Structure of cCF10, a Peptide Sex Pheromone Which Induces Conjugative Transfer of the *S. faecalis* Tetracycline Resistance Plasmid, pCF10*

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The peptide pheromone, cCF10, which induces aggregation and high frequency plasmid transfer in *S. faecalis* cells carrying the tetracycline resistance plasmid, pCF10, was isolated and its structure determined. The molecular weight of cCF10 is 789, and its amino acid sequence is H-Leu-Val-Thr-Leu-Val-Phe-Val-OH. Pheromone activity, as determined by a clumping induction assay, was detectable at a concentration of 2.5 × 10⁻¹ⁱ M. A peptide of the same sequence as that of the cCF10 produced by *S. faecalis* cells was synthesized by the liquid-phase method. The synthetic pheromone showed biological activity and chromatographic behavior that was identical to that of the cCF10 of bacterial origin. When the response of *S. faecalis* cells to various concentrations of synthetic cCF10 was monitored by measuring both the frequency of plasmid transfer and the synthesis of pheromone-inducible antigens, an excellent correlation was observed between donor ability and the appearance of a 150-kilodalton protein that appears to be involved in formation of mating aggregates. The dose-response data in the range of concentrations where the amount of pheromone became limiting (10⁻¹¹–10⁻¹² M) were consistent with the notion that as few as one or two molecules per donor cell may be sufficient to induce a mating response.

In *S. faecalis*, conjugal transfer of certain plasmids can be enhanced by low molecular weight, heat-stable, protease-sensitive sex pheromones (1–3). The pheromones are excreted by the recipient cells, and their interaction with plasmid-containing donor cells induces expression of several surface antigens that are involved in processes such as formation of mating aggregates (clumps) (4, 5) and entry exclusion of incoming plasmids in donor cells (6). In addition, one or more plasmid transfer functions, distinct from aggregation, are also induced (7). If a pheromone-inducible plasmid is transferred into a pheromone-producing strain, culture filtrates of the new donor strain do not contain detectable pheromone activity, when assayed against responder strains carrying the same plasmid. However, the strain continues to excrete pheromones active against strains carrying unrelated conjugative plasmids (2, 8). Thus, there seems to be a plasmid-encoded inhibitor function that specifically interferes with production of the pheromone to which the plasmid determines a response. Recent evidence indicates that the donor cell produces a plasmid-encoded peptide that competes with active pheromone for binding to responder cells, but fails to induce a response (9–11). The pheromones, also termed clumping-inducing agents, or CIs (1) are named after the plasmid whose transfer functions they induce, e.g., pheromone cAD1 induces clumping and transfer in cells carrying the plasmid, pAD1, etc. (12). Similarly, the inhibitor peptide for pAD1 is termed iAD1 (9). Several pheromones and two inhibitors have been purified, their structures determined, and they have been synthesized chemically (9, 13–15). All of these compounds seem to be short, very hydrophobic peptides which exhibit biological activity at extremely low concentrations. In this paper we describe the isolation, amino acid sequence, and synthesis of cCF10, the pheromone that induces transfer of the tetracycline resistance plasmid, pCF10. This plasmid was the first R-factor described that encoded pheromone response functions (3), and its conjugal transfer system has been the subject of a considerable amount of genetic, molecular, and immunological analysis (16). We also compare the sequence of cCF10 to those of previously analyzed pheromones and inhibitors, we demonstrate directly that the synthetic pheromone induces clumping, mating, and synthesis of surface antigens, and (using synthetic cCF10 in dose-response experiments) we provide evidence that a single pheromone molecule may be sufficient to induce a mating response in a *S. faecalis* cell carrying pCF10.

**MATERIALS AND METHODS**

**Bacterial Strains, Media, and Bioassays for Pheromone Activity—**

The responder strain used for the assay of cCF10 activity was OGISs (pCF10), which carries the 58-kilobase tetracycline resistance plasmid, pCF10 (3). Strain FA2-2 (pAM351), which harbors the unrelated conjugative plasmid, pAM351 (5), was used for pCF10 production. Strain OGIS1RF (1), which carries chromosomal mutations to rifampin and fusidic acid resistance, was used as the recipient in conjugal mating experiments. For all mating experiments and clumping-inducing (CIA) assays, BYGT medium (brain-heart infusion, yeast extract, glucose, Tris-HCl) was employed as previously described (3). THG medium (18.2 g of Todd-Hewitt Broth (Oxoid), 20 g of glucose, 1 liter of distilled H₂O) was used to cultivate cells for pCF10 production. All cultures were incubated at 37°C.

1 The abbreviations used are: CIA, clumping-inducing agents; RP HPLC, reverse-phase high performance liquid chromatography; FAB, fast atom bombardment; OEt₂, oxybenzyl.

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CIA activity was assayed using the microtiter assay described previously (2). One unit of activity was defined as the smallest amount of material that induced clumping of responder cells in the 100 μl volume used in the microtiter assay. For mating experiments, over-night cultures of donor (OG1SSp (pCF10)) and recipient (OG1RF) cells were diluted (separately) 10-fold to fresh medium. The cells in the medium contained approximately 10⁶ cCF10 activity. For any specific experiment, the times of pheromone induction and mating were kept constant.

Analysis of Pheromone-induced Cell Surface Antigens—Synthesis of cell surface antigens by responder cells exposed to synthetic cCF10 was monitored by Western blot analysis using a slight modification of a technique described previously (4). A fresh overnight culture of responder cells was diluted 10-fold into fresh BYGT containing 1 volume of the donor culture, and 1 volume of the donor culture was added to 9 volumes of the recipient culture. After 15 min incubation, the mixtures were plated on media selective for donors (1000 μg/ml streptomycin + 20 μg/ml spectino-myacin) or transconjugants (100 μg/ml tetracyclin + 25 μg/ml fusidic acid + 10 μg/ml tetracyclin). For any specific experiment, the purity of the donor and recipient was determined by silver staining of the SDS-PAGE. The activity fraction was reserved at 1 ml/min. For the final purification step, the active fractions from three 20-liter batches were combined in a volume of 2.8 ml and divided into 400-μl aliquots. Each aliquot was then purified on a Senshupak SC-1251 (4.6 × 250 mm, Senshukagaku) RP HPLC column with an isocratic elution of 35% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. The purification steps are outlined in Table I. During this procedure, the active fractions were never evaporated to dryness, since this compound is very hydrophobic, and it was impossible to recover complete activity if fractions were dried.

**RESULTS**

Purification, Structural Analysis, and Synthesis of cCF10—The S. faecalis sex pheromone cCF10 was purified from 60 liters of culture filtrate by the eight steps shown in Table I and described under “Materials and Methods.” We achieved a 1.45 × 10⁴-fold purification and obtained 4.1 μg of pure compound. The biological activity after each purification step was monitored by a clumping induction assay, and the total weight estimated, as shown in Table I. The specific activity of pure cCF10, 2 μg/unit, is similar to the values determined for other S. faecalis sex pheromones (13-15).

Because treatment of partially purified cCF10 with either

### Table I

| Purification step | Total weight | Total activity | Specific activity |
|------------------|--------------|---------------|------------------|
| *Dry weight.*    | mg           | units         | ng/unit          |
| Cultured broth   | 2,800,000°   | 9,600,000     | 290,000          |
| Amberlite XAD-2  | 19,000°      | 4,000,000     | 400              |
| DEAE-Sephadex    | 4,800°†      | 4,000,000     | 1,000            |
| LRP-2            | 150°†        | 2,400,000     | 62               |
| SSC-ODS-742      | 36°†         | 2,400,000     | 12               |
| CN-4251-N        | 4.4°†        | 2,400,000     | 1.8              |
| SSC-ODS-262 (1)  | 0.12°†       | 2,400,000     | 0.05             |
| SSC-ODS-262 (2)  | 0.009°†      | 2,400,000     | 0.038            |
| sc4-1251 (1)     | 0.0041°†     | 2,100,000     | 0.002            |

° Calculated from absorbance at 280 nm.
† Calculated from absorbance at 220 nm.

(See Table I for purification steps.)
Purified cCF10 showed no CIA activity against responder strains for the other pheromones. Although cCF10 actually shows some amino acid sequence homology with the inhibitor peptides iAD1 and iPD1 (see Fig. 2), it displayed no inhibitor activity in the appropriate assays. However, iAD1 did show a very weak (100 ng/unit) CIA activity against a responder strain carrying pCF10. Two cCF10 analogs, H-Val-Ala-Thr-Leu-Val-Phe-Val-OH and H-Ala-Leu-Gly-Leu-Val-Phe-Val-OH, were synthesized, and only the former showed any CIA activity (30 ng/unit).

**Dose-Response Analysis using Synthetic cCF10**—Exposure of S. faecalis cells to culture filtrates containing CIA has a number of effects on the responder cells, as noted in the Introduction. Most of the genetic and biochemical analyses of pheromone-inducible plasmid transfer systems to date support the notion that cell clumping, synthesis of unique surface antigens, and enhanced plasmid transfer are all components of a single, coordinated response to a particular peptide pheromone. The availability of large quantities of synthetic cCF10 in pure form made it possible to test this notion more thoroughly for the pCF10 system and to compare quite precisely the pheromone induction process as measured by different criteria.

We exposed identical cultures of S. faecalis responder cells to various concentrations (based on the results of clumping inducing assays) of synthetic cCF10 and measured both the donor potential of the cells and the production of surface antigens previously associated with the pheromone response. A typical result of the mating assay is depicted in Fig. 3. At concentrations of pheromone above \(2 \times 10^{-11} \text{ M}\), the responder cells are essentially saturated and the induction is maximal. As the pheromone is further diluted and becomes limiting, there is a decline in mating induction, presumably due to the lack of a sufficient number of pheromone molecules to interact with all of the cells. The linear portion of the dose-response curve was in the range of \(10^{-11} - 10^{-12} \text{ M}\). Interestingly, we observed a >40-fold increase in donor potential over uninduced cells at a cCF10 concentration of \(3.8 \times 10^{-12} \text{ M}\) (or about \(2 \times 10^6\) molecules/ml), and nearly a 10-fold increase at \(1.9 \times 10^{-12} \text{ M}\). Since the concentration of donor cells in our induction mixtures is \(1-5 \times 10^6\) colony-forming units/ml, these results provide further evidence for the extreme sensitivity of the responder cells to the pheromone. As can be seen in Fig. 3, the slope of portion of the dose-response curve below the saturation point is about 2.

We also looked at the response of S. faecalis cells to synthetic cCF10 by examining the effects of pheromone induction on the bacterial cell surface. Previous results (4-6) have indicated that the S. faecalis pheromone response is associated with the appearance of novel proteinaceous antigens on the cell surface. In the case of cells carrying pCF10, these include...
Tra130, which mediates pheromone-inducible surface exclusion (6), and Tra150 which, based on recent cloning and immunological studies, is directly involved in formation of mating aggregates. Neither of these antigens is produced by plasmid-free cells. Cells carrying pCF10 produce several lower molecular weight forms (120–130 kilodaltons) of Tra130 (pre-Tra130) constitutively. Upon exposure to CIA, the amount of this protein increases, and the 130-kilodalton mature Tra130 form becomes predominant. Our previously published data (6) indicate that the change in molecular weight, which seems to be required for biological activity, may be the result of a pheromone-inducible post-translational modification. In contrast, Tra150 has only been detected on the surface of pheromone-induced donor cells. We used Western blot analysis of cell surface antigen extracts to examine the antigenic changes in the donor cell surface resulting from exposure to various amounts of synthetic cCF10. The results of this analysis are shown in Fig. 4. While the Tra130 antigen shows a slight increase in amount and size at the higher cCF10 concentrations, the most striking result of this study was the direct relationship between the concentration of cCF10 used in the induction and the amount of Tra150 that could be extracted from the cells. There was an excellent correlation between the mating response of cells and the synthesis of Tra50 constitutively. Upon exposure to CIA, the amount of Tra50 that could be extracted from the cells increased in amount and size at the higher cCF10 concentrations; however, there was no cCF10 even though the three sex pheromones isolated previously contain a Leu in the 3rd residue from the carboxyl terminus, whereas the carboxyl-terminal amino acid residue of the other peptides in this group are hydrophilic (Gly or Ser), that of cCF10 is Val. The three sex pheromones isolated previously contain a Leu in the 3rd residue from the carboxyl terminus, while cCF10 contains a Val. Comparison of the sequences of the various pheromone and inhibitor peptides, along with the analysis of the specificity of the CIA activity of the various peptides shows that, in spite of the extreme hydrophobicity of all of these compounds, the precise amino acid residue sequence of cCF10 is Val.
The dose-response analyses presented in Figs. 3 and 4 provide illustrations of the extreme sensitivity of \textit{S. faecalis} sex pheromone induction. We previously published the response of \textit{S. faecalis} donor cells to crude pheromone preparations utilizing an enzyme-linked immunosorbent assay for the Tra130 antigen as a measure of the response (19). These data indicated that the pheromone response might follow single-hit kinetics, which would give a slope of 1, in contrast to the slope of 2 that was observed in the linear portion of the curve shown in Fig. 3. Although it is possible that the differences between the previous and current results could be due to experimental error, both the previously published data (19) and the experiment shown in Fig. 3 were repeated several times with very similar results. An alternative explanation for the differences is that, in measuring mating frequency, there may be chain length effects. In any case Fig. 3 shows clearly that there is a significant donor response at 1-10 cCF10 molecules/donor cell.

There was a very good correlation between induction of the Tra150 antigen on the surface of donor cells (Fig. 4). Interestingly, the appearance of Tra150 and the increase in the amount of Tra130 also correlated with the disappearance of a 80-kilodalton antigen. We have not previously detected a reproducible pheromone-inducible antigenic change in this molecular mass range. It is likely that the use of the pure synthetic cCF10 in the inductions reduced the antigenic background in these immunoblots, since they are of better quality than any similar blots we have prepared from cells induced with crude CIA from culture filtrates.

The isolation and synthesis of \textit{S. faecalis} sex pheromones, and the demonstration of their biological activities as reported here and previously (13-16), has served to support the relationship of clumping, synthesis of novel surface antigens, and plasmid transfer as originally postulated (2). We feel that further investigation of the mechanism of pheromone binding and signal transduction would be of considerable interest, in light of the extreme sensitivity and specificity of the recognition system that has been demonstrated in these studies. The results reported here should provide a good foundation for such an investigation.

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