An Enterococcus faecalis plasmid, pAM373, has a high frequency of transfer in a liquid medium when induced by a recipient-produced sex pheromone, cAM373. The sex pheromone inhibitor against cAM373, termed iAM373, was isolated from a culture supernatant of E. faecalis harboring pAM377 (=pAM373::Tn917), and its structure was identified as a heptapeptide, H-Ser-Ile-Phe-Thr-Leu-Val-Ala-OH.

Certain plasmids in Enterococcus faecalis encode a mating response to recipient-produced sex pheromones. \(^{11}\) E. faecalis encodes chromosomally multiple sex pheromones. \(^{21}\) A given pheromone (cX) specifically activates the conjugal transfer system of the corresponding plasmid (pX), including the formation of sexual aggregates of donor and recipient cells. The structures of the pheromones cPDI, \(^{35}\) cAD1, \(^{45}\) cCF10, \(^{55}\) and cAM373 \(^{55}\) have been found to be very hydrophobic linear peptides composed of seven or eight amino acids. Once a recipient acquires a plasmid, it becomes a donor for the given plasmid while it continues to behave as a recipient for other plasmids. In the culture filtrate of the transconjugant, the activity of the corresponding pheromone disappears, while the activity of other pheromones continues. Two kinds of genetic functions encoded by plasmids can explain the disappearance of pheromone activity. One is production of a peptide inhibitor (iX) competitive to a pheromone (cX), and the other is repression of the pheromone production, the so-called “pheromone shutdown.” \(^{7,9}\) However, the structures of inhibitors pPDI, \(^{11}\) iAD1, \(^{10,11}\) and iCF10 \(^{12}\) have been found to be hydrophobic linear peptides similar in structure to the pheromone.

The plasmid pAM373 is a 37-kb plasmid responsive to the sex pheromone cAM373. \(^{6,13}\) Its encoding phenotype is cryptic. \(^{13}\) The plasmid pAM373 is a unique plasmid the transfer of which can be induced by a substance secreted from other species of bacteria, Staphylococcus aureus, Streptococcus sanguis, and Enterococcus hirae, as well as cAM373 secreted from E. faecalis. \(^{13}\) However, pAM373 does not transfer from E. faecalis to these bacteria. In this paper, we describe the isolation and structural elucidation of the sex pheromone inhibitor, iAM373.

pAM373 has been believed to encode the iAM373 peptide in the view that a pheromone plasmid generally encodes a pheromone inhibitor. However, we could not detect the inhibitor activity in the culture filtrate of E. faecalis FA2-2(pAM373), \(^{13}\) which harbors pAM373. Pheromone binding proteins have been cloned from pAD1 and pCF10, which reduce the activities of pheromone and inhibitor in the broth, and are expected to function as initial receptors for pheromone. \(^{14,15}\) Since the cAM373 activity decreased after incubation with FA2-2(pAM373) cells, it was thought that pAM373 also encodes the binding protein and it absorbed the self-produced iAM373. Plasmid pAM377 is a derivative of pAM373 having a transposon, Tn917. \(^{13}\) FA2-2(pAM377) failed to absorb cAM373 activity. The insertion of Tn917 might stop expression of the pheromone-binding protein, and if so, it was expected that FA2-2(pAM377) would also fail to absorb iAM373 activity. As expected, the iAM373 activity was detected in the culture filtrate of the strain FA2-2(pAM377). JSS2-2(pAM377) \(^{13}\) produced iAM373 more than FA2-2(pAM377). Thus, we used JSS2-2(pAM377) for the production of inhibitor iAM373.

During the purification, the inhibitor activity was assayed by

<table>
<thead>
<tr>
<th>Table</th>
<th>Isolation Procedure for iAM373</th>
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</thead>
<tbody>
<tr>
<td>Purification step</td>
<td>Total weight (mg)</td>
</tr>
<tr>
<td>Culture supernatant (8 liters)</td>
<td>292,000(^{6})</td>
</tr>
<tr>
<td>1. XAD-7</td>
<td>ND(^{*})</td>
</tr>
<tr>
<td>2. QAE-Sephadex A-25</td>
<td>3,300(^{6})</td>
</tr>
<tr>
<td>3. ODS-SS-1020T (i)</td>
<td>940(^{6})</td>
</tr>
<tr>
<td>4. ODS-SS-1020T (ii)</td>
<td>82(^{6})</td>
</tr>
<tr>
<td>5. ODS-H-5251 (i)</td>
<td>19(^{6})</td>
</tr>
<tr>
<td>6. ODS-H-5251 (ii)</td>
<td>4.6(^{6})</td>
</tr>
<tr>
<td>7. CN-4251-N</td>
<td>0.46(^{6})</td>
</tr>
<tr>
<td>8. VP-304-423</td>
<td>0.29(^{6})</td>
</tr>
<tr>
<td>9. ODS-2101-D (i)</td>
<td>0.12(^{6})</td>
</tr>
<tr>
<td>10. ODS-2101-D (ii)</td>
<td>0.028(^{6})</td>
</tr>
<tr>
<td>11. ODS-2101-D (iii)</td>
<td>0.003(^{6})</td>
</tr>
</tbody>
</table>

The culture supernatant was passed through an Amberlite XAD-7 column (5.0 x 25 cm; Organo), and the activity adsorbed was recovered with 1.5 liter of 80% EtOH (Step 1). The eluate was diluted four-fold and chromatographed on an anion-exchange QAE-Sephadex column (3 x 20 cm, acetate form; Pharmacia). The column was eluted with a 300-ml gradient of 0.02 to 0.2 M ammonium acetate in 20% ethanol (Step 2). The active fractions were combined and purified further by 9 steps (Step 3 to 11) using reverse-phase HPLC. Every HPLC column (purchased from Senshukakaku) was eluted with a gradient of acetonitrile concentration. The eluates were collected, freeze-dried and stored at room temperature (Step 4 to 11).

- Dry weight. Calculated from absorbance at 280 nm. Calculated from absorbance at 220 nm. One unit of the activity was defined as the lowest amount that inhibits self-clumping induced with 100 pg/ml cAM373. ND means not determined.
observing the inhibition against cAM373-inducible aggregation of
donor cells, a method similar to that previously reported for the
isolation of iPDI.9 In the assay, FA2-2(pAM373) was used as the
donor, and synthetic cAM373 was added to THB medium to
a final concentration of 100 pg/ml.

JSS2-2(pAM373) was anaerobically cultured with gentle stirring
at 37°C in 8 liters of THB medium (1% inoculation, for 5h) and
the cells were removed by centrifugation. The inhibitor iAM373
was isolated from the culture supernatant by a purification
procedure consisting of 11-step column chromatography, as shown
in Table. On the last HPLC, active material was eluted in a single
peak with a retention time of 10 min. About 3 µg of iAM373 was
obtained through the 12,000,000-fold purification and recovery of
the activity was 12.5%. The isolated iAM373 inhibited cAM373
(10 µg/ml)-induced aggregation of the donor cells at a concentra-
tion of 150 pg/ml (2 x 10⁻¹⁰ M).

The amino acids of the isolated iAM373 (0.3 µg) were analyzed
by a gas-phase protein sequencer (Applied Biosystems 477A). The
sequence H-Ser-Ile-Phe-Thr-Leu-Val-Ala- was identified through
7 cycles, and no amino acid could be detected after cycle 8. In a
fast atom bombardment (FAB) mass spectrum of iAM373, the
quasi-molecular ion (M + H)⁺ was observed at m/z 750, indicating
that the molecular weight of the peptide was 749. Furthermore,
some peaks in the spectrum could be assigned to the N-terminal
fragments of B₁₋₅, types from this sequence. These results
showed that iAM373 was the heptapeptide having the sequence
with a free C-terminus. A heptapeptide with this structure was
chemically synthesized by the solid-phase method using the Fmoc
strategy (the peptide synthesis kit ‘Kokku-san’, Kokusan Kagaku).
The synthetic peptide showed the same biological activity and
chromatographic behavior as the naturally obtained iAM373.

The amino acid sequences of four pheromones and their
inhibitors in E. faecalis have been chemically identified as shown
in the Figure. The Thr-Leu-Val sequence of iAM373 was in iPDI
and iAD1. There is a similar sequence, Thr-Leu-Ile, in iCF10. All
these compounds are unusually lipophilic peptides containing no
acidic or basic amino acid residues. The structure similarity
between pheromone and inhibitor suggests that the inhibitor may
act as an antagonist of the pheromone.

Acknowledgments. We thank D. B. Clewell for providing us E. faecalis
strains. This work was supported in part by a Grant from the Ministry

References
1) D. B. Clewell, in “Bacterial Conjugation,” ed. by D. B. Clewell,
2) G. M. Dunny, R. A. Craig, R. L. Caron, and D. B. Clewell, Plasmid,
3) A. Suzuki, M. Mori, Y. Sakagami, A. Isogai, M. Fujino, C. Kitada,
4) M. Mori, Y. Sakagami, M. Narita, A. Isogai, M. Fujino, C. Kitada,
5) M. Mori, Y. Sakagami, Y. Ishii, A. Isogai, C. Kitada, M. Fujino, J.
C. Adsit, G. M. Dunny, and D. B. Clewell, J. Biol. Chem., 263,
6) M. Mori, H. Tanaka, Y. Sakagami, A. Isogai, M. Fujino, C. Kitada,
9) M. Mori, H. Tanaka, Y. Sakagami, A. Isogai, M. Fujino, C. Kitada,
10) M. Mori, A. Isogai, Y. Sakagami, M. Fujino, C. Kitada, D. B.
12) J. Nakayama, R. E. Ruhfel, G. M. Dunny, A. Isogai, and A. Suzuki,
13) D. B. Clewell, F. Y. An, B. A. White, and C. Gawron-Burke, J.
14) R. E. Ruhfel, D. A. Manners, and G. M. Dunny, J. Bacteriol., 175,
15) K. Tanimoto, F. Y. An, and D. B. Clewell, J. Bacteriol., 175,