Amino acid sequence of pheromone-inducible surface protein in *Enterococcus faecalis*, that is encoded on the conjugative plasmid pPD1

Jiro Nakayama[1], Hiromich Nagasawa[1], Akira Isogai[1], Don B. Clewell[2] and Akinori Suzuki[1]

[1] *Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan* and [2] *Departments of Oral Biology and Microbiology/Immunology, School of Dentistry and Medicine, The University of Michigan, Ann Arbor, MI 48109, USA*

Received 16 April 1990

The major pheromone-inducible protein, PD78, believed to contribute to bacterial conjugation, was purified from *Enterococcus* (formerly *Streptococcus*) *faecalis* cells containing the plasmid pPD1. A cloned *Eco*RI-*Bgl*II 3.6-kbp fragment of the plasmid pAM351 (pPD1::Tn*916*) contained an open reading frame corresponding to 467 amino acid residues representing PD78. In a central region of the deduced protein, there is a repeated sequence of X-X-Pro that is repeated 15 times. This is analogous to the Gln-Gln-Pro repeat in the C-terminal region of TraD product encoded on the R100 plasmid in *Escherichia coli*

Sex pheromone, Conjugative plasmid; Plasmid transfer; Adhesin; Aggregation substance, *Enterococcus faecalis*

1. INTRODUCTION

Certain conjugative plasmids in *Enterococcus* (formerly *Streptococcus*) *faecalis* encode a mating response to specific sex pheromones [1–4] excreted by plasmid-free recipient cells [5,6]. The pheromone response involves an induced aggregation of donors and recipients as well as transfer of plasmid DNA [5,6]. It has been proposed that aggregation is caused by binding between an adhesin designated 'aggregation substance' (AS) on the donor surface and a receptor designated 'binding substance' (BS) on the recipient [6]. Self-aggregation or clumping can also occur when donor cells alone are exposed to a culture filtrate (i.e. pheromone) of the recipient cells. BS was presumed to be chromosomally determinant and commonly present on the surface of both donor and recipient [6]. On the other hand, AS was presumed to be encoded on the plasmid and induced by the corresponding sex pheromone [6].

AS was presumed to be a protein, since the formation of aggregates was sensitive to trypsin, pronase, SDS and heat, but insensitive to the lysozyme or lipase [7]. Four novel proteins appeared on surface of pPD1-carrying donor cells after exposure to the corresponding sex pheromone, cPD1, and these were designated PD157 (157 kDa), PD153 (153 kDa), PD130 (130 kDa) and PD78 (78 kDa) [8]. These proteins were believed to contribute to the mating response as they appeared 30–45 min after induction by sex pheromone, when aggregation first became apparent [8,9]. Furthermore, because antiserum raised against these proteins blocked cell aggregation, one or some of them were thought to be AS [9].

In the present study, we purified PD78 which was predominant in these inducible proteins and also cloned and sequenced a segment of plasmid DNA that encodes this protein.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

PD78 and plasmid pAM351 (pPD1::Tn*916*) were prepared from a strain *E. faecalis* OG1X (pAM351) [10]. *E. coli* strain JM109 and plasmid vector pUC118 have been previously described [11,12].

2.2. *Purification of PD78*

In each step of the purification, PD78 was followed by monitoring the pheromone-inducible 78 kDa band on SDS-polyacrylamide gel electrophoresis [8]. All extraction and purification procedures were performed at 4°C. OG1X (pAM351) cells were cultured for 4 h in the Todd-Hewitt Broth (Oxoid) medium, and self-aggregation was induced by synthetic sex pheromone cPD1 [4]. The amount of cPD1 added was 16-fold in excess of the minimum amount required for clumping. The aggregated cells were harvested by decantation followed by centrifugation at 7000 × *g* for 30 min. PD78 was extracted with 50 mM Tris-HCl buffer (pH 8.4) for 2 h with gentle shaking of the cells. The extract was purified by two-step DEAE-Sepharose CL-6B column chromatography in 50 mM Tris-HCl: in the first (3.3 × 13 cm), elution was with 0.3 M NaCl; in the latter (1.4 × 18 cm), elution involved a linear gradient 0 to 0.3 M NaCl. The final step utilized Sephacryl S-300 gel chromatography (1.9 × 98 cm) in 0.2 M ammonium acetate, pH 7.2.

Correspondence address: A. Suzuki, Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Abbreviations: SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; kDa, kilodalton; kbp, kilobase pair; N-terminal, amino terminal; C-terminal, carboxyl terminal

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.3. *Amino acid sequence analysis*

Amino acid sequence analysis was performed on a gas-phase sequencer (Applied Biosystems 470A or 477A) equipped with an on-line PTH-amino acid analyzer (Applied Biosystems 120A). Samples (100–200 pmol) were applied to the polybrene-coated glass filter.

2.4. *Lysyl endopeptidase digestion*

PD78 (20 μg) was digested with lysyl endopeptidase (Wako-junyaku) according to the methods reported by Takayama et al. [13]. The resulting peptides were separated by reversed-phase HPLC on a Senshu Pak SC4-1251 column.

2.5. *Cloning and sequencing of the PD78 gene*

An oligonucleotide probe for Southern hybridization was synthesized on the basis of 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]AA3′, [ ] means mixture) corresponding to Phe-2 to Lys-7 of the N-terminal amino acid sequence of PD78.

Plasmid pAM351 was prepared by the alkaline method described by Dunny et al. [14]. Plasmid DNA from *E. coli* was prepared by the alkaline method [15]. The pAM351 was digested with *Eco*RI and *Bgl*II. A 3.6-kbp *Eco*RI-*Bgl*II fragment which hybridized with the probe was subcloned into the plasmid pUC118. Deletion mutants for sequencing were constructed by exonuclease III digestion [11]. Sequence analysis utilized the dideoxynucleotide method of Messing [16].

3. RESULTS AND DISCUSSION

3.1. *Extraction and purification of PD78*

PD78 was easily extracted by gentle washing of the cells with buffer, and without addition of detergent or EDTA. Therefore, it is presumed that PD78 is attached to the cell surface very weakly – probably through hydrophobic and/or ionic interaction. 3 mg of PD78 was obtained from 180 g wet OG1X (pAM351) cells using 3 steps of column chromatography. The purified preparation of PD78 revealed a single band upon SDS polyacrylamide gel electrophoresis.

3.2. *Amino acid sequencing of PD78*

The N-terminal amino acid analysis of PD78 revealed the sequence Ala-1 to Pro-17 (Fig. 2).

In order to determine regions within PD78, the protein was enzymatically cleaved by lysyl endopeptidase. 23 peptide fragments were sequenced and totally 341 amino acid residues were identified (Fig. 2).

3.3. *Cloning and sequencing of PD78 gene*

Southern hybridization using an oligonucleotide complementary to the N-terminal amino acid sequence of PD78 specifically revealed a 3.6-kbp *Eco*RI-*Bgl*II fragment (Fig. 1A) of pAM351. This fragment was subcloned into sequencing vector pUC118 (pJN1; Fig. 1B). Fig. 2 shows the DNA sequence from pJN1 and the deduced amino acid sequence. pJN1 had an open reading frame corresponding to encode the entire PD78 sequence, which was preceded by potential Shine-Dalgarno sequence and terminated by TAA stop codon. However, no obvious RNA polymerase binding sequence was present. There is a palindromic sequence,

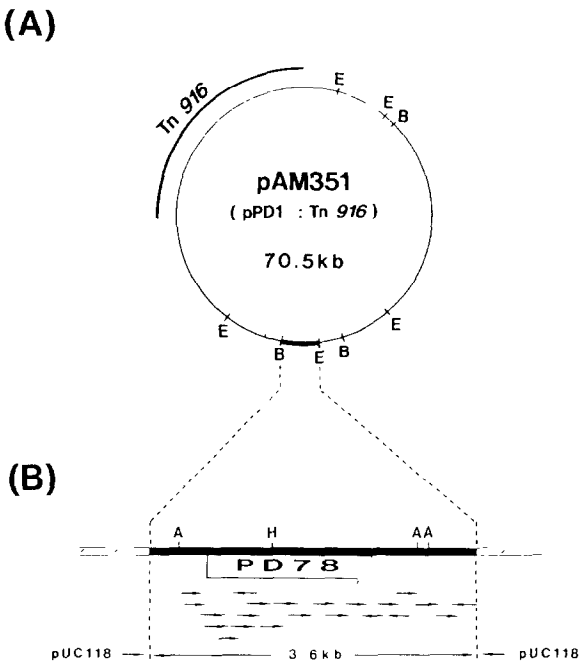

Fig. 1. (A) A circular map of pAM351. pAM351 was mapped with respect to sites cleaved by *Eco*RI and *Bgl*II and the map of pPD1 described by Yagi et al. [7]. Tn*916* is located in a 26.5-kbp *Eco*RI fragment (bold line). The wider line is the 3.6-kbp *Eco*RI-*Bgl*II fragment encoding PD78. (B) Structure of pJN1. The thick arrow indicates the direction of transcription and the region of the open reading frame of 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.

which could be related to regulation of PD78 expression, upstream (−66 to −30) of the open reading frame. The N-terminal and lysyl endopeptidase peptide sequences determined were fully contained in the amino acid sequence deduced from the nucleotide sequence. There is a hydrophobic sequence consisting of 33 amino acids preceding the N-terminus of PD78. Considering the fact that PD78 is an extracellular protein, this is probably a signal sequence. One of the lysyl endopeptidase peptides was fully consistent with the deduced C-terminal amino acid sequence. This result indicates that PD78 is not processed on its C-terminus.

There is a large discrepancy between the apparent molecular size of PD78 based on its mobility in SDS polyacrylamide gels (78 kDa, [9]) and the size calculated from the sequence data present here (53846 Da). The reason for this anomalous behavior in SDS polyacrylamide gel may be due to its unusual amino acid composition that is very rich in glutamic acid (13.8%) and lysine (11.5%) [17].

Interestingly, PD78 contains a 15 times tandem repeated sequence of X-X-Pro (amino acid residue 146–190) analogous to a 10 times repeated sequence of Gln-Gln-Pro in the C-terminal region in the product of *TraD* encoded on R100 plasmid in *E. coli* [18]. The TraD protein is believed to contribute to the transport

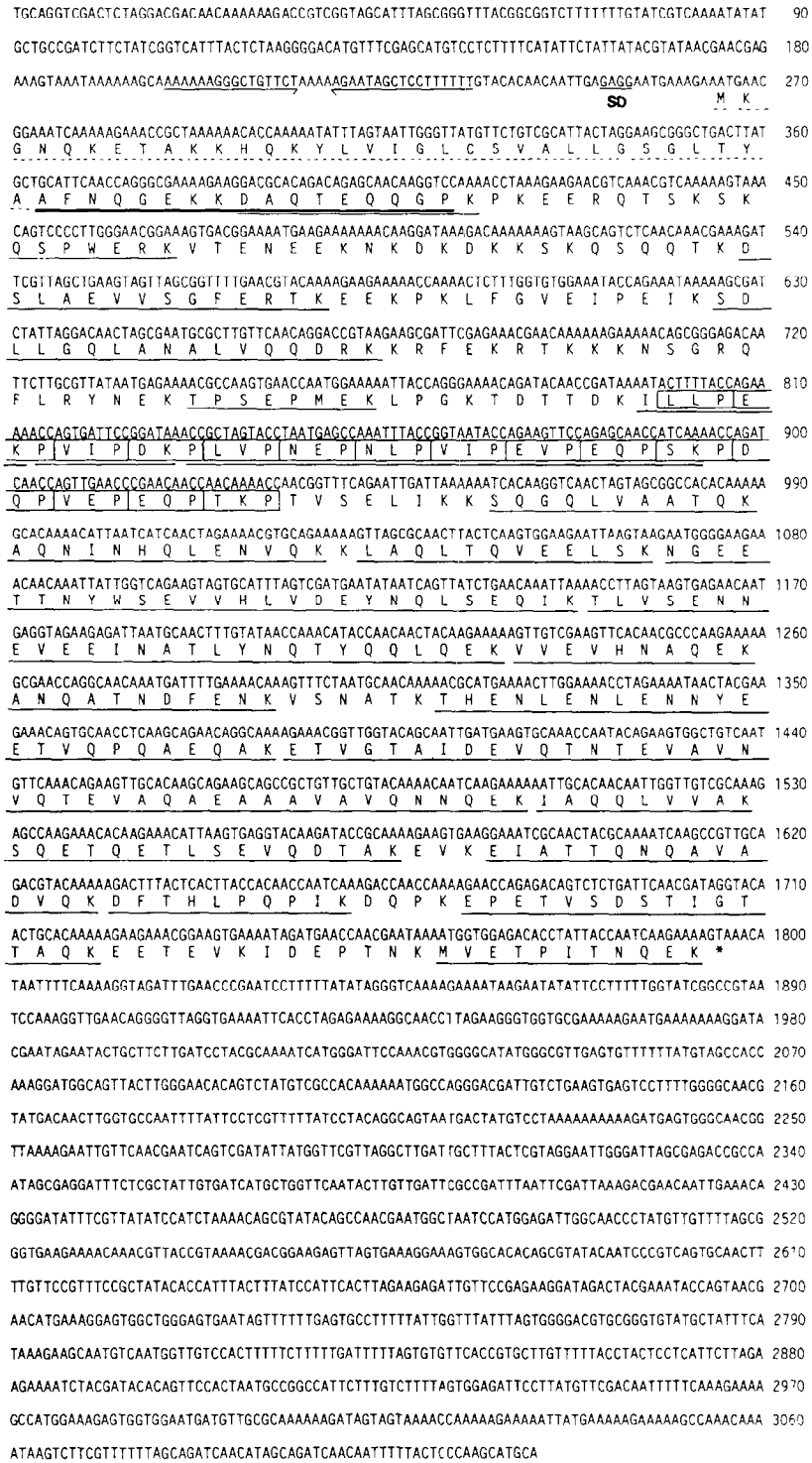

Fig. 2. DNA sequence and the deduced amino acid sequence of PD78. A pair of arrows show a palindromic sequence. SD indicates a Shine-Dalgarno sequence. Signal sequence is shown by dotted line. N-terminal amino acid sequence is shown using the bold line. The sequence identified from lysyl endopeptidase peptides is underlined. X-X-Pro repeated sequence is enclosed by boxes.

of plasmid DNA through the cell envelope or strand separation [19]. PD78 is believed to be involved with sexual aggregation but not necessarily plasmid transfer in *E. faecalis*. Such a repeated sequence has not been found in other proteins and its function is still uncertain. In efforts to study the mechanism of DNA transfer, it might be important to elucidate the function of these proline repeated sequences. The role of PD78 in cell aggregation and mating is being further investigated.

Acknowledgements: We thank Dr Ohtsubo for helpful discussions and Drs Chieko Kitada and Masahiko Fujino for providing the synthetic peptides cAD1 and cPD1. This work was partly supported by the grant from the Ministry of Education, Science and Culture, Japan and by Public Health Service Grant GM33956 from the National Institutes of Health, USA.

REFERENCES

[1] Mori, M., Sakagami, Y., Narita, M., Isogai, A., Fujino, M., Kitada, C., Craig, R.A., Clewell, D.B. and Suzuki, A. (1984) FEBS Lett. 178, 97–100.
[2] Mori, M., Tanaka, Y., Sakagami, Y., Isogai, A., Fujino, M., Kitada, C., White, B.A., An, F.Y., Clewell, D B. and Suzuki, A. (1986) FEBS Lett. 206, 69–72.
[3] Mori, M., Sakagami, Y., Ishii, Y., Isogai, A., Kitada, C., Fujino, M., Adsit, J.C., Dunny, G.M. and Suzuki, A. (1988) J. Biol. Chem. 263, 14574–14578.
[4] Suzuki, A., Mori, M., Sakagami, Y., Isogai, A., Fujino, M., Kitada, C., Craig, R.A. and Clewell, D.B. (1984) Science 226, 894–895.
[5] Dunny, G.M., Brown, B.L. and Clewell, D.B. (1978) Proc. Natl. Acad. Sci. USA 75, 3479–3483.
[6] Dunny, G.M., Craig, R.A., Carron, R.L. and Clewell, D.B (1979) Plasmid 2, 454–465.
[7] Yagi, Y., Kessler, R.E., Shaw, J.H., Lopatin, D.E., An, F.Y. and Clewell, D.B. (1983) J. Gen. Microbiol. 129, 1207–1215.
[8] Ehrenfeld, E.E , Kessler, R.E. and Clewell, D.B. (1986) J. Bacteriol. 168, 6–12.
[9] Kessler, R.E. and Yagi, Y. (1983) J. Bacteriol. 117, 360–372.
[10] Ike, Y., Craig, R.A., White, B.A., Yagi, Y. and Clewell, D.B. (1983) Proc. Natl. Acad. Sci. USA 80, 5369–5373.
[11] Yanishi-Peron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[12] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[13] Takayama, S., Isogai, A , Tsukamoto, C., Ueda, Y., Hinata, K., Okazaki, K. and Suzuki, A. (1987) Nature 326, 102–104.
[14] Dunny, G., Funk, C. and Adsit, J (1981) Plasmid 6, 270–278.
[15] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[16] Messing, J. (1983) Methods Enzymol. 101, 20–78.
[17] Burton, Z., Burgress, R R., Lin, J., Moore, D., Holder, S. and Gross, C.A. (1981) 9, 2889–2903.
[18] Yoshioka, Y , Fujita, Y. and Ohtsubo, E. (1990) J. Mol. Biol., in press.
[19] Kingsman, A. and Willetts, N. (1978) J. Mol Biol. 122, 287–300.