ISOLATION AND PARTIAL CHARACTERIZATION OF A CERULENIN-SENSITIVE MUTANT OF *PSEUDOMONAS AERUGINOSA*

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(Received for publication June 1, 1983)

Sensitivity of *Pseudomonas aeruginosa* to cerulenin was first tested. The result indicated that this bacterium is resistant to cerulenin. Cerulenin-sensitive mutants were isolated from *P. aeruginosa* PML 1552 by 1-methyl-3-nitro-1-nitrosoguanidine treatment and following carbenicillin plus D-cycloserine screening. Isolated mutants were designated CSM-1 to CSM-19, and some characters of CSM-19, which showed rapid growth almost as well as parent strain in the medium without cerulenin, were examined. The cell growth of CSM-19 was greatly inhibited by 50 μg/ml of cerulenin, but when the mixture of cellular fatty acids or both *cis*-vaccenic acid and palmitic acid were added to the medium, the growth was partially recovered. Incorporation of radioactivity into fatty acids from [1-^{14}C]acetate was lowered by cerulenin. Those results mean that the fatty acid synthesis of CSM-19 was decreased by cerulenin. Although cellular fatty acid composition and amount were not notably different between CSM-19 and PML 1552, CSM-19 had less phosphatidylethanolamine, and more phosphatidylglycerol and cardiolipin than PML 1552. CSM-19 was also supersensitive to several other antibiotics, especially to carbenicillin and tetracycline, when compared with PML 1552, although both strains showed identical sensitivity to D-cycloserine, polymyxin B, and chloramphenicol.

Cerulenin is an antibiotic that inhibits condensation enzyme (β-keto-acyl thioester synthetase) in fatty acid synthesis[1,2]. This antibiotic appears to be a very useful compound to study fatty acid metabolism. In spite of the *in vitro* potent inhibitory effect, however, the degree of cell growth inhibition by cerulenin varies according to the species of the microorganisms. It shows strong inhibition on the growth of yeasts such as *Candida albicans* or *Saccharomyces cerevisiae*, and yeast-like fungi. Among bacteria, it is also highly inhibitory to the growth of the strains belonging to the genera Mycobacterium, Nocardia and Streptomyces. However, there are many species which are relatively resistant to cerulenin[3,4]. Consequently, only cerulenin-sensitive or moderately sensitive species such as *Escherichia coli*[5,6] or *Bacillus subtilis*[7] have been adopted in the study of bacterial fatty acid metabolism using cerulenin.

We have studied the synthesis of hydroxyl fatty acids of *Pseudomonas ovalis*[8] and *P. aeruginosa*, and it was necessary to control *de novo* synthesis of fatty acids of the bacteria. Cerulenin was examined for this purpose, but it could not inhibit the growth of *P. aeruginosa*. It is therefore required to obtain cerulenin-sensitive mutants. *P. aeruginosa* PML 1552 was chosen as the parent strain since it is an outer membrane esterase defective mutant isolated by OHKAWA *et al.*[9] (outer membrane esterase hydrolyzes fatty acyl-CoA, and then disturbs enzymatic experiments of lipid metabolism). This paper describes the isolation and partial characterization of a cerulenin-sensitive mutant of *P. aeruginosa* PML 1552.

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Materials and Methods

Organisms and Cultivation

Pseudomonas aeruginosa PML 1552, *P. aeruginosa* PAO 1, and *P. aeruginosa* PAT 2004 were kindly given to us by Dr. M. KAGEYAMA of Mitsubishi-Kasei Institute of Life Science. Other strains used in this study were obtained from IAM Culture Collection, The University of Tokyo.

Cells were cultivated in N-medium, which consists of 1% glucose, 0.5% yeast extract (Difco, Detroit, U.S.A.), 0.5% Casamino Acids (Difco), 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4 and 0.02% $MgSO_4 \cdot 7H_2O$. When cerulenin or fatty acids were dissolved in the N-medium, 0.025% Triton X-100 was added (N+Triton medium). For isolation of the mutants, A-medium and G-medium were used. A-medium consists of 0.2% sodium acetate, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.4 ml of 0.4% $FeCl_2 \cdot 6H_2O$ solution per liter, and G-medium had the same composition except that sodium acetate was replaced by 0.2% glucose.

Source of Chemicals

Cerulenin either was obtained as a kind gift of Dr. S. ŌMURA of Kitasato University or was purchased from Sigma, St. Louis, U.S.A. Authentic fatty acids were also purchased from Sigma. Carbenicillin and D-cycloserine were purchased from Fujisawa Pharmaceutical Co. (Osaka, Japan) and Nakarai Co. (Kyoto, Japan), respectively. Other antibiotics were obtained from Sigma, Boehringer Mannheim Co. (Mannheim, F.R.G.), Meiji Seika Kaisha Co. (Tokyo, Japan) and Banyu Pharmaceutical Co. (Tokyo, Japan). [1-^{14}C]Acetate was purchased from Radiochemical Centre (Amersham, England).

Isolation of Mutants

P. aeruginosa PML 1552 was inoculated in 10 ml of N-medium and cultured at 30°C with shaking. Cells were harvested at the logarithmic phase, then washed with and suspended in 0.1 M citrate buffer (pH 6.0) to a density of 7×10^8 cells/ml. To this cell suspension 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was added at the final concentration of 20 μg/ml, and incubated at 37°C for 45 minutes. After removal of MNNG by centrifugation, the treated cells were allowed to grow in A-medium for 10 hours for several division cycles. The culture was centrifuged, washed and starved in A-medium without sodium acetate for 3 hours, then transferred to A-medium containing 1 mg/ml of carbenicillin, 1 mg/ml of D-cycloserine and 20 μg/ml of cerulenin. After overnight cultivation the survived cells were washed several times with A-medium solution and spread on agar plates containing G-medium. The plates were incubated at 30°C for 2 to 3 days, and colonies which appeared were transferred to both cerulenin-containing and cerulenin-free plates, and the mutants unable to grow on the former were picked up. Cerulenin-containing plates were prepared from 10 ml of heated N+Triton medium containing 3% agar, and 10 ml of N+Triton medium containing 200 μg/ml of cerulenin by mixing both solutions in the Petri dish quickly before the agar solidified.

Preparation of Cellular Fatty Acid Solution

Cells of *P. aeruginosa* PML 1552 (100 mg dry weight) were hydrolyzed in 4 M HCl at 100°C for 10 hours, and fatty acids were extracted twice with 10 ml of diethyl ether. The ether layers were combined and evaporated to dryness and dissolved in 2 ml of methanol.

Measurement of *In Vitro* Fatty Acid Synthesis

Fatty acid synthesis was measured using [1-^{14}C]acetate. Four drops of precultured cell suspension were added to 10 ml of N+Triton medium. Then cellular fatty acid solution (200 μl) and [1-^{14}C]-acetate (0.5 μCi) was also added. Cells were grown until optical density of 660 nm reached 0.7, and harvested. Cells washed three times with acetone were dried and hydrolyzed with 4 M HCl at 100°C for 10 hours. Fatty acids were extracted with petroleum ether from the hydrolysate, and the non-polar fatty acids and hydroxyl fatty acids were separated each other with small column of Silica gel 60 (70~230 mesh ASTM, Merck, Darmstadt, F.R.G.) after methyl esterified. An aliquote was submitted to the liquid-scintillation counter (Beckmann LS-230) and the rest to the radio-gas chromatography system (Aloka). Fatty acid methyl esters were separated on gas chromatography with 2 m glass column packed with 10% diethylene glycol succinate/Chromosorb W-AW (80~100 mesh).

Analysis of Phospholipid Composition

Lyophilized cells (1 g) were extracted three times with $CHCl_3$ - CH_3OH (1: 3), and the combined solvent fraction was evaporated. An aliquot was spotted on the precoated Silica gel 60 plate (Merck) and developed with the solvent system, $CHCl_3$ - CH_3OH - CH_3COOH (65: 25: 10). Lipids on the plate were visualized by spraying with ammonium sulfate - conc. H_2SO_4 solution, and heating at 180°C for 30 minutes. Each spot on the plate was identified by the Rf value.[10] Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) were detected also by spraying with DITTMER reagent[11]. Spots corresponding to PE, PG and CL were scraped off from the plate, and the phosphorus content was determined by the method of BARTLETT[12].

Results

Cerulenin Sensitivity of *P. aeruginosa*

Several strains of *P. aeruginosa* and two taxonomically similar species *P. fluorescens* and *P. ovalis* were determined on the cerulenin sensitivity. As shown in Table 1, most strains belonging to those species grew well even in the medium containing 100 μg/ml of cerulenin. On the other hand, the growth of *E. coli* K-12 IAM 1264 was completely inhibited at this concentration of cerulenin. This result indicates that *P. aeruginosa* and the related species are very resistant to cerulenin.

Isolation of Mutants

Many cerulenin-sensitive mutants have been isolated from *P. aeruginosa* PML 1552 by the method described above. All the mutants retained outer membrane esterase-defective character of the parent strain. Those mutants were designated CSM-1 to CSM-19, with the growth rate of each mutant being

Table 1. Effect of cerulenin on the growth of strains of *P. aeruginosa* and related species.

Strains	Cerulenin (100 μg/ml)	Growth (OD_{660})	Ratio of OD_{660} (+cerulenin/−cerulenin)
P. aeruginosa PML 1552	+	0.59	
	−	1.06	0.56
P. aeruginosa PAO 1	+	0.50	
	−	0.65	0.77
P. aeruginosa PAT 2004	+	0.61	
	−	0.82	0.74
P. aeruginosa IAM 1007	+	0.52	
	−	0.97	0.54
P. aeruginosa IAM 1052	+	0.18	
	−	1.04	0.17
P. aeruginosa IAM 1275	+	0.51	
	−	0.76	0.67
P. aeruginosa IAM 1514	+	0.63	
	−	0.80	0.79
P. ovalis IAM 1177	+	0.12	
	−	0.85	0.14
P. ovalis IAM 1219	+	0.49	
	−	0.61	0.80
P. fluorescens IAM 12022	+	0.18	
	−	0.47	0.38
E. coli K-12 IAM 1264	+	0.02	
	−	0.39	0.05

One ml of N+Triton medium with or without cerulenin was poured into the tubes and one drop of cell suspension was inoculated. *P. aeruginosa* PML 1552 and *E. coli* K-12 IAM 1264 were cultivated at 30°C for 20 hours with shaking, and other strains were cultivated under the same conditions for 27 hours. Cell suspensions were diluted 10 fold to measure the absorbance at 660 nm.

Fig. 1. Recovery of cell growth by the addition of fatty acids.

To 10 ml of N+Triton medium, one drop of precultured cell suspension (overnight culture) was added, and incubated at 30°C with shaking. Absorbance at 660 nm was measured every hour directly with culture test tubes (ϕ18 mm).

○, PML 1552 without cerulenin; △, CSM-19 without cerulenin; ▲, CSM-19 with 50 μg/ml of cerulenin; □, CSM-19 with 50 μg/ml of cerulenin and 200 μl of cellular fatty acid mixture (equivalent to 10 mg dry weight cells) prepared by the method described in the text; ■, CSM-19 with 50 μg/ml of cerulenin and 500 μg of *cis*-vaccenic acid and 200 μg of palmitic acid. Fatty acids were dissolved in 200 μl of methanol when added.

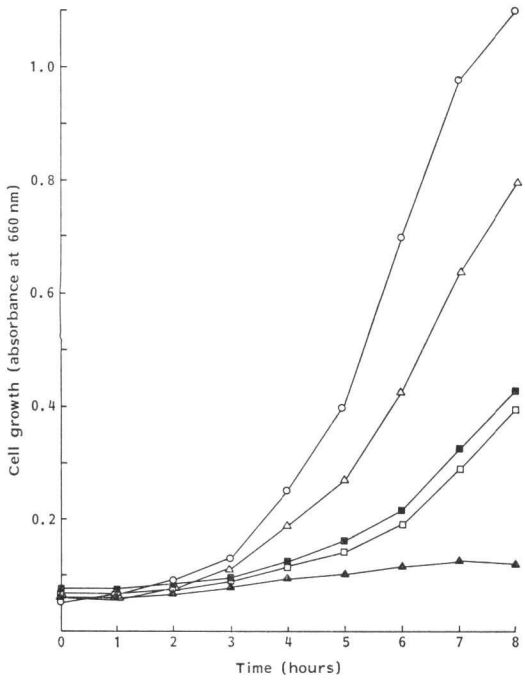

Table 2. Effect of cerulenin on the incorporation of radioactivity from [1-^{14}C]acetate into cellular fatty acids of CSM-19.

Fatty acids	Specific radioactivity (dpm/μg)		Ratio (B)/(A)
	Without cerulenin (A)	50 μg/ml cerulenin (B)	
$C_{16=0}$	730	130	0.18
$C_{16=1}$	780	130	0.17
$C_{18=1}$	490	110	0.22
3-OH-C_{10}	890	250	0.28
2-OH-C_{12}	960	420	0.44
3-OH-C_{12}	980	330	0.34

CSM-19 was cultivated with 50 μg/ml of cerulenin or without cerulenin in the medium containing cellular fatty acids and [1-^{14}C]acetate. Fatty acids were extracted from the cells and analyzed with radio-gas chromatography. The detailed procedure is described in Materials and Methods.

tested. Distinct from the parent strain PML 1552, the growth of all 19 mutants was extremely limited in medium containing 50 μg/ml of cerulenin. Among them, strain CSM-19 was chosen and further characterized because the growth rate of this mutant was almost as good as that of the parent strain in N-medium.

Recovery of Cell Growth by the Addition of Fatty Acids

Minimum inhibitory concentration of cerulenin for CSM-19 was first estimated. Though only 10 μg/ml of cerulenin considerably affected the growth of CSM-19, 50 μg/ml of cerulenin was needed for complete inhibition of the growth. Recovery experiments were thus carried out with this concentration of cerulenin.

Fig. 1 shows the time course of cell growth under several conditions. The growth of CSM-19 was completely depressed by 50 μg/ml of cerulenin throughout the incubation period, while without cerulenin the mutant grew rapidly and reached 0.8 of OD_{660} after 8 hours of incubation. PML 1552 grew faster than CSM-19 in the medium without cerulenin, while both strains showed about the same growth rate in N-medium (data not shown). This suggests that 0.025% Triton X-100 in the medium inhibited the growth of CSM-19. When cellular fatty acid extract or a mixture of *cis*-vaccenic acid and palmitic acid was added to the cerulenin-containing medium, the cell growth was partially recovered (Fig. 1). When *cis*-vaccenic acid alone was added, a little effect was observed on the recovery, but palmitic acid alone had no effect (data not shown).

Table 3. Determination of phospholipid composition.

Phospholipid	Rf value	PML 1552 (mol/mg cells)	CSM-19 (mol/mg cells)
Phosphatidylethanolamine	0.33	8.3×10^{-8}	6.8×10^{-8}
Phosphatidylglycerol	0.44	1.2×10^{-8}	1.5×10^{-8}
Cardiolipin	0.61	3.5×10^{-9}	4.7×10^{-9}

Cells were extracted with $CHCl_3$ - CH_3OH (1 : 3) and the concentrated lipid solution was spotted on the silica gel plate. The lipids were developed with the solvent system $CHCl_3$ - CH_3OH - CH_3COOH (65 : 25 : 10). Three types of phospholipid were scraped off and the molar ratio was determined by the method described in the text.

Decrease of Fatty Acid Synthesis by Cerulenin

Incorporation of [1-^{14}C]acetate into cellular fatty acids of CSM-19 was measured under the condition of growing with or without cerulenin as described in Materials and Methods. The total radioactivity incorporated into lipid fraction was decreased from 1.6×10^5 cpm to 3.0×10^4 cpm by addition of 50 μg/ml of cerulenin. The specific radioactivity of each fatty acid was also decreased by cerulenin as shown in Table 2.

Table 4. Minimum inhibitory concentration of other antibiotics.

Antibiotics	MIC (μg/ml)		MIC ratio (A)/(B)
	PML 1552 (A)	CSM-19 (B)	
Carbenicillin	5.0	0.078	64
Ampicillin	5.0	1.25	4
D-Cycloserine	62.5	62.5	1
Polymyxin B	0.63	0.63	1
Streptomycin	25000	6250	4
Kanamycin	125	31.3	4
Tetracycline	10.0	0.16	63
Novobiocin	125	7.80	16
Rifampicin	12.5	6.25	2
Chloramphenicol	62.5	62.5	1

In 5 ml of N-medium, each antibiotic was dissolved at various concentrations and precultured cells were inoculated at a density of 10^5 to 10^6 cells/ml. Those cell suspensions were cultivated at 30°C for 18 hours with shaking, and the concentration of antibiotics at which no cell growth was recognized was estimated as MIC.

Lipid Analysis of CSM-19

The lipid composition of CSM-19 was investigated because cerulenin is a hydrophobic antibiotic and its uptake into the mutant cell may have a relation to a certain alteration of the membrane lipid. First, when we compared the fatty acid composition of the cells of PML 1552 and CSM-19, the relative amount of the fatty acids was not notably different each other. Second, the phospholipid profile was examined. Three types of phospholipid, PE, PG and CL were determined. The mutant strain CSM-19 had less PE (about 80% of that of PML 1552), which is the major phospholipid of this bacterium. On the contrary, more PG and CL were detected in CSM-19 cells than in PML 1552 cells (Table 3).

Sensitivity of CSM-19 to Other Antibiotics

Minimum inhibitory concentration (MIC) of other antibiotics was estimated (Table 4). CSM-19 was about 60 fold more sensitive to carbenicillin and tetracycline than PML 1552. The mutant was also much more sensitive to novobiocin (16 fold), and a little more sensitive to ampicillin, streptomycin, kanamycin and rifampicin. CSM-19 and PML 1552 showed the same MIC to D-cycloserine, polymyxin B, and chloramphenicol.

Discussion

Though cerulenin is a useful biological compound for studying fatty acid metabolism, there are

many microorganisms resistant to cerulenin, and *P. aeruginosa* is confirmed to be one of them (Table 1). In this study some cerulenin-sensitive mutants were obtained from *P. aeruginosa* PML 1552. CSM-19, one of those mutants, could grow rapidly as well as PML 1552 in N-medium, but the growth was completely inhibited with 50 μg/ml of cerulenin.

The advantage of using cerulenin is that this antibiotic can inhibit fatty acid biosynthesis. It is therefore important to clarify whether *de novo* synthesis of fatty acids is really depressed by cerulenin when it is added to the medium. In some bacteria the growth inhibition by cerulenin is known to be partially recovered by the addition of fatty acids.[6,13] We examined whether CSM-19 also showed this character. As shown in Fig. 1, the growth of CSM-19 was partially recovered by cellular fatty acids or authentic fatty acids. Incorporation of radioactivity into cellular fatty acids from [1-^{14}C]-acetate was also examined. Total radioactivity in fatty acid fraction or specific radioactivity of each fatty acid markedly decreased by cerulenin (Table 2). These results demonstrated that the growth inhibition of CSM-19 by the antibiotic is caused by retardation of *de novo* fatty acid synthesis.

It is noteworthy that the specific radioactivity of hydroxyl fatty acids were 2 to 3 fold higher than those of non-polar fatty acids in the cells when the bacteria were grown in the medium containing [1-^{14}C]acetate under the presence of cerulenin and extract of the cellular fatty acids (Table 2). This suggests that the added hydroxyl fatty acids were hardly incorporated into the cellular hydroxyl fatty acids, while the non-polar fatty acids were subjected to the corresponding fatty acids. The hydroxyl fatty acids in this bacterium are located in lipid A part of lipopolysaccharide, and it is therefore thought that the exogeneous hydroxyl fatty acids cannot be directly utilized for the lipid A synthesis. The less availability of supplied hydroxyl fatty acids may explain the reason why CSM-19 did not show a full recovery from cerulenin inhibition of the cell growth when cellular fatty acids were added to the medium (Fig. 1).

As shown in Table 4, the cerulenin-sensitive mutant CSM-19 showed very low MIC to carbenicillin and tetracycline when compared with that of PML 1552. One of the possible accounts for those multiple changes of sensitivity is the change of permeability in cell envelope caused by a mutation. Since cerulenin has rather a hydrophobic property, other hydrophobic antibiotics such as novobiocin, rifampicin or chloramphenicol were tested on the inhibitory effect to CSM-19. Not all of them were highly effective against CSM-19. Therefore the mutation did not simply cause the change of the membrane to ease the penetration of hydrophobic substances. But a certain alteration of the phospholipid composition (Table 3), and growth inhibition by 0.025% Triton X-100 (Fig. 1) demonstrate that some alteration may have occurred in the cell envelope of CSM-19. To further characterize the mutation, it will be necessary to obtain some revertants and to analyze other components of cell envelope, decomposing enzymes of cerulenin, and fatty acid synthetase system of PML 1552, CSM-19 and the revertants.

Acknowledgments

We are very grateful to Dr. M. KAGEYAMA, Mitsubishi-Kasei Institute of Life Science, Tokyo, Japan, for giving us strains of *P. aeruginosa*. We should also like to thank Dr. S. ŌMURA, Kitasato University, Tokyo, Japan, for giving the antibiotic cerulenin. Radio-gas chromatographic analysis was performed in Radioisotope Center, The University of Tokyo. We would like to thank very much Dr. N. MORIKAWA and other members of the Center.

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