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Regulation of the pAD1 Sex Pheromone Response in *Enterococcus faecalis*: Effects of Host Strain and *traA*, *traB*, and C Region Mutants on Expression of an E Region Pheromone-Inducible *lacZ* Fusion

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Pheromone-induced conjugal transfer of the hemolysin-bacteriocin plasmid pAD1 of *Enterococcus faecalis* is regulated by a cluster of determinants designated *traA*, *traB*, and regions C and E. The E region is believed to include a positive regulator that controls many structural genes related to conjugation. The pheromone-inducible Tn*917-lac* fusion NR5, located in the E region, is regulated by the products of *traA*, *traB*, and the C region. To more closely examine the effects of these genes on the induction of E region products, inserts in each of these genes were combined with the NR5 fusion in a novel approach involving triparental matings with a pAD1 miniplasmid and recombinational mutagenesis. Results indicate that (i) the *traA* gene product is a key repressor of the pheromone response; (ii) the *traB* gene product, in cooperation with a gene within or regulated by the E region, controls pheromone shutdown; (iii) a primary function of the C region gene product is in pheromone sensing, with secondary functions in pheromone shutdown and negative regulation; and (iv) the host in which the plasmid resides has a dramatic effect on the regulation of the NR5 fusion in *traB* and C region mutants. Numerous parallels were observed between the regulation of the NR5 fusion and the regulation of the aggregation and transfer response. These parallels aided in further defining the functions of particular regulatory determinants as well as further establishing the link between the regulation of the E region and the regulation of the aggregation and transfer response.

The *Enterococcus faecalis* plasmid pAD1 (59.6 kilobases) encodes a conjugative transfer system that is induced specifically by a small peptide pheromone, cAD1, produced by potential recipients. When exposed to pheromone, plasmid-containing cells respond by producing: (i) an aggregation substance which facilitates the formation of mating or self-aggregates, (ii) surface proteins that have been correlated with aggregation substance, and (iii) other functions believed to be essential for the actual physical transfer of the plasmid DNA. Once a recipient acquires pAD1, cAD1 activity is shut down, while the activity of pheromones specific for other plasmids is unaffected. In addition, plasmid-containing cells produce a specific peptide, iAD1, which specifically and competitively inhibits cAD1 activity (for a recent review, see reference 2).

The pAD1 pheromone response was previously shown to be regulated by a cluster of genes located in the 15-kilobase *Eco*RI B restriction fragment (4, 7, 13). Miniplasmids consisting solely of this fragment and containing pheromone-inducible *lacZ* fusions were found to regulate the fusions in a manner identical to that observed when the same fusions were present on the intact plasmid (14). These results indicated that the regulatory genes on the pAD1 *Eco*RI B fragment were not only essential but also sufficient for directing a normal pheromone response. Mutational analyses with Tn*917* and transcriptional analyses with Tn*917-lac* have identified genetic determinants specifying at least three transcripts within the regulatory cluster that control the pheromone response (4, 13; see also Fig. 1). The E transcript encodes a pheromone-inducible gene product(s) that is required for induction of the pheromone response. The A transcript is constitutively transcribed in a direction opposite to the E transcript, and the two transcripts may overlap. The A transcript reads through the previously described *traA* gene, the product of which appears to be involved in both negative regulation of the pheromone response and signal transduction. The B/C transcript is constitutively transcribed in the same direction as the E transcript and includes the previously identified C region and *traB* gene. Mutations within the C region indicated that its gene product(s) might be involved in negative regulation, signal transduction, and/or iAD1 production. The product of the *traB* gene was believed to function in cooperation with the *traA* gene product as a negative regulator. In addition, a deletion resulting in the loss of much of the B/C transcript caused the loss of cAD1 shutdown capability (2). Evaluation of miniplasmids indicated that these genetic determinants were sufficient for negative regulation of the pheromone response, signal transduction, cAD1 shutdown, and iAD1 production, although the specific function of each gene product and their relationships with each other were not identified (14).

To further investigate the function of these regulatory genes, we have developed a method whereby Tn*917* insertions in the *traA* and *traB* genes and the C region can be combined in *cis* with an inducible fusion located in the E region. By combining insertion mutants in *traA*, *traB*, and the C region with the pheromone-inducible fusion, NR5, and examining the effects of these mutants on an easily quantifiable marker of pheromone response rather than on the gross phenomena of aggregation and plasmid transfer, we

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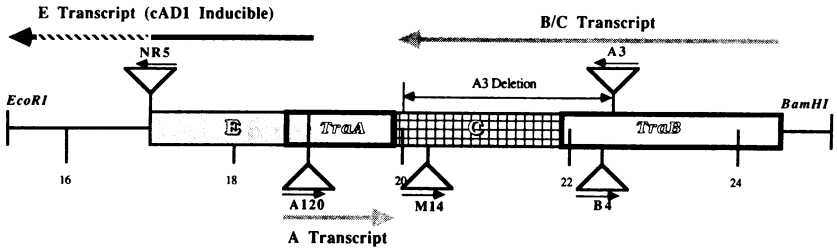

FIG. 1. pAD1 pheromone response regulatory region with inserts of interest. This map is an enlargement of that portion of the pAD1 *Eco*RI B fragment known to encode genes required for the normal regulation of the pheromone response. These genes are marked *traA*, *traB*, C, and E. The numbered kilobase markers are consistent with previous pAD1 maps. Inserts shown are those discussed in this report. Only NR5, A120, and A3 are oriented so as to produce productive *lacZ* fusions with an active promoter. The NR5 fusion is induced by cAD1, while the A3 and A120 fusions are produced constitutively at levels below uninduced NR5. The extent of the A3 deletion is also indicated. Transcript arrows represent the minimum number of transcripts produced within this region (13). The stippled arrows of the A and B/C transcripts represent their low-level constitutive production. The broken arrow of the E transcript indicates uncertainty as to the endpoint of this transcript.

have succeeded in further defining the functions of the gene products of *traA*, *traB*, and the C region. Our results are presented here and include data implicating a role for *traB* in the shutdown of endogenous cAD1 activity and involvement of a host factor or factors in the pheromone response.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this investigation are shown in Table 1. All insertion mutants contain a copy of Tn*917-lac*, a derivative of Tn*917* containing a promoterless *lacZ* gene inserted near one end (10). Only the NR5 and A120 inserts are oriented such that the *lacZ* gene is fused to an active promoter. The NR5 fusion is pheromone inducible, as previously described (13). β-Galactosidase is produced constitutively from the A120 fusion at levels below that observed in uninduced NR5. Therefore, in the NR5/A120 combination, pAM2125, and under the conditions used to assay β-galactosidase production, the A120 fusion does not make a significant contribution to enzyme activity. The *lacZ* gene of the B4 and M14 inserts is oriented in a direction opposite to the direction of transcription and, therefore, no detectable β-galactosidase is produced from these fusions (13).

The pheromone responses of all plasmids were assayed in two *E. faecalis* hosts, OG1X and FA2-2. OG1X is a streptomycin-resistant (Sm^r), extracellular protease-deficient derivative of OG1S (8). FA2-2 is a rifampin- and fusidic acid-resistant derivative (Rif^r Fus^r) derivative of JH2 (1).

Media and reagents. All pheromone induction experiments were conducted in N2GT medium (nutrient broth no. 2 [Oxoid Ltd., London, United Kingdom] supplemented with 0.1 M Tris buffer [pH 7.5] and 0.2% glucose). Cultures for plasmid preparation were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.). Solid medium was prepared by adding 1.8% agar (Difco) to either THB or N2GT; hemolysis was detected, when necessary, on THB plates containing 4% horse blood (Colorado Serum Co., Denver, Colo.). Cultures were incubated at 37°C. Antibiotics were added at the following concentrations: streptomycin, 1 mg/ml; fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; erythromycin, 10 μg/ml.

Construction of double-insert pAD1 derivatives. pAD1 derivatives containing the inducible NR5 fusion and Tn*917-lac* inserts in *traA*, *traB*, or the C region were constructed by triparental matings in which the pAD1 miniplasmid pAM2005E, containing the NR5 fusion, was mobilized by using a plasmid with the insert of interest. Thus, 50 μl of OG1X(pAM2005E); 0.5 ml of FA2-2; and 50 μl of OG1X(pAM2120), OG1X(pAM2140), or OG1X(pAM2040) containing inserts A120, M14, and B4, respectively, were added to 4.5 ml of N2GT. These mating mixtures were then collected on nitrocellulose filters. Filters were incubated on THB-blood plates overnight. Transconjugants were selected on media containing rifampin, fusidic acid, erythromycin,

TABLE 1. Bacterial strains and plasmids

Plasmid	Relevant phenotype[a]	Reference(s)
Strains		
OG1X	*str gel*	8
FA2-2	*rif fus*	1
DS16	*tet erm kan strep hem-bac*	11
Plasmids		
pAD1	Hemolysin-bacteriocin	11
pAM714	pAD1::Tn*917*, hemolysin-bacteriocin *erm* wild-type Agg Tf Isp	7, 8
pAM2005A	pAD1 derivative containing the NR5 pheromone inducible *lacZ* fusion, Agg^- Tf^- Isp^-	13
pAM2005E	Miniplasmid derived from pAM2005A	14
pAM2005K	Miniplasmid derived from pAM2005E	14
pAM2030	pAD1 containing the A3 deletion, constitutive Agg Tf Isp, cAD1 production	13
pAM2040	pAD1 containing the B4 *traB* Tn*917-lac* insert, constitutive Agg Tf Isp (but see text)	13
pAM2040E	Miniplasmid derived from pAM2040	This study
pAM2045	pAD1 containing the NR5 and B4 inserts	This study
pAM2120	pAD1 containing the A120 *traA* Tn*917-lac* insert, constitutive Agg Tf Isp	13
pAM2125	pAD1 containing the NR5 and A120 inserts	This study
pAM2140	pAD1 containing the M14 C region Tn*917-lac* insert, partial constitutive Agg Tf, decreased response (but see text)	13
pAM2145	pAD1 containing the NR5 and M14 inserts	This study

[a] Agg, Aggregation; Tf, transfer; Isp, inducible surface proteins.

and 80 ng of synthetic cAD1 (custom made by Milligen Inc., Waterton, Mass.) per ml and 100 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma Chemical Co., St. Louis, Mo.) per ml. The mobilizing plasmids were self-transmissible but produced white, dry colonies under the above-described conditions. Plasmids containing the NR5 fusion in any combination did not produce dry colonies, since the E region insert disrupts aggregation functions. Such colonies were also blue when induced with pheromone. Therefore, the rare blue colonies were purified from the dry, white background. Plasmids of interest were mobilized back to OG1X or FA2-2 in triparental matings by using OG1X (pAD1) as the helper plasmid, with selection for streptomycin-erythromycin or rifampin-fusidic acid-erythromycin, respectively. After each of these matings, it was necessary to meticulously purify the strain of interest to isolate clones having only the desired plasmid.

Routine screening of plasmid DNA from transformants was carried out by using the small-scale alkaline lysis procedure previously described (13). Plasmid DNA was analyzed by digestion with various restriction enzymes and separation of restriction fragments on 0.8% agarose. All restriction enzymes were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and reactions were carried out under the conditions recommended.

Pheromone response and β-galactosidase assays. To test the responsiveness of cells containing the plasmid of interest, 1:10 dilutions were made from an overnight culture of the appropriate strain and grown for 30 min. Cultures were then induced with 0, 2.5, 5, 10, 20, and 40 ng of synthetic cAD1 per ml for 90 min. Cell density was determined by using a Spectronic 21 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) at a wavelength of 600 nm. Cells were harvested, toluenized, and assayed for β-galactosidase activity as previously described (13). Assays were conducted with 40 μl of sample for 30 min. Assays were done with larger samples and for longer time periods to more accurately determine differences observed under uninduced conditions. Activities are expressed in Miller units (9).

Analysis of surface proteins and mating capacity. Zwittergent 3-12 (Calbiochem-Behring, La Jolla, Calif.) extracts of induced and uninduced plasmid-containing strains were prepared as previously described (5). Samples were separated on sodium dodecyl sulfate-polyacrylamide (10%) gels and then electrophoretically transferred to nitrocellulose filters and visualized by a modification of the Western blotting (immunoblotting) method of Towbin et al. (12), as described previously (5).

Mating capacity was determined in 10-min broth matings as previously described (7).

Pheromone assays. Culture filtrates (CF) were prepared from N2GT-grown cultures inoculated at 2% from cultures grown overnight in N2GT-erythromycin. Cultures were grown into early stationary phase, i.e., until no significant increase in culture density was observed over a 30-min interval. CF from pAM2040E-containing cells were prepared after the recovery period (see below). Cells were harvested by centrifugation, and the supernatant was filtered through a 0.45-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.). CF were then boiled for 10 min and stored at 4°C. Samples of the culture from which CF were prepared were plated on N2GT and then screened on N2GT-erythromycin to ensure that no significant plasmid loss which could result in pheromone production had occurred.

The concentration of cAD1 in CF was determined by the microdilution assay method described previously (3). The cAD1 titer is defined as the highest dilution of culture filtrate which induced aggregate formation in responder cells. The inhibitor iAD1 titer was determined by diluting a culture filtrate from plasmid-free cells through culture filtrates to be tested in the microdilution assay (8). The amount of inhibitor was represented as the pheromone titer in the absence of inhibitor divided by the titer in its presence. Because iAD1 production is so low in an OG1X background, iAD1 assays were done on plasmids in an FA2-2 background.

Construction and analysis of a pAD1 miniplasmid containing a *traB* insert. The construction of pAD1 miniplasmids is described in more detail elsewhere (14). Briefly, ≈1 μg of pAM2040, a pAD1 derivative containing the B4 insert, was digested to completion with *Eco*RI. The sample was then religated with T4 DNA ligase and used to transform *E. faecalis* OG1X protoplasts as previously described (15). Transformants were selected on media supplemented with 2 μg of erythromycin per ml. Plasmid DNA was screened as described above to ensure that only the pAD1 *Eco*RI B fragment was present.

RESULTS

Construction of plasmids combining the NR5 fusion with the A120, B4, or M14 insert. In order to more quantitatively examine the effects of *traA*, *traB*, and C region mutants on the pAD1 pheromone response, we developed a procedure designed to combine Tn*917-lac* inserts located in these genes with a pheromone-inducible fusion, NR5, located in the E region. That such an approach might be possible was suggested by observations concerned with mobilization of the pAD1 miniplasmid pAM2005E (Fig. 2; [14]). When pAM2005E was mobilized by pAD1 in triparental matings, the majority of transconjugants obtained after selection for the transposon-encoded Em^r were recombinants containing Tn*917-lac* on pAD1 in the same location as on the miniplasmid, as determined by restriction mapping. β-Galactosidase was also inducible by the pheromone, as would be expected from its position. The remainder of the transconjugants contained pAM2005E alone. It was reasoned, therefore, that by mobilizing the miniplasmid with pAD1 derivatives bearing inserts in *traA*, *traB*, or the C region, recombinants containing both the NR5 insert and the insert of the mobilizing plasmid could be isolated (see Materials and Methods). The mobilizing plasmids were chosen such that their inserts were in the opposite orientation to NR5 so that deletion of the intervening DNA would not occur. Inversion could presumably occur but would not likely affect the regulation of the pheromone-inducible fusion. Because of the absence of appropriate restriction sites, it was not possible to determine the frequency of inversion events on these plasmids, but similar inversions with inverted repeats of Tn*10* on the *Escherichia coli* chromosome have been observed to occur at a frequency of approximately 1×10^{-3} to 1×10^{-4} (6).

As expected, the majority of transconjugants isolated from these matings were white and dry, because of self-transfer of the mobilizing plasmid carrying its own Tn*917*-encoded Em^r. A120 is the only insert in which the *lacZ* gene is fused to an active promoter, and the level of expression of this fusion is below that necessary for detection by the approach used. However, blue colonies were observed among the white colonies in all three matings. Although it was impossible to determine transfer frequencies, it was apparent that the frequency of isolation of blue colonies was 1 to 2 orders of magnitude higher in the pAM2120 (*traA*)-mobilized matings than in the pAM2040 (*traB*)- or pAM2140 (C)-mobilized

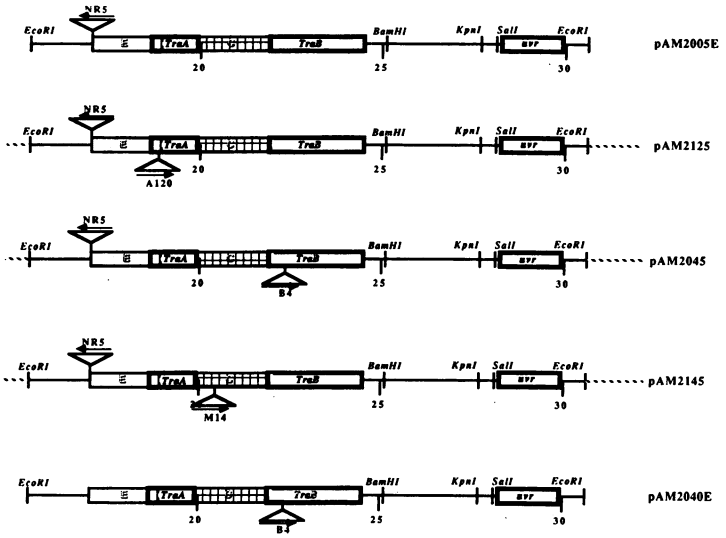

FIG. 2. Plasmid maps of double-insert combinations and related miniplasmids. Maps show only the pAD1 *Eco*RI B fragment and the relevant Tn*917-lac* inserts. Broken line extensions indicate the remainder of pAD1. Each plasmid, except pAM2040E, contains the pheromone-inducible *lacZ* fusion NR5, which results from the insertion of Tn*917-lac* in the appropriate orientation in the E region. In addition, each double insert contains a second Tn*917-lac* insert in the opposite orientation to NR5. Details of the phenotypes resulting from these inserts alone are given in reference 13. The structure of the double inserts was confirmed by using the *Bam*HI site located about 300 base pairs from the *lacZ* proximal end (at the arrow starting point) of Tn*917-lac*. In *Bam*HI-*Eco*RI double digests, novel restriction fragments corresponding to the distance between these sites and relevant plasmid *Bam*HI or *Eco*RI sites were identified. In addition, fragments corresponding to the distance between transposon *Bam*HI sites of the two inserts were also identified. No other novel fragments were observed.

matings. Curiously, once the blue colonies from the initial mating were purified and plasmid DNA was analyzed, it was found that in the case of pAM2120- and pAM2040-mobilized matings, the plasmids contained both inserts, not on the entire pAD1 as expected but on the miniplasmid. In contrast, in the pAM2140-mobilized matings, all blue colonies contained the two inserts on the intact plasmid. Indeed, despite several attempts, we have been unable to isolate a miniplasmid derivative containing the M14 and NR5 inserts on the miniplasmid. The double inserts of pAM2125 (E/*traA*) and pAM2045 (E/*traB*) were easily recombined onto the intact pAD1 by mobilizing them in triparental matings with OG1X (pAD1) as a helper plasmid.

The organization of the modified pAD1 *Eco*RI B fragments of each of the double-insert constructs is shown in Fig. 2.

Effects of *traA*, *traB*, and C region mutants on the regulation of the NR5 fusion and comparison with their effects on aggregation and transfer functions. The pheromone responses of plasmids pAM2005A (E region insertion), pAM2125 (E/*traA*), pAM2045 (E/*traB*), and pAM2145 (E/C), as measured by the induction of β-galactosidase production from the inducible NR5 fusion, is shown in Fig. 3. Responses were measured in the unrelated hosts OG1X (Fig. 3A) and FA2-2 (Fig. 3B). Several parallels were observed between the regulation of the NR5 fusion in these plasmids and the regulation of the aggregation and transfer responses in the

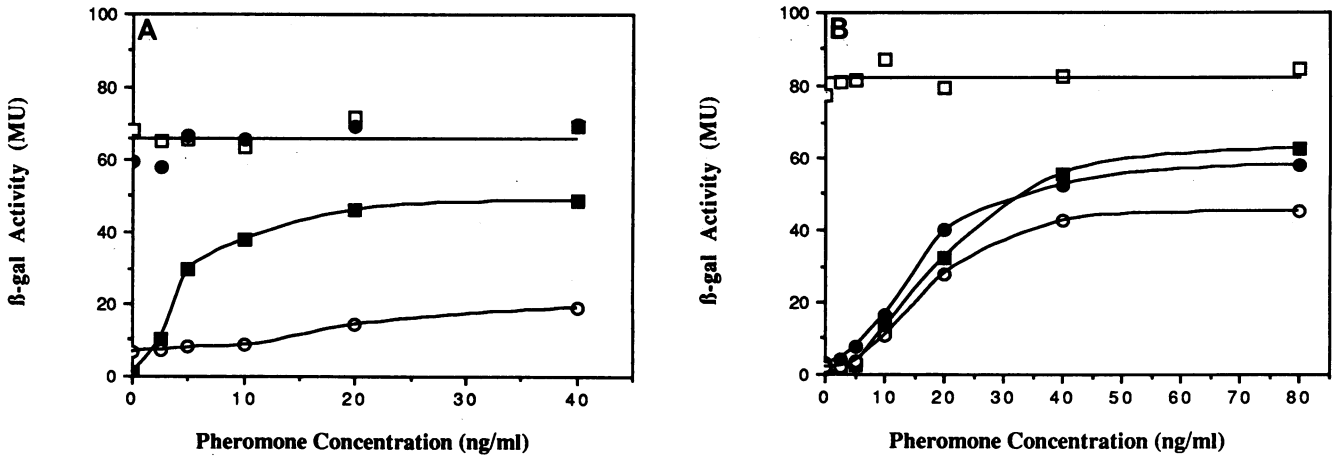

FIG. 3. Pheromone response of the NR5 fusion in the absence and presence of *traA*, *traB*, and C region inserts in an OG1X (A) and FA2-2 (B) background. Cultures were induced, and β-galactosidase assays were performed according to procedures described in Materials and Methods. β-Galactosidase activities are expressed as Miller units, a unit normalized to culture optical density at 600 nm. Lines are representative of at least three experiments. Symbols: ■, pAM2005A (NR5 E region fusion alone); □, pAM2125 (NR5/*traA*); ●, pAM2045 (NR5/*traB*); ○, pAM2145 (NR5/C).

TABLE 2. Transfer frequency of *traA*, *traB*, and C region mutants from OG1X and FA2-2[a]

Host/strain	pAM2120 (*traA*)	pAM2040 (*traB*)	pAM2140 (C)
OG1X	4.2×10^{-4}	2.4×10^{-3}	2.5×10^{-4}
FA2-2	3.0×10^{-4}	2.3×10^{-4}	2.4×10^{-5}

[a] These results are representative of at least three independent experiments. When OG1X was the donor host, FA2-2 was the recipient and vice versa.

parental plasmids, pAD1, pAM2120 (*traA*), pAM2040 (*traB*), and pAM2140 (C), respectively. These parallels will be noted below in relation to the appropriate double-insert plasmid. (It should be recalled that the NR5 fusion disrupts a gene or genes required for aggregation and transfer; therefore, none of the double-mutant plasmids were capable of inducing an aggregation response or of independent transfer.)

Examination of the response curves of the various plasmids not only revealed significant effects of mutations in each region on the regulation of the NR5 fusion but also significant host-dependent differences. Host-dependent differences were apparent even in the regulation of the NR5 fusion on pAM2005A. Most obvious was a difference in pheromone sensitivity. In OG1X, significant induction (>10 fold) was always seen at 2.5 ng of pheromone per ml and levels of β-galactosidase rose rapidly between 0 and 5 ng/ml (Fig. 3A). In an FA2-2 background, no significant induction was observed until between 5 and 10 ng of cAD1 per ml and the level of induction increased more gradually (Fig. 3B). In addition, uninduced β-galactosidase levels were slightly (two- to fourfold) but consistently higher in OG1X than in FA2-2, but maximally induced levels were higher in FA2-2 (see also pAM2125, Fig. 3, and below).

The host-dependent sensitivity differences were mirrored by differences in the sensitivity of the aggregation response in wild-type pAD1-containing cells. Thus, when OG1X (pAD1) was used as a responder in a microdilution CIA assay, it responded to a culture filtrate from plasmid-free OG1X diluted up to 256-fold, while FA2-2(pAD1) responded only up to a dilution of 16, indicating that the OG1X(pAD1) strain was 15-fold more sensitive than FA2-2(pAD1). Two other host-dependent differences may relate to this observation. First, pAD1 and its derivatives (except C region mutants, see below) produced higher levels of iAD1 in an FA2-2 background (titer, 8) than in an OG1X background (titer, ≤2). This higher production of inhibitor might interfere with the inducing action of cAD1. Second, it has been observed that OG1X(pAD1) is capable of removing exogenous pheromone from the media by a cell-dependent mechanism (i.e., culture supernatants do not inactivate pheromone) (14). This binding reaction was not observed in an FA2-2 background, perhaps indicating that the FA2-2 receptor has a lower affinity for the pheromone.

In both backgrounds, the A120 *traA* insert resulted in constitutive derepression of the NR5 fusion present on pAM2125. Similarly, pAM2120, like most other *traA* mutants (7), conferred a constitutive aggregating phenotype on host cells of either strain. Thus, both OG1X(pAM2120) and FA2-2(pAM2120) produced macroscopic aggregates in broth and dry colonies on plates in the absence of exogenous pheromone. We also observed that pAM2120-containing cells constitutively produced the four inducible surface proteins (5) (data not shown) and transferred plasmid DNA constitutively in short (10-min) broth matings (Table 2) in both hosts. Levels of β-galactosidase produced from pAM2125 were consistently 20 to 60% higher (see Fig. 3) in the FA2-2 background than in OG1X, indicating that the inducible promoter may be capable of higher expression in FA2-2 than in OG1X.

In contrast to pAM2125, expression of β-galactosidase from the NR5 fusion of pAM2045 depended heavily on the host (Fig. 3). In OG1X, β-galactosidase production from the NR5 fusion of pAM2045 was constitutively derepressed, as in pAM2125. However, in the FA2-2 background, the NR5 fusion was regulated almost normally. Some derepression was observed in the FA2-2 host (≈sevenfold in the absence of pheromone; 0.3 and 2.2 Miller units for pAM2005A and pAM2045, respectively, in 2-h β-galactosidase assays with 100-μl samples), but the effect of the *traB* insert was not generally apparent in the FA2-2 host. These host-related differences in β-galactosidase expression were paralleled by differences in the aggregation and transfer response of *traB* mutants like pAM2040. For example, *traB* mutants present in FA2-2 had a normal colony morphology, but in OG1X, colonies were ringed with a dry center surrounded by a watery edge, evidence of constitutive aggregation (7, 13). In broth, constitutive aggregation was observed in FA2-2 strains containing *traB* mutants but this aggregation was qualitatively different from that in OG1X (7). In addition, pAM2040 was observed to transfer in short broth matings from FA2-2, but at a frequency at least 1 order of magnitude lower than from OG1X (Table 2). Finally, inducible surface protein production by *traB* mutants was also different in the two hosts. As shown in the Western blot in Fig. 4, while the inducible surface proteins were produced constitutively in OG1X(pAM2040), they were not present in the absence of pheromone in FA2-2(pAM2040). Similar results were observed with pAM2300, another *traB* mutant containing the B30 Tn*917-lac* insert (13) (data not shown). We also note here the presence of a previously unidentified inducible surface protein of ≈52 kilodaltons, designated AD52, in plasmid-containing FA2-2 strains. This protein was either not produced in an OG1X background or was obscured by other protein bands.

Host-related differences were also observed in the regulation of the NR5 fusion of pAM2145, the E/C region combination. In OG1X, the M14 C region insert resulted in an average sevenfold derepression of the NR5 fusion in the absence of cAD1 and a significant decrease in pheromone sensitivity. β-Galactosidase activity increased only 3-fold, from 0 to 40 ng/ml in pAM2145, compared with a 50-fold increase in pAM2005A. In FA2-2, a three-fold derepression (barely noticeable in Fig. 3B) was observed in the absence of pheromone and some difference in sensitivity was also observed. However, the derepression and decreased sensitivity were not as apparent in FA2-2 as in OG1X. Again, the effects of the M14 insert on the regulation of the NR5 fusion paralleled its effects on aggregation and transfer in the absence of an E region insert. Indeed, partial constitutive aggregation (observed as viscous, semidry colonies in the absence of exogenous pheromone) and decreased pheromone sensitivity (observed as a failure to produce hard, dry colonies in the presence of pheromone) were the very characteristics used to select the PR/M class of C region inserts in an OG1X background (13). These mutants, of which M14 is an example, are unique among C region inserts because of their constitutive aggregation and transfer in OG1X. However, in FA2-2 no constitutive aggregation was observed, and constitutive transfer was at least 1 order of magnitude lower than in OG1X (Table 2). Little decreased

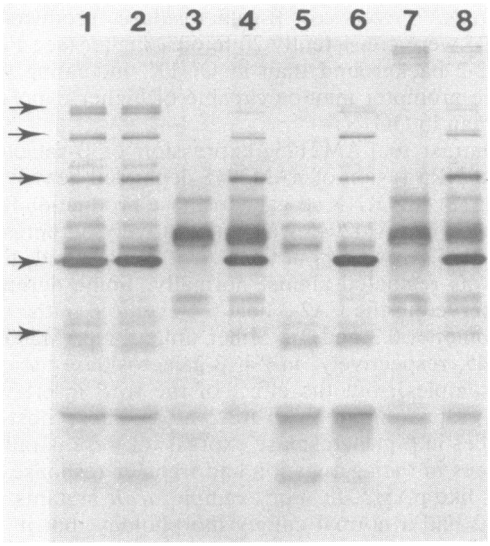

FIG. 4. Western blot of cell surface proteins extracted from induced and uninduced cells containing pAD1 wild-type and *traB* mutant plasmids. Extracts of induced and uninduced OG1X plasmid-containing strains were obtained as previously described (5). Extracts of induced and uninduced FA2-2 plasmid-containing strains were prepared in a similar manner, with the following modifications. Cultures were grown in THB, because growth rates in N2GT were too low to obtain sufficient amounts of protein at equivalent induction times. Induced cultures were exposed to 100 ng of pheromone per ml to compensate for the host-related and medium-related loss of sensitivity. To ensure that medium effects did not change inducible surface protein effects, Western blots of OG1X strains cultured in THB and induced (when appropriate) with 100 ng of cAD1 per ml were also performed. No medium effect was observed (data not shown). The five pheromone-inducible surface proteins, AD157, AD153, AD130, AD74, and AD52, are labeled (arrows, top to bottom, respectively). (Recent determinations indicate that AD157 and AD153 have masses of ≈190 and 170 kilodaltons, respectively, rather than the previously reported values of 157 and 153 kilodaltons.) Lanes: 1 (uninduced) and 2 (induced), OG1X (pAM2040); 3 (uninduced) and 4 (induced), FA2-2 (pAM2040); 5 (uninduced) and 6 (induced), OG1X (pAM714); 7 (uninduced) and 8 (induced), FA2-2 (pAM714).

sensitivity was observed with pAM2140 in an FA2-2 background.

One further host-related difference in the phenotype of C region mutants is worth mentioning. In OG1X, C region mutants resulted in an increase in iAD1 production, typically from a titer of ≤2 to a titer of 8 (4). In FA2-2, no significant increase in iAD1 titer was observed; C region mutant-containing strains, as well as strains containing any other type of pAD1 mutant or wild-type plasmid, exhibited an iAD1 titer of about 8. These results may in turn be related to observed differences in pheromone binding between the two hosts, as described above (see discussion). It is also interesting that OG1X strains containing C region mutants were defective in pheromone binding. After 1 h of exposure to cAD1 at a titer of 32, culture filtrates of cells containing pAD1 C region mutants still had a cAD1 titer of 16, whereas culture filtrates from exposed wild-type pAD1-containing cells had a titer of ≤2. This lack of binding may be related to the decreased pheromone sensitivity of C region mutants.

In summary, the regulation of the NR5 fusion was affected both by mutations in the neighboring regulatory genes and the host. In the absence of other mutations, an increased repression in the absence (and at low levels) of pheromone and increased maximal levels of expression were observed in FA2-2 compared with OG1X. With the *traA* mutation, the NR5 fusion was maximally expressed in both hosts but expression was higher in FA2-2. With the *traB* mutation, derepression in the absence of cAD1 was clearly observed in OG1X but was hardly noticeable in FA2-2. The C region mutant resulted in derepression and a decreased sensitivity of the NR5 fusion in OG1X, but, again, repression at low pheromone levels and maximal expression were greater in FA2-2.

TABLE 3. cAD1 and iAD1 production in FA2-2 and OG1X in the presence and absence of various plasmids[a]

Plasmid	Genotype[b]	OG1X		FA2-2	
		cAD1	iAD1[c]	cAD1	iAD1
None		256		32	
pAD1	WT[d]	<2	≤2	<2	8
pAM2125	*traA*/E	<2	<2	<2	8
pAM2120	*traA*	<2	2	<2	8
pAM2045	*traB*/E	32		<2	2
pAM2040	*traB*	<2	2	<2	8
pAM2145	C/E	4		<2	8
pAM2140	C	<2	8	<2	8
pAM2030	*traB*/C	64		<2	4
pAM2040E[e]	*traB*	<2	<2	ND[f]	ND

[a] Culture filtrates of all strains were prepared as previously described (3, 8). DS16 was used as the responder in both cAD1 and iAD1 assays. OG1X CF diluted 1:4 was used as the reference CF in iAD1 assays. A concentration of 40 ng of cAD1 per ml equals a titer of 64 to 128.

[b] Genes and/or regions containing Tn*917-lac* inserts are indicated.

[c] iAD1 titers were not determined in strains producing cAD1, since pheromone interferes with inhibitor detection.

[d] WT, Wild type.

[e] pAM2040E is a pAD1 miniplasmid consisting of the pAD1 *Eco*RI B fragment and the B4 *traB* insert.

[f] ND, Not determined.

cAD1 production by double mutants in OG1X and FA2-2. Table 3 shows the results of cAD1 and iAD1 assays performed on culture filtrates from plasmid-free OG1X and FA2-2 strains and strains containing each of the three double-insert plasmids and pAM2030. (pAM2030 contains the A3 deletion [see Fig. 1] and was previously shown to allow cAD1 production [2, 13]). As shown in the table, two of the double-insert plasmids, pAM2045 and to a lesser extent pAM2145, allowed production of cAD1 in an OG1X background. These results were somewhat surprising, since no single E or C region insert or *traB* single insert which results in cAD1 production has been isolated. The A3 deletion present on pAM2030 disrupts both the C region and *traB* (2), providing yet another mutant combination resulting in failure to shut down cAD1. The two combinations involving *traB* (pAM2030 and pAM2045) were least effective in pheromone shutdown. Levels of cAD1 produced by OG1X(pAM2145) were variable and produced responder (DS16) aggregates much finer and much more difficult to observe than in the other pheromone-producing strains. The *traA*-E region combination, pAM2125, had no effect on pheromone shutdown.

Host-dependent differences in cAD1 production can also be seen in Table 3. Even in plasmid-free strains, pheromone production was lower in FA2-2 than in OG1X. In FA2-2 strains containing the plasmids observed to allow production of cAD1 in OG1X, no pheromone was apparent in cAD1 assays. However, decreases in iAD1 titers observed in pAM2030- and pAM2045-containing strains may be indica-

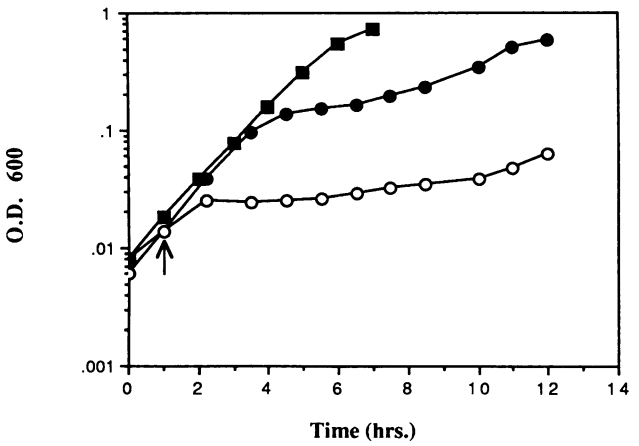

FIG. 5. Growth characteristics and pheromone inhibition of pAM2040E-containing strains. OG1X(pAM2040E) was grown overnight in N2GT-erythromycin and diluted 1:100 in fresh N2GT or N2GT-erythromycin. To test pheromone inhibition, synthetic cAD1 was added to a concentration of 40 ng/ml after 1 h of growth. A typical growth curve of OG1X(pAM2005K) in the absence of pheromone is provided for comparison. Symbols: ■, OG1X (pAM2005K); ●, OG1X(pAM2040E); ○, OG1X(pAM2040E) plus cAD1.

tive of an increased production of competing cAD1. No effect on either cAD1 or iAD1 levels was observed in FA2-2 strains containing either pAM2125 or pAM2145. These host-related differences in pheromone production are very likely related to the differences in β-galactosidase production observed between the two hosts (see Discussion).

Characterization of pAM2040E, a miniplasmid containing the *traB* insert B4. Since the NR5 insert affects expression of a large number of pheromone-inducible genes around the pAD1 plasmid, we constructed a pAD1 miniplasmid containing only the *traB* B4 insert to determine whether the NR5 disrupted gene required for pheromone shutdown is located on the pAD1 *Eco*RI B fragment (see Fig. 2). As shown in Table 3, OG1X strains containing this miniplasmid, pAM2040E, showed no evidence of cAD1 production. Therefore, the gene product affected by the NR5 insertion which results in failure to shut down pheromone in the presence of a *traB* insert must be encoded on the *Eco*RI B fragment.

Interestingly, pAM2040E-containing cells showed unusual growth characteristics. OG1X(pAM2040E) grew at a normal rate until it reached an optical density of about 0.150 at a wavelength of 600 nm (Fig. 5). At that point, generation time increased from ≈55 min to ≈8 h. After 3 to 4 h, some recovery was observed, shortly before the culture reached stationary phase. The presence or absence of antibiotic selection had no effect on the onset or magnitude of inhibition, but recovery was slightly more rapid in its absence. More than 85% of colonies isolated following recovery retained the plasmid, and all plasmid-containing isolates displayed the same unusual growth pattern, indicating that recovery was not due to accumulation of plasmid-free cells or the selection of mutants with an increased growth rate. Growth of pAM2040E-containing cells was also inhibited by the addition of exogenous cAD1, suggesting that the observed self-inhibition may be due to production of undetected cAD1 because of loss of *traB* function.

DISCUSSION

Previous work had identified four genetic determinants required for the regulation of the pAD1 pheromone response (4, 7, 13). We have constructed a number of double-insert plasmids which have allowed us to assess the effects of three of these determinants, *traA*, *traB*, and the C region, on the regulation of the fourth, the E region. These results have provided further information on the functions of the gene products encoded by each of these regions.

Results obtained with the *traA*/NR5 double insert, pAM2125, clearly indicate that the product of the *traA* gene is a key negative regulator of the pAD1 pheromone response, repressing transcription of E region genes which are essential for activation of the aggregation response (4, 13). Thus, β-galactosidase production from the NR5 fusion of pAM2125 was constitutively derepressed in both *E. faecalis* hosts, OG1X and FA2-2. While it is technically possible that the A120 insert could form a fortuitous promoter that transcribes E region genes, no outward-reading promoters have been identified on Tn*917* and no activation of genes neighboring Tn*917* or Tn*917-lac* has been reported, to our knowledge. Interestingly, it has been previously proposed that the *traA* gene product may also be involved in signal transduction, based on the characteristics of certain mutations mapping within the *traA* gene (2, 13). These mutations resulted in incomplete derepression of the aggregation and transfer response which could not be further induced by pheromone. Therefore, it appears that the *traA* gene product may interact in some way with the cAD1 signal receptor.

A negative regulatory function has also been proposed for the *traB* gene product (7). Results obtained here with the plasmid pAM2045, however, indicate that the primary function of the *traB* gene product may be in pheromone shutdown. Thus, differences in cAD1 production between the two host strains were sufficient to account for the effect of the *traB* mutation on the regulation of β-galactosidase production from the NR5 fusion of pAM2045. OG1X(pAM2045) produced significant levels of endogenous cAD1 and the NR5 fusion maximally induced in this host. FA2-2 (pAM2045) produced no detectable cAD1, and very little expression of the NR5 fusion was observed in the absence of exogenous pheromone.

Cells containing pAD1 derivatives with only *traB* inserts do not produce endogenous cAD1 to levels readily apparent in cAD1 assays but do aggregate and transfer constitutively. Because of this, it has previously been suggested that the *traB* product functions as a negative regulator, perhaps in cooperation with the *traA* product. Results with pAM2045 and pAM2030, however, suggested to us another possible explanation: that *traB* mutants respond to low levels of cAD1 that are not apparent, because of E and/or C region function. This hypothesis is strengthened by the observation that derepression of aggregation and transfer functions is less apparent in the FA2-2 host, which produces less cAD1 than OG1X. Thus, constitutive production of normally inducible surface proteins and constitutive colony aggregation were observed in OG1X but not FA2-2 strains containing *traB* mutations. Constitutive transfer and aggregation in *traB* mutation-containing FA2-2 strains were also reduced, compared with OG1X strains. These differences paralleled host-related differences (described above) in derepression of the NR5 fusion of pAM2045, a phenomenon clearly correlated with cAD1 production. The most likely explanation for these observations is that the *traB* mutation results in partial failure to shut down cAD1 activity; derepression is more

apparent in OG1X because OG1X makes more cAD1 than does FA2-2. The observed self-inhibition of OG1X (pAM2040E) may also be related to a response to endogenous cAD1.

Cells containing pAM2045, having inserts in both *traB* and the E region, produce readily detectable cAD1, while single insertions in either region do not. Therefore, a determinant either encoded within the E region or regulated by an E region product must also be involved in pheromone shutdown. The failure of OG1X(pAM2040E) to produce detectable levels of cAD1 indicates that this determinant is located on the pAD1 *Eco*RI B fragment. The fact that the E region products themselves are pheromone inducible makes it tempting to suggest that the related E region-controlled product is involved as part of a regulatory feedback mechanism that inactivates pheromone. Alternatively, basal-level expression of the E region product may be sufficient to act in cAD1 shutdown.

The function of the C region gene product(s) has been and remains the most elusive. Although the data presented here do not allow us to conclusively assign a function to this region, analysis of the expression of the NR5 gene fusion of pAM2145 and several host-dependent differences in strains containing C region mutants allow us to narrow the possibilities somewhat. The production of low levels of cAD1 by OG1X(pAM2145) would appear to rule out one possible explanation for the loss of sensitivity observed in C region mutants, that is, the increased production of iAD1. Thus, the C region insert of pAM2145 resulted in a decreased sensitivity to exogenous cAD1, even though the addition of the E region insert apparently caused the elaboration of sufficient endogenous cAD1 to counteract the effects of any excess iAD1 produced. Furthermore, pAM2145 showed some decrease in pheromone sensitivity, even in an FA2-2 background, in which C region mutants showed no detectable increase in iAD1 production. Therefore, loss of pheromone sensitivity appears to be a primary characteristic of C region mutants, not a by-product of increased iAD1 production.

One theory of C region function that satisfactorily accounts for both the decrease in sensitivity and the variability of iAD1 production proposes that the C region product enhances the affinity of the cAD1 receptor for its ligand, facilitating the binding of the pheromone in an OG1X background. Indeed, pheromone binding was considerably decreased in OG1X strains containing C region mutants. Since iAD1 is a competitive inhibitor of cAD1, it would be expected that the same protein(s) that resulted in cAD1 binding also bound iAD1. Therefore, the increase in iAD1 concentration observed in OG1X C region mutants might relate to a loss of ability to bind the inhibitor. The fact that iAD1 levels produced in OG1X C region mutants are similar to those produced by any pAD1 derivative in FA2-2 might relate to the observation that FA2-2(pAD1) does not efficiently bind pheromone. C region mutations might have less of an effect in FA2-2 because the C region product does not significantly increase the affinity of the receptor in this host. While it is possible that multiple host factors may affect pheromone sensitivity, binding, and inhibitor production, this explanation is the simplest which accounts for all of the observations. It seems unlikely that the entire receptor is encoded within the C region, since C region mutants, particularly in FA2-2, are capable of responding to pheromone. It is possible, however, that the C region product is a component of the receptor.

The reason for the failure of pAM2145 to shut down pheromone completely is unclear. One possibility is that the combination of the loss of pheromone binding due to the C region insert and the loss of the E region function results in the elaboration of detectable pheromone. OG1X(pAM2030), which contains a deletion affecting both the C region and *traB*, produced more pheromone than any other pAD1 derivative-containing strain, even though the E region product(s) was apparently constitutively produced, since this mutation causes constitutive aggregation. Like the E region product, the C region product(s) may function to mask endogenously produced pheromone, perhaps by binding or by cooperating with the *traB* product in some other way to accomplish pheromone shutdown.

Finally, as has been alluded to throughout this discussion, the expression of the NR5 fusion of the various plasmids was affected by the host in which it was located. The most dramatic effects were observed with pAM2045 and pAM2145, the *traB*/NR5 and C/NR5 combinations, respectively. Assuming that the primary function of the product of the *traB* gene is to shut down cAD1, it is not surprising that the host should affect the phenotype of *traB* mutants. Indeed, the lower production of pheromone by plasmid-free FA2-2, as compared with plasmid-free OG1X, may result in a proportionately lower production of pheromone in the presence of pAM2045. This, along with the lower sensitivity of the FA2-2 host, might be sufficient to account for the host-dependent differences in NR5 expression. Even if this simple explanation is not the case, it would not be surprising if the plasmid-encoded pheromone shutdown functions would interact differently with the chromosomally encoded pheromone production apparatus in unrelated hosts. By analogy, the host-dependent differences in expression of the NR5 fusion from pAM2145 suggest that the C region product may interact with the product of a chromosomal determinant. Since the function of the C region product appears to be primarily related to pheromone sensing, this suggests that a host factor is involved in this aspect of the pheromone response. This possibility is consistent with the hypothesis, expounded elsewhere, that the pheromone-related plasmids evolved to take advantage of a system, already available in the host, that resulted in the elaboration of these peptides (2). It would not be surprising if plasmid-free cells themselves had some sort of pheromone receptor, perhaps serving to control pheromone synthesis via a feedback circuit.

In conclusion, we have constructed plasmids with a pheromone-inducible *lacZ* fusion in the E region in combination with inserts in the *traA*, *traB*, or C region and examined their effects on β-galactosidase production in two unrelated hosts. By analyzing these results and comparing them with the effects on the pheromone response of single inserts in the same regions, we have arrived at the following conclusions: (i) the product of the *traA* gene is a key negative regulator of the pAD1 pheromone response and probably functions as the signal transducer; (ii) the product of the *traB* gene is important for the shutdown of pheromone production; (iii) a determinant encoded by or regulated by a product of the E region and located on the *Eco*RI B fragment is also important in pheromone shutdown; (iv) the primary function of the C region product most likely relates to pheromone sensing; and (v) a host factor may also be involved in pheromone sensing.

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