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Isolation and Structure of the Sex Pheromone Inhibitor, iPD1, Excreted by *Streptococcus faecalis* Donor Strains Harboring Plasmid pPD1

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The sex pheromone inhibitor iPD1, which is excreted by *Streptococcus faecalis* donor strains harboring bacteriocin plasmid pPD1 and which inhibits sex pheromone cPD1, was isolated, and its structure was determined. Its molecular weight is 828, and its amino acid sequence is H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser-OH.

Conjugative transfer of certain plasmids in *Streptococcus faecalis* is induced by peptidal sex pheromones excreted by recipient cells (1–4). Five or more pheromones, each specific for a donor strain harboring a particular plasmid, are produced by a single recipient. An induced donor synthesizes an adherent protein (termed aggregation substance) on the cell surface which facilitates the formation of mating aggregates (1–4). We have isolated and sequenced the pheromones cPD1, cAD1, and cAM373, which induce the transfer systems of the conjugative plasmids pPD1, pAD1, and pAM373, respectively (9–11).

Whereas a donor cell still releases pheromones unrelated to the plasmid it harbors, the related pheromone activity cannot be detected in culture filtrates (1–4). It was suggested that the shutting off of endogenous pheromone production by plasmid acquisition was the result of a plasmid-determined modification of the peptide (6); however, more recent data have shown that the inhibitor is a different peptide (8). We have recently isolated the sex pheromone inhibitor, iAD1, produced by donors harboring the plasmid pAD1 and have shown it to be an octapeptide (8). We have also obtained evidence that the inhibitor is plasmid-encoded (D. B. Clewell, F. Y. An, M. Mori, Y. Ike, and A. Suzuki, Plasmid, in press). Here we report the isolation, structure elucidation, and total synthesis of another sex pheromone inhibitor, iPD1, which is produced by pPD1-harboring donor strains and inhibits cPD1 activity.

During the purification, the inhibitory activity was assayed by means of a modified microtiter method similar to that previously reported for the isolation of iAD1 (8). *S. faecalis* 39-5Sα (12) harboring bacteriocin plasmid pPD1 (56.7 kilobases) was used as a responder, and sex pheromone cPD1 was added to the assay medium to a final concentration of 80 pg/ml. For the production of inhibitor iPD1, we employed the donor strain JH2-2(pAM351), which harbors a derivative of pPD1, pAM351 (pPD1::Tn*916*) (6). The bacterium was anaerobically cultured with gentle stirring at 37°C in THG medium (18.2 g of Todd-Hewitt broth [Oxoid Ltd.] and 20 g of glucose in 1 liter; 20 liters per batch) to stationary phase (1.0% inoculum for 20 h), and the cells were removed by centrifugation.

The culture supernatant (20 liters) was passed through a charcoal column (5.8 by 23 cm; Wakojunyaku), and the activity adsorbed was recovered with 50% pyridine. The eluate was diluted fivefold and subjected to anion-exchange chromatography with a DEAE-Sephadex column (3.4 by 17 cm, acetate form; Pharmacia). The active fractions eluted from the column with a 225-ml gradient of 0.05 to 0.2 M ammonium acetate in 20% ethanol were combined and purified further by two cycles of reversed-phase high-performance liquid chromatography (HPLC) on an LRP-2 column (2 by 30 cm; Whatman) at 10 ml/min with gradients of 15 to 45% acetonitrile in 10 mM ammonium acetate for 60 min and 20 to 50% acetonitrile in 0.1% heptafluorobutyric acid for 60 min. The active material obtained in the preceding step was divided into two aliquots, and each (equivalent to 10 liters of culture broth) was submitted to the following purification procedure. The material was first subjected to reversed-phase HPLC on an SSC-ODS-742 column (1 by 25 cm; Senshukagaku), and the column was eluted with a gradient of 15 to 24% (5 min) and 24 to 29% (25 min) acetonitrile in 0.1% trifluoroacetic acid at 4 ml/min. The active eluate was then charged on a Senshupak CN-4251-N column (1 by 25 cm; Senshukagaku) and the fractions showing iPD1 activity, which were obtained with a gradient of 5 to 13% (5 min) and 13 to 18% (25 min) acetonitrile in 0.1% trifluoroacetic acid at 4 ml/min, were further purified by reversed-phase HPLC with an SSC-ODS-262 column (0.6 by 10 cm; Senshukagaku) and a gradient of 15 to 24% (5 min) and 24 to 28% (20 min) acetonitrile in 10 mM ammonium acetate at 1 ml/min. Although the active material obtained in this step had been significantly purified (16,000,000-fold), it was not pure enough for structural analysis. We therefore attempted a final purification by HPLC on a Senshupak SC4-1251 (0.46 by 25 cm; Senshukagaku) with an isocratic elution of 30% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. A sample containing the partially purified iPD1 corresponding to 3.3 liters of culture broth was applied to the column for a single cycle of purification, and iPD1 appeared as a single peak with a retention time of 12.0 min. About 6.3

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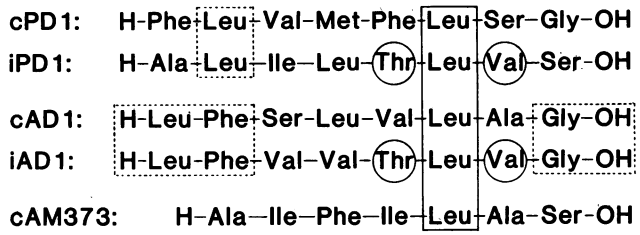

FIG. 1. Amino acid sequences of *S. faecalis* sex pheromones and sex pheromone inhibitors. The residue common to all five peptides, Leu^6, (or Leu^5 for cAM373) is boxed in with a solid line, and coincident residues in an inhibitor(s) and its corresponding pheromone are circled or boxed in with a dotted line, respectively.

μg of iPD1 was obtained through a 34,000,000-fold purification from 20 liters of culture supernatant, and the recovery of the activity was 25%. The isolated iPD1 inhibited cPD1-induced clumping of responder cells at a concentration of 8 pg/100 μl of assay medium in a microtiter dilution well in the presence of 8 pg of cPD1 in 100 μl. Thus, an equal amount of iPD1 was enough to inhibit cPD1 activity.

Since iPD1 was believed to be a peptide based on inactivation experiments with proteolytic enzymes (6), the active substance was subjected to an amino acid sequence analysis by a manually operated Edman method (9). Phenylthiohydantoin amino acid obtained at each degradation cycle was identified by HPLC on a Senshupak SEQ-4(K) column (0.46 by 30 cm; Senshukagaku) with a multistep gradient of acetonitrile containing 2-propanol (5%) and 1-chlorobutane (1.5%), 40 mM sodium acetate (pH 4.9) in 1% tetrahydrofuran, and 1% tetrahydrofuran from 35:24:41 to 65:5:30 for 15 min at 1 ml/min. As a result of analysis, the sequence H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser- was identified through cycle 8, and no amino acid could be detected after cycle 9. In a fast atom bombardment mass spectrum of iPD1 measured in a matrix of diethanolamine with a JMS DX-303 mass spectrometer (JEOL) with xenon as the fast atom, the quasi-molecular ion $(M + H)^+$ was observed at *m/z* 829, indicating that the molecular weight of the peptide was 828. These results revealed that iPD1 was the octapeptide possessing the above-mentioned sequence with a free C terminus.

The protected octapeptide, *t*-butoxycarbonyl-Ala-Leu-Ile-Leu-Thr(benzyl)-Leu-Val-Ser(benzyl)-*O*-benzyl, corresponding to the entire amino acid sequence of iPD1 was synthesized by the fragment condensation between the N-terminal tetrapeptide and C-terminal tetrapeptide by using the HONB (*N*-hydroxy-5-norbornene-2,3-dicarboximide)-DCC (*N,N'*-dicyclohexylcarbodiimide) method (7). Each fragment peptide was synthesized in solution by the stepwise HONB active-ester method (5). After treatment with trifluoroacetic acid followed by hydrogenolysis, the resulting material was precipitated from water to yield the octapeptide, iPD1. The retention time on HPLC and biological activity of the synthetic replicate were indistinguishable from those of native iPD1. Accordingly, the chemical structure of iPD1 was unambiguously confirmed.

The amino acid sequences of three pheromones and two inhibitors involved in plasmid transfer systems in *S. faecalis* have been chemically characterized (Fig. 1; 8–11). These compounds are all unusually lipophilic peptides containing no acidic or basic amino acid residues, and they commonly possess Leu at residue 3 from the C terminus. Despite these similarities it is noteworthy that each peptide is highly specific. Although iPD1 could inhibit the cPD1 activity at a pheromone/inhibitor ratio of about 1:1, which was comparable to the value observed in the interaction of cAD1 and iAD1 (8), iPD1 does not show as strong a resemblance to cPD1 in amino acid sequence as iAD1 does with cAD1 (iPD1 shares two coincident residues with cPD1, whereas iAD1 and cAD1 have four identical residue positions). Interestingly, iPD1 exhibits high homology with iAD1 in the C-terminal region corresponding to Thr^5-Leu^6-Val^7. Whether such a common structure of the inhibitors is important to their inhibitory activity is an interesting question. It is noted that the inhibitors have a bulky residue, Val, at position 2 from the C terminus where small residues, Ser or Ala, exist in the corresponding pheromones.

Although the physiological role of the inhibitor in the mating system of *S. faecalis* remains unclear, one possible purpose for inhibitor activity could be to prevent a mating response to concentrations of pheromone that are too low, meaning that recipients are too far away to encounter donor cells by random collision. It could also serve to prevent self-induction by low levels of endogenous pheromone (i.e., in the event that expression is not completely shut down by the plasmid) or other peptides (yet to be identified) which may have some cross-reactivity with the specific receptor site. That the inhibitor iAD1 is encoded by plasmid pAD1 is suggested by the observation that introduction of a pAD1 derivative into *S. faecium* 9790, which does not excrete cAD1 (or iAD1), led to production of iAD1 (Clewell et al., in press). It seems likely, in this regard, that the inhibitor iPD1 is determined by plasmid pPD1.

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