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Characterization of the Determinant (*traB*) Encoding Sex Pheromone Shutdown by the Hemolysin/Bacteriocin Plasmid pAD1 in *Enterococcus faecalis*

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pAD1 is a hemolysin/bacteriocin plasmid originally identified in *Enterococcus faecalis* DS16. It encodes a mating response to a peptide sex pheromone, cAD1, secreted by recipient bacteria. Once pAD1 is acquired, production of the pheromone ceases—a trait related in part to a determinant designated *traB*. Here we report the nucleotide sequence of *traB* and the position of several transposon insertions resulting in the characteristic self-induction phenotype. The deduced product has a mass of 43.7 kDa with the C-terminal third consisting primarily of hydrophobic amino acid residues.

Certain conjugative plasmids in *Enterococcus faecalis* encode a mating response to specific peptide sex pheromones secreted by potential recipient cells. (For recent reviews see Clewell, 1990, 1993a,b; Dunny, 1990). One such element is pAD1, a hemolysin/bacteriocin plasmid (60 kb) originally identified in the clinical isolate DS16 (Tomich *et al.*, 1979) and representative of a large family of related hemolysin plasmids (Colomar and Horaud, 1987; Ike and Clewell, 1992). The recipient-produced pheromone, designated cAD1, has been isolated and characterized (Mori *et al.*, 1984). When pAD1 is acquired, production of the endogenous host-determined peptide is shut down. Pheromones specific for different plasmid systems, however, continue to be secreted.

The pAD1 mating response is characterized by the synthesis of a surface "aggregation substance" designated Asa1 (Ehrenfeld *et al.*, 1986; Galli *et al.*, 1989, 1990). This protein binds to the surface of recipients as well as other donors. Thus, donors exposed to culture filtrates of recipients undergo a "clumping" response, which is easily visualized and is the basis of a microtiter dilution assay for quantitating pheromone (Dunny *et al.*, 1979). Plasmid-containing cells that have been plated on media containing cAD1 give rise to colonies with a characteristic "dry" morphology (Weaver and Clewell, 1988). This is related to an induced aggregation occurring within the colony.

Early studies on the regulation of the pheromone response found that transposon (Tn*917*) insertions in two loci, designated *traA* and *traB*, resulted in a constitutive clumping response (Ike and Clewell, 1984). These derivatives also transfer plasmid DNA in short (e.g., 10 min) matings at frequencies much higher (e.g., over a 1000-fold) than the wildtype, which normally takes well over 20 min to become fully induced. *traA* mutants exhibited the dry colony morphology on solid media in the absence of pheromone. The product of this determinant has been shown to represent a key negative regulator of *traE1*, whose product in turn positively regulates all or most of the structural genes related to conjugation (Clewell and Weaver, 1989; Ehrenfeld and Clewell, 1987; Galli *et al.*, 1992; Pontius and Clewell, 1992a, 1992b; Tanimoto and Clewell, 1993; Weaver and Clewell, 1988, 1989, 1990).

In contrast to the case for *traA* mutations, colonies of *traB* derivatives had a "ringed" morphology on plates without pheromone;

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these corresponded to a dry area in the middle of the colony with the outside portion (i.e., the ring) having a wildtype appearance (Weaver and Clewell, 1991). The *traB* determinant is believed to encode a product that is involved in shutdown of endogenous cAD1 production, although additional factors are also involved (Weaver and Clewell, 1990, 1991). Culture filtrates of *traB* mutants have barely detectable amounts of cAD1, but enough to result in self induction. The ring appearance of colonies is probably due to the fact that pheromone production is affected by oxygen (Weaver and Clewell, 1991), with higher levels of cAD1 being produced (as much as 16-fold more) by cells in a more anaerobic environment. Since the center of the colony is more anaerobic, more pheromone is produced resulting in self aggregation.

The regulatory region for the pAD1 pheromone response is organized as *-traE1-iad-traA-traC-traB* [see Fig. 1 (*repA, repB,* and *repC* in Fig. 1 represent determinants relating to replication and maintenance)]. *iad* (Clewell *et al.,* 1990) is the determinant for a peptide iAD1 which is a competitive inhibitor of cAD1, whereas *traC* encodes a surface (extracellular) protein involved in binding pheromone and iAD1 (Weaver and Clewell, 1988; Tanimoto *et al.,* 1993). All but *traA* are transcribed right to left. The nucleotide sequence of all but *traB* has been reported elsewhere (Clewell *et al.,* 1990; Pontius and Clewell, 1992a,b; Tanimoto *et al.,* 1993; Weaver *et al.,* 1993). Here we report the sequence of *traB* and identify the precise location of specific mutational lesions. Where not specifically noted or cited, details of cloning, nucleotide sequence determination, introduction of plasmids into bacteria by conjugation or electroporation, and other aspects of the methodology were essentially as previously described (Ausubel, *et al.,* 1987; Cruz-Rodz and Gilmore, 1990; Pontius and Clewell, 1992a; Su *et al.,* 1991; Wirth *et al.,* 1986).

The sequence was determined (both strands) using derivatives (nested deletions) of the *Escherichia coli* DH5α clones pAM7500, pAM2603, and pAM3101 indicated in Fig. 1. The vector pBluescript SK+ II was utilized in the case of pAM7500 and pAM3101, whereas pBluescript KS+ II was involved in the case of pAM2603 (Pontius and Clewell, 1992a; Weaver *et al.,* 1993). In some cases synthetic oligonucleotides served as primers in sequencing reactions. The location of Tn*917lac* insertions was determined using primers that direct synthesis out the ends of the transposon (Pontius and Clewell, 1992a).

Computer analysis revealed a large open reading frame (ORF)[1] (Fig. 2) starting with ATG and corresponding to a protein with 388 amino acid residues with a molecular weight of 43,687 and a p*I* value of 9. A good potential ribosome binding site (GGAGG) is located eight nucleotides upstream. Although an in-frame ATG is located 5 "codons" upstream, the absence of a related ribosome binding site implies that it is not a functional translational start site. The only other reading frames detected corresponding to greater than 25 amino acid residues were on the opposite strand and had no related ribosome binding sites. [They were located within the above-noted ORF and corresponded to 29 and 133 residues (not shown).] The positions of five previously reported (Weaver and Clewell, 1988) Tn*917lac* insertions resulting in the *traB* phenotype were found here to be spread through a significant portion of the large ORF (Figs. 1 and 2). It was therefore concluded that the ORF corresponded to *traB*. The 5′ end was 46 bp downstream from the 3′ terminus of *repA* (Weaver *et al.,* 1993); and the 3′ end was 29 bp from the 5′ end of *traC* (Tanimoto *et al.,* 1993). A potential sigma-70-type promoter sequence is located upstream from the start codon, and a 16-base region with dyad symetry is located between the promoter and ribosome binding site. It is conceivable that the latter is a target for a regulatory protein, although there is currently no evidence that TraB is regulated. The absence of any obvious transcription-termination site near the 3′ region of *traB* suggests that transcription might extend into

[1] Abbreviation used: ORF, open reading frame.

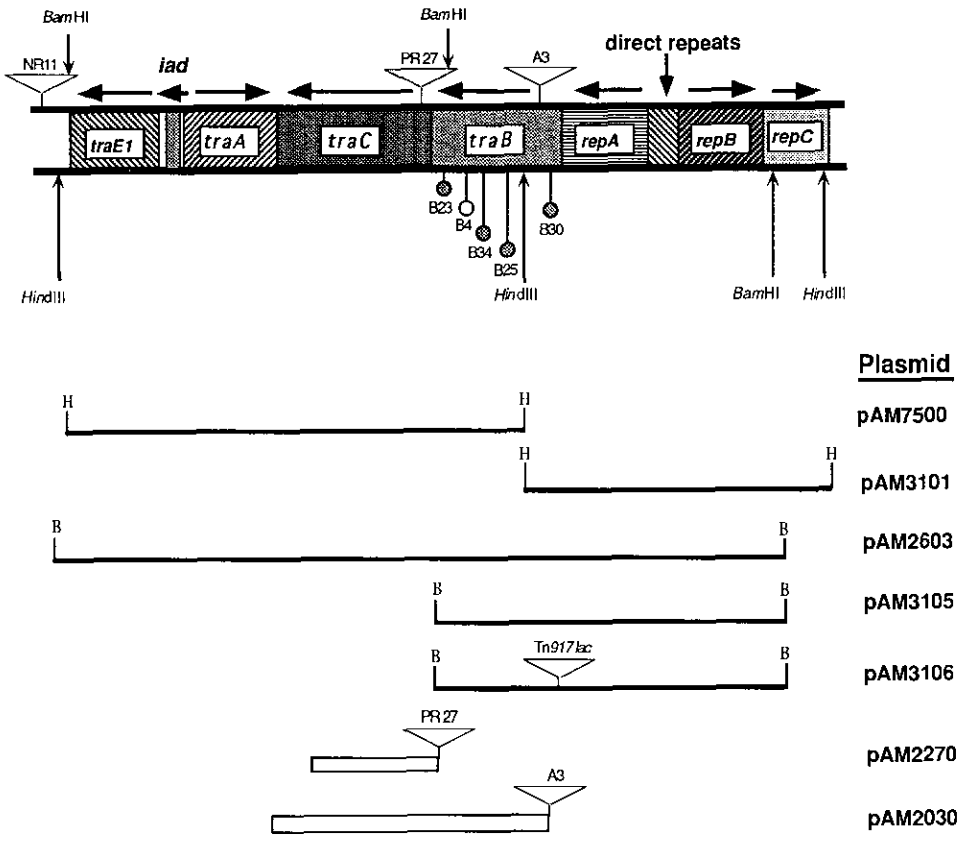

FIG. 1. Map of pAD1 region carrying *traB* and flanking genes. The arrows reflect 5′ to 3′ orientation above the labeled genes. The filled circles (B23, B34, B25 and B30) represent insertions of Tn*917lac* in *traB* which are oriented such that LacZ is expressed under the transcription of *traB*. The open circle, representing B4, corresponds to an insertion in the opposite orientation. The triangles corresponding to NR11 (see Weaver and Clewell, 1988), PR27, and A3 are also Tn*917lac* insertions with orientations similar to those of the filled circles. [pAM2270 (PR27) and pAM2030 (A3) represent deletions involving the indicated regions.] The location of certain restriction sites used in the cloning efforts is also shown. (H and B refer to *Hin*dIII and *Bam*HI sites, respectively.) Cloned segments (noted for pAM7500, pAM3101, and pAM2603) in the plasmid vector pBluescript (*E. coli* DH5α) were used for DNA sequencing analyses. The plasmids, pAM3105 and pAM3106, were derivatives of the *E. coli–E. faecalis* shuttle plasmid pAM401 (Wirth *et al.*, 1986) carrying the noted segment. (The left-end *Bam*HI sites correspond to sites in the transposon inserted in PR27.) The transposon insertion in pAM3106 is the same as that of B30 and was generated via allelic exchange by first introducing pAM2300 into OG1X(pAM3105) by conjugation, selecting on both chloramphenicol (for the vector marker) and erythromycin (for the transposon marker on pAM2300), and then using a plasmid preparation from a resulting transconjugant to transform a plasmid free OG1X strain selecting for erythromycin and chloramphenicol resistance and screening for absence of hemolysin expression. The plasmids pAM2270 and pAM2030 are derivatives of pAD1 carrying the designated Tn*917lac* insertions (PR27 or A3) with the adjacent deletions noted.

traC. A high degree of hydrophobicity in the C-terminal third of the deduced TraB suggests an association of this region with the membrane.

The orientation of the Tn*917lac* insertions corresponding to B30 (pAM2300), B25 (pAM2250), B34 (pAM2340), and B23 (pAM2230) (see Figs. 1 and 2) are consistent with their giving rise to light blue colonies on plates containing X-gal, reflecting the low constitutive expression of TraB from a promoter upstream of the 5′ end; cells carrying the oppositely oriented B4 (pAM2040) gave rise to white colonies (Weaver and Clewell,

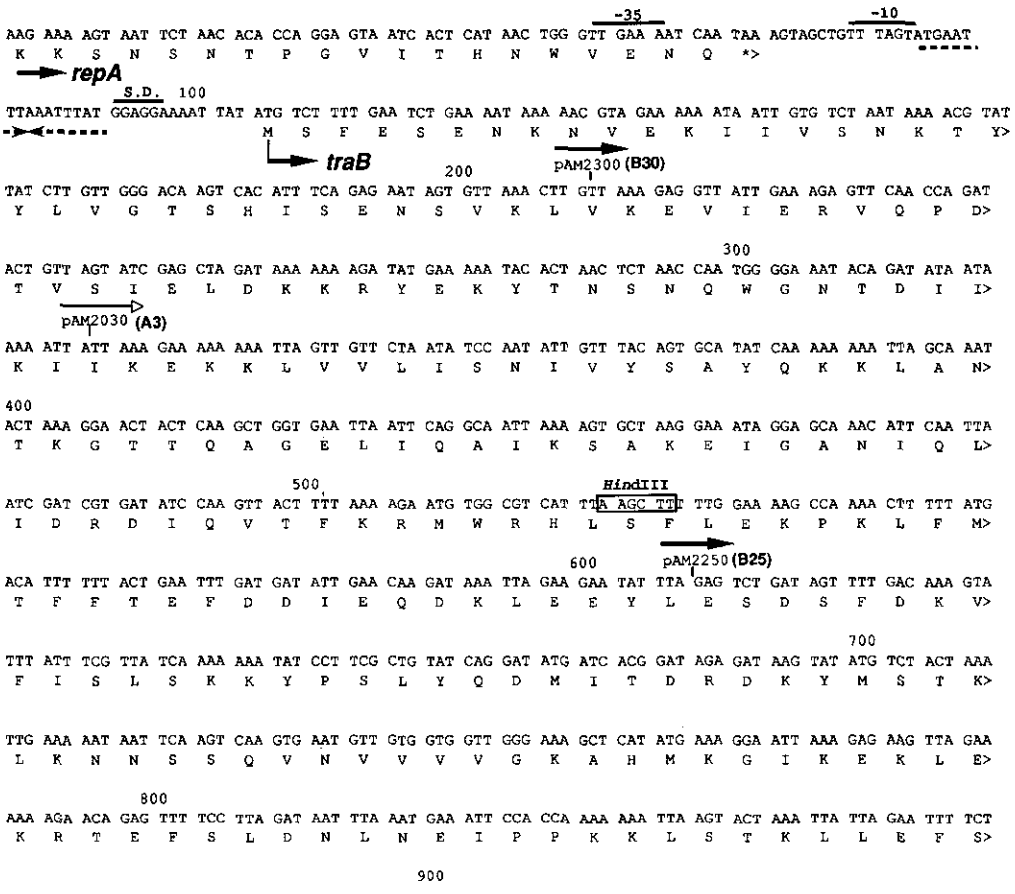

FIG. 2. Nucleotide sequence of region of pAD1 containing *traB* and portions of flanking determinants. To view the determinant in the more convenient 5′ to 3′ orientation, the sequence is reversed compared to that of Fig. 1. The position of the various Tn*917lac* insertions, with their orientations indicated relative to the ability of LacZ to be expressed via transcription of *traB*, and the names of the corresponding pAD1 derivatives are indicated. The positions of potential ribosome binding sites [S.D. (Shine and Dalgarno, 1975)] and sigma-70-like promoters (−10 and −35 hexamers) are overlined. The dashed arrows reflect a region of dyad symetry just upstream of *traB*. The GenBank accession number for the sequence is U00681.

1988, 1990). The position of two insertions, A3 and PR27, which were associated with deletions (Clewell and Weaver, 1988; Weaver and Clewell, 1988, 1990), was also determined by sequence analysis (Figs. 1 and 2). The A3 derivative corresponding to pAM2030 was deleted for most of *traB*, all of *traC*, and through the codon for the eighth amino acid residue in from the C-terminus of *traA*. The deletion corresponding to PR27 (pAM2270) is actually located downstream of *traB* and completely within *traC*. It begins at TraC amino acid residue 60 and ends with residue 384. LacZ expression in both the A3 and PR27 derivatives was constitutive at a low level (Weaver and Clewell, 1988). A3 was shown previously to exhibit the *traA* phenotype, whereas PR27 was a "partial responder" to pheromone (Weaver and Clewell, 1988).

The plasmids pAM3105 and pAM3106 (Fig. 1) represent clones of *traB*-containing DNA segments in the *E. coli–E. faecalis* shuttle vector pAM401 (Wirth *et al.*, 1986). Culture filtrates of late exponential phase *E. faecalis* OG1X cells carrying pAM3105 showed a pheromone (cAD1) titer of 8, compared to 256 for a filtrate of cells harboring

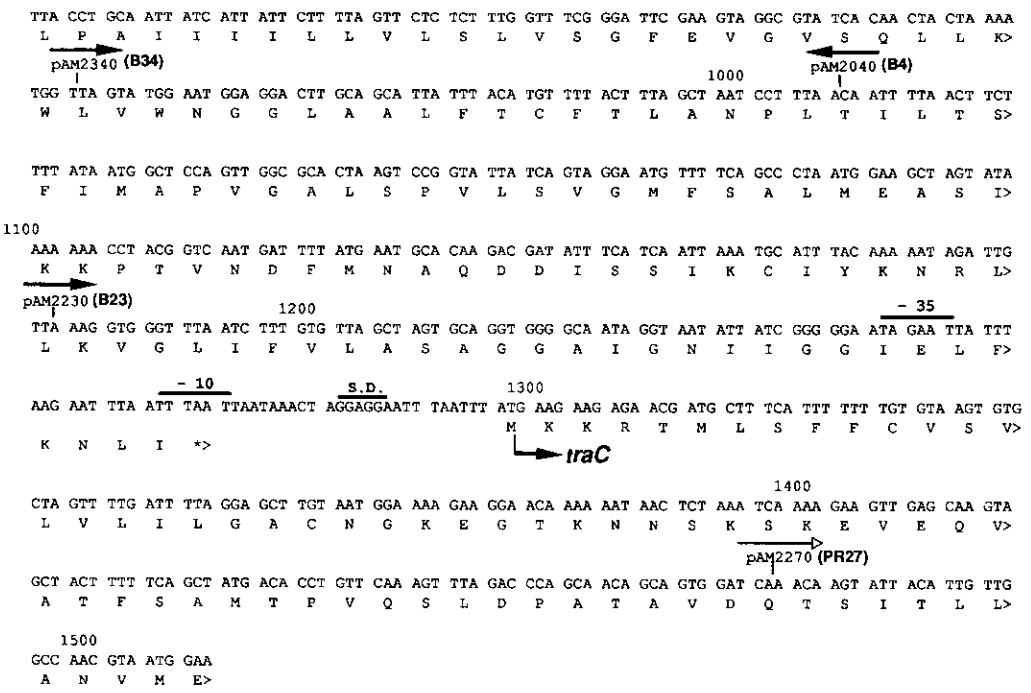

FIG. 2—*Continued*

just the vector. As expected, in the case of pAM3106, which has a transposon insertion in *traB,* the titer was the same as for the vector alone. Consistent with these results is the observation that pAM3105 appears to complement, *in trans,* the *traB* phenotype associated with pAM2300 (Tn*917lac traB* insertion B30 in pAD1; see Fig. 2). OG1X(pAM2300) exhibited the "ringed colony" phenotype on solid media, whereas OG1X(pAM2300, pAM3105) colonies appeared similar to wildtype pAD1 (data not shown). Homologous recombination was not ruled out unambiguously here since the host was not recombination-deficient; however, plasmid isolation and analyses using *Eco*RI and *Bam*HI revealed the presence of the two plasmids at their expected sizes. [The only *E. faecalis* Rec-deficient host available is UV202 (Yagi and Clewell, 1980), a nonisogenic host unable to support a pAD1 pheromone response (Ike and Clewell, 1984) and therefore not useful for the above test.]

The transposon-generated deletions associated with PR27 (carries pAM2270) and A3 (carries pAM2030) (Figs. 1 and 2) were previously reported (Weaver and Clewell, 1988) to give rise to titers of <2 and 64; these values were confirmed here when determined in experiments done in parallel with those involving pAM3105 and pAM3106 (see above). In the case of pAM2030, the observed apparent fourfold reduction (i.e., from 256 down to 64), even though a significant portion of *traB* was missing, is believed due to the associated dysfunction of *traC.* The *traC* phenotype is generally characterized by a fourfold increase in iAD1 in culture filtrates due to a reduction in peptide binding (Weaver and Clewell, 1988); this results in a masking of the cAD1 titer.

Figure 3 shows a dot matrix plot demonstrating significant similarity between *traB* and a determinant on the pheromone-responding plasmid pCF10 designated *prgY* (Ruhfel *et al.,* 1993). The size of the gene products are very similar and, like the case for TraB, the putative *prgY* product exhibits strong hydrophobicity in the C-terminal third of its structure. Significant homology

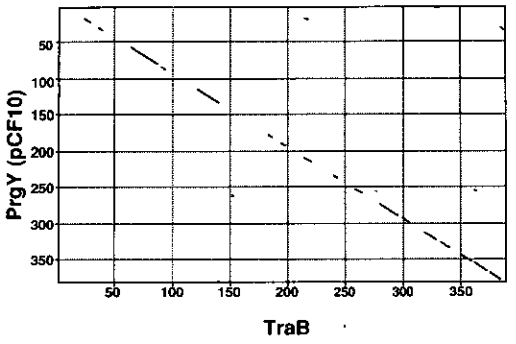

FIG. 3. Homology matrix plot comparing TraB with PrgY of pCF10. A MacVector (IBI) program was used to generated the comparisons. Each point reflects at least 60% homology within an eight amino acid alignment.

with other sequences in the GenBank data base was not detected.

It is not known how many sex pheromones are secreted by plasmid-free strains of *E. faecalis*. There are at least five and probably many more (see Clewell, 1993b). It has been speculated (Clewell, 1981, 1985) that these peptides may originally have had, and perhaps continue to have, different functions and that plasmids evolved to take advantage of them as mating signals. Since a given plasmid generally shuts down production of only the corresponding pheromone, its related determinant(s) must be specific. The homology shared by *traB* and a pCF10 reading frame *prgY* suggests that the latter might be involved with shutdown of endogenous cCF10 (Ruhfel *et al.*, 1993); however, there is no functional evidence yet for such a role. The two determinants appear to have evolved from a common ancestor. It is noteworthy that pAD1 and pCF10 also exhibit significant homology with respect to genes determining aggregation substance (Galli *et al.*, 1990; Kao *et al.*, 1991), surface exclusion (Kao *et al.*, 1991; Weidlich *et al.*, 1992), and pheromone binding (Ruhfel *et al.*, 1993; Tanimoto *et al.*, 1993).

The nature of the shutdown process is not known. Indeed, genetic determinants for enterococcal sex pheromones have not yet been identified, and thus nucleotide sequence information that might provide clues regarding their regulation is not available.

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