Pheromone-Regulated Expression of Sex Pheromone Plasmid pAD1-Encoded Aggregation Substance Depends on at Least Six Upstream Genes and a cis-Acting, Orientation-Dependent Factor

ALBRECHT B. MUSCHOLL-SILBERHORN

Universität Regensburg, NWFIII-Mikrobiologie, D-93053 Regensburg, Germany

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Conjugative transfer of Enterococcus faecalis-specific sex pheromone plasmids relies on an adhesin, called aggregation substance, to confer a tight cell-to-cell contact between the mating partners. To analyze the dependence of pAD1-encoded aggregation substance, Asa1, on pheromone induction, a variety of upstream fragments were fused to an α-amylase reporter gene, amyL, by use of a novel promoter probe vector, pAMY-eml. For pheromone-regulated α-amylase activity, a total of at least six genes, traB, traC, traA, traE1, orfY, and orf1, are required: TraB efficiently represses asa1 (by a mechanism unrelated to its presumptive function in pheromone shutdown, since a complete shutdown is observed exclusively in the presence of traC); only traC can relieve traB-mediated repression in a pheromone-dependent manner. In addition to traB, traA is required but not sufficient for negative control. Mutational inactivation of traE1, orfY, or orf1, respectively, results in a total loss of α-amylase activity for constructs normally mediating constitutive expression. Inversion of a fragment covering traA, P0, and traE1 without disrupting any gene or control element switches off amyL or asa1 expression, indicating the involvement of a cis-acting, orientation-dependent factor (as had been shown for plasmid pCF10). Unexpectedly, pAD1 represses all pAMY-eml derivatives in trans, while its own pheromone-dependent functions are unaffected. The discrepancy between the new data and those of former studies defining TraE1 as a trans-acting positive regulator is discussed.

Facultatively pathogenic microorganisms differ largely with respect to the mechanisms by which they affect their host. However, they all face the common problem that they have to strictly distinguish between two totally different “lifestyles,” i.e., between living as commensals or even free in nature and being involved in the process of colonizing tissues or blood, which demands an alternate equipment of cell surface components, exoproteins, and metabolic enzymes.

The gram-positive bacterium Enterococcus faecalis is a commensal of the intestine, but under different circumstances may infect the urinary tract, blood, or endocardium. The conditions indispensable for the infectious pathway are still unknown, and there is no common factor identified for all clinical isolates. However, sex pheromone plasmid-encoded aggregation substance is a widespread adhesin shown to be involved in the colonization of various tissues (21, 28, 30). In addition, it plays an essential role in the conjugative transfer of the sex pheromone plasmid on which it is encoded in that it confers a tight contact between donor and recipient cells, visible as large clumps. (For reviews of the sex pheromone system, see references 7, 10, and 37.)

Therefore, regulation of aggregation substance is on three different levels. Under normal growth conditions, its expression is totally shut down. During the operation of the infectious pathway, there may be various environmental factors inducing aggregation substance, among them a component of blood serum (23) and several antibiotics (16, 39). For conjugative plasmid transfer, the corresponding gene is transcribed in response to a plasmid-specific oligopeptide, called sex pheromone, secreted by recipient cells not containing the corresponding plasmid. The latter phenomenon has been known for a long time and has been investigated by several groups (7, 10, 37). Especially for two different plasmids, pAD1 and pCF10, rather detailed data on the regulatory circuits are available. Surprisingly, despite a similar overall organization of the regulatory genes and highly homologous DNA regions (15), induction of aggregation substance seems to involve two mechanistically distinct strategies. While for the pAD1-encoded aggregation substance, Asa1, a trans-acting protein, TraE1, obviously serves as the general inducer of transcription (25, 32) (a survey of the genetic data available for pAD1 is given in Fig. 1), the pCF10-encoded aggregation substance, Asc10, is expressed via transcriptional readthrough which involves a cis-acting, orientation-dependent factor (6), a regulatory RNA molecule interacting with ribosomal proteins, and a small proteincaceous regulator (4, 5).

The question is whether two related plasmids may have developed totally different strategies to regulate the same adhesin, or whether there are common basic pathways only slightly modified to ensure a specific response to the corresponding sex pheromone. This study presents data on the regulation of sex pheromone plasmid pAD1, supporting in part the second idea and addressing the function of several pAD1-specific genes. These data were obtained by use of a newly constructed α-amylase-based promoter-probe vector.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Enterococcus faecalis strains OG1X (19) and OGIX(pAD1) were grown in Todd-Hewitt-broth (THB; Oxoid). Escherichia coli cloning strain TOP10F (Invitrogen) was grown in Luria-Bertani broth (24). Selective antibiotics were added as follows: erythromycin, 800 μg/ml for E. coli and 20 μg/ml for E. faecalis; chloramphenicol, 20 μg/ml for both organisms.
Constitution of pAMY-em1 vector and derivatives containing pAD1 fragments. pAMY-em1 is composed of the same genetic elements used for the construction of expression vector pPBM-ex1 (27). In addition, a promoterless chloramphenicol acetyltransferase gene (cat) was introduced that converted the fourth trbA and reintroduced into the original constructs partially digested with E. coli I digestion, normally causing a blunt-ended cut within trbA, suggested the values have to be multiplied by a factor of 0.085 [calculated from 

\[ \text{OD}_{500} \times 0.085 = \text{cell densities} \]

at 37°C, cultures were placed on ice, and 200 μl of each cell suspension was removed for the determination of turbidity at a wavelength of 600 nm. (Note that to dissolve cell aggregates grossly influencing the turbidity, suspensions are mixed with 1 ml of 8 M urea; this treatment does not detectably lyse E. faecalis cells or break cell chains.) Turbidity should not exceed a value of about 0.7 to ensure that cells are still exponentially growing. The rest of the cultures were centrifuged at 4°C, and 700 μl of supernatant was mixed with 200 μl of a precooled slurry of Phadebas reagent (Pharmacia) in H2O. The mixtures were heavily stirred at 75°C on an Eppendorf shaker (Thermomix 5436) until not more than half of the slurry had lost its blue color (in order to guarantee linearity of activity values). The reaction was stopped by placing the suspensions on ice for several minutes, spinning them briefly in a cooled centrifuge, and transferring 700 μl of supernatant to a plastic cuvette containing 500 μl of 0.5 M NaOH (which stops the reaction). Optical densities at 620 nm (OD620) were determined, and the resulting values were equalized to standard conditions (i.e., cell densities of 1 OD620 = 10 and 15 min of amylase reaction time). 

Activities in units per liter were deduced from the conversion table supplied by the manufacturer (which, taking into account the change in assay conditions, suggested the values have to be multiplied by a factor of 0.085 [calculated from the altered dilution rates]). One unit is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of glucosidic linkage per min.

Clumping assay. Supernatants from overnight cultures of OG1X strains containing pAMY-em1 constructs were boiled for 5 min to kill residual E. faecalis cells and diluted with fresh THB in steps of 1:2 with 24-well microtitre plates used as a reservoir. After transformation into E. coli Top10F', only a derivative containing a deletion of about 300 bp within trbA (not affecting neighboring sequences) was obtained (pBEYP1).

α-Amylase activity assay. Overnight cultures of E. faecalis strains containing pAMY-em1 constructs were inoculated 1:100 into 1 ml of fresh THB medium supplemented with 20-μg/ml concentrations of chloramphenicol and erythromycin (with or without synthetic sex pheromone cAD1). After ca. 5 h of incubation

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>pBCAEYP1</td>
<td>Complete sequence of regulatory regions* from trbA (BspHI site) to the asaI start codon (BspHI site)</td>
<td>trbCAE,orfY1</td>
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<tr>
<td>pCAEYP1</td>
<td>ΔtrbA (PvuI/PvuI fragment deleted from pBCAEYP1)</td>
<td>trCAE,orfY1</td>
</tr>
<tr>
<td>pBAEYP1</td>
<td>ΔorfY (PvuII fragment deleted from pBCAEYP1)</td>
<td>trBAE,orfY1</td>
</tr>
<tr>
<td>pBEYP1</td>
<td>ΔorfY (ca. 300-bp deletion within trbA introduced into pBEYP1)</td>
<td>trBAE,orfY1</td>
</tr>
<tr>
<td>pAEYP1</td>
<td>Complete sequence of regulatory region from trbA (HincII site) to the asaI start codon (BspHI site)</td>
<td>trBAE,orfY1</td>
</tr>
<tr>
<td>pP7-P1</td>
<td>Complete sequence of regulatory region from P7 (NsiI site) to the asaI start codon (BspHI site)</td>
<td>trE,orfY1</td>
</tr>
<tr>
<td>pP7-YP1</td>
<td>Complete sequence of regulatory region from P7 (NsiI site) to the asaI start codon (BspHI site)</td>
<td>orfY</td>
</tr>
<tr>
<td>pAEY-1</td>
<td>Complete sequence of regulatory region from trbA (HincII site) to orfY (Sau3a site) through trbA</td>
<td>trAE,orfY1</td>
</tr>
<tr>
<td>pP1</td>
<td>Fragile fragment from P1 (HpaII site) to the asaI start codon (BspHI site)</td>
<td>orfl</td>
</tr>
<tr>
<td>pEA/Y-1</td>
<td>pAEPY1 with inversion of a HincII-NsiI fragment covering trbA through trbA</td>
<td>trAE,orfY1</td>
</tr>
<tr>
<td>pEA/P1</td>
<td>orfY (Sau3a site) fragment deleted from pEA/Y-1</td>
<td>orfY</td>
</tr>
<tr>
<td>pAE/RBSamy</td>
<td>Complete sequence of regulatory region from trbA (HincII site) to orfY (Sau3a site) through trbA</td>
<td>orfY</td>
</tr>
<tr>
<td>pP3</td>
<td>Fragile fragment from P3 (HpaII site) to the asaI start codon (BspHI site)</td>
<td>orfY</td>
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<td>orfY</td>
</tr>
<tr>
<td>pP2</td>
<td>pP2,orfY1 containing a 2 frameshift within trbA</td>
<td>trAE,orfY1</td>
</tr>
<tr>
<td>pP2</td>
<td>pP2,orfY1 with orfY 3-end deleted (AecI-Sau3a fragment)</td>
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* “Complete sequence of regulatory regions” indicates that the original sequence within the given fragment remained unchanged, with the following exceptions: (i) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (ii) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (iii) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (iv) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (v) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (vi) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (vii) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site); (viii) the NsiI site downstream of trbA was inactivated as follows. The pAEYP31 construct was linearized by NsiI digestion linearizing all plasmids containing the correct FspI site and selecting for by use of the same antibiotics. The recombinant product obtained after NsiI digestion (Inv) was cloned into pUC19 linearized with NsiI to the NheI site).
different methods: (i) Northern blotting (2, 13, 25) (Fig. 1), (ii) clumping assays using aggregation substance itself as a marker (11, 25), and (iii) transcriptional lacZ fusions based on a Tn917-derivative bearing a lacZ gene at one end (29, 34). Transcriptional analysis is too time-consuming for routine investigations and difficult to quantify. Clumping assays are easy to execute, but quantification is even less sensitive than Northern blotting. The use of Tn917lac suffers from the random location of transposition sites and from the polar effect of the 8.5-kb insert inactivating the possibly essential downstream sequence. Furthermore, only the transposition site itself was analyzed for transcription, while the effects of mutations on distant DNA regions are difficult to address. Our own previous attempts to present the lacZ gene on a definite promoter probe vector failed because of the instability of constructs. In addition, for assaying very weak promoter activities, β-galactosidase may be inadequate, since this cytoplasmic enzyme possibly is not quantitatively extracted from the highly rigid E. faecalis cells.

Here, a new vector, pAMY-em1 (Fig. 2), was constructed by using α-amylase (amyL) from Bacillus licheniformis (14) as a quantifiable marker. This enzyme has been successfully used previously in E. faecalis (17) and combines several advantages. As an exoprotein, its activity can be determined from the supernatant without cell extraction by using a very simple assay (Phadebas reagent from Pharmacia). Since AmyL is a thermostable enzyme with a temperature optimum of 75°C, a quantitative removal of residual cells (which might change results by enzyme production during long-run assays) is not necessary. The promoterless amyL gene was cloned adjacent to a large multiple cloning site (MCSI) containing many restriction sites for the integration of DNA fragments. To allow analysis of countertranscription from the same culture, a promoterless cat gene (1) was introduced in the opposite orientation to MCSI. (This marker gene was not used in the present work.) An erythromycin resistance gene (ermAM) downstream of amyL allows selection in E. coli and many gram-positive bacteria (9), and a second MCS protected on both sides by strong transcriptional terminators the integration of species-specific plasmid replicons. A similar vector, pEPPS-ex1, has been constructed for the purpose of gene expression (27).

After testing the vector for the absence of nonspecific promoter activity—neither E. faecalis nor E. coli expressed detectable amounts of α-amylase—a variety of DNA fragments derived from the pAD1 sequence upstream of asa1 were cloned into pAMY-em1. In most cases, the start codon of asa1 was fused to the start codon of amyL by use of the BspHI sites covering both ATG initiation sites. Thus, true translational fusions were created retaining all original nucleotides upstream of asa1, including the ribosome binding site (RBS). Only for those constructs designed to test the role of the region between orf1 and asa1 was the RBS of amyL used (RBSamy). From all constructs, the 3-kb gene for surface exclusion protein, sea1 (36), was excluded, since it encodes a cell surface protein very probably not involved in asa1 regulation. A survey of all constructs is given in Fig. 3 (also see Table 1). To propagate the plasmids in E. faecalis, they were integrated into the low-copy shuttle vector pWM401 (see Materials and Methods). Culture supernatants of E. faecalis OG1X or OG1X(pAD1) containing one of the constructs were submitted to amylase assays as described in Materials and Methods. Each construct was tested at least three times.

traB and traC are both required for sex pheromone-dependent asa1 expression. In the first approach, the complete transcriptional units defined by the promoters determined previously for the regulatory region of pAD1 (13) were successively added to the asa1-amylase fusion (Fig. 4). P3 and P1/2 turned out to be largely inactive. With the addition of P3-traE1, α-amylase was constitutively expressed at a high level (pEYP1). These data are consistent with previous results showing that activation of the P3 promoter (responsible for asa1 transcription) is dependent on the activity of traE1 which was transcribed from P3 in the absence of the negative regulator TraA (25, 32). Addition of the traA gene (pAEYP1) reduced amyL expression only slightly; this is not surprising, since the strains all produce sex pheromone, cAD1, which inactivates TraA (12).

Complementation of the constructs with original pAD1 should change results fundamentally; since transcription from P1 was shown to be activated in trans by TraE1 gene product (25), even the smallest fragment (pP1) originating at P3 should allow inducible α-amylase expression via pAD1-born TraE1. Surprisingly, not only cAD1-induced OGIX(pAD1/pP1) failed to produce detectable amounts of α-amylase (not shown); pAD1 shut down amyL activity of all pAMY-em1 derivatives—even those which per se resulted in strong constitutive expression (e.g., pAEYP1). This effect is not due to plasmid incompatibility or instability, since in all cases, the intact pAMY-em1 constructs could be isolated and retransformed into E. coli TOP10F, where α-amylase activity was restored. (In E. coli, amyL expression probably originates non-
specifically from one of the many promoter-like structures found in the low-G+C DNA of *E. faecalis.* In all of these strains, pAD1-encoded aggregation substance remained normally inducible by cAD1, since the characteristic cell clumping was observed after exposure to sex pheromone.

In the next step, the pAD1 sequence fused to *amyL* was further extended for two key elements involved in sex pheromone control: *traC,* encoding a cell surface lipoprotein responsible for sex pheromone sensing (31), and *traB,* with a presumptive function of the gene product in the shutdown of chromosomally encoded cAD1 (35). With these additional genes, *amyL* expression was considerably reduced (about 14-fold) when compared to that expressed by pAEYP 31 (Fig. 4). Addition of cAD1 to the nutrient broth increased expression about 10-fold, but to a level below that of the constitutively expressing construct pAEYP 31. Deletion of *traC* from the *traB-traC* operon (without affecting the common promoter) totally shut down *amyL* expression, irrespective of the presence of sex pheromone, but with dependence on *traA,* the additional deletion of which (pCAEYP 31) completely relieved repression. A deletion of *traB* (pCAEYP 31) further increased constitutive expression compared to that of pAEYP 31.

These data still may be explained with the predicted functions of *traB* and *traC* (compare with the results in Fig. 1): *traB* gene product switches off cAD1 production and therefore self-inducibility. If externally added cAD1 is internalized exclusively by TraC, the deletion of *traC* would make the cells totally insensitive to sex pheromone induction. However, some doubts about this interpretation emerge when the supernatants of the corresponding strains are tested for cAD1 content: OG1X(pBAEYP 31) containing the complete *traB* gene still secretes active sex pheromone (although in a markedly reduced amount, since the supernatant was active down to a 1:64 dilution, compared to a still active 1:256 dilution for OG1X(pAEYP 31) supernatant). Very surprisingly, the only strains completely defective in cAD1 production are OG1X(pCAEYP 31) and OG1X(pBCAEYP 31) expressing TraC. Here, no clumping could be induced even when the pAD1-containing cells were grown in undiluted or slightly THB-enriched supernatant. This total lack of active cAD1 cannot be explained by a possible overproduction of inhibitory pheromone iAD1: when supernatants from OG1X(pAEYP 31) are mixed 1:1 with supernatants from OG1X(pBCAEYP 31), the minimal inductive concentration is reduced by a factor of about (or slightly greater than) 2, as expected for a diluent lacking both cAD1 and iAD1.

*asa1* regulation involves a *cis*-acting factor in an orientation-dependent manner. The most obvious discrepancy between the regulation models of pAD1 and pCF10 lies in the fact that pCF10 is submitted to the action of a *cis*-acting factor probably tracking along the DNA in an orientation-dependent manner (6), while for pAD1, the TraE1 protein was shown to act in trans as a diffusible activator of the P3 promoter (25, 32). To verify the model for pAD1 by using the *amyL*-based assay system, a fragment of the constitutive pAEYP 31 construct covering the complete sequence from *traA* to *traE1* was inverted relative to the *amyL* gene (Fig. 5). A strain containing the resulting construct (pEA/YP31) failed to produce *amyL* (irrespective of the presence of cAD1). Further removal of the *P1/2-orfY* and *P3-orf1* regions (pEA/YP1, pEA/P31, and pEA/
FIG. 3. Survey of pAMY-em1 constructs used in this study. (Fusions involving the ermAM promoter [P_{erm}] were constructed by use of the related vector pERM-ex1 [27].) The nomenclature of constructs is given as follows: genes are indicated by a one-letter code (B, traB; C, traC; A, traA; E, traE1; Y, orfY; and 1, orf1); promoters (P) and terminator T1 (TTS1) are given only when they border disruptions of the original sequence; genes given in parentheses have been destroyed by point mutations (graphically marked by black triangles), while primes indicate the removal of a few nucleotides from the 5’ or 3’ ends, respectively. The diagrams of the constructs (in this and the following figures) are aligned to the graphical representation of the pAD1 sequence given on the top. Only complete genes (and those disrupted by point mutations) are accented by thick lines. Restriction sites involved in the various constructions are indicated.
RBS\textsubscript{amy} did not restore \(\alpha\)-amylase activity. The possibility that the disruption of the original sequence necessary for the rearrangement may have affected an essential structure can be largely excluded, since the used \textit{NsiI} site (i) lies within a sequence lacking any open reading frame (ORF) or striking nucleotide sequence motif and (ii) even in the highly active constructs contains some additional nucleotides from a polylinker used for intermediate cloning. Only when part of \textit{traA} and its downstream terminator were deleted in addition (pEP0/P31) could some basic \(\alpha\)-amylase activity be measured, probably representing the constitutive countertranscription from the bidirectional P0 promoter responsible for permanent \textit{traA} expression.

Therefore, a \textit{cis}-acting orientation-dependent factor similar to that of pCF10 has to be claimed for pAD1 too. Whatever its nature may be, it obviously is encoded within the 2.3-kb region from \textit{traA} to \textit{traE1} or at least initiates its \textit{cis}-acting function within this region.

\textit{traE1, orfY, and orf1 regions are essential}. There are at least three small ORFs carried on pAD1 which are not present on pCF10. While \textit{traE1} and \textit{orf1} are unique to pAD1 (and related \textit{cAD1}-inducible plasmids) (15), \textit{orfY} is ubiquitous for most sex pheromone plasmids, but at least in the special case of pCF10, it is disrupted by a stop codon, leaving only a truncated 198-bp ORF (\textit{prgT}) (20). These ORFs might be responsible for pheromone specificity of induction. To test their involvement in \textit{asa1} regulation, each of them (in common with some bordering sequences) was independently deleted from the constitutive construct pAEYP31. As shown in Fig. 6a, none of them could be removed without a nearly complete loss of \(\alpha\)-amylase activity.

\textit{traE1, orfY, and orf1 gene products are essential}. The essential roles of \textit{traE1}, \textit{orfY}, and \textit{orf1} regions need not necessarily be connected with the respective gene products, but might be dependent on other genetic information encoded on the corresponding sequence. It therefore was necessary to specifically mutate the peptides without grossly changing the nucleotide sequence. \textit{traE1} was mutated by the introduction of a 2 bp frameshift, and \textit{orf1} was mutated by creating a stop codon near its 5' end (see Materials and Methods). In the case of \textit{orfY}, the complete 3' end downstream of the unique \textit{AciI} site (84 bp) was removed. Again, OG1X strains containing any of these constructs [and a double mutant, pA(E)Y\textit{P31}, given in Fig. 3] largely failed to produce \(\alpha\)-amylase (Fig. 6b), which implies an indispensable positive involvement of all three translation products in \textit{asa1} expression.

\textit{orfY and \textit{traA} contribute to negative regulation}. Interestingly, \textit{orfY} seems to exhibit additional negative effects: Fig. 7a shows a pair of constructs differing with respect to the presence of \textit{orfY}. Here, expression was achieved by removal of transcriptional terminators TTS1 and TTS2 along with \textit{traE1}. In these cases, the presence of \textit{orfY} reduced rather than increased \(\alpha\)-amylase activity. The same may hold true for \textit{orf1}, but measurable effects are so weak that activity values may be considered as nonsignificant (not shown). \textit{traE1} could not be tested for possible negative effects, since removal of one or both of the upstream terminators without deleting \textit{traE1} has toxic effects on \textit{E. faecalis}.

\textit{TraA} has been known for a long time as a key negative regulator of aggregation substance; its disruption within pAD1 results in a constitutively clumping strain (18, 29). In this study, these data could largely be confirmed. It was shown that (in the

\begin{figure}
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\includegraphics[width=\textwidth]{figure4}
\caption{Effects of various pAD1 fragments confined by presumptive pAD1-specific promoters in the presence or absence of sex pheromone \textit{cAD1}. Black bars, \(\alpha\)-amylase activities of noninduced \textit{E. faecalis} OG1X cultures containing one of the pAMY-em1 constructs; grey bars, \(\alpha\)-amylase activities of \textit{E. faecalis} cultures induced by synthetic pheromone \textit{cAD1} (with pheromone concentration about 100-fold of the minimal inductive concentration). Activities (units per liter) always refer to the constructs given immediately on the right.}
\end{figure}
absence of the traB-traC operon) TraA is not sufficient for a shutdown of asa1 expression, but helps to keep the TTS1 and -2 terminators locked. These effects become visible when traE1, known to exhibit autoinduction (25), is deleted from several constructs (three pairs of constructs differing with respect to the presence of traA are shown in Fig. 7b). The site of action is not located within the terminator region, since the constitutive amyL expression observed when both terminators are deleted still is modified by traA. This is consistent with data defining a binding site for TraA in the P0 promoter region (32).

**DISCUSSION**

In the present study, it could be shown that regulation of pAD1-encoded aggregation substance at least involves a total of 10 kb upstream of the corresponding asa1 gene. All ORFs of >100 bp within this region, except for sea1 encoding the surface exclusion protein, are required for sex pheromone-controlled asa1 expression. On the other hand, several important structures were excluded from a detailed analysis: iad (8) coding for inhibitory sex pheromone; traD, which encodes immediately downstream from iad but is transcribed in the opposite direction (2, 33); and a small ORF immediately upstream of asa1, which encodes an RAPC amino acid motif conserved for most, if not all, sex pheromone plasmids (27). The roles of these ORFs were not addressed independently of the genes investigated in detail; therefore, their functions might further complicate the picture of pAD1 regulation presented here.

Mutation of any of the genes tested either results in constitutive expression (traB or traA) or totally shuts down expression (traC, traE1, orfY, orf1). As for pCF10, regulation involves a cis-acting orientation-dependent factor encoded—or at least initiating its function—within the 2.3-kb region covering traA through traE1, since its pure inversion results in a total loss of expression. In probably all cases, the proteins encoded by the ORFs rather than their nucleotide sequences are necessary for regulation. For orfY, a specific role of the deleted 3'-terminal 84 bp on the nucleotide level cannot totally be excluded, although there are no hints at all from the DNA sequence (e.g., repeats, conserved motifs, or possible secondary structures).

orfY (and possibly orf1) plays an ambiguous role in that it exhibits an additional negative effect. The function of TraA as a negative regulator of asa1 expression could be established; however, it was not sufficient for a complete repression, since it probably is inactivated by cAD1 still produced by the tested strains—provided that the pheromone is internalized despite the lack of a specific uptake system (see below). If this is the case, nonspecific internalization must be rather efficient, since addition of cAD1 to the culture broth of OG1X(pAEYP31) does not significantly raise α-amylase activity (Fig. 4).

The role of the remaining genes which have been investigated previously will have to be modified or newly defined according to the data presented. TraB efficiently represses aggregation substance, but not simply by avoiding self-induction via its assumed function as a repressor of cAD1 production. This can be excluded, since constructs containing traB still promote expression and secretion of active cAD1 in E. faecalis. Only the addition of traC, irrespective of the presence of traB, switches off cAD1 production, an effect that cannot simply be explained by the primary function of TraC lipoprotein as a pheromone-specific oligopeptide transporter. It may be argued that TraC surface protein traps all self-produced cAD1 from the supernatant—but why does this not induce asa1 expression (in the case of pBCAEYP1 [Fig. 4]) while externally added pheromone does?

Last but not least, and contrary to our own previous results (25), TraE1 cannot be simply a trans-acting protein directly effecting transcription from the P3 promoter. If this were the case, the inversion of a fragment containing the complete traE1 gene, including its original upstream sequence, which covers...
the corresponding P₀ promoter, should not alter activity. Instead, this manipulation switches off asa₁ expression, proving the involvement of an orientation-dependent mechanism, as has been shown for pCF10 (6).

The most puzzling result was that even complete pAD1 cannot activate the P₃ promoter in trans, a strategy which has proven successful previously (25, 26). The fact that pAD1 inactivates the otherwise constitutive pAMY-em1 constructs would imply the existence of a pAD1-encoded, trans-acting super-repressor. However, even the control plasmid (pPermRBSamy) constitutively expressing amyL and not containing any pAD1 sequence is affected by pAD1, in that α-amylase activity is reduced to about 50% of its normal value (data not shown). Maybe the cellular secretion system mediating α-amylase export out of the cell is influenced by pAD1 (possibly via simple competition by pAD1-encoded surface proteins). This effect should be independent of pAD1-specific sequences and therefore cannot explain either why amyL preceded by pAD1 sequences is reduced to about 50% of its normal value (data not shown).

Updating of the model for asa₁ regulation. How can the data presented here and in previous publications be integrated into a conclusive regulation model? The following view combines all of the negative and positive effects shown for the various regulatory genes. Under noninducing conditions, transcription within the regulatory region is locked by a cooperative action of TraB and TraA. While TraA binds directly to the P₀ promoter region (32), TraB acts in a still unclear way (but not simply by the shutdown of sex pheromone production). OrfY and Orf1 proteins could occupy specific positions upstream from their own coding regions, thus contributing to repression by preventing accidental transcription events. Additional factors help to keep the TTS1 and TTS2 terminators locked. Inhibitory pheromone iAD1 (8) competes with trace amounts of the sex pheromone cAD1, possibly present in the environment or produced by the cell itself, and traD countertranscription (2) interferes with transcription from the P₀ promoter in the direction of traE₁.

TraA does not prevent transcription from P₀, since both iad and traA itself are constitutively expressed (however, transcription stops at the transcriptional terminators located downstream of these genes); it is more likely that TraA prevents residual TraE₁ molecules from binding to its recognition site in the P₀ region. This at the same time is the clue to the rapid induction by very low concentrations of sex pheromone: the affinity of TraA for its DNA binding site is directly relieved by cAD1 binding to TraA protein, and the site becomes accessible for TraE₁. DNA-bound TraE₁ modifies RNA polymerase in that it now can pass over the downstream terminators. Transcription of traE₁ reinforces the initiation of transcription by positive feedback (25). When the active complex meets with OrfY and Orf1 gene products bound to their specific sites on the DNA (or to a DNA-binding cofactor), the proteins form an active complex initiating transcription at P₂ and P₃, respectively. (P₁ is a weakly constitutive promoter causing a basic orfY-sea₁ transcription terminating downstream of sea₁ [13].) If only one of the proteins is lacking, transcription of asa₁ is prevented.

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**FIG. 6.** Deletions of traE₁, orfY, and orf1 genes from the constitutive pAEYP₁ construct. (a) Gross deletions covering a great part of the gene (traE₁) or the complete DNA region (orfY or orf1). (b) Point mutations of traE₁ and orf1 and 3' truncation of orfY (see Materials and Methods for details).
Alternatively, the active complex may be required for the extension of a super-transcript initiating at P₀. Such a mechanism is discussed for sex pheromone plasmid pCF10 (in references 3 and 4). If this were the case, however, the transcript would be processed immediately after RNA polymerase has transcribed the processing sites, since a precursor transcript cannot be detected by Northern blotting of mRNA isolated shortly after induction (13).

In addition, the involvement of a pAD1-encoded RNA molecule or molecules as for the pCF10 system (4) must be considered. The pCF10-encoded prgQ transcripts have been shown to interact directly with ribosomal proteins, which probably results in a posttranscriptional control of Asc10 expression (the pCF10-encoded aggregation substance).

prgQ is carried within the region most conserved among all sex pheromone plasmids (15). It seems unlikely that a function attributed to this region for one plasmid should be totally irrelevant to the others. This mechanism may be rather a common principle of the regulation of all sex pheromone plasmids, while specificity is guaranteed by minor sequence variations or specific proteins (such as TraE1 and Orf1 gene products in the pAD1 system).

The attractivity of the suggested model lies in the fact that it supports both possible functions of TraE1 as a trans-acting and cis-acting factor: As a typical protein, it diffuses readily through the cell, but only in its DNA-bound conformation is able to interact with its cofactors to form an active transcription initiation complex. This conformation is permanently maintained when TraE1 tracks along the DNA. Nevertheless, the idea of TraE1 having a dual function as a cis- and trans-acting factor is still speculative and must be checked on a molecular level.

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