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NOTES

Characterization of the *traC* Determinant of the *Enterococcus faecalis* Hemolysin-Bacteriocin Plasmid pAD1: Binding of Sex Pheromone

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pAD1, a conjugative, 60-kb, hemolysin-bacteriocin plasmid in *Enterococcus faecalis*, encodes a mating response to a small peptide sex pheromone, cAD1, secreted by potential recipient bacteria. A gene, *traC*, encoding a 60.7-kDa protein with a typical amino terminal signal peptide, was identified within a region that appears to encode a product that binds to exogenous pheromone. A cloned segment of DNA containing *traC* resulted in specific binding of cells to synthetic cAD1. The putative *traC* product has strong similarity to a product of the *E. faecalis* plasmid pCF10 as well as oligopeptide binding proteins of *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis*.

pAD1 (60 kb) is a conjugative, hemolysin-bacteriocin plasmid in *Enterococcus faecalis*; it encodes a mating response to the small peptide sex pheromone cAD1 (18) secreted by recipient (plasmid-free) bacteria (for reviews, see references 3, 4, 6, and 8). The response involves synthesis of Asa1, or aggregation substance (11, 13, 14), which facilitates the initial contact with recipient cells in broth. When pAD1 is acquired by the recipient, it shuts down production of cAD1; however, pheromones specific for different plasmid systems continue to be secreted. Genes involved in the regulation of the pheromone response have been identified and include determinants for positive (TraE1) and negative (TraA) regulatory proteins (22, 29, 31). TraA negatively regulates expression of *traE1* and is also sensitive, at least indirectly, to the mating signal. TraE1 is believed to positively regulate in *trans* all or most of the structural genes involved in the conjugative process (10, 29, 31). The nature of the regulation of *traE1* via control of read-through of transcriptional terminators located upstream has recently been reported (12, 22, 27). The upstream transcript includes a determinant, *iad*, for a specific peptide, iAD1, which acts extracellularly as a competitive inhibitor of cAD1.

The mechanism by which the cAD1 signal is delivered is not known. It is presumed that there is some recognition of the pheromone on the cell surface, and there is genetic evidence for a pAD1 determinant encoding the ability to bind to cAD1 (31). Transposon inserts in what was designated the C region, henceforth designated *traC*, have been shown to cause the loss of sensitivity to pheromone in *E. faecalis* OG1X (31). These mutants all exhibit a four- to eightfold increase in iAD1 detectable in culture supernatants, a phenomenon attributed to less binding of the inhibitor to the cell surface. The arrangement of the *traC* determinant relative to its neighboring genes is shown in Fig. 1. All but *traA* are transcribed right to left. (*traB* is involved in pheromone shutdown [32].) Transcriptional fusions in *traC* indicate that expression occurs constitutively at a relatively low level (29). The nucleotide sequence for *traE1-iad-traA* has been reported (5, 21, 22), as has the *traB* sequence (1).

In this communication, we report the nucleotide sequence of *traC* and the location of some previously derived (29) transposon inserts. We also present the results of complementation and provide additional supporting evidence that TraC is involved in binding cAD1.

Where not specifically noted or cited, details of cloning,

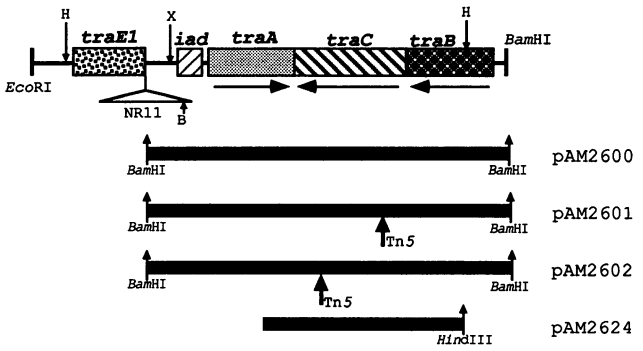

FIG. 1. Map of the pAD1 pheromone response regulatory region. The *Eco*RI-*Bam*HI segment corresponds to about 7 kb. X and H correspond to *Xba*I and *Hin*dIII sites, respectively. NR11 represents a Tn*917lac* insert with the location of a *Bam*HI (B) site shown within the transposon. The arrows represent the direction of transcription for the indicated genes. Segments representing various clones with their corresponding plasmid designations are shown. The plasmids represent clones in the *E. coli*-enterococcus shuttle vector pAM401 (33). The bordering arrows note the restriction enzyme sites used for cloning the DNA segment. The absence of a bordering arrow means that the end was generated by the nested deletion process.

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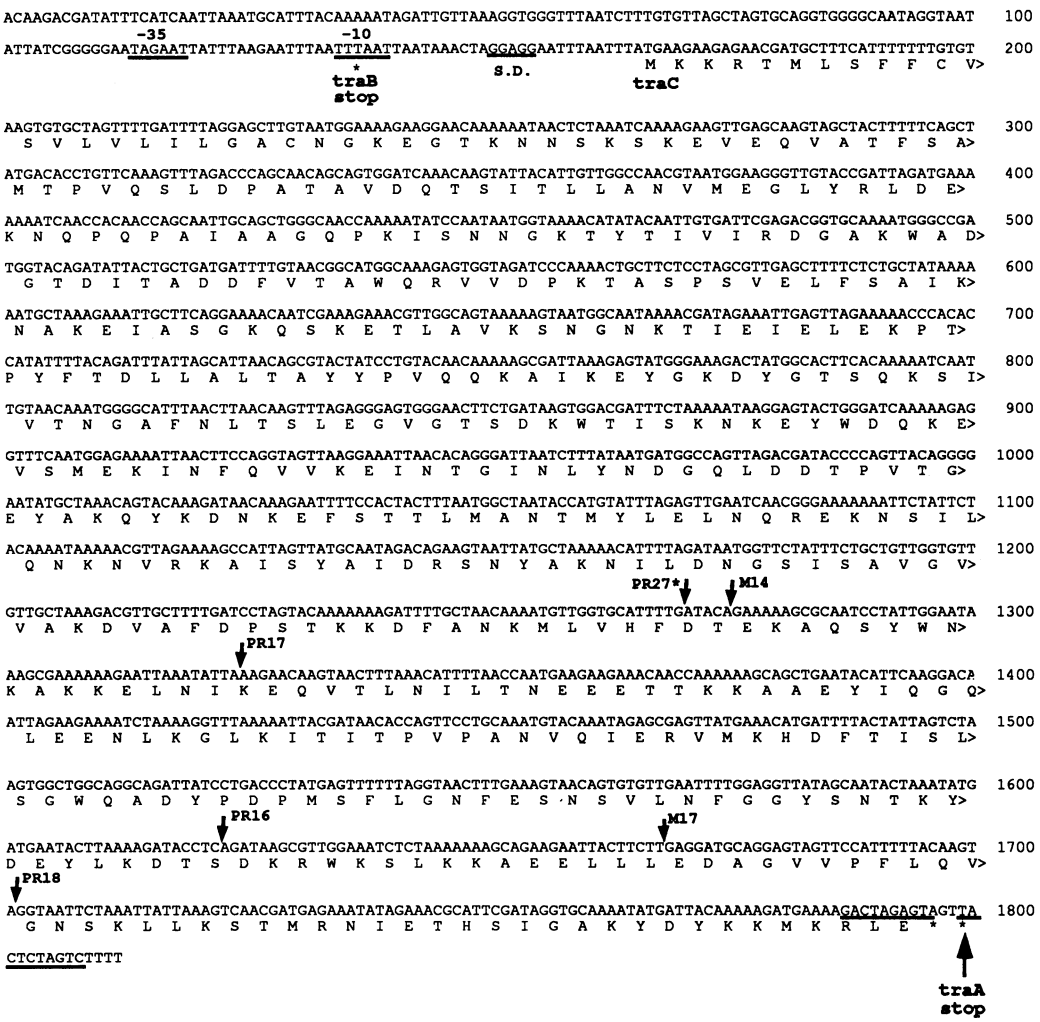

FIG. 2. Nucleotide sequence of segment of pAD1 containing *traC* and its deduced amino acid sequence. Potential promoter (−10 and −35) and ribosome binding (S.D., Shine-Dalgarno [25]) sequences are underlined. A potential transcription termination site for *traC*, which may also serve as a terminator for the oppositely oriented *traA*, is underlined, indicating the related inverted repeats. Vertical arrows mark the location of various Tn*917lac* insertions.

nucleotide sequence determination, introduction of plasmids into bacteria by conjugation or electroporation, and other aspects of the methodology were essentially as previously described (2, 7, 21, 26).

The nucleotide sequence. The nucleotide sequence of the region of pAD1 corresponding to *traC* is shown in Fig. 2. A computer-assisted open reading frame analysis revealed only one open reading frame encoding more than 50 amino acids; it encoded 543 residues with a molecular mass of 60.7 kDa. Its apparent start codon, ATG, is preceded by a good potential ribosome binding site (GGAGG) 10 bp upstream. A possible sigma 70-like promoter (−10 and −35 sequences) is located within the upstream *traB* reading frame. The open reading frame is followed by a sequence that was previously identified as a possible transcription terminator for the oppositely oriented *traA* determinant located downstream (21). It may serve as a bidirectional terminator.

The deduced protein, TraC, has a span of hydrophobic residues typical of a signal sequence at its amino terminus, and there is a potential signal peptidase processing site (19) corresponding to the L-G-A sequence at positions 19 through 21. The protein is basic, with a pI of 8.3, and there are no significant stretches of hydrophobic residues that would indicate a transmembrane region. Interestingly, seven of the C-terminal 11 residues are charged. The data are consistent with TraC being an extracellular protein which, on the basis of previous genetic studies, is associated with the cell surface.

Figure 2 also shows the positions of previously derived (29) transposon insertions (Tn*917* and Tn*917lac*) associated with the *traC* mutant phenotype. Interestingly, all of them mapped in the 3′ third of the determinant. The PR27 derivative is actually a deletion that eliminates *traC* DNA to the left of the insertion.

Evidence for TraC binding to pheromone. The plasmid pAM2600 (Fig. 1) represents a fragment containing *traC* subcloned from the miniplasmid pAM2011K (30), which contained a Tn*917lac* insertion (NR11), into the shuttle plasmid pAM401 (33). This plasmid and derivatives of it, pAM2601 and pAM2602, each containing a Tn*5* insertion within *traC* (generated in *Escherichia coli* by a previously described procedure [27, 28]), were tested for their ability to

TABLE 1. Pheromone binding

Plasmid in OG1X	Pheromone titer[a]	
	cAD1 (128)[b]	cPD1 (64)[b]
pAD1	8	32
pAM2600	8	NT[c]
pAM2601	128	NT
pAM2602	128	NT
pAM401	128	NT
pAM2140 (M14)	128	64

[a] OG1X cells carrying the specified plasmid were grown to mid-exponential phase after which 1 ml of the culture was mixed with 1 ml of synthetic pheromone in N2GT broth (31). After 1 h, the cells were pelleted, the supernatant was boiled for 10 min, and the titer was determined with a microtiter serial twofold dilution assay (9). The titer is the greatest dilution that still results in a clumping response by pAD1-containing cells.

[b] The number in parentheses corresponds to 40 ng of synthetic pheromone (prepared by the University of Michigan core peptide facility) per ml in N2GT broth (31).

[c] NT, not tested.

promote binding to cAD1 in an *E. faecalis* OG1X host. We note that in the case of all three plasmids, no endogenous cAD1 was detectable in culture supernatants, presumably because of the additional presence of *traB* as well as *iad*. (pAM2624 [Fig. 1] could not be used for such an experiment, since endogenous pheromone is secreted.) As shown in Table 1, when a portion of mid-exponential-phase cells containing pAM2600 was mixed with an equal volume of medium containing synthetic cAD1 (18), incubated for 1 h, and then pelleted by centrifugation, the pheromone titer in the supernatant was reduced from 128 to 8. The same result was obtained when cells harbored the wild-type pAD1. In contrast, cells harboring pAM2601 or pAM2602 had no effect on the cAD1 titer. Cells carrying pAM2140, which represents the Tn*917lac traC* M14 insert (Fig. 2) in full-sized pAD1, also showed no reduction. It is noteworthy that binding is pheromone specific. For example, pAD1-containing cells did not significantly reduce the titer of synthetic pheromone cPD1 (9); titers remained unchanged or were reduced by only 50% (e.g., from 64 to 32). The data support the view that TraC, located on the cell surface, is able to bind specifically to cAD1.

Complementation studies. As noted above, all *traC* mutants give rise to an elevated level of iAD1 detectable in culture supernatants. In the *E. faecalis* OG1X background, for example, titers from late-exponential-phase cultures in N2GT broth are generally 8 to 16, compared with ≤2 when wild-type pAD1 is present. Examples of such mutant plasmids are pAM2140 (M14) and pAM2170 (PR17), whose transposon insertion locations are indicated in Fig. 2. When present together with the derivative pAM2624 (a segment containing *traC* cloned in pAM401 as shown in Fig. 1), the iAD1 titer present in supernatants dropped to <2. This study did not make use of a Rec$^-$ host (an isogenic recombination deficient host is not currently available); thus, it cannot be unambiguously concluded that the effect was due to complementation. The data are consistent with such a conclusion, however. (The *E. faecalis* Rec$^-$ derivative UV202 [35] was not suitable for use in this study because of a general inability of the host strain to interact with either cAD1 or iAD1 [16].)

Homology with other proteins. Comparison of the amino acid sequence of TraC with that of PrgZ (24), a protein associated with the *E. faecalis* plasmid system pCF10, showed very significant similarity, with more than 70% of

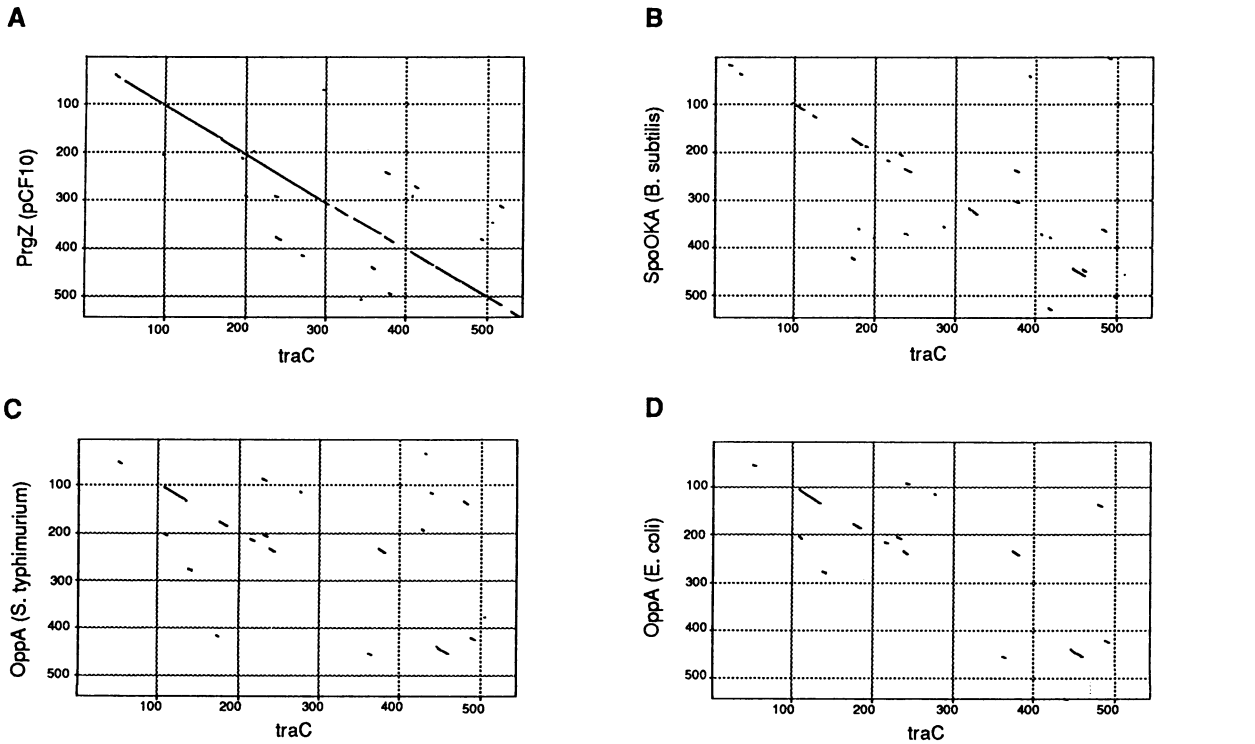

FIG. 3. Homology matrix plots comparing the amino acid sequence of TraC with those of PrgZ (A), Spo0KA (B), OppA of *S. typhimurium* (C), and OppA of *E. coli* (D). Each point represents at least 60% homology within an eight-residue alignment with the corresponding protein.

aligned amino acid residues being identical. pCF10 encodes a mating response to the sex pheromone cCF10. It is likely that PrgZ has a function similar to TraC, except for a difference in specificity. Other similarities between pAD1 and pCF10 involving determinants for aggregation substance and surface exclusion determinants have recently been reviewed (4, 8).

A computer-assisted search with a GenBank data base (issue no. 71) also revealed similarity with oligopeptide binding proteins from *Salmonella typhimurium* (15), *E. coli* (17), and *Bacillus subtilis* (20, 23). In the case of *B. subtilis*, the protein corresponds to Spo0KA (OppA), which is involved in both sporulation and competence. Figure 3 shows homology matrix plots comparing TraC with these proteins, all of which are similar in size (about 60 kDa).

In the case of the *Bacillus* Spo0KA, a potential lipoprotein modification site (34) is present near its amino terminus (20, 23). TraC exhibits a similar site (L-G-A-C-N) starting at amino acid residue 19 (see Fig. 2), as does PrgZ (24). It is therefore possible that the pheromone-binding proteins are anchored in the cell membrane by lipid.

Concluding remarks. We have provided evidence that TraC is involved in binding the pheromone cAD1. The similarity of TraC to a determinant on the *E. faecalis* plasmid pCF10 and to certain oligopeptide binding proteins is supportive of this view and presumably reflects evolution from a common ancestor. Whereas TraC appears to enhance the pheromone sensitivity of the donor strain, it is important to note that *traC* mutants can still be induced with cAD1. As reported previously (31), there can be significant host differences with regard to the function of TraC. Indeed, when present in a different strain of *E. faecalis*, FA2-2 (nonisogenic to OG1X), *traC* mutants do not exhibit a distinguishable phenotype. FA2-2(pAD1) donors are significantly less sensitive to cAD1 than are OG1X(pAD1) cells, and iAD1 is found in culture supernatants at titers of 8 to 16 in the case of both wild-type and *traC* mutants. [OG1X(pAD1) cells give rise to titers of only <2 to 2.] An independent receptor or transport system may be present on the host surface, enabling uptake of the pheromone. Such a system may be host encoded. As noted elsewhere (4, 31), it is possible that plasmid-free strains have a specific pheromone receptor located on the cell surface, which could be utilized as part of a feedback control signal for regulating pheromone synthesis. It could be speculated that such a protein excludes or prevents the function of TraC in FA2-2 but not OG1X. Such differences could also relate to why plasmid-free OG1X cells secrete about eightfold more cAD1 than FA2-2.

It is important to recall that while all *traC* mutants exhibit the elevated iAD1 titer detectable in supernatants of plasmid-containing OG1X cultures, some also exhibit a partially derepressed (for mating functions) phenotype (29). It is conceivable that the cause of this relates in some way to an effect on *traA* expression, perhaps by loss of some stability of the *traA* transcript due to reduction of what normally might be a stabilizing interaction with *traC* transcripts—again noting that both genes appear to share a weak bidirectional terminator.

Previously reported transcriptional fusion data indicated a similar constitutive low level of expression for both *traC* and *traB* (29), suggesting that these determinants are cotranscribed. However, the pAM2624 clone, which did not include the *traB* promoter, appeared able to complement the *traC* mutation, suggesting independent transcription of *traC*.

Nucleotide sequence accession number. The nucleotide sequence GenBank accession number is L19532.

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REFERENCES

1. **An, F. Y., and D. B. Clewell.** Submitted for publication.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moor, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Clewell, D. B.** 1993. Bacterial sex pheromone-induced plasmid transfer. Cell **73**:9–12.
4. **Clewell, D. B.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*, p. 349–367. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York.
5. **Clewell, D. B., L. T. Pontius, F. Y. An, Y. Ike, A. Suzuki, and J. Nakayama.** 1990. Nucleotide sequence of the sex pheromone inhibitor (iAD1) determinant of *Enterococcus faecalis* conjugative plasmid pAD1. Plasmid **24**:156–161.
6. **Clewell, D. B., and K. E. Weaver.** 1989. Sex pheromones and plasmid transfer in *Enterococcus faecalis*. Plasmid **21**:175–184.
7. **Cruz-Rodz, A. L., and M. S. Gilmore.** 1990. High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. **224**:152–154.
8. **Dunny, G. M.** 1990. Genetic functions and cell-cell interactions in the pheromone-inducible plasmid transfer system of *Enterococcus faecalis*. Mol. Microbiol. **4**:689–696.
9. **Dunny, G. M., R. A. Craig, R. L. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. Plasmid **2**:454–465.
10. **Ehrenfeld, E. E., and D. B. Clewell.** 1987. Transfer functions of the *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. J. Bacteriol. **169**:3473–3481.
11. **Ehrenfeld, E. E., R. E. Kessler, and D. B. Clewell.** 1986. Identification of pheromone-induced surface proteins in *Streptococcus faecalis* and evidence of a role for lipoteichoic acid in formation of mating aggregates. J. Bacteriol. **168**:6–12.
12. **Galli, D., A. Friesenegger, and R. Wirth.** 1992. Transcriptional control of sex pheromone-inducible genes on plasmid pAD1 of *Enterococcus faecalis* and sequence analysis of a third structural gene for (pPD1-encoded) aggregation substance. Mol. Microbiol. **6**:1297–1308.
13. **Galli, D., F. Lottspeich, and R. Wirth.** 1990. Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. Mol. Microbiol. **4**:895–904.
14. **Galli, D., R. Wirth, and G. Wanner.** 1989. Identification of aggregation substances of *Enterococcus faecalis* cells after induction by sex pheromones—an immunological and ultrastructural investigation. Arch. Microbiol. **151**:486–490.
15. **Hiles, I. D., M. P. Gallagher, D. J. Jamieson, and C. F. Higgins.** 1987. Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. J. Mol. Biol. **195**:125–142.
16. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158**:777–783.
17. **Kashiwagi, K., Y. Yamaguchi, Y. Sakai, H. Kobayashi, and K. Igarashi.** 1990. Identification of the polyamine-induced protein as a periplasmic oligopeptide binding protein. J. Biol. Chem. **265**:8387–8391.
18. **Mori, M., Y. Sakagami, M. Narita, A. Isogai, M. Fujino, C. Kitada, R. Craig, D. B. Clewell, and A. Suzuki.** 1984. Isolation and structure of the bacterial sex pheromone, cAD1, that induces plasmid transfer in *Streptococcus faecalis*. FEBS Lett. **178**:97–100.
19. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39**:615–648.
20. **Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch.** 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. Mol. Microbiol. **5**:173–185.

21. **Pontius, L. T., and D. B. Clewell.** 1992. Regulation of the pAD1-encoded sex pheromone response in *Enterococcus faecalis*: nucleotide sequence analysis of *traA*. J. Bacteriol. **174:**1821–1827.
22. **Pontius, L. T., and D. B. Clewell.** 1992. Conjugative transfer of *Enterococcus faecalis* plasmid pAD1: nucleotide sequence and transcriptional fusion analysis of a region involved in positive regulation. J. Bacteriol. **174:**3152–3160.
23. **Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman.** 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. J. Bacteriol. **173:**1388–1398.
24. **Ruhfel, R. E., D. A. Manias, and G. M. Dunny.** 1993. Cloning and characterization of a region of the *Enterococcus faecalis* conjugative plasmid, pCF10, encoding a sex pheromone-binding function. J. Bacteriol. **175:**5253–5259.
25. **Shine, J., and L. Dalgarno.** 1975. The 3′ terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
26. **Su, Y. A., M. C. Sulavik, P. He, K. K. Makinen, P.-L. Makinen, S. Fiedler, R. Wirth, and D. B. Clewell.** 1991. Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. *liquefaciens*. Infect. Immun. **59:**415–420.
27. **Tanomoto, K., and D. B. Clewell.** 1993. Regulation of the pAD1-encoded sex pheromone response in *Enterococcus faecalis*: expression of the positive regulator TraE1. J. Bacteriol. **175:**1008–1018.
28. **Tanimoto, K., and T. Iino.** 1985. Additional genes essential for replication of the mini-F plasmid from origin I. Mol. Gen. Genet. **198:**358–359.
29. **Weaver, K. E., and D. B. Clewell.** 1988. Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: construction and characterization of *lacZ* transcriptional fusions in a key control region of the plasmid. J. Bacteriol. **170:**4343–4352.
30. **Weaver, K. E., and D. B. Clewell.** 1989. Construction of *Enterococcus faecalis* pAD1 miniplasmids: identification of a minimal pheromone response regulatory region and evaluation of a novel pheromone-dependent growth inhibition. Plasmid **22:**106–119.
31. **Weaver, K. E., and D. B. Clewell.** 1990. Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: effects of host strain and *traA*, *traB*, and C region mutants on expression of an E region pheromone-inducible *lacZ* fusion. J. Bacteriol. **172:**2633–2641.
32. **Weaver, K. E., and D. B. Clewell.** 1991. Control of *Enterococcus faecalis* sex pheromone cAD1 elaboration: effects of culture aeration and pAD1 plasmid-encoded determinants. Plasmid **25:**177–189.
33. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.
34. **Wu, H. C.** 1987. Posttranslational modification and processing of membrane proteins in bacteria, p. 37–71. *In* M. Inouye (ed.), Bacterial outer membranes as model systems. Wiley, New York.
35. **Yagi, Y., and D. B. Clewell.** 1980. Recombination-deficient mutant of *Streptococcus faecalis*. J. Bacteriol. **143:**966–970.