

Construction of *Enterococcus faecalis* pAD1 Miniplasmids: Identification of a Minimal Pheromone Response Regulatory Region and Evaluation of a Novel Pheromone-Dependent Growth Inhibition

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Several pAD1 miniplasmids were constructed that consisted of all or a portion of the pAD1 *EcoRI* B fragment with pheromone-inducible "E region" *lacZ* transcriptional fusions. Miniplasmids containing the entire *EcoRI* B fragment (the "E miniplasmids") were found to regulate LacZ expression normally, indicating that sufficient information is present on this fragment to regulate a pheromone response. These plasmids also encoded normal replication functions. The E miniplasmids were further reduced by deleting a *KpnI* fragment. These "K miniplasmids" were able to perform most of the functions central to a pheromone response but failed to induce the fused transcripts to levels observed in the parental plasmids. This defect was found to be due to a pheromone-dependent growth inhibition of cells containing the K miniplasmids. Evidence indicated that this inhibition was due to transcriptional readthrough beyond the *lacZ* gene and into the putative replication region of the plasmid. Possible mechanisms of this inhibition as well as its potential usefulness in further examining the characteristics of the pheromone response are discussed. © 1989 Academic Press, Inc.

Conjugative transfer of a group of plasmids found in the opportunistic pathogen *Enterococcus faecalis* is induced by small, hydrophobic peptide pheromones (Dunny *et al.*, 1978; Mori *et al.*, 1984, 1988; Suzuki *et al.*, 1984). Each pheromone is specific for a particular plasmid species and is produced only by cells which do not contain the related plasmid (Dunny *et al.*, 1979). Potential donors respond to pheromone by producing a proteinaceous adhesion, called "aggregation substance," which appears on the surface of induced donor cells (Yagi *et al.*, 1983). Aggregation substance facilitates the formation of mating aggregates by binding to a "binding substance" present on the surface of recipient cells. Since plasmid-containing cells also express binding substance, self-aggregation can be induced by exposure to pheromone. Induction involves synthesis of a number of surface proteins, at least some of which are likely components of aggregation substance (Ehrenfeld *et al.*, 1986;

Tortorello and Dunny, 1985). Expression of an inducible surface (entry) exclusion has also been associated with one of these proteins in the plasmid pCF10 (Dunny *et al.*, 1985). In addition, functions necessary for transfer of the plasmid itself appear to be inducible (Clewell and Brown, 1980). Thus, when cells containing two different pheromone plasmids are induced by pheromone specific for only one of the plasmids, productive mating aggregates are formed but transfer of only the specifically related plasmid is induced (Ehrenfeld *et al.*, 1986). Once the plasmid has been transferred to the recipient, the production of pheromone activity specific for that plasmid is shut down. Pheromones specific for other plasmids not present in the transconjugant continue to be produced. In addition, transconjugants begin to produce a small peptide which competitively inhibits the action of the pheromone specific for that plasmid (Ike *et al.*, 1983; Mori *et al.*, 1986). Therefore, at least four basic conjugation-related functions must be determined by the plasmid DNA: (i) the detection and transduction of the pheromone signal; (ii) the

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synthesis and/or regulation of the aggregation and transfer functions; (iii) the specific shut-down of endogenous pheromone activity; and (iv) the production of inhibitor.

Plasmid pAD1 (≈ 60 kb) encodes a response to the sex pheromone cAD1. It also encodes a hemolysin–bacteriocin (Tomich *et al.*, 1979; Clewell *et al.*, 1982) as well as resistance to ultraviolet light (Clewell *et al.*, 1986). The hemolysin–bacteriocin has been implicated as a virulence factor in *E. faecalis* (Ike *et al.*, 1984, 1987). Previous work with this plasmid defined a region of approximately 31 kb (Fig. 1) required for a normal pheromone response (Ehrenfeld and Clewell, 1987). For simplicity, determinants within this region can be categorized into three broad groups: (i) those affecting plasmid transfer but not aggregation or inducible surface protein production (comprising the previously defined G and H regions); (ii) those affecting aggregation and/or inducible surface protein production but not transfer

if cells are artificially “aggregated” by pressing them together on a solid surface (comprising the previously defined and recently extended F region); and (iii) those affecting both aggregation and transfer (Fig. 1). This latter group represents a cluster of determinants (*traA*, *traB*, and the E and C regions) located near one end of the 31-kb region. These genes are essential for the normal regulation of the pheromone response. The E region is believed to encode a positive regulator since mutations within this region result in an inability of plasmid-containing cells to aggregate or transfer the plasmid in response to pheromone (Ehrenfeld and Clewell, 1987). In addition, production of β -galactosidase from *lacZ* fusions within this region, constructed using Tn917-*lac* (Perkins and Youngman, 1986), is exquisitely sensitive to the presence of pheromone (Weaver and Clewell, 1988). The *traA* and *traB* genes appear to be negative regulators of the pheromone response, as mutations within

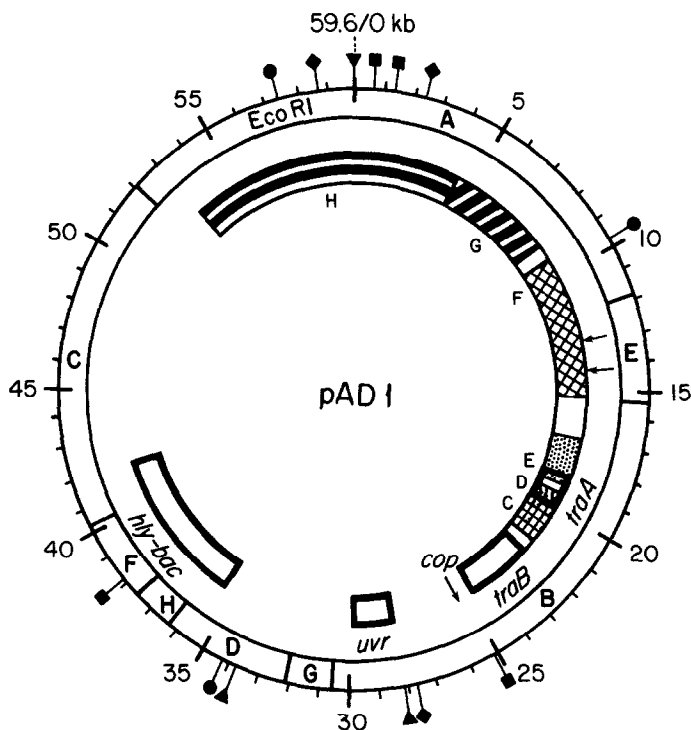


FIG. 1. Physical and functional map of pAD1. Regions important for transfer are indicated as shaded or open boxes on the interior of the map and labeled *traA*, *traB*, and C through H. Regions *hly-bac*, *uvr*, and *cop* represent genes for hemolysin–bacteriocin, uv resistance determinants, and copy number control, respectively. *EcoRI* fragments are labeled within the outer circle. Other restriction sites are (▼), *SalI*; (■) *BamHI*; (◆) *KpnI*; and (●) *PstI*.

these genes result in constitutive aggregation and rapid transfer of plasmid DNA in matings too short to allow normal pheromone induction (Ike and Clewell, 1984). In addition, abnormalities in the expression of transfer functions and inducible surface proteins observed in some *traA* mutants suggest that the product of this gene is also required for signal transduction (Weaver and Clewell, 1988). The function of the C region has yet to be determined, but mutations within this region result in an increased production of iAD1 and a decreased sensitivity to pheromone (Ehrenfeld and Clewell, 1987). A subset of mutants within the C region also results in "semiconstitutive" aggregation and transfer (Weaver and Clewell, 1988). Finally, a 2.5-kb deletion within this cluster of genes, removing a portion of *traB* and most of the C region, results in the inability of the plasmid to shut down pheromone production (Weaver and Clewell, 1988; K. E. Weaver, unpublished results). Therefore, genes present within this regulatory cluster are implicated in each of the four expected plasmid-encoded functions mentioned above.

To determine if the genes within the regulatory cluster are sufficient for the regulation of the pheromone response and to facilitate further investigations on the interactions of the regulatory factors, we have constructed "miniplasmids" consisting of all or a portion of the *EcoRI* B fragment of pAD1. Evidence presented in this report indicates that all genetic information necessary to regulate pheromone-inducible genes, as well as determinants required for replication, are present on this fragment. In addition, we report the construction of a miniplasmid derivative which resulted in pheromone-dependent growth inhibition of plasmid-containing cells. Possible mechanisms of this unique observation and its implications to pAD1 replication and the pheromone response are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used and their relevant characteristics are shown in Table 1. Except where noted plasmid characteristics were determined in an

E. faecalis OG1X background. Plasmids used or constructed in this investigation are shown in Table 1.

Media and reagents. All strains were grown in Antibiotic Medium No. 3 (Difco Laboratories, Detroit, MI) for ethidium bromide-CsCl gradient preparation of plasmid DNA and Todd-Hewitt broth (THB)³ (Difco) for rapid plasmid isolation. All other experiments were conducted in N2GT medium (Nutrient Broth No. 2 (Oxoid Ltd., London, UK) supplemented with 0.1 M Tris buffer (pH 7.5) and 0.2% glucose). Solid medium was prepared by adding agar (1.8%) (Difco); and hemolysis was detected, when necessary, on THB plates containing 4% horse blood (Colorado Serum Co., Denver, CO). Cultures were incubated at 37°C. Where appropriate, the following antibiotics were added at the indicated concentrations: streptomycin, 1 mg/ml; fusidic acid, 25 µg/ml; rifampin, 25 µg/ml; erythromycin, 10 µg/ml; tetracycline, 10 µg/ml. Other antibiotic concentrations are noted when appropriate.

Construction and physical analysis of pAD1 miniplasmids. In order to isolate a functional miniplasmid two requirements must be met: (i) the plasmid fragment selected must contain sufficient information for stable replication and inheritance; and (ii) the plasmid fragment must contain a selectable marker. In addition, since our intention was to determine the ability of regulatory genes on the miniplasmid to properly direct a pheromone response, some means of measuring that response was also required. Isolation of a Tn917 insertion located near the *traB* gene on the pAD1 *EcoRI* B fragment that resulted in a fivefold increase in plasmid copy number was previously reported (Ike and Clewell, 1984; see also Fig. 2), indicating that pAD1 replication functions might be encoded on this fragment. Inducible *lacZ* fusions, such as Tn917-*lac* inserts NR11 and NR5 (Weaver and Clewell, 1988), within this fragment would provide both the antibiotic selection and a method for quantifying the pheromone response. Therefore, it was con-

³ Abbreviations used: THB, Todd-Hewitt broth; MU, Miller unit.

TABLE 1

E. faecalis STRAINS AND PLASMIDS

Strain or plasmid	Relevant phenotype ^a	Reference
Strains		
OC1X	<i>str gel</i>	(Ike <i>et al.</i> , 1983)
FA2-2	<i>rif fus</i>	(Clewell <i>et al.</i> , 1982)
DS16	<i>tet erm</i>	(Tomich <i>et al.</i> , 1979)
CG110	<i>tet</i> , high frequency donor of Tn916	(Gawron-Burke and Clewell, 1982)
Plasmids		
pAD1	Hemolysin-bacteriocin	(Tomich <i>et al.</i> , 1979)
pAM2005A	pAD1 derivative containing the NR5 pheromone-inducible <i>lacZ</i> fusion, <i>agg</i> , <i>tf</i> , <i>isp</i> ⁺	(Weaver and Clewell, 1988)
pAM2005E	Miniplasmid derived from pAM2005A	This report, see Fig. 2
pAM2005K	Miniplasmid derived from pAM2005E, pheromone-inhibited growth	This report, see Fig. 2
pAM2005KR	Spontaneous pheromone-resistant derivative of pAM2005K	This report
pAM2516KT	Tn916-derived pheromone-resistant derivative of pAM2005K (KT16 insert)	This report, see Fig. 2
pAM2524KT	Tn916-derived pheromone-resistant derivative of pAM2005K (KT24 insert)	This report, see Fig. 2
pAM2011A	pAD1 derivative containing the NR11 pheromone-inducible <i>lacZ</i> fusion, <i>agg</i> , <i>tf</i> , <i>isp</i> ⁺	(Weaver and Clewell, 1988)
pAM2011E	Miniplasmid derived from pAM2011A	This report, see Fig. 2
pAM2011K	Miniplasmid derived from pAM2011E, pheromone-inhibited growth	This report, see Fig. 2
pAM211	pAD1::Tn916 <i>EcoRI</i> F fragment insert nonhemolytic	(Gawron-Burke and Clewell, 1982)

^a *agg*, aggregation; *tf*, transfer; *isp*, inducible surface proteins.

sidered possible that miniplasmids could be constructed from the *EcoRI* B fragment of plasmids containing one of these inserts.

In order to construct these derivatives, plasmids pAM2005A and pAM2011A (pAD1 derivatives containing the NR5 and NR11 inserts, respectively) were purified by ethidium bromide-CsCl ultracentrifugation as previously described (Clewell *et al.*, 1974). Approximately 1 µg of DNA was digested with *EcoRI*, precipitated, and religated with T4 DNA ligase. Religated DNA was then used to transform OG1X protoplasts as previously described (Wirth *et al.*, 1986). Transformants were selected on media supplemented with 2 µg of erythromycin per milliliter. Routine screening of plasmid DNA from transformants

was carried out using the small-scale alkaline lysis procedure previously described (Weaver and Clewell, 1988). Plasmid DNA was analyzed by digestion with various restriction enzymes and electrophoretic separation of restriction fragments on 0.8% agarose. All restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, MD) and reactions carried out in the buffers provided under the conditions recommended. These derivatives, the E miniplasmids, were designated pAM2005E and pAM2011E to indicate that they consisted of the entire *EcoRI* B fragments of their respective parental plasmids (Fig. 2).

To further reduce the size of the miniplasmids a similar isolation procedure was carried

out with *KpnI*-cleaved pAM2005E and pAM2011E. *KpnI* sites are located just outside of the plasmid-encoded *uvr* gene and just downstream of the Tn917 *erm* gene (Shaw and Clewell, 1985). Therefore, such constructs would not be expected to disturb the antibiotic resistance or *lacZ* determinants. These derivatives, the K miniplasmids, were mapped and designated pAM2005K and pAM2011K to indicate that they consisted of the *KpnI* fragment of their respective parental plasmids (see Fig. 2).

Determination of plasmid copy number, incompatibility, and stability. The copy number of pAM2005A, its miniplasmid derivatives, and pAM2011K were determined using [³H]thymidine-labeled cultures as previously described (Clewell *et al.*, 1974). To determine plasmid incompatibility, pAM211 was transferred by filter mating (Clewell *et al.*, 1985) into OG1X strains carrying the plasmid to be tested. Transconjugants were selected on tetracycline-containing medium to select for the incoming plasmid and then screened for maintenance of the resident plasmid on erythromycin-containing plates. The rate of plasmid loss (i.e., plasmid stability) was determined after cells containing the plasmid were grown from single colony inoculum overnight in selective medium. The cells were washed once and then diluted in fresh nonselective medium. The culture was maintained in log phase by periodic dilution for at least 100 generations or until <1% of colony isolates contained the plasmid. Samples were taken periodically and screened for the percentage of plasmid-containing cells based on the retention of the *erm* determinant. After 100 generations, any remaining erythromycin-resistant colonies were screened for expected plasmid-determined characteristics (e.g., pheromone-dependent growth inhibition or pheromone induction of the *lacZ* fusion) to ensure that transposition of Tn917 or plasmid mutation had not occurred.

Pheromone assays. The concentration of cAD1 was determined by the microtiter assay method described previously (Dunny *et al.*, 1979). The cAD1 titer was 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 microtiter assay (Ike *et al.*, 1983). The inhibitor titer was represented as the pheromone titer in the absence of inhibitor divided by the titer in its presence. Because iAD1 production is very low in an *E. faecalis* OG1X background, all plasmids were transferred to *E. faecalis* strain FA2-2 to ensure that iAD1 production was normal. In the case of nonconjugative pAD1 derivatives and miniplasmids, this was accomplished by performing triparental matings between the plasmid-containing strain and FA2-2, using OG1X(pAD1) as the helper. Transconjugants were selected on plates containing fusidic acid, rifampin, and erythromycin and then screened for pheromone-induced production of β -galactosidase or pheromone inhibition. In addition, miniplasmid-bearing transconjugants were checked for hemolysin production, transmissibility, and plasmid content to ensure that no pAD1 copies coexisted in the cell.

Pheromone response and pheromone-dependent growth inhibition. To test the pheromone responsiveness of cells containing miniplasmids and their parental plasmids, 1:10 dilutions were made from an overnight culture of the strain to be tested and grown for 30 min. Cultures were then induced with 0, 2.5, 5, 10, 20, and 40 ng/ml of synthetic cAD1 (custom made by Milligen Inc., Watertown, MA) for 90 min. Cell density was determined using a Spectronic 21 colorimeter (Bausch & Lomb, Inc., Rochester, NY) at a wavelength of 600 nm. Cells were harvested, toluenized, and assayed for β -galactosidase activity as previously described (Weaver and Clewell, 1988). Assays were conducted with 40 μ l of sample for 30 min. Activities are expressed in Miller units (MU; Miller, 1972). Plasmid-containing strains were routinely checked for pheromone-dependent growth inhibition by exposing early log phase cultures to 20–40 ng of cAD1 per milliliter and following growth at OD 600 as above. Specific conditions of various inhibition experiments are described in the text.

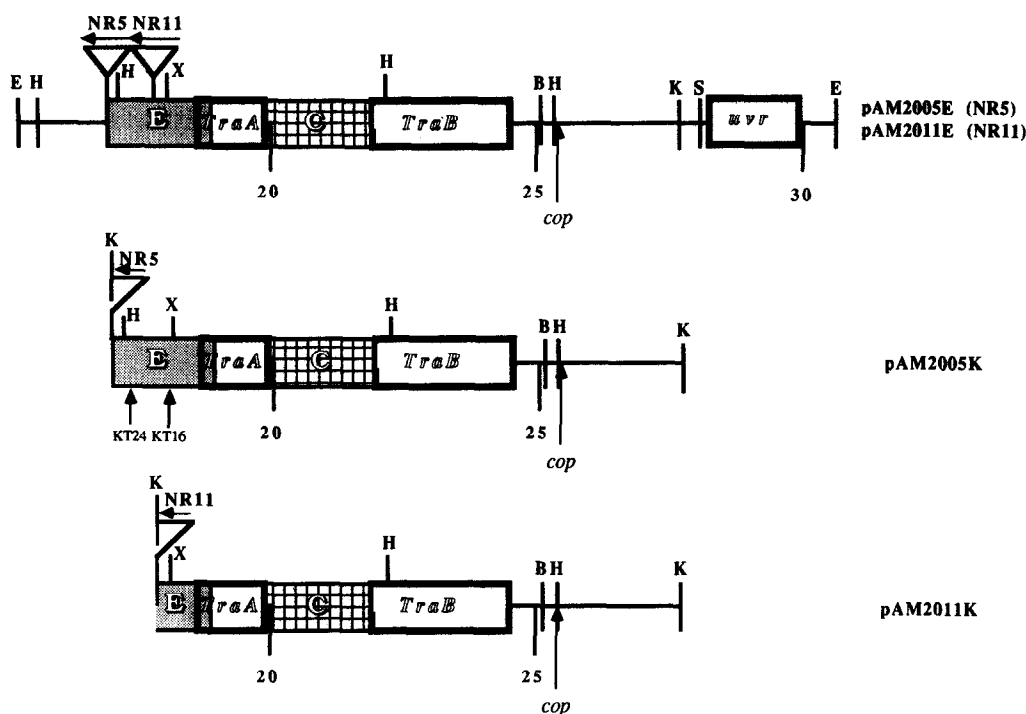


FIG. 2. Plasmid maps of pAD1 miniplasmids. All four plasmids consist of portions of the pAD1 *EcoRI* B fragment and each contains an inducible *lacZ* fusion, NR5 or NR11, indicated by the insertion triangle. pAM2005E and pAM2011E differ only in the site of the Tn917-*lac* insertion, NR5 and NR11, respectively. The regions designated *traA*, *traB*, C, and E have previously been shown to be important for pheromone regulation. The *uvr* gene is required for the plasmid-encoded uv resistance. Restriction sites are designated above the map and kb markers below. Restriction sites mapped are E, *EcoRI*; H, *HindIII*; X, *XbaI*; B, *BamHI*; K, *KpnI*; S, *SalI*. Tn917-*lac* contains several *HindIII* and *SalI* sites that are not shown and a single *KpnI* site which was used in the construction of pAM2005K and pAM2011K. Also shown on the pAM2005K map are the sites of two Tn916 inserts, KT16 and KT24, which relieve the pheromone-dependent growth inhibition. The deletion present in pAM2005KR is located between the *HindIII* and the *KpnI* sites on the far right end of pAM2005K. The site marked *cop* is a formerly mapped locus at which a Tn917 insert results in a fivefold increase in plasmid copy number.

Mutagenesis and selection of pheromone resistant mutants of pAM2005K. Spontaneous pheromone-resistant mutants of pAM2005K were selected simply by streaking single colonies of OG1X (pAM2005K) on plates containing 20–40 ng of cAD1 and 100 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Sigma Chemical Co., St. Louis, MO) per milliliter. Colonies that grew faster than the background and produced a blue pigment, indicating induction of the E region *lacZ* fusion, were selected for further analysis. Tn916 transposon mutants were selected following an overnight filter mating of the plasmid-containing strain with CG110. Transconjugants were selected on tetracycline- and strepto-

mycin-containing plates supplemented with 20–40 ng of cAD1 per milliliter. Colonies growing faster than background were selected and purified for further characterization.

RESULTS

Replication and Pheromone Induction Characteristics of pAD1 Miniplasmids

Miniplasmids were constructed from the pAD1::Tn917-*lac* derivatives pAM2005A and pAM2011A as described under Materials and Methods. Physical and functional maps of the miniplasmids are shown in Fig. 2. All miniplasmid-containing strains failed to produce cAD1 and excreted normal levels of iAD1.

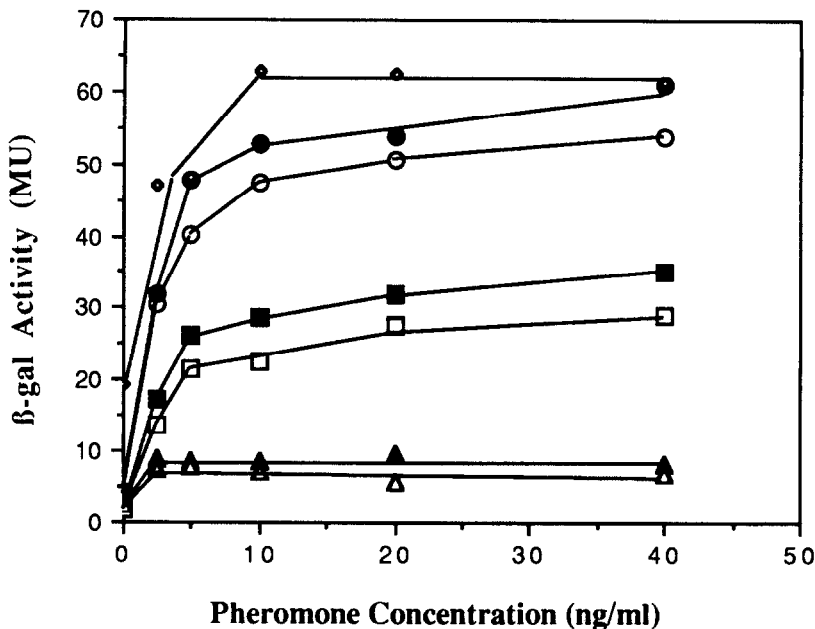


FIG. 3. Pheromone response of miniplasmids and their parental plasmids. OG1X strains containing the various plasmids were cultured and induced as described under Materials and Methods. β -Galactosidase activity is expressed in Miller units, a unit of measure normalized to culture optical density. Each line is an average of three independently conducted experiments. Symbols: (○) pAM2005A; (●) pAM2005E; (▲) pAM2005K; (◇) pAM2005KR; (□) pAM2011A; (■) pAM2011E; (△) pAM2011K.

Comparison of the parental pAD1::Tn917-*lac* plasmid, pAM2005A, with its two miniplasmid derivatives, pAM2005E and pAM2005K, revealed that maintenance was near normal in all cases. Copy numbers of all three plasmids were two to four per chromosome equivalent. In addition, no significant difference in expression of incompatibility was observed between the miniplasmids and pAM2005A following uptake and selection for pAM211 (see Materials and Methods). In all cases, loss of the unselected *erm* determinant was greater than 90%. Uptake frequencies of pAM211 were one to two orders of magnitude higher when the recipient contained one of the miniplasmids, compared to pAM2005A-containing recipients ($\approx 10^{-4}$ vs 10^{-5} per donor cell). The presence of an as yet unidentified surface exclusion function on pAM2005A that is absent on the miniplasmids could account for this difference. Similar copy number and incompatibility characteristics were observed for the smaller miniplasmid, pAM2011K.

The pAM2005E derivative was slightly less stable than its parent, pAM2005A. Thus,

pAM2005E was lost at $\approx 0.1\%$ per generation in the absence of selection while no loss of pAM2005A was observed. This may be less due to deletion of maintenance functions than to the loss of the hemolysin-bacteriocin determinant from the miniplasmid. The bacteriocin might be expected to quickly kill any plasmid-free segregant, resulting in an apparent increase in plasmid stability. Stability of pAM2005K was highly variable, ranging from greater than 99% plasmid loss within 50 generations to less than 5% loss over 100 generations. In one experiment the plasmid was maintained in 90% of cells over 40 generations and then was lost from >99% of cells over the next 30 generations. In all cases, erythromycin-resistant strains remaining near the end of the experiment were indistinguishable, phenotypically or in plasmid content, from those in the inoculum. The basis of this unusual stability pattern is due to the unique characteristics of the K miniplasmids addressed below and under Discussion.

Figure 3 shows the pattern of pheromone-inducible β -galactosidase production in cells

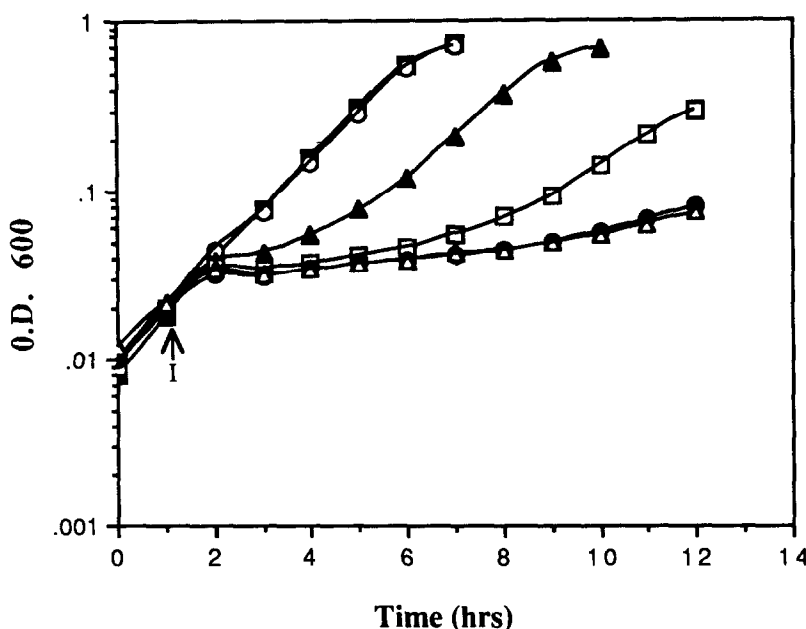


FIG. 4. Pheromone-dependent growth inhibition of pAM2005K-containing cells. OG1X(pAM2005K) was grown overnight in N2GT/Em and then diluted to 1% in fresh N2GT/Em. After 1 h pheromone was added to the indicated concentration: (■) 0 ng/ml; (○) 0.6 ng/ml; (▲) 1.25 ng/ml; (□) 2.5 ng/ml; (●) 5 ng/ml; (△) 10 ng/ml. The arrow indicates the point at which pheromone was added.

containing the miniplasmids and their parental plasmids. Both pAM2005E and pAM2011E regulated the production of β -galactosidase from their respective fusions in a manner nearly identical to that observed in the parental plasmids, pAM2005A and pAM2011A, respectively, although activity levels were consistently 10–20% higher in strains containing the miniplasmids. Regulation of the β -galactosidase production by pAM2005K and pAM2011K was clearly not normal. The pheromone-inducible promoter appeared to be normally repressed in this plasmid; and, upon exposure to pheromone, some increase in the production of β -galactosidase was observed. However, β -galactosidase activity levels reached a maximum at or before pheromone concentrations of 2.5 ng/ml and were not increased by further addition of pheromone.

Pheromone-Dependent Growth Inhibition of Cells Containing pAM2005K and pAM2011K

Following the 1.5-h induction period used to generate the pheromone response curves

shown in Fig. 3, it was observed that the culture densities of pAM2005K- and pAM2011K-containing cells induced with >2.5 ng of cAD1 per milliliter were nearly half those of uninduced cultures. No such difference was observed with either of the E miniplasmids or the parental plasmids. Therefore, it seemed possible that a pheromone-inducible growth inhibition was in some way linked to, or responsible for, the observed failure of the K miniplasmids to normally induce the *lacZ* fusions.

To test this hypothesis we examined the effects of various pheromone concentrations on pAM2005K-containing cells. Results are shown in Fig. 4. Cultures exposed to pheromone concentrations of greater than 1 ng/ml showed a detectable decrease in growth rate within 30–40 min of induction. At pheromone concentrations above 2.5 ng/ml, growth inhibition persisted, resulting in a fairly constant growth rate approximately $\frac{1}{10}$ of normal. The generation time in the absence of pheromone is ≈ 1 h and was increased to greater than 10 h at pheromone concentrations of 5 and 10

ng/ml. At pheromone concentrations below 5 ng/ml, cultures were observed to recover at a period of time after induction that was dependent on the concentration of inducing pheromone (i.e., the higher the pheromone concentration the greater the lag period before recovery). At these lower pheromone concentrations, recovery was not due to selection of pheromone-resistant mutants since greater than 95% of isolates from cultures grown to stationary phase could still be inhibited by pheromone. Recovery was also observed after prolonged incubation following induction with pheromone concentrations of 5 ng/ml and higher, but in this case the majority of isolates from such recovered cultures were pheromone resistant (data not shown). As can be seen from Fig. 4 the effects of pheromone on growth rate are persistent. Even cultures induced with 1.2 ng/ml pheromone fail to return to a normal growth rate prior to reaching stationary phase and the higher the pheromone the greater the effect. For example, a maximum growth rate of 80 min is observed for cultures recovering from induction at 1.2 ng/ml and 100 min for cultures recovering from induction at 2.5 ng/ml, compared to 60 min for uninduced cultures. Similar results were obtained with cultures containing pAM2011K, but no significant pheromone-dependent growth inhibition was observed in cultures containing either of the E miniplasmids (data not shown).

Reversibility of inhibition was tested by washing induced pAM2005K-containing cells 1 h after induction and resuspending in pheromone-free medium. Washed cultures still grew slowly but recovered more quickly than unwashed cultures (data not shown). For example, the generation time of cultures washed and allowed to recover after induction with 2.5 ng of pheromone per milliliter reached 90 min 5 to 9 h after induction, compared to 100 min 7 to 11 h after induction for unwashed cultures. One observation that may be connected to the nearly irreversible nature of pheromone inhibition was that pAD1-containing cells are capable of binding and removing exogenous pheromone from the medium. Thus, if pAM2005A-containing cells were diluted 1:10 from a stationary phase cul-

ture, allowed to grow 1 h, and then exposed to 20 ng of pheromone per milliliter within 1 h after induction the pheromone titer of the culture filtrate dropped from 64 to 2. Incubation of pheromone with the supernatant of similarly grown cells had no effect on the pheromone titer. In the case of pAM2005K-containing cells exposed to 2.5 ng of pheromone per milliliter (titer of four) no pheromone was detectable after 1 h. Therefore, the relative ineffectiveness of washing to reverse inhibition may have been due to the fact that the majority of pheromone was already bound to receptors on the cell surface; washing removed only the residual, unbound pheromone.

Comparable pheromone-dependent growth inhibition occurred in the absence of antibiotic selection. Thus, the generation time of cultures induced with >10 ng of cAD1 per milliliter in the absence of erythromycin increased from 48 min to >10 h. Cultures induced with 2.5 ng of pheromone per milliliter were initially inhibited and then recovered to a maximal growth rate of 84 min before entering stationary phase. This recovery at 2.5 ng of cAD1 per milliliter is not due to selection of plasmid-free cells, as 96% of colonies isolated following recovery remain erythromycin resistant (and pheromone inhibitable). However, when plated on solid media the majority of colonies isolated following recovery at pheromone concentrations above 5 ng/ml had lost the plasmid. Therefore, pheromone-dependent growth inhibition does not require continued selection for plasmid maintenance.

The effects of pheromone induction on the viability of pAM2005K-containing cells is shown in Fig. 5. Just 20 min after induction with 10 ng of pheromone per milliliter viable counts dropped to 66% of counts obtained prior to induction. Approximately 1 h after induction viable counts were reduced by 50% and then stabilized or increased slightly. In addition, colonies from cultures induced with pheromone took up to a day longer to become visible on plates than colonies from uninduced cultures, again indicating the slow recovery from growth inhibition even after cells are removed from pheromone-containing media. It

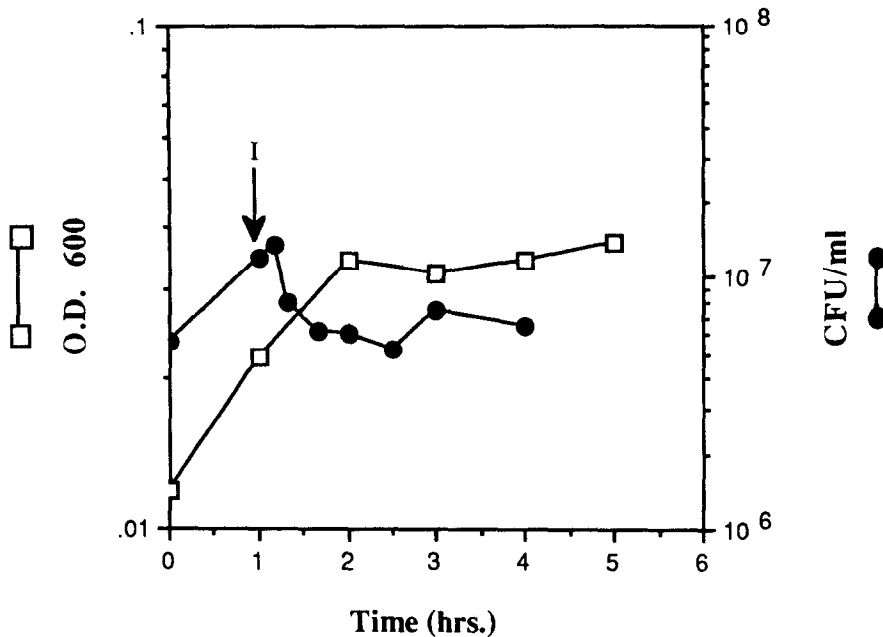


FIG. 5. Pheromone-induced loss of viability. OG1X(pAM2005K) was grown overnight in N2GT/Em, diluted to 1% in fresh N2GT/Em, and induced after 1 h of growth with 10 ng of pheromone per milliliter. Samples were taken periodically and plated on N2GT/EmSm plates to follow viability. Symbols (□) culture optical density at OD 600; (●) viable counts.

should be noted that the majority of cell death occurred during the period when the optical density of the culture was still increasing, indicating that while cells may increase in mass and actually divide, a significant proportion of new cells is nonviable. This decrease in viability was not accompanied by any visible changes in cell morphology when samples were examined under a phase contrast light microscope.

The effect of pheromone induction on plasmid copy number was also determined. After 1 h of induction with 40 ng of pheromone per milliliter no detectable change was observed in plasmid copy number.

All of the data presented above indicated that pheromone-dependent growth inhibition was due to the induction of one or more gene products encoded on the K miniplasmids. In cells containing the E miniplasmids either this gene product is not induced or its effects are moderated via genetic material deleted from the K miniplasmids. It should be noted here that the Tn917-*lac KpnI* site used in constructing both K miniplasmids is located

within a putative transcription terminator; in addition a transcription termination site at the distal end of the transposon was removed (Shaw and Clewell, 1985). Since the inserted *lacZ* gene contains no terminator sequences and no other potential terminators have been identified which precede the one interrupted by *KpnI*, it is possible that expression downstream (as well as upstream) of the *lacZ* gene could account for the observed growth inhibition. To further localize the offending gene we isolated pheromone-resistant revertants of pAM2005K-containing cells either by Tn916 mutagenesis or by selection of spontaneous mutants on pheromone-containing plates (see Materials and Methods).

Two Tn916 inserts were isolated that resulted in pheromone resistance of plasmid-containing cells. The location of these inserts is shown in Fig. 2. Although both inserts were mapped to regions upstream of the *lacZ* gene, both resulted in an inability to induce the *lacZ* fusion. KT24, the insert closest to the NR5 insert, resulted in total loss of β -galactosidase expression; activity levels observed were be-

tween 0.2 and 0.4 MU—less than that observed in uninduced pAM2005K. KT16 allowed expression of β -galactosidase to uninduced levels (2–3 MU) but did not allow significant induction to occur. Therefore, inducible expression both upstream and downstream of the *lacZ* fusion was affected by these insertions. Furthermore, KT24 is clearly located between the positions of the NR11 and NR5 inserts. Thus, any putative inhibitory gene product disrupted by KT24 would also be absent in pAM2011K. Since growth of pAM2011K-containing cells was inhibited by pheromone, it is likely that the pheromone resistance of cells containing pAM2524KT is due to the effects of the KT24 insert on expression of the region downstream of the *lacZ* gene.

Results obtained with a spontaneous pheromone-resistant derivative of pAM2005K support this conclusion. Several pheromone-resistant derivatives were isolated that were capable of induction of the *lacZ* fusion. The majority of these showed no obvious alteration in plasmid DNA, but one derivative was isolated that contained an \approx 200-bp deletion within the *KpnI*–*HindIII* fragment downstream of the *lacZ* and *erm* genes. The growth rate of strains containing this plasmid, designated pAM2005KR, was not affected by the presence of pheromone even though the promoter controlling *lacZ* expression is significantly induced. Indeed, as can be seen in Fig. 3, induction of the *lacZ* fusion in this mutant occurred to levels higher than those observed in any of the other fusions. The pAM2005KR fusion was also significantly derepressed in the absence of pheromone, producing β -galactosidase to levels six- to sevenfold higher than those in uninduced pAM2005K.

Since the deletion present in pAM2005KR is located within the region believed to be required for plasmid replication, the plasmid was examined for any changes in maintenance properties. No effect on average plasmid copy number or expression of incompatibility was observed. A slight but consistent decrease in plasmid stability was observed. In the absence of selection, pAM2005KR was lost at a rate of 0.5–1.0% per generation. The deletion had

no effect on the shutdown of cAD1 or on the production of iAD1. Thus, the pheromone-dependent growth inhibition of pAM2005K- and pAM2011K-containing cells may be due to expression or overexpression of a plasmid stability determinant.

DISCUSSION

Previous work has identified a cluster of genetic determinants located on the pAD1 *EcoRI* B fragment that are essential for normal regulation of the pheromone response. Results presented in this report indicate that these genes are not only essential but, at least in the case of the pheromone-inducible E region product(s), they are also sufficient. Thus, miniplasmids consisting entirely of the pAD1 *EcoRI* B fragment were (i) capable of normally regulating two different inducible *lacZ* fusions within the E region; (ii) able to shut down cAD1 activity; and (iii) able to produce normal amounts of iAD1. In addition, although it was unable to regulate the fusion normally, it is clear that the smallest miniplasmid isolated, pAM2011K (see Fig. 2), contains determinants sufficient to negatively regulate the pheromone-inducible promoter and sense/transduce the pheromone signal. Cells containing pAM2011K also shut down cAD1 activity and produce iAD1. It should be noted that these results do not rule out the possible involvement of host (chromosomally encoded) factors in controlling the pheromone response. Also, whereas the E region is believed to encode a positive regulator(s), it is possible that additional “regulators” may be necessary for induction of aggregation and transfer functions.

Results indicate that sufficient information for normal plasmid maintenance is also present on the miniplasmids. Thus, miniplasmids were maintained at a normal copy number and expressed normal incompatibility characteristics. Although stability of the K miniplasmids seemed variable from experiment to experiment, the fact that pAM2005K was stably maintained for 40 to 100 generations in some cases indicates that information sufficient for stable maintenance is also present.

Variability was most likely due to exaggeration of the rate of loss due to the inhibitory effects of pheromone produced by plasmid-free segregants on the remaining plasmid-containing cells. The smallest miniplasmid, pAM2011K, is approximately 14.8 kb, 4.8 kb of which represents the remaining Tn917-*lac* DNA. Of the remaining 10 kb of plasmid DNA, approximately 7 kb has been previously identified as important for the pheromone response. Assuming that there are no overlapping functions, this leaves approximately 3 kb of plasmid DNA for carrying out the required maintenance functions.

The K miniplasmids failed to induce their respective fusions to levels observed in either the parental or the E miniplasmids, presumably because growth of cells containing these plasmids was inhibited under the conditions used to induce the cells. The dose-related response to pheromone and the isolation of Tn916 inserts in pAM2005K which interfere with both pheromone-related growth inhibition and production of β -galactosidase indicates that growth inhibition is related to transcription initiated at the promoter(s) controlling transcription of the NR5 and NR11 fusions. The positions of the Tn916 inserts and the isolation of a spontaneous pheromone-resistant mutant containing an \approx 200-bp deletion in the area suspected of encoding replication functions suggest that inhibition may be due to transcriptional readthrough from an E region promoter into the *rep* region. This is possible because the Tn917-*lac* remnant present in the K miniplasmids does not have a functional transcription terminator. Because growth inhibition occurs in the absence of selection for plasmid maintenance it is unlikely that it is due to transcriptional interference with plasmid replication alone. In the absence of selection such interference might lead to the rapid accumulation of plasmid-free segregants but with little or no effect on culture growth rates. However, at least three low copy number plasmids, F, R100, and R1, have been found to encode determinants (*ccd*, *pem*, and *hok*, respectively) which function to enhance plasmid stability by killing plasmid-free segregants (Hiraga *et al.*, 1986; Tsuchimoto *et al.*,

1988; Gerdes *et al.*, 1986). If such a system were operative in pAD1, transcriptional interference with pAM2005K plasmid replication could lead to inhibition of growth indirectly by giving rise to plasmid-free segregants which are then killed. Alternatively, if such a gene were fused directly to the pheromone-induced transcript, overproduction of its gene product could lead to cell death, as has been observed when the R1 *hok* gene is overproduced (Gerdes *et al.*, 1986). The observed decrease in viability of pAM2005K-containing cells following pheromone induction and the slight but consistent decrease in plasmid stability of the spontaneous pheromone-resistant mutant pAM2005KR are consistent with this hypothesis. In addition, both *ccd* and *pem* are located near the respective plasmid replication regions, as is the deletion in pAM2005KR. Alternatively, overexpression of some other replication function could lead to growth inhibition. Since there is no significant increase in plasmid copy number in induced pAM2005K-containing cells, it is unlikely that inhibition is due to runaway plasmid replication.

One may wonder why erythromycin itself does not induce growth inhibition since the *erm* gene is located downstream of the *lacZ* gene and resistance is known to be inducible. However, the Tn917 *erm* gene is one of a family of inducible erythromycin resistance genes that are regulated by translational attenuation (Gryczan *et al.*, 1980; Horinouchi and Weisblum, 1980). Thus, erythromycin induction of the *erm* gene affects only translation of its own gene product and would not be expected to increase the production of other gene products with their own ribosome binding sites encoded on the same transcript.

Three intriguing aspects of the pheromone-dependent growth inhibition phenomenon may have relevance to the pheromone response. First, induced cells were able to recover from pheromone inhibition. At low concentrations of pheromone this recovery was not due to selection of either plasmid-free or pheromone-resistant cells. The gradual nature of recovery indicates that it may not be due to outgrowth of uninduced, and therefore un-

inhibited, cells. One possible explanation is that induced cells have a mechanism for terminating the pheromone signal, either by inactivation of the pheromone itself or by modifying the receptor or signal transducer in some unknown way. The observed pheromone binding may also be important for moderating the pheromone signal. Second, the effects of the KT24 and KT16 Tn916 inserts have implications regarding the organization and regulation of the E region gene products. The KT24 insert resulted in the virtual elimination of transcription of the *lacZ* fusion, implying that this insert is located between the *lacZ* gene and the pheromone-inducible promoter. The KT16 insert, however, allowed transcription of the *lacZ* gene at uninduced levels but did not allow pheromone induction of the fusion. Outward reading promoter-like sequences have been observed in the right end of Tn916 (Clewell *et al.*, 1988), but in pAM2516KT the transposon is inserted in the wrong orientation for these putative promoters to affect *lacZ* expression. Therefore, the KT16 insert may be located in a regulatory site or gene required for induction. Alternatively, the left end of Tn916 may have an as yet unidentified promoter. Finally, the deletion present in plasmid pAM2005KR not only resulted in pheromone resistance but also affected production of β -galactosidase from the inducible fusion. Higher levels of expression were observed in the absence of pheromone as well as following pheromone induction. No significant increase in plasmid copy number was observed, making it unlikely that a related increase in gene dosage could account for the observed six- to sevenfold increase in uninduced levels of β -galactosidase production. This is the first indication that genetic information encoded in this region can effect the pheromone response and may indicate a link between plasmid replication and pheromone induction. Further investigation of this and other spontaneous pheromone-resistant mutants of the K miniplasmids may be useful in establishing such a link.

In conclusion, in this report we describe the isolation of a number of pAD1 miniplasmids which encode sufficient information to regu-

late a pheromone-inducible gene and to control plasmid replication. These plasmids will prove very useful for further studies on the control of the pheromone response and on the examination of the replication properties of plasmid pAD1.

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