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# Cloning and Characterization of a Region of the *Enterococcus faecalis* Conjugative Plasmid, pCF10, Encoding a Sex Pheromone-Binding Function

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**In order to investigate the mechanism by which peptide sex pheromones induce expression of the conjugation functions of certain *Enterococcus faecalis* plasmids, a biological assay was developed to measure the ability of cells carrying the conjugative plasmid pCF10 to bind the sex pheromone cCF10. The data indicated that pCF10 endows its host *E. faecalis* cell with the ability to specifically remove (apparently by irreversible binding) cCF10 activity from culture medium. The pCF10 DNA encoding this ability was localized to a 3.4-kb segment within a region involved in negative control of expression of conjugal transfer functions. This segment also encoded ability to bind the pheromone inhibitor peptide iCF10. DNA sequencing revealed three open reading frames, which have been denoted *prgW* (pheromone responsive gene *W*), *prgZ*, and *prgY*. The deduced product of *prgW* resembled regulatory proteins from other bacteria and eucaryotes, with a very high degree of identity within a putative DNA-binding domain. The *prgY* gene actually extended into an adjacent region of pCF10 and could encode a protein with significant similarity to a protein called TraB, believed to be involved in shutdown of pheromone cAD1 production by cells carrying the pheromone-inducible hemolysin plasmid pAD1, according to F. Y. An and D. B. Clewell (Abstr. Gen. Meet. Am. Soc. Microbiol. 1992, H70, 1992). The *prgZ* gene product showed significant relatedness to binding proteins encoded by oligopeptide permease (*opp*) operons in gram-positive and gram-negative bacteria and is highly similar to a pAD1-encoded protein, TraC, which is believed to mediate sex pheromone cAD1 binding (K. Tanimoto, F. Y. An, and D. B. Clewell, submitted for publication). A Tn*5* insertion into *prgZ* abolished cCF10 binding ability.**

In *Enterococcus faecalis*, peptide pheromones produced by recipient cells induce expression of the conjugal transfer functions encoded by certain plasmids, resulting in efficient transfer of the plasmids when the host (donor) bacterial cells are in close proximity to potential recipients (7, 10, 11). Most of the analysis of the regulation of expression of conjugal transfer functions of the pheromone-inducible plasmids has focused on the hemolysin-bacteriocin plasmid pAD1 (7) and the tetracycline resistance plasmid pCF10 (10). In both cases, expression of transfer functions seems to be controlled by a complex mechanism involving overlapping positive and negative control circuits. In wild-type donor cells grown in the absence of pheromone, a negative control mechanism appears to block expression of transfer functions (10). The top portion of Fig. 1 shows a genetic and physical map of the transfer region of pCF10, with some of the genes and their apparent functions, based on genetic and physical analysis (3–5, 19, 25). Addition of pheromone or genetic inactivation of the negative control region by insertion or deletion allows a novel, *cis*-acting, orientation-dependent positive control system to activate expression of genes mediating formation of mating aggregates and other transfer functions (5).

Biochemical and genetic analysis by Suzuki and coworkers and Clewell et al. (7, 12, 24, 31) of pheromone-inducible conjugation systems encoded by several *E. faecalis* plasmids suggests that there are a number of chromosomally encoded peptides which may function as sex pheromones. In the case of pCF10, a heptapeptide, cCF10 ($H_3N$-Leu-Val-Phe-Leu-Val-Thr-Val-COOH), has been shown to be the active pheromone (23). Each pheromone-inducible plasmid apparently confers a host response to a specific peptide produced by recipient cells, and at least some of the plasmids also encode a distinct but related peptide which acts as a competitive inhibitor of the pheromone; the latter peptide seems to be involved in modulation of the pheromone response or in negative control of expression of transfer functions (6, 7). The specificity of each system suggests that a distinct, plasmid-determined binding function should be associated with each transfer system and that the gene(s) conferring pheromone-binding activity for a particular plasmid might also mediate binding to the corresponding inhibitor. In order to test this hypothesis for the pCF10 system, we set out to identify a specific cCF10 (pheromone)- and iCF10 (inhibitor)-binding locus on the plasmid.

## MATERIALS AND METHODS

**Bacterial strains and culture media.** *Escherichia coli* DH5α was used in all cloning experiments and was grown in Luria-Bertani medium (29) unless otherwise indicated. All strains of *E. faecalis* used here were derived by introduction of plasmids into OG1RF (11) by conjugation (9) or transformation (13). These strains were grown in M9-YE or BYGT medium as described previously (9). We used the following concentrations of antibiotics: spectinomycin, 50 μg/ml in *E. coli* and 1,000 μg/ml in *E. faecalis*; chloramphenicol, 10 μg/ml; and kanamycin, 50 μg/ml in *E. coli* and 1,000 μg/ml in *E. faecalis*.

**Genetic manipulations, DNA sequencing, and recombinant DNA techniques.** Plasmids pCF10, pINY1801, pINY1825,

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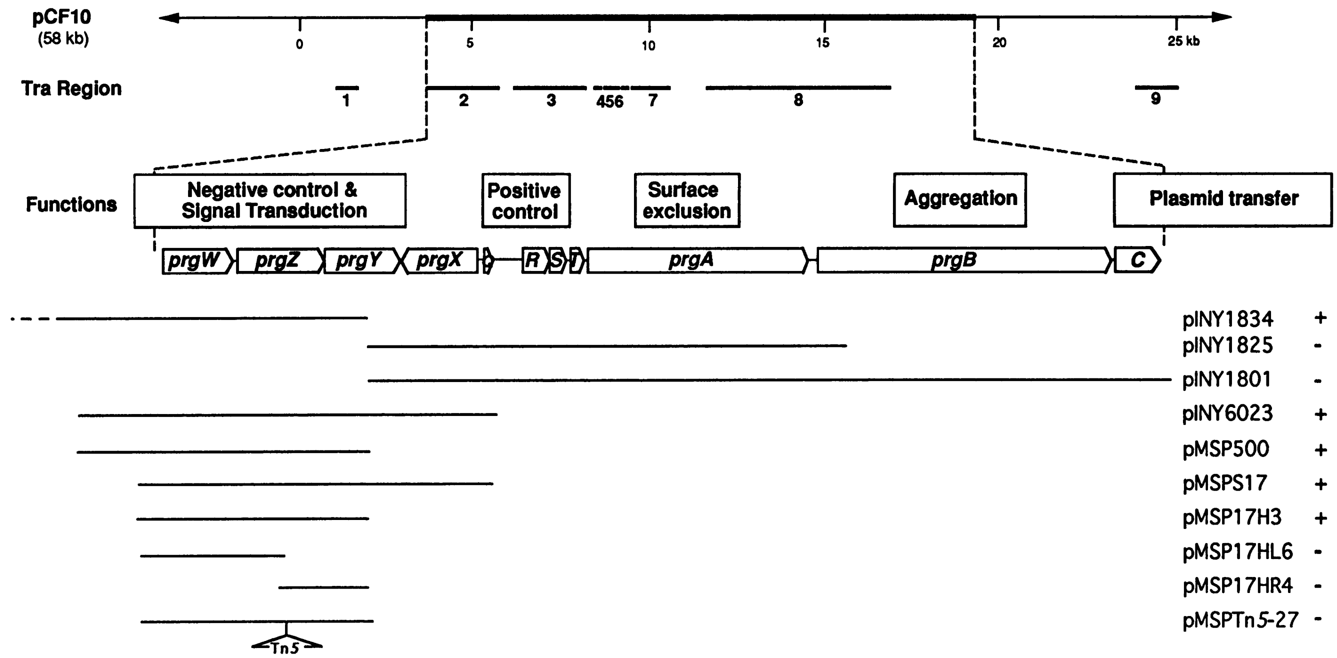


FIG. 1. Genetic and physical map of the pheromone-inducible transfer (Tra) region of pCF10. The top portion of the figure depicts the genetic and physical map of the transfer region of pCF10, indicating the approximate locations and functions of regulatory and structural genes determined in previous studies. The portion of pCF10 which has been sequenced is indicated by the heavy line (the sequences reported in this paper correspond to 3.5 kb at the left end of this line). The results of genetic and molecular analysis of the positive control region which activates transcription of the *prgB* gene, encoding the aggregation substance protein, have been reported recently (5). In cells carrying wild-type pCF10, the negative control region appears to block this activation in the absence of pheromone. The Tra segments are regions of pCF10 identified by Tn*917* insertional mutagenesis in the initial stages of characterization of the transfer region (3). The lower set of lines indicates the positions of subcloned fragments that were tested in this study for their ability to confer pheromone-binding ability. Plasmid pMSPTn5-27 is a derivative of pMSP17H3 with an insertion of Tn*5* in the position indicated by the vertical line. The + and − signs indicate the results of pheromone-binding assays described in the text and presented quantitatively in Table 1. The three ORFs corresponding to *prgW*, *prgZ*, and *prgY*, as determined by DNA sequencing of the pCF10 insert in pMSP17H3 (see Fig. 2 for the complete sequence), are described in the present study; the remaining genes were sequenced previously (19).

pINY1834, and pAD1 have been described previously (4, 7, 9). The *E. coli-E. faecalis* shuttle vectors pWM402 (35), pDL276, and pDL278 (13, 22) have been used extensively in our laboratory for cloning pCF10 DNA (4, 5, 13, 19). Construction of pINY6023 was carried out by removing the 7.5-kb *Sal*I-*Xba*I fragment of pCF10 indicated in Fig. 1, blunting the ends by treatment with Klenow's fragment, and inserting the DNA into the unique *Hin*cII site of pDL276 by blunt-end ligation. The portions of pCF10 DNA contained in the recombinant plasmids used in this study are shown in Fig. 1. In order to generate nested deletions of cloned fragments in vitro, plasmid pINY6023 (Fig. 1) was digested with either *Bgl*II to delete from the right end or *Xba*I to generate deletions from the left end, and the digested DNA was subjected to treatment with the *E. coli* exonuclease III and S1 enzymes, by the method of Henikoff (16), as described in reference 2. Deleted plasmids were propagated in *E. coli* and analyzed by gel electrophoresis of minipreps obtained with the method of alkaline lysis (29), and selected plasmids were transferred to *E. faecalis* by electroporation (13). Insertions of Tn*5* into pMSP17H3 were generated and mapped in *E. coli* as described previously (4, 19) and were transformed into *E. faecalis* for analysis of pheromone binding. DNA sequencing and computer analysis were carried out with double-stranded plasmid DNA as previously described (19). Synthetic primers were made (with an Applied Biosystems 391 automated DNA synthesizer) near the ends of previously sequenced regions to extend the sequence. Both strands were completely sequenced, and the position of the transposon insertion in pMSPTn5-27 and the extent of nested deletions were also determined by sequence analysis.

**Assays of pheromone or inhibitor binding.** In order to assay for pheromone binding, a synthetic pheromone preparation was diluted in M9-YE medium to give a titer in a clumping-inducing microtiter plate assay (12) of approximately 64/128, as determined experimentally. A volume of 30 μl of this diluted material was mixed with an equal volume of medium containing $6 \times 10^8$ exponentially growing *E. faecalis* cells per ml to be tested for pheromone binding. After a 15-min incubation at 37°C, the bacteria were removed by centrifugation in a Baxter Microfuge at 13,000 rpm. The pheromone activity remaining in the supernatant was assayed with a standard microtiter plate clumping assay (12) and compared with that in control preparations diluted and incubated in parallel in the absence of bacteria.

Our group, in collaboration with J. Nakayama and A. Suzuki (University of Tokyo), has recently determined that pCF10 encodes synthesis of a peptide inhibitor (iCF10), which appears to act as a competitive inhibitor of cCF10 in modulation of the pheromone response (28), similar to the iAD1 inhibitor which competes with the cAD1 pheromone in the pAD1 system (6). To test strains for ability to bind inhibitor, synthetic iCF10 (generously provided by J. Na-

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TABLE 1. Pheromone-binding activity conferred by various plasmids[a]

| Pheromone or plasmid | Pheromone titer | Binding capacity of bacterial strain |
|---|---|---|
| Pheromone | 64/32 | |
| None | 64/32 | <2 |
| pDL278 | 64/32 | <2 |
| pCF10 | 4/4 | 16/8 |
| pINY1825[b] | 64/32 | <2 |
| pINY1801[b] | 64/32 | <2 |
| pINY1834[b] | 8/8 | 8/4 |
| pINY6023[c] | 16/8 | 4/4 |
| pMSPS17[c] | <4/<4 | >16 |
| pMSP17H3[d] | <4/<4 | >16 |
| pMSP17HL6[d] | 64/32 | <2 |
| pMSPTn5-27[e] | 64/32 | <2 |
| pAD1 | 16/16 | 4/2 |

[a] All plasmids were inserted into *E. faecalis* OG1RF by conjugation (9) or electroporation (13). The resulting strains were assayed for binding by assessing the ability of the bacteria to reduce the pheromone titer of a preparation of cCF10 (previously diluted to a titer of 32 to 64) by the assay described in Materials and Methods. The values listed for binding capacity indicate the reduction in pheromone titer observed after incubation of the pheromone with the bacterial strain indicated. The sets of numbers separated by slashes represent results for replicate assays done on two separate occasions.

[b] Plasmid contains a fragment of DNA representing the segment of pCF10 indicated in Fig. 1 cloned into pWM402.

[c] Plasmid contains a fragment of DNA representing the segment of pCF10 indicated in Fig. 1 cloned into pDL276.

[d] Plasmid contains a fragment of DNA representing the segment of pCF10 indicated in Fig. 1 cloned into pDL278. Neither this plasmid nor pMSP17HR4 (Fig. 1) ever conferred binding ability in repeated trials.

[e] Derivative of pMSP17H3 with Tn*5* inserted in the position shown in Fig. 1.

TABLE 2. Binding of the inhibitor peptide iCF10 by *E. faecalis* cells[a]

| Incubated with bacteria (plasmid) | Inhibitor present | Pheromone titer | Fold reduction in titer | Inhibitor-binding capacity |
|---|---|---|---|---|
| No | No | 64 | | |
| No | Yes | 4 | 16 | |
| Yes (none) | Yes | 4 | 16 | |
| Yes (pDL278) | Yes | 4 | 16 | |
| Yes (pAD1) | Yes | 4 | 16 | |
| Yes (pCF10) | Yes | 8 | 8 | 2 |
| Yes (pMSP17H3) | Yes | 64 | ≤2 | ≥16 |

[a] As described in Materials and Methods, inhibitor assays involve testing the ability of a solution containing the putative inhibitor to reduce the titer of a pheromone preparation in a clumping assay. For the data reported in this table, derivatives of strain OG1RF containing the various plasmids indicated were incubated for 15 min with a solution of inhibitor sufficient to reduce the titer of a cCF10 preparation from 64 to 4. The bacteria were then removed by centrifugation, and the remaining inhibitor activity was assayed. The first two rows of data indicate the titer of the pheromone preparation used in the presence or absence of an inhibitor solution that had not been incubated with bacteria.

kayama, University of Tokyo) was mixed with bacteria, incubated, and centrifuged exactly as described above for the pheromone-binding assay. This supernatant was added to microtiter plates as a diluent, and cCF10 preparations were diluted serially through the solutions to be tested for inhibitor activity as described by Clewell et al. (6). For both the inhibitor-binding and pheromone-binding assays, the final pheromone (clumping-inducing) assays utilized OG1RF(pCF10) cells as responders (9).

**Nucleotide sequence accession number.** The sequences used in this study have been deposited in GenBank under accession number L14285.

## RESULTS

**Identification of a region of pCF10 conferring pheromone-binding activity.** To identify a locus responsible for pCF10-encoded pheromone binding, we developed a biological assay for binding of the pheromone, cCF10. The assay involved testing the ability of isogenic *E. faecalis* strains carrying various plasmids to specifically remove pheromone activity from culture medium during a 15-min incubation (see Materials and Methods). Incubation of plasmid-free OG1RF cells or OG1RF cells carrying the cloning vector pDL276, pDL278, or pWM402 with medium containing pheromone at a known concentration had no effect on the titer of the pheromone (Table 1). On the other hand, isogenic cells containing pCF10 consistently reduced the titer of pheromone preparations (Table 1). In control experiments, we made culture supernatants of cells showing binding activity and found that these supernatants did not reduce the titer of pheromone preparations, indicating that the reduction in activity was not due to secretion of a soluble protease by the bacteria. In the initial studies of pheromone binding, we used a solution of synthetic cCF10 at a concentration of $3.05 \times 10^{-10}$ M. This concentration of pheromone had a titer of 32 to 64 when assayed against OG1RF(pCF10) responder cells. Given the concentration of cells in the binding assay and the fact that the titer was typically reduced by 8- to 16-fold during the binding assay, we calculate the pheromone-binding capacity of OG1RF(pCF10) to be about 150 to 300 molecules per CFU.

Preliminary studies indicated that insertions of Tn*917* in a region of pCF10 called the Tra2 segment (3) eliminated or reduced binding. To confirm the involvement of this region in binding and to define the minimal amount of pCF10 DNA required for binding, we examined a number of cloned fragments of pCF10 for expression of binding activity in *E. faecalis*. These results, shown in Fig. 1 and Table 1, confirmed that binding ability was expressed by clones containing the Tra2 region, which is also believed to be involved in negative regulation of transfer functions (10). None of these clones conferred binding in *E. coli*.

By generating nested deletions in the cloned DNA in pINY6023, as described in Materials and Methods, we were able to localize more precisely the DNA sequences required for binding. The smallest fragment (3.4 kb) that retained binding ability was contained in pMSP17H3. A stable Tn*5* derivative, pMSPTn5-27, lost binding ability (Fig. 1). As described below, this Tn*5* insertion interrupts the open reading frame (ORF) believed to represent the binding gene. As expected, insertion of Tn*5* into the vector sequences had no effect on pheromone-binding ability (data not shown). Interestingly, a strain carrying pAD1, which encodes ability to respond to a distinct pheromone, cAD1 (24), showed a slight amount of binding to cCF10; the possible significance of this result is addressed in the Discussion.

**Inhibitor binding.** We also assayed strains carrying the Tra2 region for their ability to bind the recently identified inhibitor peptide iCF10 (28). As shown in Table 2, strains carrying cloned pCF10 DNA, and which bound cCF10, also bound iCF10. The strain carrying pAD1 exhibited no detectable binding of iCF10, in contrast to the apparent low level of binding of cCF10 observed with the same strain (Table 1).

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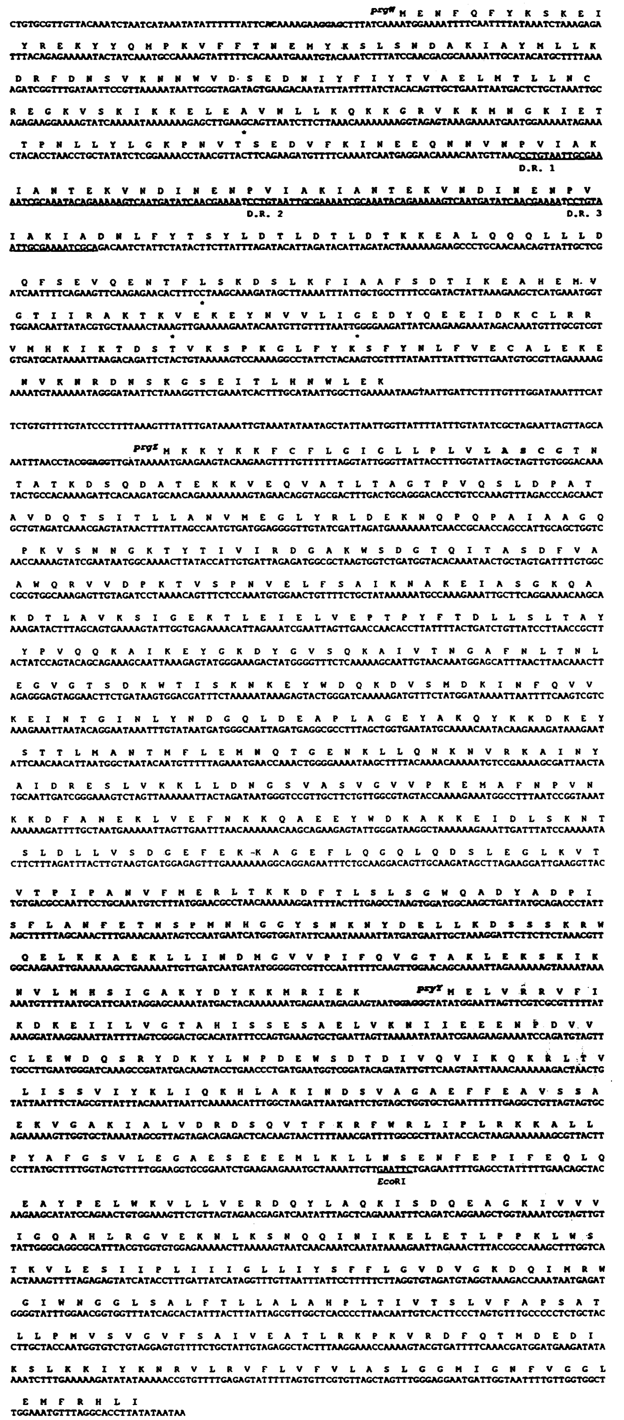

FIG. 2. DNA and protein sequences of the region of pCF10 encoding pheromone-binding ability. The nucleotide sequence of a 4,029-bp segment, including all of the cloned pCF10 DNA in pMSP17H3 and an additional 0.7-kb segment of the adjacent *Eco*RI c fragment of pCF10, whose sequence was reported previously (19), is shown. The deduced amino acid sequences of the ORFs corresponding to the *prgW*, *prgZ*, and *prgY* genes are indicated in the single-letter code. The nucleotides shown in boldface represent potential ribosome binding sites, while the amino acid residues shown in boldface represent a potential lipid attachment site which is also found in the *Bacillus* Spo0KA protein, as noted in reference 26 and the text. The *prgW* and *prgZ* genes were preceded by sequences resembling promoter regions identified in other procaryotes (8) and in other pCF10 *prg* genes (19). A repeated sequence, D.R., is indicated in the *prgW* gene and is described in the text. The underlined sequence in the *prgY* gene represents the *Eco*RI site separating the *Eco*RI b and c fragments of pCF10. The stars below some *prgW* sequences indicate the endpoints of nested deletions from the left which were constructed in *E. coli* but which could not be inserted into *E. faecalis*.

From these results, we conclude that the pheromone-binding machinery encoded by the 3.4-kb insert in pMSP17H3 also confers ability to bind inhibitor.

**DNA sequence analysis.** The complete nucleotide sequence of the 3.4-kb insert contained within pMSP17H3 was determined as described in Materials and Methods. We also sequenced several hundred bases flanking the junctions between the pCF10 sequences and the Tn*5* sequences in pMSPTn5-27. We found three ORFs of substantial length. The first ORF, denoted *prgW*, could encode a 38.9-kDa (333-amino-acid) protein with a pI of 7.2. The second ORF, henceforth designated *prgZ*, would code for a 535-amino-acid protein with a predicted molecular weight of 61,000 and a pI of 8.98. The third ORF, henceforth denoted *prgY*, initiated 9 bp downstream from the *prgZ* stop codon and extended to the right-hand end of the *Eco*RI b fragment. Analysis of previously sequenced pCF10 DNA in the adjacent *Eco*RI c fragment indicated that the 3′ end of this ORF (provisionally denoted ORF1 by Kao et al. [19]) extends for 657 bp, to give a total length of 1,152 bp, with the translated gene product having a predicted size of 43.4 kDa and a pI of 6.29. As indicated in Fig. 2, each of the three ORFs was preceded by potential ribosome binding sites. The close spacing between *prgZ* and *prgY* suggests that these genes might be translated from the same message. Within the *prgW* sequence, there was a 54-bp sequence directly repeated one time, followed by an additional repeat of the first 21 bp. Several potential stem-loop structures with fairly high thermodynamic stability ($\Delta G$ values between $-20$ and $-30$ kcal/mol [ca. $-84$ and $-126$ kJ/mol]) could be formed within this region.

**Similarity of the *prgW* and *prgY* gene products to other proteins.** On the basis of the deduced amino acid sequence, the *prgW* gene product is predicted to be a predominantly helical, basic, cytoplasmic protein. A 30- to 50-amino-acid sequence near the amino-terminal end of the *prgW* gene product was very similar to a region of the mouse interferon response binding factor-1, believed to be involved in activation of expression of interferon-inducible genes (33). The relevant region of interferon response binding factor-1 is postulated to contain the DNA-binding domain. The *prgW* gene product is also similar in this region to a protein of unknown function encoded by a *Lactobacillus* plasmid, which is likely involved in plasmid replication (32). Recently, Weaver et al. described genetic and molecular analysis of a region of pAD1 believed to function as a replicon (34a). The *prgW* gene product shows significant similarity to the RepA protein of pAD1 and could represent the pCF10 homolog of this protein.

The product of the *prgY* gene is predicted to be hydrophobic, especially in the carboxy-terminal half of the molecule. This protein did not resemble any sequences in the data bases except for the *traB* gene of the pheromone-inducible hemolysin plasmid, pAD1. Clewell and coworkers (1) have suggested that the *traB* gene may shut down production of

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1   MKKYKKFCFLGIGLL-PLVLASCGTNTATKDSQDATEKKVEQVATLTAGTP
    ||  |:       |   |||::||   :  :::  : |     : :  |
1   MK--KRWSIVTLMLIFTLVLSACGFGGTGSNGEGKKDSKGKTTLNINIKTE

51  VQSLDPATAVDQTSITLLANVMEGLYRLDEKNQPQPAIAAGQPKVSNNGKT
      || |: | |  |  :  :  ||| | ::  :|: : |:     |::|||
50  PFSLHPGLANDSVSGGVIRQTFEGLTRINADGEPEEGMAS-KIETSKDGKT

102 YTIVIRDGAKWSDGTQITASDFVAAWQRVVDPKTVSPNVELFSAIKNAKEI
    ||  ||||:|||:|  :|| ||  ||  ::||:: |  :     || | :
100 YTFTIRDGVKWSNGDPVTAQDFEYAWKWALDPNNESQYAYQLYYIKGAEAA

153 ASGKQAKDTLAVKSIGEKTLEIELVEPTPYFTDLLSLTAYYPVQQKAIKEY
    ::|| : | :|||:: :||| :|| :||||||:| :  :| |:::|
151 NTGKGSLDDVAVKAVNDKTLKVELNNPTPYFTELTAFYTYMPINEKIAEKN

204 GKDYGVSQKAIVTNGAFNLTNLEGVGTSDKWTISKNKEYWDQKDVSMDKIN
     |    :  : |:||:|::|:    |:    | :|| :|||   | : ||:
202 KKWNTNAGDDYVSNGPFKMTAWKHSGSI---TLEKNDQYWDKDKVKLKKID

255 FQVVKEINTGINLYNDGQLDEAPLA-GEYAKQ----YKKDKEYSTTLMANT
      :::: ||  : :::|:|| | :: |: : :     |||        |
250 MVMINNNNTELKKFQAGELDWAGMPLGQLPTESLPTLKKDGSLHVEPIAGV

301 MFLEMNQTGENKLLQNKNVRKAINYAIDRESLVKKLLDNGSVASVGVVPKE
         |   |:| |:| |:||| :|: ||:| ||:: :   ::::::||
300 YWYKFN--TEAKPLDNVNIRKALTYSLDRQSIVKNVTQGEQMPAMAAVPPT

352 M-AFNPVNKKDFANEKLVEFNKKQAEEYWDKAKKEIDLSKNTSL---DLLV
    | :|   ||  : ::: |    | | || :|: ||  |||   |    |
350 MKGFED-NKEGYFKDNDV----KTAKEYLEKGLKEMGLSKASDLPKIKLSY

399 SDGEFEKKAGEFLQGQLQDSLEGLKVTVTPIPANVFMERLTKKDFTLSLSG
    :  :   |  :: :|     :| |: | :     ||: ::|   |:  :  |
396 NTDDAHAKIAQAVQEMWKKNL-GVDVELDNSEWNVYIDKLHSQDYQIGRMG

450 WQADYADPISFLANF-ETNSPMNHGGYSNKNYDELLKDS-----SSKRWQE
    | :|::|||:||: | : |:  |  | :| ::  ||::|      :|| :
446 WLGDFNDPINFLELFRDKNGGNNDTGWENPEFKKLLNQSQTETDKTKRAEL

495 LKKAEKLLINDMGVVPIFQVGTAKLEKSKIKNVLMHSIGAKYDYKKMRIEK
    |||||   |::| |:||:     : :: :: | | | : |: | :::
497 LKKAEGIFIDEMPVAPIYFYTDTWVQDENLKGVIMPGTGEVY-FRNAYFK
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FIG. 3. Alignment of the deduced *prgZ* gene product with the Spo0KA protein from *B. subtilis*. The complete deduced amino acid sequences of both proteins are presented with the *prgZ* product on the upper line. Identical residues are connected by solid lines, and conservative substitutions are indicated by dots. The boldface letters represent a putative covalent lipid modification site in the *Bacillus* protein (26) which is also present in a similar site in the *prgZ* protein.

the chromosomally encoded cAD1 pheromone in donor cells carrying pAD1.

**Predicted structural properties of the *prgZ* gene product and similarity to other proteins.** The deduced amino acid sequence of PrgZ indicates that the protein would be mostly hydrophilic, but with a hydrophobic domain near the amino-terminal end which could play a role in secretion of the protein. However, the carboxy-terminal portion of this protein does not contain the predicted wall-spanning and membrane anchor motifs found in numerous cell surface proteins of gram-positive cocci (15), including three pCF10 gene products (19). Computer searches indicated that the deduced sequence of the product of *prgZ* was related to a variety of oligopeptide binding proteins encoded by oligopeptide permease (*opp*) operons in gram-positive and gram-negative bacteria (17, 18, 26). Figure 3 shows an alignment between the *prgZ* gene product and the *Bacillus subtilis spo0KA* gene product (26). There is considerable similarity throughout the length of the proteins, but the regions between amino acid residues 100 and 200 show a particularly high degree of relatedness. The *spo0KA* gene has been shown to be a part of an operon comprising the *spo0K* locus, which is involved in the initiation of sporulation as well as the expression of competence (26, 27). Like the previously characterized oligopeptide permease (Opp) systems of *E. coli* and *Salmonella typhimurium* (17, 26), the *Bacillus* system consists of five genes encoding a binding protein (Spo0KA), two membrane-spanning proteins (Spo0KB and C) predicted to form a channel, and two membrane-associated cytoplasmic proteins (Spo0KD and F) with ATPase activity. Interestingly, none of the other pCF10 genes in the vicinity of *prgZ* resemble *opp* genes of other organisms.

According to the model of Higgins and coworkers (17, 18), the OppA binding protein is periplasmic in gram-negative bacteria. In *Bacillus* spp., a potential site for covalent attachment of a lipid which could anchor the Spo0KA protein in the membrane has been identified near the amino-terminal end of the molecule (26). As indicated in Fig. 3, this same motif is present in the *prgZ* gene product.

In the accompanying paper from Tanimoto et al. (34), the sequence of the *traC* gene of pAD1 is presented, along with evidence that *traC* encodes a binding function for the pheromone cAD1. The pAD1 *traC* and the pCF10 *prgZ* gene products show a high degree of similarity, with almost complete identity in the amino-terminal halves of the proteins (34).

## DISCUSSION

The results presented here indicate that a 3.4-kb region of pCF10 confers on *E. faecalis* host cells the ability of the bacteria to specifically remove sex pheromone activity from culture medium. The simplest interpretation of these results is that the pheromone is irreversibly bound or internalized by the bacteria, and this notion is supported by the DNA sequence analysis of the genes contained within this region of pCF10 DNA. Although it is conceivable that the removal of pheromone is due to some sort of cell-associated enzymatic inactivation of the pheromone which is not directly related to the induction of conjugation, we feel that this is unlikely. The binding activity was also active against the inhibitor peptide iCF10. Isogenic cells carrying the plasmid pAD1, which determines a mating response to a distinct pheromone called cAD1, showed a low level of binding to cCF10 and no observable binding of iCF10. Previous analysis of the biological activities of various peptide pheromones and inhibitors (23) indicated that the iAD1 peptide, which acts as an inhibitor in the pAD1 system, had a very low level of pheromone activity against cells carrying pCF10. This previous observation, as well as the similarity of the two binding proteins (34), may account for the observation that pAD1 confers a slight ability to bind cCF10.

Clones such as pMSP17H3, which were deleted for a major portion of the 3′ end of *prgY*, conferred binding ability. In contrast, deletion or interruption of *prgZ* (the position of the Tn*5* insert in pMSPTn5-27 is indicated in Fig. 1) resulted in loss of binding. Because *prgZ* is present in all constructs which conferred pheromone binding and interruption of this gene by insertion or deletion abolished binding, we infer that this gene is necessary for cCF10 binding in *E. faecalis*. The sequence comparisons described above also support the contention that the product of *prgZ* is required and suggest that it is directly involved in pheromone binding.

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It is possible that the *prgW* product (acting in concert with the *prgZ* product) could also play a direct role in pheromone binding; alternatively, *prgW* (or sequences immediately upstream from *prgW*) could be required for *prgZ* expression in an *E. faecalis* host. As yet, we have been unable to test this directly because all of our clones containing deletions of *prgW* that retained an intact *prgZ* gene were unstable in *E. faecalis* (Fig. 2).

The *prgZ* gene product resembles oligopeptide binding proteins from *B. subtilis* and other gram-positive and gram-negative bacteria, as well as the TraC protein encoded by pAD1 (which was demonstrated by Tanimoto et al. [34] to be involved in binding of the pheromone cAD1). The *E. faecalis* pheromone-binding proteins share about 35% identical amino acids with the *B. subtilis* binding protein and over 70% identity with one another. The two *E. faecalis* proteins are virtually identical in the amino-terminal regions and are somewhat less similar in the carboxy-terminal region. Thus, the specificity for recognition of the two pheromones could lie in the carboxy portion of the binding proteins. The *prgZ* product appears to have a structural motif near the amino end which could serve as a site for covalent attachment of a lipid that would anchor the protein to the external face of the cell membrane, where it would be available to interact with pheromone molecules. Preliminary data (not shown) indicate that a 61-kDa protein identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) in *E. coli* maxicells (21, 30) and in cell wall extracts (4, 14) of *E. faecalis* cells carrying pMSP17H3 is the *prgZ* gene product (data not shown). The transposon insertion in pMSPTn5-27 which abolished binding ability also blocks production of this protein, supporting this notion.

In the oligopeptide permease systems of other bacteria, the binding protein is part of a complex of at least five molecules, including two membrane-spanning proteins that appear to form a channel and two membrane-associated cytoplasmic ATPases which couple ATP hydrolysis to the import of the peptide (which is initially bound to the cell surface via the binding protein). The bacterial *opp* operons described above, and others, appear to belong to a superfamily of ATP-dependent transport systems found in both procaryotes and eucaryotes (17). Interestingly, we have not found any other *opp*-like genes in adjacent regions of pCF10. Therefore, it is possible that a chromosomally encoded Opp system of *E. faecalis* interacts with the *prgZ* gene product to form the apparatus for internalizing pheromone, with the specificity being supplied by the plasmid-encoded binding protein. The initial pheromone-binding event would presumably result in either transduction of a signal or internalization of the pheromone and binding to a intracellular target that would lead to inactivation of repression of transfer functions. Alternatively, the binding protein may somehow function in the absence of additional Opp proteins or may act in concert with other plasmid-encoded gene products to effect the biological processes that result from pheromone binding. With regard to the latter hypothesis, several adjacent genes in the region of *prgZ*, including *prgW*, *prgY*, and *prgX*, could play a role. All three of these genes have homologs in the pAD1 system (1, 7, 34), and in some cases, genetic evidence suggesting a role for some of these genes in signal transduction has been obtained (7, 34). The fact that deletions from the 5′ end of *prgW* that removed all or part of this gene but left the putative *prgZ* promoter intact resulted in instability in an *E. faecalis* host could indicate that the *prgW* product interacts with the *prgZ* promoter region. Alternatively, *prgW* could be more directly involved in the signalling process or replication (34a).

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