

# Isolation and structure of the bacterial sex pheromone, cAD1, that induces plasmid transfer in *Streptococcus faecalis*

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The *Streptococcus faecalis* sex pheromone cAD1, which is involved in the conjugative transfer of the hemolysin plasmid pAD1, has been isolated and its structure determined. Its  $M_r$  is 818 and its amino acid sequence is H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH. A replicate of the pheromone synthesized by the liquid-phase method showed the same biological activity and chromatographic behavior as the isolated cAD1.

Pheromone activity was detectable at a concentration of  $\sim 5 \times 10^{-11}$  M.

*Streptococcus faecalis*      Sex pheromone      Conjugative plasmid transfer      Amino acid sequence

## 1. INTRODUCTION

In *Streptococcus faecalis*, conjugative plasmids fall into two general categories. Members of one group transfer at a relatively high frequency ( $10^{-3}$ – $10^{-1}$  per donor) in culture broth; whereas the others transfer rather poorly in broth ( $< 10^{-6}$  per donor) but efficiently ( $\geq 10^{-4}$  per donor) when matings are conducted on solid surfaces [1]. In the systems where plasmids transfer readily in culture broth, recipient strains excrete multiple peptidal sex pheromones specific for donor strains harboring certain conjugative plasmids [2–4]. Donor cells induced by the pheromone synthesize an adherent surface structure which facilitates the formation of

mating aggregates [5]. If exposed to a culture filtrate of the recipient, donors are induced to self-clumping; for this reason, sex pheromones have also been called clumping-inducing agents (CIA) [3].

Recently, we have isolated the *S. faecalis* sex pheromone cPD1 ( $M_r$  912), which is involved in the conjugative transfer of plasmid pPD1 (36 MDa; determines bacteriocin), and determined its amino acid sequence (see fig.1(I)) [6]. This is the first characterization of a conjugation-related sex pheromone from a prokaryote. We describe here the isolation, structure elucidation and synthesis of a second sex pheromone, cAD1, which induces a mating response in cells harboring the conjugative plasmid pAD1 (38 MDa; determines hemolysin) [7].

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains and media

The responder strain for assaying cAD1 was *S. faecalis* OG1S(pAD::Tn917), which harbors a

**Abbreviations:** CIA, clumping-inducing agent; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid

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derivative of conjugative hemolysin plasmid pAD1 carrying the erythromycin resistance transposon *Tn917* [8,9]. Plasmid-free strain *S. faecalis* JH2-2 [10] was used for production of cAD1.

Todd-Hewitt Broth (Oxoid) was used in the CIA assays. For cAD1 production, BPGS medium (5 g meat extract, 10 g peptone, 20 g glucose, 1 g NaCl, 1 g NaHCO<sub>3</sub>, 0.2 g Na<sub>2</sub>HPO<sub>4</sub>, 1 l distilled water, pH 7.7) was employed.

## 2.2. Bioassay

The CIA activity was assayed using the microtiter dilution method [4]. One unit of activity was defined as the lowest amount that could induce clumping of responder cells in 100  $\mu$ l of assay medium in a microtiter dilution well.

## 2.3. Isolation of cAD1

The pheromone-producing strain JH2-2 was grown (1.25% inoculum) in BPGS medium (150 l per batch) with gentle stirring under anaerobic condition at 37°C for 20 h (late log phase), and two batches (300 l) were combined and passed through an Amberlite XAD-2 column (Rohm and Haas, 15 l). The active material was adsorbed and recovered with 80% ethanol. The eluate was concentrated in vacuo to remove the ethanol, and the residual syrup was extracted twice with *n*-butanol. The *n*-butanol layers were combined and concentrated in vacuo. The concentrate was dissolved in 20% ethanol in 0.05 N acetic acid and applied to an SP-Sephadex C-25 column (Pharmacia, 5.0  $\times$  23.0 cm, Na<sup>+</sup> form); the column was eluted with a gradient of 0.05 (500 ml) to 1.0 (500 ml) M NaCl in 0.05 M sodium citrate buffer (pH 4.0). The active fractions were combined and mixed with ethanol to a final concentration of 25% (ethanol). After this step, a series of purification operations was performed using an aliquot of this solution corresponding to 100 l of culture broth.

The active material in 25% ethanol obtained in the preceding step was subjected to reverse-phase HPLC on an LRP-2 column (Whatman, 2.0  $\times$  30 cm) and the column was eluted with a gradient of 20–50% acetonitrile in 10 mM ammonium acetate for 60 min at 10 ml/min. The active fractions were 1.5-fold diluted with 0.1% HFBA and charged on the LRP-2 column again at 10 ml/min. The column was eluted with a gradient of 20–50% acetonitrile in 0.1% HFBA for 60 min at 10 ml/min and

the active fractions were combined. The active material thus obtained was further purified using 3 cycles through reverse-phase HPLC on an SSC-ODS-742 column (Senshukagaku, 1.0  $\times$  25.0 cm) at 4 ml/min with gradients of (i) 30–40% acetonitrile in 0.1% TFA for 20 min, (ii) 30–32% acetonitrile in 10 mM ammonium acetate for 20 min, and (iii) 27–32% acetonitrile in 0.1% TFA for 25 min.

## 2.4. Amino acid analysis

The isolated cAD1 (~4 nM) was hydrolyzed with 5.7 N HCl at 110°C for 20 h and the hydrolysate was applied to a Shimadzu amino acid analyzing system utilizing *o*-phthalaldehyde postcolumn derivatization [11].

## 2.5. FAB mass spectrum

2  $\mu$ l of 50% acetonitrile solution containing cAD1 (~2  $\mu$ g) was added to a matrix of diethanolamine on a stainless steel probe tip and the tip was introduced into the ion source of a mass spectrometer. Analysis was performed with a JEOL JMS DX-300 mass spectrometer using xenon as the fast atom.

## 2.6. Amino acid sequence analysis

About 10 nM of cAD1 was degraded by manually operated Edman method according to [12], and the resulting anilinothiazolinone was converted to a phenylthiohydantoin with 30% TFA at 58°C for 10 min. The phenylthiohydantoin amino acid derivatives were identified by HPLC on an Ultrasphere ODS (Altex) with a gradient of 10–50% acetonitrile in 10 mM sodium acetate (pH 4.5).

## 2.7. Synthesis of cAD1 replicate

Protected octapeptide was synthesized in solution by fragment condensation employing the HONB (*N*-hydroxy-5-norbornene-2,3-dicarboximide)-DCC (*N,N'*-dicyclohexylcarbodiimide) method [13] between N-terminal tetrapeptide and C-terminal tetrapeptide which were separately prepared by the stepwise chain elongation method. After TFA treatment followed by hydrogenolysis, the resulting material was purified by column chromatography on Sephadex LH-20 (Pharmacia) using 5.0 N acetic acid as solvent.

Table 1  
Isolation of cAD1

Purification step	Total weight (mg)	Total activity (units)	Specific activity ( $\mu\text{g}/\text{unit}$ )
Culture broth (300 l)	9 400 000 <sup>a</sup>	192 000 000	49
Amberlite XAD-2	180 000 <sup>a</sup>	96 000 000	1.8
<i>n</i> -BuOH extr.	53 000 <sup>a</sup>	96 000 000	0.55
SP-Sephadex C-25	25 000 <sup>b</sup>	96 000 000	0.26
LRP-2 (i)	5300 <sup>b</sup>	96 000 000	0.055
LRP-2 (ii)	1400 <sup>b</sup>	48 000 000	0.029
SSC-ODS-742 (i)	110 <sup>b</sup>	48 000 000	$2.4 \times 10^{-3}$
SSC-ODS-742 (ii)	8.3 <sup>c</sup>	38 400 000	$2.2 \times 10^{-4}$
SSC-ODS-742 (iii)	0.2 <sup>c</sup>	38 400 000	$5 \times 10^{-6}$

<sup>a</sup>Dry wt<sup>b</sup>Calculated from absorbance at 280 nm<sup>c</sup>Calculated from absorbance at 220 nm

### 3. RESULTS AND DISCUSSION

The isolation procedure of cAD1 is summarized in table 1. In each step of the purification, the biological activity was monitored by the CIA assay. Finally we obtained  $\sim 200 \mu\text{g}$  of pure cAD1 from 300 l of culture broth. The specific activity of the purified cAD1 was  $5 \mu\text{g}/\text{unit}$  ( $\sim 5 \times 10^{-11} \text{ M}$ ), a value similar to that found for cPD1 [6].

The FAB mass spectrum of cAD1 showed the two quasi-molecular ion peaks at  $m/z$  819 ( $M + H$ )<sup>+</sup> and 841 ( $M + Na$ )<sup>+</sup>; consequently, the  $M_r$  of cAD1 was deduced to be 818. On amino acid analysis of the acid hydrolysate of cAD1, the following amino acids (molar ratio to Ala = 1.0) were detected; Ser (0.8), Gly (1.1), Ala (1.0), Val (0.9), Leu (3.3), Phe (1.3). Considering the  $M_r$  of cAD1, this peptide should be composed of 1 mol each of Ser, Gly, Ala, Val and Phe and 3 mol of Leu with free N- and C-termini. The amino acid sequence of cAD1 was determined by a direct Edman method with HPLC identification of the phenylthiohydantoin amino acid derivative obtained from each degradation step. The results showed the amino acid sequence of cAD1 to be that shown in fig.1(II).

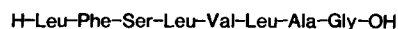
A replicate of this peptide was synthesized by the liquid-phase method. The retention time on HPLC and the clumping-inducing activity of the synthetic octapeptide were fully identical with those of native cAD1.

Whereas the cAD1 showed its activity at a considerably low concentration against OG1S(pAD1::Tn917), it failed to induce the self-clumping of pPD1-containing cells at a concentration as high as  $\sim 1 \times 10^{-6} \text{ M}$ . These results are comparable with the ineffectiveness of the cPD1 to pAD1-harboring strain [6].

The comparison of the amino acid sequences of cAD1 with that of cPD1 (fig.1) revealed the following structural resemblances: (i) Gly as the C-terminus and Leu as the 6th residue, (ii) absence of acidic and basic residues, (iii) unusual hydrophobicity of the molecules, and (iv) Ser as the only hydrophilic residue. In spite of these structural similarities, cross-activity was never observed between these pheromones. The presence of a single serine in both cAD1 and cPD1 is noteworthy in that these residues may represent the sites for pheromone modification (inactivation) in donor cells. It is noted that donor cells excrete a substance that competitively inhibits the correspond-



(I)



(II)

Fig.1. Amino acid sequences of cPD1 (I) and cAD1 (II).

ing sex pheromone, and the inhibitor can be converted to active pheromone by treatment with phosphodiesterase II [14].

Earlier  $M_r$  estimates based on relative elution positions from a Bio-Rad P2 acrylamide gel column suggested cAD1 had a higher  $M_r$  than cPD1 [14]. As shown here, cAD1 actually has a lower  $M_r$ ; the earlier elution from the P2 column probably reflects different degrees of hydrophobic interaction with the acrylamide gel, i.e., possibly related to the fact that cPD1 has two phenylalanine residues compared to one for cAD1.

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#### REFERENCES

- [1] Clewell, D. (1981) *Microbiol. Rev.* 45, 409-436.
- [2] Clewell, D., Yagi, Y., Ike, Y., Craig, R., Brown, B. and An, F. (1982) in: *Microbiology-1982* (Schlessinger, D. ed.) pp. 97-100, American Society for Microbiology, Washington, DC.
- [3] Dunny, G., Brown, B. and Clewell, D. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3479-3483.
- [4] Dunny, G., Craig, R., Carron, R. and Clewell, D. (1979) *Plasmid* 2, 454-465.
- [5] Yagi, Y., Kessler, R., Shaw, J., Lopatin, D., An, F. and Clewell, D. (1983) *J. Gen. Microbiol.* 129, 1207-1215.
- [6] Suzuki, A., Mori, M., Sakagami, Y., Isogai, A., Fujino, M., Kitada, C., Craig, R. and Clewell, D. (1984) *Science*, in press.
- [7] Clewell, D., Tomich, P., Gawron-Burke, C., Franke, A., Yagi, Y. and An, F. (1982) *J. Bacteriol.* 152, 1220-1230.
- [8] Tomich, P., An, F. and Clewell, D. (1980) *J. Bacteriol.* 141, 1366-1374.
- [9] Clewell, D. and Brown, B. (1980) *J. Bacteriol.* 143, 1063-1065.
- [10] Jacob, A. and Hobbs, S. (1974) *J. Bacteriol.* 117, 360-372.
- [11] Ishida, Y., Fujita, T. and Asai, K. (1981) *J. Chromatogr.* 204, 143-148.
- [12] Kimura, S. (1974) *Bunseki Kagaku* 23, 563-575.
- [13] Fujino, M., Kobayashi, S., Obayashi, M., Fukuda, T., Shinagawa, S. and Nishimura, O. (1974) *Chem. Pharm. Bull. (Tokyo)* 22, 1857-1863.
- [14] Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. and Sugiura, H. (1967) *Bull. Chem. Soc. Jap.* 40, 2164-2167.