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A Phase Variation Event That Activates Conjugation Functions Encoded by the *Enterococcus faecalis* Plasmid pAD1

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Enterococcus faecalis cells carrying the conjugative plasmid pAD1 undergo several related changes when induced by the sex pheromone cAD1. Included are the production of novel surface proteins, the formation of cellular aggregates in broth cultures, the ability to transfer the plasmid at high frequency in broth matings, and the change from a soft to a "dry" colony morphology. Spontaneous, constitutively dry colony (Dry^c) variants of *E. faecalis* (pAD1) were found to arise at a frequency of 10^{-4}–10^{-2}. Dry^c phase variants constitutively expressed aggregation and plasmid transfer functions typically expressed only under cAD1-inducing conditions. Reversion of Dry^c variants to a cAD1-inducible phenotype (Dry$^+$) occurred at a similar frequency. Tn*917-lac* mutagenesis of regions of pAD1 previously shown to be involved in plasmid transfer revealed that in Dry$^+$ cells these regions were transcribed only when the inducer, cAD1, was present. In Dry^c variants the regions were transcribed constitutively. A pAD1 miniplasmid containing determinants regulating cAD1 inducible plasmid transfer and a cAD1-inducible *lacZ* transcriptional fusion displayed phase variation in LacZ expression at a rate similar to the Dry$^+$/Dry^c phase variation. These results suggest that the site of mutation(s) resulting in the Dry^c phenotype is within the regulation-related region of pAD1. Complementation tests showed that this region, when supplied in *trans*, complemented the Dry^c phenotype. Phase variation affecting mating functions represents an alternative (pheromone independent) method of regulating pAD1 transfer. © 1991 Academic Press, Inc.

pAD1 is a 60-kb conjugative plasmid originally found in *Enterococcus faecalis* DS16 (Tomich *et al.*, 1979). Dissemination of the plasmid is of clinical interest due to a hemolysin–bacteriocin determinant which may be a significant virulence factor (Ike *et al.*, 1984, 1987). Like a number of other conjugative plasmids found in *E. faecalis*, transfer functions are induced by a sex pheromone excreted by potential recipients (reviewed in Clewell and Weaver, 1989). The transfer functions, in this case, are specifically induced by the small peptide, cAD1 (Mori *et al.*, 1984).

Cells carrying pAD1 undergo a number of changes when stimulated by cAD1, including production of several novel surface proteins. Recent studies have indicated at least one of these proteins is plasmid encoded and corresponds to a cellular adhesin called aggregation substance (Galli, *et al.*, 1989, 1990). Production of the latter allows pAD1-containing cells to coaggregate with *E. faecalis* cells which do not contain pAD1 (i.e., recipients). Cellular clumping is readily observed when pAD1-containing cells are grown in broth in the presence of synthetic cAD1 or culture filtrate from cells which produce cAD1. In a related phenomenon pAD1-containing cells grown on solid media, which normally give rise to colonies that are dull and soft or fluid in consistency, characteristically give rise to bright, fracturable colonies when cAD1 is present (Weaver and Clewell, 1988).

In addition to inducing expression of aggregation substance, cAD1 also induces the expression of genes required for plasmid DNA transfer (Clewell and Brown, 1980; Ehrenfeld and Clewell, 1987; this study). *E. faecalis* (pAD1) cells that are preinduced

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with cAD1 are capable of transferring the plasmid at high frequency in short broth matings (e.g., 10 min) (Ike and Clewell, 1984). After receiving a copy of pAD1, endogenous cAD1 production by the recipient is shut down, although pheromones specific for other plasmids continue to be produced. A plasmid-encoded competitive inhibitor of cAD1, designated iAD1, is produced (Ike *et al.*, 1983; Mori *et al.*, 1986; Clewell *et al.*, 1990) which acts to prevent induction of transfer functions if cAD1 levels are low.

Previous studies have shown that approximately half of pAD1 encodes products necessary for the mating response. Structural genes are believed to be coded for within regions F, G, and H (Ehrenfeld and Clewell, 1987; see Fig. 1). Aggregation functions are encoded within the F region (Ehrenfeld and Clewell, 1987; Galli *et al.*, 1990), while the G and H regions appear to encode functions required for stabilization of mating aggregates and DNA transfer (Ehrenfeld and Clewell, 1987). Plasmid determinants responsible for regulating expression of transfer and aggregation have been localized to a 6-kb segment of the plasmid referred to as the regulatory region (Fig. 1). Areas within the regulatory region have been characterized by Tn*917* and Tn*917-lac* mutagenesis (Ike and Clewell, 1984; Ehrenfeld and Clewell, 1987; Weaver and Clewell, 1988). The product of *traB* is believed to be involved in the shutdown of cAD1 production (Weaver and Clewell, 1990, 1991). The *traA* product acts to repress expression of aggregation and transfer functions when cAD1 is not present and is thought to play a role in pheromone sensing (Weaver and Clewell, 1988; Clewell and Weaver, 1989). Insertion mutations within either of these regions derepresses expression of aggregation and transfer functions. C region product(s) is believed to play a role in pheromone sensing and possibly in cAD1 shutdown. C region mutants are phenotypically variable but all have elevated levels of iAD1 expression in an *E. faecalis* OG1X host background. This characteristic is hypothesized to be due to the inability of OG1X cells containing C region mutants to bind iAD1 (Weaver and Clewell, 1990). *traB, traA,* and the C region are constitutively transcribed (Weaver and Clewell, 1988). The E region is transcribed only in the presence of pheromone (Weaver and Clewell, 1988) and is thought to encode product(s) which act as positive regulators of proteins needed for aggregation and transfer (Weaver and Clewell, 1988). In addition, the gene encoding iAD1, the competitive inhibitor of cAD1, maps within the regulatory region (Clewell *et al.*, 1990).

In the present study we have characterized a class of spontaneous mutants of *E. faecalis* (pAD1) which constitutively express cellular aggregation and plasmid transfer functions. The mutation(s) occurred at a frequency higher than would be expected for normal spontaneous mutation, and reversion was found to occur at approximately the same frequency as the forward mutation. This phase variation phenomenon was found to be related to a heritable change in pAD1, and evidence is presented suggesting the mutation(s) responsible occur within a portion of the pAD1 transfer regulatory region. Potential sites for the mutations responsible for phase variation within the regulatory region are discussed as well as implications of regulation of transfer via phase variation on the dissemination of pAD1.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* OG1X was used as a host strain unless otherwise noted. pAM714, a pAD1::Tn*917* derivative that exhibits normal conjugation, was used instead of pAD1 in conjugation experiments because of the ease of selection for Em^r. The dry colony (Dry^c) variants pAM714SW2 and pAM714SW3 were derived in separate experiments. OG1RF(pAM211) was used as the donor strain in surface exclusion experiments.

pAM7701 is a recombinant consisting of the shuttle vector pAM401 (Wirth *et al.*,

TABLE 1

STRAINS AND PLASMIDS

Strain or plasmid	Relevant characteristics	Ref.
Strains		
E. faecalis		
OG1X	*str*	Ike *et al.* (1983)
OG1RF	*rif fus*	Oliver *et al.* (1977)
FA2-2	*rif fus*	Clewell *et al.* (1982)
DS16	*tet erm*	Tomich *et al.* (1979)
JH2-2	*rif fus* Rec+	Jacob and Hobbs (1974)
UV202	*rif fus* Rec$^-$ (derived from JH2-2)	Yagi and Clewell (1980)
E. coli		
DH5α	F$^-$ 80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end* A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 λ^-*thi*-1 *gyr*A	Bethesda Research Labs
Plasmids		
pAD1	Hemolysin–bacteriocin, Agg$^+$, Tra$^+$.	Tomich *et al.* (1979)
pAM211	pAD1::Tn*916*, *tet*, Agg$^+$, Tra$^+$.	Gawron-Burke and Clewell (1982)
pAM401	*E. coli–E. faecalis* shuttle vector, *cat*	Wirth *et al.* (1986)
pAM714	pAD1::Tn*917*, *erm*, Agg$^+$, Tra$^+$.	Ike *et al.* (1983)
pAM714SW2	Dry^c phase variant of pAM714.	This study
pAM714SW3	Dry^c phase variant of pAM714.	This study
pAM714SW2R	Dry$^+$ revertant of pAM714SW2.	This study
pAM2011E	Miniplasmid derived from pAM2011, *erm*, Agg$^-$, Tra$^-$ (see Results, Fig. 3B).	Weaver and Clewell (1989)
pAM2120	pAD1 with a Tn*917-lac* insert in *tra*A. Constitutive for aggregation and transfer.	Weaver and Clewell (1988)
pAM7016	pAD1 with a Tn*917-lac* insert in the mapping at 15.0 kb on the pAD1 map, Agg$^+$, Tra$^+$.	This study
pAM7227	pAD1 with a Tn*917-lac* insert in the I region, Agg$^+$, Tra$^+$ (see Table 4).	This study
pAM7245	pAD1 with a Tn*917-lac* insert in the I region, Agg$^+$, Tra$^+$ (see Table 4).	This study
pAM7500	*Amp* (The *Hin*dIII fragment of pAD1 spanning 17.2–22.2 on the pAD1 map, inserted into the *Hin*dIII site of pBluescript (Stratagene).	(Pontius and Clewell, manuscript in preparation)
pAM7607	Dry^c variant of pAM7016, (Tra$^+$).	This study
pAM7608	Dry^c variant of pAM7245, (Tra$^+$).	This study
pAM7609	Dry^c variant of pAM7227, (Tra$^+$).	This study
pAM7701	pAM401 with an insert of pAD1 DNA [including *tra*A, *iad*, the C region and a portion of *tra*B and the E region, Tra$^-$, Agg$^-$ (see Materials and Methods)].	This study

Note. Agg$^+$, capable of aggregation in response to cAD1; Agg$^-$, incapable of aggregation in response to cAD1; Tra$^+$, transfer proficient; Tra$^-$, incapable of self-transfer.

1986) and a portion of the pAD1 regulatory region, specifically the *Hin*dIII fragment spanning 17.2–22.2 kb on the pAD1 map, which includes a portion of the E region, *iad*, *traA*, *traB*, and the C region (Weaver and Clewell, 1989; Clewell *et al.*, 1990) (see Fig. 3). pAM7701 was constructed by first digesting pAM7500 (pBluescript containing the pAD1 *Hin*dIII fragment of interest (Pontius, manuscript in preparation) with *Bam*HI and *Sal*I. These fragments were cloned into *Bam*HI/*Sal*I-digested pAM401 using standard cloning procedures (Maniatus *et al.*, 1982). The resulting ligation mixture was used to transform *Escherichia coli* strain DH5α, and Cm^r isolates were selected. Iso-

lates were then screened for Tet[s] and Amp[s]. Plasmid DNA was collected (by the method of Ish-Horowitz and Burke, 1981) from selected transformants and restriction analyses were performed to confirm that the recombinant contained the desired insert.

Reagents. Antibiotics were generally used in the following concentrations: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 1000 μg/ml; chloramphenicol, 25 μg/ml; and erythromycin, 10 μg/ml. In the case of *E. faecalis* transformations, an erythromycin concentration of 2 μg/ml and a chloramphenicol concentration of 15 μg/ml were used. Media for culture of *E. faecalis* strains were THB (Todd-Hewitt Broth, Difco Laboratories, Detroit, MI) or N2GT [Nutrient Broth No. 2, Oxoid Ltd., London, England), supplemented with 0.2% glucose and 0.1 M Tris–HCl (pH 7.5)]. Media for culture of *E. coli* was LB (Davis *et al.,* 1980). When solid media was required 1.5% agar (Difco) was added. Synthetic cAD1 was generally used at a concentration of 40 ng/ml and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)[1] (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 100–200 μg/ml. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA) and were used under conditions recommended by the manufacturer.

Frequency of phase variation. Frequency of *E. faecalis* OG1X (pAD1) and OG1X (pAM714) phase variation was determined after suspending a single colony of each strain in N2GT broth with appropriate antibiotics, incubating overnight, and plating out dilutions of the overnight culture on solid media. Screening for variants was performed on the basis of colony morphology. The rate of variation of OG1X (pAM2011E) was determined by suspending a block of agar with one 36-h-old colony in broth and immediately plating dilutions on solid media containing X-gal. This method allows calculation of rate of phase transition in variants/cfu/generation (Eisenstein, 1981). Screening was based on colony color after 36 h. Values of frequency and rates of variation of *E. faecalis* cells given in Tables 2 and 5 should be viewed as approximations. While every effort was made to vortex cells vigorously prior to plating it is doubtful that cell chains or even cell aggregates were completely dispersed prior to plating.

Conjugation and transformation. Ten-minute broth matings were done as previously described (Ehrenfeld and Clewell, 1987). Nonconjugative introduction of plasmid DNA into OG1X was accomplished using a protoplast transformation procedure (Wirth *et al.,* 1986). Transformation of *E. coli* was done as previously described (Gawron-Burke and Clewell, 1984). Transformation of strain UV202 was performed using a procedure developed for electroporation (Cruz-Rodz and Gilmore, 1990).

Surface exclusion experiments. Experiments were designed as previously described (Dunny *et al.,* 1985). Overnight cultures of donors were diluted 1:10 in fresh N2GT with cAD1 and preincubated for 1 h at 37°C. Overnight cultures of recipients were diluted in a like manner and preincubated in N2GT with or without cAD1. Following preincubation, a 15-min broth mating was performed. Transconjugants were selected on the basis of resistance to streptomycin (1000 μg/ml) and tetracycline (10 μg/ml).

iAD1 assays. iAD1 assays (Ike *et al.,* 1983) were performed as previously described using *E. faecalis* DS16 as the responder strain. Twofold dilutions of each filtrate were tested for the ability to inhibit the clumping response of the responder strain in a solution containing cAD1. One unit of iAD1 activity is defined as the amount required to reduce a given cAD1 titer by half (Ike *et al.,* 1983). Culture filtrates were prepared when cultures reached late exponential growth phase (a reading of 90 using a Klett-Summerson colorimeter (Klett Manufacturing Co., Long Island City, NY), with a number 54 filter).

[1] Abbreviations used: X-gal, 5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside; CC, counterclockwise.

Tn917-lac mutagenesis of pAD1. Tn917-*lac* mutagenesis of pAD1 was accomplished using the delivery vector pTV32Ts provided by P. Youngman (Perkins and Youngman, 1986; Youngman, 1987). Mutagenesis and mapping of transposon inserts was performed as previously described (Weaver and Clewell, 1988) in an *E. faecalis* host background. Mutants used in this study were selected randomly and on the basis of reduction in plasmid transfer efficiency, the inability to aggregate in response to cAD1, or cAD1-inducible production of β-galactosidase. To determine the latter, cells were placed on solid media containing X-gal with or without cAD1. Colony color was observed after 24 and 48 h incubation at 37°C.

RESULTS

Aggregation characteristics of Dry^c variants. OG1X(pAD1) colonies typically appear dull and are soft or fluid in consistency when grown on solid media. If grown on solid media containing cAD1, colonies appear dry and fracturable (Weaver and Clewell, 1988). This cAD1-inducible change in colony morphology is referred to here as Dry$^+$. At a frequency higher than would be expected for normal spontaneous mutation constitutively bright, fracturable colonies, designated Dry^c, were found. Dry^c cells reverted back to wild-type (Dry$^+$)—also at a relatively high frequency. The frequencies of variation observed between Dry$^+$ and Dry^c states in experiments using OG1X(pAD1) and OG1X-(pAM714) are given in Table 2. Both strains gave rise to Dry^c variants at a frequency of 10^{-4}–10^{-2}. The frequency of reversion of Dry^c variants to the Dry$^+$ phenotype was similar. Previous studies have linked a dry, fracturable colony morphology with the cAD1-inducible expression of cellular aggregation functions (Ike and Clewell, 1984; Weaver and Clewell, 1988). It was therefore of interest to determine whether the Dry$^+$ $\leftrightarrow$ Dry^c phase variation affected expression of these aggregation factors. Indeed, when grown in broth, Dry^c cells aggregated with an appearance similar to that of Dry$^+$ cells that were exposed to cAD1. Revertants of Dry^c variants showed the pheromone-inducible expression of aggregation factors typical of cells carrying wild-type pAD1. The appearance of four novel surface proteins has previously been correlated with pheromone-inducible aggregation in an OG1X *E. faecalis* background (Ehrenfeld and Clewell, 1987; Weaver and Clewell, 1988). Western blot analysis showed that Dry^c variants constitutively produce these proteins, whereas Dry$^+$ revertants produced these proteins only when grown in the presence of cAD1 (not shown).

Plasmid transfer characteristics of Dry^c variants. Previous studies (Clewell and Brown, 1980; Ehrenfeld and Clewell, 1987) showed that in addition to induction of aggregation, pheromone also induced functions required for DNA transfer. It was therefore of interest to determine whether phase variants were altered in expression of plasmid transfer functions or if the phenomenon was linked only to a change in cellular aggregation characteristics. OG1X(pAM714) and a Dry^c variant of this strain were tested for the ability to transfer the plasmid in 10-min broth matings. Normally, significant plasmid transfer in such matings occurs only when donors are preinduced with cAD1 (Ike and Clewell, 1984). As shown in Table 3, Dry^c variants were found to transfer the plasmid efficiently, at a frequency at least 200-fold higher than a Dry$^+$ strain. A Dry$^+$ revertant of OG1X-(pAM714SW2) that was tested for ability to transfer in 10-min broth matings showed no detectable transfer (transfer of $<10^{-7}$–10^{-6} transconjugants/donor), which is typical of the wild-type phenotype [since induction by pheromone requires at least 20–30 min (Ike and Clewell, 1984)]. Dry$^+$ revertant plasmids transferred at wild-type levels (approximately 10^{-3} transconjugants/donor) in 1-h broth matings, indicating a return to normal, cAD1-inducible, regulation of transfer function expression. Thus, expression of plasmid transfer functions, as well as cellular aggregation functions, are linked to phase variation.

TABLE 2

PHASE VARIATION FREQUENCY OF OG1X(pAD1) AND OG1X(pAM714)

Plasmid	Phase transition		Variant colonies/ total colonies	Frequency
	$Dry^+ \rightarrow Dry^c$			
pAD1		Expt 1	9/7688	1.2×10^{-3}
		Expt 2	6/3322	1.8×10^{-3}
		Expt 3	1/3275	3.1×10^{-4}
pAM714		Expt 1	1/6852	1.5×10^{-4}
		Expt 2	169/2944	5.7×10^{-2}
	$Dry^c \rightarrow Dry^+$			
pAD1		Expt 1	40/1715	2.3×10^{-2}
		Expt 2	13/425	3.1×10^{-2}
		Expt 3	33/3506	9.4×10^{-3}
pAM714		Expt 1	3/4127	7.3×10^{-4}
		Expt 2	1/3009	3.3×10^{-4}

Tn917-lac mutagenesis of pAD1. To gain additional evidence that pAD1-encoded mating response genes in addition to aggregation factors are expressed under noninducing conditions by Dry^c phase variants, Tn*917-lac* mutagenesis of pAD1 was undertaken, and several of the derivatives were tested for phase variation. The position of each Tn*917-lac* insert obtained is shown in Fig. 2. Specific map positions and the orientation of the *lacZ* fusion created by the insert relative to the pAD1 map are listed in Table 4.

In all cases where the orientation of the fusion was such that potential transcription of *lacZ* could occur in a counterclockwise orientation (CC), relative to the pAD1 map shown in Fig. 2, pAD1::Tn*917-lac* mutants gave rise to blue colonies on X-gal media containing cAD1 and white colonies on media devoid of the pheromone. When *lacZ* fusions were oriented in the opposite direction no transcription was detected on either medium. Thus, transcription could only be detected as occurring in a CC direction and only in the presence of cAD1. Interestingly, pheromone-inducible transcription was observed in the region counterclockwise to the H-region, now designated I, where associated phenotypes are not presently known. It was also observed that transcription levels, as judged qualitatively from the intensity of colony color on exposure to cAD1, appeared lower in the latter area compared to the F, G, or H regions.

In order to examine transcription at various insertion points, Dry^c variants of several of the newly generated Tn*917-lac* deriva-

TABLE 3

TRANSFER FREQUENCY OF DRY^+ AND DRY^c OG1X(pAM714)[a]

Plasmid	Transfer frequency (in Transconjugants/donor)	
	10-min broth mating	1-h broth mating
pAM714 (Dry^+)	$<5.0 \times 10^{-7}$	6.1×10^{-3}
pAM714SW2 (Dry^c)	4.7×10^{-4}	5.9×10^{-3}
pAM714SW2R2 (Dry^+)[b]	$<5.0 \times 10^{-7}$	3.3×10^{-3}

[a] Ten-minute and 1-h broth matings were performed between Dry^+ or Dry^c OG1X(pAM714) and FA2-2.
[b] pAM714SW2R2 is a Dry^+ revertant derived from pAM714SW2.

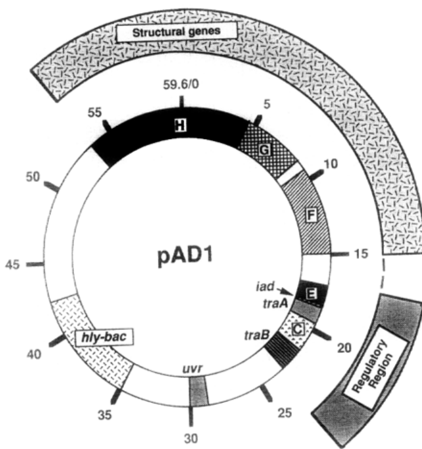


FIG. 1. Map of pAD1. Regions believed to encode the structural genes required for the mating response are labeled F, G, and H. The region responsible for regulating expression of the mating response is also indicated. It includes *traA, traB, iad,* and the C and E regions. *hly-bac* indicates the location of the hemolysin-bacteriocin determinant. *uvr* indicates the position of an ultraviolet light resistance determinant.

tives were obtained. No F region derivatives were tested; since unlike other Tn*917-lac* mutants obtained, these strains were incapable of aggregating in broth or expressing a dry colony morphology in response to cAD1 (data not shown) and should therefore be incapable of expressing a Dry^c phenotype. Strains tested included those Tn*917-lac* mutants with cAD1-inducible *lacZ* fusions indicated by an asterisk in Fig. 2.

Selected OG1X(pAD1::Tn*917-lac*) derivatives were plated on solid media, and phase variants of each strain were selected on the basis of a Dry^c colony morphology. These arose at a frequency of 10^{-5}–10^{-3}. The derivatives were then plated on media containing X-gal but no cAD1. In each case the colonies that arose were blue. Thus, transcript(s) normally made only in the presence of pheromone were constitutively produced by Dry^c variants. These results provide additional evidence indicating that expression of structural genes involved in the mating response, in addition to aggregation factors, are affected by phase variation.

Localization of DNA responsible for phase variation. Results indicating that both transfer and aggregation functions were constitutively expressed in Dry^c variants led to the hypothesis that the mutation(s) responsible for phase variation occurred on pAD1, possibly within the regulatory region, rather than the chromosome of the host strain. It was not surprising then to find that when plasmid DNA was obtained from both a Dry^c variant of OG1X(pAM714) and a revertant strain and introduced into plasmidless strains of OG1X by protoplast transformation, transformants exhibited the phenotype of the strain from which the particular plasmid was isolated (not shown). Thus, phase variation is due to heritable changes in pAD1 DNA.

To determine if the mutations relating to phase variation were in the regulatory region of pAD1 (i.e., between 17.0 and 23.0 kb on the pAD1 map) a miniplasmid derivative, pAM2011E, was tested for phase variation (Weaver and Clewell, 1989) (depicted in Fig. 3B). The miniplasmid consists of a 15-kb region of pAD1 DNA that includes the regulatory region. In addition, the miniplasmid carries a Tn*917-lac* insert that interrupts the E region, which is essential for positive regulation of the pheromone response. The *lacZ* fusion created by the Tn*917-lac* insertion (Weaver and Clewell, 1989) normally produces LacZ only when cAD1 is present. Phase variation to the Dry^c phenotype in

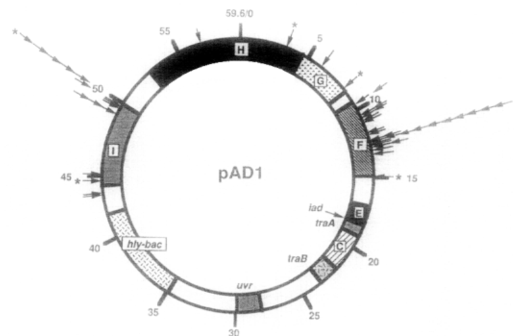


FIG. 2. Position of Tn*917-lac* inserts in pAD1. The arrows mark the position of inserts obtained and asterisks mark derivatives tested for phase variation.

TABLE 4

POSITION OF Tn917-*lac* INSERTS IN pAD1[a]

Inserts with *lacZ* fusions in a counterclockwise orientation[b]
pAM7241 (44.1), pAM7248 (44.4), pAM7245 (44.7), pAM7203 (48.9), pAM7219 (49.8), pAM7233 (49.8), pAM7215 (50.0), pAM7227 (50.0), pAM7229 (50.0), pAM7214 (50.0), pAM7201 (50.0), pAM7137 (3.3), pAM7142 (6.1), pAM7109 (8.6), pAM7131 (9.8), pAM7144 (9.8), pAM7136 (11.0), pAM7104 (11.1), pAM7110 (11.1), pAM7044 (11.9), pAM7035 (12.5), pAM7020 (12.8), pAM7028 (12.8), pAM7021 (12.9), pAM7001 (12.9), pAM7022 (12.9), pAM7085 (12.9), pAM7040 (12.9), pAM7014 (12.9), pAM7033 (12.9), pAM7042 (12.9), pAM7061 (12.9), pAM7018 (12.9), pAM7025 (13.2), pAM7030 (13.2), pAM7004 (13.4), pAM7016 (15.0)

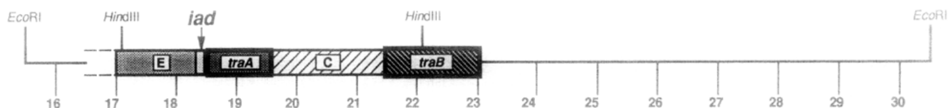
Inserts with *lacZ* fusions in a clockwise orientation[c]
pAM7275 (45.1), pAM7243 (48.9), pAM7225 (48.9), pAM7261 (49.2), pAM7226 (49.5), pAM7114 (57.0), pAM7102 (11.0), pAM7027 (12.1), pAM7034 (12.4), pAM7024 (13.0), pAM7049 (13.1), pAM7077 (13.6)

[a] Map positions of Tn917-*lac* inserts in pAD1 correspond to those shown on the pAD1 map (Fig. 2).
[b] Indicates potential transcription across the *lacZ* fusion would occur in a counterclockwise direction in relation to the pAD1 map (Fig. 2).
[c] Indicates potential transcription across the *lacZ* fusion would occur in a clockwise direction in relation to the pAD1 map (Fig. 2).

OG1X(pAM2011E) could not be observed because pAD1 genes necessary for expression of aggregation and plasmid transfer functions had been deleted. OG1X(pAM2011E) was tested for phase variation by observing changes in colony color when the strain was plated on solid media containing X-gal. The results are shown in Table 5. Blue colony variants occurred at a frequency of approximately 10^{-4}–10^{-3}, and white colony revertants appeared at a similar frequency. An estimate of the actual rate of phase transition, found to be 10^{-5}–10^{-4}/CFU per generation, could be made in this case (see Materials and Methods) because, unlike pAD1 or pAM714, pAM2011E is incapable of self-transfer during the experiment. When white colony revertants were streaked on plates containing X-gal and cAD1, the colonies that arose were blue indicating the revertants had returned to normal pheromone-inducible regulation of the *lacZ* fusion.

Protoplast transformation experiments were performed to determine whether the

A. pAD1 Regulatory Region.



B. pAM2011E.

C. pAM7701.

FIG. 3. (A) The regulatory region of pAD1. (B) Corresponding DNA present on the miniplasmid pAM2011E (Weaver and Clewell, 1989) with the position of a Tn*917-lac* insert indicated. (C) Portion of pAD1 included in the recombinant pAM7701.

TABLE 5

RATE OF PHASE VARIATION OF OG1X(pAM2011E)[a]

	Rate of phase variation (Variants/CFU/generation)	
	White → Blue	Blue → White
Expt 1	1.8×10^{-3}	2.7×10^{-4}
Expt 2	6.2×10^{-5}	1.6×10^{-4}
Expt 3	1.1×10^{-4}	1.5×10^{-4}

[a] Colonies 36 h old were suspended and immediately plated on solid media (THB with agar) containing 100 μg/ml X-gal and appropriate antibiotics. Colony color determinations were made after 36 h incubation at 37°C. Frequencies are reported in variants/CFU/generation; number of generations varied from 23 to 28.

phase transition observed was plasmid directed. Plasmid DNA was isolated from blue colony variants, white colony revertants, as well as the original OG1X(pAM2011E). When pAM2011E was used to transform OG1X, 114 transformants were obtained. On X-gal-containing media 105 of these transformants gave rise to white colonies while 9 gave rise to blue colonies. When plasmid DNA from a blue colony variant was used, 38 of 39 transformants gave rise to blue colonies. When plasmid DNA from a white colony revertant was used to transform OG1X, 321 of 326 transformant colonies were white; the remaining 5 were blue. All transformants gave rise to blue colonies when plated on media containing X-gal and cAD1. Since the majority of transformants were true to parental type, it was concluded that the observed phase change of OG1X(pAM-2011E) is plasmid directed.

Because the frequency of white ↔ blue phase variation of pAM2011E is similar to that for Dry^+ ↔ Dry^c phase variation of OG1X(pAD1) and because in each case genes necessary for expression of plasmid-regulated aggregation and transfer functions are affected by phase variation, we believe the two phenomena are related. The data imply that the mutations responsible for phase variation occur within the region of the plasmid previously shown to regulate the pheromone response—between 17.0 and 23.0 kb on the pAD1 map (Fig. 1 and Fig. 3A).

Complementation analyses. To further examine the nature of the mutation(s) responsible for phase variation a portion of the regulatory region of pAD1 was cloned and tested for the ability to complement the Dry^c phenotype in *trans.* pAD1 DNA corresponding to the region 17.2–22.2 kb on the pAD1 map (Fig. 1 and Fig. 3C) was inserted into an appropriate shuttle vector (pAM401) and the recombinant plasmid, pAM7701, was introduced via electroporation into the Rec^- *E. faecalis* strain UV202. A control strain, UV202(pAM401), was also constructed. OG1X strains harboring pAM714, pAM-7227, pAM7245, or pAM7016 and Dry^c variants of each strain were then used as donors in matings with the recipients UV202-(pAM401) and UV202(pAM7701). Transconjugants were observed for colony morphology and, when appropriate, for colony color on media containing X-gal. Transconjugants resulting from matings with the recipient UV202(pAM401) were true to the original donor phenotype. pAD1 derivatives, including those from Dry^c donors, when introduced into UV202(pAM7701), however, showed a soft colony morphology. Those carrying LacZ fusions gave rise to white colonies on plates containing X-gal. Thus, the Dry^c phenotype was complemented (or suppressed) in *trans* by a product(s) from the portion of the pAD1 regulatory region present on the recombinant plasmid pAM7701 [17.2–22.2 kb (Fig. 3C)].

Surface exclusion characteristics of Dry^c variants and a traA mutant. Previous reports showed that surface exclusion functions of pAD1 (Clewell and Brown, 1980) and pCF10 (another conjugative, pheromone-inducible *E. faecalis* plasmid) were pheromone inducible. In the case of pCF10 regulation of surface exclusion was not necessarily linked to regulation of transfer and aggregation functions; a transfer constitutive, aggregation constitutive mutant of pCF10 did not constitutively express surface exclusion functions

TABLE 6

EXPRESSION OF SURFACE (ENTRY) EXCLUSION FUNCTIONS BY DRY[c] VARIANTS AND A *traA* MUTANT OF pAD1[a]

Plasmid content of the recipient			Transconjugants/recipient		P Value[d]
			−cAD1[b]	+cAD1[c]	
pAM714	Expt 1		6.4×10^{-4}	9.9×10^{-6}	
	Expt 2		7.7×10^{-4}	5.0×10^{-7}	
	Expt 3		7.6×10^{-4}	5.8×10^{-6}	
	Expt 4		1.7×10^{-4}	4.4×10^{-5}	
	Expt 5		2.0×10^{-4}	3.1×10^{-5}	
		Mean	5.1×10^{-4}	1.8×10^{-5}	<0.005
pAM2120	Expt 1		5.6×10^{-6}	2.6×10^{-5}	
	Expt 2		4.5×10^{-6}	5.3×10^{-6}	
	Expt 3		5.8×10^{-6}	5.9×10^{-5}	
	Expt 5		2.7×10^{-5}	9.4×10^{-6}	
		Mean	1.1×10^{-5}	2.5×10^{-5}	N.S.
pAM714SW2	Expt 3		7.1×10^{-4}	5.0×10^{-5}	
	Expt 4		1.2×10^{-3}	2.5×10^{-4}	
	Expt 5		1.2×10^{-4}	1.4×10^{-5}	
		Mean	6.8×10^{-4}	1.0×10^{-4}	$0.05 < P < 0.1$[e]
pAM714SW3	Expt 1		3.2×10^{-4}	1.7×10^{-5}	
	Expt 2		1.5×10^{-5}	5.7×10^{-5}	
	Expt 3		8.4×10^{-4}	1.0×10^{-5}	
		Mean	3.9×10^{-4}	2.8×10^{-5}	$0.1 < P < 0.375$[e]

[a] Experiments were performed as described under Materials and Methods. The donor strain, OG1RF(pAM211), was preincubated for 1 h in N2GT broth containing cAD1, prior to matings. Matings were for 15 min.

[b] Recipients were preincubated for 1 h in N2GT broth prior to matings.

[c] Recipients were preincubated for 1 h in N2GT broth with cAD1 prior to matings.

[d] *P* values are for *t* test (unpaired) comparisons for each isolate with vs without cAD1. Transformation of the data and use of geometric means rather than arithmetic means does not change results obtained substantially. N.S., not significant.

[e] If the data for pAM714SW2 and pAM714SW3 are combined the $P < 0.025$.

(Dunny *et al.*, 1985). Phenotypic observations and results of complementation tests suggested that mutations within the *traA* locus (which encodes a negative regulator of aggregation and transfer gene expression) might be responsible for phase variation. If so, expression of surface exclusion functions of *traA* mutants and Dry[c] variants should be the same under both inducing and noninducing conditions. Experiments were performed to determine whether this was the case, and the results are shown in Table 6. OG1X (pAM714), used here as the control or "wild-type" recipient, consistently exhibited a significant ($P \leqslant 0.005$) reduction in uptake of donor plasmid DNA when it (the recipient) had been preincubated with cAD1 prior to mating. OG1X(pAM2120), containing pAD1 with a Tn*917-lac* insert in *traA*, was reduced in plasmid entry under both inducing and noninducing conditions at the approximate level of induced OG1X(pAM-714). The Dry[c] variants resembled that of wild type—with a reduction in uptake only if preincubated with cAD1 ($P \leqslant 0.025$ for the combined data from pAM714SW2 and pAM714SW3). Thus, while surface exclusion functions of the *traA* insertion mutant was constitutively expressed, this was not the case for the Dry[c] variants.

Comparative restriction analysis. Phase variation phenomena are known in some cases to involve DNA rearrangements (e.g., inversions, insertions/excisions, etc), and

comparative restriction fragment analyses proved useful in identifying the region of DNA affected (Abraham *et al.,* 1985; Marrs *et al.,* 1988; Komano *et al.,* 1986, 1990; Bartlett *et al.,* 1988; Bartlett and Silverman, 1989). With the hope of gaining insight into the nature and location of the mutations responsible for phase variation, wild-type pAD1 and plasmid DNA from OG1X-(pAD1) Dry^c variants, as well as pAM2011E and plasmid from blue colony variants of OG1X(pAM2011E), were analyzed using a number of restriction enzymes. Restriction enzymes used included *Alu*I, *Bam*HI, *Eco*RI, *Hae*III, *Hinc*II, *Hind*III, *Hpa*I, *Hpa*II, *Mse*I, *Pvu*II, *Rsa*I, *Sal*I, *Sau*3A, and *Taq*I. Digests were compared using both agarose and polyacrylamide gel electrophoresis. No differences in restriction patterns were detected between plasmid from variant cells and plasmid obtained from cells with nonvariant phenotype.

cAD1 and iAD1 levels of Dry^c variants. Previous studies indicated that the relative level of cAD1 to iAD1 is critical in determining whether plasmid transfer functions are expressed (Ike *et al.,* 1983; Mori *et al.,* 1986; Ehrenfeld and Clewell, 1987). One easily testable hypothesis to explain the observed phase variation was that Dry^c phase variants expressed less iAD1, and/or that cAD1 was being produced at levels that would cause self-induction. Either of these occurrences would be detected as an apparent decrease in iAD1 production in an iAD1 microtiter assay. However, when iAD1 levels of OG1X *E. faecalis* strains containing variant plasmids were tested, they were found to be the same as for OG1X containing wild-type pAD1.

Phase variation in a Rec$^-$ host. To determine if homologous recombination played a role in phase variation, UV202(pAM714) and UV202(pAM7016) were examined and compared to Rec$^+$ isogenic control strains JH2-2(pAM714) and JH2-2(pAM7016). UV202(pAM7016) was found to change to the Dry^c phenotype at a frequency of 9.2×10^{-6} in one experiment. No mutants were detected (a frequency of $<10^{-6}$) in two other experiments. In a JH2-2 background pAM-7016 changed to the Dry^c phenotype at an average frequency of 3.5×10^{-6} in three experiments, but no mutants were detected (a frequency of $<10^{-6}$) in two additional experiments. Results for UV202(pAM714) and JH2-2(pAM714) were similar. The data imply that phase variation is Rec-independent; however, it appears to occur at a lower frequency in the JH2-2 host (the Rec$^+$ parental strain of UV202) compared to the nonisogenic OG1X.

DISCUSSION

In this study we have found that OG1X(pAD1) undergoes a heritable, reversible, phenotypic change from Dry$^+$ to Dry^c colony morphology at a frequency of 10^{-4}–10^{-2}. In addition to a change in colony morphology Dry^c variants constitutively express aggregation and plasmid transfer functions that are normally expressed only in the presence of cAD1. Restriction fragment polymorphism analysis with a number of restriction enzymes have revealed no detectable changes in restriction patterns. These results make it appear unlikely that insertion and excision of an IS element, or a gross DNA rearrangement is responsible for the changes observed during phase variation. Results indicate that the mutations responsible for phase variation occur within the regulatory region of pAD1. Sequence analysis of a portion of the regulatory region (Clewell *et al.,* 1990; Pontius and Clewell, manuscript in preparation) reveals the presence of large numbers of direct and indirect repeats where small insertion/deletion events resulting in frameshift mutations might occur (reviewed in Levinson and Gutman, 1987; Ripley, 1990).

The Dry^c phenotype could be complemented in *trans* with a portion of the regulatory region from pAD1. The construct tested contained portions of the E region and *traB,* as well as *iad,* the C region, and *traA*. *traB* is believed to produce products necessary for cAD1 shutdown; *traB* mutants have a "ringed" colony morphology (Weaver and

Clewell, 1988) which is not typical of Dry^c phase variants, making it seem unlikely that the mutations responsible for phase variation occur within *traB*. It is also unlikely that an alteration of the E region, which encodes determinant(s) necessary for positive regulation of the pheromone response, is responsible for phase variation; E region product in *trans* would not be expected to complement (cause apparent repression of) the Dry^c phenotype. The level of the inhibitor, iAD1, produced by variants was identical to wild type, so mutations affecting *iad* are not responsible for phase variation.

Changes affecting the negatively acting *traA* product would predictably result in many of the phenotypic changes observed during phase variation to the Dry^c state. However, surface exclusion functions of a *traA* mutant are fully derepressed while those of Dry^c variants are not; the latter appear to remain inducible. Additional data (not shown) indicate pAD1 transfers from Dry^c variants during 10-min broth matings at an even higher frequency if the donors are preinduced with pheromone. cAD1-inducible transfer is not characteristic of pAD1::Tn*917-traA* and pAD1::Tn*917-lac-traA* mutants (Ike and Clewell, 1984; Weaver and Clewell, 1988). These results suggest that TraA may be partially functional in Dry^c variants. A mutation resulting in a reduced activity or level of TraA might result in a Dry^c phenotype.

A mutation affecting C region product alone is unlikely to be responsible for phase variation. All C region insertion derivatives obtained to date express elevated levels of iAD1 in an OG1X *E. faecalis* host background, a characteristic which is not typical of Dry^c phase variants. Tn*917-lac* insertions within the C region mapping near *traA* show a partial derepression of aggregation and transfer functions (Weaver and Clewell, 1988). These findings suggest the possibility that efficient production of TraA might be affected by mutations within the C region. Thus, the results obtained to date suggest that the mutations responsible for phase variation directly or indirectly affect TraA production. Comparative nucleotide sequencing analyses (underway) should reveal the precise location of the responsible mutations.

Phase variation provides an alternative mechanism for regulating pAD1 fertility functions and allows plasmid transfer to occur in the absence of pheromone. pAD1 could then be introduced into recipient strains that do not excrete cAD1 thereby extending its host range. Another possible role for phase variation stems from the recent observation by Galli *et al.* (1990) that the pAD1 aggregation substance (the product of *asal*) contains two tetra-amino acid motifs that have been shown in other systems to contribute to binding to eukaryotic cells. Since pAD1 is already believed to contribute to virulence as a result of its hemolysin-related determinant (Ike *et al.*, 1984, 1987), the possibility that phase variants constitutively expressing aggregation substance might more readily colonize human/animal tissue is worthy of consideration.

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