Short Communication

Isolation and Structure of the Streptococcus faecalis Sex Pheromone Inhibitor, iAD1, That Is Excreted by the Donor Strain Harboring Plasmid pAD1

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Recipient strains of Streptococcus faecalis excrete small peptidal sex pheromones which induce a mating response in donor strains harboring certain conjugative plasmids. (For a recent review of this phenomenon, see ref. 1.) A single plasmid-free strain excretes 5 or more different pheromones, each specific for a different donor plasmid. We have recently isolated two of these peptides, cPD1 and cAD1, which specifically induce the mating response of donor strains harboring plasmids pPD1 (determines bacteriocin) or pAD1 (determines hemolysin), respectively. The amino acid sequences were found to be H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH for cPD1 and H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH for cAD1.

When a recipient acquires a copy of the conjugative plasmid, production of the related pheromone ceases and the strain becomes responsive to the exogenous pheromone. There is evidence that the "shutting off" of endogenous pheromone production by plasmid acquisition is probably through a plasmid-determined modification of the peptide. In addition, an inhibitor of the sex pheromone to which the donor cells respond was revealed to be present in its culture filtrate. Here, we report the isolation, structural elucidation and synthesis of the sex pheromone inhibitor, iAD1, which is produced by the pAD1-harboring donor strains and inhibits the related sex pheromone, cAD1.

For the production of iAD1, we used the donor strain FA2-2(pAM727) which harbors the plasmid pAM727 (=pAD1::Tn977), an insertional mutant of pAD1, and excretes 4~5 times amount of the inhibitor than normal strains. The bacterium was cultured to a stationary phase (0.5% inoculum, 20 hr) in 20 liters of BPGS medium at 37°C with gently stirring under anaerobic conditions. The grown cells were pelleted by centrifugation and the supernatant was passed through an Amberlite XAD-7 column (Rohm and Haas, 5.3 x 19cm), which was then eluted with EtOH-pyridine-water (8:1:1). The eluate was diluted 3-fold with water and added with pyridine to a final concentration of 5%, the solution being applied to a DEAE-Sephadex A-25 column (Pharmacia, 3.2 x 7cm, Cl− form). The column was eluted with a gradient of 0.05 M (160 ml) to 0.2 M (160 ml) NaCl in 25% EtOH. The active fractions were combined, subjected to reverse-phase HPLC on an LRP-2 column (Whatman, 2 x 30 cm), and eluted with a gradient of 20~50% acetonitrile in 10 mM AcONH4 for 60 min at 10 ml/min. The active material obtained in the preceding step was further purified by the following 3 cycles through reverse-phase HPLC on an SSC-ODS-742 column (Senshukagaku, 1 x 25 cm) at 4 ml/min with gradients of (i)

Abbreviations: HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Boc, t-butoxycarbonyl; Bzl, benzyloxycarbonyl.
28~38% acetonitrile in 0.1% heptafluorobutyric acid for 20 min, (ii) 28~32% acetonitrile in 10mM AcONH₄ for 20 min, and (iii) 28~32% acetonitrile in 0.1% TFA for 20 min. The partially purified iAD1 was then subjected to ion exchange HPLC on a TSK gel SP-5PW column (Toyosoda, 0.75 x 7.5 cm, Na⁺ form), the column being eluted with a gradient of 5~500mM AcONa (pH 4) in 20% acetonitrile for 20 min at 1 ml/min. The active fraction thus obtained was applied to final purification with reverse-phase HPLC on an SSC-ODS-262 column (Senshukagaku, 0.6 x 10 cm), and a gradient of 20~40% acetonitrile in 0.1% TFA for 20 min at 1 ml/min afforded iAD1 as a single peak. About 20μg of iAD1 was obtained through a 10,000,000-fold purification from 20 liters of culture broth, and the recovery of activity was about 25%.

In each step of purification, the inhibitory activity was monitored using a modification of the microtiter dilution method, which relates to the ability of donor cells alone to undergo a "clumping response" when exposed to cAD1.²⁻⁴ To serially diluted (2-fold) sample solutions (50 μl) in Todd-Hewitt broth (Oxoid) on titerplates, we added 50 μl aliquots of the responder cells, OG1S(pAD1::Tn917),⁷ representing a 1:10 mixture of an overnight culture of cells and Todd-Hewitt broth containing cAD1 (ca. 160 pg/ml). The plates were incubated for 2.5~3 hr at 37°C, and the inhibition of cAD1-induced clumping of responder cells was observed for the samples with inhibitory activity. The isolated iAD1 prevented the self-clumping of OG1S(pAD1::Tn917) cells at least at a concentration of 25 pg per 100 μl in the presence of 8 pg of cAD1 in 100 μl; consequently, a 3-fold exess of iAD1 was sufficient to inhibit cAD1 activity.

Since inactivation experiments of iAD1 with proteolytic enzymes showed that the active substance should be a peptide,⁵ the isolated iAD1 (ca. 5 μg) was subjected to sequence analysis by a manually operated direct Edman method³, accompanied by the identification of PTH amino acids with HPLC.⁸ From the 1st to the 8th step of analysis, the amino acid sequence, H-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-, was identified, and no amino acids were detectable after the 9th step. In the fast atom bombardment mass spectrum of iAD1, the spectrum was terminated by two peaks at m/z 869 and 891, which were assignable as the sodium-clustered quasi-molecular ion peaks, (M+Na)⁺ and (M+2Na–H)⁺, respectively. Thus the molecular weight of iAD1 was 846, which explains the above-mentioned sequence with a free C-terminus. Accordingly, the structure of iAD1 was determined as shown in Fig. 1. On an amino acid analysis of the acid hydrolysate of iAD1 by the PTC method,⁹ the following amino acids (molar ratio to Leu = 2.0) were detected: Gly (1.2), Thr (0.7), Val (2.7), Leu (2.0) and Phe (1.1). These were consistent with the results of the sequence analysis.

The protected octapeptide, Boc-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-OBzl, corresponding to the proposed amino acid sequence of iAD1 was prepared in solution by fragment condensation between the N-terminal pentapeptide and C-terminal tripeptide. Each protected fragment was synthesized by the stepwise chain elongation method. After removing both the Boc and Bzl groups from the protected octapeptide, the resulting material was purified by washing with both 1 n AcOH and 20% MeOH, and was then dried to give the octapeptide, iAD1. The retention time on HPLC and the inhibitory activity of the synthetic octapeptide were identical with those of native iAD1. Hence, the chemical structure of iAD1 was unambiguously established.

Four out of 8 residues in the amino acid sequence of the inhibitor iAD1 coincide with those of the sex pheromone cAD1 (Fig. 1). This structural similarity of the inhibitor with the sex pheromone accounts for inhibition via the competition for a receptor site, although

<table>
<thead>
<tr>
<th>iAD1:</th>
<th>H-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-OBzl</th>
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<tbody>
<tr>
<td>cAD1:</td>
<td>H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OBzl</td>
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**Fig. 1.** Amino Acid Sequences of iAD1 and cAD1. Coincident residues are boxed.
the mode of sex pheromone reception by the donor cells remains unclear. The physiological role of the inhibitor in the mating system of *S. faecalis* is obscure. It is conceivable, however, that it may serve to desensitize the donor to "very low" concentrations of endogenous (unmodified) or exogenous pheromone. Low exogenous pheromone levels would mean that the corresponding recipient is far away or in too low a concentration (relative to the donors); a response under these conditions might lead the donor cells to nonproductive self-clumping.

It was previously reported\(^5\) that the modified endogenous pheromone itself might be the sex pheromone inhibitor; this was based on the observation that a culture filtrate containing inhibitor activity could give rise to some cAD1 activity if treated with phosphodiesterase II. However, it is evident from the present results that the inhibitor is not a modified form of the pheromone.\(^10\) These results, nevertheless, do not contradict a modification mechanism,\(^5\) but simply imply the participation of a novel factor, the sex pheromone inhibitor, in the interaction between the pheromone, plasmid and donor cell.

Recent studies have now shown that *S. faecium* 9790 (which does not produce cAD1) will excrete iAD1 if a pAD1 derivative is introduced into it,\(^11\) which suggests that iAD1 could be plasmid encoded.

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**REFERENCES AND NOTES**


8) PTH amino acids were identified by HPLC on a Senshupak AQUASIL SEQ-4 column (Senshukagaku, 0.46×30cm) with a multistep gradient of acetonitrile-40 mM AcONa (pH 4.9)—water from 36:26:38 to 65:5:30 for 13 min at 1 ml/min.


10) Since the iAD1-containing fractions were the sole portion which showed inhibitory activity in each isolation step, the possibility that the modified pheromone possessed iAD1 activity can be ruled out.