STUDIES ON PHEROMONE RESPONSIVE

(*Enterococcus faecalis* の性フェロモン応答性接合伝達プラスミドに関する研究)

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IN *ENTEROCOCCUS FAECALIS*

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JIRO NAKAYAMA

中山二郎

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INTRODUCTION

Multiple antibiotic resistance is a serious clinical problem. Conjugative plasmids and transposons play a key role in the acquisition of not only drug resistance but also certain virulence traits (e.g., hemolysin determinants). How these plasmids and transposons move is of increasing interest, because recovery from certain diseases depends completely on antibiotic chemotherapy. For this reason, for example, infective endocarditis is one of the most feared diseases caused by *Enterococcus* (formerly *Streptococcus*) *faecalis*, a Gram-positive bacterium commonly found in the human intestine and frequently involved in urinary tract and other infections (Clewell 1981; Clewell 1990a).

There are two kinds of conjugative plasmids in *Enterococcus faecalis* (Clewell 1981). One, about 20-30 kb in size, is unable to transfer in liquid culture whereas can transfer into other species of hemolytic bacteria on a membrane filter, by so-called "filter mating". The other, about 50 kb in size, can transfer at high frequency in liquid culture. An interesting feature of the latter type of plasmid is the role played by sex pheromones. Bacterial sex pheromones are thus far unique to enterococci. The sex pheromone was revealed to be a "clumping inducing agent (CIA)" and a "fertility boosting ingredient (FBI)" (Dunny *et al.* 1978). It was found that culture filtrate from plasmid-free *E. faecalis* induced aggregation of bacteria harboring a certain plasmid (donor) and plasmid-free bacteria (recipient). The filtrate also induced aggregation of donor cells in the absence of the recipient, which is so-called self-clumping (Fig. 0-1). These results indicated that recipient cells secrete a clumping-inducing-agent (CIA). When donor and recipient cells were induced to aggregate, the harbored plasmids were transferred in broth at quite high frequency (10^{-1} to 10^{-2} per donor) as compared to the uninduced (10^{-7} to 10^{-8} per donor). These data indicated

that the recipient secretes a fertility-boosting-ingredient (FBI). About twenty plasmids responsive to the recipient culture filtrate have been reported (Table 0-1). Among these plasmids, hemolysin/bacteriocin plasmid pAD1 (60 kb), tetracycline resistance plasmid cCF10 (54 kb), bacteriocin plasmid pPD1 (56 kb), and unknown phenotype plasmid pAM373 (36 kb) have been well studied.

Sex Pheromones, cAD1, cPD1, cAM373, and cCF10, were isolated by monitoring the CIA activities during purification from donor strains harboring pPD1 (Suzuki *et al.* 1984), pAD1 (Mori *et al.* 1984), pAM373 (Mori *et al.* 1986b), and pCF10 (Mori *et al.* 1988), respectively. Sex pheromones for plasmid pX were designated cX, where c represents CIA. All isolated pheromones showed both activities of CIA and FBI specific to the donor harboring the corresponding plasmid at a concentration of about 10^{-10} to 10^{-9}M. The pheromones were found to be small hydrophobic peptides composed of seven or eight amino acid residues (Table 0-2). The structurally defined pheromones had different amino acid sequences and there was no cross-activity among them. These data suggest that each sex pheromone corresponds specifically to a certain plasmid. Thus, the number of sex pheromones may be similar to the number of pheromone-responsive plasmids existing in *E. faecalis*. The multiple pheromones are chromosomally encoded in *E. faecalis* and potentially each cell may secrete the multiple sex pheromones.

Pheromone shutdown and pheromone inhibitor are putatively encoded on the pheromone-responsive plasmid to prevent host donor from self-induction of the host donor by endogenous pheromone. Once the recipient acquires a copy of plasmid, it becomes a donor for the given plasmid while it continues to behave as recipient for other plasmids. Indeed, in culture filtrates

of the transconjugant donor, the activity of the corresponding pheromone disappears while the activity of other pheromones continues to be elaborated. This event seems to function to prevent self-induction of the donor by endogenous pheromone.

The pheromone shutdown model proposed by Dunny *et al.* (1979) could explain this phenomenon as shown in Fig. 0-2. This model was that a substance encoded on the plasmid represses (or inactivates) endogenous pheromone. However, inhibitory activity against pheromone activity has been found in culture filtrates from donor cells (Ike *et al.* 1983). Thus, not pheromone shutdown but the pheromone inhibitor, representing the inhibitory ativity, has seemed to be explained by the disappearance of the pheromone activity. The inhibitors against cAD1 (Mori *et al.* 1986a) and cPD1 (Mori *et al.* 1987) were isolated and each was shown to be a hydrophobic octapeptide similar in structure to the pheromone. These were termed iAD1 and iPD1, respectively: inhibitor against cX was designated iX, where i represents inhibitor. The structural similarity between pheromone and inhibitor suggests that the inhibitor may act as a competitive antagonist of the pheromone (Table 0-2). Indeed, the amount of inhibitor required to block the pheromone activity varied in proportion to the amount of pheromone (Nakayama *et al.* 1994b). The pheromone inhibitor, iAD1, was found to be encoded on pAD1 (Clewell *et al.* 1987) and the encoded gene was cloned (Clewell *et al.* 1990b). On the other hand, it remains unclear whether pheromone shutdown occurred or not, since the true (absolute) titer of pheromone could not be measured in the presence of inhibitor.

In chapter I, the author developed a method for quantitative analysis of the absolute titer of the pheromones. The production of pheromones by donor cells was not shut off completely but was limited to a level where the inhibitors could overcome the activity of the corresponding pheromone. In other words, the pheromone-responsive plasmid encodes double systems of

pheromone inhibitor and pheromone shutdown in order to prevent self-induction of the host by endogenous pheromone.

Genetics of the pheromone-responsive plasmids has been extensively developed in the past decade. The transposon Tn*917* (Tomich *et al.* 1980) was used to generate many mutations in pAD1 (Clewell *et al.* 1982; Ike *et al.* 1984; Ehrenfeld *et al.* 1987; Ike *et al.* 1990) and pCF10 (Christie *et al.* 1986). The mutated plasmids displayed various phenotypes indicating loss of functions or gain of functions related to the pheromone-inducible conjugative process. It was found that more than a half of the mutations in these plasmids influenced pheromone-inducible conjugation. In the case of pAD1, various functional regions have been identified as depicted in the map shown in Fig. 0-3 (Ehrenfeld *et al.* 1987). Recent research has been focused on the region designated F and the so-called regulatory region. In the case of pCF10, two regions analogous to the F region and the regulatory region of pAD1, respectively, exist and have been well studied (Christie *et al.* 1988; Kao *et al.* 1991). The F region of pAD1 is responsible for formation of cell aggregates and has been shown to encode an aggregation substance (Galli *et al.* 1990). The regulatory region is involved in regulation of the pheromone response. This region has been shown to contain genes encoding a pheromone binding protein (*traC*) (Tanimoto *et al.* 1993a), a pheromone shutdown (*traB*) (An *et al.* 1993), a pheromone inhibitor (*iad*) (Clewell *et al.* 1990b), and negative (*traA*) (Pontius *et al.* 1992a) and positive regulators (*traEI*) (Pontius *et al.* 1992b, Tanimoto *et al.* 1993b, Muscholl *et al.* 1993) of the aggregation substance gene.

The TraC protein exhibits homology with known oligopeptide-binding proteins in *B. subtilis* (Pergo *et al.* 1991, Rudner *et al.* 1991), *E. coli.* (Kashiwagi *et al.* 1990), and *S. typhimurium* (Hiles *et al.* 1987) and it displays a strong ability to reduce titers of both cAD1 and iAD1 in broth.

This indicates that TraC acts as an initial receptor for cAD1. Its strong effect on both titers complicated and interrupted the research of pheromone shutdown, as will be described in chapter I. In the case of cCF10, the author found that pheromone shutdown did not occur, as will be shown in chapter I. On the other hand, with pPD1, pheromone shutdown was clearly detected, while the TraC-like effect was not detected. These findings indicated that pPD1 would be a more appropriate plasmid to study the system of pheromone shutdown than either pAD1 or pCF10. However, since this transpositional mutagenesis of pPD1 generated only a few kinds of mutants, any genetic study has not been done.

In chapter II, the iPD1 gene (*ipd*) was cloned by monitoring the production of iPD1 in *E. coli.* Then a segment corresponding to the pAD1 regulatory region was tagged by *ipd,* since the pPD1 regulatory region was expected to be located upstream from *ipd* in analogy with pAD1 and pCF10. This segment had an ORF-organization similar to pAD1 and pCF10. Each ORF was disrupted by site-directed mutagenesis using homologous recombination. The *traB*-disrupted mutant underwent self-clumping constitutively. This is probably induced by high level of endogenous pheromone, suggesting that *traB* may encode cPD1 shutdown.

Sexual aggregation is the most important process in the conjugative process. Fig. 0-4 shows a model for pheromone-inducible sexual aggregation. The donor strain forms a visible aggregate within 30-45 min after exposure to the corresponding pheromone. It seems likely that several events occur on the surface of pheromone-induced cells, and cell-to-cell contact is one of the most crucial events. Dunny et al. suggested that the cell-to-cell contact is mediated by a pheromone-inducible cell surface adhesin termed "aggregation substance"(AS) (Dunny *et al.* 1979). According to this hypothesis, pheromone induces expression of the AS gene

encoded on the corresponding plasmid and AS binds to a substance referred to as "binding substance"(BS) on the recipient surface. The mating pair networks of donor and recipient cells formed in this process result in visible aggregation. Pheromone-inducible aggregation was sensitive to trypsin, pronase, sodium dodecyl sulfate (SDS) (0.05 %), and heat, whereas treatment with lysozyme, lipase, and glutaraldehyde or the presence of a number of different sugars had no effect (Yagi *et al.* 1983). From these data, it was inferred that AS was a protein. Immunoelectron microscopic studies of induced cells revealed the presence of a uniform coating of fuzzy surface material which was absent from uninduced cells (Yagi *et al.* 1983; Galli *et al.* 1989; Wanner *et al.* 1989). This material has been referred to as AS.

There is a line of evidence that BS may in part be made up of lipoteichoic acid (LTA), since purified LTA was able to inhibit aggregation at very low concentrations (Ehrenfeld *et al.* 1986). This is also consistent with the observation that induced donor cells form aggregates by "self-clumping" in the absence of recipient cells; LTA corresponds to the Lancefield group D antigen on the surface of *E. faecalis*. Indeed, the author has found that LTA purified from *E. faecalis* OG1X harboring pAM351 (pPD1::Tn916) inhibited aggregation to the same extent as LTA from plasmid-free JH2-2 cells did (Nakayama, 1988). Self-clumping is believed to be biologically disadvantageous because it consumes energy of the host cell in vain. Plasmid transfer between donors harboring the same plasmid is inhibited by a system of surface exclusion (Dunny *et al.* 1985). Genes designated, *sea1* (Weidlich *et al.* 1992) and *sec10* (Kao *et al.* 1991) have been cloned as a gene encoding surface exclusion protein from pAD1 and pCF10, respectively. It seems likely that surface exclusion is a third system to prevent self-mating in additon to the pheromone inhibitor and pheromone shutdown.

Many events other than cell-to-cell contact and surface exclusion are believed to occur in the conjugation process. The synthesis of a channel for transfer of plasmid DNA and stabilization of the mating pair are proposed to exist, in analogy with the case of the F plasmid in *E. coli.* (Willetts *et al.* 1984; Panicker *et al.* 1985).

An immunological study of pheromone-induced surface antigen has been reported (Kessler *et al.* 1983). In this study, rabbits were immunized with pheromone-induced and glutaraldehyde-fixed pPD1-donor cells and the antiserum obtained was absorbed with uninduced cells. The resulting antiserum was expected to react specifically to pheromone-inducible surface substance. Immunoelectrophoresis using this antiserum revealed a 78 kDa protein induced on the donor surface and this protein was associated with cell-aggregation formation. This 78-kDa protein was later designated PD78. Four proteins, AD157 (157 kDa), AD153 (153 kDa), AD130 (130 kDa), and AD74 (74 kDa), were found to be induced on the surface of pAD1-donor cells as indicated by western blotting with an antiserum specific for the cAD1-induced surface substance (Ehrenfeld *et al.* 1986). In the extract of cPD1-induced cell surface, three high-molecular-weight proteins were found to cross-react with the anti-cAD1-induced-cell antiserum. On the other hand, there was no cross-reactivity between the low-molecular-weight proteins, AD74 and PD78.

In chapter III, the author reports purification and characterization of PD78 and AD74 by means of structural, immunological, and genetic studies. AD74 was found to be derived from the N-terminal half of pAD1-encoded AS. PD78 was found to have a function other than AS, perhaps contributing to plasmid transfer as a stabilizer of mating pair.

In the past few years, research of pheromone-responsive plasmids has progressed dramatically. In this thesis, the author will discuss about these recent investigations in addition to the results obtained here.

Fig. 0-1. Liquid culture of pheromone-uninduced (left) and -induced (right) *E. faecalis* cells harboring pheromone-responsive plasmid.

Table 0-1. *Enterococcal* plasmids known to encode a pheromone response

Plasmid	Size(kb)	Original host	Phenotype encoded[a]	Related pheromone	References
pAD1	60	DS16	Hly/Bac, UV^r	cAD1	(Dunny, 1975)
pPD1	56	39-5	Bac	cPD1	(Tomich, 1979)
pAM373	36	RC73	?	cAM373	(Clewell, 1985)
pCF10	54	SF-7	Tc^r	cCF10	(Dunny, 1981)
pAMβ1	60	DS5	Hly/Bac, UV^r	cAD1	(Clewell, 1982)
pAMβ2	60	DS5	Bac	cAMβ2	(Clewell, 1982)
pAMβ3	60	DS5	?	cAMβ3	(Clewell, 1982)
pOB1	71	5952	Hly/Bac	cOB1	(Oliver, 1977)
pJH2	59	JH1	Hly/Bac	cAD1	(Jacob, 1974)
pBEM10	70	HH22	Pn^r, Gm^r, Km^r, Tm^r	cAD1	(Murray, 1988)
pAM323	66	HH22	Em^r	cAM323	(Murray, 1988)
pAM324	53	HH22	?	cAM324	(Murray, 1988)
pHKK100	55	228	Hly, Vm^r	cHKK100	(Handwerger, 1990)

a Hly : hemolysin ; Bac : bacteriocin ; UV^r : ultraviolet light resistance ;
Tc^r : tetracycline resistance ; Pn^r : penicillin resistance ; Gm^r : gentamicin resistance ;
Km^r : kanamycin resistance ; Tm^r : tobramycin resistance ; Em^r : erythromycin resistance ;
Vm^r : vancomycin resistance ; ? : cryptic

Table 0-2. Structures of sex pheromones and inhibitors

Name	Pepide structure	Reference
cPD1	Phe-Leu-Val-Met-Phe-Leu-Ser-Gly	(Suzuki *et al.* 1984)
iPD1	Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser	(Mori *et al.* 1987)
cAD1	Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly	(Mori *et al.* 1984)
iAD1	Leu-Phe-Val-Val-Thr-Leu-Val-Gly	(Mori *et al.* 1986)
cAM373	Ala-Ile-Phe-Ile-Leu-Ala-Ser	(Mori *et al.* 1986)
iAM373	Ser-Ile-Phe-Thr-Leu-Val-Ala	(Nakayama *et al.* 1994)
cCF10	Leu-Val-Thr-Leu-Val-Phe-Val	(Mori *et al.* 1988)
iCF10	Ala-Ile-Thr-Leu-Ile-Phe-Ile	(Nakayama *et al.* 1994)

Outlined letter represents coincidental residues between pheromone and inhibitor

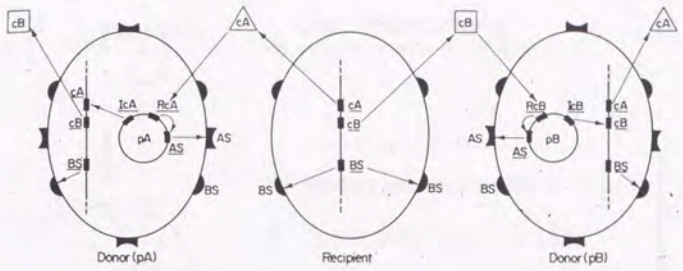
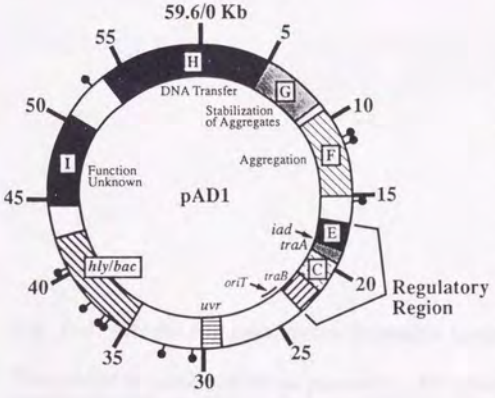


Fig. 0-2. A model for pheromone shutdown proposed by Dunny *et al.* (1979).

A model showing various donor and recipient relationships with respect to the synthesis of and response to sex pheromones. cA and cB are the determinants of sex pheromones cA and cB. BS represents the determinant for binding substance (BS) which is located on the cell surface. IcA and IcB are determinants for substances which repress (or inactivate) endogenous cA or cB. RcA and RcB are determinants of regulatory proteins which respond respectively to cA or cB resulting in a "turning on" of the determinant AS which produces aggregation substance (AS) which locates itself on the cell surface. Once a donor has responded to a sex pheromone, AS can now bind to BS which is located on recipients and also donors.



Map of pAD1. Regions associated with various functions are indicated as shaded areas. The hemolysin/bacteriocin determinant is indicated as *hly/bac*, and *uvr* indicates a region that confers increased resistance to UV. *traA*, *traB*, and regions C and E are related to regulation of the mating process. This region also contains the determinant *iad* for the pheromone inhibitor peptide iAD1. Regions F, G, H, and I include various structural genes that are induced as a result of exposure to pheromone. (The determinants and their functions are discussed in the text.) The markers on the outer circle indicate *Eco*RI restriction sites.

Fig. 0-3. Genetical map of pAD1 (from Clewell 1993a)

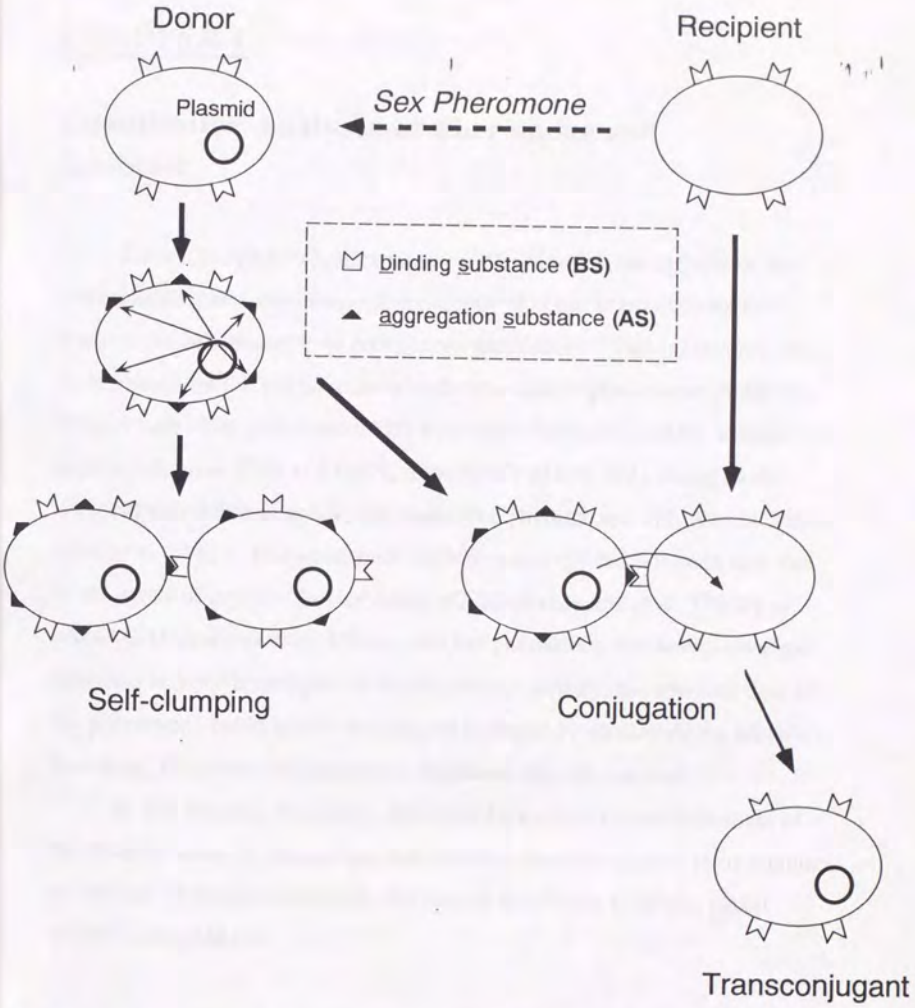


Fig. 0-4. Model for pheromone-inducible conjugation and self-clumping.

Plasmid-free recipient secretes sex pheromone. Sex pheromone induces expression of aggregation substance (AS) on the surface of donor. Binding between AS of donor and BS of recipient initiates conjugation. Binding between AS and BS of donor leads to self-clumping. In conjugation, a copy of the plasmid is transferred into recipient. After recipient obtains the copy, it becomes a donor for the given plasmid, and is known as a transconjugant.

CHAPTER I

Quantitative Analyses of Pheromone and Inhibitor

Once a recipient acquires a copy of the plasmid, the activity of the corresponding sex pheromone is apparently shut out to prevent the host donor from self-response to endogenous pheromone. This mechanism may be explained by the sex pheromone inhibitor and/or pheromone shutdown. Donors harboring pPD1 and pAD1 have been shown to produce a small peptide inhibitor iPD1 and iAD1, respectively (Table 0-2), being likely competitive inhibitors against the respective pheromones (Mori *et al.* 1986a; Mori *et al.* 1987). However, such inhibitory activity has not been detected in the broth of donor cells harboring pCF10 (Nakayama *et al.* 1994b) or pAM373 (Nakayama *et al.* 1994a). As for pheromone shutdown, since the inhibitor activity is antagonistic to pheromone activity, the absolute titer of the pheromone could not be determined in donor broth containing inhibitor; therefore, the nature of pheromone shutdown remains unclear.

In this chapter, the author established a method for measurement of the absolute titers of pheromone and inhibitor and investigated their amounts in cultures of various recipients and donors in relation to pPD1, pAD1, pCF10, and pAM373.

1. MATERIALS AND METHODS

1.1. Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used, together with their characteristics, related references, and sources, are listed in Appendix 1. All *E. faecalis* strains were grown at 37°C. All pheromones and inhibitors were prepared from cultures grown in N2GT medium (see Appendix 2). In bioassays for pheromone or inhibitor, either N2GT or THB medium was employed (see Appendix 2). pAM351 is a derivative of pPD1 having an insertion of tetracycline resistance transposon Tn*916* (Franke *et al.* 1981; Senghas *et al.* 1988) in the *Eco* RI B-fragment (Ike *et al.* 1983). OG1X(pAM351) showed the same phenotype as OG1X(pPD1) relating to the pheromone-inducible aggregation and plasmid transfer. pAM714 is a derivative of pAD1 having an insertion of erythromycin resistance transposon Tn *917* (Tomich *et al.* 1980) in the *Eco* RI B-fragment (Ike *et al.* 1984). OG1X(pAM714) and FA2-2(pAM714) showed the same phenotype relating to pheromone-inducible aggregation and plasmid transfer. pAM377 is a derivative of pAM373, having an insertion of Tn *917* in the *Eco* RI A-fragment (Clewell *et al.* 1985). JH2SS(pAM377) is less sensitive than FA2-2(pAM373) to cAM373.

1.2. Separation of pheromone and inhibitor

All pheromones and inhibitors were produced and isolated essentially in the same way. A 5 ml inoculum of overnight culture of each strain was transferred to 200 ml of fresh N2GT medium. The bacteria were cultured to a final OD_{600}=1.0 with gentle stirring under anaerobic conditions at 37°C. The grown cells were removed by centrifugation at 7000 x g. The culture filtrate was passed through an Amberlite XAD-7 column (Rohm and Haas, 5 ml, ø 12 x 50 mm). The column was washed with 15 ml of distilled water and eluted with 30 ml of 60% ethanol. The eluate was threefold

diluted and mixed with pyridine (final concentration 5 %). This solution was applied to a QAE-Sephadex A-25 column (Pharmacia, Cl^- form, 5ml, ø 18 x 20 mm). After washing with 15 ml of distilled water containing 5 % pyridine, the column was eluted with 30 ml of 0.1 M NaCl. The eluate was then passed through an Amberlite XAD-2 column (Rohm and Haas, 5 ml, ø 12 x 50 mm). After washing with 15 ml of distilled water, the column was eluted with 30 ml of 80 % ethanol. The eluate was evaporated to approximately 1.5 ml and then 100 μl of this solution was applied to a reverse-phase HPLC column (SSC-ODS-262, Senshukagaku, ø 6 x 100 mm). The column was eluted with a linear acetonitrile gradient of 20 to 30 % for 5 min and then 30 to 33 % for 30 min in 0.1 % trifluoroacetic acid. Fractions were collected at 0.5 min intervals. Each fraction was bioassayed for pheromone and inhibitor. cPD1 and iPD1 were eluted at 22 min and 15 min, respectively. cAD1 and iAD1 were eluted at 16 min and 17 min, respectively. cCF10 and iCF10 were eluted at 20 min and 27 min, respectively. cAM373 and iAM373 were eluted at 8 min and 13 min, respectively.

1.3. Bioassay for pheromone and inhibitor

See Appendix 3

2. RESULTS

2.1. Methods for measurement of absolute titers of pheromone and inhibitor

Since all pheromones and inhibitors are lipophilic oligopeptides, a reverse-phase HPLC was used to separate them. cPD1 and iPD1 could be separated as shown in Fig. 1-1. The other pairs of pheromone and inhibitor, cAD1 and iAD1, cCF10 and iCF10, cAM373 and iAM373, also could be separated under the same conditions of HPLC. Each fraction was applied to the bioassay for pheromone or inhibitor. The bioassay was performed by observing the self-clumping of donor cells in a microtiter plate (Dunny *et al.* 1979). The inhibitor assay was performed in the presence of 100 pg/ml of pheromone. One titer unit of pheromone represents the minimum concentration required for induction of self-clumping. One titer unit of inhibitor represents the minimum concentration required to block the effect of one titer unit of pheromone. It was assumed that pheromone inhibitor acts competitively to block pheromone activity, an inhibitor titer equal to the pheromone titer would be necessary to block the pheromone activity. With the exception of cPD1, pheromones and inhibitors were recovered with yields of about 100 %. cPD1 was recovered with a yield of about 30 %.

2.2. Titers of cPD1 and iPD1

Fig. 1-2 shows the absolute titers of cPD1 and iPD1 observed in various strains. iPD1 was detected in the broth of all strains harboring pAM351. pAM351 is a derivative of pPD1 having an insertion of tetracycline-resistance transposon Tn*916* in the *Eco* RI B-fragment (Ike *et al.* 1983). OG1X(pAM351) showed the same phenotype as OG1X(pPD1) relating to pheromone-inducible aggregation and plasmid transfer. In

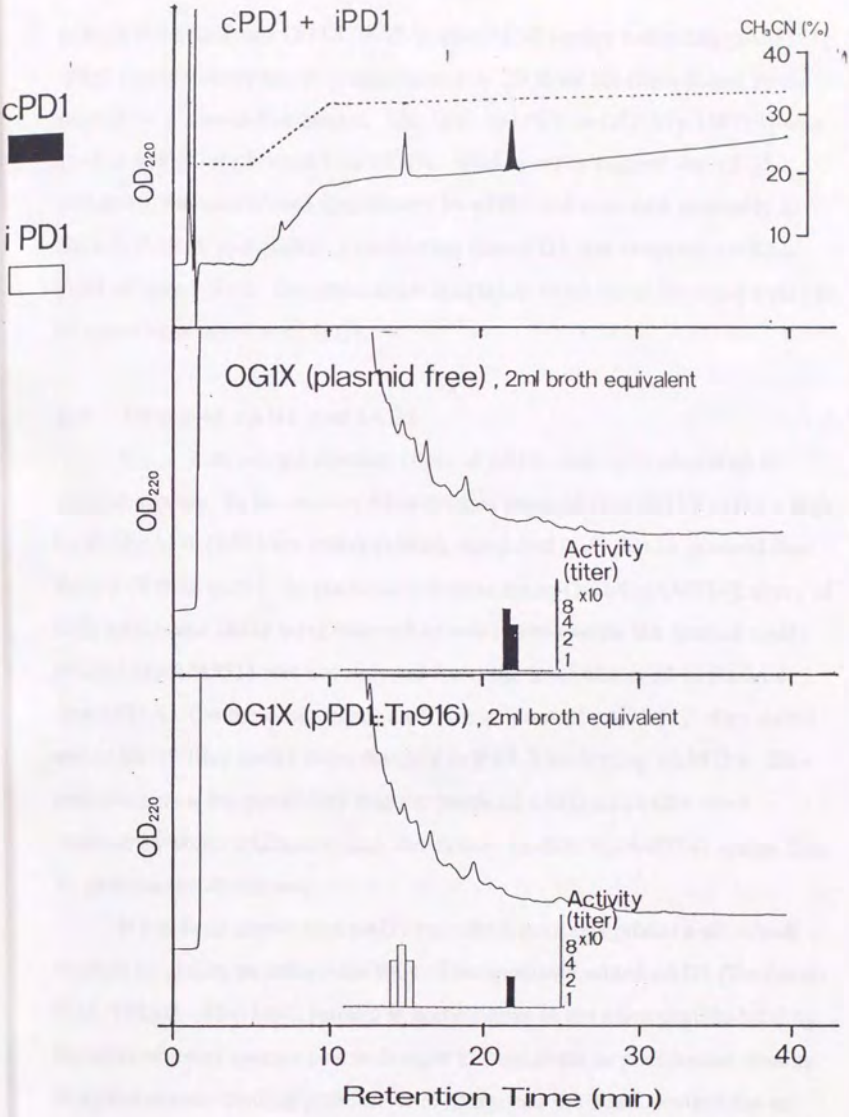


Fig. 1-1. Separation of cPD1 and iPD1 by RP-HPLC.

Dotted line indicates the percent of acetnitrile. Bar-graph indicates the titers of cPD1 and iPD1.

culture filtrates from OG1X, FA2-2, and JH2-2 strains harboring pAM351, cPD1 titers were reduced to approximately 20 % of the titers found in the respective plasmid-free strains. The titer of cPD1 in OG1X(pAM714) was similar to that in plasmid-free OG1X. These results suggest that cPD1-shutdown was performed specifically by pPD1 and occurred generally in OG1X, FA2-2, and JH2-2. Considering that cPD1 was recovered with a yield of about 30 %, the actual titer of cPD1 is expected to be about equal to or more than the titer of iPD1.

2.3. Titers of cAD1 and iAD1

Fig. 1-3 shows the absolute titers of cAD1 and iAD1 observed in various strains. In the culture filtrate from plasmid-free OG1X cells, a high level of cAD1 (134 titer units) existed, compared with that in plasmid-free FA2-2 (9 titer units). In the culture filtrate from OG1X(pAM714), titers of both cAD1 and iAD1 were reduced to nearly zero while the titer of cAD1 in OG1X(pAM351) was not reduced from the level observed in plasmid-free OG1X. On the other hand, a certain amount of cAD1 (4.5 titer units) and iAD1 (9 titer units) were detected in FA2-2 harboring pAM714. This result suggests the possibility that the levels of cAD1 and iAD1 were reduced by some pAD1-encoded mechanism in OG1X(pAM714) rather than by pheromone shutdown.

It has been shown that pAD1 encodes a gene designated *traC* which showed an ability to reduce the titer of exogenously added cAD1 (Tanimoto *et al.* 1993a). The TraC protein is homologous to the oligopeptide-binding proteins of other species and is thought to contribute to pheromone sensing as a pheromone-binding protein. It is likely that the TraC protein has an antagonistic site for iAD1 and exhibits the ability to bind iAD1 as well as cAD1. The binding of both cAD1 and iAD1 to the TraC protein probably resulted in the loss of both cAD1 and iAD1 in the broth. It has been shown

that *traC* is inactive in FA2-2 harboring pAD1 (Weaver *et al.* 1990). This coincides with the fact that levels of both cAD1 and iAD1 were not reduced to nearly zero; certain amounts of cAD1 and iAD1 were detected in strain FA2-2(pAM714).

Fig. 1-4 shows the absolute titers of cAD1 and iAD1 in a strain carrying pAM2270 having a transposon generated deletion derived from pAD1 (Weaver *et al.* 1990). This deletion in pAM2270 is located completely within *traC*. In the culture broth from OG1X(pAM2270), titers of both cAD1 and iAD1 were enhanced (cAD1: 6 titer units, iAD1: 18 titer units) compared with those of OG1X(pAM714) (cAD1: undetectable, iAD1: 1 titer unit). Considering that OG1X(pAM2270) lacked the ability to bind the pheromone, these values may represent the gross amount of cAD1 and iAD1 secreted from the donor in the absence of pheromone/inhibitor binding. If this is the case, the difference in cAD1 titer between OG1X(pAM714) and OG1X(pAM2270) would indicate the existence of cAD1-shutdown.

2.4. Titers of cCF10 and iCF10

The existence of iCF10 has been unclear, because inhibitory activity could not be detected in culture broth from donor strains harboring pCF10. In this study, fractions eluted at a retention time of 27 min from the reverse-phase HPLC column showed inhibitory activity against cCF10, revealing the existence of iCF10. Fig. 1-5 shows the absolute titers of cCF10 and iCF10 found in plasmid-free OG1RF strains and OG1RF strain harboring pCF10. The titer of cCF10 observed in OG1RF(pCF10) was not reduced from that found in plasmid-free OG1RF, showing that cCF10-shutdown did not occur in the pCF10-donor. However, OG1RF(pCF10) did not undergo clumping constitutively. These findings indicate that pheromone shutdown is not always necessary to shield the donor from self-induction by the endogenous

pheromone. Indeed, the donor produced a sufficient titer of iCF10 to block the activity of cCF10 produced in the absence of pheromone shutdown.

2.5. Titers of cAM373 and iAM373

Fig. 1-6 shows the absolute titers of cAM373 and iAM373 found in various strains. With FA2-2(pAM373), titers of both cAM373 and iAM373 were reduced to nearly zero, as occurred with OG1X(pAM714). FA2-2(pAM373) also showed an ability to reduce the activity of exogenously added cAM373, suggesting that pAM373 encodes a TraC-like pheromone-binding protein. pAM377 is an insertional mutant generated with transposon Tn917. FA2-2(pAM377) lacked the ability to reduce the activity of exogenously added cAM373. Therefore, it seems likely that the insertion disrupts the *traC*-like gene in pAM373. JH2SS(pAM377) secreted 50 titer units of iAM373 and 9 titer units of cAM373. These values might represent the gross titers secreted from the donor in the absence of pheromone/inhibitor binding and this result demonstrates that cAM373-shutdown did not occur. The donor produced a sufficient amount of iAM373 to block the activity of cAM373. Thus, similar to the case of pCF10, it seems that cAM373-shutdown was not necessary to prevent the self-response of he host to the endogenous pheromone.

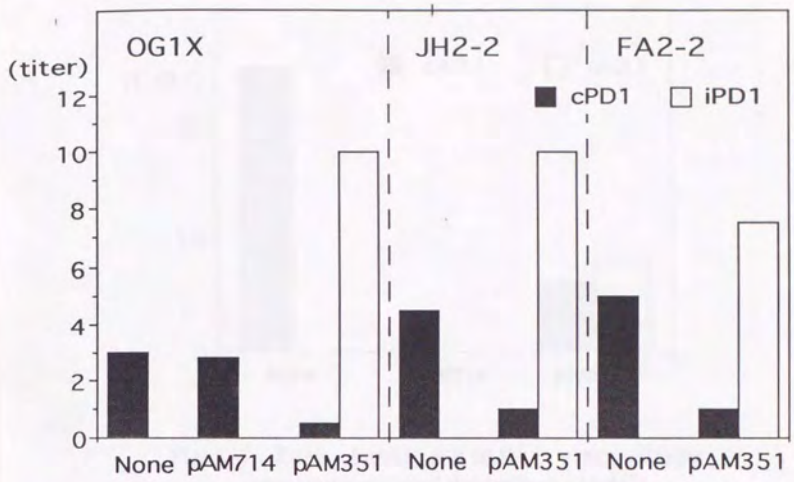


Fig. 1-2 Titer of cPD1 and iPD1 in various strains

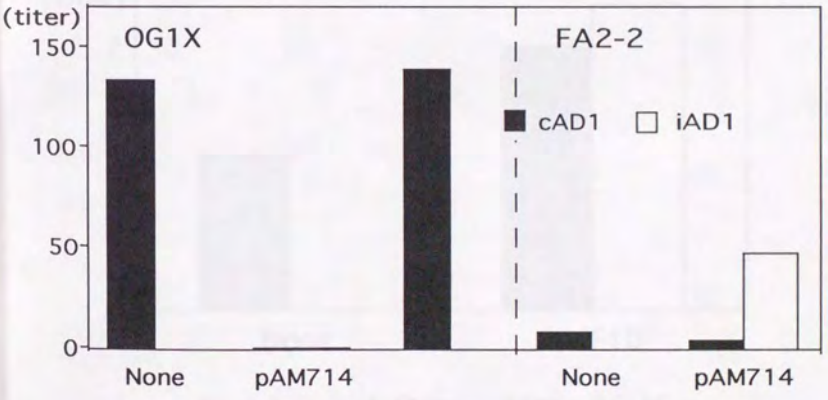


Fig. 1-3 Titer of cAD1 and iAD1 in various strains

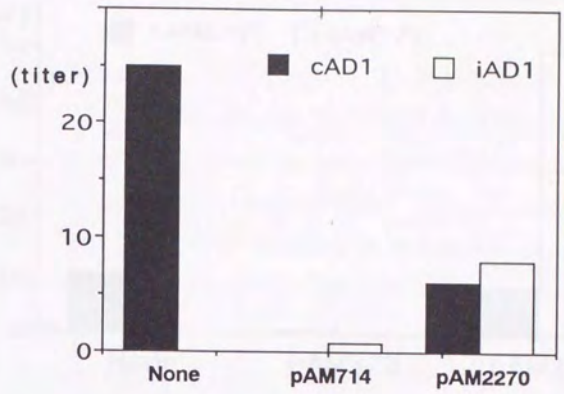


Fig. 1-4 Titers of cAD1 and iAD1 in various strains containing mutated derivatives of pAD1

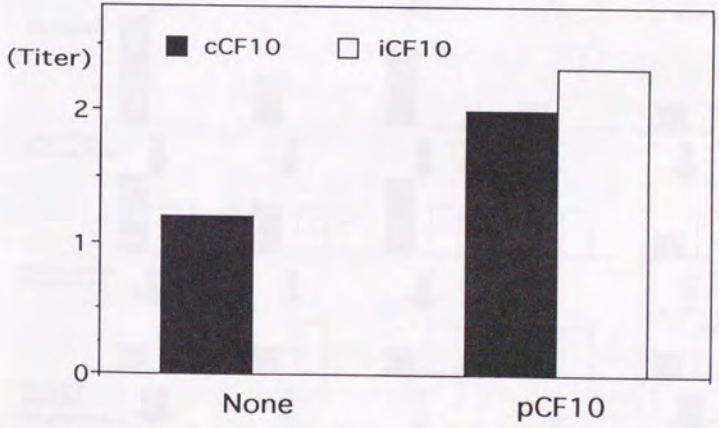


Fig. 1-5 Titers of cCF10 and iCF10 in OG1RF and OG1RF(pCF10)

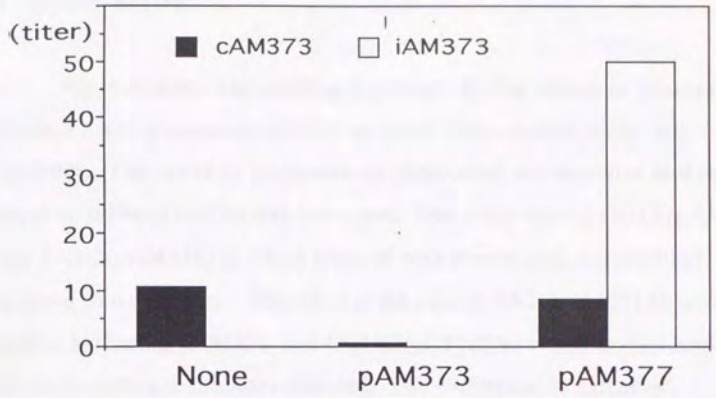


Fig. 1-6 Titer of cAM373 and iAM373 in various strains

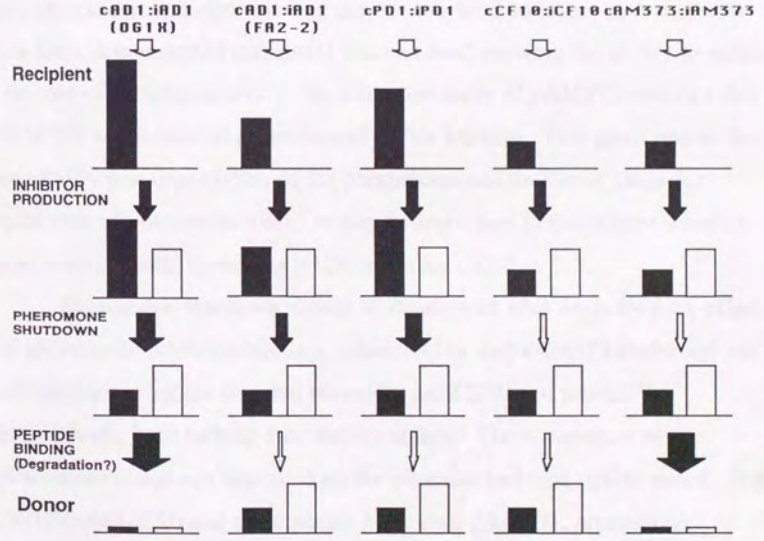


Fig. 1-7. Working hypothesis for the effects of pheromone shutdown and pheromone binding on titers of pheromone/inhibitor

3. DISCUSSION

Fig. 1-7 shows the working hypothesis for the effects of pheromone shutdown and pheromone binding on broth titers of pheromone and inhibitor. The extent of expression of pheromone and inhibitor in donor broth could be classified into two types. One is the case of OG1X(pAM714) and FA2-2(pAM373) in which titers of both pheromone and inhibitor were reduced to nearly zero. The other is the case of FA2-2(pAM714), several strains harboring pAM351, and OG1RF(pCF10), in which certain amounts of pheromone and inhibitor detected. This difference in extent of expression may result from a difference in pheromone/inhibitor binding. In the former case, the pheromone/inhibitor binding may be so strong that free pheromone/inhibitor was absent from the culture broth. In the latter case, the pheromone/inhibitor binding may be too weak to affect both titers. It has been demonstrated that pAD1 encodes *traC* showing the ability to reduce the titer of exogenous cAD1. Recently our study of pAM373 revealed that cAM373 was degraded proteolytically after binding. This gives rise to the possibility that degradation of the pheromone was the direct cause for reduction of pheromone titer. It may be important to investigate whether pAD1-donor cells harboring pAD1 degrades cAD1 or not.

Pheromone shutdown should be considered after excluding the effect of pheromone/inhibitor binding. Thus, cAD1- and cAM373-shutdown was investigated using the mutated plasmids, pAM2270 and pAM377, respectively, both lacking this binding ability. The occurrence of pheromone shutdown depended on the plasmids and host strains tested. With OG1X(pAM2270) and some strains harboring pAM351, pheromone shutdown was clearly detected. With FA2-2(pAM714), pheromone shutdown was weak. With OG1RF(pCF10) and JH2-2(pAM377), pheromone shutdown was not detected. In this case, a sufficient amount of

inhibitor was produced to block the activity of the pheromone. Therefore, it seems likely that the system of pheromone shutdown is not expressed, when not necessary. This hypothesis is supported by data indicating that the potential shutdown gene, *traB,* was inactive in the strain FA2-2 which produced low levels of cAD1 compared with OG1X (Weaver *et al.* 1990); pheromone shutdown might be regulated via a feedback mechanism after the pheromone induction.

In any case, it seems likely that pheromone-responsive plasmids potentially encode double systems, pheromone shutdown and pheromone inhibitor in order to shield the host cell from self-response to endogenous pheromone.

CHAPTER II

Genetic Analyses of a Region Containing Pheromone Inhibitor (*ipd*), Sensitivity (*traC*), and Shutdown (*traB*) Genes

Recent genetic analysis of pheromone responsive plasmids has focused on aggregation substance (AS) gene, surface exclusion gene, and some regulatory genes involved in the pheromone response. The regulatory region of pAD1 has been shown to contain the iAD1 gene (*iad*) (Clewell *et al.* 1990b), a potential pheromone shutdown gene (*traB*) (An *et al.* 1993), pheromone binding protein gene (*traC*) (Tanimoto *et al.* 1993a), and negative (*traA*) (Pontius *et al.* 1992a) and positive (*traEI*) (Pontius *et al.* 1992b; Tanimoto *et al.* 1993b; Muscholl *et al.* 1993) regulators of the AS gene. The regulatory region of pCF10 has been shown to contain the iCF10 gene (*icf*) (Nakayama *et al.* 1994b), a pheromone-binding protein (*prgZ*) (Ruhfel *et al.* 1993), and negative (*prgY*) (Kao *et al.* 1991) and positive (*prgX, R, S, T*) (Kao *et al.* 1991; Chung *et al.* 1992) regulators of the AS gene. There is strong homology between *traC* and *prgZ* (Tanimoto *et al.* 1993a), and between *traB* and *prgY* (An *et al.* 1993). Thus, the proteins encoded by these genes are expected to have the same functions. It has been suggested that the *traC* and *prgZ* genes encode pheromone/inhibitor binding proteins. The function of *prgY* remains unclear, however, it seems likely that *traB* encodes a protein involved in pheromone shutdown (An *et al.* 1993).

In the quantitative study of pheromone and inhibitor described in Chapter I, pAD1 and pCF10 showed an ambiguous phenotype relating to pheromone shutdown. With OG1X strain harboring pAD1, the activities of

both pheromone and inhibitor were reduced to nearly zero , probably due to the combined effects of pheromone/inhibitor binding and pheromone shutdown. In the case of pCF10, pheromone shutdown was not detected. In the case of pPD1, however, pheromone shutdown was clearly detected, while the binding of pheromone/inhibitor was very weak and did not affect the titers of pheromone and inhibitor. Thus, pPD1 is considered to be an appropriate plasmid for the study of pheromone shutdown.

Transposon Tn*917* generated only a few mutants of pPD1, thus a genetic map of this plasmid could not be constructed and research in the plasmid has not progressed well. The only genes of pPD1 cloned were *asp1* (Galli *et al.* 1992) and *pd78* (Nakayama *et al.* 1990). Strong homology exisits between *asp1* to *asa1,* indicating that *asp1* encodes the AS. *pd78* was cloned in the study described in Chapter III.

In this chapter, the inhibitor gene of pPD1 (*ipd*) was cloned by monitoring the production of iPD1 in *E. coli.* Since the pPD1 regulatory region was expected to be located upstream from *ipd* in analogy with pAD1 and pCF10, the regulatory region was tagged by *ipd*. The auther suggests that the regulatory region contains inhibitor, pheromone shutdown, and pheromone sensitivity genes.

1. MATERIALS AND METHODS

1.1. Bacterial strains, plasmids, and media

The bacterial strains and plasmids used, together with their characteristics, related references, and sources, are listed in Appendix 1. All *E. faecalis* strains were grown at 37°C. In bioassays for cPD1 and iPD1, N2GT medium was employed (see Appendix 2).

E. coli JM109 and HB101 strains were grown in Luria-Bertani (LB) broth at 37°C (see Appendix 2). *E. coli* JM109 containing recombinant

pUC118 or pUC119 was identified as white colonies on LB solid medium containing 0.5 mM isopropyl-ß-D-galactopyranoside and 12 mM 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (both from Sigma).

The concentrations of antibiotics used in selective media are indicated in Appendix 2.

1.2. DNA manipulations

Restriction digestions were performed with restriction endonucleases purchased from TaKaRa, Toyobo, or Nippon Gene, under the conditions recommended by suppliers. DNA ligations were done with T4 DNA ligase according to the protocol of a ligation kit (TaKaRa). DNA fragments to be used for cloning and hybridization probes were isolated from agarose using Gene Clean II as recommended by the supplier (BIO I01 Inc.). Blunting of cohesive DNA ends was done with T4 DNA polymerase according to the protocol of a DNA blunting kit (TaKaRa). PCRs were performed in a Atto Zymoreacter with Taq DNA polymerase purchased from TaKaRa (AmpliTaq^{TM})

1.3. Transformation of bacteria

DNA was introduced into *E. coli* by the method of Hanahan (1983) or by the electroporation method of Dower *et al.* (1988). Transformation of *E. faecalis* was done according to the procedure of Appendix 5, which was based on the method of Cruz-Rodz *et al.* (1990)

1.4. Preparation of DNA

The alkaline lysis procedure was used to isolate plasmids from *E. faecalis* (Appendix 4) (Anderson *et al.* 1983) and *E. coli* (Sambrook *et al.* 1989).

1.5. Nucleotide sequence determination

For sequence determination, 5.4-kb *Eco*RV-*Hin* cII region was divided into three fragments (0.8-kb *Eco* RV-*Pst* I, 2.9-kb *Pst* I-*Spe* I, and 2.3-kb *Hin* cII-*Hin* cII) and each fragment was cloned into pUC118 or pUC119

sequencing vectora. The resultant plasmids were designated pEVP5, pPS32, pHH21, respectively. Nested deletions were performed on pPS32 and pHH21 using the Deletion kit for kilo-sequence (TaKaRa) together with exonuclease III, mung bean nuclease, and klenow fragment according to the supplier-recommended protocol based on the methods of Yanishi-Peron *et al.* (1985). This generated inserts of various lengths successively deleted every 100-300 base pair in both orientations.

Double strand DNAs (>1.5-kb insert) or immobilized single strand DNAs (<1.5-kb insert) were applied to Taq dyedeoxy primer cycle sequencing or Taq dyedeoxy terminator cycle sequencing. The immobilized single strand DNAs were prepared by PCR using biotinylated primer and magnetic separation according to the protocol of Dynabeads template preparation kit (Dynal) (Hultman *et al.* 1991; Hultman *et al.* 1989). Cycling conditions were as follows for 30 cycles; denaturation at 96°C for 0.5 min, annealing at 65°C for 1 min and extension at 72°C for 2 min. Taq dye primer cycle sequencings were performed by using FAM, JOE, TAMRA, and ROX dye -21M13 or M13 reverse primers (Applied Biosystems). The cycling condition was as follows for 30 cycles; denaturation at 98°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 70°C for 2 min. Taq dye terminator cycle sequencing was performed using -21M13 or M13 reverse primer and FAM, JOE, TAMRA, ROX dyedeoxy terminator (Applied Biosystems). The cycling condition was as follows for 25 cycles; denaturation at 98°C for 0.5 min, annealing at 50°C for 0.3 min, and extension at 60°C for 4.5 min. The products of the sequencing reactions were resolved in an Applied Biosystems Model 373A DNA sequencer.

The sequence was determined for both DNA strands. Each sequenced segment was completely overlapped. The DNA sequence was assembled and analyzed using Genetyx-Mac (Software Development).

1.6. Gene disruption

pUCC, a chimeric plasmid consisting of pUC119 and a 2.4-kb *Eco* RV-*Sna* BI fragment, was digested with *Pst* I, then blunted with T4 DNA polymerase, and self-ligated. The resultant plasmid was designated pUCCM, having a 4-bp deletion at the *Pst* I site. pUCB, a chimeric plasmid consisting of pUC118 and a 2.6-kb nested-deleted segment generated from a 2.9-kb *Pst* I-*Spe* I fragment was digested with *Sna* BI and ligated with an 8-bp *Pst* I linker d(GCTGCAGC) purchased from TaKaRa. The resultant plasmid was designated pUCBM. A *Hin* dIII-*Cla* I 1.5-kb segment of pVA891, which encodes a selectable Em^r marker in *E. faecalis*, was blunted with T4 DNA polymerase and ligated into the *Sma* I site of the residual multiple cloning site in the vector moiety of pUCCM and pUCBM. The resultant plasmids were designated pUCCMEm and pUCBMEm, respectively. Both plasmids were transformed into *E. faecalis* OG1X(pAM351) by electroporation (see Appendix 5). The Em^r transformants were selected on THB agar plates containing 50 μg/ml of erythromycin (Sigma). Passage cultures were performed in liquid THB medium without erythromycin. Approximately a thousand colonies were replicated from non-selective plates onto Em^r selective plates and Em^s colonies were selected.

1.7. Bioassay for absolute titers of cPD1 and iPD1

See Chapter I.

2. RESULTS

2.1. AL-fragment (16 kb of *Eco* RI-*Sal* I fragment) encoding both iPD1 and cPD1 shutdown

Eco RI digested pPD1 to generate five major fragments which were named alphabetically according to size, beginning with the largest fragment. A-fragment (23 kb) was divided into 16-kb and 7-kb fragments at its *Sal* I site. These subfragments were named AL (large of A) and AS (small of A). In previously reported maps of pPD1 (Yagi *et al.* 1983 and Galli *et al.* 1992), the AL-fragment was adjacent to the E-fragment. However, the sequence at the *Eco* RI side of the AL-fragment (data not shown) was identical to the 5' end sequence of *asp1* (aggregation substance gene of pPD1) (Galli *et al.* 1992), which extends over the region of the A, G and C fragments. This indicates that the AL-fragment is adjacent to the G-fragment. The revised map of pPD1 is shown in Fig. 2-1.

Each fragment has been ligated into the *Esherichia coli-E. faecalis* shuttle vector pAM401 (Wirth *et al.* 1986) and the resulting chimeric plasmids have been transformed into *E. faecalis* OG1X (Clewell *et al.*, unpublished). The cPD1 activity in the culture filtrate from each transformant was assayed. The result is shown in Fig. 2-2. pAM351 is a derivative of pPD1 having an insertion of tetracycline-resistance transposon, Tn*916* in the *Eco* RI B-fragment (Ehrenfeld *et al.* 1986). OG1X(pAM351) has been shown to display the same phenotype relating to pheromone-inducible aggregation and plasmid transfer as the pPD1-donor strain and it was used as a control in this study. OG1X(pAM401AL) shut out the cPD1 titer to the same extent as OG1X(pAM351) and the cPD1 titer was unaffected in other instances (Fig. 2-2). This result indicates that the AL-fragment encodes iPD1 and/or cPD1 shutdown. Fig. 2-3 shows the absolute titers of cPD1 and iPD1 in the culture filtrate from OG1X(pAM401AL).

These titers were determined after cPD1 and iPD1 were separated by HPLC according to the method described in Chapter I. The AL-fragment reduced the titer of cPD1 to 20 % of that found in plasmid-free OG1X whereas the amount of iPD1 was similar to that of strains carrying pAM351. This revealed that the AL-fragment contains both the iPD1 gene designated *ipd* and the cPD1-shutdown gene.

2.2. Cloning of *ipd*

The AL-fragment was digested with various restriction endonucleases and the generated fragments were ligated into the *E. coli* cloning vector pUC118 or pUC119 (Vieira *et al.* 1987). A physical map of the AL-fragment is shown in Fig. 2-1. All regions of the AL-fragment except for 3.8-kb *Sal* I-*Eco* RV region were cloned into *E. coli.* Cloning of the 3.8-kb *Sal* I-*Eco* RV segment was unsuccessful despite several attempts. Some strains of *E. coli* transformed with pPH53, which was chimeric plasmid consisting of a 5.4-kb *Pst* I-*Hin* dIII fragment and pUC118, showed the iPD1 activity in broth. Strangely, strains JM109 and DH5 carrying pPH53 produced 128 titer units of iPD1 whereas HB101 having the same plasmid produced only 2 titer units. *E. coil* JM109 carrying pHH21 produced 256 titer units of iPD1. In order to determine in more detail the location of *ipd,* nested deletions of pHH21 were constructed by partial digestion with exonuclease III. As shown in Fig. 2-1, JM109 carrying deletion derivatives of pHH21 having an insert bigger than 1.7 kb showed 256 titer units of iPD1 activity, while JM109 carrying deletions derivatives of pHH21 having an insert smaller than 1.5 kb showed no inhibitor titer. These results indicate that *ipd* is located around 1.6-kb downstream from the left *Hin* cII site of the two as shown in Fig. 2-1.

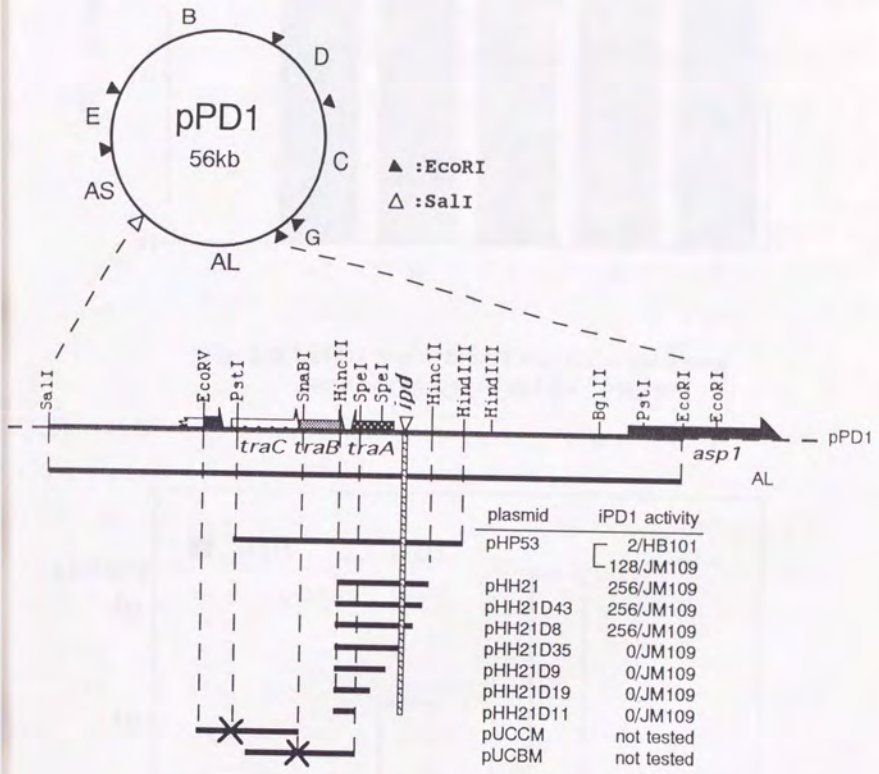


Fig. 2-1. Physical map of pPD1 and AL-fragment and insert structures of some plasmids used in this study

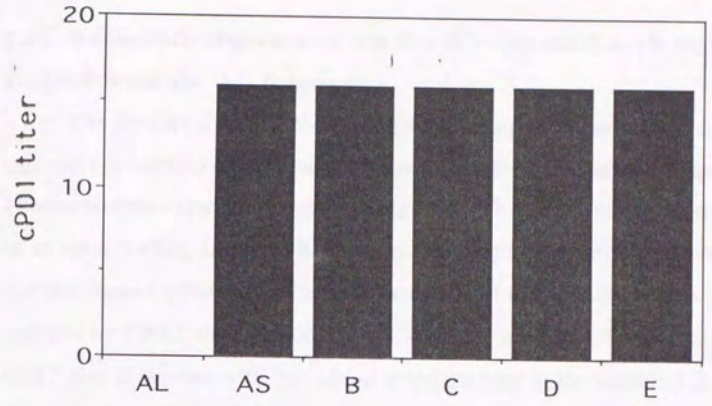


Fig. 2-2. cPD1 titer in broth from strains harboring each cloned *Eco* RI and *Sal* I fragment

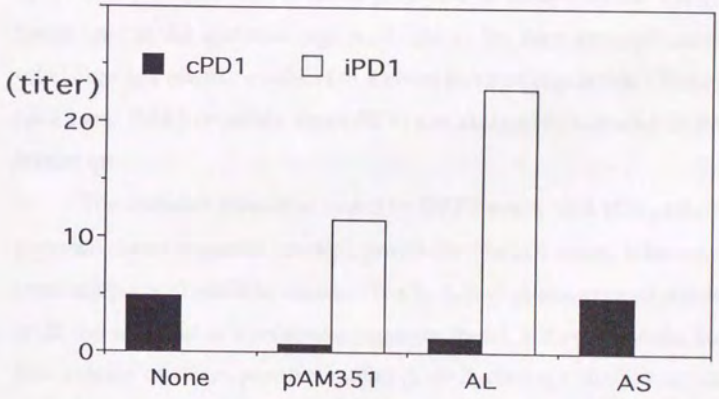


Fig. 2-3. Titers of cPD1 and iPD1 in strains carrying None, AL-fragment, AS-fragment, and pAM351

2.3. Nucleotide sequence of the *Eco* RV-*Hin* cII 5.4-kb segment derived from the AL fragment

The *Eco* RV-*Hin* dIII 5.4-kb segment having *ipd* located near its 3' end was expected to correspond to the regulatory region and was sequenced. The nucleotide sequence is shown in Fig. 2-4. This segment contained a part of an open reading frame (ORF1) at its 5' end and four entire putative open reading frames (ORF2-5). The last 8 residues of the deduced product encoded by ORF5 were identical to the sequence of iPD1. The location of ORF5 also coincided with that of *ipd* as determined in the section 2.2. From these results, ORF5 was identified as *ipd*. ORF4 was encoded in the reverse direction relative to the other ORFs. Potential Shine-Dalgarno ribosomal binding sequences were found to precede ORF2-5 optimally. The potential promoter consensus sequence (having a score of more than 60 as calculated by the software GENETYX-MAC [Software Development Co. Ltd.]) was found only in the upstream region of ORF5. So, the transcriptions of genes other than *ipd* might be subject to a novel form of regulation. The adjacent ORF2 and ORF3 (possibly also ORF1) are likely to be encoded on the same transcript.

The deduced product encoded by ORF2 was a 60.8 kDa protein with a potential signal sequence having a positively charged amino terminus and containing a hydrophobic stretch. The L-A-S-C-G sequence of residues 19 to 23 are identical to a consensus sequence found in membrane-anchored lipoproteins of Gram-positive species (L-*Y*-*Z*-cleavage site-C-*y*-*z*; where *Y* is A, S, V, Q, T; *Z* is G or A; *y* is S, G, A, N, Q, D; *z* is S, A, N, Q) (Gilson *et al.* 1988). These results suggest that the ORF2 product is an extracellular protein anchored onto the membrane via an amino terminal lipid group.

The deduced product encoded by ORF3 was a 43.5 kDa protein without a potential signal sequence. The C-terminal one-third of the ORF3 product has a high degree of hydrophobicity. This observation suggests that

the ORF3 product might associate with the inner membrane through this region.

The deduced product encoded by ORF4 was a 37.7 kDa protein without a potential signal sequence, suggesting that the putative product was a cytosol protein.

2.4. Comparison of the *Eco* RV-*Hin* cII 5.4-kb segment with the corresponding regulatory region of pCF10 and pAD1

As expected, the *Eco* RV-*Hin* cII segment was homologous to the regulatory region of pAD1 and pCF10 as indicated by the nucleotide sequence, ORF organization, deduced amino acid sequence, and molecular profile such as *M*r and hydrophobicity of each ORF product.

Fig. 2-5 shows HarrPlots comparing the nucleotide sequence of the *Eco* RV-*Hin* cII segment to the corresponding regulatory region of pCF10 or pAD1. These data show that this region of pPD1 is more homologous to pCF10 than to pAD1. Especially, DNA sequence of more than 3-kb spanning the region encoding ORF1-3 is highly (more than 85 %) homologous to that of the corresponding region of pCF10. Downstream from *ipd,* there is a small region (300 bp) conserved among the three plasmids. This region is 95% identical between pAD1 and pCF10, and 75% identical between pPD1 and pCF10/pAD1. Two potential inverted repeat sequences, which are predictly to form stable structures (ΔG<-30 kcal), are conserved among the three plasmids as shown in Fig. 2-6. One is located between two confronting ORFs, and it likely acts as a transcription terminator of either or both ORF(s). The other is located downstream from the inhibitor gene. This region of pAD1 was named TTS1 (Pontius *et al.* 1992b) and was found to be involved in control of transcription of the downstream gene containing structural genes, such as the AS gene. The AS

gene of pPD1, *asp1,* is located 3-kb downstream from the inverted repeat (Fig. 2-1) similar to the case of pAD1..

Fig. 2-6 shows the ORF profiles in the sequenced segment of pPD1 and the corresponding regions of pCF10, and pAD1. The hydrophobicity profiles and molecular weights of the products coded by ORF2, 3, and 4 were well conserved in pCF10 and pAD1. Thus, ORF2 corresponds to *prgZ* of pCF10 and *traC* of pAD1. ORF3 corresponds to *prgY* of pCF10 and *traB* of pAD1. ORF4 was assigned to *prgX* of pCF10 and *traA* of pAD1. The ORF organization of pPD1 was the same as that of pCF10. Compared with pAD1, ORF2 and ORF3 were interchanged. The author assigned the designations*traB* for ORF2, *traC* for ORF3, and *traA* for ORF4, according to the nomenclature of pAD1. Henceforth, each gene or its product will be mentioned b its name/plasmid; for example, TraB of pPD1 will be mentioned as TraB/pPD1.

TraC/pPD1 has significant homology to PrgZ/pCF10 with 86.6 % identical amino acids, and is 70.5 % identical to TraC/pAD1. A consensus sequence involved in N-terminal membrane anchor is conserved in PrgZ/pCF10 (Ruhfel *et al.* 1993) and TraC/pAD1 (Tanimoto *et al.* 1993a).

TraB/pPD1 has significant homology to PrgY/pCF10 with 76.7 % identical amino acids and is 47.6 % identical to TraB/pAD1. The hydrophobicity profiles in the C-terminal of one-third are strikingly similar among these proteins.

TraA/pPD1 has low homology to PrgX/pCF10 with 21.7 % identical amino acids, and is 36.6 % identical to TraA/pAD1. The hydrophobicity profiles are not very similar among these proteins. A hydrophobic region around residue 100 is common among these proteins.

The inhibitor genes, *ipd, iad,* and *icf* encode extremely small peptides consisting of 21, 22, and 23 amino acid residues, respectively. The last 7 or 8 residues comprise the inhibitor peptides, iPD1, iAD1, or iCF10,

respectively. The peptides preceding the mature inhibitors are likely to function as signal peptides, athough their hydrophobic stretches are too short to act as a signal peptide (see Discussion).

Legend to Fig. 2-4 (Next Page).

The putative products of ORF1, -2, -3, -5 are indicated in single (uppercase)-letter notation. ORF4 is encoded on the complementary strand and the putative product is shown from right to left in single (lowercase)-letter notation. ORF2, -3, -4, -5 were termed *traC, traB, traA,* and *ipd.* Potential inverted repeat structures showing a calculated energy of less than -30 kcal are indicated by a pair of arrows. The Shine-Dalgarno potential ribosome binding sites are indicated by bold underline. Potential promoter sequenced showing a score of more than 60 (see text) are indicated by thick underline.

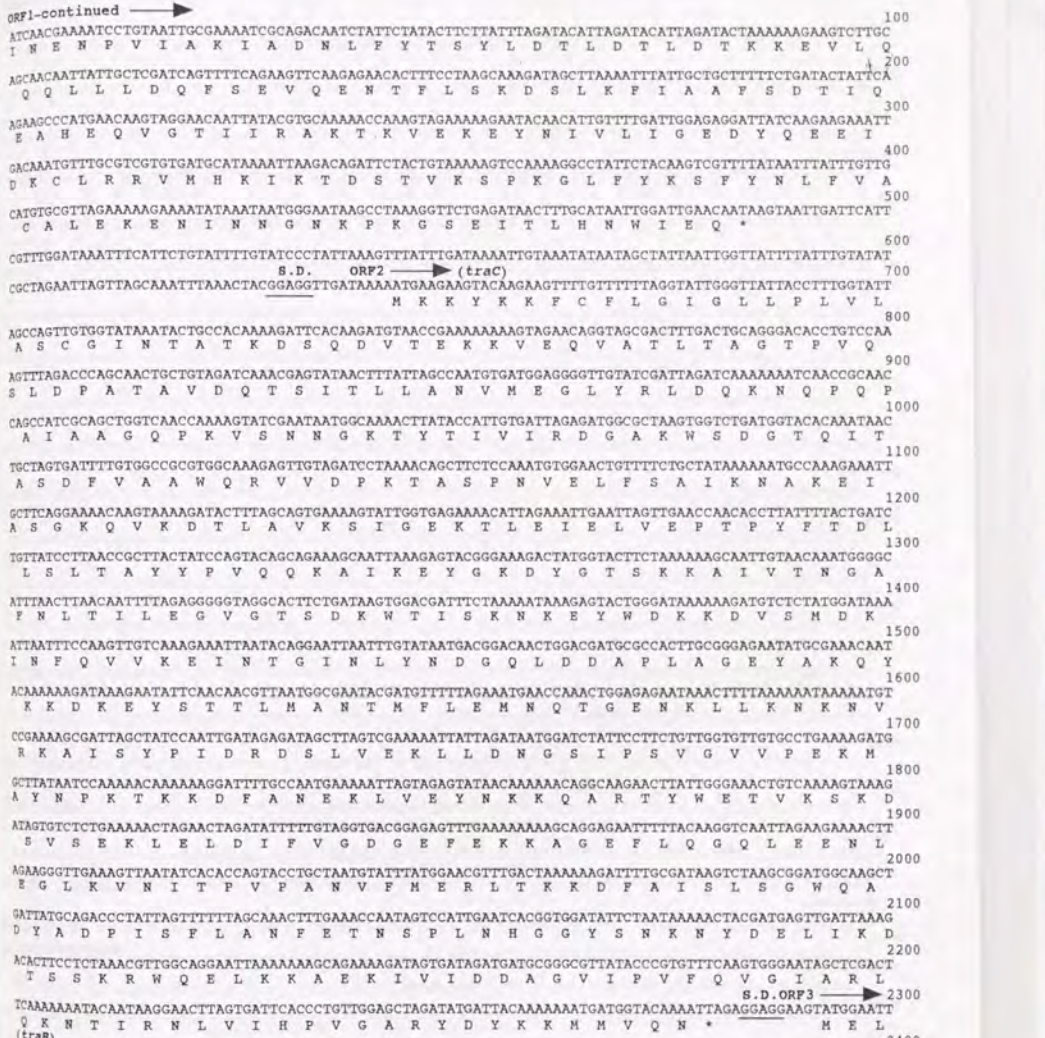


Fig. 2-4 Nucleotide sequence of *Eco* RV-*Hin* cII 5.4 kb region

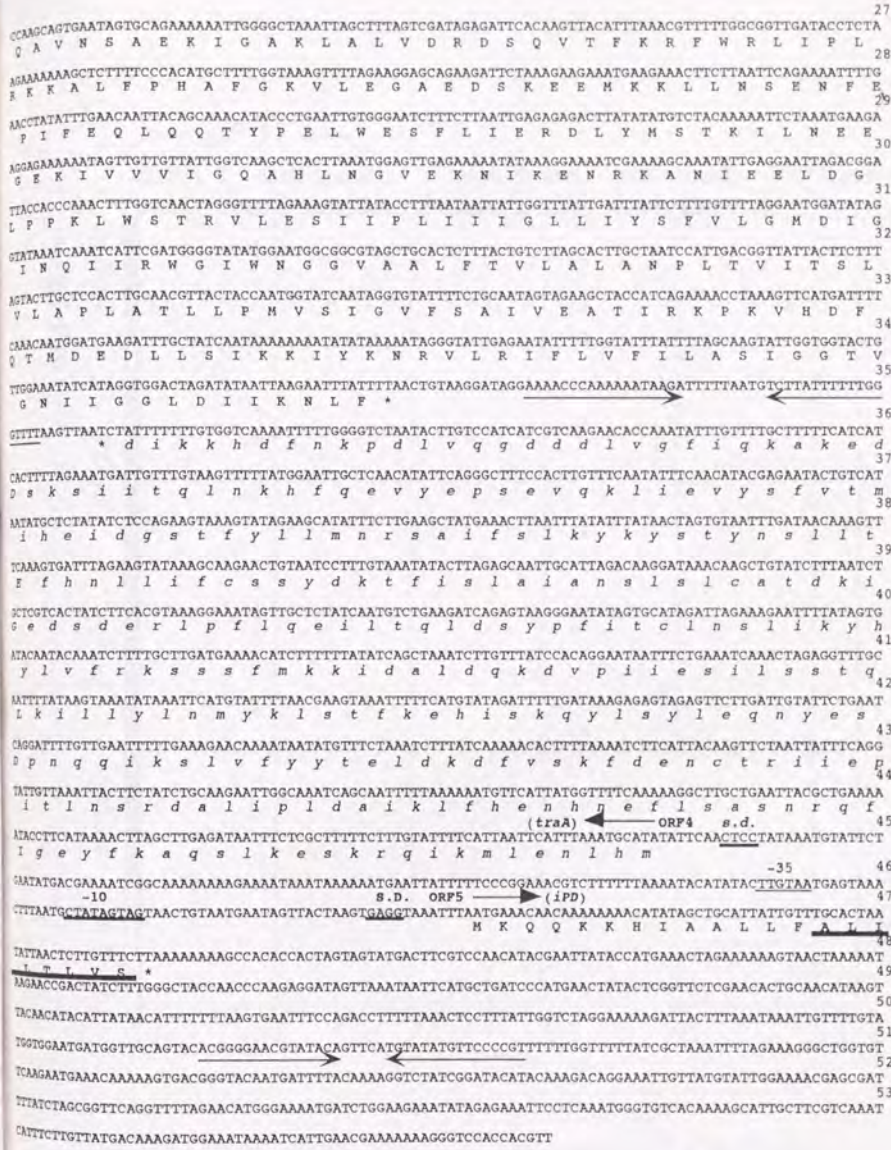


Fig. 2-4 - Continued

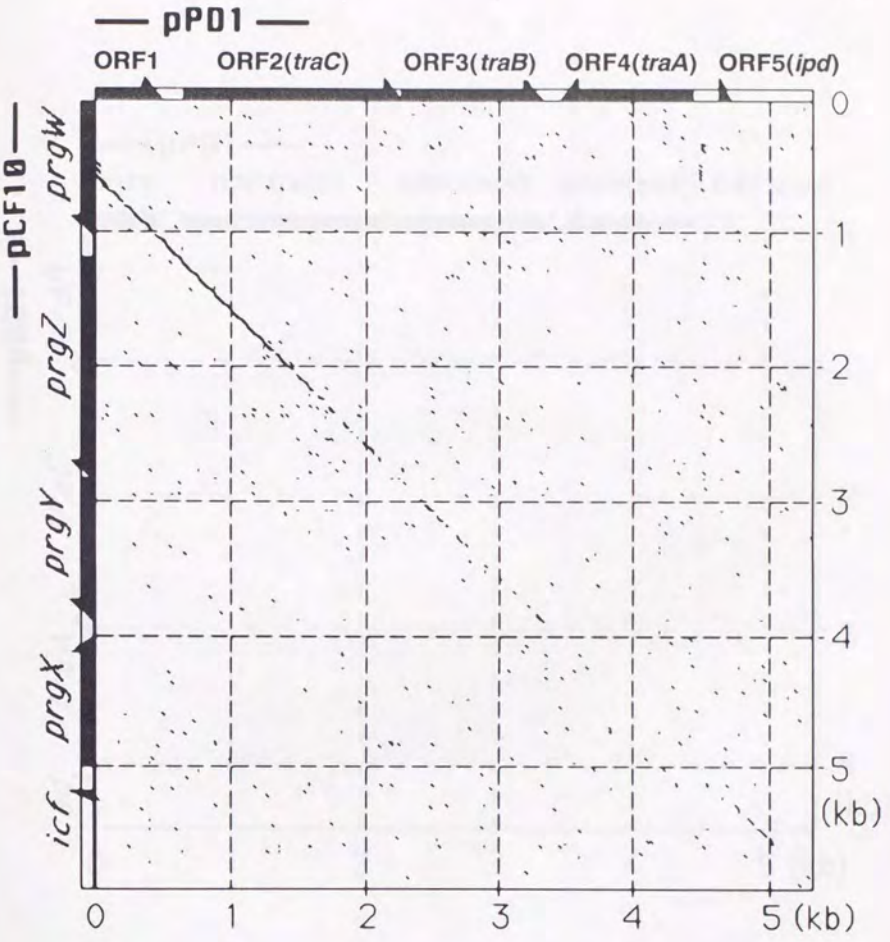


Fig. 2-5 (A). HarrPlot between nucleotide sequence of 5.4-kb *Eco* RV-*Hin* cII segment of pPD1 and the corresponding region of pCF10.

Each point represents at 100 % homology within an nine-bases alignment.

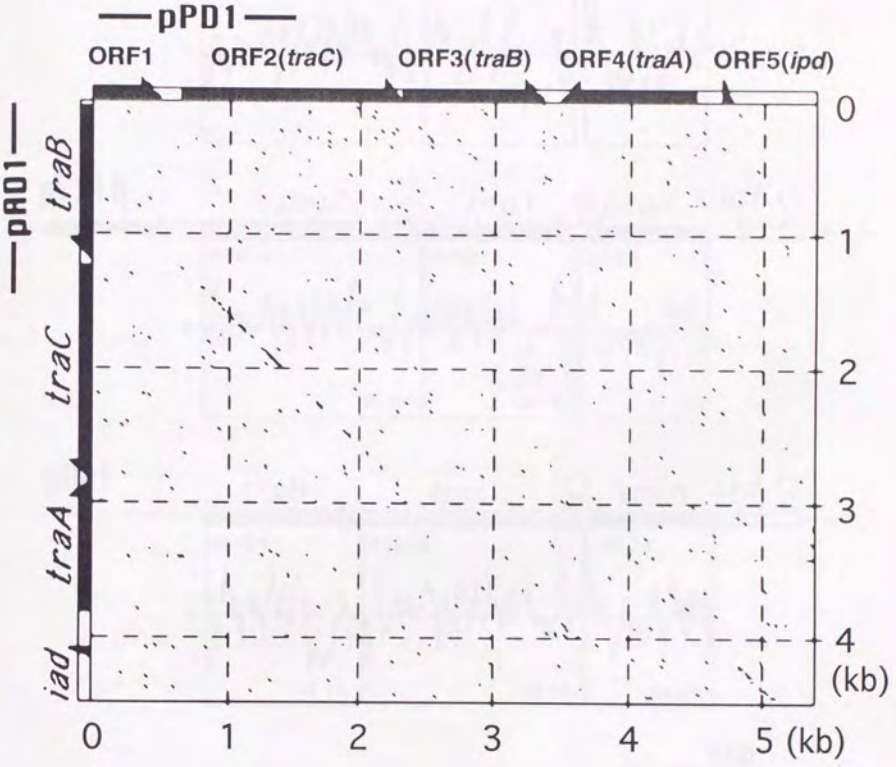


Fig. 2-5 (B). HarrPlot between nucleotide sequence of 5.4-kb *Eco* RV-*Hin* cII segment of pPD1 and the corresponding region of pAD1.

Each point represents at 100 % homology within an nine-bases alignment.

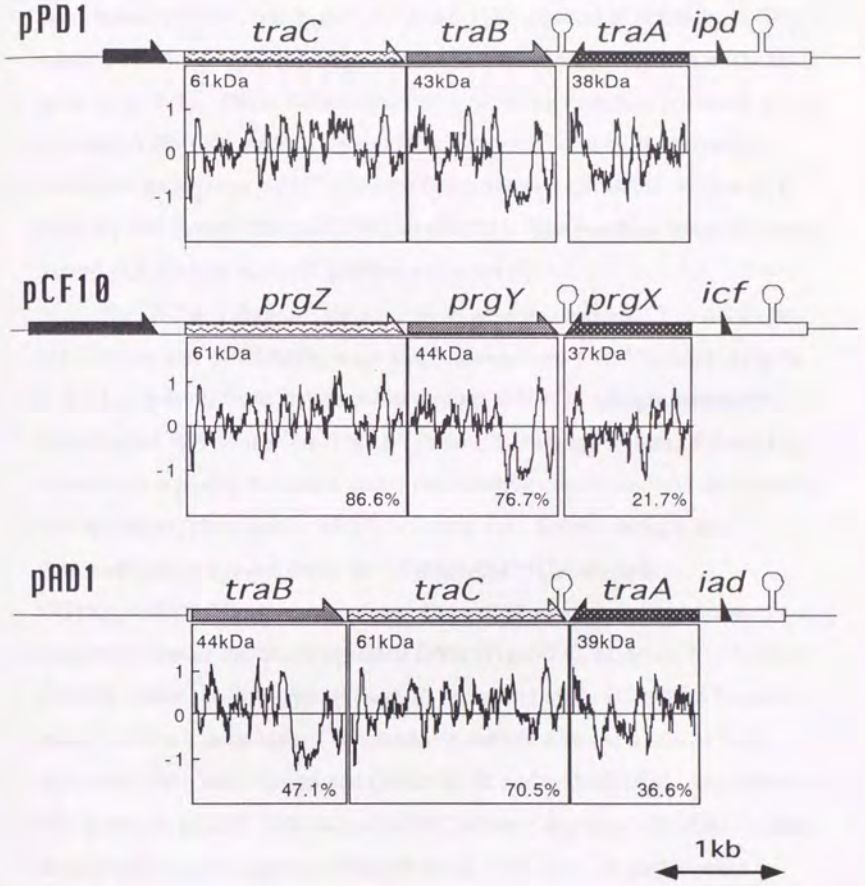


Fig. 2-6. ORF profiles of 5.4-kb *Eco* RV-*Hin* cII segment of pPD1 and the corresponding region of pCF10 and pAD1.

The percent values indicate the ratio of identical amino acid residues of each putative product relative to the corresponding gene product on pPD1. Ω indicates the potential inverted repeat structures showing the calculated energy less than -30 kcal.

2.5. Gene disruptions on *traC* and *traB* loci

The plasmid pUCCM has a 4-bp deletion at the *Pst* I site which results in a frameshift mutation in the *traC* locus. The plasmid pUCBM has a 8-bp insertion in the *Sna* BI site which results in a frameshift mutation in the *traB* locus (Fig. 2-1). These lesions generate a nonsense mutation proximal to the translation start site of *traC* and *traB*, respectively. An erythromycin-resistance gene from pVA891, which functions as a selectable marker in *E. faecalis*, was ligated into pUCCM and pUCBM. The resultant plasmids were named pUCCMEm and pUCBMEm, respectively.

Fig. 2-7 is a diagram showing these gene disruptions. The plasmids, pUCCMEm and pUCBMEm were used to transform OG1X(pAM351) cells to Em^r, resulting from its integration into pAM351 via single cross-over homologous recombination (Fig. 2-7 A or B). Passage culture of these Em^r strains was repeated ten times under nonselective conditions. As the result, a few erythromycin sensitive (Em^s) colonies were found among a few thousand colonies tested from the OG1X(pAM351BEm) and OG1X(pAM351CEm), respectively. These Em^s strains resulted from recombination of the direct repeated DNA (Fig.2-7 C, D, E, or F). In this process, either the wild-type (D and F) or mutant allele (C and E) from the suicide vector can be lost. The mutated plasmids with the *traC*- or *traB*-disrupted allele were designated pAM351CM and pAM351BM, respectively. The plasmids pAM351BM and pAM351CM were digested with *Pst* I or *Sna* BI and analyzed by agarose electrophoresis (Fig. 2-8). A single point mutation at the *Pst* I site was confirmed by the loss of 6-kb and 9-kb fragments and generation of a 15 kb fragment. A single point mutation of the *Sna* BI site was confirmed by the loss of 7-kb and 10-kb fragments and generation of a 17-kb fragment. OG1X(pAM351BM) was further mutated at the *Sna* BI site of pUCCM by the same method, thereby generating pAM351BCM, a plasmid having disruptions of both *traB* and *traC* locus.

2.6. Phenotype of the *traC*- and/or *traB*-disrupted mutants

OG1X cells carrying pAM351CM could undergo cPD1-inducible self-clumping, but required a 5 times higher concentration of cPD1 than observed with OG1X cells carrying pAM351. Apparently, OG1X(pAM351CM) was less sensitive to the pheromone, indicating that TraC contributes to pheromone sensitivity but is not essential to pheromone-inducible aggregation.

OG1X(pAM351BM) underwent constitutive clumping in the absence of exogenous pheromone. There are two possible explanations for this phenotype. One is that *traB* encodes a negative regulator of transcription of the AS gene, and the loss of TraB gives rise to constitutive expression of AS. The other is that *traB* encodes a protein required for pheromone shutdown, and OG1X(pAM351BM) is induced constitutively to clump by the high level of endogenous pheromone in the absence of a functional pheromone shutdown system. However, a large amount of iPD1 inhibited the constitutive clumping. Since the inhibitor is probably competitive antagonist against the pheromone, it was thought that the inhibitor is unable to recover the function of the negative regulator. Thus, the hypothesis that the *traB* encodeds the negative regulator could be denied.

Fig. 2-9 shows the absolute titers of cPD1 and iPD1 in culture filtrates from these mutated strains. In OG1X(pAM351CM), the titers of cPD1 and iPD1 were similar to those in OG1X(pAM351). This indicates that TraC/pPD1 has no ability to reduce the titers of pheromone or inhibitor as TraC/pAD1 did (Tanimoto *et al.* 1993a). In the culture filtrate from OG1X(pAM351BM), the titer of cPD1 remained at a level similar to that in plasmid-free OG1X, while the amount of inhibitor was similar to that in OG1X(pAM351). The titer of cPD1 from OG1X(pAM351BCM) was also similar to that in plasmid-free OG1X. These findings indicate that *traB*

encodes not pheromone/inhibitor binding but pheromone shutdown. OG1X(pAM351BCM) showed a partially clumping phenotype. This coincides with the conclusion that TraC and TraB contribute to pheromone sensitivity and pheromone shutdown, respectively; OG1X(pAM351BCM) lacked pheromone sensitivity and therefore it could not be induced completely despite its high level production of cPD1.

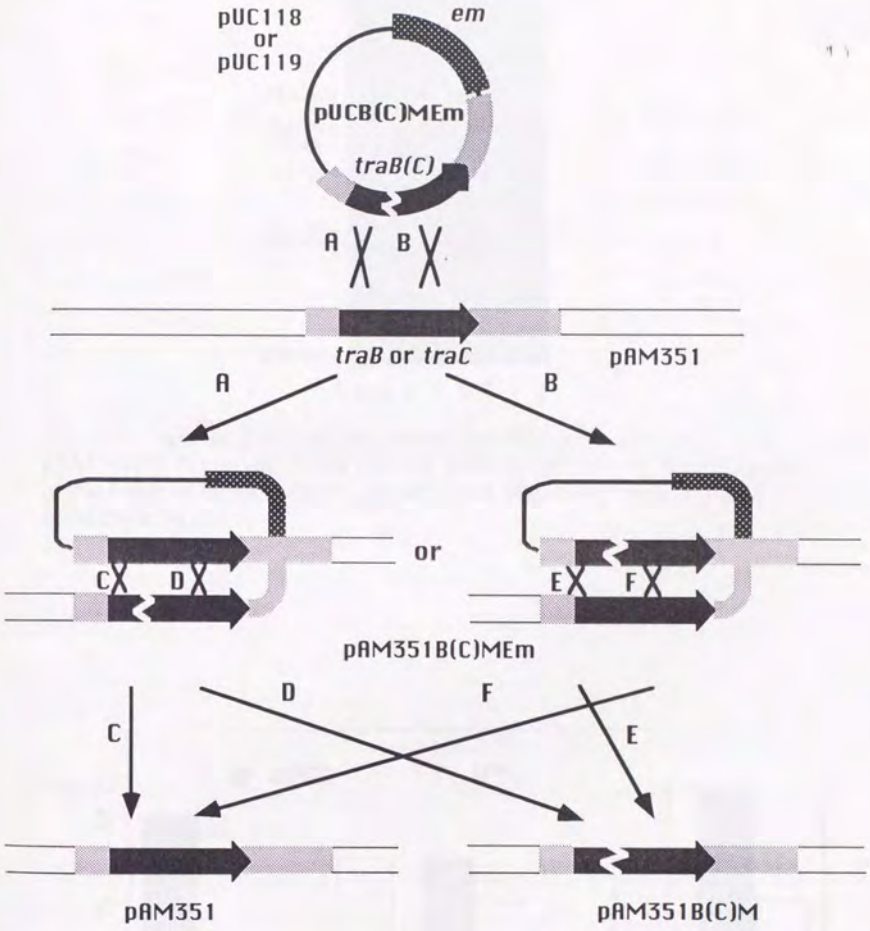


Fig. 2-7. Strategy for gene disruption of the *traB* or *traC* locus.

The suicide vector pUCBMEm or pUCCMEm was transformed into OG1X(pAM351). The first recombination occurred at A(upstream of the lesion) or B(downstream of the lesion). Both recombinations at A and B generate the Em^r plasmid pAM351B(C)MEm. The second recombination occurred at C, D, E, or F, to generate the Em^s plasmid. The recombinations at D and F generate pAM351B(C)M having a mutant allele. The recombinations at C and F generate wild-type pAM351.

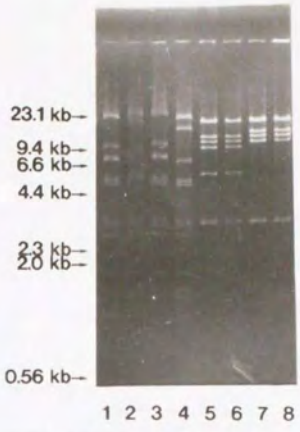


Fig. 2-8. Agarose gel electrophoresis of *Sna* BI digests of pAM351 (1), pAM351BM (2), pAM351CM (3), and pAM351BCM(4), and *Pst* I digests of *Pst* I digests of pAM351 (5), pAM351BM (6), pAM351CM (7), and pAM351BCM (8).

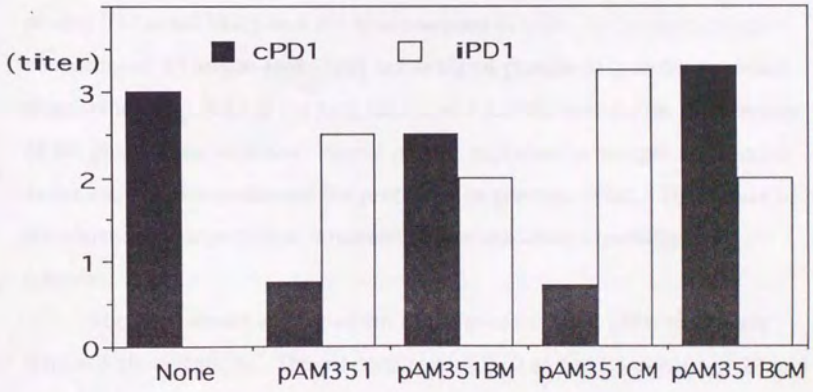


Fig. 2-9 Amount of cPD1 and iPD1 in strains harboring mutated derivatives of pAM351.

3. Discussion

3.1. *ipd* locus

E. coli transformed with *ipd* produced and secreted iPD1 into the culture broth. This shows that the -35 and -10 sequence upstream from *ipd* acts as a general promoter which leads to constitutive transcription of *ipd,* and the processing of the precursor peptide to generate iPD1 is not performed by a particular system but a general system. Considering that iPD1 is a secreted peptide, the peptide preceding the mature iPD1 might act as a signal peptide. However, the central hydrophobic region composed of six amino acid residues is too short to span the membrane. In *E. coli,* more than seven amino acids were necessary to process and translocate a pro-protein (Hikita *et al.* 1992). In Gram-positive bacteria, the average length of the hydrophobic region was 15 (Heijne *et al.* 1989). Phenylalanine is not a conserved amino acid at position -1 of the cleavage site of signal peptides (Heijne *et al.* 1989). Shen et al. showed that signal peptidase of *E. coli* can process only a C-terminus comprised of small amino acid residues such as alanine, glycine, and serine (Shen *et al.* 1991). Considering all of the above points, it is more likely that the entire sequence, with a hydrophobic region consisting of 13 amino acids, may act as signal peptide to span the bacterial membrane. Fig. 2-10 is the proposed model for the mechanism of secretion of the pheromone inhibitor. Signal peptide peptidase is thought to function as the enzyme that processed the precursor to generate iPD1. The nature of the signal peptide peptidase remianed to be elucidated, especially in *E. faecalis.*

Fig. 2-11 shows a comparison of the precursors of iPD1-precursor with iCF10, and iAD1. The precursors of iCF10 and iAD1 consist of 23 and 22 amino acid residues, respectively. The last 7 and 8 residues comprise iCF10 and iAD1, respectively. As in the case of iPD1, the hydrophobic stretches preceding iAD1 and iCF10 were short. Furthermore, the amino

acids at position -1 of iAD1 and iCF10 were threonine and isoleucine, respectively. Neither of these is a conserved amino acid at the cleavage site of signal peptides. However, *icf* and *iad* expressed iCF10 and iAD1 respectively in *E. coli.* As in the case of iPD1 encoded by *ipd,* the whole iCF10 and iAD1 precursor peptides are thought to act as signal peptides which span the bacterial membrane and are then processed by some peptidase to generate the mature inhibitors.

3.2. *traC* locus

E. faecalis transformed with *prgZ* /pCF10 or *traC*/pAD1 reduced the titer of added exogenous pheromone (Ruhfel *et al.* 1993; Tanimoto *et al.* 1993a). This observation might be explained by absorption or binding of pheromone to the TraC and PrgZ products. That is, the proteins encoded by *traC*/pAD1 and *prgZ*/pCF10 are thought to have the ability to bind pheromone. TraC/pPD1, TraC/pAD1, and PrgZ/pCF10 have a significant homology to oligopeptide-binding proteins of *Bacillus subtilis* (Perego *et al.* 1991; Rudner *et al.* 1991), *Salmonella typhimurium* (Hiles *et al.* 1986; Hiles *et al.* 1987), and *E. coli.* (Kashiwagi *et al.* 1990) These bacterial proteins are encoded in the oligopeptide permease (Opp) operon involved in an uptake of oligopeptides. OppA of *B. subtilis* is an extracellular protein anchored in the membrane (L-*Y*-*Z*-cleavage site-C-*y*-*z* is conserved in OppA) and those of Gram-negative bacteria are periplasmic proteins. The OppAs are presumed to act as an initial receptor for extracellular oligopeptides and to deliver them to a complex of membrane-associated proteins encoded in the Opp operon. TraC/pAD1, TraC/pPD1, and PrgY/pCF10 might also act as initial receptors to capture the respective pheromones on the cell surface. Then the pheromones might be subject to a certain delivery system and be taken up into the cells. However, the observation that OG1X(pAM351CM) could undergo self-clumping in the

presence of a high concentration of pheromone indicates that pheromone binding by TraC/pPD1 is not essential for pheromone-induction in donor cells. This coincides with the fact that OppAs were also not necessary for uptake of most kinds of peptides. The group proteins including TraC and OppA might facilitate the function of some transporter by binding the peptides.

The sequence alignment of these proteins is shown in Fig. 2-12. All regions except the N-terminal and C-terminal regions are well conserved. In the middle region from residue 260 to 300, there is a region showing many gaps between the *E. faecalis* TraC group and other bacterial OppA groups. This might explain the difference in ranges of peptides bound. Proteins of the OppA group nonspecifically bind peptides composed of up to five amino acid residues. On the other hand, TraC group proteins specifically bind a certain pheromone composed of seven or eight amino acid residues. Two variable regions exist within all proteins of the TraC group, one extending from residues 370 to 390, and the other extending form residue 490 to the C-terminus. These two variable regions might contribute to specific discrimination of the structurally similar pheromones. The C-terminal variable region is rich in basic amino acids. This feature is not observed in OppA proteins.

3.3. *traB* locus

In a previous study on pAD1, the titer of cAD1 activity was reduced by transformation of a *traB*-ligated multicopy plasmid into *E faecalis* (An *et al.* 1993). On the other hand, with a pCF10-donor, cCF10-shutdown was not detected as described in Chapter I (Fig. 1-5) (Nakayama *et al.* 1994b). The titer of iCF10 produced by this pCF10-donor was enough to block the activity of cCF10 which the host strain produced without pheromone shutdown. Evidently, the pCF10-donor does not require pheromone

shutdown to escape from self-induction. However, pCF10 encodes PrgY which is significantly homologous to TraB/pPD1 and TraB/pAD1. Thus, PrgY/pCF10 might be degenerated to the inactive protein or transcription of *prgY* might be repressed. *traB*/pAD1 does not function in FA2-2(pAD1) while it functions in OG1X(pAD1) (Weaver *et al.* 1990). Strain FA2-2 produced much less cAD1 than strain OG1X and FA2-2(pAD1) did not require pheromone shutdown to the extent that OG1RF(pCF10) did. Considering all of the above points, the potency of pheromone shutdown seems to be controlled in compliance with the demand for pheromone shutdown. The control mechanism might involve feedback regulation of *traB* transcription after pheromone induction. Further investigation of transcription of *traB* or *prgY* is important to clarify the control mechanism.

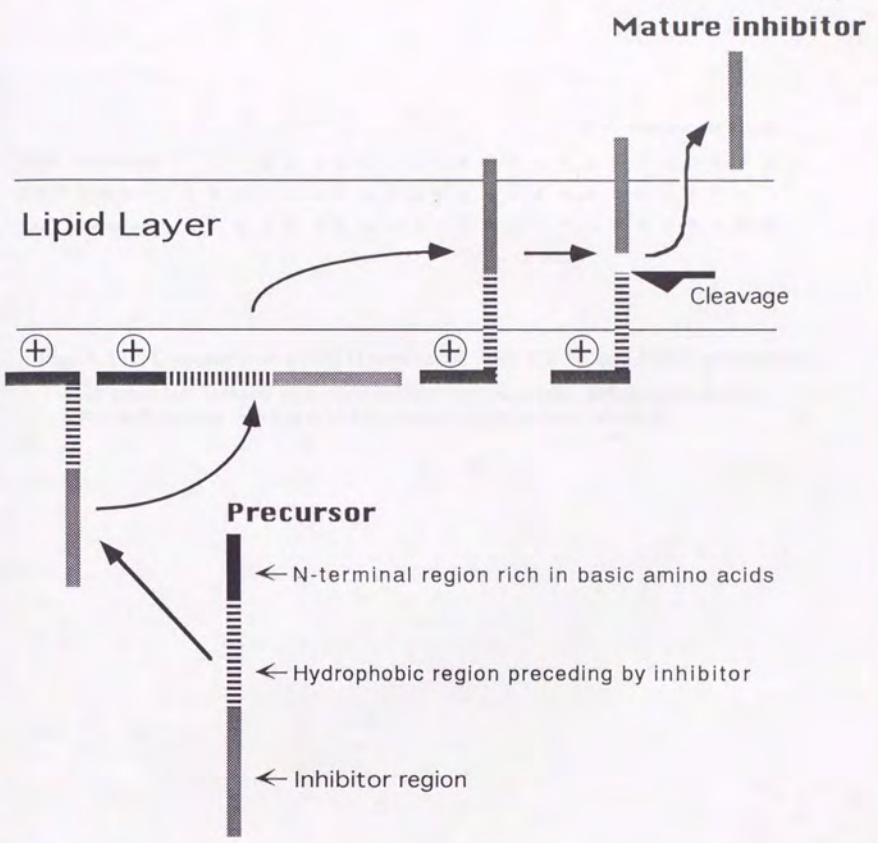


Fig. 2-10. Model for secretion of inhibitor

The precursor attaches onto the inner membrane through its N-terminal positive charge. Then the precursor spans the membrane through its C-terminal hydrophobic stretch including the inhibitor region. The precursor is then cleaved by some peptidase and the mature inhibitor generated is excreted out of the cell.

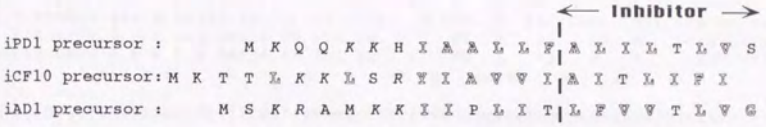


Fig. 2-11. Comparison of iPD1 precursor with iCF10 and iAD1 precursors.

Slant letters indicate basic amino acid residues. Encircled letters indicate hydrophobic amino acid residues. Vertical dotted line indicates N-terminus of inhibitors.

Legend to Fig. 2-12 (Next page). Amino acid sequence alignment of TraC/pPD1 (a), PrgZ/pCF10 (b), TraC/pAD1 (c), OppA of *B. subtilis* (d), of *S. typhimurium* (e), and *E. coli* (f). Asterisks under the sequences represent identical residues among the six proteins. Dots over the sequences indicate variable residues among the *E. faecalis* proteins (a-c).

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CHAPTER III

Pheromone-Inducible Surface Proteins

The pheromone responsive plasmid is thought to encode several surface proteins involved in the conjugative process. An immunological study revealed a pheromone-induced surface antigen (Kessler *et al.* 1983). In this study, a rabbit was immunized with glutaraldehyde-fixed pheromone-induced pPD1-donor cells and the antiserum obtained was absorbed with uninduced cells. The resulting antiserum should be specifically reactive to pheromone-inducible surface substance. The antiserum reacted with a 78-kDa protein, later termed, that was induced on the donor surface after induction of aggregation. Immunoelectron microscopy revealed a proteinaceous microfibrillar substance induced on the surface pPD1-donor cells (Yagi *et al.* 1983). Anti-cAD1-induced-cell antibody was similarly prepared. The antibody reacted with four proteins, AD157 (157 kDa), AD153 (153 kDa), AD130 (130 kDa), and AD74 (74 kDa), which were induced on the surface of pAD1-donor cells (Ehrenfeld *et al.* 1986). The antibody cross-reacted with three high-molecular-weight proteins, PD157(157 kDa), PD153(153 kDa), and PD130 (130 kDa), in the extract of cPD1-induced cell surface. There was no cross-reactivity between the low-molecular-weight proteins, AD74 and PD78. These two proteins were more abundant as compared with the high-molecular weight proteins and are considered likely to be AS.

In this chapter, the author reports purification and characterization of PD78 and AD74 by means of structural and immunological studies. With regard to PD78, gene cloning and gene disruption are also reported.

1. MATERIALS AND METHODS

1.1 Bacteria, plasmid, and medium.

The *E. faecalis* strains and plasmids used in this chapter are listed in Appendix 1. All strains were grown in THB medium (36.4 g of Todd-Hewit broth [Oxoid Ltd.] per liter) at 37°C.

1.2. Extraction and purification of PD78 and AD74.

In each step of extraction and purification, PD78 or AD74 was monitored by SDS-PAGE (Laemmli, 1970). The extractions and purifications of both proteins were done essentially in the same way. OG1X(pAM351) and OG1X(pAM714) cells were cultured for 4hr in THB medium (20 liters per batch) in the presence of 0.5 ng/ml cPD1 and 5 ng/ml of cAD1, respectively, to induce aggregation. Aggregated cells were harvested by decantation followed by centrifugation at 7,000 x g for 30 min. PD78 and AD74 were extracted with 100 ml of 50 mM Tris-HCl buffer (pH 8.4) for 2 hr at 4°C with gentle stirring from the aggregated cells of OG1X(pAM351) and OG1X(pAM714), respectively.

The crude extract was absorbed at 4°C to a column of DEAE-Sepharose CL-6B (Pharmacia, 3.3 x 13 cm) that had been equilibrated with 50 mM Tris-HCl, pH 8.4. After being washed with the same buffer, the column was eluted with 0.3 M NaCl in 50 mM Tris-HCl, pH 8.4. The PD78 or AD74-rich fractions were collected and concentrated to about 2 ml by ultrafiltration (YM-10 membrane, Amicon), and then absorbed at 4°C to a column of DEAE-Sepharose CL-6B (1.4 x 18 cm) that had been equilibrated with 50 mM Tris-HCl, pH 8.4. When no more protein was eluted by the same buffer, the column was eluted with a 300 ml linear gradient of 0 to 0.3 M NaCl in 50 mM Tris-HCl, pH 8.4. The PD78 or AD74-rich fractions were concentrated to about 2 ml by ultrafiltration.

The concentrated eluate from the DEAE-Sepharose CL-6B column was put onto a column of Sephacryl S-300 superfine (Pharmacia, 1.9 x 98 cm) previously equilibrated with 0.2 M ammonium acetate, pH 7.2, and was eluted with the same buffer at 4°C. The eluate was collected in 2.3-ml fractions and was monitored by absorbance at 280 nm. Fractions 51-54 or 54-58 containing PD78 or AD74, respectively, were collected, lyophilized. These samples were used for amino acid sequencing and immunological research.

1.3. Amino acid sequence analysis

Amino acid sequence analysis was performed on an Applied Biosystems 470A or 477A gas-phase sequencer with an on-line 120A PTH analyzer, or a Shimadzu PSQ-1 gas phase sequncer with an on-line Shimadzu LC-6A HPLC system. Samples (100-200 pmol) were applied to the polybrene-coated glass filter.

1.4. Lysyl endopeptidase digestion

The purified PD78 (20 μg) and AD74 (20 μg) were digested in 100 μl of lysyl endopeptidase solution (80 pmol/ml in 20 mM Tris-HCl buffer, pH 9.0). Proteolysis was allowed to proceed for 15hr at 37°C and stopped by adding 12 μl of 2 % TFA to the reaction mixture.

1.5. BrCN digestion

The purified PD78 (140 μg) and AD74 (15 μg) were dissolved in 100 μl of 70 % formic acid and treated with 1 mg of BrCN at room temperature for 24hr. The reaction mixtures were diluted with 900 μl of water and lyophilized. C-terminal peptides of AD74 and PD78 were purified by reverse phase HPLC with a column of Senshu Pak ODS VP 318.

1.6. LSIMS (liquid secondary ion mass spectrometry) analysis

MS analyses were done using a JEOL SX102 double-focusing mass spectrometer with a cesium ion source. A 1:1 mixture of glycerol and

thioglycerol was used as the matrix. Approximately 100 pmol of a sample (1 μl) was mixed with the matrix (1 μl) on the probe tip and was bombarded with a cesium ion beam of 25 keV.

1.7. LC/MS analysis

The HPLC system for LC/MS was a HP1090 liquid chromatograph (Hewlett Packard) with a column of Senshu Pak ODS (VP318-MB151, 2.1 x 150 mm). The column was eluted with a linear gradient of 0 % to 50 % acetonitrile containing 0.1 % TFA in 50 min at a flow rate of 150 μl/min. As a matrix in the mass spectrometry, 50 μl/min of 3 % glycerol in MeOH was mixed into the effluent and the mixing flow was split to 5 μl/ml (1:40) by a splitter (JEOL). The split flow was carried by a fused-silica capillary tubing to a JEOL Frit-FAB interface consisting of a 0.25 mm thick stainless steel frit. The opposite site of frit, where the effluent oozed out, was bombarded with a cesium ion beam of 16 keV.

1.8. Generation and characterization of anti-AD74 or anti-PD78 polyclonal antibody

New Zealand White rabbits were immunized at 2-week intervals by four subcutaneous injections of the purified PD78 or AD74 (200 μg in 0.5 ml of physiological saline/rabbit) in complete Freund's adjuvant, followed two weeks later by an intramusclar injection of the same sample. The rabbits were bled two weeks after the last injection. Antibodies were purified by precipitation of the serum with 15 % sodium sulfate to yield the IgG fraction and used in this study.

To characterize anti-PD78 and anti-AD74 antibody, competitive ELISA was done by the methods described in Mizoguchi *et al.* (1987).

1.9. Western blotting of cell wall extracts

Cell wall extracts were made by the lysozyme extraction methods described by Galli *et al.* (1990). A mid-log-phase culture of each strain was inoculated into 10 volumes of fresh THB medium and then was

incubated for 3hr with or without induction with a pheromone (5 ng/ml). Cells were harvested by centrifugation at 2,000 x g. One gram of wet cells was washed twice with PBS (10 mM, 0.75 % NaCl, pH 7.5) and suspended in 1 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF, and 0.5 M sucrose, pH 8.0, containing 5 mg of lysozyme. Extraction was performed by shaking 1-ml portions for 60 min at 37°C. After extraction, cells were removed by centrifugation and the supernatant corresponding to the extract from 1.6 ml of culture was used for SDS-PAGE. After electrophoresis, the gels were soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11.0) for 5 min, and then placed on filter papers (Bio-Rad) wetted with the transfer buffer. A Gene Screen Plus nylon membrane (NEN) saturated with the transfer buffer was placed on the gel and covered with buffer-wetted filter paper. The gel-filter assembly was set in a blotting apparatus (Nihon-eido), and electroblotted for 30 min at 0.5 A in the transfer buffer. After blotting, the membrane was washed with Tris-HCl buffered saline (TBS) (50 mM Tris-HCl, 0.2 M NaCl, pH 7.4), blocked with 10 % bovine serum albumin (BSA) in TBS for 12hr and exposed overnight to the anti-AD74 or anti-PD78 antibody (10 μg/ml). After it was washed three times with TBS containing 0.05 % Tween 20 (TTBS), the membrane was exposed to peroxidase-labeled antibodies to rabbit IgG (2 μg/ml) for 1hr. It was then washed and developed with 4-chloro-1-naphthol.

1.10. Assay for blocking of self-clumping by antibody

Mid-log-phase cultures of OG1X(pAM714), OG1X(pAM351), OG1SSp(pCF10), and FA2-2(pAM373) were transferred into 5-volumes of fresh THB medium containing 5 ng/ml of cAD1, cPD1, cCF10, and cAM373, respectively, and further incubated for 30 min at 37°C without shaking. Then, 1 ml of culture was placed in a tube containing various concentrations of anti-AD74 antibody and shaken for 15 min (for cAD1,

cPD1, or cCF10-induced self-clumping) or for 60 min (for cAM373-induced self-clumping). Then, the cultures were vortexed for 3 sec and were further incubated with shaking for 5 min at 37°C. After the cultures were permitted to stand for 5 min, the turbidities (optical density at 660 nm) were measured.

1.11. Assay for blocking of plasmid transfer by antibody

An overnight culture of OG1X(pAM351) or OG1X(pAM714) cells as donor, or JH2-2 cells as recipient was transferred into 10 volumes of fresh THB medium and cultured at 37°C. At 3hr of cultivation (mid-log-phase), the donor cell culture was transferred into 10 volumes of fresh THB medium containing 0.5 ng/ml of cPD1 or cAD1 and grown as an induction culture for 1hr. The mating mixture consisted of 0.1 ml of the induced donor cell culture and 0.1 ml of a mid-log-phase culture of JH2-2 cells in 1 ml of THB that included 1 mg of anti-PD78 or anti-AD74 antibody. After a 15-min incubation at 37°C, the mating mixtures were cooled to 4°C, vortexed to dissociate clumped cells , diluted appropriately, and spread (0.1 ml) on plates containing antibodies to select donors and transconjugants. After incubation at 37°C for 24-48hr, the colonies formed were counted. The antibiotics used were tetracycline (5 μg/ml) for donors OG1X(pAM351), erythromycin (50 μg/ml) for donors OG1X(pAM714), rifampin (25μg/ml) plus tetracycline (5 μg/ml) for transconjugants JH2-2 carrying pAM351, and rifampin (25 μg/ml) plus erythromycin (50 μg/ml) for transconjugants JH2-2 carrying pAM714.

1.12. Cloning and sequencing of *pd78*

An oligonucleotide probe for southern hybridization was synthesized (Applied Biosystems 380B) on the basis of the N-terminal amino acid sequence of PD78. The probe was a 17-mer antisense oligonucleotide (5' TT[TC]TC[ATGC]CC[TC]TG[AG]TT[AG]AA 3', [

]means mixtures) corresponding to Phe-2 to Lys-7 of the N-terminal amino acid sequence of PD78 and it was labeled with [∂-^{32}P]ATP (111TBq/mmol) and T4 polynucleotide kinase (TaKaRa).

Plasmid pAM351 was prepared by the alkaline method (see Appendix 4) (Anderson *et al.* 1983). Plasmid DNA from *E. coli* was prepared by the alkaline method (Sambrook *et al.* 1988). The pAM351 was digested with *Eco* RI and *Bgl* II and separated by agarose gel electrophoresis. The gel was blotted onto a GeneScreen filter (NEN Research Products). The filter was washed with 6 x SSC, 0.1 % SDS at 37°C. A 3.6-kb *Eco* RI-*Bgl* II fragment which hybridized with the probe was extracted from an agarose gel using the GeneClean II kit (BIO 101 Inc.) and was ligated into the *Eco* RI-*Bam* HI multiple cloning site of the plasmid pUC118. The resultant chimera designated pJN1 was transformed into *E. coli* JM109. Deletion mutants for sequencing were constructed by exonuclease III digestion (Yanisch-Peron *et al.* 1985). Sequence analysis utilized the dideoxynucleotide method of Messing (Messing 1983).

1.13. Gene disruption of *pd78*

pJN1 was digested with *Acc* III, then blunted with T4 DNA polymerase according to the method described in the TaKaRa DNA Blunting Kit, and self-ligated with T4 DNA ligase (TaKaRa). The region of the resultant plasmid designated pJNM1 having the mutation was sequenced. Unexpectedly, a spontaneous deletion (nucleotide no. 829-1403) was found as shown in Fig. 2. A *Hin* dIII-*Cla* I 1.5 kb segment of pVA891, which encodes a selectable Em^r marker in *E. faecalis*, was blunted with T4 DNA polymerase and ligated into the *Hin* cII site of pJNM1 and resulted in pJNM1Em. pJNM1Em was transformed into *E. faecalis* OG1X(pAM351) by the electroporation method of Cruz-Rodz *et al.* (1990) (see Appendix 5). The transformant

OG1X(pAM351PD78MEm) was selected on THB agar plates containing 50 μg/ml of erythromycin (Sigma). OG1X(pAM351PD78Em) was cultured to mid-log-phase in THB liquid medium not containing erythromycin. Approximately a thousand colonies were replicated from non-selective plates onto Em^r selective plate and four Em^s colonies were found.

2. RESULTS

2.1 Extraction and purification of PD78 and AD74

PD78 and AD74 were prepared from pheromone-induced aggregated cells of strains OG1X(pAM351) and OG1X(pAM714), respectively. pAM351 is a derivative of pPD1 having an insertion of tetracycline-resistance transposon Tn*916* in the *Eco* RI B-fragment (Ike *et al.* 1983). pAM714 is a derivative of pAD1 having an insertion of erythromycin-resistance transposon Tn*917* (Ike *et al.* 1984). OG1X(pAM351) and OG1X(pAM714) showed the same phenotype relating to pheromone-inducible clumping and plasmid transfer. Both proteins were extracted with 50 mM Tris-HCl buffer, pH 8.4. Fig. 3-1 shows the SDS-PAGE profile of the crude extracts and arrows indicate cPD1-induced and cAD1-induced AD74 (lane 2 and lane 7).

Each proteins was purified by three steps of column chromatography as shown in Fig. 3-1. Upon the last Sephacryl S-300 (gel filtration) chromatography, PD78 and AD74 were each eluted in a single peak corresponding to a molecular mass of 540 kDa and 360 kDa, respectively. This suggested that each protein did not aggregate non-uniformly but formed a uniform oligomer. Each purified preparation of PD78 and AD74 after the Sephacryl S-300 column showed as a single band on SDS-PAGE (lanes 5 and 10 in Fig. 3-1). The yield of the PD78 protein was approximately 3 mg from 80 liter culture of cPD1-induced cells and that of AD74 was approximately 2 mg from 20 liters culture of cAD1-induced cells.

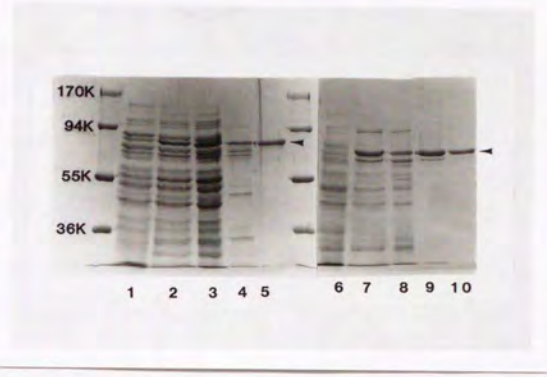


Fig. 3-1. SDS-PAGE showing the purification profile of PD78 and AD74.

Electrophoresis was done with 8.2 % polyacrylamide in the resolving gel and with 4.5 % in the stacking gel. Just, before electrophoresis, samples were boiled in the presence of SDS and ß-mercaptoethanol. Each sample was 30 μg equivalent of the crude extract as protein. The gels were stained with Coomassie brilliant blue R-250. Lanes 1 to 5 relate to PD78 and 6 to 10 to AD74. Arrows indicate PD78 and AD74. Lanes 1 and 6, Tris-HCl buffer- extracted proteins from the pheromone-uninduced cells of strain OG1X that carried plasmids pAM351 and pAM714, respectively; 2 and 7, Tris-HCl buffer-extracted proteins from the pheromone-induced cells; 3 and 8, after the first DEAE-Sepharose CL-6B column chromatography; 4 and 9, after the second DEAE-Sepharose CL-6B column chromatography; 5 and 10, after Sephacryl S-300 column chromatography.

2.2. Amino acid sequence of AD74

The N-terminal sequence of AD74 was AELDTQPGTTT. This sequence was identical to the deduced N-terminal sequence of *asa1* cloned from pAD1 by Galli *et al.* (1990). The *asa1* encodes a 137 kDa protein (Asa1) having a membrane-anchored sequence at the C-terminus that is conserved in Gram-positive bacteria (Fischetti *et al.* 1990). Fig. 3-2 shows the deduced amino acid sequence. AD74 showed a molecular size of 74 kDa, and AD74 was probably derived from N-terminal half of Asa1 by enzymatic cleavage.

In order to determine the structure of the AD74 moiety, peptide mapping of AD74 was performed. The purified AD74 was digested with lysyl endopeptidase or chemically cleaved by BrCN. The digested peptides were analyzed mainly by liquid chromatography/mass spectrometry (LC/MS) (Nakayama *et al.* 1992). Molecular ions identical to the calculated molecular mass of underlined peptides in Fig. 3-2 were detected. This showed that these peptides fragments were generated from AD74 and the C-terminus of AD74 was proximal to fragment 32. The C-terminal peptide generated by BrCN cleavage showed a molecular mass of 2,286 Da which corresponded to that of W-492 to K-510. Amino acid sequence analysis of the BrCN peptide confirmed the sequence of W-492 to K-510. These results indicated that AD74 was generated by specific cleavage between K-510 and G-511 in Asa1. The calculated molecular mass was 55,574 Da, which is much smaller than that estimated by SDS-PAGE. This abnormal behavior on SDS-PAGE may possibly be explained by its feature of being very rich in basic amino acid residues. AD74 has no cysteine residues. This suggests that AD74 may form a hair-like structure and its tertiary structure feature might also cause this abnormal behavior on SDS-PAGE.

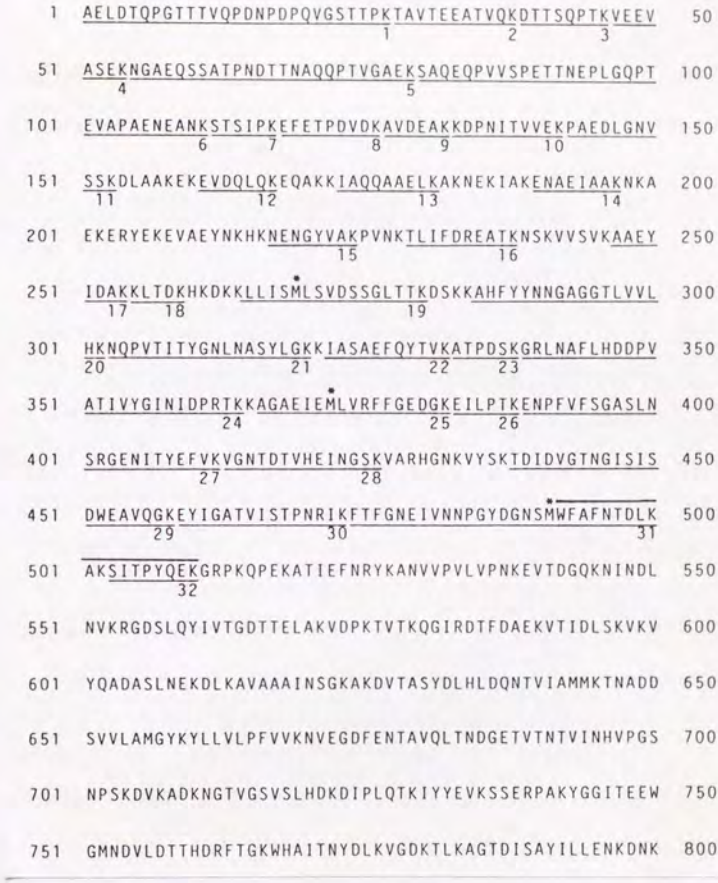


Fig. 3-2. Sequence of Asa1 (1-800) and the peptide fragments of AD74.

Identified lysyl endopeptidase-generated fragments are underlined and numbered in series from the N-terminus. Methionines are marked by stars. The line over the sequence 492-510 indicates the C-terminal fragment produced by BrCN digestion.

2.3. Immunological study with anti-AD74

Specific antiserum against PD78 and AD74 was raised by immunization of rabbits with the purified PD78 and AD74, respectively. Western blotting experiments showed that each antiserum specifically reacted to PD78 or AD74 (Fig. 3-3). Competitive ELISA showed that there was no cross-reactivity between anti-PD78 and anti-AD74 antibody (Fig. 3-4).

As shown in lane 1 of Fig. 3-5, it was found that there was a 153-kDa protein reactive with anti-AD74 antibody in the cell wall extract from the cAD1-induced cells; this protein probably represents Asa1. This result coincides with sequence data indicating that the C-terminal region of the Asa1 product was spanning the peptideglycan. In a similar size range, proteins of cPD1-induced OG1X(pAM351) cells and cCF10-induced OG1SSp(pCF10) were also reactive to the anti-AD74 antibody. In the extract from cAM373-induced cells, no reactive proteins were detected. These data suggest that sex pheromone induced the expression of the Asa1-like protein generally on the donor cell surface, except in the case of the pAM373-donor. In the extract from OG1X(pAM714) cells, AD74 was also detected in the 70-80 kDa range and other minor proteins were in the range of 100-150 kDa. These might be proteolytic products of Asa1 during extraction. A protein of 70-80 kDa in the extract from OG1SSp(pCF10) cells was also found to be a proteolytic product of pCF10-AS, which may represent SA73 (Tortorello *et al.* 1985). In the extract from OG1X(pAM351) cells, a protein similar in size to AD74 was not found.

Fig. 3-5 shows the effect of anti-AD74 antibody on self-clumping. If self-clumping was inhibited, the cell could not make

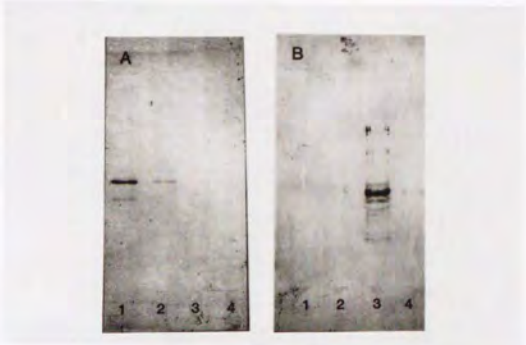


Fig. 3-3. Western blotting of cell surface extract from cAD1 or cPD1 induced OG1X(pAM351) with anti-PD78 or anti-AD74 antibody.

Lanes: 1, 0.1 μg of Tris-HCl buffer-extracted protein from pheromone-induced cells of strain OG1X(pAM351); 2, 0.1 μg of Tris-HCl buffer-extracted proteins from pheromone-uninduced cells of strain OG1X(pAM351); 3, 0.1 μg of Tris-HCl buffer extracted proteins from pheromone-induced cells of strain OG1X(pAM714); 4, 0.1 μg of Tris-HCl buffer-soluble proteins from pheromone-uninduced cells of strain OG1X(pAM714).

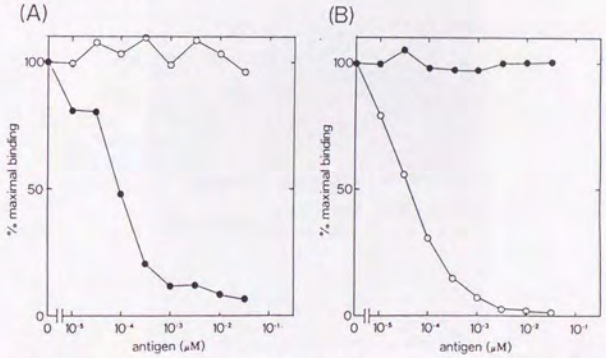


Fig. 3-4. Binding activity of (A) Anti-PD78 and (B) Anti-AD74 antibody as assessed by competitive ELISA to PD78 and AD74, with PD78 (-•-) or AD74 (- -).

ELISA plate was precoated with 50 μl of 10^{-10} M/liter of PD78(A) and AD74(B) as a competitive antigen and competitive ELISA was done on this plate in the presence of anti-AD74(A) or anti-PD78(B) antibodies corresponding to 1/3 x 10^4 and 1/2 x 10^3 dilutions of a 5 mg/ml IgG preparation, respectively.

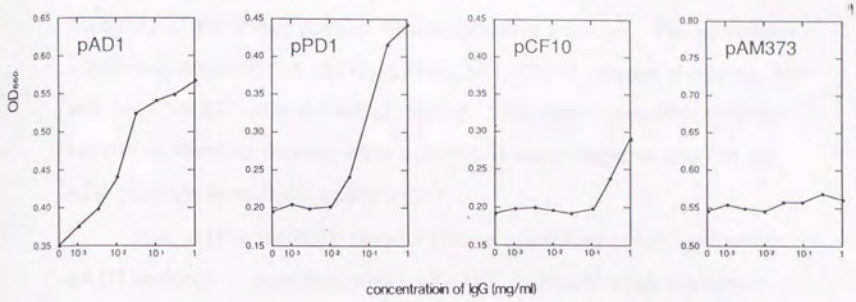


Fig. 3-5. Effect of anti-AD74 antibody on self-clumping of the strain OG1X(pAM714) (A); OG1SSp(pCF10) (C); FA2-2(pAM373) (D). The values of OD_{660} are the average of two experiments.

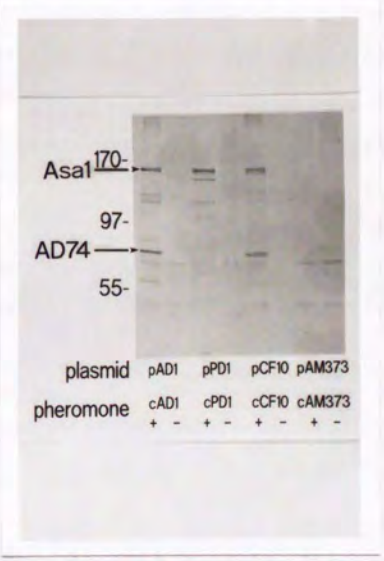


Fig. 3-6. Western blotting of cell wall extracts with anti-AD74 antibody.

Lanes: 1 (pheromone-induced) and 2 (uninduced), OG1X(pAM714) cells; 3 (induced) and 4 (uninduced), OG1X(pAM351) cells; 5 (induced) and 6 (uninduced), OG1SSp(pCF10) cells; 7 (induced) and 8 (uninduced), FA2-2(pAM373) cell. SDS-PAGE was done with 7.5 % polyacrylamide in the resolving gel. Numbers on the left side indicate molecular masses in kilodaltons.

aggregates that would normally fall to the bottom of the tube; the turbidity of the upper portion would therefore increase. The inhibitory effect was detected for cAD1-, cPD1-, and cCF10-induced clumping, but not for cAM373-induced self-clumping. This result coincides with the results of Western blotting shown in Fig. 3-6 and suggests that the 153 kDa proteins have the function of AS.

Anti-AD74 antibody showed also an inhibitory effect against the cAD1-induced transfer frequency of pAD1 and very weak inhibitory effect against pPD1-transfer (Table 3-1).

2.4. Amino acid and nucleotide sequence of PD78

N-terminal amino acid analyses of PD78 revealed the sequence Ala-1 to Pro-17 (Fig. 3-7). In order to determine the endo-sequence of PD78, the protein was enzymatically cleaved by lysyl endopeptidase. 23 peptide fragments were sequenced and a total of 341 amino acid residues were identified (Fig. 3-7)

PD78 was expected to be encoded by pPD1. Southern hybridization using an oligonucleotide complementary to the N-terminal amino acid sequence of PD78 specifically revealed a 3.6-kb *Eco* RI-*Bgl* II fragment (Fig. 3-8A) of pAM351. This fragment was subcloned into sequencing vector pUC118 (pJN1; Fig. 3-8B). Fig. 3-2 shows the DNA sequence from pJN1 and the deduced amino acid sequence. pJN1 had an open reading frame designated *pd78,* corresponding to the entire PD78 sequence, which was preceded by a potential Shine-Dalgarno (SD) sequence and terminated by TAA stop codon. However, no obvious promoter sequence was

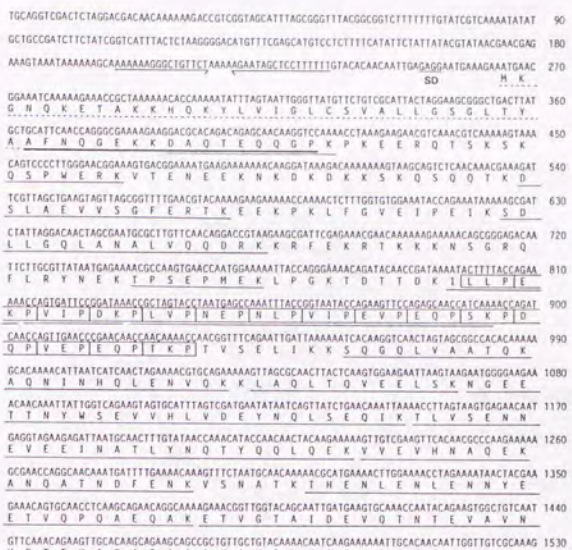


Fig. 3-7. DNA sequence and the deduced amino acid sequence of PD78

A pair of arrows show a inverted repeat sequence. SD indicates a Shine-Dargarno sequence. Signal sequence is indicated by dotted line. The identified N-terminal amino acid sequence is indicated by bold line. The sequence identified from lysyl endopeptidase are underlined. X-X-Pro repeated sequence is enclosed by boxes.

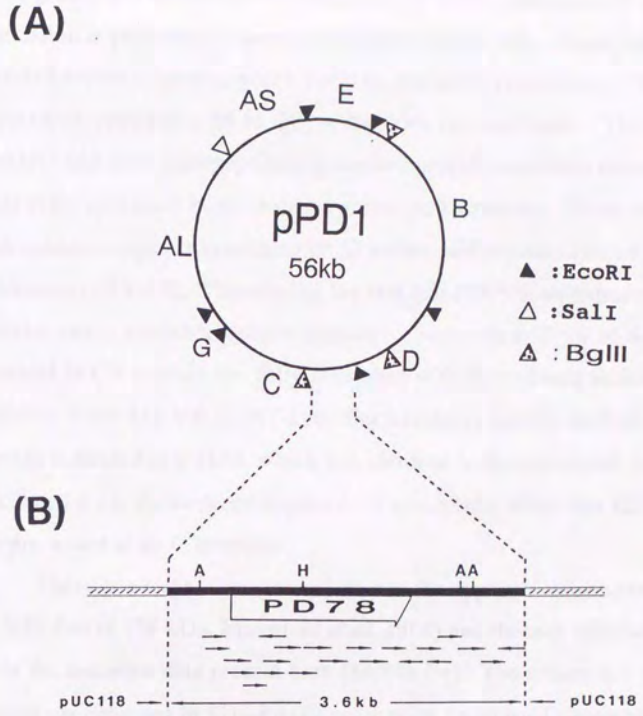


Fig. 3-8.

(A) A circular map of pAM351.

pAM351 was mapped with respect to sites cleaved by *Eco* RI and *Bgl* II and the map of pPD1 described by Yagi et al (Yagi *et al.* 1983). Tn*916* is located in a 26.5-kb *Eco* RI fragment (bold line). The wider line is the 3.6-kb *Eco* RI-*Bgl* II fragment encoding PD78.

(B) Structure of pJN1.

The thick arrow indicates the direction of transcription and the region of the open reading frame designated *pd78*. The strategy used to determine the nucleotide sequence is shown by the short arrows. E, *Eco* RI; B, *Bgl* II; A, *Acc* I; H, *Hin* cII.

present. There are two in-frame ATGs (bp 256-258, 265-267) immediately after the SD sequence. Considering the spacings between the SD sequence and the two ATGs (Singer *et al.* 1981), initiation of translation is presumed to occur at the downstream one. There is an inverted repeat sequence, which could be related to regulation of PD78 expression, upstream (-66 to -30) of the open reading frame. The N-terminal and lysyl endopeptidase-generated peptide sequences determined were fully contained in the deduced amino acid sequence. There is a hydrophobic sequence consisting of 33 amino acid residues preceding the N-terminus of PD78. Considering the fact that PD78 is an extracellular protein, this is probably a signal sequence. Sequence analysis of the C-terminal BrCN peptide was fully consistent with the deduced amino acid sequence from 458-Val to 467-Lys. Furthermore, LSIMS analysis of the peptide indicated m/z 1158, which was identical to the molecular weight calculated from the deduced sequence. These results show that PD78 is not processed at its C-terminus.

There is a large discrepancy between the apparent molecular size on SDS PAGE (78 kDa, Ehrenfeld *et al.* 1986) and the size calculated from the sequence data present here (53,846 Da). The reason for this anomalous behavior in SDS-PAGE gel may be its unusual amino acid composition that is very rich in glutamic acid (13.8 %) and lysine (11.5 %) (Burton *et al.* 1981). PD78 has no cysteine residues like the case of AD74. This suggests that PD78 may form a hair-like structure and its tertiary structure might also cause this abnormal behaviors on SDS-PAGE.

PD78 contains a fifteen times tandem repeated sequence of X-X-Pro (amino acid residue 146-190). The similar repeated sequence has been found in several gene products described in Table 3-1. The author will discuss about this sequence in the section of discussion.

Table 3-1. List of proline-rich repeated sequence

Protein	Repeated sequence (x times)	MW	Localization	Origin	Function	Reference
PD78	X-X-Pro (x 15)	53kDa	extracellular	*E. faecalis* (pPD1)	this study	this study
TraD	Gln-Gln-Pro (x 10)	84kDa	inner membrane	*E. coli* (R100)	plasmid transfer or strand separation	Yoshioka *et al.*, (1990)
ORF C	Val-Asp-Pro (x 11) Thr-Glu-Pro (x 35)	31kDa	membrane-anchored	*E. faecalis* (pAMß1)	plasmid transfer	Swinfield *et al.*, (1990)
Prg C	X-X-Pro (x 40)	30kDa	membrane-anchored	*E. faecalis* (pCF10)	plasmid transfer	Kao *et al.*., (1991)
US11	Arg-X-Pro (x 24)	18kDa	nucleus	Herpes simplex virus	DNA binding	McGeoch *et al.*, (1985)
PRO21	Pro-Thr-Ser-Pro-Ser-Tyr-Ser (x 26)	191kDa	nucleus	*S. cerevisiae*	RNA polymerase	Allison *et al.*, (1985)

2.5. Immunological study with anti-PD78

There was no protein detected that cross-reacted with anti-PD78 in the cell wall extract. This result indicates that PD78 has neither cell-wall spanning region nor a membrane-anchoring region such as Asa1 did.

Anti-PD78 antibody also exerted an inhibitory effect to cPD1-induced clumping and partially inhibited cAD1-induced clumping (Fig. 3-9). These results suggest that PD78 contributes to pheromone-inducible clumping and PD78-like protein exists on the surface of cAD1-induced donor cells. However, no PD78-like protein was found on the surface of donor induced by other pheromones.

Anti-PD78 antibody exerted an inhibitory effect against cPD1-induced transfer of pPD1 and a weak effect against transfer of pAD1 (Table 3-2).

2.6. Gene disruption of *pd78*

Fig. 3-10 is a diagram showing the gene disruption of *pd78*. The plasmid pJNM1 is a chimeric plasmid consisting of pUC118 and having *pd78* a deletion extending from nucleotide 829 to 1403 as shown in Fig. 3-7. This deletion results in a loss of the major C-terminal part of PD78. An erythromycin-resistance gene from pVA891, which can act as a selective marker in both *E. coli* and *E. faecalis,* was ligated into pJNM1. This new plasmid was designated pJNM1Em. It was used to transform OG1X(pAM351) to Em^r, resulting from its integration into pAM351 via reciprocal recombination (Fig.3-10, A or B). One transformant obtained was chosen for study and was designated OG1X(pAM351PD78Em). Growth of the resultant Em^r strain under nonselective conditions 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. 3-10, C, D, E, or F). In this process, either the wild-type (D and F) or the mutant *pd78* allele of pJNM1Em (C and E) can be lost. Two of the four resultant Em^s strains were the same as OG1X(pAM351) and others were designated OG1X carrying pAM351PD78M which has a mutant allele of *pd78.* Agarose gel electrophoresis analysis of pAM351PD78M *Eco* RI digests showed that the plasmid has a deletion in the *Eco* RI-C fragment which resulted from a deletion in *pd78* (Fig 3-11). This results confirm that pAM351PD78M has a mutant allele of *pd78.*

OG1X(pAM351PD78M) showed the same phenotype as OG1X(pAM351) relating to pheromone-inducible self-clumping. On the other hand, pAM351PD78M was transferred at a frequency of 1.70×10^{-5}/donor when induced by cPD1, while pAM351 was transferred at 8.3×10^{-4} /donor (when uninduced, pAM351 transferred at $<3.4 \times 10^{-7}$). This

result indicates that PD78 contributed to plasmid transfer but was not essential to plasmid transfer.

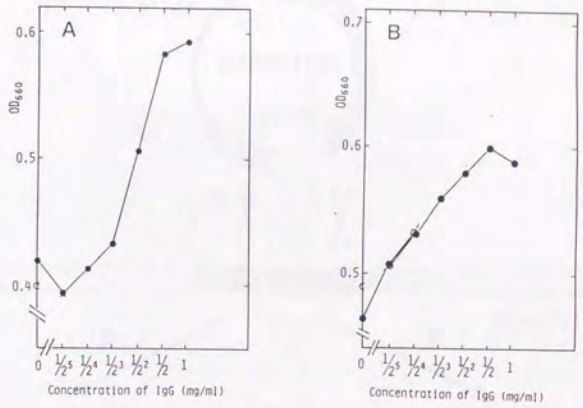


Fig. 3-9. Effects of anti-PD78 antibodies on self-clumping of strains OG1X(pAM351) induced by cPD1 (A), and strain OG1X(pAM714) induced by cAD1 (B).

Table 3-2. Effects of anti-PD78 and anti-AD74 antibody on plasmid transfer.

Addition of IgG	Frequency[a]	
	pAM351/donor	pAM714/donor
None	5.5×10^{-3} (100%)	3.4×10^{-3} (100%)
Anti-PD78	5.1×10^{-4} (9.2%)	1.9×10^{-3} (57.7%)
Anti-AD74	4.2×10^{-3} (76.2%)	8.1×10^{-5} (2.4%)

[a] Number of transconjugants containing the indicated plasmid per total number of donor cells. The numbers in parentheses are the percent relative to non-addition.

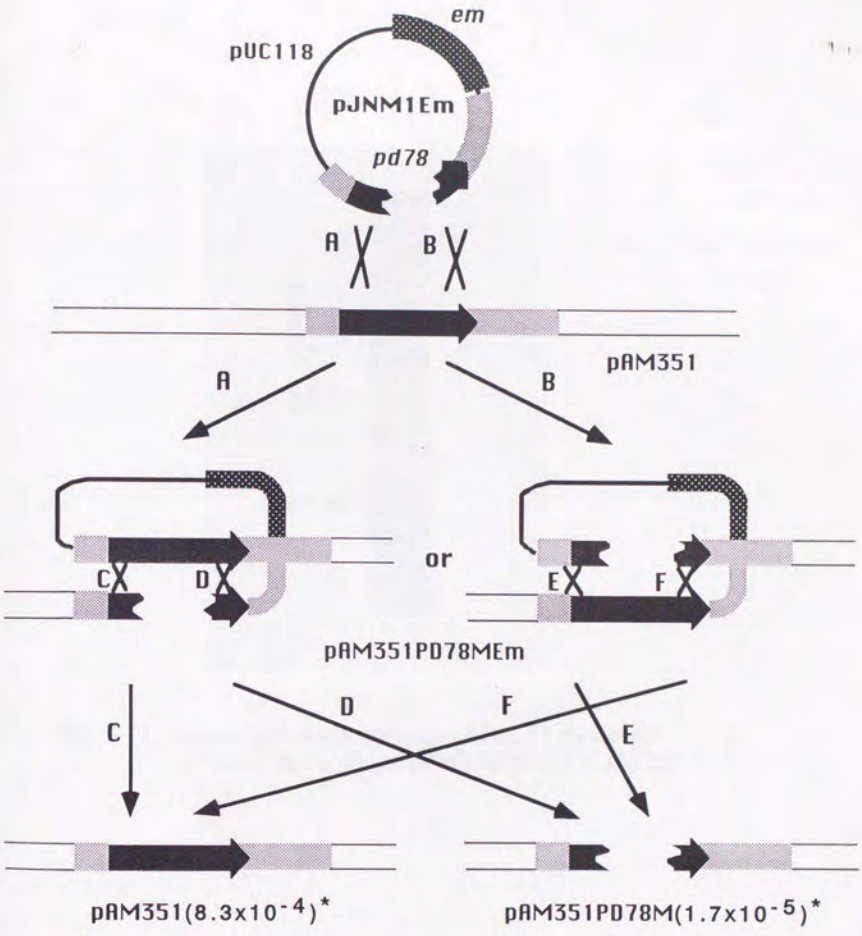


Fig. 3-10. Strategy for gene disruption of *pd78*

The suicide vector pJNM1Em was transformed into OG1X(pAM351). The first recombination occurred at A(upstream of the deletion) or B(downstream of the deletion) and generated the Em^r plasmid pAM351Em. The second recombination at C or F generated the wild-type pAM351. The recombination at D or E generated pAM351M1 having the mutant allele. *Values in parentheses indicate transfer frequency per donor.

Fig. 3-11 Agarose gel electrophoresis of *Eco* RI digests of pAM351 (Lane 1) and pAM351PD78M (Lane 2).

3. DISCUSSION

3.1. AD74

The nucleotide sequence and amino acid sequence revealed that AD74 is derived from the N-terminal half of the Asa1 protein which is anchored onto the membrane. The gene encoding the Asa1 protein, designated *asa1,* was cloned from the *Eco* RI-f region of pAD1 (Galli *et al.* 1990). This region was found to be required to form pheromone-inducible aggregates by mutagenesis with Tn*917* (Ehrenfeld *et al.* 1987). In this study, anti-AD74 polyclonal antibody blocked cAD1-induced self-clumping. These data proved that Asa1 acts as the aggregation substance (asa1 means aggregation substance coded by pAD1). The anti-AD74 antibody also blocked cAD1-induced pAD1 transfer. It seems inevitable that the blocking of aggregation reduced the transfer frequency of the plasmid. The anti-AD74 antibody blocked the transfer frequency of pPD1 very weakly (Table 3-1) in spite of the blocking of cPD1-inducible self-clumping as shown in Fig. 3-5. The reason might be that the antibody could not block the AS completely and the residual free AS led to a small aggregate, which did not sediment, and plasmid transfer occurred.

Anti-AD74 antibody cross-reacted with 153 kDa proteins in cPD1- or cCF10-induced cell wall extract and blocked inducible self-clumping. These results indicate that asa1-like proteins act as aggregation substance generally in strains carrying the pheromone responsive plasmid. However, the anti-AD74 antibody did not cross-react with any protein in cAM373-induced cell wall extract and could not block self-clumping of pAM373-harboring cells. These results coincide with the data of Galli *et al.* indicating that there was a segment highly homologous (more than 80 %) to *asa1* in every pheromone responsive plasmid except for pAM373 (Galli *et al.* 1991).

Recently, the genes of these Asa1-like proteins, *asp1* (Galli *et al.* 1992) and *asc10* (Kao *et al.* 1991; Olmsted *et al.* 1991), have been cloned and sequenced. These aggregation substance proteins, Asa1, Asp1, and Asc10, exhibited the amino acid motifs Arg-Gly-Asp-Ser and Arg-Gly-Asp-Val. These motifs have been identified as recognition sequences for eukaryotic membrane receptors, the so-called integrins (Pierschbacher *et al.* 1984; Hynes *et al.* 1987; Ruoslahti *et al.* 1988). This has opened the very interesting possibility that *E. faecalis* aggregation substances mediate binding to eukaryotic cells, indicating a possible role of the adhesin as a virulence factor.

3.2. PD78

The nucleotide sequence and amino acid sequence of PD78 indicates that PD78 undergoes signal sequence processing, but it has no membrane-anchored consensus sequence (Fischetti *et al.* 1990) and no membrane spanning hydrophobic region. Indeed, PD78 was extracted in solution without detergent. Thus, it seems likely that PD78 is localized noncovalently in the capsular layer on the cell surface.

Gene disruption of *pd78* did not affect cPD1-inducible self-clumping. This result indicates that PD78 was not responsible for pheromone-inducible self-clumping. However, the anti-PD78 antibody inhibit cPD1-induced self-clumping. The effect of the antibody on self-clumping might be caused by steric hindrance of the immunoglobulin molecules bound to the cell surface. On the other hand, the gene-disruption of *pd78* reduced the cPD1-induced transfer frequency to 10 % of the wild-type level. This result indicates that PD78 contributes but is not essential to plasmid transfer. There are two possible explanation for these results. One is that PD78 may contribute to form the channel for transfer of plasmid DNA. However, this seems unlikely because

pAM351PD78M could undergo cPD1-induced transfer at a frequency higher than 10^3 times that of cPD1-uninduced pAM351. The other explanation is that PD78 may be involved in stabilization of the mating pair while plasmid DNA is transferred. This is likely similar to the case of the TraN product of the F plasmid of *E. coli* (Maneewannakul *et al.* 1992). The TraN product is an outer-membrane protein of *M*r. 66,000 required to stabilize mating aggregates while interacting with an inner-membrane protein TraG. However, there was no sequence similarity between PD78 and TraN.

PD78 contains a fifteen times tandem repeated sequence of X-X-Pro (amino acid residue 146-190). The similar repeated sequence has been found in several gene products described in Table 3-1. TraD protein is incorporated into the cell envelope and is believed to contribute to transport of plasmid DNA through the cell envelope or strand separation (Kingsman *et al.* 1978). ORF C and PrgC have a protein similarity with predicted molecular weights and the deduced amino acid sequence exhibiting some motif sequences of signal sequence, membrane-anchored sequence and membrane-associated sequence. The strong similarity between ORF C and PrgC suggests that they may function in a step of plasmid transfer that is common both to pheromone-inducible plasmids which transfer efficiency in liquid and to broad-host-range plasmids such as pAMß1 (Clewell *et al.* 1974), which generally requires solid surfaces to transfer. US11 and PRO21 have a function of DNA binding. Considering all of the above things, it is likely that the proline-rich repeated sequece of PD78 interacts with DNA.

In comparison with AD74, some mutated derivatives of pAD1 having an insertion in the G region showed the same phenotype as pAM351PD78M. The G region is located downstream from the aggregation substance gene, as is *pd78* (Fig. 0-3), suggesting the G region may encode the PD78-like

protein. In the southern blotting analysis, the *Eco* RI-A fragment of pAD1 containing the G region hybridized very weakly with the labeled nucleotide probe of *pd78* (data not shown). Analysis of this region would be helpful to consider the function of PD78.

SUMMARY

The process of sexual recognition, of which partner male or female, is a basic problem encountered in the search for an appropriate sex partner. In *E.faecalis,* the sex pheromone plays a key role and pheromone-responsive plasmids possess ingenious systems to use the pheromone efficiently. Apparent pheromone shutdown is one of these systems. With this system, once a recipient acquires a copy of the plasmid, the transconjugant apparently shuts down the activity of the corresponding pheromone. This represents a sort of sexual diversion, that is, the transconjugant no longer behave as a recipient for the given plasmid. In another view, this system functions to shield the host cell from self-response to endogenous pheromone.

In Chapter I, quantitative analyses of pheromones and inhibitors were performed in an effort to determine the true nature of the apparent pheromone shutdown. pAM351(pPD1::Tn916) reduced the true titer of cPD1 to 20 % of the level found in recipient cells and produced a certain amount of iPD1. This indicated that pPD1 encodes the pheromone inhibitor and the true pheromone shutdown. pAM714(pAD1::Tn917) in OG1X cells reduced titers of both cAD1 and iAD1 to nearly zero. This was thought to be caused by the TraC product which is a putative binding protein for pheromone/inhibitor. The *traC*-disrupted pAD1 (pAM2270) in OG1X cells reduced the titer of cAD1 to 20 % of the level found in recipient cells and produced a certain amount of iAD1, similar to pAM351. The titers in pAM2270-carrying cells should represent the real amounts of pheromone and inhibitor secreted from donor cells and indicated that pAD1 also encodes the pheromone inhibitor and the proteins responsible for true pheromone shutdown. pCF10 did not reduce the titer of cCF10. pAM714 in FA2-2 cells also did not so much

reduce the titer of cAD1 (about 50 % of recipient level). Both plasmids produced enough iCF10 to block the activity of the secreted pheromones. Considering that pAD1 and pCF10 encode the putative pheromone shutdown genes, *traB* and *prgY*, these results suggest that pheromone shutdown is controlled by some mechanism such as feedback regulation of the pheromone response. In summary, the apparent pheromone shutdowns was caused by the pheromone inhibitor and/or the true pheromone shutdown. Conceivably, the pheromone responsive plasmids encode double systems, pheromone shutdown and pheromone inhibitor. In another view, the plasmids seem to control the level of pheromone, limiting it to a range where the inhibitor is able to overcome the activity of the pheromone.

In Chapter II, the so-called regulatory region of pPD1, which had been expected to contain the pheromone inhibitor gene and pheromone shutdown genes, was analyzed. The region was located in the AL-fragment (16-kb *Eco* RI-*Sal* I). A *Eco* RV-*Hin* cII 5.4 kb segment of the AL-fragment was sequenced and found to be organized as *-traC-traB-traA-ipd-* which is same as the corresponding region of pCF10 and similar to that of pAD1 (*-traB-traC-traA-iad*). *ipd* encodes a peptide consisting of 21 amino acids, of which the C-terminal eight residues possess the sequence of iPD1. *E. faecalis* and *E. coli* transformed with a vector plasmid containing *ipd* showed the activity of iPD1 in the culture broth. The *traC* gene encodes a 60.8 kDa protein with a potential signal sequence. The putative TraC product has a strong similarity to oligopeptide binding proteins of *Escherichia coli, Salmonella typhimurium,* and *Bacillus subtilis* as well as those of pCF10 and pAD1. Genetic disruption of *traC* reduced the sensitivity of the host to cPD1. These data suggest that the TraC product contributes to pheromone sensitivity as a pheromone-binding protein but it is not essential for the

pheromone response. The *traB*/pPD1 encodes a 43.5 kDa protein having no potential signal sequence but containing a very hydrophobic region on its C-terminal one-third. Donor cells carrying *traB*-disrupted pPD1 produced the same level cPD1 as found in plasmid-free recipient cells and self-clumping occurred constitutively, showing that TraB/pPD1 is responsible for pheromone shutdown. TraB/pAD1 and PrgY/pCF10 are expected to function similarly in pheromone shutdown. As described above, the expression of pheromone shutdown seems to be controlled. The products encoded by the putative shutdown genes might be regulated, possibly at the level of the transcription or post-translation.

In Chapter III, the pheromone-inducible surface proteins, AD74 and PD78, which are thought to be responsible for some events involved in conjugative plasmid transfer, were characterized. PD78 was encoded on *Eco* RI-C fragment of pPD1. The calculated molecular mass was 53 kDa which was much different from 78 kDa estimated by SDS-PAGE. The extraction experiment and sequencing data suggest that PD78 is localized in the capsule layer on the surface of cPD1-induced donor cells. Anti-PD78 antibody exerted an inhibitory effect against cPD1-induced self-clumping and pPD1 transfer. Strain OG1X carrying *pd78*- disrupted pAM351, designated pAM351PD78M, showed the same phenotype as OG1X(pAM351) relating to cPD1-inducible self-clumping. On the other hand, transfer of pAM351PD78M occurred at about 10 % of the level of pAM351 in broth. Conceivably, PD78 is involved in the stabilization of aggregates and/or the formation of the conjugation bridge. In the *Eco* RI-A fragment of pAD1, there was found a segment, designated the G region, potentially having a function similar to PD78.

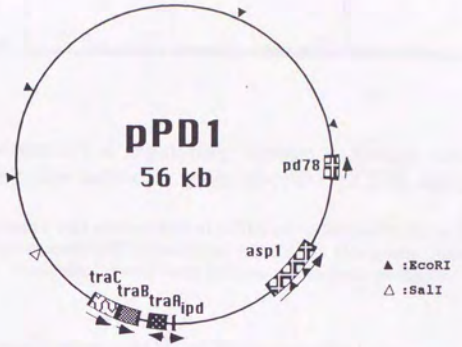
AD74 was found to represent the N-terminal half (Ala-1 from 510-Lys) cleaved from the Asa1 protein. The Asa1 product had a calculated molecular weight of 137 kDa (153 kDa on SDS-PAGE) and had a potential membrane-anchoring sequence and a peptidoglycan spanning sequence. Indeed, Asa1 was extracted by lysozyme digestion. Western blotting with anti-AD74 antibody revealed that there were Asa1-like proteins in the cPD1- and cCF10-induced cell wall extract. The anti-AD74 antibody could block cAD1-, cPD1-, and cCF10-induced self-clumping. These data suggest that Asa1-like protein acts as aggregation substance (AS), which mediates cell-to-cell contact in sexual aggregation, generally in the case of the pheromone conjugative plasmids.

Fig. 4-1(A) is a map of pPD1 showing the location of genes found in previous and this study. Fig. 4-1(B) is a map of pAD1 reported by Clewell (1993b). The organization of regulatory region and AS gene in pPD1 was found to be similar to the corresponding regions in pAD1 and pCF10 as shown in Fig. 4-2. In analogy with pAD1 and pCF10, the non-sequenced region between *asp1* and *ipd* was thought to contain positive-regulator gene to activate AS gene, and a gene encoding surface exclusion protein. Table 4 summarizes profiles of the reading frames described in Fig. 4-2.

Fig. 4-3 is a model summarizing this study and showing the plasmid-encoded products and their role in the pheromone response. In *E. faecalis* multiple sex pheromones, cX, cY, cZ, and so on are chromosomally encoded. A certain pheromone-responsive plasmid pX encodes genes for pheromone inhibitor (iX) and pheromone shutdown (TraB), that both function to prevent the host from self-response to the endogenous pheromone. Pheromone shutdown does not completely suppress the production or secretion of the pheromone cX but controls its

level below the range where the secreted iX can overcome the cX. When the recipient comes close to the donor, the cX secreted from the recipient outcompetes the iX. The TraC product acts as an initial receptor for cX and contributes to pheromone sensitivity. However, the TraC products is not essential to the cX response. That is, the cX-signal may be transmitted into the plasmid inside of cell membrane in the absence of the TraC product. The signal of cX triggers the expression of surface proteins involved in the conjugative process such as AS (Asa1-like protein) and PD78(-like protein).

(A)



(B)

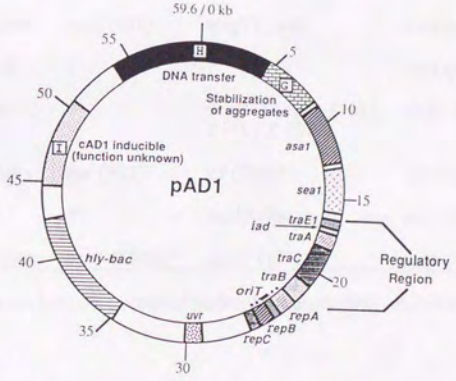


Fig. 4-1. Map of pPD1 showing the location of genes found in previous and this study (A) and map of pAD1 reported by Clewell (1993b).

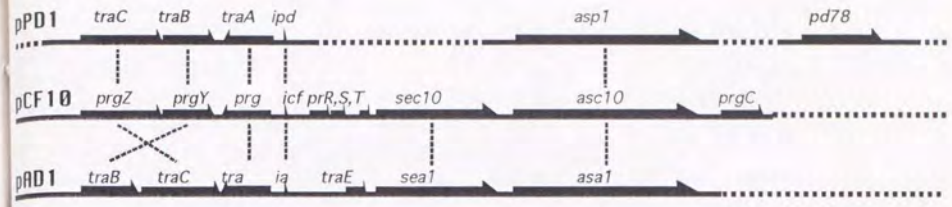


Fig. 4-2. Constructions of regulatory, surface exclusion, and aggregation substance genes of pPD1, pCF10, and pAD1.

The location orientation, and relative size of ORFs are indicated by the arrows. Related information for each ORF is tabulated in Table 4. Horizontal lines indicate the sequenced region. Horizontal dotted lines indicate not-sequenced region.

Table 4. ORF profiles of pAD1, pCF10, and pPD1.

	Determinant		
Function/Role	pPD1	pCF10	pAD1
Pheromone sensing	*traC* (61k)	*prgZ* (61k)	*traC* (61k)
Pheromone shutdown	*traB* (43k)	*tprgY?* (44k)	*traB* (44k)
Negative regulation	*?*	*?*	*traA* (39k)
Positive regulation	*?*	*prgR* (14k), *S*, (10k), *T* (8k), *X* (37k)	*traE1* (14k)
Pheromone inhibitor	*ipd* (912)*	*icf* (789)*	*iad* (818)*
Surface exclusion	*?*	*sec10* (98k)	*sea1* (100k)
Aggregation substance	*asp1* (138k)*	*asc10* (137k)*	*asa1* (137k)*

Values in parentheses indicate calculated molecular weight (Da). Asterisks indicate the mature proteins.

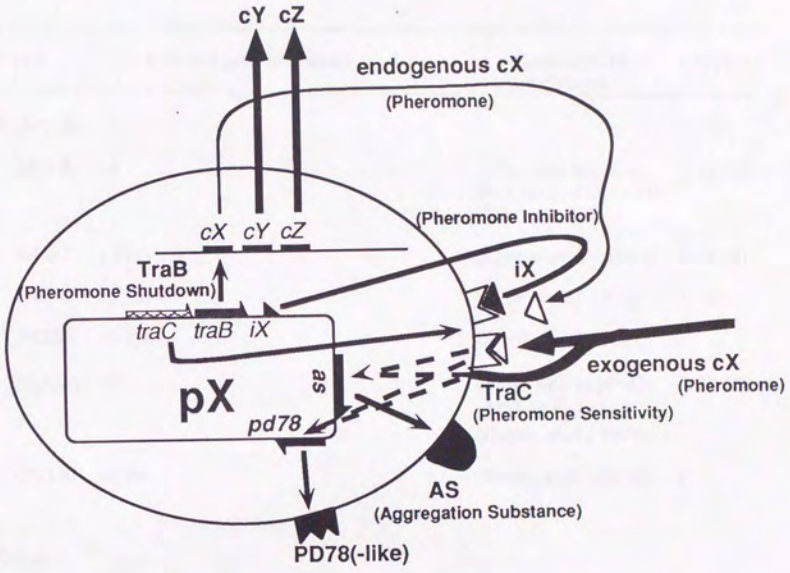


Fig. 4-3. Model showing the plasmid-encoded products and their role in the pheromone reponse

Appendix 1 Bacterial strains and plasmids

Abbreviations used in this section: *str,* streptomycin resistance; *rif,* refampin resistance; *fus,* fusidic acid resistance; *spc,* spectinomycin resistance; *tet,* tetracycline resistance.

1. Bacterial Strains

Strain	Relevant genetic markers	Comments or reference	Chapter
E. faecalis			
OG1X	*str*	Gelatinase-negative derivative of OG1-10, (Ike *et al.*, 1983)	I, II, III
FA2-2	*rif fus*	(Clewell *et al.*, 1982)	I, II, III
JH2-2	*rif fus*	(Jacob *et al.*, 1974)	I, III
JH2SS	*str spc*	(Franke *et al.*, 1981)	I
39-5Sα	*str*	carries pPD1, pPD2, pPD3 and pAMα1 (Dunny *et al.*, 1979)	I, II, III
OG1RF	*rif fus*	(Dunny *et al.*, 1978)	I
E. coli			
HB101	*supE44, hsdS20*($r_B^-m_B^-$), *recA13, ara-14, proA2, lacY*, *galk2, rspL20, xyl-5, mtl-1*	(Bolivar *et al.*, 1979)	II
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*)F' [*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	(Yanisch-Peron *et al.*, 1985)	II, III
DH5	*supE44, hsdR17, recA1, gyrA96, thi-1, relA1*	(Low *et al.*, 1968)	II

2. Plasmids (*E. faecalis*)

Plasmid	Relevant genetic marker	Sex pheromone	Comments and reference	Chapter
pPD1	Bacteriocin	cPD1	(Tomich, 1979)	
pAM351	Bacteriocin, *tet*	cPD1	pPD1::Tn916 (Tomich, 1979)	I, II, III
pAM351BM	Bacteriocin	cPD1	constitutive clumping (Δ*traC*), this study	II
pAM351CM	Bacteriocin	cPD1	less sensitive to cPD1(Δ*traB*), this study	II
pAM351BCM	Bacteriocin	cPD1	partially/constitutively clumping (Δ*traBC*), this study	II
pAM351PD78M	Bacteriocin	cPD1	low level transfer (Δ*pd78*), this study	III
pAD1	Hemolysin/bacteriocin	cAD1	(Dunny, 1975)	
pAM714	Hemolysin/bacteriocin, *erm*		(Ike, 1984)	I, III
pAM2270	Hemolysin/bacteriocin	cPD1	PR27 deletion (Δ*traC*) (Weaver ,1990)	I
pCF10	*tet*	cCF10	(Dunny, 1981)	I
pAM373	cryptic	cAM373	(Clewell, 1985)	I
pAM377	cryptic, *erm*	cAM373	pAM373::Tn917, less sensitive to cAM373 (Clewell, 1985)	I

3. Plasmid (*E. coli-E. faecalis*; used in Chapter II)

Plasmid	Description	Reference or source
pAM401	*tet cat*(*E. coli*), *cat* (*E. faecalis*)	(Wirth *et al.*, 1986)
pAM401AL	*Eco* RI-*Sal* I 16-kb of pPD1 cloned into pAM401	(Univ of Michigan) (Clewell *et al.*, unpublished)
pAM401AS	*Eco* RI-*Sal* I 6-kb of pPD1 cloned into pAM401	(Clewell *et al.*, unpublished)
pAM401B	*Eco* RI-b of pPD1 cloned into pAM401	(Clewell *et al.*, unpublished)
pAM401C	*Eco* RI-c of pPD1 cloned into pAM401	(Clewell *et al.*, unpublished)
pAM401D	*Eco* RI-d of pPD1 cloned into pAM401	(Clewell *et al.*, unpublished)
pAM401E	*Eco* RI-e of pPD1 cloned into pAM401	(Clewell *et al.*, unpublished)

4. Plasmid (*E. coli*)

Plasmid	Description	Reference or source	Chapter
pUC118	*amp*	(Messing, 1983)	II, III
pUC119	*amp*	(Messing, 1983)	II
pVA891	*cat* (*E. coli*), *erm* (*E. faecalis*)	(Macrina, 1983)	II
pPH53	5.3-kb *Pst* I-*Hin* dIII of AL-fragment cloned into pUC118	this study	II
pHH21	2.3-kb *Hin* cII-*Hin* cII of pPH53 cloned into pUC118	this study	II
pPS32	2.9-kb *Pst* I-*Spe* I of pHP53 cloned into pUC119	this study	II
pEVP5	0.8-kb*Eco* RV-*Pst* I of AL-fragment cloned into pUC118	this study	II
pUCBM	*traC* (2.4-kb *Eco* RV-*Sna* BI of AL-fragment) having a lesion at *Pst* I site cloned into pUC119	this study	II
pUCCM	*traB* (2.9-kb *Pst* I-*Spe* I of pPH53) having a lesion at *Sna* BI xite cloned into pUC119	this study	
pUCBMEm	*term* of pVA891 cloned into pUCBM	this study	II
pUCCMEm	*term* of pVA891 cloned into pUCCM	this study	II
pJN1	*pd78* (3.7-kb *Bgl* II-*Eco* RI of pPD1) cloned into pUC118	this study	III
pJN1MEm	*pd78* having a lesion at *Acc* III site and *erm* of pVA891 cloned into pUC118	this study	III

Appendix 2 Media and antibiotics

1. MEDIA

LB medium (Luria-Bertani Medium)

bacto-trypton	10 g
bacto-yeast extract	5 g
NaCl	10 g
	1 liter

For *E. coil*

THB medium (Todd-Hewitt Broth medium)

Todd-Hewitt Broth (Oxoid)	36.4 g
	1 liter

For *E. faecalis*

N2GT medium

Nutrient Broth no. 2 (Oxoid)	25 g
Glucose	2 g
Tris-HCl	0.1 M
	1 liter

For *E. faecalis*

*For solid plate, bacto-agar used at 1.5%

2. ANTIBIOTICS

Antibiotic	*E. coli*	*E. faecalis*
Ampicillin	60 μg/ml	-
Chloramphenicol	25 μg/ml	25 μg/ml
Tetracycline	50 μg/ml	10 μg/ml
Erythromycin	150 μg/ml	50 μg/ml

Appendix 3 Bioassay of Pheromones and Inhibitors

Medium and donor strains used in each bioassay of pheromone or inhibitor are described in Table 1.

Pheromone/Inhibitor	Donor strain	Medium
cPD1/iPD1	39-5Sα	N2GT
cAD1/iAD1	OG1S(pAM714)	THB
cCF10/iCF10	OG1RF(pCF10)	THB
cAM373/iAM373	FA2-2(pAM373)	THB

(1) Sample solutions are added to U-shaped wells of a bottom microtiter plate. If sample is aqueous solution, the solution is diluted (two-fold) using 0.05-ml microdiluters. If sample contains organic solvent, the sample is diluted into 50 % acetonitrile and evaporated.

(2) The donor cells from mid- or late-log phase are diluted and added to each well to a final A_{660} of 0.1. For inhibitor assay, the corresponding pheromone was added to be 100 pg/ml. The titer was read after 120 min incubation at 37°C on a shaker.

Appendix 4 Preparation of Plasmid from *E. faecalis*

Step	Detail of following protocol	
	Screening	Preparative
Grow cells to A600=0.2-0.4	10-20 ml	600 ml
Harvest grown cells by centrifugation	5000 x g	7000 x g
Resuspend pelleted cells in 6.7 % sucrose-50 mM Tris-1 mM EDTA, pH 8.0	379 µl	30 ml
Warm to 37°C		
Add lysozyme (10 mg/ml in 25 mM Tris, pH8.0)	96.5 µl	7.5 ml
Incubate for 5 min at 37°C		
Add 0.25 M EDTA-50 mM Tris, pH 8.0	48.6 µl	3.75 ml
Add sodium dodecyl sulfate (20 % [wt/vol] in 50 mM Tris-20 mM EDTA, pH 8.0)	27.6 µl	2.25 µl
Mix immediately		
Incubate for 5 to 10 min at 37°C to complete lysis		
Vortex at highest setting for 30 s in an appropriate tube	1.5-ml Eppendorf	15 ml per tube (50 ml Centrifuge Tube [Corning])
Add fresh 3.0 N NaOH	2.6 µl	2.40 ml
Mix gently by intermittent inversion or swirling for 10 min	Inversion	Swirl in 300-ml centrifuge bottle
Add 2.0 M Tris-HCl, pH 7.0	49.6 µl	3.90 ml
Continued gentle mixing for 3 min		
Add 5.0 M NaCl	717 µl	5.7 ml
Add phenol saturated with 3 % NaCl; mix thoroughly	700 µl	5.7 ml
centrifuge	5 min	7000 x g, 10 min
Remove upper phase and extract with the saturated phenol-chloroform-isoamyl alcohol (25:24:1)	700 µl	55.8 ml
Remove upper phase, precipitate with 1 vol of isopropanol		
Incubate at 0°C	>30 min	>60min
Centrifuge	5 min at 16,000 rpm	20 min at 10,000 x g
Remove excess isopropanol and add EtOH	1 ml	60 ml
Centrifuge	5 min at 16,000 rpm	10 min at 10,000 x g
Remove excess EtOH and evaporate in vacuo.		

Modified from Anderson *et al.*

Appendix 5 Transformation of *Enterococcus faecalis* by electroporation

1. Grow *E. faecalis* cells overnight in Todd-Hewitt Broth containing 0.5 M sucrose and 6% Glycine.
2. Harvest cells by centrifugation at 2000 x g.
3. Wash the cells twice with ice-cold electroporation solution (0.5M sucrose and 10% glycerol).
4. Resuspend the cells in 1/100 the original volume of electroporation solution and freeze them at -80°C. Both fresh and frozen cells are highly competent for electroporation.
5. Electroporation is carried out with the Gene Pulser (Bio-Rad Laboratories). Frozen cells are thawed on ice. One microliter of DNA is mixed in the 0.2 cm cuvette with 50-100 μl of competent cells immediately prior to electroporation. Electroporation is carried out at single pulse, 2.5 kV, 25 μF, 200 Ω.
6. The discharged cell suspension is immediately diluted to 1.0 ml with ice-cold THB medium containing 0.5 M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and kept it on ice for 5 min and then incubated for 2 hr at 37°C.
7. The cells are then spread on THB agar plate containing appropriate antibiotics to select for transformants.

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CHAPTER I

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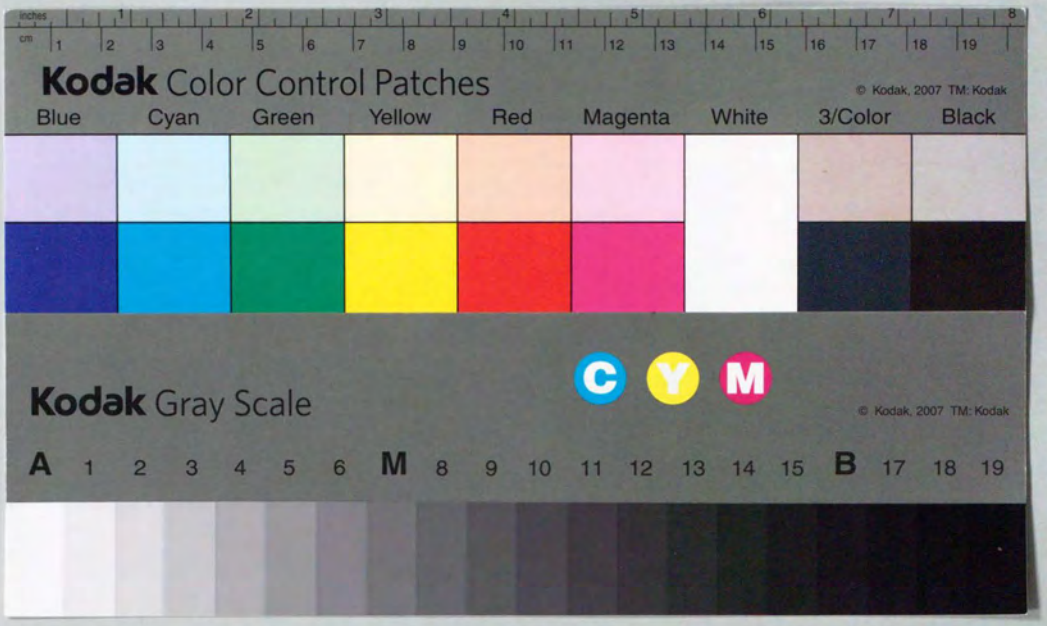
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