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Cloning and Expression of Genes Encoding Pheromone-Inducible Antigens of *Enterococcus (Streptococcus) faecalis*

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Fragments, generated by restriction enzyme digestion, of the 58-kilobase *Enterococcus (Streptococcus) faecalis* tetracycline resistance plasmid pCF10 were cloned and introduced into *Escherichia coli* and *E. faecalis* to characterize the pheromone-inducible conjugation system encoded by this plasmid. Western blot (immunoblot) analyses revealed that a 130-kilodalton (kDa) antigen, identical to the Tra130 antigen shown previously to be involved in pCF10-mediated pheromone-inducible surface exclusion, was produced by both bacterial hosts carrying the recombinant plasmid pINY1825 (cloned *Eco*RI C fragment). Both bacterial hosts carrying pINY1825 also produced various amounts of immunologically related 118- to 125-kDa antigens (designated pre-Tra130) that resembled antigens produced by *E. faecalis* cells carrying pCF10. An additional 150-kDa antigen, Tra150, probably involved in pheromone-induced cellular aggregation, was produced by *Escherichia coli* and *E. faecalis* hosts carrying pINY1801 (cloned *Eco*RI C and E fragments). The coding sequences for the Tra150 and Tra130 antigens were further localized in the *TRA* region of pCF10 by transposon insertion mutagenesis. Western blot analyses of the recombinant strains, and of strains carrying derivatives of pCF10 or various recombinant plasmids containing Tn*5* or Tn*917* insertions, suggested that the portion of pCF10 comprising the *tra3* through *-6* segments (previously defined by Tn*917* insertional mutagenesis) contained several genes that are involved in regulating the synthesis of Tra130 and Tra150.

Pheromones excreted by *Enterococcus (Streptococcus) faecalis* recipient cells elicit a complex physiological response among donor cells carrying a certain class of conjugative plasmids that results in enhanced aggregation of donors and recipients and high levels of donor conjugal DNA transfer (for reviews, see references 7 and 11). In response to pheromones, also called clumping-inducing agents (CIAs), donor cells produce several proteinaceous cell surface antigens, of which one or more are believed to promote cellular aggregation (18, 23, 26). We have identified three pheromone-inducible surface antigens associated with the 58-kilobase (kb) tetracycline resistance plasmid pCF10 (11, 23). In the absence of pheromone, *E. faecalis* cells carrying pCF10 produce several different, antigenically related forms of a surface protein which migrate on sodium dodecyl sulfate-polyacrylamide gels with apparent molecular masses of 120 to 130 kilodaltons (kDa). The total amount of this material, as well as the relative amount of each antigenic band, varies considerably in different antigen extracts from cells grown in the absence of pheromone. Exposure of these cells to pheromone results in an increase in the total amount of antigenic material in this molecular mass range and a shift in the relative amounts of the various forms of this protein such that the 130-kDa protein (Tra130) becomes the predominant form. Previous genetic, immunological, and biochemical experiments indicated that the shift from the smaller to the larger forms of this protein might result from a pheromone-induced posttranslational modification of lower-molecular-weight precursor forms (that we denote pre-Tra130) to the mature Tra130 molecule (22). Alternative hypotheses to account for the different molecular weight forms of the antigen include multiple translational start sites in the structural gene encoding Tra130 or differences in the proteolysis of antigens from cells grown in the presence or absence of pheromone. Regardless of the reason for the different molecular weight forms of this antigen, our previous experiments indicated clearly that the 130-kDa Tra130 protein (but not the lower-molecular-weight pre-Tra130 forms) is involved in a pheromone-inducible surface exclusion process that prevents induced donor cells from acting as conjugal recipients for plasmids related to pCF10 (13).

Two additional surface antigens of 73 kDa (Tra73) and 150 kDa (Tra150) are detected only on pheromone-induced cells carrying pCF10 (11, 22). The Tra130 and Tra150 antigens apparently are different proteins, as determined by Western blot (immunoblot) analyses and enzyme-linked immunoassays using various polyclonal and monoclonal antibody reagents (13, 22, 23), but the possible relationship of either of these proteins to Tra73 is not clear. While recent evidence suggests a possible role for Tra150 in the aggregation of donor and recipient cells (11, 19a; this study), the function of Tra73 is not known.

We began molecular studies of the pheromone-inducible conjugation system by mutagenizing pCF10 with the transposon Tn*917* (5, 21). Mutant analysis revealed that pCF10 encodes two independent conjugal DNA transfer systems (5, 6). The tetracycline resistance transposon Tn*925* (16 kb) associated with pCF10 encodes a transfer system which mediates the conjugative transfer of Tn*925* between *E. faecalis* and *Bacillus subtilis* cells (6). A second conjugation system, localized in a region of ~25 kb (designated *TRA*) of pCF10, determines pheromone-dependent conjugal plasmid transfer between *E. faecalis* cells. The *TRA* region consists of at least nine distinct segments (tra) which confer various phenotypes on host cells (5). Cells carrying pCF10 derivatives with insertions in tra1 through -3 and tra7 through -9 exhibit a clumpy appearance in broth culture and transfer the mutated plasmid at elevated frequencies relative to cells

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TABLE 1. Bacterial strains and plasmids used

Strain	Plasmid	Description	Reference
Enterococcus faecalis			
OG1RF	None	Chromosomal Rif^r Str^r	10
OG1SSp	None	Chromosomal Str^r Spc^r	10
	pCF10	Tet^r, pheromone dependent, conjugative	12
	pCF11	Tet^r, pCF10 derivative, causes host cells to aggregate in the absence of pheromone	12
	pAMα1	Tet^r, nonconjugative, used as vector in these studies	3
	pINY1852	*Bam*HI B fragment (18.5 kb) of pCF10 cloned into pAMα1	This study
Escherichia coli			
JM109	None	*recA1 gyrA96 thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q} ZΔM15]	27
VJS470	None	F$^-$ λ$^-$ r$^+$ m$^+$ *proC*::Tn*5*	V. Stewart, personal communication
DH5α	None	F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	Bethesda Research Laboratories, Inc.
E. faecalis and *Escherichia coli*			
	pWM401	Cat^r Tet^r (*Escherichia coli*); Cat^r (*E. faecalis*)	24
	pINY1801	*Eco*RI C and E fragments (11.6 kb) of pCF10 cloned into pWM401	This study
	pINY1825	*Eco*RI C fragment (7.5 kb) of pCF10 cloned into pWM401	This study
	pINY1802	*Eco*RI E fragment (4.1 kb) of pCF10 cloned into pWM401	This study
	pINY1842	*Eco*RI C fragment (7.5 kb) of pCF10 cloned into pWM401 (opposite orientation from pINY1825)	This study

carrying the wild-type plasmid. Cells harboring plasmids with insertions in tra4 and tra6 do not clump in response to pheromone and transfer the mutated plasmid at very low frequencies. Insertions in tra5 completely abolish aggregation and plasmid transfer. These findings suggested that the tra4 through -6 segments (~1.5 kb) may encode a regulatory function involved in controlling expression of plasmid transfer-related genes.

We have examined functions of the various tra segments in greater detail by introducing cloned restriction fragments of pCF10 into *Escherichia coli* and *E. faecalis* cells. We show that two surface antigens normally produced by pheromone-induced *E. faecalis* cells carrying pCF10 are synthesized by both *Escherichia coli* and *E. faecalis* hosts carrying the cloned tra7 through -8 segments of the *TRA* region. We also used Tn*5* mutagenesis of cloned fragments of pCF10 in *Escherichia coli* to target regions in which we were unable to obtain insertions of Tn*917*. Our data provide the first direct evidence that the pheromone-induced antigens are plasmid encoded and also provide new insights into the complex nature of a pCF10-encoded regulatory system that enables donor cells to respond to pheromone.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The bacterial strains used in this study and their plasmid contents are described in Table 1. *E. faecalis* cells were grown in BYGT (23) or in M9-YE medium (23). *Escherichia coli* cells were grown in LB medium (19). Selective agar plates contained the following concentrations of antibiotics, purchased from Sigma Chemical Co. (St. Louis, Mo.): tetracycline (10 μg/ml) chloramphenicol (25 μg/ml), erythromycin (50 μg/ml), rifampin (50 μg/ml), fusidic acid (10 μg/ml), streptomycin (500 μg/ml), and spectinomycin (500 μg/ml). Sources of lysozyme, restriction enzymes, T4 DNA ligase, nick translation kits, and [α-^{32}P]dCTP (800 Ci/mmol) were described previously (5, 6). Protein molecular weight standards conjugated to Remazol brilliant blue R were purchased from Sigma. Horseradish peroxidase-conjugated anti-rabbit and anti-rat immunoglobulin G antibodies were obtained from Boehringer Mannheim Biochemicals Indianapolis, Ind. *N*-Acetylmuramidase SG (mutanolysin) was purchased from Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.

Plasmid purification and recombinant DNA techniques. Plasmid DNA was isolated from *E. faecalis* and from *Escherichia coli* as described previously (4, 5). *Escherichia coli* cells were transformed by the method of Hanahan (16). Protoplasts from *E. faecalis* cells were transformed and regenerated as described by Wirth et al. (25). Conditions for restriction endonuclease digestions, DNA blot transfer, nick translations, and Southern hybridizations were described previously (5, 6).

Insertions mutagenesis with Tn*5*. *Escherichia coli* VJS470, a derivative of W3110 that contains a single chromosomal copy of Tn*5* (2) in the *proC* gene, was provided by Valley Stewart, Cornell University, Ithaca, N.Y. (Table 1). This strain was transformed with either pINY1801, pINY1825, or pINY1842. Approximately 10^9 CFU of each transformant strain was plated on LB agar containing neomycin (200 μg/ml). The several hundred colonies that grew on the plates were pooled, and plasmid DNA was extracted. This DNA was used to transform strain JM109 or DH5α by selecting for high-level neomycin (or kanamycin) resistance. Transformants were screened for plasmid content, and presumptive inserts of Tn*5* were identified by looking for an ~6-kb increase in one of the fragments obtained by digesting the original recombinant plasmid with *Eco*RI (Tn*5* contains no *Eco*RI sites). The rationale for this technique was that transposition of Tn*5* from the chromosome to the plasmid would increase the copy number of the transposon and give rise to a higher level of antibiotic resistance (9). We found that >90% of the transformants contained Tn*5* inserts into the plasmid that ~50% of these inserts were in the portions

derived from pCF10. The positions of the inserts were determined by restriction enzyme analysis.

Protein extraction procedures. Surface antigens were extracted from *E. faecalis* cells as follows. Cells from a 10-ml culture grown to mid-log phase were pelleted and suspended in 0.1 ml of lysozyme (5 mg/ml of H_2O) and Tris-buffered protease inhibitors (22) and then incubated for 45 min at room temperature with gentle rocking. These conditions were shown to release antigens from the enterococcal cell surface. The mixture was centrifuged in a microfuge (Fisher Scientific Co., Pittsburgh, Pa.) for 3 min, and the supernatant was immunoprecipitated or analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. *Escherichia coli* proteins were obtained by sonicating cells from 3 ml of an overnight culture. Cells were pelleted, suspended in 100 μl of phosphate-buffered saline (0.14 M NaCl, 1.5 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4), and disrupted on ice with three 20-s bursts at setting 60, using a Virsonic model 16-850 cell disrupter. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4°C. Antigenic material in the sonic fluid was immunoprecipitated as described below.

Immunoprecipation. Polyclonal antiserum, raised in rabbits by immunization with whole pheromone-induced pCF10-carrying *E. faecalis* cells (23), and monoclonal antibodies prepared as described previously by Tortorello et al. (22) were used to immunoprecipitate enterococcal surface antigens. Protein extracts, obtained from *Escherichia coli* by sonicating cells from a 3-ml culture or from *E. faecalis* by lysozyme treatment of cells from a 10-ml culture (see above), were incubated in the presence of the polyclonal (20 μl) or monoclonal (50 μl) antibodies for 1 h at room temperature with gentle rocking. Anti-rabbit or anti-rat immunoglobulin G antibodies conjugated to horseradish peroxidase (1,000 U) were added, and the reaction mixture was incubated for 1 h at room temperature. Immune complexes were pelleted by centrifugation in a Fisher microfuge for 1.5 h at 4°C. Immune complexes were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis as described below.

Electrophoresis of proteins and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Tortorello and Dunny (23). Gels were constructed with 7.5% polyacrylamide in the resolving portion and 4.5% in the stacking portion. After electrophoresis, proteins were transferred to nitrocellulose in cold 20 mM phosphate buffer, pH 6.5, with a Hoefer Transfor apparatus (Hoefer Scientific). The nitrocellulose blots were incubated for 1 h at room temperature in buffer (50 mM Tris [pH 7.4], 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100) containing polyclonal antiserum against whole, pheromone-induced *E. faecalis* cells (22) or monoclonal antibodies against Tra130 (23). Further immunoenzymatic development with horseradish peroxidase-conjugated antibodies and color development reagents were as described by Tortorello and Dunny (23). Molecular sizes of immunoreactive antigens were estimated by inclusion of protein standards conjugated to Remazol brilliant blue R when the gel was run.

RESULTS

Cloning restriction fragments of pCF10 in *Escherichia coli* and *E. faecalis*. Fragments of pCF10 obtained by *Eco*RI digestion were introduced into *Escherichia coli* and *E. faecalis* by cloning into the transgeneric shuttle plasmid pWM401 (24). This plasmid consists of a fusion of the *Escherichia coli* vector pACYC184 and plasmid pGB351, a derivative of the broad-host-range streptococcal plasmid pIP501 (1). The pACYC184-derived tetracycline (Tet^r) and chloramphenicol (Cat^r) resistance genes are expressed in *Escherichia coli*, and a second Cat^r gene, associated with the pGB351 portion of the vector, is expressed in *E. faecalis* (24). Of nine recombinant plasmids isolated, seven consisted of vector and single *Eco*RI restriction fragments of pCF10. Two additional plasmids each harbored two adjacent *Eco*RI fragments that were aligned in the same orientation in the recombinant plasmids as in pCF10, as determined by restriction endonuclease mapping. The nine recombinant plasmids were introduced into *E. faecalis* cells by protoplast transformation, and transformants were selected on regeneration plates containing chloramphenicol (5 μg/ml; see Materials and Methods). *Bam*HI restriction fragments of pCF10 also were cloned and introduced into *E. faecalis*, with the nonconjugative tetracycline resistance enterococcal plasmid pAMα1 (3) used as a vector. The recombinant plasmids obtained in these studies are described in Table 1, and the physical locations of most important cloned regions, relative to the pCF10 map, are summarized in Fig. 1. Since the only assay for gene expression of the cloned DNA fragments available at the time this work was done was the production of specific antigens, the remainder of this article focuses on the cloned regions which expressed proteins previously associated with pheromone induction.

Production of pCF10-encoded antigens by *Escherichia coli* and *E. faecalis*. Chimeric plasmid DNA containing the various cloned fragments described above was introduced into several different strains of *Escherichia coli* in order to determine the best genetic background to use to detect expression of the cloned genes. The recombinant plasmids were also introduced into *E. faecalis* hosts and assayed for expression. Protein extracts were subjected to Western blot analysis, using monoclonal and polyclonal antibodies directed against pheromone-inducible antigens produced by *E. faecalis* cells carrying pCF10 (23) for immunodevelopment. Figure 2 shows a comparison of the antigenic profiles of *E. faecalis* OG1SSp(pCF10) cells grown in the absence (lane 6) or presence (lane 7) of CIA; *E. faecalis* cells carrying pCF11, a mutant derivative of pCF10 that confers a constitutively clumpy and high-frequency-transfer phenotype on its host cell (12) (lane 9); and various *Escherichia coli* and *E. faecalis* strains carrying either vector plasmids or chimeric plasmids containing cloned fragments of pCF10. In this antigenic preparation, the *E. faecalis* cells carrying pCF10 produced a small amount of the 130-kDa Tra130 antigen in the absence of CIA and a barely detectable amount of the smaller pre-Tra130 antigen. When these cells were exposed to CIA, there was a substantial increase in the amount of both antigens, particularly Tra130, and also the appearance of the 150-kDa Tra150 protein. The antigenic profiles of the cells carrying pCF11 were similar to those of the induced cells carrying pCF10, except that the amounts of Tra130 and Tra150 were somewhat reduced. *E. faecalis* cells carrying pINY1801 (cloned *Eco*RI C and E fragments; Fig. 2, lane 9) constitutively produced antigen profiles that were essentially identical to those from the cells containing pCF11; i.e., both the Tra130 and Tra150 antigens were present. In contrast, *E. faecalis* cells carrying the cloned *Eco*RI C fragment contained within pINY1825 (lane 10) appeared to express Tra130 but not Tra150. Interestingly, *Escherichia coli* cells containing these recombinant plasmids also produced antigenic proteins that were very similar to those detected in the

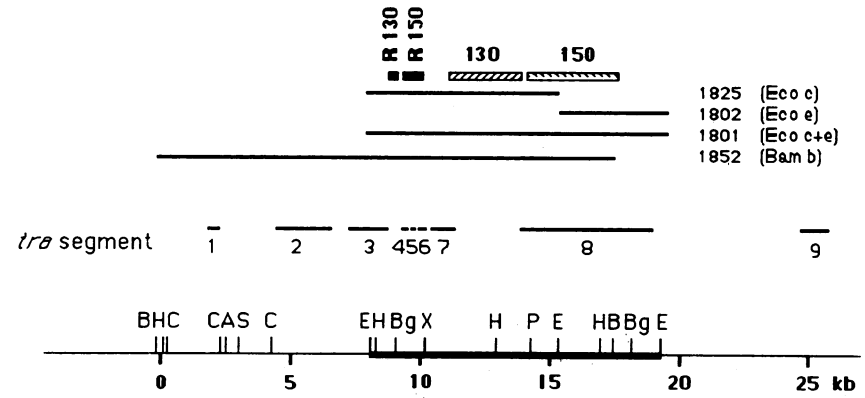

FIG. 1. Organization of the transfer region of pCF10. The horizontal line at the bottom represents a 25-kb region of pCF10 shown previously (5) to contain genes involved in pheromone-inducible conjugation. The cloned fragments of pCF10 generated by specific restriction enzyme cleavage are shown at the top. Abbreviations for and the positions of various restriction enzyme cleavage sites, and the putative locations of the structural genes for the Tra130 and Tra150 antigens, are indicated as in Fig. 3. The region that was subjected to Tn*5* insertional mutagenesis as described in the legend to Fig. 3 is indicated by the heavy line at the bottom.

E. faecalis preparations (Fig. 2, lanes 2 and 3). The presence of pINY1825 in *Escherichia coli* (lane 3) resulted in production of a series of antigenic bands with a size range of 100 to 130 kDa. The largest pair of these bands comigrated with the pre-Tra130 and Tra130 antigens produced by *E. faecalis*. The *Escherichia coli* cells carrying the larger pINY1801 (lane 2) produced an identical set of antigen in this size range and additional higher-molecular-weight proteins, the largest being very similar to the Tra150 antigen of *E. faecalis*. When

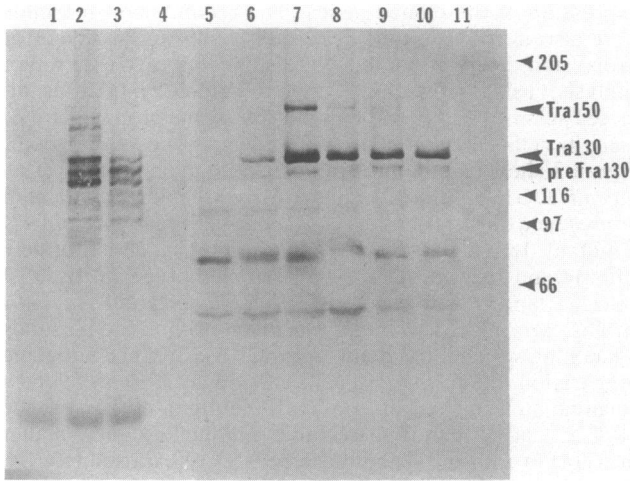

FIG. 2. Western blot of antigenic proteins from *Escherichia coli* and *E. faecalis* strains with recombinant plasmids constructed during these studies. Proteins were extracted from *Escherichia coli* by sonication and immunoprecipitated with antiserum raised against pheromone-induced *E. faecalis* cells (27); the blot was then developed with the same antiserum, as described in the text. *E. faecalis* cell surface antigens were extracted by limited lysozyme digestion and prepared for immunoblotting as described in the text. Lanes: 1, DH5α(pWM401); 2, DH5α(pINY1801); 3, DH5α(pINY1825); 4, molecular size standards (numbers on the right indicate mass in kilodaltons); 5, OG1SSp(pWM401); 6, OG1SSp(pCF10) grown in the absence of CIA; 7, OG1SSp(pCF10) induced by CIA; 8, OG1SSp(pCF11), uninduced; 9, OG1SSp(pINY1801), uninduced; 10, OG1SSp(pINY1825), uninduced; 11, size standards. The positions of Tra130, pre-Tra130, and Tra150 are also indicated on the right.

blots identical to those shown in Fig. 2 were developed with a monoclonal antibody specific for Tra130 (13), the bands in both *Escherichia coli* and *E. faecalis* preparations corresponding to pre-Tra130 and Tra130 showed reactivity (data not shown). Since we have observed identical protein production by both *Escherichia coli* and *E. faecalis* cells carrying either pINY1825 or pINY1842 (which represent the same fragment of pCF10 cloned into pWM401 in both orientations), we infer that the Tra130 protein is being expressed from its natural promoter in both bacterial hosts.

Although we do not have a monoclonal antibody that develops Tra150 directly on a blot, we feel that the 140- to 150-kDa proteins showing antigenic reactivity to the polyclonal antibodies raised against pheromone-induced *E. faecalis* cells that were produced by the *Escherichia coli* cells carrying pINY1801, as well as the 150-kDa antigen produced by the *E. faecalis* cells harboring the same plasmid, very likely resulted from expression of the structural gene for Tra150 in both hosts. Supporting this notion was the observation that all *E. faecalis* cells producing the 150-kDa antigen showed a clumpy appearance in broth culture, which is consistent with previous observations (19a), and indicates the involvement of Tra150 in clumping.

In contrast to the constitutive production of antigens conferred by plasmids pINY1801, -1825, and -1842, we found that *E. faecalis* cells carrying pINY1852, which contained a larger region of pCF10 DNA (Fig. 1), displayed an antigenic profile essentially identical to that of cells carrying pCF10 (Fig. 2); i.e., CIA induction resulted in production of Tra150 and an increased production of Tra130. This finding, coupled with previous observations showing that Tn*917* insertions into the tra2 or tra3 region of pCF10 resulted in elevated transfer in the absence of CIA (5) and constitutive expression of Tra150 (Table 2; P. J. Christie, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1987) suggest that a region to the left of tra4 may encode a negative regulatory gene that blocks expression of a number of the *TRA* genes in the absence of pheromone.

Location of the structural genes encoding the Tra130 and Tra150 antigens. *E. faecalis* strains carrying transposon insertions in the tra segments of pCF10 were examined for production of the pheromone-inducible antigens. Since there were gaps in the *TRA* region where no Tn*917* insertions were generated during our previous mutagenesis experiments (5),

TABLE 2. Production of antigens by *E. faecalis* strains (wild type and pCF10 derivatives) carrying various plasmids

Plasmid content of strains carrying:	Production[a] of:					
	Pre-Tra130		Tra130		Tra150	
	−CIA	+CIA	−CIA	+CIA	−CIA	+CIA
No insertions						
pCF10	+/−	+	+	++	−	+
pWM401	−	−	−	−	−	−
pINY1801	++	+	+	++	+	+
pINY1825	++	+	+	++	−	−−
pINY1842	++	+	+	++	−	−
pINY1852	++	+	+	+	−	+
Tn*917* insertions in pCF10[b]						
pCF204 (tra1)	+/−	+	+/−	++	−	+
pCF43 (tra2)	++	++	+	+	++	++
pCF53 (tra3)	++	++	+	+	++	++
pCF272 (tra4)	+	+	+	+	−	−
pCF67 (tra5)	+/−	+/−	+	+	−	−
pCF243 (tra6)	+/−	+/−	+	+	−	−
pCF38 (tra7)	+	+	+	+	+/−	+/−
pCF249 (tra8)	+	+	+	+	−	−
pCF121 (tra8)	+/−	+/−	+	+	−	−
pCF281 (tra9)	+/−	+/−	+	+	+	+
Tn*5* insertions in pINY1801[c]						
pINY4509	++	++	++	++	−	−
pINY4501, 4513	++	++	++	++	−	−
pINY4515	−	−	−	−	++	++
pINY4503	−	−	−	−	++	++
pINY4507	++	++	++	++	−	−
pINY4505	++	++	++	++	+/−	+/−
Tn*5* insertions in pINY1825[d]						
pINY4153	++	++	++	++		
pINY4154	++	++	++	++		
pINY4175	++	++	++	++		
pINY4141, 4148	−	−	−	−		
pINY4169	++	++	++	++		
pINY4162	−	−	−	−		
Tn*5* insertions in pINY1842[e]						
pINY4245	−	−	−	−		
pINY4252	−	−	−	−		
pINY4253	+[e]	+[e]	+[e]	+[e]		
pINY4261	+[e]	+[e]	+[e]	+[e]		
pINY4270	−	−	−	−		

[a] Determined by Western blot analysis and denoted as follows: −, no antigen; +/−, low level; +, moderate level; ++, high level of antigen. Antigen production of cells carrying wild-type pCF10 is indicated for comparison.

[b] Insertions are in the tra segments indicated in parentheses. They are described in reference 5.

[c] Insertions are in the positions indicated in Fig. 3.

[d] Insertions are in the positions indicated in Fig. 3. Tra150 was not produced by either pINY1825 or pINY1842, since they lack the structural gene for this antigen (see Fig. 1 and 3 and the text).

[e] Truncated protein; see Fig. 4 and text.

we constructed additional insertions in the *TRA* region by mutagenizing pINY1801, pINY1825, and pINY1842 with transposon Tn*5* in *Escherichia coli*. Derivatives of these three plasmids, harboring Tn*5* insertions in the cloned *Eco*RI C and E fragments, were isolated and introduced into *E. faecalis* to facilitate antigenic analysis (see Materials and Methods). Figure 3 shows the physical locations of various Tn*5* insertions within the *Eco*RI C and E fragments. Results of Western blot analyses of some *E. faecalis* strains carrying Tn*5* insertions in the *TRA* region are presented in Fig. 4. These results, as well as similar analyses of additional Tn*5* insertions and previous antigenic analyses of pCF10::Tn*917* derivatives carrying insertions in each of the tra segments (5; Christie, Ph.D. thesis), are summarized in Table 2. Tn*5* insertions that abolished production of both Tra130 and pre-Tra130 were mapped in a region of approximately 3 kb (bounded by insertions 4169 and 4507) between tra7 and tra8 and in a second region, designated R130, of less than 1 kb (bounded by insertions 4175 and 4509) between tra3 and tra4. Interestingly, we had not previously isolated Tn*917* insertions in either region (5; Fig. 1). Insertions of Tn*917* that abolished production of Tra150 were mapped in tra4 through -6, which is identical to the segment designated R150 in Fig. 3 (in the region to the left of insertion 4169 and to the right of insertion 4245) defined by Tn*5* mutagenesis, and in tra8 (between insertions 4261 and 4505); we believe that insertion 4505 is very close to one end of the structural gene for Tra150, since this insertion greatly reduces Tra150 production. The R130 region between tra3 and tra4, where Tn*5* insertions abolished Tra130 production, and the tra4 through -6 (R150) region, where Tn*917* or Tn*5* insertion prevented expression of Tra150, were too small to encode proteins of 130 and 150 kDa. In contrast, the regions extending from tra7 into tra8 (Fig. 1 and 3), where transposon insertions abolished production of one or the other antigen, were of sufficient size to encode these proteins. The smaller regions apparently represent regulatory segments necessary for expression of the proteins. Because the plasmids (pINY1825 and pINY1842) in which the Tn*5* insertions in the R130 region were constructed lack the structural gene for the Tra150 antigen, it is not known whether synthesis of Tra150 also requires this segment of pCF10 DNA.

The Western blot analysis of the Tn*5*-containing recombinant plasmids provides strong supporting evidence for the putative locations of the regulatory and structural genes encoding Tra130 and Tra150 that are shown in Fig. 1 and 3. Insertions in the R130 region (e.g., 4141, 4148, and 4245) and in the central region of the putative Tra130 structural gene (e.g., 4141, 4245, and 4252) abolished production of both Tra130 and pre-Tra130. Two insertions near the end of the Tra130 structural gene, 4253 and 4261, appeared to produce truncated derivatives of Tra130, with deletions of approximately 28 and 18 kDa, respectively. (Although the strain with insertion 4162 produced immunoreactive proteins of approximately 80 and 100 kDa, we feel that these antigens may not represent truncated forms of Tra130, since we have previously observed similar bands in some antigenic extracts of cells carrying pCF10 and pCF11.) These results suggest that transcription of this gene proceeds from left to right on the maps depicted in Fig. 1 and 3. The distance between insertions 4253 and 4261 as determined by restriction enzyme analysis of the DNA agrees very well with the apparent reductions in sizes of the proteins. The sizes of the deletions predicted by the protein analysis would place the 3′ end of the gene about 400 to 500 base pairs to the right of insertion 4261, thus overlapping the structural gene for Tra150. Regardless of whether the genes actually overlap, or whether there are small errors in the current map, we feel that the locations of the genes shown are sufficiently accurate to initiate DNA sequence analysis, which should resolve any discrepancies. It should also be noted that a mutant carrying a Tn*917* insertion near the end of tra8 that is

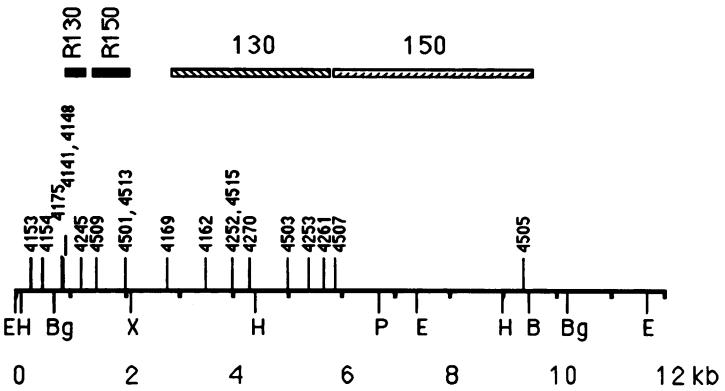

FIG. 3. Tn*5* insertional mutagenesis of the cloned *Eco*RI C and E fragments of pCF10. Shown are the positions of Tn*5* insertions within cloned regions of pCF10 that were derived by insertional mutagenesis of chimeric plasmids pINY1801, pINY1825, and pINY1842. Abbreviations for restriction enzyme cleavage sites: E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; X, *Xba*I; P, *Pst*I; B, *Bam*HI; C, *Cla*I; A, *Ava*I; S, *Sal* I). The distances in kilobase pairs are indicated at the bottom. The positions of Tn*5* insertions in the various recombinant plasmids described in Table 2 are indicated by the vertical lines and numbers; the putative locations of the structural genes and regulatory genes required for synthesis of the Tra130 and Tra150 antigens are indicated at the top.

adjacent to tra9, in a location similar to that of Tn*5* insertion 4505 (5; Christie, Ph.D. thesis), produced a predominant immunoreactive antigen of ~140 kDa that was probably a product of a truncated structural gene for the Tra150 protein, supporting the gene location for Tra150 depicted in Fig. 1 and 3.

DISCUSSION

The molecular cloning studies presented here demonstrate that the region defined by the *Eco*RI C fragment (7.5 kb) of pCF10 encodes a 130-kDa surface antigen, Tra130, previously shown to mediate pheromone-induced surface exclusion (13). The region defined by the *Eco*RI C and E fragments (11.6 kb) also encodes a surface antigen, Tra150 (11), whose function as an aggregation substance has been implicated. A more thorough understanding of the molecular arrangement of genes involved in the pheromone-inducible conjugation system encoded by pCF10 was gained by combining results of the molecular cloning studies with data obtained from transposon mutagenesis. All of our results are consistent with the model shown in Fig. 1, in which the structural gene encoding pre-Tra130 is localized between the tra7 and tra8 segments and the coding sequence for Tra150 is localized in the tra8 segment. These results also clarify the relationship between the Tra130 protein and antigenically related lower-molecular-weight proteins that we have collectively termed pre-Tra130. Previously, we hypothesized that the pre-Tra130 antigens were converted to active Tra130 by posttranslational modification. Although our current results do not rule out this possibility, they do suggest that the pre-Tra130 antigens could also result from proteolysis of Tra130, from the use of more than one initiation codon, or from differences in transcription termination. In any event, our analysis of Tn*5* insertion mutations into cloned fragments encoding these antigens (Fig. 3 and 4) indicated that all insertions that abolished Tra130 production, or resulted in production of truncated derivatives of Tra130, also eliminated pre-Tra130. Therefore, the pre-Tra130 and Tra130 antigens are almost certainly different forms of the same gene product, regardless of the mechanism by which each form is generated. To verify the proposed locations of both the Tra130- and Tra150-encoding sequences, we are constructing deletions of the cloned fragments in vitro and subcloning the appropriate regions for DNA sequencing. The products of these genes are also being purified for biochemical analysis.

Our studies have also contributed information concerning the regulation of genes expressed in response to pheromones. Our previous mutational analysis of the *TRA* region indicated that the tra4 through -6 segments of pCF10 could represent an important regulatory region (5). We report here that mutants carrying Tn*917* insertions in tra4 through -6 failed to produce Tra150 (Table 2), and we have independently identified the same region (R150; Fig. 1 and 3) by Tn*5* mutagenesis, providing further evidence for a role of this region in the regulation of genes expressed in response to pheromone. Interestingly, tra4 through -6 (R150) mutants still produce Tra130, suggesting that a regulatory function encoded by tra4 through -6 may control expression of some, but not all, of the pheromone-inducible genes. Two lines of evidence indicate that the tra 4 through -6 region does not simply provide a promoter for transcription of the structural gene for Tra150. First, Tn*5* inserts into the structural gene for Tra130 had no polar effects on the expression of Tra150, second, Tn*917*-induced tra7 mutations had no polar effects on Tra150 expression. Therefore, the tra4 through -6 region may encode a *trans*-acting regulatory factor that controls expression of Tra150 protein.

A second regulatory region, R130, required for expression of Tra130, was also identified between tra3 and tra4, where a gap existed in the map of the *TRA* region that was regenerated by Tn*917* mutagenesis (4). Because insertions in the tra4 through -6 region (located between the putative R130 regulatory region and the structural region for Tra130) had no effect on Tra130 expression, it also appears that this segment could encode a factor that acts in *trans*.

Our studies suggest that a third regulatory locus encoded by the tra1 through -3 segments may be involved in pheromone-inducible production of Tra150 (and an inducible increase in Tra130 production). When the tra1 through -3 segments are absent, as is the case for cells carrying pINY1801, Tra150 is produced independently of exposure of cells to pheromone. However, when these segments are present, as for cells carrying pINY1852, Tra150 production is strictly dependent on exposure to pheromone. In addition, *TRA* mutants carrying transposon insertions in the tra2 and

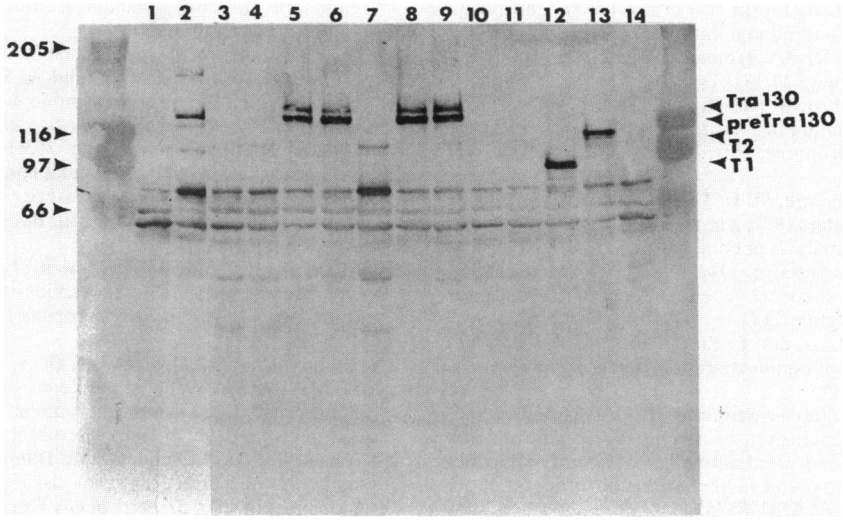

FIG. 4. Western blot analysis of *E. faecalis* strains carrying various pINY1825::Tn*5* or pINY1842::Tn*5* derivatives. Recombinant plasmids derived by Tn*5* mutagenesis of pINY1825 and pINY1842 were introduced into *E. faecalis* by protoplast transformation, and the resulting strains were subjected to Western blot analysis. The positions of the specific Tn*5* inserts are indicated in Fig. 3. The antigenic profiles of these strains were compared with those of OG1SSp(pCF11) (positive control) and OG1SSp(pWM401) (negative control). The plasmid content of the strain whose antigen profile is shown in each lane is as follows: 1, pWM401; 2, pCF11; 3, pINY4141; 4, pINY4148; 5, pINY4153; 6, pINY4154; 7, pINY4162; 8, pINY4169; 9, pINY4175; 10, pINY4245; 11, pINY4252; 12, pINY4253; 13, pINY4261; 14, pINY4270. The molecular masses (in kilodaltons) of the size standards are indicated at the left, and the positions of pre-Tra130, Tra130, and truncated derivatives (T1 and T2) produced by strains 4253 and 4261 are indicated at the right.

tra3 segments produce Tra150 constitutively. These findings are consistent with the hypothesis that a function encoded by this region of pCF10 represses expression of the gene encoding Tra150 (and, to a lesser extent, Tra130) in the absence of pheromone but that the synthesis or activity of this putative repressor is inhibited upon exposure of cells to pheromone. Preliminary complementation experiments (Christie, Ph.D. thesis) suggest that tra3 may actually encode the repressor activity.

The results presented here suggest that pheromone-inducible conjugation is subject to a complex and interesting regulatory mechanism. Recent studies demonstrated that conjugal transfer and pheromone response functions of another pheromone-dependent plasmid pAD1, are also encoded by several distinct regions, which led to speculation that these genes are probably controlled by different promoters (8, 14, 15, 17). Our findings have supported this interpretation and allowed us to focus on specific regions of pCF10 that are likely to encode structural and regulatory components of the pheromone induction system. To gain a better understanding of this system, we are introducing promoterless, readily assayed reporter genes carried by transposons (20) into the *TRA* region to identify promoters and examine the expression of genes residing in this region. We are also initiating complementation analyses to characterize the presumptive regulatory regions more extensively. The combined use of our collection of *TRA* mutants and recombinant strains carrying cloned restriction fragments of pCF10 should greatly facilitate these studies.

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