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Targeted disruption of the PD78 gene (*traF*) reduces pheromone-inducible conjugal transfer of the bacteriocin plasmid pPD1 in *Enterococcus faecalis*

Jiro Nakayama [a,*], Don B. Clewell [b], Akinori Suzuki [a]

[a] *Department of Applied Biological Chemistry, Division of Agriculture and Agricultural Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan*

[b] *Department of Biologic and Materials Sciences, School of Dentistry and Department of Microbiology / Immunology, School of Medicine, The University of Michigan, Ann Arbor, MI 48109-1078, USA*

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Abstract

Bacterial sex pheromone, cPD1, induces sexual aggregation of *Enterococcus faecalis* harboring the bacteriocin plasmid, pPD1, and enables pPD1 to transfer at high frequency in a liquid culture. PD78 is a cPD1-inducible cell surface protein encoded by pPD1. The PD78 gene, *traF*, was disrupted by homologous recombination between pPD1 and an artificial vector having a deletion in the middle portion of *traF*. The disruption of *traF* did not affect the cPD1-inducible aggregation but reduced the transfer frequency of pPD1 to 2% of the wild-type level.

Keywords: Enterococcus faecalis; Conjugative plasmid; Gene disruption; Bacterial sex pheromone; *traF*; pPD1

1. Introduction

Bacteriocin plasmid of *Enterococcus faecalis*, pPD1 (54 kb) [1,2], encodes a mating response to the peptide sex pheromone, cPD1 [3], secreted from the plasmid-free recipient bacteria. cPD1 induces synthesis of a surface adhesin, termed Asp1 (aggregation substance coded by pPD1, 138 kDa), which leads to sexual aggregation of recipient and donor cells [4]. The formation of the mating aggregate facilitates the high frequency transfer of pPD1 in a liquid medium [3,5].

PD78 is also cPD1-inducible protein located on the cell surface and is detected as a 78-kDa protein based on SDS-PAGE [6,7]. The PD78 gene, henceforth designated *traF*, was cloned from pPD1; and its nucleotide sequence revealed that PD78 is actually a 54-kDa protein [8]. The discrepancy of the apparent molecular size in SDS-PAGE compared to the sequence data was presumed to be due to its unusual amino acid composition that is very rich in glutamic acid and lysine. The fact that Anti-PD78 polyclonal antibody blocked the cPD1-induced aggregation suggested that PD78 might contribute to the formation of sexual aggregation [9]. However, there was a possibility that the blocking effect was caused by steric hindrance of the immunoglobulin molecules. Therefore, the real function of PD78 has been unclear.

* Corresponding author. Tel: +81 (3) 3812-2111 ext. 5133; Fax: +81 (3) 3812-0544; e-mail: jiro-n@tansei.cc.u-tokyo.ac.jp.

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Use of the transposon Tn*917* [10] to generate many mutations in pAD1 and pCF10 has contributed much to this field [11,12]. However, genetic research on pPD1 has not progressed as well as might has been desired, since the generation of pPD1::Tn*917* derivatives has been somewhat difficult. In this study, we conducted a targeted disruption of *traF* using homologous recombination, and investigated the function of PD78.

2. Materials and methods

2.1. Bacterial strains and media

Escherichia coli JM109 [13] containing recombinant plasmids was grown in Luria-Bertani broth. *E. faecalis* strains OG1X(pAM351) [14], OG1X-(pAM351FM) [this study] and JH2-2 [15] were grown in Todd-Hewitt broth (Oxoid). The antibiotics used in the selective media for *E. coli* were ampicillin (60 μg/ml) and erythromycin (150 μg/ml), and for *E. faecalis* were erythromycin (50 μg/ml), tetracycline (10 μg/ml), and rifampicin (25 μg/ml).

2.2. Plasmid

The maps of the plasmids used in this study were shown in Fig. 1. Plasmid DNA was isolated by the alkaline method from *E. coli* [16] and *E. faecalis* [17]. pAM351 is a derivative of pPD1 having an insertion of tetracycline-resistance transposon Tn*916* in the *Eco* RI B-fragment [14]. OG1X(pAM351) showed the same phenotype as OG1X(pPD1) relating to pheromone-inducible aggregation and plasmid transfer. pJN1 was a chimeric plasmid consisting of pUC118 [13] and a 3.6-kb *Bgl*II-*Eco*RI segment containing *traF* of pAM351 [8]. pJN1 was digested with *Acc*III(TaKaRa), treated with T4 DNA polymerase (TaKaRa), and self-ligated by T4 DNA ligase (TaKaRa). The resultant plasmid was designated pJNM1. The region including the mutation of pJNM1 was sequenced. Unexpectedly, a spontaneous deletion (nucleotide no. 827-1401 in ref. [8]) was found as shown in Fig. 1. The 5′ end of the deletion corresponded to the *Acc*III site and the 3′ end was included in a palindoromic sequence CAATTG (corresponding to *Mun*I recognition sequence), which was probably digested by some nuclease contaminated from the *E. coli* lysate. A *Hin*dIII-*Xba*I 1.7-kb segment of pVA891 [18], which encodes a selective Em^r marker in *E. faecalis*, was ligated into the *Hin*dIII-*Xba*I site of residual multiple cloning site in the vector moiety of pJNM1. The resultant plasmid was designated pJNM1Em.

2.3. Transformation of bacteria

E. coli was transformed by an electroporation method described in ref. [16]. *E. faecalis* was transformed by electroporation method modified by a procedure of Cruz-Rodz et al. [19]. OG1X(pAM351)

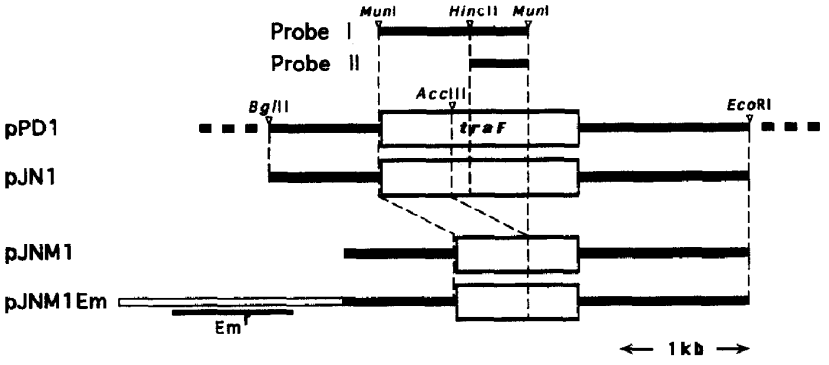

Fig. 1. Maps of plasmids and probes used in this study. The insert of pJN1 consists of PD78-coding region (*traF*) indicated by hatched box and its upstream and downstream regions indicated by bold lines. pJNM1 has a deletion of the *Acc*III-*Mun*I segment in *traF*. The open bar in the upstream region of pJNM1Em was derived from a *Hin*dIII-*Xba*I fragment containing Em^r of pVA891. Probe I and probe II were used in Southern hybridization shown in Fig. 3B and 3C, respectively.

cells were grown overnight in Todd-Hewitt Broth containing 0.5M sucrose and 6% glycine. The cells were harvested by centrifugation at $2000 \times g$ and then washed twice with ice-cold electroporation solution (0.5 M sucrose and 10% glycerol). Finally, the cells were resuspended in 1/100 the original volume of electroporation solution. Fifty microliters of the suspension and 1 μg of pJNM1Em were mixed in the 0.2 cm cuvette and were exposed to a single electric pulse (peak voltage, 2.5 kV; capacitance, 25 μF; pulse controller, 200 Ω) in a Bio-Rad Gene Pulser and pulse controller. The discharged cell suspension was immediately diluted in 0.96 ml of ice-cold Todd-Hewitt Broth containing 0.5M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$, and was kept on ice for 5 min. Then, the cells were incubated at 37° C for 2 h. The erythromycin resistant transformants were selected on the agar plate containing erythromycin at 37° C within 24–48 h.

2.4. Southern hybridization

DNA was cut with *Eco*RI and run on a 0.6% agarose gel, transferred to nylon membrane (Hybond-N; Amersham), hybridized under stringent condition to random-prime-^{32}P labeled probe, and autoradiographed, as described in ref. [15]. Probes were prepared by Random primer DNA labeling kit (TaKaRa).

3. Results

Fig. 2 is a diagram showing the gene disruptions of *traF* of pAM351. The plasmid pJNM1 is a chimeric plasmid consisting of pUC118 and *traF* having a deletion in the middle portion of its open reading frame. This deletion also generates frame-shift and results in loss of the C-terminal two-third of PD78. An erythromycin-resistance gene from pVA891, which can act as a selective marker in *E. faecalis*, was ligated into pJNM1. This new plasmid, pJNM1Em, could not be autonomously replicated in *E. faecalis*. However, introduction of pJNM1Em into *E. faecalis* cells by electroporation transformed some OG1X(pAM351) cells to Em^r, resulting from its integration into pAM351 via reciprocal recombination (Fig. 2, A or B). One transformant obtained was

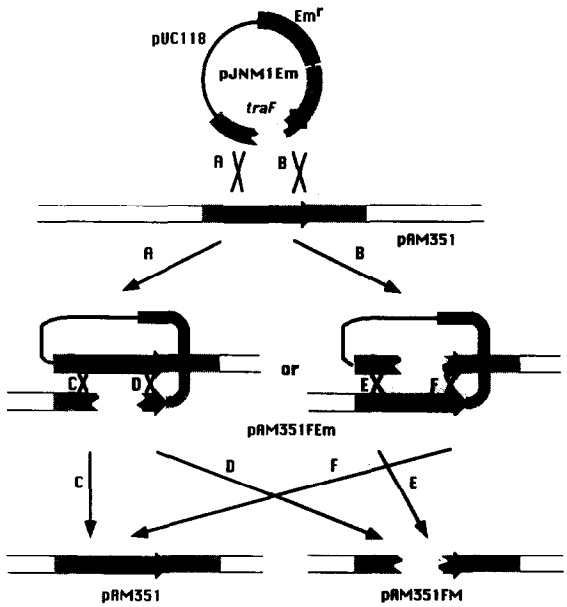

Fig. 2. Schematic showing the strategy used to disrupt *traF*. The suicidal vector pJNM1Em was introduced into OG1X(pAM351) by electroporation. The first recombination occurred at A (upstream of the deletion) or B (downstream of the deletion) and generated the Em^r plasmid pAM351FEm. The second recombination at C or F generated the wild-type pAM351. The recombination at D or E generated pAM351FM having the mutant allele.

chosen for study and was designated OG1X-(pAM351FEm). Growth of the resultant Em^r strain under nonselective condition gave rise to two kinds of Em^s strains. These strains resulted from the loss of the integrated chimera mediated by recombination of the flanking homologous DNA (Fig. 2, C, D, E, or F). In this process, either the wild-type or the mutant *traF* allele of pJNM1Em can be lost. Two of the four resultant Em^s strains were the same as OG1X(pAM351) and others were OG1X carrying pAM351FM which has a mutant allele of *traF*. The plasmids pAM351 and pAM351FM were digested with *Eco*RI and analyzed by agarose gel electrophoresis (Fig. 3A) and Southern hybridization (Fig. 3B and 3C) Probe I (Fig. 1) hybridized to a 8.6-kb fragment of pAM351 and a 8.0-kb fragment of pAM351FM (Fig. 3B). Probe II (Fig. 1) hybridized to the wild-type 8.6-kb fragment, but did not hybridize to the mutant 8.0-kb fragment (Fig. 3C). These results indicate that pAM351FM has the deletion in *traF*.

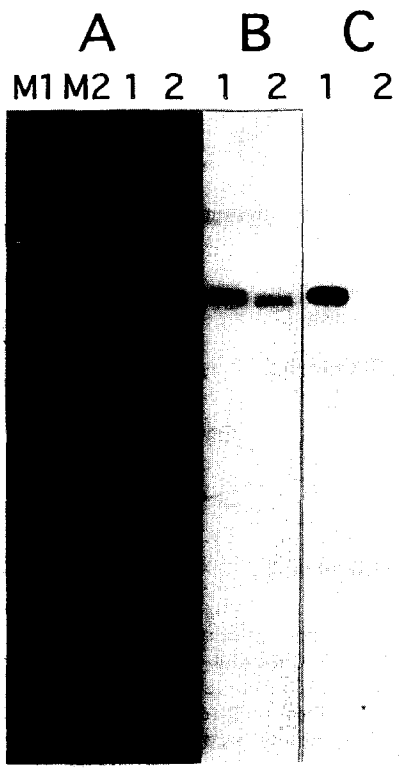

Fig. 3. Agarose gel electrophoresis and Southern hybridizations showing the deletion within *traF*. Lane M1 shows molecular masses standards 23.1 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb and 2.03 kb of λ-*Hin*dIII digests. Lane M2 shows molecular masses standards 8.45 kb, 7.24 kb, 6.39 kb, 5.69 kb, 4.82 kb, 3.68 kb, 2.32 kb, 1.93 kb, 1.37 kb, 1.26 kb of λ-*Bst*PI digests. Lanes 2 and 3 show *Eco*RI digests of pAM351 and pAM351FM, respectively. Digests were separated on 0.6% agarose gel and stained with ethidium bromide (**A**), transferred to a nylon membrane, hybridized under stringent condition to random-prime-^{32}P labeled probe I (**B**) and probe II (**C**).

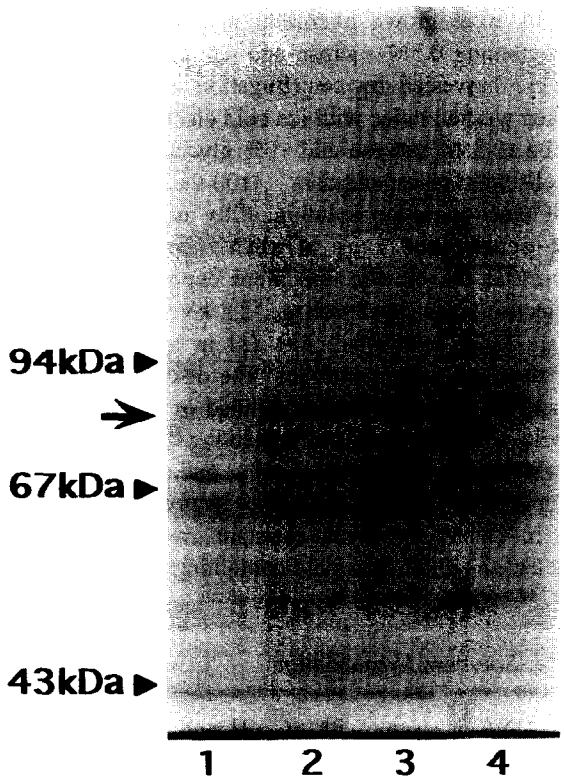

Fig. 4. SDS-PAGE of cell surface extracts. Arrow indicates PD78. Lane 1, cPD1-noninduced OG1X(pAM351); 2, cPD1-induced OG1X(pAM351); 3, cPD1-noninduced OG1X(pAM351FM); 4, cPD1-induced OG1X(pAM351FM). Extracts were prepared by solubilizing the cell surface proteins with 50 mM Tris-HCl (pH 8.4) containing 0.1% Zwittergent 3-12 (Calbiochem) [9]. Pheromone induction was done with THB medium containing 0.1 ng/ml cPD1 for 2 h.

Fig. 4 shows SDS-PAGE of cell surface extract from OG1X(pAM351) and OG1X(pAM351FM) strains. The cPD1-induced OG1X(pAM351) cells produced PD78 (Fig. 4, lane 2). The extract from cPD1-noninduced OG1X(pAM351) cells contained a low amount of PD78 (Fig. 4, lane 1). Our previous study showed that OG1X(pAM351) cells secrete a low level of cPD1 [20]. The endogenous pheromone

Table 1
Pheromone sensitivity and transfer frequency of plasmids

Plasmid	Pheromone sensitivity [a] (pg/ml)	Transfer frequency [b] (transconjugant/donor)	
		−cPD1	+cPD1
pAM351	100	6.2×10^{-6}	8.3×10^{-4}
pAM351PD78M	100	3.8×10^{-7}	1.7×10^{-5}

[a] The value represents the minimum concentration required for the induction of aggregation.

[b] Mating experiment was done according to the methods described in ref. [3]. Donor strain OG1X(pAM351) or OG1X(pAM351FM) was exposed to 0.5 ng/ml synthetic cPD1 for 45 min before mixing with recipient JH2-2 (+cPD1). For the control experiment, the donor strains were grown without the pheromone induction (-cPD1). Mating time was 15 min. Transconjugant cells were selected on the plate containing tetracycline (10 μg/ml) plus rifampicin (25 μg/ml). Donor cells were selected on the plate containing tetracycline (10 μg/ml).

probably induced PD78. Indeed, PD78 was not detected when the OG1X(pAM351) was exposed to the pheromone inhibitor iPD1 (data not shown). PD78 was not detected in the extract from either cPD1-noninduced (lane 3) or cPD1-induced (lane 4) OG1X (pAM351FM) cells. This confirmed that pAM351FM had a mutant allele of *traF*.

OG1X(pAM351FM) cells did exhibit pheromone-inducible aggregation as did OG1X(pAM351). The minimum concentration of cPD1 required for the induction of OG1X(pAM351FM) was the same as that of OG1X(pAM351), as shown in Table 1. This result indicates that PD78 did not relate to the aggregate formation. The transfer frequency of pAM351FM was enhanced by two orders of magnitude by the pheromone induction. However, the transfer frequency of pheromone-induced pAM351FM was 2% of that of pAM351. These results indicate that PD78 is not essential but contributes to the pheromone-inducible plasmid transfer. Under the non-inducing condition, the transfer of pAM351 occurred at a 16-fold higher frequency than that of pAM351FM. As shown in Fig. 4, OG1X(pAM351) produced low level of PD78 without pheromone induction. This low level of PD78 probably caused the enhancement of the transfer frequency of pheromone-uninduced pAM351. Indeed, in the presence of pheromone inhibitor, iPD1 [21], the transfer frequency of pAM351FM was similar to that of pAM351 (data not shown).

4. Discussion

The targeted disruption experiment of *traF* showed that PD78 is involved in the enhancement of transfer frequency of pPD1 by the mechanism other than the cPD1-inducible aggregate formation. PD78 contains the sequence of X-X-Pro repeated 15 times in a central region. Similar repeated sequences have been found in TraD encoded by the *E. coli* R100 plasmid (84 kDa) [22]. From the findings that *traD* was required for plasmid transfer [23] and incorporated into the cell envelope [24], it may be required for transport of plasmid DNA through the cell envelope or strand separation [25]. As PD78 is not transmembrane protein [8], and as the *traF* mutant, pAM351FM, still can be transferred at high frequency (Table 1), PD78 is considered to be nonessential to plasmid transfer and not equivalent component to TraD. PD78 might be involved in stabilization of the mating pair while plasmid DNA is transferred. This is similar to the case of the *traN* product coded by F plasmid of *E. coli* [26]. The *traN* product is an outer membrane protein of M_r. 66,000 required to stabilize mating pair while interacting with an inner membrane protein TraG [27].

In the case of the pheromone responsive plasmid pAD1, some mutated derivatives from pAD1 having a transposon insertion in the G-region showed the same phenotype as the *traF* mutant [28]. The G region is located downstream of the aggregation substance gene (*asa1*), as is *traF*, suggesting that the G region may encode the PD78-like protein. Indeed, in the Southern hybridization analysis, the *Eco* RI-A fragment of pAD1 containing the G region hybridized weakly with the labeled nucleotide probe of *traF* (unpublished data). Furthermore, Galli et al. have noted about the existence of a gene homologous to *traF* in the downstream region of *asa1* [4]. Considering the above information, the *traF* determinant encoding PD78-like protein may be generally encoded by pheromone-responsive plasmids.

The method of the targeted gene recombination established here could be applied to construct many kinds of mutant genes coding truncated protein, chimera protein, and one amino acid substitution etc., and would be effective to investigate functions of not only PD78 and also other proteins encoded on pPD1.

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