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Regulation of the *Enterococcus faecalis* pAD1-related sex pheromone response: analyses of *traD* expression and its role in controlling conjugation functions

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Summary

The *Enterococcus faecalis* haemolysin plasmid pAD1 (60 kb) confers a conjugative mating response to an octapeptide sex pheromone (cAD1) secreted by plasmid-free strains. The response involves two plasmid-borne regulatory determinants: *traE1*, whose product positively regulates all or most conjugation-related structural genes; and *traA*, whose product negatively regulates *traE1* by controlling transcriptional read-through of an upstream termination site (TTS1/TTS2). TraA binds to the promoter region of *iad*, which encodes a pheromone-inhibitor peptide, iAD1; and TTS1/TTS2 tightly terminates transcription arriving from this promoter during the uninduced state. A determinant, *traD*, appearing to encode a small peptide (23 amino acids), is located just downstream of *iad* and is in the opposite orientation. Transcripts of *traD* were identified and found to be present at a relatively high level in cells not expressing conjugation functions; the amount of RNA was greatly reduced, however, upon induction of the pheromone response. The decrease in *traD* RNA was not a consequence of the induced activity of TraE1, as it also occurred in a *traE1* insertion mutant. A mutation in *traD* that would eliminate translation but that did not affect transcription had no apparent effect on the cell phenotype, indicating that RNA was likely to be the functional product. This was consistent with our finding that synthesis of *traD* RNA containing the translational defect was able to complement, *in trans,* a temperature-sensitive *traD* mutation. Thus, transcription of the *traD* determinant is significantly involved in down-regulation of the pAD1 pheromone response.

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Introduction

The *Enterococcus faecalis* plasmid pAD1 (Tomich *et al*., 1979; Clewell *et al*., 1982) encodes a haemolysin/bacteriocin (cytolysin) shown to contribute to virulence in animal models (Ike *et al*., 1984; Jett *et al*., 1992; Chow *et al*., 1993), and haemolytic enterococci harbouring related plasmids are commonly associated with human parenteral infections (Ike *et al*., 1987; Ike and Clewell, 1992). pAD1 (60 kb) is highly conjugative and encodes a mating response to an octapeptide sex pheromone (Mori *et al*., 1984) secreted by plasmid-free strains of *E. faecalis*. The peptide, designated cAD1, induces synthesis of a plasmid-encoded proteinaceous 'aggregation substance' (Asa1) and activates various structural genes that facilitate pAD1 transfer; once recipients acquire a copy of the plasmid they 'shut down' the secretion of the chromosome-encoded cAD1 pheromone. However, other sex pheromones specific for different donors carrying unrelated conjugative plasmids continue to be secreted. For background and reviews of pAD1 and other pheromone-responding systems in *E. faecalis*, see Clewell (1993a,b, 1998), Dunny and Leonard (1997) and Wirth (1994).

A region on pAD1 that contains the pheromone-response regulatory determinants is organized as shown in Fig. 1. *traA* encodes a negatively acting regulatory protein; mutations in this determinant result in constitutive expression of conjugation functions and donor clumping (Ike and Clewell, 1984; Pontius and Clewell, 1992a). TraA (38 kDa) prevents expression of *traE1*, which encodes a positive regulator necessary for the activation of all or most structural genes involved in the mating response (Ehrenfeld and Clewell, 1987; Weaver and Clewell, 1988 1989 1990; Muscholl *et al*., 1993). Induction of the pheromone response involves interaction of TraA with the pheromone signal, which, in turn, leads to the expression of *traE1*.

traC encodes a protein located on the cell surface, which enhances sensitivity to exogenous cAD1 (Tanimoto *et al*., 1993), and *traB* is involved in 'shut-down' of endogenous

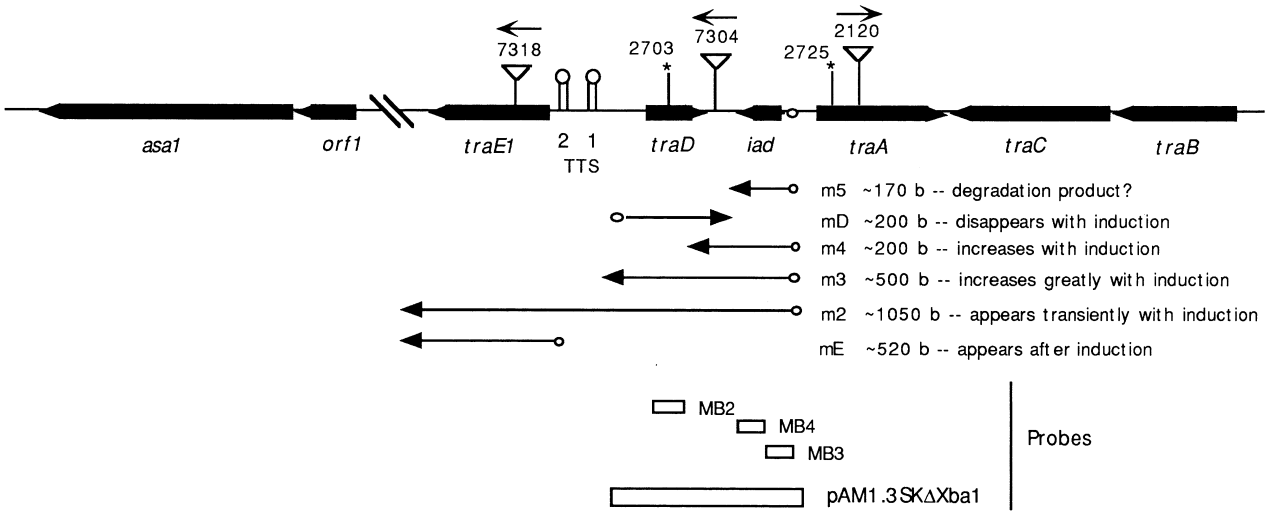

Fig. 1. Map of the pAD1 regulatory region. The various determinants and their relative orientations are indicated as heavy bold (solid). The arrows indicate the various transcripts and their orientation; these are noted with their approximate sizes. The triangles above the map point to the position of specific Tn*917lac* insertions. The asterisks mark the position of temperature-sensitive point mutations in *traD* and *traA*. MB2, MB3 and MB4 represent single-stranded probes designed to detect mD or possible extensions of this transcript (see Fig. 4). Note that there is break between *traE1* and *orf1* and that the unshown region includes the surface exclusion determinant *sea1* and a short open reading frame *orfy*. Parts of the map, which covers an overall size of about 13 kb, are not drawn to scale.

pheromone (An and Clewell, 1994). *iad* encodes an octapeptide (iAD1) that acts as a competitive inhibitor of cAD1 (Mori *et al*., 1986; Clewell *et al*., 1990), and *traD* corresponds to a small recently identified determinant (Bastos *et al*., 1997) that is the primary subject of this communication.

There are two apparent transcription–termination sites, TTS1 and TTS2, relatively close to each other between *traD* and *traE1* (Pontius and Clewell, 1992b). There are no known open reading frames in the 125 nucleotides separating TTS1 and TTS2, although there is a possible short open reading frame (15 amino acid residues) starting within TTS1 and extending a short distance beyond the terminator; it is believed that the second terminator (TTS2), while somewhat weaker than the first based on energy considerations, helps to prevent readthrough of transcription from the *iad* promoter into *traE1* (Pontius, and Clewell, 1992b; Tanimoto and Clewell, 1993). Thus, the primary basis of control involves regulation of transcription into *traE1* from the *iad* promoter; and TraA is a major element in governing this process. Once a minimal amount of TraE1 is made via readthrough of TTS1/TTS2, the protein upregulates itself from its own promoter located in part within TTS2, resulting in a burst of TraE1 synthesis (Tanimoto and Clewell, 1993); this, in turn, activates conjugation functions. TraE1 is probably unstable, and its ability to continue regulating itself positively may depend on maintaining a minimal level of TTS1/TTS2 readthrough from upstream.

Recent evidence has revealed that exogenous pheromone is actually internalized and binds directly to TraA causing a release of the protein from its binding site at the *iad* promoter (Fujimoto and Clewell, 1998). It is not clear whether the 'induction' of transcription into *traE1* results primarily from an increase in transcription from the *iad* promoter (which does occur) or whether TraA itself enhances the TTS1/TTS2 termination event by a process that is stopped upon exposure to cAD1. It is noteworthy that a sequence TTATTTTTATTT located within the *iad* promoter that contains the site at which TraA has been observed to bind (Tanimoto and Clewell, 1993) is also located close to the *traE1* promoter (Pontius and Clewell, 1992b; noted in Fig. 4); however, TraA binding at the *traE1*-proximal site was not detected previously.

Recently, we reported on the generation of several thermolabile pAD1 point mutations that resulted in activation of the mating response when cells were shifted from 32°C to 42°C (Bastos *et al*., 1997). Some of these mapped within *traA*, as had been expected; one such variant, with the most dramatic temperature-inducible conjugation phenotype (in two non-isogenic *E. faecalis* hosts), was designated pAM2725. Some mutations, however, mapped outside of *traA*; and, in two cases, the lesion was within a small potential open reading frame for a 23-amino-acid residue peptide that was subsequently designated *traD*. As noted above, this reading frame is located downstream of *iad* and is in the opposite orientation (see Fig. 1).

In this communication, we report on a further examination of *traD* and show that it is transcribed readily in uninduced cells but not in cells exposed to pheromone. In addition, we present evidence that it is the RNA product rather than a TraD peptide that is an active regulatory

component. Genetic analyses provide insight into how the transcript may function, and a hypothetical model is discussed.

Results

Identification of a traD *transcript that is greatly reduced upon induction of the pheromone response*

According to earlier results reported by Tanimoto and Clewell (1993), three relatively short transcripts, designated m3, m4 and m5, could be detected in RNA preparations extracted from uninduced OG1X/pAD1 cells when using a probe corresponding to the region between the *iad* promoter and a point 80 nucleotides from TTS1 (see Fig. 1). After a 20 min exposure of cells to cAD1 (40 ng ml^{-1}), two additional transcripts, designated m1 [about 4400 nucleotides, extending beyond *traE1* and through the downstream determinant *sea1* (not shown)] and m2 (about 1050 nucleotides), were also detected. All the RNAs were considered likely to have similar start sites but different 3′ ends (Tanimoto and Clewell, 1993); although the possibility that certain transcripts, for example m5, might be degradation products of others has not been ruled out. Because the probe in those studies involved labelled, double-stranded DNA, it would not have distinguished a transcript representing the recently identified *traD* in the opposite orientation. Thus, we used a synthetic oligonucleotide (32-mer) probe MB2 (see Fig. 1), designed specifically to detect a *traD* signal.

Figure 2A shows the results of Northern blot analyses on RNA extracted from *E. faecalis* OG1X cells carrying the plasmid pAM714 (pAD1::Tn*917* with wild-type mating properties). Lane 1 shows a significant band corresponding to about 200 nucleotides in addition to bands that probably correspond to 16S and 23S ribosomal RNA species. Samples extracted after 5, 10, 20 and 40 min of exposure to cAD1 are seen in lanes 2–5 respectively; the 200-nucleotide band is significantly reduced after 5 min of cAD1 exposure and is barely detectable after 10 min. Figure 2B shows similar results using cells carrying the temperature-inducible plasmid pAM2725 (mutation in *traA*) at 32°C (lane 1) and exposure to 42°C for 20, 40, 60 and 80 min (lanes 2–5 respectively). Again, the 200-nucleotide band decreases significantly during induction, although not as quickly as for the pAM714 plasmid. Also, the uninduced pAM2725-containing cells (i.e. those at 32°C) appeared to express at a significantly higher level compared with the uninduced wild type (pAM714) shown in lane 6; it is not known yet if this relates to the fact that the latter cells had been at a higher temperature (i.e. 37°C). In addition, the RNA species was not observed in cells carrying pAM2120, which constitutively expresses conjugation functions because of a transposon insertion in *traA* (lane 8).

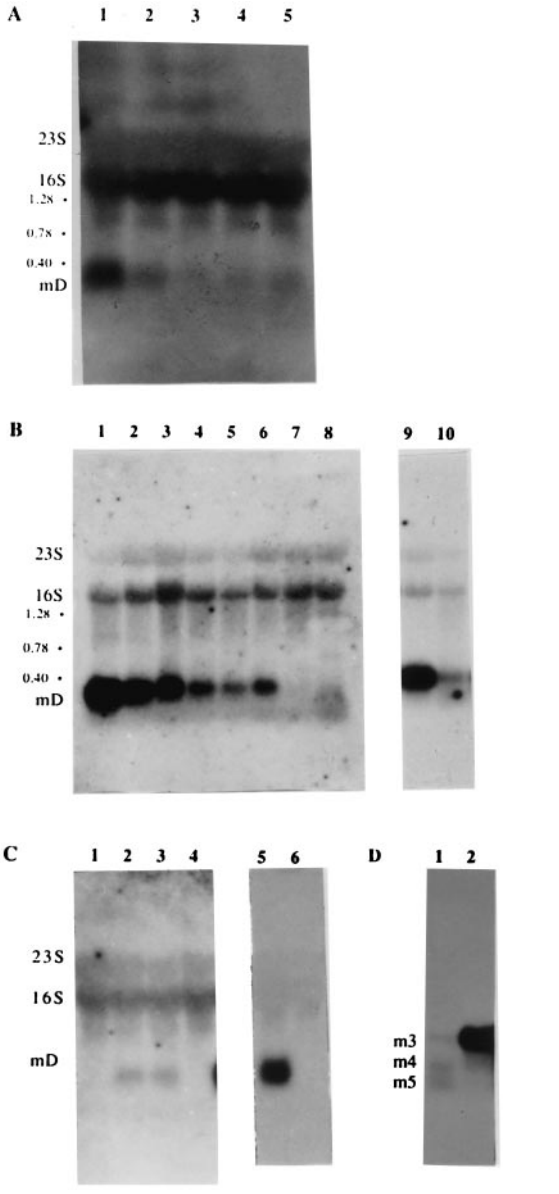

Fig. 2. Northern blot hybridization of RNA preparations. In (A), (B) and (C), the single-stranded MB2 probe designed to detect the mD transcript was used. In (D), the probe was pAM1.3SKΔXba1, which corresponds to the double-stranded DNA of that noted in Fig. 1.
A. Lanes 1–5 correspond to OG1X/pAM714 grown at 37°C in the presence of cAD1 (100 ng ml^{-1}) for 0, 5, 10, 20 and 40 min respectively.
B. Lane 1 corresponds to OG1X/pAM2725 grown at 32°C, whereas lanes 2–5 correspond to the same cells grown at 42°C for 20, 40, 60 and 80 min respectively. Lane 6 represents OG1X/pAM714 cells uninduced (37°C), and lane 7 represents exposure to cAD1 (40 ng ml^{-1}) for 40 min. Lane 8 corresponds to OG1X/pAM2120. Lanes 9 and 10 represent OG1X/pAM2703 at 32°C and 42°C (40 min) respectively.
C. Lane 1, OG1X plasmid-free cells; lane 2, OG1X/pAM714; lane 3, OG1X/pAM714-d1HT; lane 4, OG1X/pAM401; lane 5, OG1X/pAM-HT; lane 6, OG1X/pAM-KT. The position of RNA markers are noted on the left in (B) (in kb).
D. OG1X/pAM2703 at 32°C (lane 1) and 42°C (lane 2).

Lanes 9 and 10 of Fig. 2B represent cells carrying the plasmid pAM2703, which contains a temperature-sensitive lesion [a GAT (aspartate) substituted for a GGT (glycine)] within *traD* (Bastos *et al.*, 1997) at 32°C and 42°C (40 min) respectively. In this case, expression of the transcript is similar to that for the temperature-sensitive *traA* mutation (pAM2725); that is, an increase in temperature was associated with a reduction in the transcript. The material migrating in the position of 16S ribosomal RNA had a similar intensity in all cases. Using a probe that detects transcription from the *iad* promoter demonstrated that transcription was greatly enhanced upon shifting OG1X/pAM2703 cells from 32°C to 42°C (Fig. 2D), a result typically associated with induction (Tanimoto and Clewell, 1993).

The data indicate that the more rapidly migrating RNA species is expressed at a relatively high level when the cells are in an uninduced state; whereas it decreases or disappears when conjugation functions are induced. The RNA is of a size similar to that of the previously reported m4 (see Fig. 1); however, as m4 could be detected with a double-stranded probe in cAD1-induced OG1X/pAM714 cells and in OG1X/pAM2120 (not shown; see Tanimoto and Clewell, 1993), it is clearly different from that species. We have, therefore, designated the newly resolved species mD.

To estimate better the general location of the 3′ end of mD, the oligonucleotides MB3 and MB4 located 140 and 90 nucleotides downstream of MB2, respectively, with the same orientation (see Figs 1 and 4) were used as probes. No transcripts were detected in either case (data not shown), suggesting that mD terminates upstream of the MB4-related sequence.

Transcriptional initiation site of mD

Figure 3 shows the results of a primer extension analysis performed on RNA prepared from OG1X/pAM2725 and OG1X/pAM2700, each grown at 32°C. Whereas pAM2725 encodes a temperature-sensitive TraA, the temperature-sensitive lesion in pAM2700 is in an unknown position outside the region between and including *traA* and *traE1*. In both cases, a transcript with two apparent start sites 2 nucleotides apart and starting with T was observed; another possible initiation site, involving a G, was observed 8 nucleotides downstream from the second site. [Essentially identical results were obtained in the case of OG1X cells carrying pAM714 at 37°C (not shown).] It is conceivable that the second and third sites reflect degradation from the 5′ end corresponding to the first site. Initiation of RNA synthesis in this region and termination in a region well upstream of the area covered by the MB4 probe (which did not detect a signal) would give rise to a transcript corresponding in size to mD (about 200 nucleotides) terminating close to the 3′ end of *iad* (see Fig. 4). A possible −10/−35 hexanucleotide pair is noted upstream of *traD* in Fig. 4; an obvious factor-independent transcription termination site downstream was not evident.

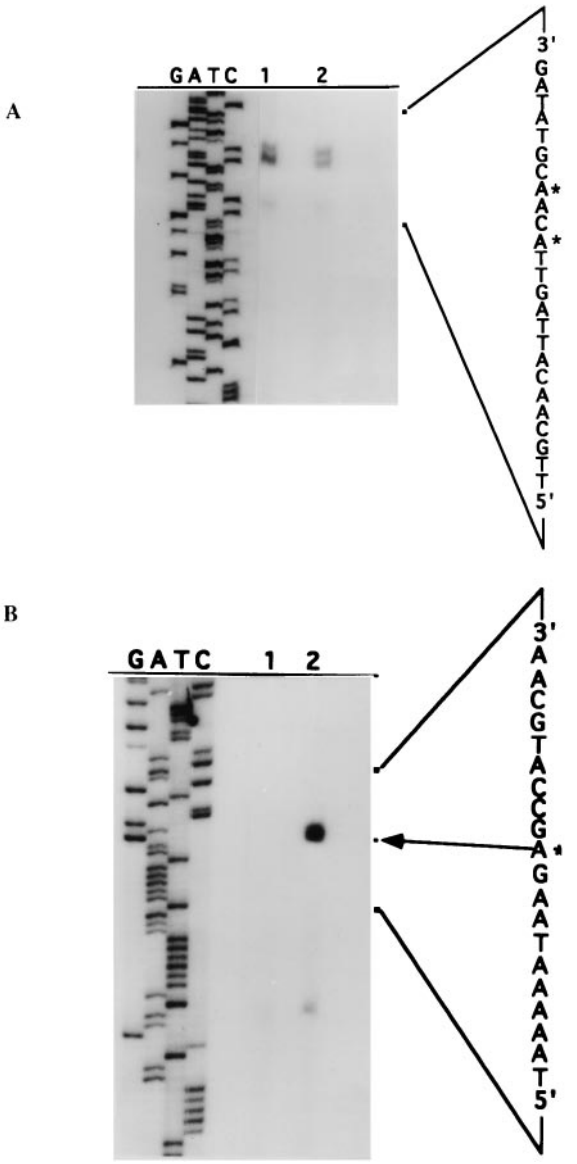

Fig. 3. Determination of the 5′ end of mD and mE transcripts by primer extensions. In each case, a DNA sequencing preparation was run in parallel using the same primer. The asterisks correspond to points within the corresponding sequence representing the apparent 5′ ends.
A. Lanes 1 and 2 represent strains OG1X/pAM2700 and OG1X/pAM2725, respectively, grown at 32°C.
B. Lanes 1 and 2 represent OG1X/pAM7318 and OG1X/pAM714 respectively. Both were exposed to cAD1 (100 ng ml^{-1}) for 40 min, a condition that gives rise to LacZ expression in the case of the Tn*917lac* insertion of OG1X/pAM7318. (Note the primer represented a region upstream of the insertion.)

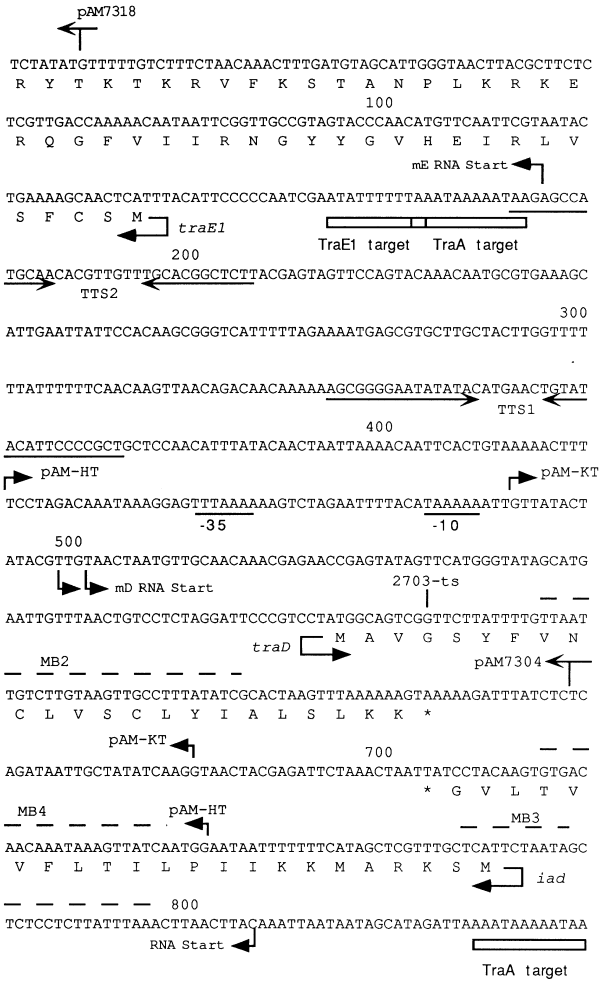

Fig. 4. Nucleotide and protein sequences corresponding to *traD*, *iad* and flanking regions including a portion of *traE1*. The position of RNA start sites, insertional and temperature-sensitive mutations, as well as the boundaries of the pAM-HT and pAM-KT clones, are indicated. The bold dashed lines indicate the position of specific probes used for Northern blotting.

traD *mutant defective in translation appears to have a normal phenotype*

The nature of the lesion of pAM2703 suggested that the related temperature sensitivity may result from the change from a glycine to an aspartate codon in the fourth amino acid residue in from the amino terminus of a TraD peptide; alternatively, the lesion could involve an effect on the secondary structure of the transcript. To explore this question further, we constructed a derivative containing a substitution of an arginine codon for the presumptive methionine start codon at the *traD* translational start site – a change expected to eliminate the production of a 'TraD' peptide (see *Experimental procedures*). Interestingly, the mutation (pAM714-d1HT) had no apparent effect on the phenotype. Mating in broth occurred normally and was pheromone inducible. Induction of clumping by cAD1 in microtitre assays occurred at a level of peptide sensitivity similar to that of the wild type; and Northern blot analysis showed a *traD* transcript indistinguishable from that of the wild type (Fig. 2C, lanes 2 and 3). The data suggested that a TraD peptide product, if it is indeed synthesized in wild-type cells, does not itself play a major functional role.

Plasmid chimera producing traD *transcript complements* in trans

In an earlier report (Bastos *et al*., 1997), we showed that the temperature-sensitive *traD* lesion of pAM2703 could be complemented *in trans* by a segment of DNA that extended from just upstream of *iad* to TTS2. To focus further on the complementing DNA, two smaller chimeras were constructed. pAM-HT and pAM-KT contain segments of DNA representing *traD* cloned in the shuttle vector pAM401. (See Fig. 4 for regions represented.) pAM-HT contained a larger segment than pAM-KT and included significantly more DNA upstream of *traD* (64 vs. 13 nucleotides upstream of the *traD* transcriptional start site respectively); it probably contained the upstream sequence necessary for appropriate *traD* promoter activity (see below). pAM-HT was able to complement the *traD* temperature-inducible mutant represented by pAM2703 *in trans*. At 42°C (using the FA2-2 host), there was no clumping, and mating potential did not increase in a 10 min mating after cells were held at 42°C for 1 h. These cells exhibited a mating response upon exposure to cAD1. A derivative of pAM-HT containing the methionine-to-arginine change at the *traD* translational 'start' site (pAM-HTd1) did not affect the ability to complement. In contrast, the pAM-KT chimera, which contained only a short region upstream of *traD* and is assumed to lack important promoter-related sequences, was not able to complement the thermolabile mutation at the elevated temperature. Northern blot experiments, whereby pAM-HT or pAM-KT were present alone in OG1X, showed that, in the case of pAM-HT, the *traD*-related RNA was produced at a high level, whereas none was detected in the case of the pAM-KT-containing strain (see Fig. 2C, lanes 5 and 6). The data again support the notion that synthesis of an intact *traD* transcript, but not a translational product, is necessary for maintaining a 'repressed' state.

Analyses of mD in traE1 *and* traD *insertion mutants*

pAM7318 is a pAD1 derivative with a Tn*917lac* insertion within *traE1* (see Figs 1 and 4), which expresses a high level of LacZ only upon exposure of cells to cAD1 (Pontius and Clewell, 1992b). Figure 3B shows that, while transcription starts 41 nucleotides upstream of the *traE1* translational start site in the case of pheromone-induced cells carrying the 'wild-type' pAM714 (lane 2), it does not occur

in the case of the *traE1* insertion (lane 1). The data support the previous evidence (Tanimoto and Clewell, 1993) that TraE1 is involved in activating its own expression after enough protein is synthesized via transcriptional read-through of TTS1/TTS2.

To determine if the defect in *traE1* had any effect on the expression of *traD*, Northern blot analyses using the MB2 probe were conducted; the results are shown in Fig. 5 (lanes 3 and 4). The mD transcript was similar to that for the control [pAM714 (wild-type properties); lanes 1 and 2], in that it was present in uninduced cells and absent in cells exposed to pheromone. Thus, TraE1 made during induction was not responsible for the reduction in synthesis of the *traD* transcript.

The Tn*917lac* insertion 7304 (on pAM7304; see Figs 1 and 4) is located downstream of *traD* but is presumed to occur within the 3′ region of the mD transcript. This derivative expresses LacZ constitutively from the *iad* promoter at a level equivalent to maximum expression (Pontius and Clewell, 1992b). Interestingly, there is no significant change in the level of mD transcript detected in Northern blots after cells are exposed to pheromone (Fig. 5, lanes 5 and 6). Whereas the size of the transcript appears to be similar to that of mD, we assume that the 3′ end is different and represents a portion of the transposon. It is noteworthy that this insertion would also affect the structure of the m4 transcript. (Of course, the m2 and m3 transcripts would also be affected.) When pAM-HT was present together with pAM7304, there was no effect on LacZ expression (not shown); β-galactosidase continued to express at a high level in the absence of pheromone.

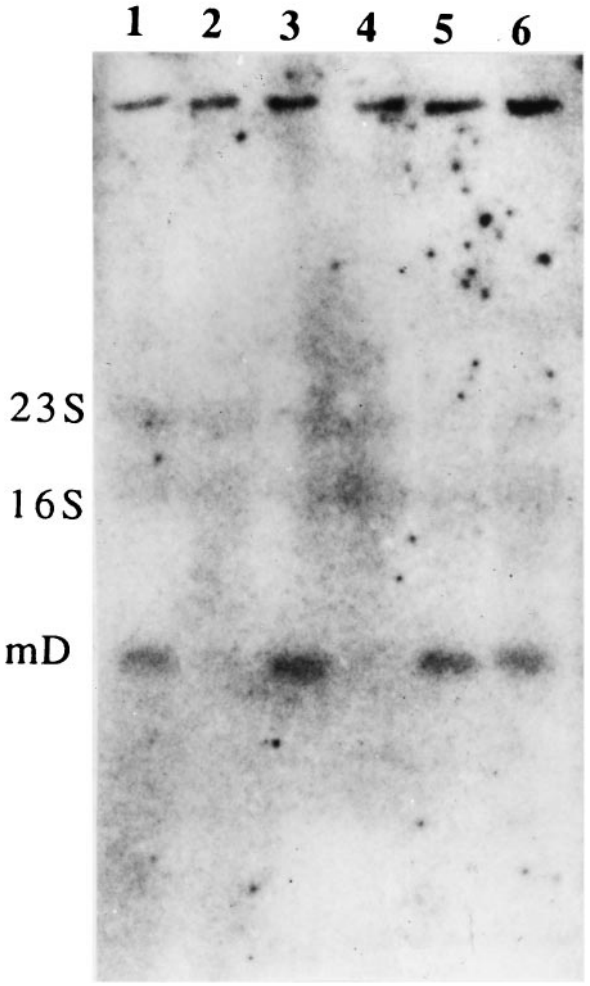

Fig. 5. Northern blot of pAD1 derivatives carrying transposon insertions. Lanes 1 and 2 correspond to OG1X/pAM714 unexposed and exposed, respectively, to cAD1 (100 ng ml^{-1} for 40 min). Similarly treated were OG1X/pAM7318 (lane 3, unexposed; lane 4, exposed) and OG1X/pAM7304 (lane 5, unexposed; lane 6, exposed). The single-stranded DNA probe was MB2.

Discussion

A short (approximately 200 nucleotide) pAD1-encoded transcript (mD) corresponding to *traD* has been identified and found to express at a high level when conjugation-related structural genes are inactive. Expression of the mating system as a result of pheromone exposure or as a result of mutations (thermolabile or transposon insertion) in *traA* resulted in a significant reduction in or disappearance of mD. The fact that a mutation designed to eliminate translation of the putative 23-amino-acid TraD polypeptide did not alter the mating phenotype or mD transcription implied that it was the transcript rather than a related protein product that was of primary functional significance.

The decrease in mD associated with induction of conjugation functions was not the result of a feedback inhibition caused by the pheromone-induced increase in TraE1, as the reduction was also observed in a mutant unable to express TraE1. The lowering of mD probably involves a falloff of initiation from the *traD* promoter caused by disruption by the significantly increased converging transcription from the *iad* promoter. As the temperature-sensitive *traD* derivative (pAM2703) exhibited enhanced expression from the *iad* promoter (typical of induction) at the elevated temperature, it is likely that the altered mD was responsible for this upregulation, which, in turn, resulted in its own reduction. This view is also supported by the observation that a transposon insertion within the region corresponding to the 3′ end of mD (pAM7304) prevented mD from decreasing upon pheromone exposure, a result probably resulting from the absence of transcription from the *iad* promoter beyond the transposon. Interestingly, this insertion resulted in a maximum transcription (derepression) from the *iad* promoter, suggesting that the transposon-related alteration in the structure of mD is responsible. Compared with wild-type behaviour, this mutant is reduced by four orders of magnitude in its ability to transfer DNA in a filter mating

Fig. 6. Potential complementarity of the 5′ end of mD and the 3′ end of an *orf1* transcript. The arrows correspond to inverted repeats.

(Pontius and Clewell, 1992b). The data suggest that the mD transcript itself acts at the *iad* promoter to effect, together with or via interaction with TraA, the reduced (basal) transcription associated with the uninduced state. Such an interaction could involve binding of the RNA to TraA. The fact that a cloned fragment able to provide mD, but not a translated 'TraD', *in trans* could complement a temperature-sensitive point mutation within *traD* (pAM2703) supports this view. However, the inability of the same clone to complement the insertion mutation between *iad* and *traD* (i.e. pAM7304) suggests that the co-existing disruption of m4/m3 may affect transcription from the *iad* promoter as well (see Fig. 1 and below).

It has been noted previously (Pontius and Clewell, 1992b) that there are sequences within the region between the *iad* promoter and TTS1 that are similar in a segment of DNA found about 25 nucleotides upstream of the ribosome binding site of *asa1*. Interestingly, these are within the 5′ end of mD. As shown in Fig. 6, an RNA sequence corresponding the 5′ untranslated region of the *asa1*-encoding transcript, which is initiated from the promoter of the adjacent upstream *orf1* determinant (Galli *et al*., 1992), might easily interact (hybridize) with mD. It is conceivable that an RNA::RNA interaction of this nature could contribute to the regulation of Asa1 expression; and, if so, the elevated level of mD in the uninduced state could help to downregulate *asa1* expression by having an 'attenuational' or other inhibitory effect on the extension of transcription of a 'preinitiated' mRNA (see below). Induction would relate to mD no longer being available to do this.

Overall, the data are consistent with the hypothetical model illustrated in Fig. 7 in which the 3′ ends of mD and m4 are shown overlapping (annealing). The mD structure is estimated to extend about 40 nucleotides beyond the *traD* translational stop site (see Fig. 4), placing its 3′ end about 15 nucleotides downstream of the stop site of

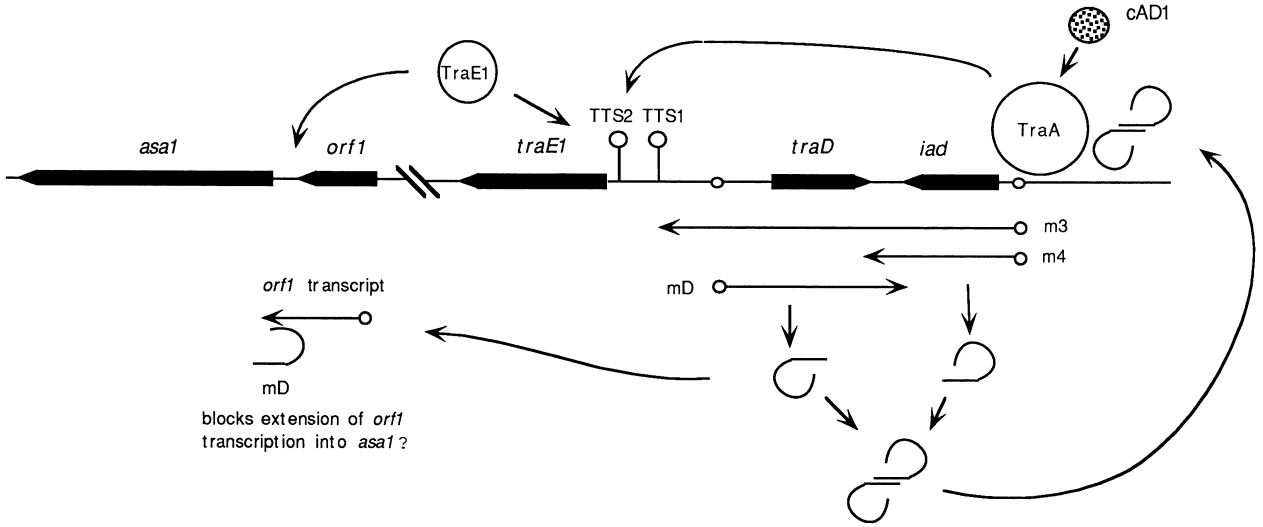

Fig. 7. A. Hypothetical model reflecting the uninduced state. The mD transcript is viewed as interacting with m4 and/or m3, resulting in a complex able to bind with TraA at the *iad* promoter to facilitate the basal (repressed) state. mD is also viewed as possibly being able to interact with a transcript of *orf1* via complementarity (e.g. see Fig. 6), in such a way as to somehow reduce the expression of Asa1. Induction would correspond to the binding of cAD1 to TraA, resulting in a release of TraA from the promoter, giving rise to a higher level (burst) of transcription that would disrupt the initiation of transcription from the *traD* promoter, resulting in a downregulation of mD synthesis.

iad; thus, the m4 transcript, estimated at approximately 200 nucleotides, would overlap mD by about 80 nucleotides. The two transcripts should be able to anneal within the sequence overlap; and the complex may interact with TraA at the *iad* promoter to facilitate the appropriate level of expression consistent with the uninduced state. (The model in Fig. 7 illustrates the point with mD::m4, but it could alternatively involve mD and m3 with a more extensive annealing than would be expected for an mD::m4 complex.) The temperature-sensitive *traD* defect (pAM2703) may involve a change in the secondary structure of mD, disrupting the 'repressed' state; this is easily envisioned as being restored by providing a normal mD *in trans* (i.e. pAM-HT or pAM-HTd1). It can also be seen why pAM-HT would not complement the insertion of pAM7304, because, in this case, m4 (m3) as well as mD is disrupted. The normal mD provided by the co-resident plasmid chimera (pAM-HT) would not have its normal annealing target. (The insertion is estimated to be close to the middle of the mD::m4, 80 nucleotide overlap.)

According to the model, the mD::m4 (or mD::m3) complex is viewed as downregulating activity at the *iad* promoter to facilitate the 'basal' (uninduced) level of transcription; this could involve direct or indirect interaction with TraA or RNA polymerase. While initiation of the mating response is triggered by the interaction of cAD1 with TraA (Fujimoto and Clewell, 1998), the resulting enhancement of transcription leads to the disruption of converging transcription from the *traD* (mD) promoter. This lowers the level of mD, which, in turn, gives rise to even further enhancement of transcription from the *iad* promoter.

The model also depicts mD as being able to interact at its 5′ end with the 3′ untranslated end of an *orf1* transcript via complementarity in such a way as to affect transcription into *asa1*. It is not clear how this would work, but one could envision that mD bound to nascent RNA interacts with RNA polymerase in such a way that transcription downstream of the RNA::RNA annealing region is inhibited. In any case, the effect would be eliminated upon induction, which causes a reduction in mD via the burst of transcription from the *iad* promoter. At the same time, enhanced transcriptional readthrough of TTS1/TTS2 results in expression of TraE1, which is believed to activate transcription of itself from its own promoter within TTS2 (Tanimoto and Clewell, 1993), as well as transcription of aggregation substance determinant *asa1*. [Muscholl *et al*. (1993) have reported that TraE1 positively regulates *asa1* expression from the *orf1* promoter *in trans*.] There is an 11 nucleotide sequence TTAAAAAATAT located 21 nucleotides upstream of the translational start site of *asa1*, which is also located 18 nucleotides upstream of the translational start site of *traE1* (Galli *et al*., 1990; Pontius and Clewell, 1992b); it is a reasonable possibility, therefore, that this sequence is the target of TraE1. Interestingly, the *asa1*-proximal 'target' sequence is 4 nucleotides outside of where the 5′ end of mD and the 3′ end of an *orf1* transcript are shown to combine, suggesting that there could be a linkage between TraE1 binding to DNA and reduced interaction of the two RNAs just upstream. In this case it is presumed that transcription of *orf1* occurs to some extent in the uninduced state but without extension into *asa1*. In a study reported by Galli *et al*. (1992), such a transcript was not noted; however, its small size would have precluded its detection under the conditions used. Preliminary Northern blotting studies (H. Tomita) show that an *orf1* transcript is indeed present in uninduced cells.

It is clear that regulation of the pAD1 pheromone response is a complex process and that mD may play multiple roles. Complementation studies in which mD is provided *in trans* to examine the effect on transcription at different sites on pAD1 are currently underway. The potential secondary structures of the various transcripts deriving from the region between the *iad* promoter and TTS2 are probably of significance, although it is impossible at this time to invoke function to specific structures. It is worth noting, however, that a computer analysis of the mD transcript using the RNA folding program MFOLD version 2.3 by Zucker and Turner (http://www.ibc.wustl.edu/~zuker/rna/form1.cgi) found that the three most stable structures predicted to occur at 42°C exhibited energies between -41.1 and -40.4 kcal mol^{-1}; whereas the transcript of pAM2703 (thermolabile) was unable to form any structures with an energy above -36.0 kcal mol^{-1} (not shown). It would appear that there are specific mD structures that are important for interaction with TraA or RNA polymerase to facilitate negative regulation, and perhaps such complexes cannot form at high temperature in the case of pAM2703. It will be interesting to determine if mD RNA, or an mD::m4 complex, is able to influence the strength of binding of TraA to DNA within the *iad* promoter and/or whether it affects the affinity of TraA for pheromone (Fujimoto and Clewell, 1998). It will also be interesting to see if TraA acquires an affinity for the *traE1*-proximal sequence that resembles the *iad*-proximal target site (see *Introduction* and Fig. 4) when RNA is provided. Such studies are currently in progress.

Finally, it is interesting that the sequence from near the beginning of the *traD* translational start site to TTS2 is almost identical to that in pCF10 (Kao *et al*., 1991), a plasmid in a different incompatibility group, which makes use of a different sex pheromone, cCF10. Within the corresponding pCF10 region, significant potential of 'folded' RNA to interact with ribosomal components has been demonstrated (Bensing *et al*., 1997; Bensing and Dunny, 1997). [See Bensing and Dunny (1997) for an example of the type of extensive secondary structure that a transcript encoded by this region might exhibit.] Although an equivalent to mD or TraA has not yet been noted in the pCF10 system, it is very possible that the region common

to the two systems is involved in a similar function for both. Another interesting aspect of the pCF10 system is evidence recently reported suggesting that expression of the aggregation substance determinant (*prgB*) is controlled post-transcriptionally (Bensing *et al*., 1997). The mechanism involved, however, appears to be somewhat different from that of pAD1. [See Clewell (1998) for a recent discussion of the differences and similarities between pAD1 and pCF10.]

Experimental procedures

Bacterial strains, plasmids, reagents and general methodology

The strains and plasmids used in this study are listed in Table 1. Plasmid pAM714 (Ike and Clewell, 1984) is a derivative of pAD1 that carries an insertion of the erythromycin (Em) resistance transposon Tn*917* (Tomich *et al*., 1980) in a region that is not involved in transfer. This plasmid was routinely used to represent 'wild-type' conjugation properties. pAM2120 represents a previously derived *traA* mutant containing the transposon Tn*917lac* inserted into the 5′ region of *traA* (Pontius and Clewell, 1992a); it is fully derepressed for clumping and plasmid transfer. Matings and pheromone sensitivity assays were conducted as reported previously (Dunny *et al*., 1979; Clewell *et al*., 1985).

The medium used for *E. faecalis* was Todd–Hewitt broth (THB) (Difco Laboratories). Luria–Bertani broth (Maniatis *et al*., 1982) was used for culturing *Escherichia coli*. Solid media were prepared by including 1.5% agar. Cultures were incubated at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; erythromycin (Em), 20 μg ml^{-1}; streptomycin (Sm), 1 mg ml^{-1}; kanamycin (Km), 500 μg ml^{-1}; spectinomycin (Sp), 500 μg ml^{-1}; chloramphenicol (Cm), 50 μg ml^{-1} in the case of *E. coli* and 20 μg ml^{-1} in the case of *E. faecalis*; rifampicin (Rif), 25 μg ml^{-1}; and fusidic acid (Fus), 25 μg ml^{-1}. Xgal (Gibco BRL) was used at a concentration of 100 μg ml^{-1} (Miller, 1972). Synthetic cAD1 was prepared at the University of Michigan Core Peptide Synthesis Facility.

DNA techniques

Routine screening of plasmid DNA was carried out using a small-scale alkaline lysis procedure described previously (Weaver and Clewell, 1988). Purified plasmid DNA from *E. coli* was prepared using the Plasmid Midi Kit (Qiagen) as recommended by the manufacturer. Plasmid DNA was analysed by digestion with restriction enzymes, and separation of restriction fragments was performed in 0.8% agarose gels. All restriction enzymes were purchased from Gibco BRL, and reactions were carried out under the conditions recommended. Standard recombinant DNA techniques were used in the construction of plasmids (Ausubel *et al*., 1987). Polymerase chain reaction (PCR) was performed with a Perkin-Elmer Cetus apparatus, using conditions recommended by the manufacturer. Oligonucleotide primers and probes were synthesized by the Biomedical Research DNA Core Facility of the University of Michigan. DNA sequencing reactions were carried out as described by Chen and Seeburg (1985).

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmid	Relevant features	Source or reference
Strains		
E. faecalis		
OG1X	*str gel*	Ike *et al*. (1983)
FA2-2	*rif fus*	Clewell *et al*. (1982)
JH2SS	*str spc*	Tomich *et al*. (1980)
DS16	Clinical isolate; carries pAD1 and pAD2	Tomich *et al*. (1979)
E. coli DH5α	F$^-$ ϕ80 *lacZ* Δ*M15 endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi1* Δ*gyrA96* Δ(*lacZYA-argF*) *U169*	Promega Research Laboratories
Plasmids		
pBluescript KS+	*E. coli* plasmid vector; *amp*	Stratagene
pAM1.3SKΔXba1	A pBluescript clone containing region designated in Fig. 1	Tanimoto and Clewell (1993)
pAM1.6SK	1.6 kb fragment (*iad* through *traE1*) cloned in pBluescript	Clewell *et al*. (1990)
pSF141	Cloning vector (Km and Cm resistance); suicidal in *E. faecalis*	Tao and Ferretti (1991)
pAM401	*E. coli–E. faecalis* shuttle; *cat tet*	Wirth *et al*. (1986)
pAM714	pAD1::Tn*917*; Hly/Bac, *erm*; wild-type mating properties	Ike and Clewell (1984) insertion mapped in An and Clewell (1997)
pAM714-d1HT	pAM714 derivative with point mutation (Met to Arg) in *traD*	This study
pAM2120	pAD1 with Tn*917* insert in *traA*	Pontius and Clewell (1992a)
pAM2700	pAM714 with ts mutation (location outside of *traD* and *traA*)	Bastos *et al*. (1997)
pAM2703	pAM714 with ts mutation in *traD*	Bastos *et al*. (1997)
pAM2725	pAM714 with ts mutation in *traA*	Bastos *et al*. (1997)
pAM7318	pAD1 with Tn*917lac* insertion in *traE1*	Pontius and Clewell (1992b)
pAM7304	pAD1 with Tn*917lac* insertion between *traD* and *iad*	Pontius and Clewell (1992b)
pAM-HT	pAM401 with cloned 321 nucleotide PCR fragment carrying *traD*	This study
pAM-HTd1	Same as pAM-HT except has the d1 (*traD* Met to Arg) mutation	This study
pAM-KT	pAM401 with cloned 205 nucleotide PCR fragment carrying *traD*	This study

Analysis of transcripts

RNA was prepared in a manner similar to that described by Ladin *et al*. (1992), with minor modifications. Overnight cultures of *E. faecalis* OG1X or FA2-2 carrying the plasmids of interest were grown at 32°C in THB containing Em and 0.5–1.0% glycine. The cultures were diluted to 20% in 25 ml of the same medium, and the cells were grown at 32°C for 3 h. At this point, the cultures were either transferred to 42°C or kept at 32°C. After a specific time, the cells were harvested by centrifugation and resuspended in 300 μl of lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM glucose). Vanadyl ribonucleoside complex (10 mM; Gibco BRL) and lysozyme (2 mg; Sigma) were added, and the mixtures were maintained at 37°C for 30 min. Aliquots of 400 μl of RNAzol B (Tel-Test) and 40 μl of chloroform were added. The mixtures were vortexed for 20 s and kept on ice for 5 min. The phases were then separated by centrifugation, and the aqueous phase was extracted twice with phenol–chloroform and once with chloroform–isoamylalcohol. The RNA was recovered by isopropanol precipitation, and the pellet was resuspended in 200 μl of DEPC-treated water. RNA species (20 μg), after denaturation with glyoxal and dimethyl sulphoxide, were then separated by electrophoresis in a 1.2% agarose gel prepared in 10 mM phosphate buffer, pH 7.0, as described by Ausubel *et al*. (1987). Northern (RNA) hybridizations (Ausubel *et al*., 1987) were carried out at either 50°C or 55°C. The probes used consisted of MB2, MB3 and MB4 (Fig. 1) and corresponded to the structures 5′-GCGATATAAAGGCAACTTACAAGACAATTAAC-3′ and 5′-GTTTAAATAAGAGGAGAGCTATTAGAATGAGC-3′ and 5′-TGATAACTTTATTTGTTGTCAC-3′ respectively. MB2, MB3 and MB4 were end-labelled using 20 U of T4 polynucleotide kinase (Gibco BRL) in the presence of 80 μCi of [γ-^{32}P]-ATP (10 mCi ml^{-1}; Amersham). Nick translation was used to label the probe corresponding to pAM1.3SKΔXba1. RNA marker molecules were from Gibco BRL

Determination of the initiation site of transcription

The synthetic primer used for determining the start site of *traD* was MB2 (see above), which is located in the *traD* determinant (see Figs 1 and 4). This primer was end-labelled as described above. An aliquot of 40 μg of RNA was hybridized with 1.0 pmol of kinase-treated primer for 5 min at 50°C in 10 μl of hybridization buffer (100 mM KCl, 50 mM Tris-HCl, pH 8.3). Primer extension was conducted at 44°C for 45 min with 200 U of SuperScript II RNaseH$^-$ reverse transcriptase under the conditions recommended in the SuperScript preamplification system kit (Gibco BRL). Samples were run in a sequencing gel, and the gel was dried and autoradiographed. DNA sequencing reactions were carried out using template DNA corresponding to that of the transcript. The experiment involving the *traE1* start site was performed similarly and made use of the primer 5′-ACGCTTCTCTCGTTGACCAAAAACAATAAT-3′.

Construction of the traD *mutation of pAM714-d1HT*

This derivative was constructed as follows using the Promega Altered Sites II *in vitro* mutagenesis systems kit. pAM1.6SK is a pBluescript clone carrying a 1.6 kb pAD1 fragment extending from upstream of *iad* to downstream of *traE1* (Clewell *et al*., 1990). The 1.6 kb pAD1 DNA fragment was removed using *Bam*H1 and *Kpn*I and cloned into the *Bam*HI/*Kpn*I sites of the pALTER-1 *E. coli* vector. Site-directed mutagenesis of the construct (pALTER-1::1.6SK) was done using the kit protocol with some modification. A synthetic 21-mer primer (5′-ACCGACTGCC**C**TAGGACGGGA-3′) for making the desired *traD* mutation (ATG to AGG; Met to Arg) was used. Mutated DNA was expected to contain a newly created *Avr*II cleavage site (C**C**TAGG) that could be screened easily. A 1.6 kb *Eco*RI fragment from a mutated plasmid was subcloned into the *Eco*RI site of pSF141 (encodes Km and Cm resistance determinants that express in Gram-positive bacteria) suicide vector (Tao and Ferretti, 1991). The recombinant plasmid pSF141::1.6SKd1 was introduced into *E. faecalis* OG1X/pAM714 by electroporation (Flannagan and Clewell, 1991). Transformants carrying a co-integrate plasmid (recombinant between pAM714 and pSF141::1.6SKd1 at homologous segment) were selected on plates containing erythromycin and kanamycin. The recombinant strain was subcultured for 14 passages in THB without drug and then plated on media containing erythromycin. Colonies were replica-plated on media containing chloramphenicol, and candidates (Em^r/Cm^s) were screened using PCR amplification to obtain DNA to be examined for the presence of the newly created *Avr*II cleavage site. The two primers used for PCR and cloning for the purpose of sequence analysis were 5′-TTACGGAATTCAGATCATGCGTGTTGTGTACC-3′ (within *traA*) and 5′-ATTGCGAATTCACAGCAAACATCCCCTCAATT-3′ (within *traE1*). One candidate containing the expected mutation was designated pAM714-d1HT.

Construction of plasmid chimeras for use in complementation studies

pAM-HT was constructed by PCR amplification of a 321 bp segment of pAD1 that included *traD* (see Fig. 4) using, respectively, an upstream primer, 5′-ATTTTGGATCCTAGACAAAATAAAGGAGTTTA-3′, and downstream primer 5′-ATTTTGGATCCATTGATAACTTTATTTGTTG-3′. The incorporation of *Bam*HI sites into the 5′ termini of the primers allowed for incorporation of the PCR product into the *Bam*HI site of pBluescript-II KS+. After confirming that the fragment maintained the wild-type sequence, the DNA was subcloned into the *Bam*HI site of pAM401 shuttle plasmid. pAM-HTd1 was generated in the same manner, except that the template used for the PCR amplification was pAM714-d1HT. pAM-KT (see Fig. 4) involved generation of a 207 bp segment and used (for PCR) the upstream primer 5′-GGATCCGTTATACTATACGTTGTAAC-3′ and the downstream primer 5′-GGATCCCTTGATATAGCAATTATCTG-3′. The pAM401 shuttle chimeras were introduced into *E. faecalis* OG1X or FA2-2 cells carrying pAM2703 by electroporation, selecting for resistance to chloramphenicol and erythromycin.

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