site. The pAD1 *Hin*dIII fragment (17.2 to 22.2 kb on the map of Fig. 1)

TABLE 1. Strains and plasmids used in this study[a]

Organism, strain, or plasmid	Relevant phenotype	Reference or source
Strains		
E. faecalis		
OG1X	*str*	23
FA2-2	*rif fus*	8
E. coli DH5α	Rec$^-$	Promega, Madison, Wis.
Plasmids		
pBluescript SK	*amp*	Stratagene, La Jolla, Calif.
pAM7500	pAD1 *Hin*dIII fragment containing the C region, *traA*, *traB*, *iad*, and a portion of the E region inserted into pBluescript SK	30
pAM714	pAD1::Tn*917* (Agg^I, Tra^I)	22, 23
pAM957	pAD1::Tn*917* (Tra$^-$)	14
pAM959	pAD1::Tn*917* (Tra$^-$)	14
pAM960	pAD1::Tn*917* (Tra$^-$)	14
pAM2011	pAD1 with a Tn*917-lac* insert in the E region (the NR11 insert, Agg$^-$, Tra$^-$, β-gal^I).	37
pAM2019	pAD1 with a Tn*917-lac* insert in the E region (the NR19 insert, Agg$^-$, Tra$^-$, β-gal^{E,I})	37
pAM7304	pAD1::Tn*917-lac* (E region insert)	This study
pAM7314	pAD1::Tn*917-lac* (E region insert)	This study
pAM7318	pAD1::Tn*917-lac* (E region insert)	This study
pAM7330	pAD1::Tn*917-lac* (E region insert)	This study
pAM7337	pAD1::Tn*917-lac* (E region insert)	This study
pAM7338	pAD1::Tn*917-lac* (E region insert)	This study
pAM7343	pAD1::Tn*917-lac* (E region insert)	This study
pAM7348	pAD1::Tn*917-lac* (E region insert)	This study

[a] Agg, aggregation; Tra, transfer in 1- or 4-h broth matings; β-gal, β-galactosidase production; −, no aggregation in response to cAD1 or no plasmid transfer in 1- or 4-h broth matings; I, pheromone inducible; E, elevated basal level.

in this recombinant contains the C region, *traB*, *traA*, *iad*, and a portion of the E region.

Media, enzymes, and reagents. *Escherichia coli* was grown in Luria broth (10). *E. faecalis* strains were grown in THB (Todd-Hewitt broth; Difco Laboratories, Detroit, Mich.) or in N2GT (nutrient broth no. 2; Oxoid Ltd., London, England) supplemented with 0.2% glucose and 0.1 M Tris-HCl (pH 7.5). Agar (1.5%; Difco) was added for preparation of solid media. Antibiotics were generally used in the following concentrations; ampicillin, 120 μg/ml; erythromycin, 10 μg/ml; fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; and streptomycin, 1,000 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma Chemical Co., St. Louis, Mo.) was used at a concentration of 100 μg/ml. Synthetic cAD1 was used at a concentration of 40 ng/ml. The sources of all enzymes, reagents, and primers used for the generation of nested deletions and sequence analysis have previously been reported (30, 34). Restriction enzymes for characterization of Tn*917-lac* inserts in pAD1 were purchased from New England Biolabs (Beverly, Mass.) and were used under the conditions recommended by the manufacturer.

Construction of nested deletions and sequence analysis. Nested deletions of pAM7500 were constructed by using the exonuclease III-mung bean nuclease procedure recommended by Stratagene. Dideoxy sequencing (31) from double-stranded templates (2) was performed as previously described (30, 34). In some cases, unique synthetic primers were used. A primer, 5'CCTAAACACTTAAGAG3', within the right end of Tn*917* (33) was used to determine the junction sequence of Tn*917* and Tn*917-lac* inserts in pAD1. Analyses of the nucleotide sequence were performed by using the DNA Inspector IIe program (Textco, Lebanon, N.H.) and the MacVector program (GenBank data base release 61.0, 1990; International Biotechnologies, Inc., New Haven, Conn.).

Tn*917-lac* mutagenesis of pAD1. The Tn*917-lac* mutagenesis of pAD1 was performed as previously described (37), with the delivery vector pTV32Ts (28, 40). pTV32Ts is a temperature-sensitive transposon delivery vector carrying Tn*917-lac*. Growth of cells was maintained for 20 or more generations at an elevated temperature, and then the cells were plated on media containing erythromycin. Erythromycin-resistant colonies generally corresponded to insertions, and usually 95% of these were found on pAD1. (pAD1 is a preferred target over the chromosome.) Insertion mutants were selected for further study on the basis of an inability to aggregate in response to pheromone, inability to transfer in a 1-h broth mating, restriction digests showing inserts to map within the regulatory region, and LacZ activity data.

Aggregation in response to cAD1 was tested by streaking isolates on solid media containing cAD1 and observing the colony morphology after 36 h of growth. Previous studies showed that isolates expressing pheromone-inducible surface proteins produce colonies that are dry in appearance and fracture when touched with a toothpick (22). Colonies of isolates incapable of expressing these proteins are dull in appearance and soft (37). In all cases in which a soft colony morphology was observed, inability to aggregate in response to pheromone was confirmed by diluting overnight cultures of the test strains 1:10 in media containing cAD1 and observing the culture after 3 h of incubation at 37°C for the formation of visible mating aggregates.

Isolates were tested for the ability to transfer in 1-h broth matings as previously described (14). Plasmid DNA for restriction digestion was isolated by a modified alkaline lysis procedure (37). LacZ activity was determined qualitatively by growing isolates on solid media containing X-Gal with or without the addition of cAD1. Colony color was observed after 36 h of incubation at 37°C.

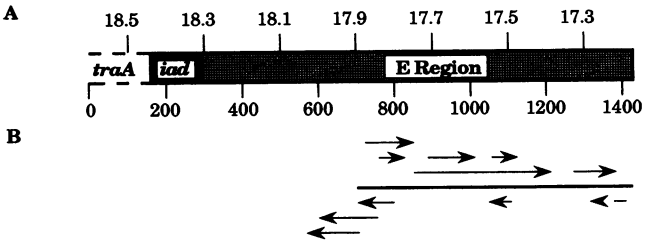

FIG. 2. ORF analysis of the E region. (A) Approximate locations of *traA*, *iad*, and the E region, as indicated by previous studies (7, 37). Numbers below the diagram refer to bases of the nucleotide sequence. Numbers above the diagram indicate the positions of the sequence (in kilobases) on the pAD1 map. (B) Locations and orientation (5'→3') of ORFs beginning with the ATG, TTG, or GTG start codon (see Results). A portion of the sequence analyzed for ORFs was previously reported (7) and was included in this analysis to locate any overlapping ORFs. A dashed arrow indicates that the complete sequence of the ORF is not known.

Characterization of pAD1::Tn*917-lac* isolates. Matings on filter membranes (filter matings) were performed as previously described (5). Pheromone-inducible production of β-galactosidase was tested as previously described (37); results are reported in Miller units (25).

Nucleotide sequence accession number. The GenBank accession number for the 3' 734-base sequence containing *traE1* (see Fig. 3) is M87836. The remaining (5') region is registered as M36625.

RESULTS

Sequence analysis of the E region. Nucleotide sequence analysis of the region from 17.2 to 17.8 kb (Fig. 1) was performed. A computer-aided search for open reading frames (ORFs) encoding 20 or more amino acids with an ATG, TTG, or GTG start codon (18) revealed 11 such ORFs, which are depicted in Fig. 2. This diagram includes ORFs initiating within the sequence reported here but overlapping with a sequence previously reported (7). Each ORF was examined for the presence of a strong translation initiation signal (GGAGG; allowing for one mismatch) located within the first 20 bases 5' of a start codon. One such ORF, designated *traE1*, was found. The nucleotide sequence of this ORF as well as the predicted amino acid sequence is shown in Fig. 3.

The nucleotide and deduced amino acid sequences of *iad* and a second ORF, now referred to as ORF-TTS1, were reported previously (7) and are also shown in Fig. 3 because of their integral relationship with the fusion data obtained in the present study (see below). Both sequences are read in a 5' → 3' (or rightward) direction in relationship to the sequence data shown in Fig. 3. *iad* encodes iAD1, the competitive inhibitor of cAD1. ORF-TTS1 would encode a protein of 15 amino acids with a molecular weight of 1,872.

***traE1*.** The 356-base *traE1* ORF was found 181 bases downstream of ORF-TTS1 (Fig. 3). A potential translation initiation signal and sequences with weak homology to *E. coli* consensus promoter sequences (−35, TTGACA; −10, TATAAT [19]) are indicated upstream of *traE1* (Fig. 3). *traE1* is deduced to encode a protein of 118 amino acids with

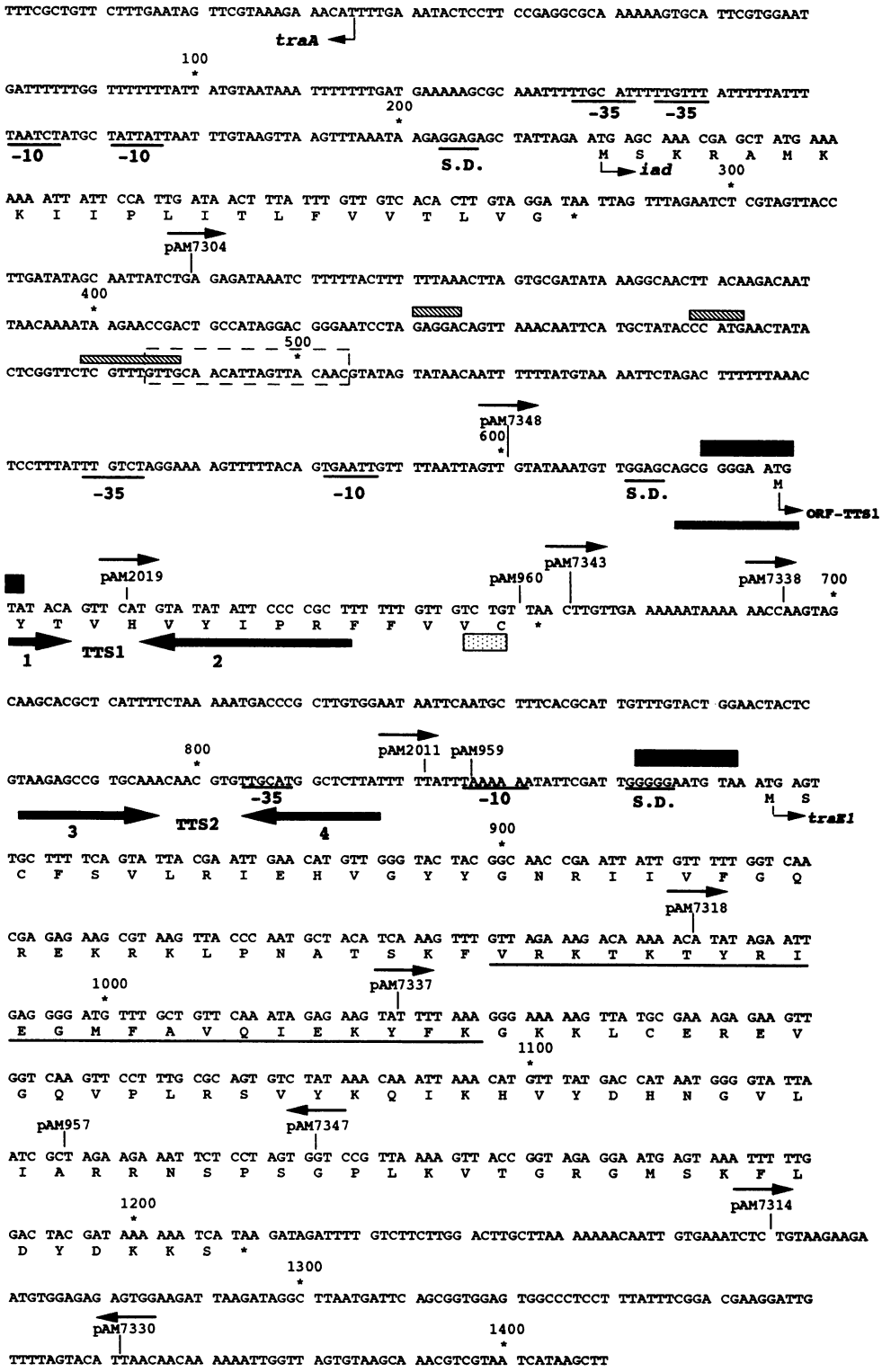

FIG. 3. Nucleotide sequence of the E region. The sequence shown overlaps with that previously reported (7, 30). The deduced amino acid sequences of *iad*, ORF-TTS1, and *traE1* are shown. Potential transcription initiation signals (−35 and −10) and ribosome binding sites (S.D.) are indicated. The positions of the Tn*917* and Tn*917-lac* inserts are indicated by vertical lines, and the designation for the plasmid bearing each insert is shown above each line. In the case of Tn*917-lac* inserts, an arrow above the plasmid designation represents the orientation of the *lacZ* fusion (5′→3′) created by the insert. Boldface arrows are used to indicate the positions of proposed transcription termination signals. ■, a sequence with homology to sequences found in other factor independent termination signals (1); [], an area of dyad symmetry; ▨, a repeated sequence (see Results); ▧, a sequence with similarity to a sequence found in the promoter region of *asa1* (see Results) (17). The underlined amino acid segment in *traE1* contains a potential helix-turn-helix binding motif (11).

a molecular weight of 13,731 and a high positive charge (+17).

A search of GenBank entries did not reveal any proteins with significant homology to TraE1; however, there was limited homology over an 83-amino-acid region to bacteriophage P22 antirepressor protein (28% identical and 16% conserved residues in the aligned sequences [32]). A portion of the TraE1 sequence also exhibited weak homology with the consensus helix-turn-helix motif of certain DNA-binding proteins (noted in Fig. 3). This protein segment scored 2.5 SD by the Dodd and Egan method of identification (11).

Potentially important secondary structure within the E region. Mapping upstream of *traE1* are two putative transcription terminations signals, TTS1 and TTS2 (Fig. 3 and 4A). Both appear to be factor-independent terminators, TTS1 consists of 17-base inverted repeats separated by 5 bases (ΔG = −34.4 kcal [ca. −143.9 kJ]) followed by five Ts (7). It also has a consensus sequence in common with other factor-independent terminators (TCTG downstream of the putative transcript termination site [indicated in Fig. 3] [1, 16]). TTS2 consists of 14-base inverted repeats separated by 8 bases (ΔG = −23.2 kcal [ca. −97.1 kJ] [35]) followed by five Ts. A number of alternate stem loop conformations within this region are possible and involve both TTS1 and TTS2 sequences. An example of one alternate structure is shown in Fig. 4B.

It is important to note the locations of these potential transcription termination signals in relationship to the ORFs under discussion. TTS1 and TTS2 are downstream of *iad*, and the short potential ORF, ORF-TTS1, is within TTS1. The start codon for *traE1* is 31 bases downstream of TTS2.

Transcriptional analysis of the E region. In a previous study the generation and analysis of *lacZ* fusions in the E region was performed by using Tn*917-lac* mutagenesis (37). The positions of some of the inserts obtained in that study as well as newly constructed inserts (this study) were determined by sequence analysis (see Table 1 for the source of each construct). The precise position of each insert is given in Fig. 3 and Table 2. LacZ activity data are also shown in Table 2. Results reported in this section are summarized pictorially in Fig. 5.

The Tn*917-lac* insert in pAM7304, which exhibits a soft colony morphology in the presence of cAD1, maps 43 bases downstream of *iad*. Transcription across the fusion occurs constitutively in a rightward direction at a relatively high level (see β-galactosidase activity data, Table 2). No transcription termination signal is apparent between the end of *iad* and the position of this insert. Data relating to pAM7348 (Table 2) indicate that transcription immediately upstream of TTS1 occurs at an elevated basal level which can be induced to higher levels by cAD1. The basal level of transcription across this fusion (12.3 Miller units), however, is much lower than that found across the fusion in pAM7304 (53.8 Miller units), indicating that some reduction of transcription must occur between the position of these inserts (upstream of TTS1).

The *lacZ* fusion created by a Tn*917-lac* insertion within TTS1, present in pAM2019 (see Table 2), is transcribed at an elevated basal level and is further inducible with cAD1. ORF-TTS1, which maps within TTS1 (as shown in Fig. 3 and 5) is interrupted by the insert in pAM2019, and the fusion in this insert should act as a transcriptional reporter for ORF-TTS1. Transcription immediately downstream of TTS1 occurs only when cAD1 is present, as indicated by results of β-galactosidase activity assays with OG1X(pAM7343). This rightward, pheromone-inducible transcription extends beyond TTS2 into *traE1* (e.g., data for pAM2011 and see below).

Plasmids with Tn*917-lac* inserts mapping within *traE1* include pAM7318, pAM7337, and pAM7347. Related β-galactosidase activity data indicated that transcription proceeds through the ORF in a rightward direction (relative to Fig. 3 and 5) and only under inducing conditions. The LacZ activity data from a Tn*917-lac* insert mapping 49 bases downstream of *traE1* (in pAM7314) suggested that a change in transcriptional activity occurred in this downstream region; transcription occurred at an elevated basal level and was pheromone inducible. The insert in pAM7330 also mapped downstream of *traE1*, but the fusion is oriented in the 3′ → 5′ (or leftward) direction relative to the sequence shown in Fig. 3. Little, if any, β-galactosidase was produced in this case, even under inducing conditions.

Further characterization of Tn*917-lac* inserts within the E region. Previous studies showed that Tn*917* and Tn*917-lac* inserts within the E region failed to undergo induced aggregation in media containing cAD1, because of the lack of expression of pheromone-inducible surface proteins (14, 37). These plasmids were also found to be unable to transfer during 1- or 4-h broth matings (14, 37). pAD1::Tn*917-lac* derivatives with inserts in the E region constructed in the present study exhibited a similar phenotype (data not shown).

Since matings on filter membranes can bypass the need for aggregation substance (14), experiments were performed with a number of pAD1 derivatives with inserts within the E region to determine whether DNA transfer functions were affected by the inserts. Results of these experiments are shown in Table 2.

The insert in pAM7304 (mapping 43 bases downstream of *iad* and 287 bases upstream of TTS1) caused a 10,000-fold reduction in plasmid transfer (1.9×10^{-5} transconjugants per donor) compared to the control plasmid, pAM714 (2.7×10^{-1} transconjugants per donor). The insert in pAM7348, which maps 16 bases upstream of TTS1, results in an even greater decrease in plasmid transfer frequency during filter matings. Plasmids with inserts mapping downstream of the insert in pAM7348 but upstream of *traE1*, pAM2019 and pAM7343, are capable of plasmid transfer during filter matings in the range of 10^{-4} to 10^{-5} transconjugants per donor. The fact that the insert in pAM2019 maps within ORF-TTS1 deems it unlikely that a product of this putative ORF is essential for plasmid transfer (see Discussion).

No detectable transfer was observed in the case of plasmids with inserts in *traE1*. This finding contrasted with those for plasmids carrying inserts mapping upstream of *traE1* (pAM7343) or downstream of *traE1* (pAM7314) which exhibited some transfer (Table 2). Thus, *traE1* appears to be essential for plasmid transfer.

Potentially significant sequences within the E region. Several interesting features of the E region sequence were noted. DNA-binding proteins are often found to bind within regions of dyad symmetry (20, 21). A number of small areas of dyad symmetry can be detected within this sequence, but one substantial region was found within positions 485 to 504, between *iad* and TTS1 (shown within a broken-line box in Fig. 3). The sequence is 20 bases in length, and the dyad symmetry is imperfect, with three mismatches.

Recently, the nucleotide sequence of the pheromone-inducible pAD1 gene *asa1* coding for aggregation substance was published by Galli et al. (17). A comparison of E region sequences with that of the promoter region of this determinant was made in an attempt to identify sequences which

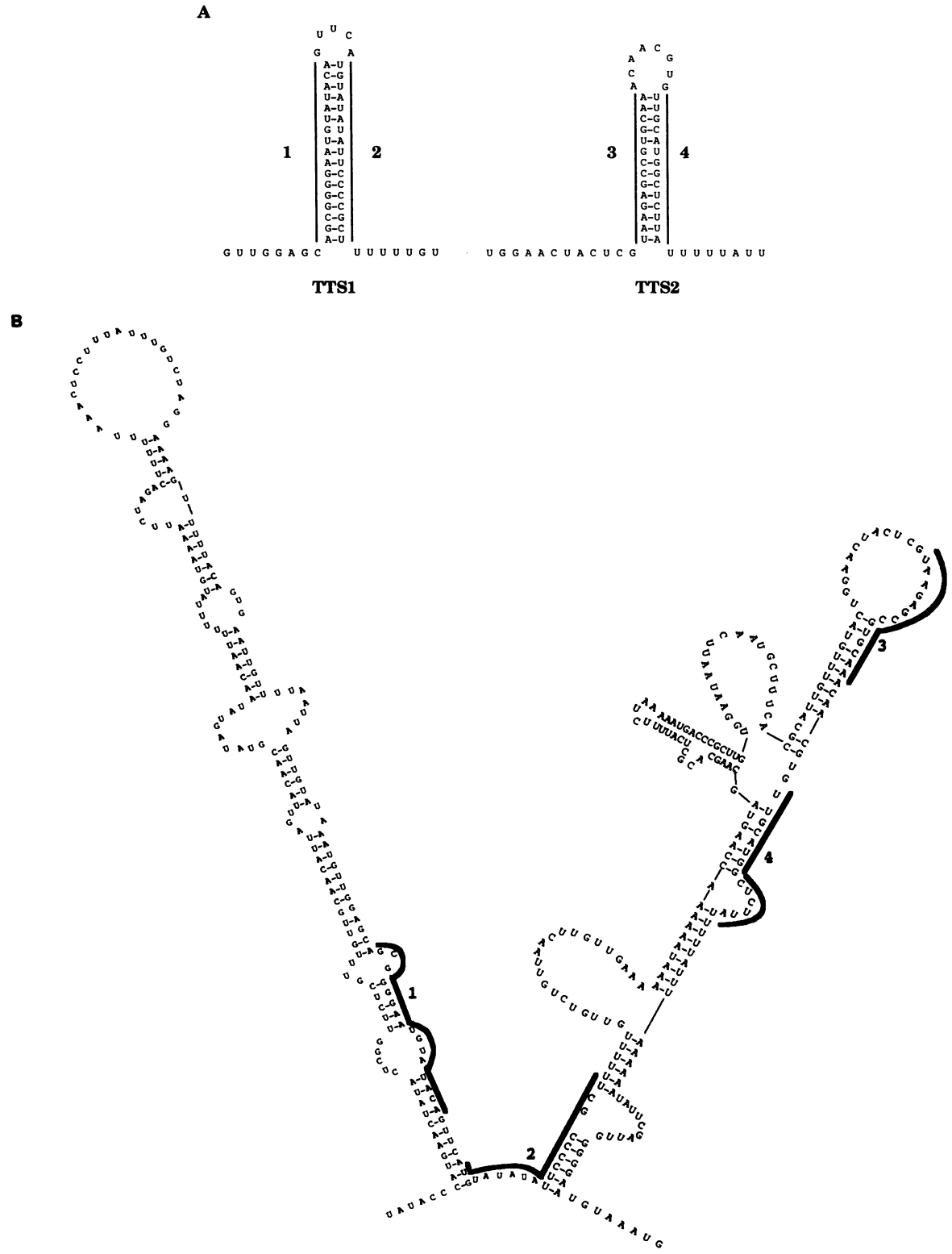

FIG. 4. Potential transcription terminators and alternate stem loop structures within the E region. (A) TTS1 and TTS2. Inverted repeats making up the proposed termination signals are indicated with solid lines and are numbered. (B) An alternate stem loop structure involving sequences in TTS1 and TTS2. Repeats corresponding to those shown in panel A are indicated by boldface lines and corresponding numbers.

TABLE 2. Characterization of plasmids with Tn*917* or Tn*917-lac* inserts mapping within the E region

Plasmid	Position of Tn*917* or Tn*917-lac*[a]	Orientation[b]	β-Galactosidase activity			Filter mating (no. of transconjugants/donor)
			Initial observation[c]	Enzyme assay[d] (Miller units)		
				− cAD1	+ cAD1	
paM714[e]						$2.7 \times 10^{-1} \pm 3.7 \times 10^{-1}$
pAM7304	329	R	C	53.8 ± 3.3	57.5 ± 5.2	$1.9 \times 10^{-5} \pm 1.9 \times 10^{-5}$
pAM7348	600	R	E + I	12.3 ± 1.0	50.2 ± 7.7	4.8×10^{-7}[f]
pAM2019	637	R	E + I	6.1 ± 0.9	18.9 ± 1.6	$4.4 \times 10^{-4} \pm 5.3 \times 10^{-4}$
pAM960	669					
pAM7343	673	R	I	1.2 ± 1.2	25.8 ± 4.8	$8.6 \times 10^{-5} \pm 1.2 \times 10^{-4}$
pAM7338	694	R	I			
pAM2011	821	R	I	1.1 ± 0.9	23.9 ± 3.3	
pAM959	826					
pAM7318	981	R	I	2.6 ± 0.2	37.2 ± 2.1	$<7.7 \times 10^{-8}$
pAM7337	1023	R	I			$<8.2 \times 10^{-8}$
pAM957	1128					
pAM7347	1148	L	−			
pAM7314	1260	R	E + I	10.2 ± 2.3	45.4 ± 5.7	$4.7 \times 10^{-5} \pm 3.2 \times 10^{-5}$
pAM7330	1361	L	−			

[a] The positions of Tn*917* or Tn*917-lac* inserts relative to the sequence shown in Fig. 3.

[b] The orientations of *lacZ* fusions was determined by sequence analysis: R, the *lacZ* fusion is oriented 5′→3′; L, the *lacZ* fusion is oriented 3′→5′, in relationship to the sequence shown in Fig. 3.

[c] Isolates were streaked on solid media containing 100 μg of X-Gal (with or without 40 ng of cAD1 per ml) per ml. Colony color was observed after 36 h of incubation at 37°C. β-Galactosidase production is indicated as follows: −, none; E, elevated basal level; I, cAD1 inducible; and C, constitutive.

[d] Enzyme assays for β-galactosidase activity were performed as described in Materials and Methods. Cells were incubated at 37°C for 90 min with (+) or without (−) cAD1 prior to enzyme quantitation.

[e] pAM714 (Table 1) is used as the wild-type control in filter mating experiments.

[f] Transfer was detected in one experiment at the frequency shown. In two other experiments with the same donor strain, no plasmid transfer was detected ($<10^{-8}$ transconjugants per donor).

may be important in regulation of pheromone-inducible transcription. The analysis revealed one striking area of sequence similarity. The sequence 5′ GAGGA-24 bases-CCATG-9 bases-TCGTTGTTTG 3′ was found 53 bases upstream of the start codon of *asa1*. A similar sequence (5′ GAGGA-23 bases-CCATG-15 bases-TCGTTTGTTG 3′, see Fig. 3) was found in the E region, downstream of *iad* but upstream of TTS1. It is interesting to note that this region of sequence similarity overlaps with the region of dyad symmetry discussed above. A similar region of dyad symmetry was not evident in the same relative position upstream of *asa1* (17).

Another potentially important sequence was identified on the basis of repetition and placement. GGGGAATGTA was found 3 bases upstream of *traE1* and also overlapping the start of ORF-TTS1 (shown in Fig. 3). Both of these ORFs are transcribed under inducing conditions.

DISCUSSION

Three ORFs with ATG start codons preceded by a strong ribosome binding site are present within the E region of pAD1. These include the previously identified *iad* (7) and a short reading frame (15 amino acids) present within a likely transcription terminator region, TTS1. Downstream from TTS1 is a second region resembling a transcription terminator (TTS2), followed eventually by the ORF designated *traE1*. TraE1 is predicted to be a positively charged 13.7-kDa protein exhibiting limited homology with an 83-amino-acid region of the bacteriophage P22 antirepressor protein (32). In addition, it contains a possible helix-turn-helix motif characteristic of certain DNA-binding proteins.

Transcriptional analyses (summarized in Fig. 5) showed that immediately downstream of *iad*, several hundred bases upstream of TTS1 (e.g., in the region of the insertion pAM7304), transcription occurred at a high level during both the absence and presence of cAD1, whereas the areas immediately upstream of TTS1 and extending through *traE1* showed pheromone-inducible expression. Since there are no obvious transcription terminators between the translational end of *iad* and TTS1, there is a reasonable likelihood that expression downstream of TTS1, and also TTS2 (see pAM2011, Fig. 5), is linked to transcription of *iad*. Induction would therefore result in an enhanced transcriptional read-through of TTS1 (and TTS2), possibly involving antitermination events. Recent Northern (RNA) blot analyses (34a) are supportive of this view. It is probably significant that transcription immediately upstream of TTS1 (e.g., in the case of pAM7348) is regulated to some extent, as LacZ expression in the uninduced state is significantly lower than that observed farther upstream (i.e., for pAM7304). The insertion within TTS1 (pAM2019) seemed similarly regulated. The region between the insertions of pAM7304 and pAM7348 therefore appears to play an integral role in regulation and may interact with the primary negative regulator (repressor) of the pheromone response, TraA. Possible sites for interaction with regulatory factors include a 20-base area of dyad symmetry (boxed by broken lines in Fig. 3) that overlaps a segment exhibiting striking homology with a sequence 53 bases upstream of *asa1*, a gene for an aggregation factor (17). Alternatively, TraA might serve to stabilize RNA stem loop structures, such as those relating to TTS1. The potential complexity of the control mechanism is illustrated by the possible secondary structure that might be involved in transcripts of this region (Fig. 4B). It is also conceivable that the putative product of ORF-TTS1 or events associated with its translation influence transcription through the TTS1.

Phenotypic data suggested that pheromone-inducible transcription within the E region may originate at the *iad* promoter or at another promoter between *iad* and TTS1 and

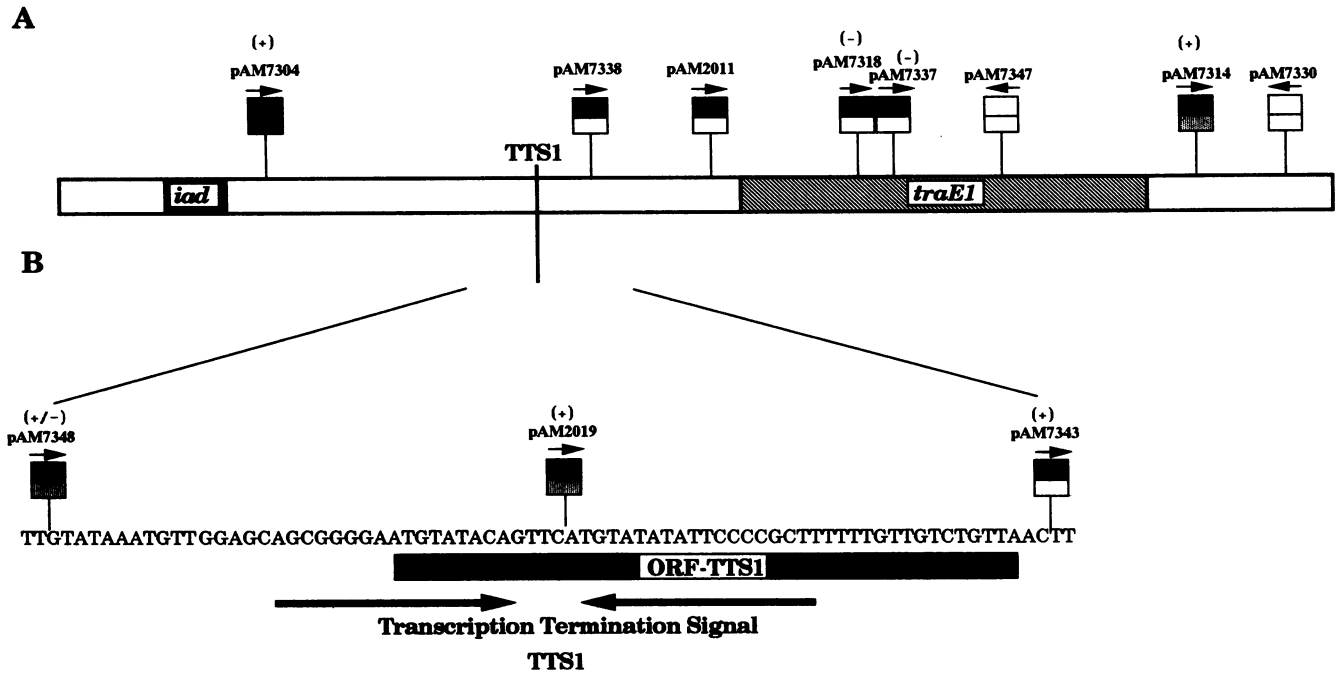

FIG. 5. A schematic diagram of the E region. (A and B) The positions of *iad*, ORF-TTS1, and *traE1* are indicated. The positions of Tn*917-lac* inserts mapping within this region are indicated by vertical lines, and the arrow above each insert indicates the orientation of the *lacZ* determinant (5′→3′). The designation for the plasmid carrying the insert is given above the arrow. ⊟, no significant β-galactosidase is produced from the *lacZ* fusion under inducing (top) or noninducing (bottom) conditions; ■, an elevated basal level of production of β-galactosidase, which is increased to a higher level when cAD1 is present; ■, β-galactosidase produced from the *lacZ* fusion at significant levels only when cAD1 is present; ■, constitutive production of β-galactosidase. Results of filter mating experiments to test the transfer of some of the plasmids are given above the plasmid designations: +, 10^{-5} to 10^{-4} transconjugants per donor; +/−, 10^{-7} or fewer transconjugants per donor; −, no detectable transfer ($<10^{-8}$ transconjugants per donor). (B) An expanded view of the region in the immediate vicinity of TTS1.

proceed through the E region. Transposon insertions throughout the E region result in strains that are unable to undergo induced aggregation and that exhibit negligible plasmid transfer in broth matings and reduced (by at least 10^3) plasmid transfer in filter matings. Filter mating data, however, show certain unexpected polarity effects. pAM7348 exhibited virtually no transfer in filter matings, while pAM7343, with a Tn*917-lac* insert downstream, does transfer to some extent in filter matings. In addition, inserts within *traE1* were found to eliminate plasmid transfer, whereas interruption upstream (pAM2019 and pAM7343) allowed a low level of transfer. These results suggest the possibility that more than one transcript may be produced from the region or perhaps that a low level of transcription is occurring out of the right end of the transposon. Further testing will be required to clarify this point.

TraE1 would appear to be an absolutely required regulatory factor for plasmid transfer. However, while inserts directly in *traE1* eliminated conjugal transfer, they also prevented expression of aggregation factors in response to cAD1. It seems unlikely that TraE1 alone is responsible for regulating expression of both aggregation substance and plasmid transfer, since an insertion downstream of *traE1* (pAM7314) also affected aggregation and was over 10^3-fold reduced in plasmid transfer in filter matings. It is conceivable that regulation affects extension of transcription further downstream into a region that influences expression of aggregation and transfer functions.

This study is a preliminary molecular characterization of the pAD1 E region. The ORFs investigated in detail include only those preceded by a strong ribosome binding site. The possibility exists that additional products (RNA or protein) encoded within this region, preceded by weaker control signals, also play significant roles. Identification of *traE1*, an ORF essential for plasmid transfer, and potentially important regulatory sequences and structures should facilitate future investigations. The E region extends at least 200 bases beyond the area characterized in this study (37), and evidence suggests that additional regulatory elements may be discovered in this downstream region.

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