Genetic Analysis of Plasmid-specific Pheromone Signaling Encoded by pPDI in *Enterococcus faecalis*

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Certain plasmids in *Enterococcus faecalis* encode a mating response to recipient-produced peptide sex pheromones. Targeted disruption of *tra* genes on pPDI suggested that TraA plays a central role in the plasmid-specific pheromone signaling pathway. TraA functioned as a negative regulator for the pheromone-inducible conjugal transfer. Complementation analysis of pPDI *tra* gene mutants by pAD1 suggested that the pheromone binding function of TraC was non-specific between these plasmids, but the function of TraA and the pheromone shutdown function of TraB are plasmid-specific.

**Key words:** *Enterococcus faecalis*; bacterial sex pheromone; *tra* genes; pheromone signaling; targeted gene disruption

Conjugal transfer of certain plasmids, such as the bacteriocin plasmid pPD1, hemolysin plasmid pAD1, and tetracycline-resistance plasmid pCF10, is controlled in *Enterococcus faecalis* by pheromone signaling.1,2) The plasmid-free recipient bacteria secrete multiple pheromones. A pheromone (cX) specifically activates the conjugal transfer system of the corresponding plasmid (pX). Five pheromones, cPD1, cAD1, cCF10, cAM373, and cOB1 have been identified as hepta- or octapeptides consisting of protein amino acids.3,4) Synthesis of a surface adhesin, termed "aggregation substance (AS)," is one of the most important and drastic processes induced by pheromones. AS leads to formation of mating aggregates of recipient and donor cells and facilitates the high frequency transfer of plasmids in a liquid medium.5) Once a recipient acquires the plasmid, it becomes a donor, but it continues to behave as a recipient for other plasmids. Two functions encoded by the plasmid, pheromone shutdown and production of a pheromone inhibitor (iX), contribute to blocking the related pheromone activity. Consequently, donor cells no longer secrete detectable pheromone, while other pheromones continue to be secreted.6)

In our previous study, we cloned and characterized a region of a bacteriocin plasmid, pPD1, organized as *traC*-traB-traA-ipd (Fig. 1).7) The open reading frames (ORFs) encode TraC (61 kDa) with a putative signal sequence, TraB (43 kDa) and TraA (38 kDa) without signal sequences, and a precursor of inhibitor peptide iPD1.7) Phenotypic analysis of the mutants generated by targeted disruption identified the functions of *traB* and *traC*. A *traB* deficient mutant, OG1X(pAM351BM), secretes a high level of pheromone and undergoes cell-clumping constitutively without exogenous pheromone.7) These facts indicate that the TraB product contributes to pheromone shutdown. TraC has much similarity to oligopeptide-binding proteins found in other bacterial species.8-11) A *traC* deficient mutant, OG1X (pAM351CM), required a concentration of cPD1 fourfold higher than that needed by the wild-type strain for induction of sexual aggregation.7) This suggests that TraC contributes to pheromone sensitivity as a peptide-binding protein on the donor cell surface. The region of pPDI organized as *traC*-traB-traA-ipd resembles corresponding regions of other pheromone plasmids, pAD1 and pCF10, in ORF organization and deduced amino acid sequence of each product.12-17) The similar region of pPDI is organized as *traB*-traC-cha-Iad, encoding pheromone shutdown, pheromone-binding protein, negative regulator, and pheromone inhibitor, respectively.12-15)

In this communication, we describe further genetic analysis of pPDI *tra* genes by targeted gene disruption. Moreover, to investigate the plasmid specificity of the *tra* genes, we did complementation analysis of *tra* gene mutants by pAD1.

**Materials and Methods**

Strains, plasmids, and media. E. faecalis strains, OG1X18) and JH2-2,19) were grown in THB medium (Todd-Hewitt broth [Oxoid]), at 37°C. A map of the plasmids used in this study is shown in Fig. pAM351 is a derivative of pPD1 with an insertion of a tetracycline-resistance transposon, Tra9/16.20) pAM714 is a derivative of pPDI with an insertion of an erythromycin-resistance transposon, Tra9/17.20) OG1X (pAM351) and OG1X (pAM714) showed the same phenotype as OG1X (pPD1) and OG1X (pAD1), respectively, relating to the pheromone-inducible aggregation and plasmid transfer. Plasmids pAM351BM, pAM351CM, and pAM351BCM have been described previously.7) pDLES23 was a chimeric plasmid consisting of pDL27620) and a 2.3-kb EcoRV-SauBI fragment of pPDI. pDLDH21 was a chimeric plasmid consisting of pDL276 and a 2.3-kb HinclI-HindII fragment of pPDI. pAM2600 and pAM2801 are referred to by Tamimoto et al.12) Strains carrying two plasmids used for complementation analysis were prepared by conjugal transfer of plasmids.

pUCAM was a chimeric plasmid consisting of pUC119 and a 1.0-kb nested-deleted segment (nucleotide no. 3782 to no. 4410)20) from a 2.3-kb HindII-HindII fragment of pPDI. pUCAIM was generated by deletion of the SpeI-SpeI 1.0-kb segment [ATraA-ipd]] from pHH21 which was a chimeric plasmid consisting of pUC119 and a 2.3-kb HindII-HindII fragment of pPDI.21) A HindII-Clod 1.5-kb segment of pVA9121) which encodes a selectable erythromycin resistant marker (erm) in E. faecalis, was blunted with T4 DNA polymerase and ligated into the Smal site of the residual multiple cloning site in the vector moiety of pUCAM and

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**Abbreviation:** OG1X (plasmid); strain OG1X carrying the plasmid.
pUCAIM.

Targeted disruption. pUCAM::erm and pUCAIM::erm were introduced into OGIX (pAM351) by an electroporation method described previously.\(^ {2,23} \) The resultant erythromycin-resistant cells were selected by 50 \( \mu g/ml \) of erythromycin. pAM351AM was generated by integration of pUCAM::erm into pAM351 via single crossover recombination. pAM351AIM was generated by further recombination of the direct repeated DNA made by integration with pUCAM::erm into pAM351. OGIX (pAM351CM) and OGIX (pAM351BCM) were further mutated by the same method with pUCAM::erm, thereby generating pAM351ACM and pAM351ABC.M.

Measurement of transfer frequency. Transfer frequency in a liquid medium was measured by the method described previously.\(^ {31} \) An overnight culture of the donor strain, OGIX carry pPD1 derivative was inoculated into 10 volumes of fresh THB medium and cultured at 37°C. At 3 h of cultivation, the donor cell culture was transferred into 10 volumes of fresh THB medium and grown for 45 min. For phenome induction, pPD1 was added to the THB medium to 2 ng/ml. One-tenth ml of the donor cells culture was mixed with 0.1 ml of a mid-log-phase culture of JH2-2 cells in 1 ml of THB medium. After a 15-min incubation at 37°C, the mating mixture was cooled to 4°C, vortexed to dissociate clumped cells, diluted appropriately, and spread (0.1 ml) on plates containing antibiotics to select donors and transconjugants. After incubation at 37°C for 24-48 h, the colonies formed were counted.

Filter mating was done by the method of Clewell et al.\(^ {29} \) One milliliter of fresh THB medium was added to a mixture of an overnight culture (0.045 ml) of donor strain, OGIX carrying a pPD1 derivative, and an overnight culture (0.45 ml) of the recipient strain, JH2-2. The cells in the mixture were collected on a membrane filter (type GTP; pore size 0.2 \( \mu m \); diameter, 13 mm; Millipore Corp.). The filter was placed on a THB agar plate and incubated at 37°C overnight. The cells were then suspended in 1 ml of broth and spread on plates containing appropriate selective drugs. After incubation at 37°C for 24-48 h, the colonies formed were counted.

The antibiotics used were tetracycline (10 \( \mu g/ml \)) for donors OGIX carrying a pPD1 derivative, rifampicin (25 \( \mu g/ml \)) plus tetracycline (10 \( \mu g/ml \)) or erythromycin (50 \( \mu g/ml \)) for transconjugants JH2-2 carrying a pPD1 derivative.

Results and Discussion

Targeted disruption of tra genes on pPD1

The Table shows results of targeted disruption on tra genes of pPD1 and phenotypic analysis relating to the clumping phenotype inducible by cPD1, phenomone production, and plasmid transfer of the mutant derivatives of pPD1. With the exception of traA deficient mutants, the transfer frequency reflects the clumping phenotype: the transfer frequency was increased from about \( 10^{-3} \) to about \( 10^{-3} \) to \( 10^{-2} \) (transconjugants per donor) when the formation of mating aggregates was accomplished. In the case of OGIX (pAM351BM), the transfer frequency was about \( 10^{-3} \) without phenomone induction. This high frequency was due to self-induction by high levels of endogenous cPD1 in the absence of a functional phenomone shutdown system encoded by traB. In the case of OGIX (pAM351CM), the transfer frequency was 10\(^{+4} \) after exposure to cPD1. This low frequency was due to disruption of the phenomone sensitivity function encoded by traC.

The traA gene was disrupted by homologous recombination via a single cross-over between pPD1 derivative, pAM351, and a suicidal vector, pUCAM carrying a fragment within the traA gene (Fig.). The resultant traA-disrupted plasmid was designated pAM351AM. OGIX (pAM351AM) underwent constitutive clumping without the induction by phenomone. This indicates that TraA functions as a negative regulator for plasmid-inducible clumping i.e., expression of AS. Despite the constitutive clumping phenotype, the transfer of pAM351AM was not detected when transconjugants were selected on tetracycline-containing agar plates. On the other hand, when erythromycin was used instead of tetracycline, a large number of resistant transconjugant colonies were grown. These observations implied that Tn916 encoding tetracycline resistance was unstable in pAM351AM during conjugal transfer. Indeed, Tn916 was deleted on the plasmid obtained from the erythromycin-resistance transconjugant. Without phenomone induction, the transfer frequency of the erythromycin resistant was as high as seen with induction by phenomone. In conclusion, TraA functions as a negative regulator for phenomone response including formation of cell clumping and plasmid transfer. These findings coincide with the result of Tanimoto et al. showing that a pPD1 traA mutant (pMG200) had a constitutive clumping phenotype and an ability to transfer at high frequencies in short mating period.\(^ {24} \)

The further disruption of tra on pAM351CM or pAM351BCM also resulted in the constitutive clumping phenotype of host cells. These results suggest that TraA functions as the negative regulator downstream of TraC and plays a central role in the cPD1-signaling pathway.

pAM351AIM was constructed by double homologous recombination via single cross-over between pAM351 and
a suicidal vector, pUCAIM, carrying a fragment with a deletion, Δ(traA-ipd), from the N-terminal part of the traA ORF to ipd as shown in Fig. OG1X (pAM351AIM) did not clump or transfer plasmids in a liquid medium containing cPDI; it was insensitive to phenomycin. This indicates that the deleted region was necessary for the phenomycin response. This finding coincides with the models proposed in the case of pAD1 [15,25] and pCF10 [26,27]. In the case of pAD1, TraA binds a promoter region of iad and negatively regulates the expression of a transcript from iad to traE1, which positively regulates in trans the expression of AS [15,25]. In the case of pCF10, a transcript including the inhibitor-encoding region involves in positive regulation for expression of AS [26,27]. Though transcriptional analysis has never been done, the case of pPD1 can be explained by a similar model: TraA binds the promoter region of ipd and negatively regulates a transcript including ipd, which positively regulates the expression of AS. As shown in Table 1, the transfer frequency of pAM351AIM was very low even in the filter mating. This suggests that the deleted region was involved in positive regulation for plasmid transfer as well as for clumping formation.

**Complementation analysis of pPD1 tra gene mutants by pAD1**

To investigate the plasmid specificity of the tra genes, a pAD1 derivative, pM714 [19], was introduced by conjugation into certain strains carrying the mutant derivatives of pPD1. OG1X (pAM351BM + pM714) produced high levels of cPDI and self-induced clumping constitutively like OG1X (pAM351BM). These suggest that the loss of traB, causing cPDI shutdown, was not complemented by pAD1 traB. The double mutant, OG1X (pAM351BCM), [15] did not have a constitutive clumping phenotype despite its high level cPDI production. This can be explained by the increased phenomycin sensitivity caused by the loss of the TraC function. When pDLES23 (a segment containing traC of pPD1 cloned into a E. coli - E. faecalis shuttle vector pDL276) was introduced into OG1X (pAM351BCM), the transformant OG1X (pAM351BCM + pDLES23) had constitutive clumping phenotype like OG1X (pAM351BM). This observation implied that the loss of the TraC function was complemented in trans by pPD1 traC. Similarly OG1X (pAM351BCM + pM714) had a constitutive clumping phenotype like OG1X (pAM351BM). This result indicates that the loss of the pPD1 TraC function could be complemented by pAD1 traC as done by pPD1 traC. To confirm this possibility, a complementation test was done using pAM2600 (a segment containing traC of pAD1 cloned into a multicopy vector, pAM401) and pAM2601 which has a Tn5 insertion within traC of pAM2600. [12] As expected, OG1X (pAM351BCM + pAM2600) exhibited the constitutive clumping phenotype while OG1X (pAM351BCM + pAM2601) did not. Considering the assumption that the TraC protein contributes...

### Table: Targeted Disruption and Complementation of tra Genes of pPD1

<table>
<thead>
<tr>
<th>Plasmid in OG1X</th>
<th>Genotype</th>
<th>Relevant phenotype*</th>
<th>Transfer frequency of pPD1 derivative (transconjugant/donor)</th>
<th>Non-induced</th>
<th>+cPDI</th>
<th>Filter mating</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAM351</td>
<td>pPD1</td>
<td>Same as wild-type</td>
<td></td>
<td>1.49 × 10⁻⁵</td>
<td>2.15 × 10⁻²</td>
<td>2.20</td>
</tr>
<tr>
<td>pAM351 + pAM714</td>
<td>pPD1 + AD1</td>
<td>Same as wild-type</td>
<td></td>
<td>1.82 × 10⁻⁵</td>
<td>8.36 × 10⁻²</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351BM</td>
<td>pPD1-traA</td>
<td>High level cPDI production constitutive clumping</td>
<td></td>
<td>1.06 × 10⁻³</td>
<td>2.76 × 10⁻²</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351BM + pAM714</td>
<td>pPD1-traA + pAD1</td>
<td>High level cPDI production constitutive clumping</td>
<td></td>
<td>N.T.²</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351CM</td>
<td>pPD1-traC</td>
<td>Less sensitive to cPDI</td>
<td></td>
<td>2.73 × 10⁻⁵</td>
<td>1.74 × 10⁻⁴</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351CM + pAM714</td>
<td>pPD1-traC + pAD1</td>
<td>Not less sensitive to cPDI</td>
<td></td>
<td>1.09 × 10⁻⁵</td>
<td>6.68 × 10⁻⁵</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351AM</td>
<td>pPD1-traA</td>
<td>Constitutive clumping</td>
<td></td>
<td>&lt;4.40 × 10⁻⁶</td>
<td>&lt;1.42 × 10⁻⁷</td>
<td>3.89 × 10⁻⁶</td>
</tr>
<tr>
<td>pAM351AM + pDLHH21</td>
<td>pPD1-traA + traA pPD1</td>
<td>Less sensitive to cPDI</td>
<td></td>
<td>3.45 × 10⁻⁴</td>
<td>6.06 × 10⁻⁴</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351AM + pAM714</td>
<td>pPD1-traA + pAD1</td>
<td>Constitutive clumping</td>
<td></td>
<td>2.07 × 10⁻⁴</td>
<td>2.36 × 10⁻⁴</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351BCM</td>
<td>pPD1-traA</td>
<td>High level cPDI production not constitutive clumping</td>
<td></td>
<td>7.09 × 10⁻⁸</td>
<td>&lt;1.26 × 10⁻⁷</td>
<td>N.T.</td>
</tr>
<tr>
<td>pAM351BCM + pAM714</td>
<td>pPD1-traA + pAD1</td>
<td>High level cPDI production not constitutive clumping</td>
<td></td>
<td>3.54 × 10⁻⁵</td>
<td>8.11 × 10⁻⁵</td>
<td>N.T.</td>
</tr>
</tbody>
</table>

*a* In this column, phenotypes relating to clumping and cPDI-production were described. “High level cPDI production” indicates that the culture filtrate from the strain showed more than 5 titer of cPDI in the reverse-phase high performance liquid chromatography assay, [7] in competition with 1 to 2 titer of wild-type strain. “Constitutive clumping” indicates that the strain clumped without addition of exogenous cPDI. “Less sensitive to cPDI” indicates that the strain required more than four-fold concentration of cPDI to clump. “Insensitive to cPDI” indicates that the strain did not clump in the presence of less than a thousand titer of cPDI.

*traA-ipd* indicates a deletion as shown in Fig.

N.T., not tested.

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a suicidal vector, pUCAIM, carrying a fragment with a deletion, Δ(traA-ipd), from the N-terminal part of the traA ORF to ipd as shown in Fig. OG1X (pAM351AIM) did not clump or transfer plasmids in a liquid medium containing cPDI; it was insensitive to phenomycin. This indicates that the deleted region was necessary for the phenomycin response. This finding coincides with the models proposed in the case of pAD1 [15,25] and pCF10 [26,27]. In the case of pAD1, TraA binds a promoter region of iad and negatively regulates the expression of a transcript from iad to traE1, which positively regulates in trans the expression of AS [15,25]. In the case of pCF10, a transcript including the inhibitor-encoding region involves in positive regulation for expression of AS [26,27]. Though transcriptional analysis has never been done, the case of pPD1 can be explained by a similar model: TraA binds the promoter region of ipd and negatively regulates a transcript including ipd, which positively regulates the expression of AS. As shown in Table 1, the transfer frequency of pAM351AIM was very low even in the filter mating. This suggests that the deleted region was involved in positive regulation for plasmid transfer as well as for clumping formation.

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Considering the assumption that the TraC protein contributes

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pheromone sensitivity as an extracellular pheromone-binding protein.\textsuperscript{7,12} This finding indicates that cPD1 could be recognized by pAD1 \textit{traC}.

The constitutive clumping phenotype of OG1X (pAM351AM) reverted to the pheromone-inducible clumping phenotype when transformed with pDLHH21 (a segment containing \textit{traA} of pPD1 cloned into pDL276). This implied that the pPD1 \textit{TraA} function was complemented in \textit{trans} by pPD1 \textit{traA}. On the other hand, the constitutive clumping phenotype of pAM351AM was maintained when harbored with pAM714. This result suggests that the function of \textit{TraA} is plasmid-specific between pPD1 and pAD1. Molecular analysis of DNA-binding property of \textit{TraA}s could explain the precise mechanism of the plasmid-specific pheromone signaling.

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\textbf{References}


