Isolation and Structure of Bacterial Sex Pheromone, cPD1

Abstract. *The* Streptococcus faecalis *sex pheromone cPD1, which induces a mating response in cells harboring the conjugative plasmid pPD1, has been isolated and its structure determined. It was found to have a molecular weight of 912, and its amino acid sequence was H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH. A synthetic octapeptide showed the same biological activity and chromatographic behavior as the isolated cPD1. Pheromone activity was detectable at a concentration of approximately* 4×10^{-11}M.

The involvement of hormonal or pheromonal signals in the life cycles of certain microorganisms is well known. Chemical mating signals in ascomycetous and basidiomycetous yeasts have recently attracted much attention; α factor in *Saccharomyces cerevisiae* (*1*), rhodotorucine A in *Rhodosporidium toruloides* (*2*), and tremerogens in several *Tremella* species (*3*), have been chemically characterized. In prokaryotic microbes, extracellular mating signals (sex pheromones), which promote the transfer of conjugative plasmids, have only been reported in *Streptococcus faecalis* (*4–7*).

In *S. faecalis*, conjugative plasmids are classified into two categories. Members of one group transfer at a relatively high frequency (10^{-3} to 10^{-1} per donor) in culture broth; whereas members of the second group transfer poorly in broth (at frequencies of less than 10^{-6} per donor) (*4*). In the latter case, mating experiments are generally conducted on a solid surface, where the frequency can be higher than 10^{-4} per donor. In systems where plasmids transfer readily in culture broth, recipient strains excrete multiple peptidal sex pheromones that induce mating responses in donors harboring certain conjugative plasmids; donors harboring different conjugative plasmids respond to different sex pheromones (*5, 7*). Induction of the mating response results in the synthesis of a proteinaceous substance on the donor surface, which facilitates the formation of mating aggregates. Since donor cells mixed with a cell-free culture filtrate of recipients undergo self-clumping, sex pheromones have also been called clumping-inducing agents (*6*). The clumping-inducing agent cPD1 induces specific responses in strains harboring a 36 megadalton plasmid, pPD1, that determines bacteriocin. It is considered to be a small peptide, based on its sensitivity to proteolytic enzymes and behavior on gel filtration chromatography (*8*).

The isolation of cPD1 is detailed in Table 1. Biological activity was measured by means of the microtiter dilution assay (*7*) with pPD1-containing strain 39-5Sα as responder cells. The responder strain is a derivative of 39-5S that also contains the nonconjugative tetracycline-resistance plasmid pAMα1 (*7, 10*). Drying treatments, including lyophilization or evaporation, were avoided and dithiothreitol was added to all the eluents (at a final concentration of 100 μg/ml) except in the final step, to protect the active substance against oxidation. It was possible to isolate 20 μg of pure cPD1 from 100 liters of cell culture, which induced the clumping of 39-5Sα cells at a concentration of 4 pg per 100 μl (approximately $4 \times 10^{-11}M$). Donor cells may be capable of responding to only a few molecules of the pheromone.

The fast atom bombardment (FAB) mass spectrum of cPD1 showed two quasi-molecular ion peaks at mass-to-charge ratios 913 $(M+H)^+$ and 935 $(M+Na)^+$ (*11*); consequently, the molecular weight of cPD1 was concluded to be 912. The isolated peptide was hydrolyzed with 5.7*N* HCl containing 4 percent thioglycolic acid at 110°C for 20 hours, and the

Table 1. Isolation of cPD1.

Purification step*	Total weight (mg)	Total activity (units)	Specific activity† (units/μg)
Culture medium (20 liter)	680,000‡	12,800,000	0.019
Charcoal		6,400,000	
QAE-Sephadex A-25		3,200,000	
LRP-2	13.2§	3,200,000	240
SSC-ODS-742 (first)	0.052‖	1,600,000	30,000
SSC-ODS-742 (second)¶	0.004‖	1,000,000	250,000

*Purification. For cPD1 production, 20 liters of late log phase *S. faecalis* JH2-2 cells (*9*) in 3.64 percent Todd-Hewitt broth (Oxoid) were prepared. The cell-free supernatant was passed through a charcoal column (Wakojunyaku), which was then eluted with 50 percent pyridine. The eluate was diluted fivefold with water and applied to a QAE-Sephadex A-25 column (Pharmacia, 3.3 by 8.5 cm, Cl^- form) equilibrated with 10 percent pyridine. The active fractions were recovered with 0.1*M* sodium chloride. Ethanol was added to the active fractions to a final concentration of 25 percent, and the solution was subjected to reversed-phase high performance liquid chromatography (HPLC) on an LRP-2 column (Whatman, 2.0 by 30 cm) at 10 ml/min. The column was washed with 20 percent acetonitrile for 20 minutes and eluted with a gradient of 20 to 50 percent acetonitrile (60 minutes; 10 ml/min). Active fractions were combined, diluted with 10 m*M* ammonium acetate, (1.5 volumes), and subjected to reversed-phase HPLC on a SSC-ODS-742 column (Senshukagaku, 1.0 by 25 cm) at 4 ml/min. The column was eluted with a gradient of 30 to 50 percent acetonitrile in 10 m*M* ammonium acetate (40 minutes; 4 ml/min), and the active eluates corresponding to five batches (100 liters total) were diluted twofold with 0.1 percent trifluoroacetic acid. This solution was again subjected to reversed-phase HPLC on SSC-ODS-742, and the column was eluted with a gradient of 32 to 35 percent acetonitrile in 0.1 percent trifluoroacetic acid for 30 minutes at 4 ml/min. The peak of cPD1 appeared when the concentration of acetonitrile reached 32.9 percent. †Bioassay. Samples dissolved in 50 μl of N2GT (Oxoid Nutrient Broth No. 2 supplemented with 0.2 percent glucose and 0.1*M* tris-HCl, *p*H 7.7) were serially diluted in 96-well microtiter plates. A suspension of 39-5Sα cells (50 μl) was added to each well. One unit of activity was defined as the lowest amount that could induce clumping of responder cells after incubation for 120 minutes at 37°C with shaking. ‡Dry weight. §Calculated from absorbance at 280 nm. ‖Calculated from absorbance at 220 nm. ¶Values relative to a 20-liter batch.

Table 2. Induction of plasmid transfer by cPD1. The plasmid-free strain JH2-2 has a chromosomal determinant for rifampicin resistance (*9*) and strain OG1S(pAD1::Tn*917*) harbors a derivative of pAD1 carrying an erythromycin resistance determinant located on the transposon Tn*917* (*17, 18*). Strain 39-5Sα harbors, in addition to the conjugative plasmid pPD1, tetracycline resistance plasmid pAMα1, which is nonconjugative but mobilized efficiently by pPD1. The donor potential of pPD1 was monitored by its ability to mobilize pAMα1 (*7, 10*). Donor cells [39-5Sα or OG1S(pAD1::Tn*917*)] were grown to late log phase and diluted into a mixture of equal volumes of THB (Oxoid Todd-Hewitt broth) and JH2-2 culture filtrate or cPD1 (2.5 ng per milliliter of THB). The cells were then incubated for 45 minutes at 37°C. At the end of the donor induction period, 0.2 ml of the donors were mixed with 1.8 ml of a log phase culture of JH2-2 cells. After 10 minutes at 37°C the cells were plated on selective media (Difco Pennassay broth, 1.0 percent agar) in the presence of rifampicin (25 μg/ml) and tetracycline (5 μg/ml) for 39-5Sα × JH2-2, or rifampicin (25 μg/ml) and erythromycin (50 μg/ml) for OG-1S(pAD1::Tn*917*) × JH2-2.

Mating system	Conjugation conditions	Transconjugants per donor
39-5Sα × JH2-2	JH2-2 filtrate	3.8×10^{-4}
	cPD1 (native)	5.0×10^{-4}
	cPD1 (synthetic)	3.6×10^{-4}
	control (THB)	$<3.4 \times 10^{-7}$
OG1S(pAD1::Tn*917*) × JH2-2	JH2-2 filtrate	1.1×10^{-3}
	cPD1 (native)	4.7×10^{-6}
	control (THB)	2.4×10^{-6}

hydrolyzate was applied to a Shimadzu amino acid analyzing system with the use of *o*-phthalaldehyde postcolumn derivatization (*12*). The proportions of amino acids in the hydrolyzate of cPD1 were serine, 1.13; glycine, 1.57; valine, 1.00; methionine, 0.88; leucine, 1.92; and phenylalanine, 1.62, or roughly, 1:1:1:1:2:2, respectively. Although the molar ratio of glycine is high, we believe that the amino acid ratios are as indicated for the reasons described in (*13*). The amino acid sequence of cPD1 was determined by a manually operated direct Edman method. The phenylthiohydantoin amino acid derivative obtained at each degradation cycle was identified by high-performance liquid chromatography (HPLC) (*14*). The results reveal that the amino acid sequence of cPD1 was H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH.

Fully protected cPD1 was synthesized in solution by a stepwise chain elongation from the carboxyl terminus. In each step, condensation was achieved by the HONB(*N*-hydroxy-5-norbornene-2,3-dicarboximide)–DCC(*N*,*N*′-dicyclohexylcarbodiimide) method (*15*). After removal of all the protective groups with hydrogen fluoride (*16*), the major product of synthesis was purified by reprecipitation from trifluoroacetic acid. Correct synthesis of cPD1 was confirmed by amino acid analysis and FAB mass spectrometry. The retention time on HPLC and the clumping-inducing activity of the synthetic octapeptide were fully identical with those of native cPD1.

It has been reported that the frequency of plasmid transfer can be increased by several orders of magnitude, by exposing donor cells to a cell-free filtrate of recipient cells for 20 to 50 minutes prior to mating (*6*, *7*). Enhancement of plasmid transfer was observed for both isolated and synthetic cPD1 adjusted to a concentration whose activity corresponded to that in a recipient cell-free filtrate (Table 2). The cPD1 did not affect mating between OG1S(pAD1::Tn*917*) and JH2-2, which involves a different conjugative plasmid (pAD1::Tn*917*) that responds to cAD1 (*17*). This was consistent with the observation that cPD1 failed to induce self-clumping of OG1S(pAD1::Tn*917*) at a concentration as high as 100 ng per 100 μl (approximately $1 \times 10^{-6} M$) in a microtiter dilution well. These results confirm the chemical specificity of characterized pheromone activity.

We believe that our findings are relevant to studies on the plasmid-determined pheromone-inactivation mechanism in donor cells. It is possible that the single serine residue is involved in the inactivation as a relationship between formation of a phosphodiester bond and pheromone-inactivaton has been reported (*8*).

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11. The isolated cPD1 (approximately 5 μg) was dissolved in 10 μl of 50 percent acetonitrile. A portion (2 μl) of sample solution was mixed with 2 μl of diethanolamine on a small stainless steel tip and then the FAB mass spectrum was measured with a JMS DX-300 mass spectrometer (JEOL) with xenon as the fast atom.
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19. We thank Drs. Masahiko Yoneda and Hisayoshi Okazaki, Central Research Division, Takeda Chemical Industries, Ltd., for a large-scale fermentation; Professor Keiji Yano, Department of Agricultural Chemistry, University of Tokyo, for suggestions at the beginning of this work; and F. An and B. White for assistance and discussion. Supported in part by NIH grants DE02731 and AI10318 (D.B.C.).

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7 June 1984; accepted 4 September 1984

Blockade of *N*-Methyl-D-Aspartate Receptors May Protect Against Ischemic Damage in the Brain

Abstract. *In rats ischemia of the forebrain induced by a 30-minute occlusion of the carotid artery, followed by 120 minutes of arterial reperfusion, produced ischemic lesions of selectively vulnerable pyramidal cells in both hippocampi. Focal microinfusion into the dorsal hippocampus of 2-amino-7-phosphonoheptanoic acid, an antagonist of excitation at the* N*-methyl-D-aspartate–preferring receptor, before ischemia was induced protected against the development of ischemic damage. It is proposed that excitatory neurotransmission plays an important role in selective neuronal loss due to cerebral ischemia.*

Cerebral ischemia and status epilepticus produce similar patterns of neuronal loss in the hippocampus (*1*), involving preferentially those pyramidal neurons (in regions CA1, CA3, and CA4) that most readily show burst firing (*2*). This led to the suggestion (*3*) that enhanced calcium entry during burst firing, in the course of either status epilepticus or the reperfusion phase after ischemia, accounts for selective neuronal vulnerability, with excessive intracellular calcium leading to cell death in a manner similar to that proposed for muscle and liver cells (*4*). We have shown that mitochondria in selectively vulnerable hippocampal neurons show massive overloading with calcium during status epilepticus and after 2 hours of reperfusion following cerebral ischemia (*5*).

Burst firing can be triggered in hippocampal, cortical, or striatal neurons by iontophoresis of aspartate or of other compounds acting on the *N*-methyl-D-aspartate (NMDA)–preferring receptor, including quinolinic acid (*6*). The excit-