JOURNAL OF BACTERIOLOGY, Apr. 1995, p. 2107–2117 Vol. 177, No. 8
0021-9193/95/$04.00+0


# Genetic Analysis of a Region of the *Enterococcus faecalis* Plasmid pCF10 Involved in Positive Regulation of Conjugative Transfer Functions

JUNGWON W. CHUNG,† BARBARA A. BENSING, AND GARY M. DUNNY*

*Institute for Advanced Studies in Biological Process Technology and Department of Microbiology, University of Minnesota, St. Paul, Minnesota 55108*

Received 25 July 1994/Accepted 10 February 1995

**The *prgB* gene encodes the surface protein Asc10, which mediates cell aggregation resulting in high-frequency conjugative transfer of the pheromone-inducible tetracycline resistance plasmid pCF10 in *Enterococcus faecalis*. Previous Tn*5* insertional mutagenesis and sequencing analysis of a 12-kb fragment of pCF10 indicated that a region containing *prgX*, *-Q*, *-R*, *-S*, and *-T*, located 3 to 6 kb upstream of *prgB*, is required to activate the expression of *prgB*. Complementation studies showed that the positive regulatory region functions in *cis* in an orientation-dependent manner (J. W. Chung and G. M. Dunny, Proc. Natl. Acad. Sci. USA 89:9020–9024, 1992). In order to determine the involvement of each gene in the activation of *prgB*, Tn*5* insertional mutagenesis and exonuclease III deletion analyses of the regulatory region were carried out. The results indicate that *prgQ* and *-S* are required for the expression of *prgB*, while *prgX*, *-R*, and *-T* are not required. Western blot (immunoblot) analysis of these mutants shows that *prgQ* is also essential for the expression of *prgA* (encoding the surface exclusion protein Sec10), which is located between *prgB* and the positive-control region. Complementation analysis demonstrates that a *cis*-acting regulatory element is located in the *prgQ* region and that pCF10 sequences in an untranslated region 3′ from *prgQ* are an essential component of the positive-control system. Analyses of various Tn*5* insertions in pCF10 genes suggest that transcription reading into this transposon is terminated in *E. faecalis* but that outward-reading transcripts may initiate from within the ends of Tn*5* or from the junction sequences.**

*Enterococcus faecalis* strains often carry conjugative plasmids whose transfer functions can be induced by small specific peptide pheromones secreted by potential recipient cells (13). In response to pheromones, donor cells express several transfer gene products, including aggregation substance. This cell surface adhesin promotes cellular aggregation between donor and recipient cells in broth culture, enabling high-frequency transfer of the conjugative plasmids (8, 11, 16, 36).

Plasmid pCF10 is a 58-kb conjugative plasmid encoding tetracycline resistance and whose transfer functions are inducible by the heptapeptide pheromone cCF10 (4). Genes involved in pheromone-inducible conjugation have been localized to a 25- to 30-kb region of pCF10 (4). Subcloning and Western blot (immunoblot) analyses demonstrated that the adjacent *Eco*RI C (7.5-kb) and *Eco*RI E (4.5-kb) fragments of pCF10 (see Fig. 1) encode two cell surface proteins, Asc10 and Sec10 (5). Asc10, encoded by *prgB*, is a 150-kDa aggregation substance protein (5, 28). Sec10, encoded by *prgA*, is a 130-kDa protein involved in surface exclusion, which reduces plasmid transfer between donor cells (17). The production of Asc10 is inducible by cCF10 in cells carrying wild-type pCF10 but constitutive in cells harboring pINY1801 (see Fig. 1) which contains the *Eco*RI C and E fragments of pCF10 cloned in shuttle vector pWM402 (4). In contrast to *prgB*, expression of *prgA* and production of Sec10 in wild-type donors occurs in the absence of pheromone; however, there is evidence that the level of expression of this protein may increase with pheromone induction (17). A negative-control region, which represses the expression of Asc10 in the absence of pheromone in wild-type pCF10, is located to the left of the *Eco*RI C fragment (4, 19).

Tn*5* insertional analysis demonstrated that expression of the *prgB* gene is positively controlled by a region located 3 to 6 kb upstream from *prgB* (22). Sequence analysis (22) indicated that the regulatory region contains several open reading frames designated *prgX*, *-Q*, *-R*, *-S*, and *-T* (see Fig. 1). Certain Tn*5* insertions in the *prgR* and *prgS* region completely eliminated the expression of *prgB* but did not abolish the expression of *prgA* (22). In addition, Tn*5* insertions in *prgA*, which is located between this regulatory region and *prgB*, did not abolish the expression of *prgB* (5, 22). Transcriptional analysis of *prgA* and *prgB* indicated that both genes are transcribed monocistronically and that the transcription of *prgB* is inducible in *E. faecalis* cells carrying pCF10 (6). Complementation studies in which several different segments of the regulatory region were cloned in a high-copy-number plasmid and assayed for their ability to activate the expression of a *prgB*::*lacZ* transcriptional fusion demonstrated that the regulatory region does not function in *trans* and that at least one *cis*-acting element is located in a region spanning *prgX* and *prgQ* (6). Analysis of the positive regulatory region in *cis* with the *prgB*::*lacZ* target gene showed that it functions in an orientation-dependent manner (6).

Because of the lethal effect of Tn*5* insertions in the *prgX* gene in an *E. faecalis* background and the lack of any transposon insertions in the *prgQ* and *prgT* genes (22), it could not be determined which specific upstream control genes were directly involved in the regulation of the expression of *prgB*. The predicted structures of *prgX*, *prgR*, and *prgS* gene products showed no significant homology with other known regulatory molecules (22). The *prgQ* gene encodes a specific peptide inhibitor, iCF10 (26) which, by analogy to the pAD1 system (9,

* Corresponding author. Mailing address: Bioprocess Technology Institute, 240 Gortner Laboratories, 1479 Gortner Ave., St. Paul, MN 55108. Phone: (612) 626-1217. Fax: (612) 625-1700. Electronic mail address: gary-d@molbio.cbs.umn.edu.

† Present address: Channing Laboratory, Boston, MA 02115.

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description[a] | Reference(s) or source |
|---|---|---|
| *E. coli* DH5α | F′ ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *recA1 hsdR17* ($r_K$ $m_K$) *supE44*$^+$ *thi-1 gyrA relA1* | Bethesda Research Laboratories |
| *E. faecalis* | | |
| INY3000 | Str$^r$ Spc$^r$ Tet$^r$ Ebs$^-$ | 35 |
| OG1RF | Rif$^r$ Fus$^r$ | 14 |
| Plasmids | | |
| pCF10 | Tc$^r$, 58-kb conjugative plasmid | 14 |
| pDL414 | Km$^r$, 5.7-kb multicopy streptococcal plasmid | 23 |
| pUT2100 | Cm$^r$ Ery$^r$, 16.8-kb *Bacillus* plasmid with temperature-sensitive replicon | 7 |
| pWM402 | Cm$^r$ Tc$^r$ (*E. coli*), Cm$^r$ (*E. faecalis*), 11.6-kb shuttle vector | 37 |
| pINY1801 | *Eco*RI C and E fragments of pCF10 cloned in the natural orientation into *Eco*RI site of pWM402 | 4 |
| pINY1825 | *Eco*RI C fragment of pCF10 cloned into *Eco*RI site of pWM402 | 4 |
| pINY4501, -4502, -4508, -4509, -4510, -4512, -4518, -4520, -4527, -4550, and -4560 | pINY1801::Tn*5* derivatives | 21 |
| pINY8101 | 3.3-kb *Sma*I fragment of pUT2100 carrying *lacZ* cloned into *Pst*I site of pINY1825 | 7 |
| pINY8104 | 1.65-kb *Sca*I fragment deleted from pINY8101 | 7 |
| pINY8109 | 8.7-kb *Xba*I-*Eco*RI fragment of pINY8101 cloned into *Eco*RV site of pWM402 | 7 |
| pINY8301, -8319, -8321, -8322, -8324, -8325, -8326, -8329, -8338, -8341, -8343, and -8348 | Plasmids containing deletions in *prgQ* and in its putative promoter region, generated by Exo III from pINY8104 | This study |
| pINY8351, -8353, -8356, -8359, -8364, and -8365 | Plasmids containing deletions in *prgT* and in its putative promoter region, generated by Exo III from pINY4527 | This study |
| pINY8402 | 4.2-kb *Eco*RI-*Pvu*II fragment of pINY1825 ligated with 5.3-kb *Eco*RI-*Hin*cII fragment of pDL414 | 7 |
| pINY8503, -8508, -8513, -8534, -8556, and -8588 | pINY8301::Tn*5* derivatives | This study |
| pINY4501 to -4560 | pINY1801::Tn*5* derivatives | 18, 19 |
| pINY4508Z | 3.3-kb *Bam*HI fragment of pUT2100 containing *lacZ* cloned into *Sma*I site of pINY4508 | This study |
| pINY4501Zs, -4503Zs, -4508Zs, -4509Zs, -4510Zs, -4518Zs, -4527Zs, -4551Zs | 5.7-kb *Xho*I fragment containing the *neo*::*lacZ* fusion of pINY4508lacZ ligated with 2.5-kb *Xho*I fragment of pINY4501, -4503, -4508, -4509, -4510, -4518, -4527, and -4551, respectively (*neo*::*lacZ* cloned in the same orientation as that of *prgB*) | This study |
| pINY4501Zo, -4503Zo, -4508Zo, -4509Zo, -4510Zo, -4518Zo, -4527Zo, -4551Zo | 5.7-kb *Xho*I fragment containing the *neo*::*lacZ* fusion of pINY4508lacZ ligated with 2.5-kb *Xho*I fragment of pINY4501, -4503, -4508, -4509, -4510, -4518, -4527, and -4551, respectively (*neo*::*lacZ* cloned in the orientation opposite that of *prgB*) | This study |

[a] Ebs, enterococcal binding substance; Cm$^r$, chloramphenicol resistance; Fus$^r$, fusidic acid resistance; Rif$^r$, rifampin resistance; Str$^r$, streptomycin resistance; Spc$^r$, spectinomycin resistance; Tet$^r$, tetracycline resistance.

10, 24), may prevent induction of donor cells by other donor cells carrying pCF10 or block self-induction by donor cells. The amino acid sequence of *prgT* showed some similarity (25 to 30%) to the *hutP* gene product of *Bacillus subtilis* which activates transcription of genes involved in utilization of histidine (27) and is also highly similar to a pAD1 sequence of unknown function called *orfY* (36).

In order to determine the involvement of these genes in positive control of *prgB* expression, additional Tn*5* insertional mutations in the regulatory region were characterized phenotypically, and the precise locations of insertions were determined by DNA sequencing. By using in vitro deletion analysis techniques, mutations in *prgX* and *prgT* were obtained. The effects of several of these mutations on the expression of the *prgA* and *prgB* genes were examined. The results presented here demonstrate that *prgX*, *-R*, and *-T* are not required for the activation of *prgB* and that the putative promoter region of *prgQ* and several hundred base pairs of DNA sequences downstream of the coding region of *prgQ* are directly involved in the expression of *prgB* and also of *prgA*. These data also confirm that the *prgS* region is essential for the expression of *prgB*. In addition, some of the genetic results suggest that Tn*5* or a junction sequence generated by Tn*5* insertion might function as an active outward-reading promoter to enhance transcription of flanking pCF10 DNA. In support of this notion, we present analysis of transcription reading into and out from Tn*5* insertions.

## MATERIALS AND METHODS

**Bacterial strains, media, and reagents.** Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium (32), and *E. faecalis* strains were grown in M9-YE medium (17). The concentrations of antibiotics in selective media are the following: tetracycline, 10 μg/ml; chloramphenicol, 15 μg/ml; and neomycin, 200 μg/ml. Restriction enzymes, exonuclease

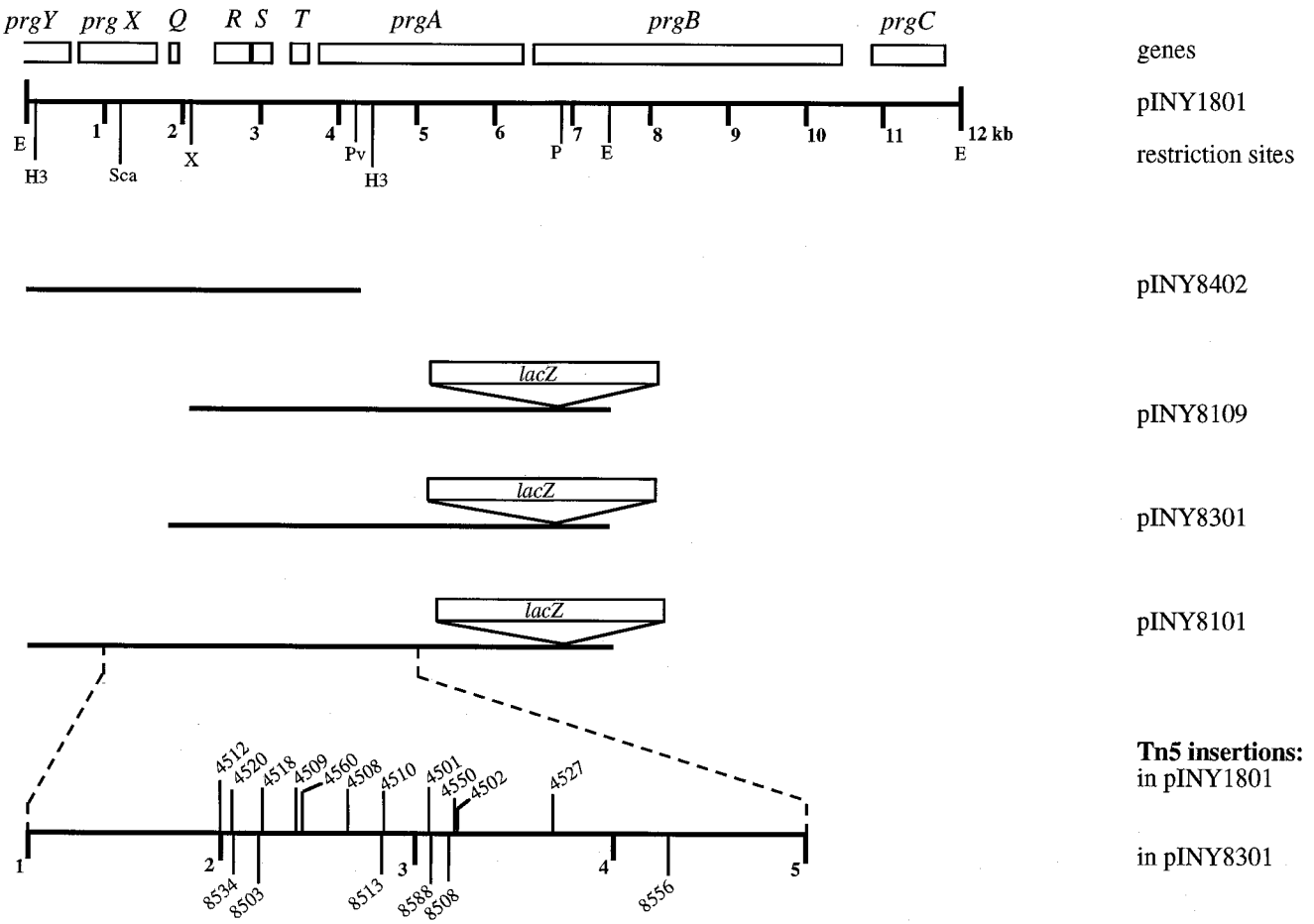


FIG. 1. Locations of insertions, deletions, subclones, and gene fusion on the genetic and physical map of the region of pCF10 containing positive regulatory genes and adjacent DNA. (Top) Linear map of the contiguous *Eco*RI C and E fragments of pCF10 containing the genes discussed in this article. Specific genes identified previously (22) by DNA sequencing and other analyses are indicated on the top line. The heavy line near the top represents the pCF10 DNA cloned in pINY1801 (5); the numbers refer to distance in kilobases. Restriction enzyme cleavage sites used for generating some constructs used in this study are designated as follows: E, *Eco*RI; Sca, *Sca*I; X, *Xba*I; Pv, *Pvu*II; P, *Pst*I; H3, *Hin*dIII. Subcloned fragments of pCF10, some containing a *prgB*::*lacZ* transcriptional fusion described previously (6) and in Materials and Methods, are shown below pINY1801. (Bottom) Positions of various Tn*5* insertions. Note that the scale has been expanded, and the numbers on the heavy line correspond to the same numbers on the kilobase scale depicted at the top of the figure in pINY1801. pINY designations are given above and below the heavy line. The exact positions of these insertions as determined by sequencing are shown in Fig. 2.

III (Exo III), DNA polymerase Klenow fragment, nuclease S1, T4 polynucleotide kinase, and T4 DNA ligase were from Gibco/Bethesda Research Laboratories. Sequenase and other sequencing reagents were from United States Biochemical Corp. [$\alpha$-$^{35}$S]dATP and [$\gamma$-$^{32}$P]ATP were acquired from Amersham. Oligonucleotide primers (15- to 17-mers) were synthesized with an Applied Biosystems 391 automated synthesizer.

**Plasmid and strain constructions.** Plasmid pINY1825 contains the *Eco*RI C fragment of pCF10 subcloned into the *Eco*RI site of the shuttle vector pWM402 (5). Plasmid pINY1801 contains the *Eco*RI C and E fragments in the same site (5). Plasmid pINY8101 (Fig. 1) contains a *prgB*::*lacZ* fusion where a promoterless *lacZ* gene (furnished with the ribosomal binding site of the *spo0V* gene) was inserted into the unique *Pst*I site of pINY1825 which is located 222 bp downstream of the start codon of *prgB* (6). The cloned pCF10 DNA segments contained in various recombinant plasmids analyzed in the present study are indicated in a linear map in Fig. 1, along with the positions of Tn*5* insertions. Initial cloning was carried out in an *E. coli* background, and then the purified plasmids were introduced into *E. faecalis*. Transformation of *E. coli* was achieved by electroporation (12). Transformation of *E. faecalis* was also done by electroporation as detailed previously (15) with the following modifications. One to five microliters of plasmid DNA (1 to 3 $\mu$g) was mixed with 30 $\mu$l of the competent cells in a BioRad Gene Pulser cuvette (0.2 cm wide). The mixture was incubated on ice for 10 min and electroporated at 2.0 kV and 200 $\Omega$. The plasmid content of the transformants was confirmed by restriction enzyme digestion and analysis of the digested DNA by agarose gel electrophoresis.

**Deletion of *prgX* and *prgT*.** Deletion of the 3′ end of the structural gene of *prgX* was accomplished by removing the 1.65-kb *Sca*I fragment from pINY8101 extending from within *prgX* into vector sequences (Fig. 1), generating plasmid pINY8104. This plasmid was linearized by *Sca*I digestion and subjected to deletion, in vitro, by using Exo III and the following procedure. Seventy-five units of Exo III per picomole of DNA was used to delete approximately 30 bp/min at 30°C from each end of the linearized pINY8104. After an 8-min incubation, 3-$\mu$l aliquots of the reaction mixture were taken at 1-min intervals for 8 min to vary the extent of Exo III digestion and treated as detailed previously (32). Transformants were screened by colony hybridization (32) with end-labelled (32) oligonucleotides A, B, and C (Fig. 2) as probes. Colonies which hybridized with probe C but not with probe A were chosen for further analysis. Colony hybridization with probe B gave an estimation of the endpoint of the deletion. After the plasmid contents of these transformants (pINY8301 to -8348) were confirmed by *Hin*dIII and *Xba*I double digestion, plasmids containing deletions of approximately 0.9 kb were sequenced to identify the endpoints of the deletions; these plasmids are shown in Fig. 2 and were given pINY designations between 8301 and 8348.

Plasmid pINY4527 contains Tn*5* between *prgT* and *prgA* (Fig. 1). To delete *prgT*, pINY4527 was linearized by *Sma*I digestion and subjected to deletion by Exo III as described above, except that 150 U of Exo III per pmol of DNA was used. Digestion with Exo III was carried out for 40 min to allow all (2.4 kb) of the DNA sequence between *prgT* and the *Sma*I site of Tn*5* to be deleted before removing aliquots of the reaction mixture at 1-min intervals. Transformants were screened by colony hybridization with end-labeled oligonucleotides D, E, and F (Fig. 2) as probes. Colonies which hybridized with probes D and F but not with probe E were chosen for further analysis. Plasmids with appropriate deletions were identified by *Hin*dIII restriction enzyme mapping and DNA sequencing; the endpoints of these deletions are shown in Fig. 2 and given pINY designations between 8351 and 8364.

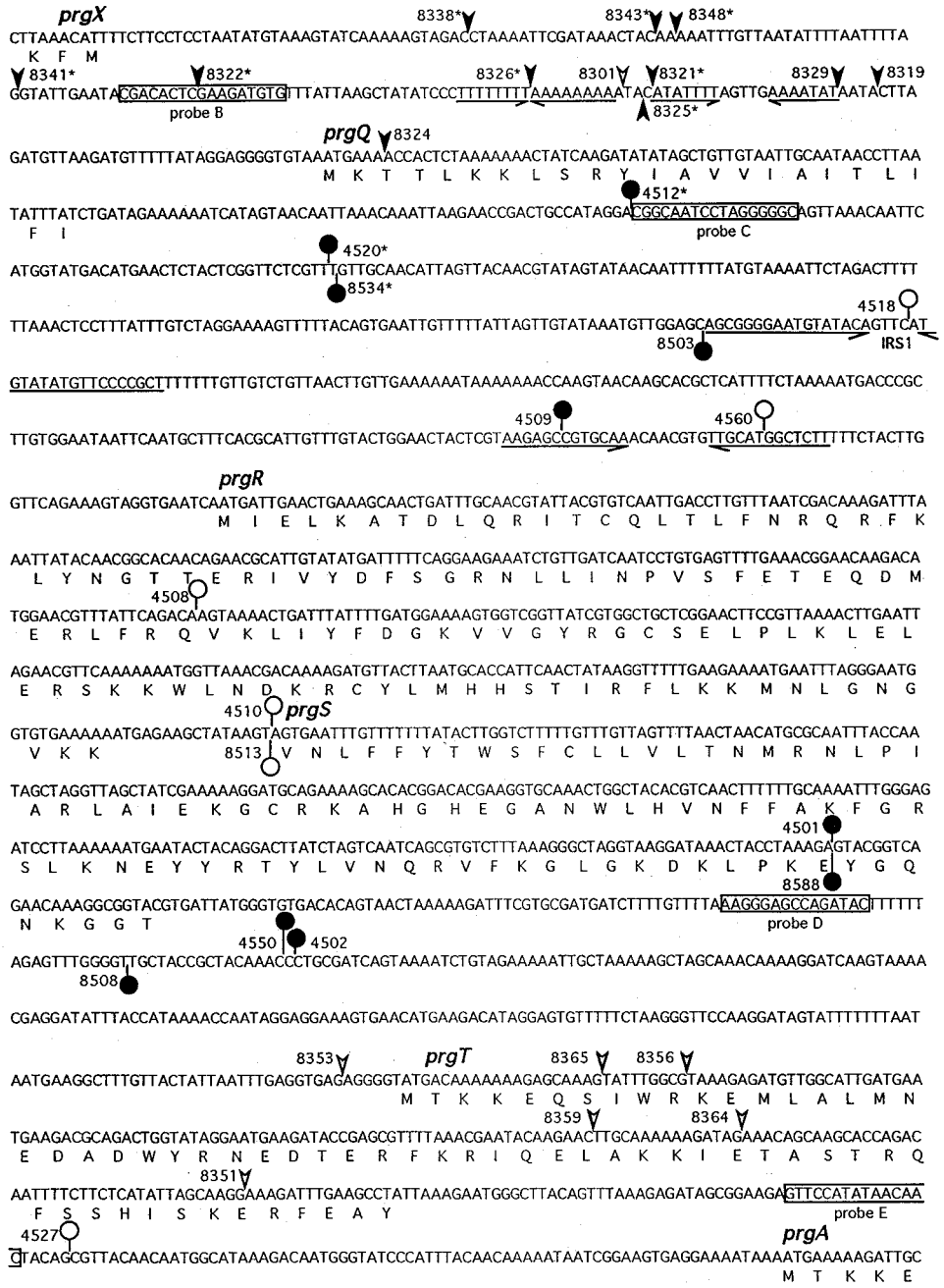


FIG. 2. Sequencing analysis of Tn*5* insertion sites or deletion endpoints of subcloned pCF10 segments carrying mutations in the positive-control region. Sequences between the start codon of *prgX* and the start codon of *prgA* are shown with the amino acid sequences of the *prgQ*, *-R*, *-S*, and *-T* genes. The locations of Tn*5* insertion sites are indicated by the markers ♀ and ♀ for the derivatives of pINY1801::Tn*5*; ♁ and ● for the derivatives of pINY8301::Tn*5*). pINY designations are given. The plasmids marked with asterisks were unstable in enterococci. Inverted repeat structures were indicated by arrows. Oligonucleotide probes used for colony hybridization analysis were shown in boxes except probes A and F. Probe A was made of the sequences between the 97th and 104th codons of *prgX* (to the left of the region shown in the figure). Probe F was made of the sequences between the 27th and 34th codons of *prgA*. The arrowheads in the top portion of the figure show the deletion endpoints of the plasmids where deletion was generated in the *prgX* region with pINY8104; pCF10 sequences to the left of each arrow are deleted in these plasmids. The arrowheads in the lower portion show the endpoints of the plasmids where deletions were generated in the *prgT* region with pINY4527 (see Materials and Methods); pCF10 sequences to the right of each arrow and to the left of the Tn*5* insertion site of pINY4527 were deleted, as well as sequences within Tn*5*. All open figures indicate normal expression, and filled figures indicate abolished or greatly reduced expression of *prgB* or *prgB*::*lacZ*.

**Insertion mutagenesis with Tn*5*.** Tn*5* insertional mutations in plasmid pINY8301 (Fig. 2) were generated as described previously (5). Plasmids containing Tn*5* insertions in the regulatory region were identified by a band shift of the 4.5-kb *Hin*dIII fragment which contains *prgQ*, *-R*, *-S*, and *-T*. Tn*5* insertion mutations in plasmid pINY1801 were generated and introduced into *E. coli* DH5α strains in an earlier study (21). This library was further screened, and plasmids (pINY4520, -4518, -4560, -4550, and -4527; Fig. 1) with Tn*5* insertions in the regulatory region were identified. Sequencing analysis was performed to determine the precise location of the Tn*5* insertions, and the precise positions are indicated in Fig. 2.

In order to study the effects of the Tn*5* insertions in the regulatory region on the expression of *prgB*, three different approaches were taken. Derivatives of pINY1801 containing Tn*5* were initially introduced into *E. faecalis* OG1RF, and the formation of cell aggregates in broth culture was examined. *E. faecalis*

INY3000 contains insertional mutations in the chromosome that abolish expression of enterococcal binding substance (EBS), which acts as the recipient receptor for the aggregation substance produced by donor cells (16, 35). The $EBS^-$ phenotype of strain INY3000 facilitates Asc10 extraction from the cell membrane. Thus, certain plasmids were introduced into INY3000 in order to analyze the production of Asc10 (the protein product of *prgB*) by Western blotting, as described below. Derivatives of pINY8301::Tn*5* (containing a *prgB*::*lacZ* transcriptional fusion) were introduced into OG1RF, and β-galactosidase activity was measured as described below.

**Determination of Tn*5* insertion sites by DNA sequencing.** Primers were made from DNA sequences located approximately 50 to 150 bp upstream or downstream of the Tn*5* insertion sites in the derivatives of plasmids pINY1801::Tn*5* and pINY8301::Tn*5*, as estimated by restriction enzyme digestion mapping. These plasmids were used as templates in sequencing reactions. Sequence analysis of both ends of Tn*5* at the junction sites indicated a duplication of a 9-bp segment flanking each Tn*5* insert, as previously demonstrated (2).

**Construction of *neo*::*lacZ* fusions in pINY1801::Tn*5* derivatives.** Plasmid pINY4508 which contains Tn*5* in *prgR* (Fig. 1) was linearized by *Sma*I restriction enzyme digestion. A unique *Sma*I restriction enzyme site is located within the *neo* gene of Tn*5*. The 3.3-kb blunted *Bam*HI fragment of pUT2100 containing a promoterless *lacZ* gene was cloned into the unique *Sma*I site of pINY4508 in the same orientation as that of the *neo* gene, generating pINY4508Z. This plasmid was then digested with *Xho*I, and the 8-kb fragment containing the *neo*::*lacZ* fusion was purified. Derivatives of pINY1801::Tn*5* (pINY4518, -4509, -4560, -4508, -4510, -4501, -4527, and -4503) were cut by *Xho*I and ligated with the 8-kb *Xho*I fragment. This resulted in plasmids containing the *Xho*I fragment in both orientations generating pINY1801::Tn*5*Zs (the *neo*::*lacZ* fusion is located in the same orientation as that of *prgB*) or pINY1801::Tn*5*Zo (the *neo*::*lacZ* fusion is located in the orientation opposite that of *prgB*); these constructs are listed in Table 1 and further described below in the text. Plasmids were introduced into *E. faecalis* OG1RF, and β-galactosidase activities of transformants were measured as described below.

**β-Galactosidase assay.** β-Galactosidase activity was measured as described previously (6). Each culture was assayed in duplicate, and reported values were averaged from at least two independent experiments. Variations in activities were less than 5%.

**Western blot analysis.** Preparation of surface proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were conducted as described previously (19). Proteins were transferred to a nitrocellulose blot in Tris-glycine buffer at 50 V for 2 h. The blot was developed by using a polyclonal antiserum raised against whole, pheromone-induced *E. faecalis* cells containing pCF11 which is a spontaneous mutant of pCF10 (34) that produces copious amounts of Asc10 and Sec10. To confirm that an equivalent amount of proteins was loaded in each lane, a duplicate gel was stained by the silver staining method (32) and the band intensities of proteins in each lane were compared (data not shown).

**Nucleotide sequence accession number.** The sequence of the region of pCF10 described in this article is deposited in GenBank under accession number M64978.

## RESULTS

**Deletion analysis of the *prgX* structural gene and its promoter region.** Mutations in *prgX* previously generated by Tn*5* insertions were lethal in an *E. faecalis* background (22). In order to determine the involvement of *prgX* in the activation of *prgB*, it was necessary to construct a stable mutation in *prgX*. A deletion mutation in *prgX* was generated by removing a *Sca*I fragment from pINY8101 (Fig. 1) which includes the 3′ end of *prgX* and 200 bp of vector DNA (see Materials and Methods). This partial deletion of the 3′ end of *prgX* (pINY8104) was unstable in *E. faecalis*, although it was maintained stably in *E. coli*. The remaining coding region and the promoter region of *prgX* in pINY8104 was then subjected to further deletion with Exo III (see Materials and Methods). Although deletion constructs pINY8338, -8343, -8348, -8341, -8322, and -8326 (Fig. 2) were also unstable in *E. faecalis*, pINY8301 (Fig. 2), a *prgB-lacZ* fusion plasmid which lacks the entire structural gene of *prgX* and an additional 147 bp upstream from the *prgX* start codon (containing the putative *prgX* promoter region), was maintained stably in *E. faecalis*. As shown in Table 2, the same level of β-galactosidase activity was detected in strain OG1RF(pINY8301) as was previously observed in the isogenic strain carrying pINY8101 (6), indicating that *prgX* is not required for the expression of *prgB*.

Interestingly, deletion of 2 or 3 bp toward the *prgQ* gene from the deletion endpoint of pINY8301 (pINY8325 and -8321; Fig. 2) resulted in instability in *E. faecalis*, whereas further deletion of 22 bp from the deletion endpoint of pINY8301 (pINY8329; Fig. 2) restored stability of the plasmid. The OG1RF strain carrying pINY8329, however, produced no β-galactosidase activity, showing that this 22-bp segment is essential for the expression of *prgB*; on the basis of additional experiments described below and in the accompanying article (7), the 22-bp deletion in pINY8329 likely affects the integrity of the *prgQ* promoter region. In order to test whether expression of β-galactosidase activity in OG1RF(pINY8329) could be activated in *trans*, pINY8402 (Fig. 1) carrying the entire regulatory region including *prgX*, *-Q*, *-R*, *-S*, and *-T* on the high-copy-number vector pDL414 (6) was introduced into OG1RF(pINY8329) by electroporation. No β-galactosidase activity (less than 1 Miller unit) was detected in OG1RF harboring pINY8329 and pINY8402, suggesting that the failure of pINY8329 to express *prgB* is due to the lack of a *cis*-acting sequence.

TABLE 2. Locations of deletions or Tn*5* insertions in pINY8301 and the effects of each insertion on the expression of the *prgB*::*lacZ* fusion[a]

| Plasmid | Mutation | Position of mutation | % of wild-type β-galactosidase expression |
|---|---|---|---|
| pINY8301 | None (wild-type construct) | | 100 |
| pINY8329 | Deletion | *prgQ* promoter | 4 |
| pINY8534 | Tn*5* insertion | 3′ from *prgQ* | 4 |
| pINY8503 | Tn*5* insertion | 3′ from *prgQ* | 4 |
| pINY8513 | Tn*5* insertion | 5′ from *prgS* | 100 |
| pINY8588 | Tn*5* insertion | *prgS*, near 3′ end | 37 |
| pINY8508 | Tn*5* insertion | 3′ from *prgS* | 12 |
| pINY8556 | Tn*5* insertion | *prgA* | 87 |

[a] Each one of these plasmids contains the identical *prgB*::*lacZ* transcriptional gene fusion, as described in Materials and Methods. The plasmids listed were derived by generation of mutations as described in the text. The positions of these mutations are shown in Fig. 1 and 2. Isogenic strains carrying each plasmid were grown under identical conditions and assayed for β-galactosidase activity as described in Materials and Methods. Under these conditions, the wild-type construct, pINY8301, produced 104.5 Miller units of activity, defined as the 100% level; the relative levels of activity of strains carrying each mutant derivative are shown as a percentage of this value.

**Effects of mutations in the *prgQ* region on *prgB* expression.** The *prgQ* gene encodes the peptide inhibitor iCF10, which specifically and competitively inhibits the activity of the pheromone cCF10 (26). Complementation studies described in a previous article (6) demonstrated that the *prgX-prgQ* region is required in *cis* for positive control of *prgB*. Because it seemed likely that the *prgQ* region provided a *cis*-acting, positive regulatory element for the activation of *prgB*, we attempted to isolate and characterize mutations in *prgQ* and in sequences 3′ from the end of the coding sequence. Plasmids containing Tn*5* insertions 48 bp (pINY4512) and 86 bp (pINY4520) downstream from the stop codon of *prgQ* (Fig. 2) exhibited instability in OG1RF similar to that of the insertion mutants of *prgX* (22). In contrast, pINY4518, which contains a Tn*5* insertion at a location 236 bp downstream from the stop codon of *prgQ* (Fig. 2), was maintained stably in OG1RF, and the cells were clumpy in broth culture, indicating that *prgB* was expressed in these cells. Western blot analysis of INY3000 cells carrying pINY4518 confirmed that Asc10 was produced (Fig. 3).

Since it was possible that the presence of *prgX* contributed to the instability of some of the constructs described above, Tn*5* mutagenesis of pINY8301 (Fig. 1) was also carried out to try

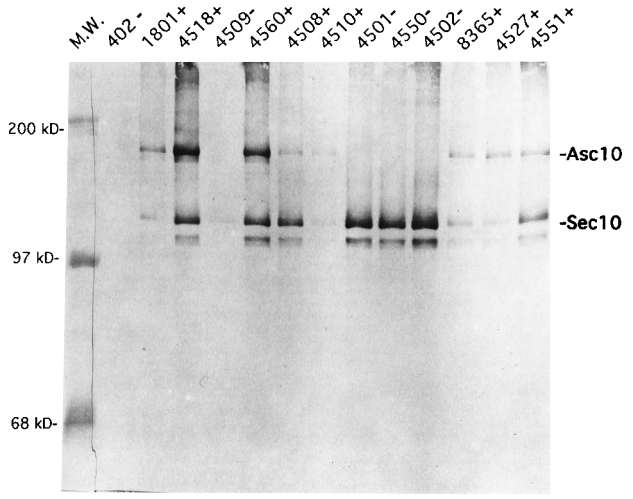


FIG. 3. Effects of Tn*5* insertions or deletions in the *prgQ*, *-R*, *-S*, and *-T* region on the production of Asc10 and Sec10. Western blot analysis of protein extracts from *E. faecalis* INY3000 strain carrying the plasmids indicated in the figure was carried out as described in Materials and Methods. Lane M.W. contains prestained protein high-molecular-weight standards obtained from Bio-Rad. The plasmid (pINY designation) content of the strain from which the surface antigen was extracted is shown at the top of each lane. The locations of Asc10 (150 kDa) and Sec10 (130 kDa) are indicated. The phenotype of the broth culture of *E. faecalis* OG1RF cells carrying each plasmid is indicated after the pINY designation as + (clumpy) or − (not clumpy) over each lane of the Western blot.

and obtain additional insertional mutations of the *prgQ* region. Although a direct Tn*5* insertion in the structural gene of *prgQ* could not be obtained, two plasmids, pINY8534 and pINY8503, which carried Tn*5* 87 and 217 bp downstream from the 3′ end of the *prgQ* structural gene, respectively (Fig. 1 and 2) were identified. Plasmid pINY8534 produced unstable transformants, while pINY8503 was maintained stably in *E. faecalis* (Fig. 2). OG1RF carrying pINY8503 completely lost β-galactosidase activity (Table 2). These results indicate that an approximately 230-bp DNA segment 3′ from the open reading frame corresponding to *prgQ* is required for the expression of *prgB*. Interestingly, there is no open reading frame with a reasonable ribosomal binding site in this region.

**Tn*5* insertional analysis of the *prgR* and *prgS* region.** We showed previously (5) that Tn*5* insertions in the *prgR* and *prgS* region of pINY1801 (pINY4509 and -4501) abolished the expression of *prgB*. In this study, the precise location of these insertions was determined by DNA sequencing. Plasmid pINY4509 proved to have Tn*5* inserted 59 bp upstream from the initiation codon of *prgR*, possibly disrupting a promoter element of *prgR*. Plasmid pINY4501 was shown to carry a Tn*5* insertion at the 82nd codon of the structural gene of *prgS* (Fig. 2), which would eliminate the production of the putative *prgS* protein product (PrgS).

Additional Tn*5* insertion mutants in the *prgR* and *prgS* region of pINY1801 and pINY8301 (Fig. 1 and 2) were also characterized. Plasmid pINY4508 carried the Tn*5* insertion in the 62nd codon of the structural gene of *prgR* and pINY4560 carried the transposon 38 bp upstream from the initiation codon of *prgR* (Fig. 2). To examine the effects of these insertions on the expression of *prgB*, the plasmids were introduced into *E. faecalis* OG1RF and INY3000. As shown in Fig. 3, INY3000(pINY4508) produced Asc10 and Sec10, indicating that the gene product of *prgR* is not required for the expression of *prgA* or *prgB*. Cells carrying pINY4560 also produced Asc10 (Fig. 3). The latter result is surprising, since the transposon insertion site in pINY4560 is only 21 bp downstream from that in pINY4509, and cells carrying pINY4509 failed to express *prgB*. Since the coding sequence for *prgR* is not required for *prgB* expression, it appears that Tn*5* insertion in pINY4509 disrupts important sequences upstream from *prgR* required to activate *prgB*.

Both pINY4510 (pINY1801::Tn*5*) and pINY8513 (pINY 8301::Tn*5*) carried Tn*5* 1 bp upstream from the start codon of *prgS* (Fig. 2), separating a potential promoter region and the ribosomal binding site from the structural gene. However, *prgB* was normally expressed in *E. faecalis* cells carrying these plasmids (Fig. 3 and Table 2), while the insertion construct pINY4501 carrying Tn*5* in the coding region of *prgS* did not produce Asc10 (Fig. 3). The possibility that DNA sequences in the 3′ end of Tn*5* might provide a functional promoter and a ribosomal binding site in *E. faecalis* for the expression of *prgS* in the pINY4510 and pINY8513 constructs is considered below. Plasmid pINY8588 (pINY8301::Tn*5*) carried Tn*5* at the same site as in pINY4501. Thirty-six Miller units of β-galactosidase activity (approximately 35% of that of OG1RF carrying pINY8301; Table 2) were detected in OG1RF(pINY8588), whereas Asc10 was not detected in INY3000(pINY4501) by Western blot analysis (Fig. 3). It is possible that a truncated form of PrgS produced from either pINY4501 or pINY8588 is partially functional in the activation of *prgB*::*lacZ* or that detection of β-galactosidase activity is more sensitive than detection of Asc10 by immunoblotting.

Interestingly, Tn*5* insertions downstream of the *prgS* structural gene also abolished the expression of *prgB*. Plasmids pINY4550 and pINY4502, both derivatives of pINY1801::Tn*5*, have the transposon inserted 103 and 105 bp downstream from the stop codon of the *prgS* structural gene, respectively (Fig. 2). Plasmid pINY8508 (pINY8301::Tn*5*) carried the transposon 90 bp downstream from the 3′ end of *prgS* (Fig. 3). These results suggest that sequences downstream of *prgS* are essential for the expression of *prgB*, possibly by maintaining the stability of the *prgS* mRNA. Although several Tn*5* insertions in the *prgS* region (pINY4501, -4550, and -4502) abolished the expression of Asc10, they appeared to increase the level of the production of Sec10 (Fig. 3).

**Deletion analysis of the *prgT* region.** Efforts to generate Tn*5* insertional mutations within the *prgT* gene failed, most likely because of the small size of the *prgT* structural gene (186 bp). The closest Tn*5* insertion to *prgT* was in pINY4527 (pINY1801::Tn*5*) where Tn*5* was inserted 55 bp downstream from the *prgT* structural gene (Fig. 2). INY3000 cells carrying pINY4527 produced normal levels of Sec10 and Asc10, as shown by Western blot analysis (Fig. 3). This indicated that Tn*5* insertion between *prgT* and *prgA* did not affect the expression of *prgA* or *prgB*. By using the unique *Sma*I site located in Tn*5* as a target site for Exo III, deletion mutants of *prgT* (Fig. 2) were generated from pINY4527. Although deletion occurred bidirectionally, *prgT* could be removed without deletion of pCF10 DNA downstream of Tn*5* toward *prgA* because of the asymmetrical location of the *Sma*I site in Tn*5* (2). This avoided an additional mutation in the *prgA* gene. Among the deletion constructs shown in Fig. 2, pINY8365, in which more than 80% of the structural gene of *prgT* was deleted from the 3′ end, was introduced into *E. faecalis* to test the effect of the deletion of *prgT*. OG1RF(pINY8365) formed aggregates in broth culture, and Western blot analysis (Fig. 3) confirmed the production of Asc10 and Sec10 in strains carrying pINY8365, indicating that *prgT* is not required for the expression of either *prgA* or *prgB*. However, if Tn*5* or the junction fragment between Tn*5* and *prgA* provided a promoter activity for downstream genes, the

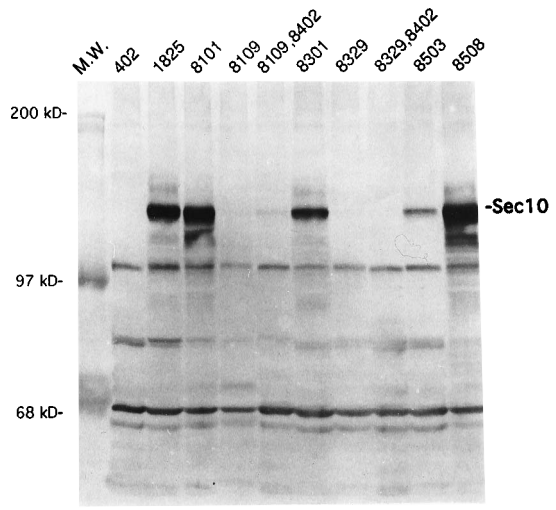


FIG. 4. Effects of mutations in the *prgX* or *prgQ* region on the expression of Sec10. Western blot analysis was performed to examine the effects of mutations in the *prgX* or *prgQ* region on the expression of Sec10 (130 kDa). Lane M.W. contains prestained protein high-molecular-weight standards (Gibco/BRL). The plasmid (pINY designation) content of the OG1RF strain from which the surface antigen was extracted is shown at the top of each lane.

gene product of *prgT* might be involved in the expression of *prgA* in wild-type pCF10; *prgT* and *prgA* might also be transcribed as an operon in the wild-type plasmid.

**Effects of mutations in the *prgX* and *prgQ* region on expression of *prgA*.** To test the effects of these mutations on the expression of *prgA*, Western blot analysis was performed with strain OG1RF carrying a plasmid (pINY8109, pINY8301, pINY8329, and pINY8503) in which the *prgX* or *prgQ* region had been deleted to different extents (pINY8109, -8301, and -8329) or Tn*5* had been inserted downstream of the *prgQ* region (pINY8503).

OG1RF carrying pINY8109 in which the entire *prgX* and *prgQ* region was deleted did not produce Sec10 (product of *prgA*), suggesting that *prgX* or *prgQ* might be involved in the expression of *prgA* (Fig. 4). OG1RF carrying pINY8301 produced Sec10 normally, indicating that *prgX* is not required for the expression of *prgA* (Fig. 4). OG1RF carrying pINY8329 in which the putative promoter region of *prgQ* had been deleted, however, did not produce Sec10 (Fig. 4). This result suggests that a gene product of the *prgQ* region is required for the expression of *prgA* or that the putative promoter region of *prgQ* is involved in the expression of *prgA*.

Tn*5* insertion in the region 3′ from *prgQ* (pINY8503; Fig. 2) abolished the activation of *prgB* (Fig. 3) and greatly reduced production of Sec10 compared with OG1RF carrying pINY8301 (Fig. 4), suggesting that this region is important for the expression of *prgA*. However, a Tn*5* insertion in *prgS* (pINY8508; Fig. 2) that reduced *prgB* expression dramatically did not abolish the expression of *prgA* (Fig. 4).

To study whether the deletion mutations in the *prgQ* region could be complemented for the production of Sec10, pINY8402 (6), in which the regulatory region containing the complete *prgX*, *-Q*, *-R*, *-S*, and *-T* genes (Fig. 1) had been cloned in a high-copy-number streptococcal plasmid, was introduced into strain OG1RF carrying pINY8109 or pINY8329 in which the *prgQ* gene or the putative promoter region of *prgQ* had been deleted. As shown in Fig. 4, no significant production of Sec10 was detected by Western blot analysis with the strain carrying either plasmid, suggesting that a sequence or a gene product encoded by pCF10 sequences, including the region between the *prgQ* promoter and about 300 bp past the 3′ end of the *prgQ* open reading frame, somehow functions in *cis* to activate the expression of *prgA*. A slight production of Sec10 detected in OG1RF carrying pINY8109 and pINY8402 was probably due to a recombination of these two plasmids which would generate the wild-type arrangement of the regulatory region. The fact that no Sec10 protein was detected in OG1RF carrying both pINY8329 and pINY8402 further supports the idea that the regulatory mechanism functions in *cis*.

**Analysis of termination of transcription reading into or out from Tn*5* insertions in *E. faecalis*.** Tn*5* is known to be polar when inserted into an operon in *E. coli* (2, 3), presumably because the abundant inverted repeat sequences located throughout Tn*5* could form stem-loop structures and serve as factor-independent transcriptional terminators (2). The notion that transcription of *prgB* is initiated near the 5′ end of its structural gene is based in part on the assumption that Tn*5* is also polar in *E. faecalis*. To examine whether transcription reading into Tn*5* is terminated in *E. faecalis* when Tn*5* is located between the upstream regulatory region and the *prgB* gene, a promoterless *lacZ* gene was cloned into the *Sma*I site of the *neo* gene in Tn*5* with pINY4508 (a derivative of pINY1801::Tn*5*). This Tn*5* derivative containing a *neo*::*lacZ* transcriptional fusion was used to replace Tn*5* in pINY4518, -4509, -4560, -4508, -4501, -4527, and -4503 in both orientations (Fig. 5). In the case of pINY4518, for example, the final constructs were named pINY4518Zs (*neo*::*lacZ* was cloned in the same orientation as that of the interrupted gene) and pINY4518Zo (*neo*::*lacZ* was cloned in the orientation opposite that of the interrupted gene). All the constructed plasmids (pINY4518Zs, -4518Zo, -4509Zs, -4509Zo, -4560Zs, -4560Zo, -4508Zs, -4508Zo, -4501Zs, -4501Zo, -4527Zs, -4527Zo, -4503Zs, and -4503Zo) were then introduced into OG1RF, and the β-galactosidase activity of each construct was measured.

Since *lacZ* is fused with the *neo* gene in Tn*5*, it was possible that *lacZ* could be transcribed from the promoter of *neo* if the promoter of *neo* is functional in *E. faecalis*. As shown in Table 3, however, all the constructs carrying the *neo*::*lacZ* fusion in the orientation opposite that of the target gene did not express any significant level of β-galactosidase activity. These results indicated that the promoter of *neo* did not function in *E. faecalis*. The data also suggested that neither the insertion sequences of Tn*5* nor sequences between the insertion sequence and *neo* provided a functional promoter activity reading into Tn*5*. Therefore, any β-galactosidase activity detected with the construct containing the *neo*::*lacZ* fusion in the same orientation as that of the target gene is likely due to a transcriptional readthrough provided by the target gene in which Tn*5* is inserted.

All the constructs carrying the *neo*::*lacZ* fusion in the same orientation as that of the target gene expressed very low levels (less than 10 Miller units) of β-galactosidase activity, regardless of the location of the Tn*5* insertion in each plasmid. OG1RF carrying pINY4518Zs produced a slightly higher level of β-galactosidase activity than the other strains (Table 3), but the β-galactosidase activity was still much lower than the level of the β-galactosidase activity produced by the *prgB*::*lacZ* fusion (pINY8101; Table 3). Although the position of a gene fusion in an operon could clearly affect the level of reporter gene activity, these fusions at eight different sites (and in both orientations at each site; Fig. 5), spread over a 5-kb region of pCF10 DNA, all resulted in levels of β-galactosidase activity less than 10% that of the *prgB*::*lacZ* fusion. This is consistent with, but does not prove the notion that the transcription of

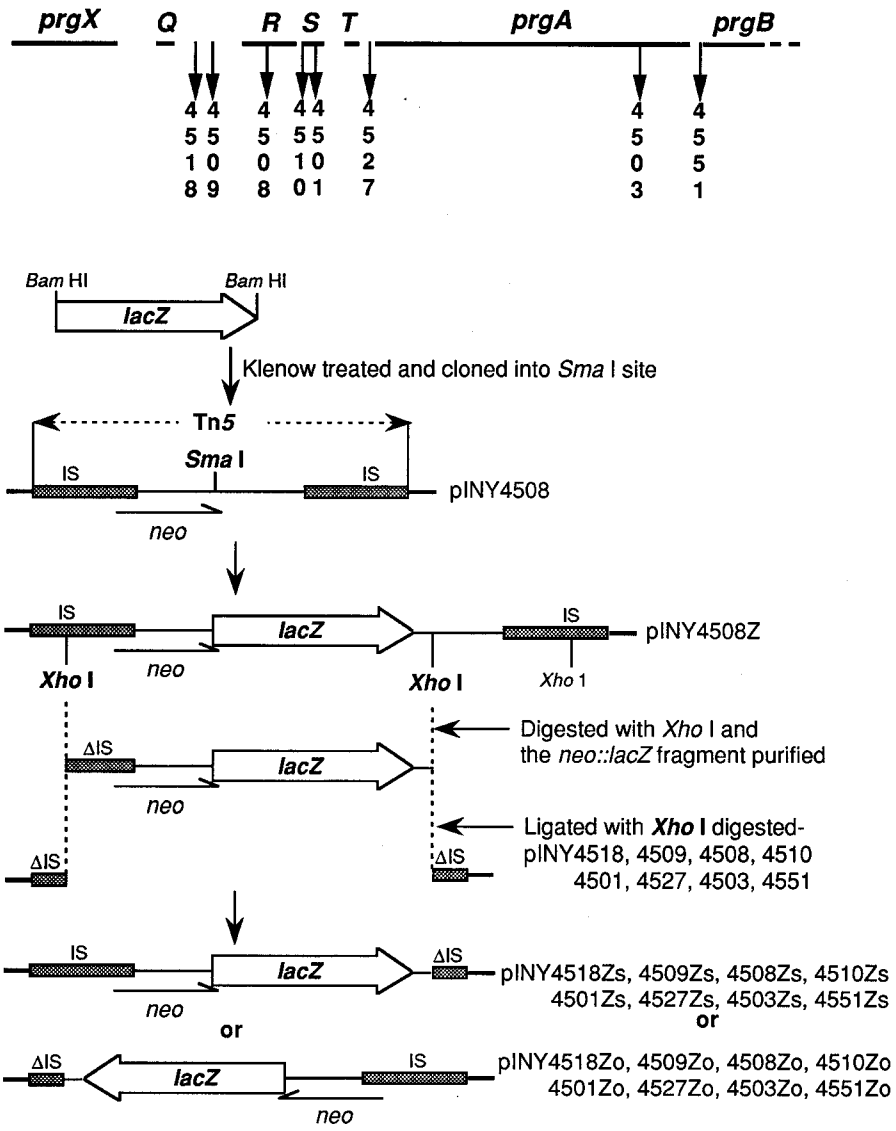


FIG. 5. Construction of the *neo*::*lacZ* fusions in pINY1801::Tn*5* derivatives. The locations of Tn*5* insertions of pINY1801::Tn*5* derivatives are indicated at the top. The Klenow fragment-treated *Bam*HI fragment of pUT2100 containing a promoterless *lacZ* gene was cloned into the unique *Sma*I site of pINY4508. The *Xho*I fragment containing the *neo* gene and the *lacZ* reporter gene was then inserted between the *Xho*I sites in the IS sequences of each Tn*5* insertion. The insertion sequences (IS) of Tn*5* are shown as dotted boxes.

*prgB* does not occur by a transcriptional readthrough generated from an upstream regulatory region (for example, *prgQ*).

Although Tn*5* is known to be polar in most cases, it has been shown that a junction fragment between Tn*5* and its target gene could sometimes provide a weak promoter activity for the transcription of downstream genes (3, 20). For this reason, the 3′ end of Tn*5* was examined for any sequence which could function as an outward-reading promoter in *E. faecalis*. In pINY4551 (a derivative of pINY1801::Tn*5*), the insertion site of Tn*5* was mapped between *prgA* and *prgB* (21). Sequence analysis revealed that Tn*5* is located 19 bp downstream from the 5′ end of the *prgB* transcription start site (Fig. 6). When pINY4551 was introduced into *E. faecalis* INY3000, Asc10 was produced, as shown by Western blot analysis (Fig. 6). The result indicated that the *prgB* gene was expressed, albeit at a slightly lower level, in spite of the insertion of Tn*5* downstream from its promoter region. To confirm that expression of Asc10 was not due to a transcriptional readthrough initiating from the *prgB* promoter in OG1RF (pINY4551), the Tn*5* in pINY4551 was replaced with Tn*5*::*lacZ* as described above. OG1RF carrying pINY4551Zs in which the *neo*::*lacZ* fusion is located in the same orientation as that of *prgB* produced less than 1 Miller unit of β-galactosidase activity (Table 3), whereas OG1RF carrying the *prgB*::*lacZ* fusion (pINY8101) produced 110.7 Miller units of β-galactosidase activity, indicating that Tn*5* terminates the inward-reading transcription initiated from the *prgB* promoter. However, broth cultures of either OG1RF(pINY4551Zs) or OG1RF (pINY4551Zo) were clumpy, indicating that the cells were synthesizing Asc10 and suggesting the existence of an outward-reading promoter near the end of Tn*5* or in the junction sequence.

Taken together, the results presented in this section suggest that transcription into Tn*5* insertions is terminated in *E. faecalis* but that either the ends of Tn*5* or junction sequences

TABLE 3. β-Galactosidase activity and expression of *prgB* in cells with pINY1801::Tn*5*::*lacZ* fusion constructs[a]

| Parental plasmid | Aggregation[b] | β-Galactosidase activity (Miller units) of: | |
|---|---|---|---|
| | | Zs construct | Zo construct |
| pINY4518 | + | 10.1 | 0.4 |
| pINY4509 | − | 4.3 | 0.3 |
| pINY4508 | + | 5.4 | 0.7 |
| pINY4510 | + | 4.6 | 0.0 |
| pINY4501 | − | 3.9 | 0.5 |
| pINY4527 | + | 3.3 | 0.3 |
| pINY4503 | + | 3.8 | 0.3 |
| pINY4551 | + | 0.8 | 0.6 |

[a] The Zs and Zo derivatives of the pINY45 series plasmids indicated were constructed as shown in Fig. 5. The plasmids were inserted into strain OG1RF and assayed for production of β-galactosidase activity with an isogenic strain containing pINY8101 assayed as a positive control. As noted in the accompanying article (7), data on this strain provide an indication of the level of *prgB* transcription in cells containing the wild-type positive-control region in the absence of negative control. The level of β-galactosidase activity observed with this positive-control strain was 110.7 Miller units.

[b] Growth of the strain in liquid culture. Symbols: +, aggregation caused by production of Asc10 by the strain; −, no aggregation.

contain a promoter functional in *E. faecalis*. This could cause expression of some pCF10 genes 3′ to certain Tn*5* insertions.

## DISCUSSION

Several previous studies from our laboratory have indicated that the region of pCF10 extending from *prgX-prgT* contained one or more genes required for activation of expression of *prgB* (4–6, 22). This activation mechanism has some novel features, including the facts that it seems to function only in *cis* and in an orientation-specific fashion (6). In this study, we attempted to define the specific pCF10 sequences required for *prgB* activation, using insertion and deletion mutagenesis. Because mutations that disrupted the coding sequences of the *prgX*, *prgR*, and *prgT* genes had no effect on *prgB* expression, we conclude that these genes are not essential components of the positive-control system. In contrast, disruption of the putative promoter of the *prgQ* gene (pINY8319 and -8329; Fig. 2), or of the open reading frame corresponding to *prgS* (pINY4501; Fig. 2) abolished expression of *prgB*.

Interestingly, certain mutations 3′ to the coding sequences of the *prgQ* and *prgS* genes also resulted in loss of *prgB* expression. In the case of *prgS*, Tn*5* insertions about 90 to 100 bp past the 3′ end of *prgS* (pINY4502, -4550, and -8508; Fig. 2) knocked out *prgB* expression. On the basis of our analysis of *prgS* transcription (1, 7), and on preliminary results suggesting that a nonsense mutation in *prgS* abolishes *prgB* expression (1), we hypothesize that the functional product of *prgS* is a polypeptide and that the mRNA encoding this protein is subject to processing. The nucleotide sequence at the 3′ end of the *prgS* message probably contributes to its stability, accounting for the observed effects of Tn*5* insertions in plasmids pINY4502, pINY4550, and pINY8508 (Fig. 2). With regard to *prgQ*, Tn*5* insertions at >265 bp (pINY8503; Fig. 2) and >450 bp (pINY4509; Fig. 2) past the 3′ end of the open reading frame corresponding to *prgQ* abolished *prgB* expression completely. These results, along with the transcriptional analysis presented in the accompanying article (7), suggest that the functional positive regulatory products encoded by this region of pCF10 may be RNA molecules whose synthesis initiates from a promoter at the 5′ end of *prgQ* and continues beyond the stop codon in the polypeptide coding sequence for the iCF10 inhibitor molecule (26). (At present it is not known whether the inhibitor plays any role in positive regulation.) Analysis of the size and pheromone inducibility of the mRNA molecules synthesized from this region of pCF10 (7) indicates that the expression of *prgB* in wild-type donor cells correlates with the presence of a pheromone-inducible ~530-nucleotide RNA molecule called $Q_L$ with a 3′ end at the approximate position of the sequence denoted IRS2 in Fig. 2. A constitutively synthesized smaller RNA, $Q_S$, with a 3′ end at the approximate position of IRS1 in Fig. 2 is present as a major species in all *E. faecalis* cells harboring plasmids containing this region of pCF10. Analysis of expression of the *prgS* and *prgA* genes in wild-type donor cells and in cells containing various subcloned fragments and mutant derivatives of pCF10 (Fig. 4) (7) suggests that *prgS* and *prgA* expression may require $Q_S$, but not $Q_L$, and that the product of *prgS* could stabilize or enhance the synthesis of $Q_L$ (see Fig. 5 of the accompanying article [7]). The negative-control region which has been recently found to include several genes that are located to the left of *prgQ* (Fig. 1), including *prgX*, appears to interfere with the activation process (19, 31). When pheromone is added to wild-type donor cells or when portions of the negative-control region are deleted, as in cells containing pINY1801 or pINY8101 (Fig. 1), the positive-control mechanism can function.

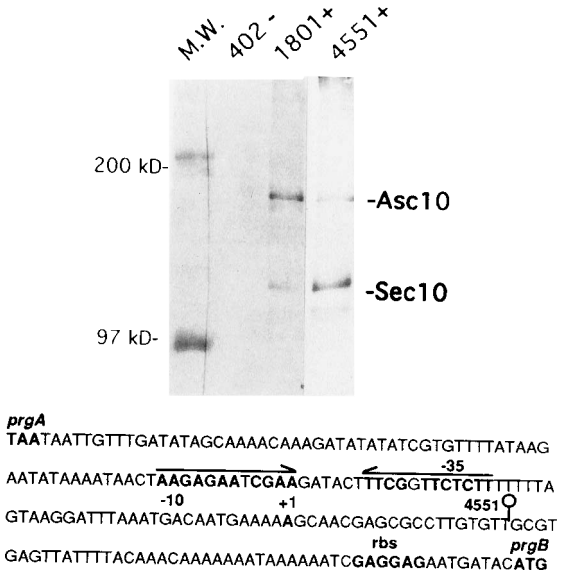


FIG. 6. Location of Tn*5* insertion site in pINY4551 and expression of Asc10. Western blot analysis of protein extracts from *E. faecalis* INY3000 strain carrying the plasmids (pINY designations) indicated at the top of each lane was carried out. Lane M.W. contains prestained protein high-molecular-weight standards. The location of Tn*5* insertion site in pINY4551 (♀) is shown. The approximate transcriptional start site of the *prgB* gene (+1), the −10 and −35 boxes of the putative promoter, and the putative ribosomal binding site are indicated. A 30-bp interrupted dyad symmetric element is indicated by inverted horizontal arrows above the sequence. The phenotype of the broth culture of *E. faecalis* OG1RF cells carrying each plasmid is indicated as + (clumpy) or − (not clumpy) after the pINY designation over each lane of the Western blot.

Most of the data presented here and in the accompanying article are consistent with the general model described above. However, several observations made in these studies raise some important questions which cannot be fully explained without additional analyses. These are discussed in the next three paragraphs.

Several constructs containing the positive-control region with deletions or insertions of Tn*5* in the *prgQ* or *prgX* gene or their promoter regions were not stable in an *E. faecalis* host

cell, although they could be maintained stably in *E. coli*. These results are consistent with earlier studies, where no Tn*917* insertions could be isolated in the corresponding regions of wild-type pCF10. The reasons for these instability effects are not clear at present, but it is unlikely that either *prgQ* or *prgX* is involved in incompatibility or plasmid replication (16, 19). It is possible that the observed effects may be related to the novel regulatory mechanism encoded by pCF10.

The data from phenotypic analysis of Tn*5* insertions within the coding sequences of the various *prg* genes is completely consistent with the model for positive control described above, and effects of the insertions 3′ from *prgS* (pINY4502, -4550, and -8508; Fig. 1 and 3) can be ascribed to alterations in mRNA stability. However, there were a few Tn*5* insertions obtained in this study which generated phenotypes that were very surprising, if it is assumed that insertion of Tn*5* would be polar (2, 3). For example, in pINY4510 and -8513 (Fig. 1 and 2), the insertion would be predicted to separate the promoter and ribosomal binding site of *prgS* from the start codon. Thus, it would be predicted that these insertions would abolish *prgS*, and therefore *prgB*, expression. Because *prgB* is transcribed normally in strains carrying these plasmids, we suggest that in at least some constructs containing Tn*5* insertions, sequences within the transposon or at the junctions serve as functional outward-reading promoters that can effect transcription of the adjacent pCF10 sequences. A similar argument could be made to explain the active expression of *prgB* in cells containing pINY4518, in which transposon insertion occurs within IRS1, believed to correspond to the region at the 3′ end of $Q_S$; it might be expected that the sequences encoding the 3′ end of $Q_L$ would never be transcribed in this construct. To address the question of whether this transposon terminates inward-reading transcription, we examined a series of *lacZ* transcriptional fusions at eight different locations over a 5-kb segment of pCF10 spanning the positive-control region (Fig. 5). These results indicate very clearly that, regardless of the orientation of the reporter gene, the ends of Tn*5* block inward-reading transcription of an internal reporter gene very effectively (Table 3). However, genetic analysis of a Tn*5* insertion (in pINY4551) between the *prgB* promoter and the ATG start codon indicated that the 3′ end (relative to the direction of transcription of pCF10 sequences) of Tn*5* could provide a functional promoter to allow for synthesis of the *prgB* gene product (Fig. 6). Importantly, when the *lacZ* reporter gene was inserted within this copy of Tn*5*, no β-galactosidase expression was detected, providing further evidence against the notion that transcription of *prgB* in wild-type pCF10 initiates far upstream from the 5′ end of the gene. Preliminary efforts to map the 5′ end of the outward-reading transcripts from Tn*5* insertions (1) indicate that they initiate within the IS*50* sequences and would thus be expected to have effects independent of the orientation of the Tn*5* insertion. It will be of considerable interest to examine the level of transcription from these Tn*5* insertions and to determine whether the positive-control genes are required to activate the promoter(s) within Tn*5*.

While the genetic analysis presented here has identified pCF10 sequences involved in positive control of *prgB* expression, it has not suggested an explanation for the *cis*-acting, orientation-dependent nature of the regulatory mechanism suggested from previous studies (6). Although regulatory gene products (proteins or RNA molecules) would generally be expected to function in *trans*, this might not be true if the tracking model proposed previously (6) and in the accompanying article (7) is valid. In this model, the $Q_L$ RNA is postulated to be the effector molecule that acts at the *prgB* promoter. All of our data suggest that the DNA encoding $Q_L$ must be in *cis*, relative to *prgB*, but it is conceivable that PrgS might function in *trans*, to enhance $Q_L$ synthesis or stability, especially if the *prgS* gene were put under control of a heterologous promoter that could drive its expression, independent from *prgQ* transcription. Because this type of construct has not been generated, a *cis*-acting requirement for PrgS remains to be demonstrated.

The pheromone-inducible conjugative hemolysin plasmid pAD1 has been analyzed in considerable detail, and it shares considerable homology to pCF10 (18, 36, 38). The *iad* gene of pAD1 encodes an inhibitor analogous to iCF10 encoded by *prgQ*, and the mRNA species transcribed from the *iad* promoter have some similarity to $Q_S$ and $Q_L$ (25, 29). However, the rest of the positive-control region is completely different in the two plasmids. In particular, pAD1 encodes a *trans*-acting positive regulatory protein called TraE1 (25, 29), which has no homolog in the pCF10 system. In addition, the genetics of the two systems are very different, and no lethal effects of transposon insertion in the *traA* gene of pAD1 have been reported (30), analogous to those observed in the *prgX* and *prgQ* regions of pCF10. It has been suggested that pAD1 and pCF10 evolved from a common ancestor (36). If this is the case, it seems likely that pAD1 may have acquired an additional regulatory protein (TraE1), which might serve to increase molecular interactions between RNA polymerase, other regulatory factors, and the target promoter.

## ACKNOWLEDGMENTS

We thank Tina Leonard and David Mills for reading the manuscript.

This work was supported by a Public Health Service grant (IR01 GM 49530-10) to G.M.D. from the NIH.

## REFERENCES

1. **Bensing, B. A., D. A. Manias, and G. M. Dunny.** Unpublished data.
2. **Berg, D. E., and C. M. Berg.** 1983. The prokaryotic transposable element Tn5. Biotechnology **1:**417–435.
3. **Berg, D. E., A. Weiss, and L. Crossland.** 1980. Polarity of Tn*5* insertion mutations in *Escherichia coli*. J. Bacteriol. **142:**439–446.
4. **Christie, P. J., and G. M. Dunny.** 1986. Identification of regions of the *Streptococcus faecalis* plasmid pCF-10 that encode antibiotic resistance and pheromone response functions. Plasmid **15:**230–241.
5. **Christie, P. J., S.-M. Kao, J. C. Adsit, and G. M. Dunny.** 1988. Cloning and expression of genes encoding pheromone-inducible antigens of *Enterococcus* (*Streptococcus*) *faecalis*. J. Bacteriol. **170:**5161–5168.
6. **Chung, J. W., and G. M. Dunny.** 1992. Cis-acting, orientation-dependent, positive control system activates pheromone-inducible conjugation functions at distances greater than 10 kilobases upstream from its target in *Enterococcus faecalis*. Proc. Natl. Acad. Sci. USA **89:**9020–9024.
7. **Chung, J. W., and G. M. Dunny.** 1995. Transcriptional analysis of a region of the *Enterococcus faecalis* pCF10 involved in positive regulation of conjugative transfer functions. J. Bacteriol. **177:**2118–2124.
8. **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.
9. **Clewell, D. B., F. Y. An, M. Mori, Y. Ike, and A. Suzuki.** 1987. *Streptococcus faecalis* pheromone cAD1 response: evidence that the peptide inhibitor excreted by pAD1-containing cells may be plasmid determined. Plasmid **17:**65–68.
10. **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.
11. **Clewell, D. B., and K. E. Weaver.** 1989. Sex pheromones and plasmid transfer in *Enterococcus faecalis*: a review. Plasmid **21:**175–184.
12. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
13. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75:**3470–3483.
14. **Dunny, G. M., C. Funk, and J. Adsit.** 1981. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. Plasmid **6:**270–278.
15. **Dunny, G. M., L. N. Lee, and D. J. LeBlanc.** 1991. Improved electroporation

and cloning vector system for gram-positive bacteria. Appl. Environ. Microbiol. **57:**1194–1201.
16. **Dunny, G. M., B. A. B. Leonard, and P. Hedberg.** 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication. J. Bacteriol. **177:**871–876.
17. **Dunny, G. M., D. L. Zimmerman, and M. L. Tortorello.** 1985. Induction of surface exclusion by *Streptococcus faecalis* sex pheromones: use of monoclonal antibodies to identify an inducible surface antigen involved in the exclusion process. Proc. Natl. Acad. Sci. USA **82:**8582–8586.
18. **Galli, D., and R. Wirth.** 1991. Comparative analysis of *Enterococcus faecalis* sex pheromone plasmids identifies a single homologous DNA region which codes for aggregation substance. J. Bacteriol. **173:**3029–3033.
19. **Hedberg, P., R. E. Ruhfel, B. A. B. Leonard, and G. M. Dunny.** Unpublished data.
20. **Hoopes, B. C., and W. R. McClure.** 1987. Strategies in regulation of transcription initiation, p. 1231–1240. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
21. **Kao, S.-M.** 1990. Localization and sequence analysis of genes involved in pheromone-inducible conjugation of pCF10. Ph.D. dissertation, Cornell University, Ithaca, New York.
22. **Kao, S.-M., S. B. Olmsted, A. S. Viksnins, J. C. Gallo, and G. M. Dunny.** 1991. Molecular and genetic analysis of a region of plasmid pCF10 containing positive control genes and structural genes encoding surface proteins involved in pheromone-inducible conjugation in *Enterococcus faecalis*. J. Bacteriol. **173:**7650–7664.
23. **LeBlanc, D. J., J. M. Inamine, and L. N. Lee.** 1986. Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin. Antimicrob. Agents Chemother. **29:**549–555.
24. **Mori, M., A. Isogai, Y. Sakagami, M. Fujino, C. Kitada, D. B. Clewell, and A. Suzuki.** 1986. Isolation and structure of the *Streptococcus faecalis* sex pheromone inhibitor, iAD1, that is excreted by the donor strain harboring plasmid pAD1. Agric. Biol. Chem. **50:**539–541.
25. **Muscholl, A., D. Galli, G. Wanner, and R. Wirth.** 1993. Sex pheromone plasmid pAD1-encoded aggregation substance of *Enterococcus faecalis* is positively regulated in trans by *traE1*. Eur. J. Biochem. **214:**333–338.
26. **Nakayama, J., R. E. Ruhfel, G. M. Dunny, A. Isogai, and A. Suzuki.** 1994. The *prgQ* gene of the *Enterococcus faecalis* tetracycline resistance plasmid pCF10 encodes a peptide inhibitor, iCF10. J. Bacteriol. **176:**7405–7408.
27. **Oda, M., A. Sugishita, and K. Furukawa.** 1988. Cloning and nucleotide sequence of histidase and regulatory genes in the *Bacillus subtilis hut* operon and positive regulation of the operon. J. Bacteriol. **170:**3199–3205.
28. **Olmsted, S. B., S.-M. Kao, L. J. van Putte, J. C. Gallo, and G. M. Dunny.** 1991. Role of the pheromone-inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. J. Bacteriol. **173:**7665–7672.
29. **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.
30. **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.
31. **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.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Tanimoto, K., F. Y. An, and D. B. Clewell.** 1993. Characterization of the *traC* determinant of the *Enterococcus faecalis* hemolysin-bacteriocin plasmid pAD1: binding of sex pheromone. J. Bacteriol. **175:**5260–5264.
34. **Tortorello, M. L., and G. M. Dunny.** 1985. Identification of multiple cell surface antigens associated with the sex pheromone response of *Streptococcus faecalis*. J. Bacteriol. **162:**131–137.
35. **Trotter, K. M., and G. M. Dunny.** 1990. Mutants of *Enterococcus faecalis* deficient as recipients in mating with donors carrying pheromone-inducible plasmids. Plasmid **24:**57–67.
36. **Wirth, R.** 1994. The sex pheromone system of *Enterococcus faecalis*: more than just a plasmid-collection mechanism? Eur. J. Biochem. **222:**235–246.
37. **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.
38. **Wirth, R., S. Olmsted, D. Galli, and G. Dunny.** 1991. Comparative analysis of cAD1- and cCF10-induced aggregation substances of *Enterococcus faecalis*, p. 34–38. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.