The *prgQ* Gene of the *Enterococcus faecalis* Tetracycline Resistance Plasmid pCF10 Encodes a Peptide Inhibitor, iCF10

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Conjugative transfer of the *Enterococcus faecalis* tetracycline resistance plasmid pCF10 is stimulated by a peptide pheromone, cCF10. Once a recipient strain acquires pCF10 and thus becomes a pheromone-responsive donor, cCF10 activity is no longer detected in culture filtrates. Here we show that pCF10 encodes a peptide inhibitor, iCF10, secreted by donor cells; this inhibitor antagonizes the cCF10 activity in culture filtrates. In order to detect and quantitate iCF10, we developed a reverse-phase high-performance liquid chromatography assay in which the inhibitor peptide elutes separately from the pheromone; this type of assay enabled us to determine that lack of pheromone activity in donor culture filtrates was due to secretion of a mixture of iCF10 and cCF10, rather than abolition of cCF10 secretion. The gene encoding iCF10, *prgQ*, is located on the *Eco*RI-C fragment of pCF10. The open reading frame comprising the *prgQ* gene encodes a 23-amino-acid precursor that resembles a signal peptide. This precursor is cleaved to the mature heptapeptide iCF10 during the secretion process.

Certain plasmids of *Enterococcus faecalis* encode a mating response to specific peptide sex pheromones secreted by potential recipient cells (3, 5). Two kinds of genetic functions have been identified which prevent the host donor cell from self-responding to endogenous pheromone. The *traB* gene of the plasmid pAD1 (1) has been identified as a "pheromone shutdown" gene whose effect is to reduce the amount of active pheromone secreted into the culture medium by pAD1-containing cells; it is not yet known whether the reduction is due to a block in synthesis or to another mechanism, such as degradation or intracellular sequestering. The second mechanism for blocking self-induction of donors is the secretion of a plasmid-encoded pheromone inhibitor, believed to act competitively against any pheromone that might escape the shutdown mechanism. Inhibitor peptides (iAD1 and iPD1, respectively) have been identified in culture filtrates of cells carrying pAD1 (11) and pPD1 (13). The sequence similarity between the pheromones and their respective inhibitors suggests that the inhibitors might be antagonists for the pheromones.

The transfer of the *E. faecalis* tetracycline resistance plasmid, pCF10, is induced by the peptide pheromone, cCF10, excreted from pCF10-free strains (7). The structure of cCF10 was determined to be a heptapeptide (12). Although little or no cCF10 activity is found in culture filtrates of pCF10-containing donor cells, inhibitor activity against cCF10 has not been detected previously. In order to determine conclusively whether pCF10-containing donor cells secrete an inhibitor or shut down extracellular pheromone production, we used high-performance liquid chromatography (HPLC) to separate and quantitate *E. faecalis* peptide pheromones and inhibitors in culture filtrates. We also report here the identification and cloning of a gene from pCF10 called *prgQ* (prg = pheromone

responsive gene) that encodes an inhibitory peptide, iCF10, and show that mature iCF10 is composed of seven amino acid residues and is found in culture filtrates from pCF10-containing donor cells.

(A preliminary report of this study was presented at the 1993 American Society for Microbiology General Meeting in Atlanta, Ga. [17].)

Identification and cloning of *prgQ*, the putative structural gene for iCF10. Kao et al. (10) reported the nucleotide sequence of the *Eco*RI-C fragment of pCF10. Two possible open reading frames (ORFs) are found in the region of pCF10 now designated *prgQ*, as shown in Fig. 1A. One, designated ORF 3 previously (10), is not preceded by a consensus ribosome binding sequence, and because its deduced amino acid sequence (upper line of amino acid sequence in Fig. 1A) is not similar to that of cCF10, it is unlikely to encode an inhibitor. The ORF corresponding to *prgQ* is much shorter but is optimally preceded by a ribosome binding site and a potential −10 and −35 promoter sequence; recent results to be reported separately have confirmed that the latter sequence actually functions as a very strong promoter (2). This ORF encodes a peptide of 23 amino acids (lower line of amino acid sequence in Fig. 1A). The seven residues at the C-terminal end seemed likely to compose iCF10, considering the similarity to the sequence of cCF10 as shown in Fig. 1B. In addition, the organization of this region of pCF10 is very similar to that of a corresponding region of pAD1 which also encodes iAD1 (1, 3, 4, 14, 15, 20).

To confirm that the *prgQ* gene actually encoded an inhibitor, we used PCR (18) to amplify a DNA segment containing *prgQ*. Primers corresponding to bases 1745 to 1760 and the inverse complement of bases 2339 to 2354 shown in Fig. 1A (as indicated by the boxes) were synthesized with an Applied Biosystems automated synthesizer. These primers were used for 30 cycles of PCR (94°C, 30 s; 50°C, 30 s; and 72°C, 2 min, for each cycle), and a 10-min final extension at 72°C). Following amplification, a product of the expected size (~582 bp) was

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A

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       y  n  l  e  q  r  i  q  k  l  v  s  g  i  k  f  m
1601 TAATTTAACTCTTGCCTTATTTGTTTCAGGACAGAACCTATCTTAAACATTTTCTTCCTCCTAATATGTAAAGTATCAAAAAGTAGACCTAAAATTCGAT
                                            ◄ prgX

1701 AAACTACAAAAATTTGTTAATATTTTAATTTTAGGTATTGAATACGACACTCGAAGATGTGTTTATTAAGCTATATCCCTTTTTTTTAAAAAAAAATACA

                    M  L  R  C  F  Y  R  R  G  V  N  E  N  H  S  K  K  T  I  K  I  Y  S  C  C
        -35                  -10                   M  K  T  T  L  K  K  L  S  R  Y  I  A  V
1801 TATTTTAGTTGAAAATATAATACTTAGATGTTAAGATGTTTTTATAGGAGGGGTGTAAATGAAAACCACTCTAAAAAAACTATCAAGATATATAGCTGTT
                                                      prgQ ►

            N  C  N  N  L  N  I  Y  L  I  E  K  I  I  V  T  I  K  Q  I  K  N  R  L  P  *
       V  I  A  I  T  L  I  F  I  *
1901 GTAATTGCAATAACCTTAATATTTATCTGATAGAAAAAATCATAGTAACAATTAAACAAATTAAGAACCGACTGCCATAGGACGGCAATCCTAGGGGGCA

2001 GTTAAACAATTCATGGTATGACATGAACTCTACTCGGTTCTCGTTTGTTGCAACATTAGTTACAACGTATAGTATAACAATTTTTTATGTAAAATTCTAG

2101 ACTTTTTTAAACTCCTTTATTTGTCTAGGAAAAGTTTTTACAGTGAATTGTTTTTATTAGTTGTATAAATGTTGGAGCAGCGGGGAATGTATACAGTTCA
                                                                                         ───────►

2201 TGTATATGTTCCCCGCTTTTTTTGTTGTCTGTTAACTTGTTGAAAAAATAAAAAAACCAAGTAACAAGCACGCTCATTTTCTAAAAATGACCCGCTTGTG
     ◄───────

2301 GAATAATTCAATGCTTTCACGCATTGTTTGTACTGGAACTACTCGTAAGAGCCGTGCAAACAACGTGTTGCATGGCTCTTTTTCTACTTGGTTCAGAAAG

            M  I  E  L  K  A  T  D  L  Q  R  I  T  C  Q  L  T  L  F  N  R  Q  R  F  K  L  Y  N  G  T
2401 TAGGTGAATCAATGATTGAACTGAAAGCAACTGATTTGCAACGTATTACGTGTCAATTGACCTTGTTTAATCGACAAAGATTTAAATTATACAACGGCAC
     prgR ►
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B

cCF10:   Leu-Val-Thr-Leu-Val-Phe-Val

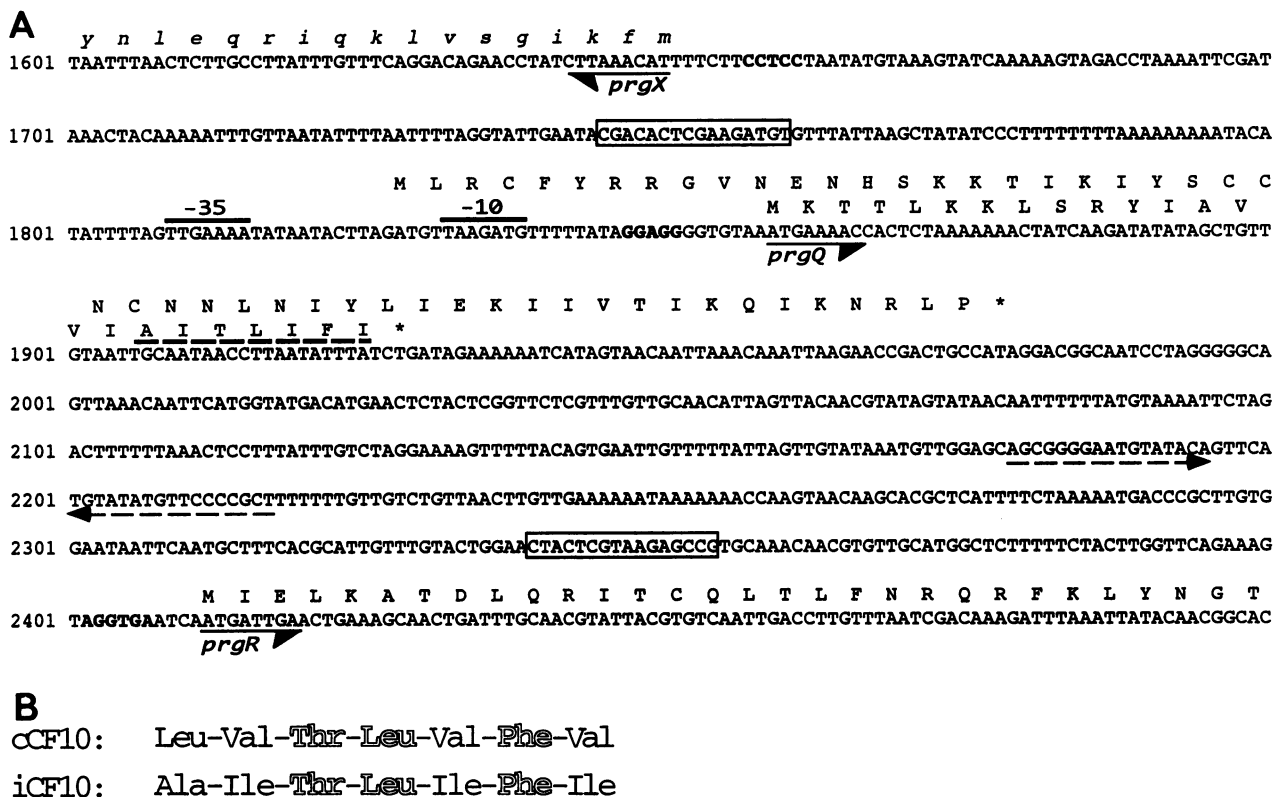iCF10:   Ala-Ile-Thr-Leu-Ile-Phe-Ile

FIG. 1. (A) Nucleotide sequence of the region of pCF10 encoding iCF10. The nucleotide sequence and the nucleotide number are from reference 9. Two different ORFs are encoded in a sequence between prgX and prgR. The start codons of various genes are indicated by arrows. The sequences used to generate the PCR primers for prgQ amplification are indicated by the boxes. The −10 and −35 regions of the prgQ promoter are indicated by the overlines. Possible ribosome binding sequences are shown by bold letters. A pair of dashed arrows show a inverted repeat sequence as a putative downstream transcription terminator. The dashed line highlights the iCF10 amino acid sequence. (B) Comparison between cCF10 and iCF10. Identical residues between cCF10 and iCF10 are noted by open letters.

detected by agarose gel electrophoresis (not shown). The PCR product was cloned into the EcoRV site of pWM401 (21) by blunt-end ligation, and the resulting plasmid, pMSP5011, was transformed into Escherichia coli DH5α and E. faecalis OG1RF. Pheromone and inhibitor activities present in culture filtrates of these strains, as well as those of OG1RF(pCF10), were separated and analyzed as described below.

Production of cCF10 and iCF10 by donor cells. Culture filtrates from OG1RF(pCF10) did not show iCF10 activity, suggesting the possibility that the OG1RF(pCF10) might produce an amount of cCF10 which masked the iCF10 activity. To test this possibility, culture filtrates were subjected to reverse-phase HPLC to achieve physical separation of iCF10 from cCF10. The column was eluted with a gradient of acetonitrile, and the resulting fractions were tested for either iCF10 or cCF10 activity. The cCF10 activity was assayed by observing self-clumping of OG1RF(pCF10) cells exposed to pheromone (6, 12), and iCF10 activity was determined by inhibition of cCF10-inducible self-clumping (see the legend to Fig. 2). A single peak consisting of three successive fractions with retention times ranging from 20.0 to 21.5 min showed the cCF10 activity and a second peak comprising two fractions with 27.0- to 28.0-min retention times showed iCF10 activity. A chemically synthesized peptide possessing the iCF10 sequence as deduced from the nucleotide sequence of prgQ eluted in a single peak of optical density at 220 nm at 27.6 min and showed iCF10 activity at concentrations greater than 0.8 ng/ml (10^{-9}

M), a value higher than observed with either iAD1 (0.25 ng/ml [11]) or iPD1 (0.08 ng/ml [13]). Synthetic cCF10 eluted from the same column at 21 min. These results indicated that OG1RF(pCF10) excretes iCF10 as a heptapeptide with the sequence H-Ala-Ile-Thr-Leu-Ile-Phe-Ile-OH. They also suggested that the apparent lack of either iCF10 or cCF10 activity in crude culture filtrates of this strain is because such filtrates actually contain a mixture of the two peptides. As shown in Fig. 1B, three of seven residues are conserved between iCF10 and cCF10.

Antagonism cCF10 by iCF10. Table 1 shows the minimum concentration of iCF10 required for inhibition of various amounts of cCF10. The concentration of iCF10 required for inhibition varied directly in proportion to the concentration of cCF10 used in the assays. This result suggests that iCF10 is a competitive inhibitor of cCF10. The ratio of iCF10/cCF10 required for inhibition is 8 to 16. In contrast, the corresponding ratio in the iAD1/cAD1 system is 2 (4, 11).

Quantitation of the amount of cCF10 and iCF10 in culture filtrates of various strains. By the HPLC method described in Fig. 2, the absolute amount of both cCF10 and iCF10 in culture broth could be measured independently. Table 2 shows the concentrations of cCF10 and iCF10 in 3-h (early log phase) cultures from various strains. These values were estimated by comparing the pheromone or inhibitor activity of the separated fractions with that of the corresponding synthetic peptides. OG1RF(pCF10) produced 640 pg of iCF10 per ml. This result

TABLE 1. Minimum amount of iCF10 required for inhibition of
self-clumping induced by various amounts of cCF10

Concn (pg/100 μl)		iCF10/cCF10 ratio
cCF10[a]	iCF10[b]	
10	80	8
20	320	16
40	640	16
80	640	8
160	1,280	8
320	2,560	8
640	5,120	8

[a] Concentration of cCF10 in the microtiter plate well.
[b] Concentration of iCF10 in the well required to block response to cCF10.

is consistent with the observation that iCF10 activity was not
detected in crude culture filtrates of OG1RF(pCF10); iCF10
showed no activity at concentrations lower than 800 pg/ml.
OG1RF(pMSP5010) produced much more iCF10 than
OG1RF(pCF10). This might be caused by the difference in
copy number; pWM401 is a multicopy plasmid (21), while
pCF10 is of lower copy number (7). The amount of cCF10 in
OG1RF(pCF10) culture filtrates was same as that of plasmid-
free OG1RF, indicating that pCF10 does not abolish secretion
of pheromone into the culture medium by its host cell. In
addition, E. coli DH5α cells carrying pMSP5011 secrete an
amount of iCF10 into the culture fluid sufficient to cause an
eightfold reduction in the pheromone titer of synthetic cCF10,
whereas the corresponding culture fluids of isogenic cells
carrying only the vector have no inhibitor activity. We also used
site-directed mutagenesis to change the prgQ sequence to one
encoding cCF10 rather than iCF10 in the same shuttle vector
system, and we found that crude culture filtrates of either E.
faecalis OG1RF or E. coli DH5α carrying this mutated prgQ
derivative have pheromone activity but no inhibitor activity. A
similar experiment in which PCR amplification of prgQ was
carried out with a primer that resulted in an adenine-for-
thymine substitution in the final codon of the prgQ ORF, thus
changing the C-terminal Ile to Asn, generated a prgQ deriva-
tive that encoded neither pheromone nor inhibitor activity.
Taken together, these results strongly indicate that the expres-
sion of prgQ is responsible for the observed inhibitor activity in
cells carrying either wild-type pCF10 or the cloned prgQ gene.

The data presented here demonstrate that the prgQ gene of
pCF10 encodes a heptapeptide, iCF10, with inhibitory activity
against the chromosomally encoded peptide pheromone,
cCF10. The fact that E. coli transformed with the cloned prgQ
gene excreted iCF10 suggests that the prgQ promoter is
functional in this host and that the processing system that
generates iCF10 from the precursor is not specific for this
peptide. It is possible that the 16-amino-acid fragment re-
moved from the precursor of iCF10 might function as a signal
peptide. However, the six-amino-acid hydrophobic region of
this peptide is likely to be too short to span the membrane,
since a length of seven or more amino acids has been shown to
be required for this function in E. coli (9, 19) and the
corresponding region in signal peptides of gram-positive or-
ganisms is even longer (8). Thus, the entire precursor might act
as a signal peptide in traversing the membrane, with subse-
quent cleavage by a membrane-associated or extracellular
protease to generate the mature inhibitor.

It has been consistently observed that acquisition of a
plasmid encoding a response to a particular pheromone results
in a decrease in the pheromone activity secreted by E. faecalis
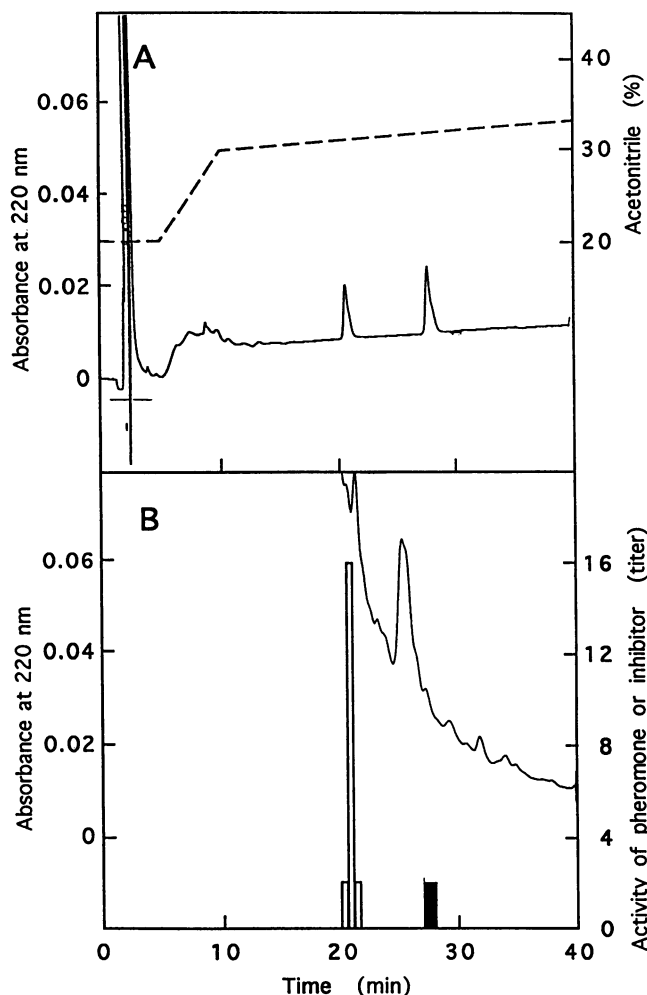

FIG. 2. Separation of iCF10 and cCF10 by reverse-phase HPLC.
HPLC was performed on a SSC-ODS-262 column (0.6 by 10 cm;
Senshukagaku) with a gradient of acetonitrile (dotted line) in 0.1%
trifluoroacetic acid at a flow rate of 1 ml/min. (A) Chromatography of
chemically synthesized iCF10 and cCF10. (B) Chromatography of
partially purified culture filtrate from OG1RF(pCF10). Five-hour
culture filtrates (10 ml from cultures grown in Oxoid Nutrient Broth
No. 2 supplemented with 0.2% glucose and 0.1 M Tris-HCl, pH 7.7) of
E. faecalis OG1RF(pCF10) was passed through an Amberlite XAD-7
column (1 ml; Rohm and Haas) and eluted with 5 ml of 80% ethanol.
The eluate was evaporated and then redissolved in 400 μl of 50%
dimethylsulfoxide, and 100 μl of this solution was applied to HPLC.
Fractions of the eluate were collected every 0.5 min. Each fraction was
bioassayed for iCF10 and cCF10 activity by the microtiter dilution
method described in reference 5. iCF10 activity was assayed in the
presence of 100 pg of cCF10 per ml. In both assays, visible clumping of
OG1RF(pCF10) cells after 2 h of incubation with the pheromone/
inhibitor preparations was used as an indicator of pheromone activity.
The vertical open bar shows cCF10 activity, and the closed bar shows
iCF10 activity.

cells (1, 3, 5). However, it has not been possible in previous
studies to determine conclusively whether this decrease was
due to a reduction in pheromone synthesis or secretion, to
increased inhibitor secretion, or to both. The development of a
quantitative HPLC method to separate pheromones and in-
hibitors was important in sorting out these phenomena in the
pCF10 system, as well as in resolving some apparent discrep-

TABLE 2. Amount of iCF10 and cCF10 in culture filtrates from
various strains

Strain	Concn (pg/ml)	
	cCF10[a]	iCF10[b]
OG1RF	16	ND[c]
OG1RF(pCF10)	16	640
OG1RF(pWM401)	16	ND[c]
OG1RF(pMSP5011)	16	15,360

[a] Estimated concentration of cCF10 in culture filtrate based on comparison of pheromone titer in culture filtrates with the titer of synthetic pheromone at various concentrations.

[b] Estimated concentration of iCF10 in culture filtrate based on comparison of the inhibitor titer of culture filtrates with that of synthetic inhibitor at various concentrations.

[c] ND = none detected; limit of detection is 80 pg/ml in this assay.

ancies between the pCF10 inhibitor/pheromone system and those associated with other *E. faecalis* plasmids. Because pCF10-containing donor cells excrete a mixture of iCF10 and cCF10, and the specific activity of iCF10 is lower than that of iPD1 or iAD1, iCF10 activity was not initially detected in culture filtrates in spite of the fact that these cells actually excrete amounts of inhibitor similar to that produced by cells carrying pPD1 or pAD1. Recently, An et al. cloned a pheromone shutdown gene coded by pAD1, designated *traB*, and found that TraB protein is highly homologous to PrgY protein encoded on pCF10 (16). However, the results of the present study show that pCF10 does not shut down the production of extracellular cCF10. Further investigation of the expression and genetic functions of *prgY* are required to explain these differences.

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