MODE OF ACTION OF A BACTERIOCIN FROM
ERWINIA CAROTOVORA III.
PROPERTIES OF PHOSPHOLIPASE A OF *ERWINIA*
CAROTOVORA AND ITS INVOLVEMENT IN
PHOSPHOLIPID DEGRADATION CAUSED
BY CAROTOVORICIN

YOSHIFUMI ITOH,¹ TOSHIHARU IWATA, KAZUO IZAKI,
AND HAJIME TAKAHASHI²

*Department of Agricultural Chemistry, Faculty of Agriculture,
Tohoku University, Sendai 980*

(Received February 3, 1981)

Phospholipase A in the outer membrane of *Erwinia carotovora* 645ArT was found to be activated by various detergents such as cholate, deoxycholate and Triton X-100, and methanol. The enzyme was inhibited by ethylenediaminetetraacetic acid, and Ca^{2+} was required for the enzyme activity. The enzyme hydrolyzed phosphatidylethanolamine and phosphatidylglycerol to form nearly equimolar amounts of free fatty acids and lysophospholipids. Cardiolipin, however, was not susceptible to the enzyme. In a mutant strain deficient in the phospholipase A activity, the rate of degradation of membrane phospholipid caused by carotovoricin Er, a bacteriocin from *Erwinia carotovora* strain Er, declined to about 1/10 of that of the parent strain under similar conditions. Lysis caused by carotovoricin Er also diminished in the phospholipase A-deficient mutant. These results imply that carotovoricin Er provokes an activation of phospholipase A in sensitive cells, and subsequently, degradation of membrane phospholipid and cell lysis result. Phospholipase A-deficient mutant, however, still retained sensitivity to killing activity of carotovoricin Er. Hence, it could be concluded that activation of phospholipase A is not related to the primary bactericidal action of the bacteriocin.

Bacteriocins are bactericidal proteinaceous substances produced by certain bacteria (*1*). Carotovoricin Er, a bacteriocin from *Erwinia carotovora* strain

¹ Present address: Department of Bacteriology, Shinshu University School of Medicine, Matsumoto 390.

² Author to whom reprint requests should be addressed.

Er, is a species of high molecular weight bacteriocin according to the classification of BRADLEY (2), and its structure consists of a contractible sheath, a core surrounded with the sheath and fibers, quite closely resembling that of a tail part of bacteriophage (3).

In previous reports, we demonstrated that carotovoricin Er caused prompt and extensive cell lysis (4) and membrane phospholipid degradation (5) of a sensitive strain. Our previous results suggested that phospholipase A of a sensitive strain might be a major cause of the phospholipid degradation and lysis of the sensitive cells treated with carotovoricin Er. From the examination of the morphology of native carotovoricin Er and carotovoricin Er adsorbed to the cell surface of a sensitive strain, infection of the bacteriocin is considered to proceed as follows: i) Specific interaction of the tips of the bacteriocin fibers with bacteriocin receptors on the cell surface. ii) Contraction of the sheath after adsorption of the fibers onto the receptor. iii) Interaction of the core with the cell envelope. During the course of infection, the fibers and core of the bacteriocin would interact with the outer membrane of sensitive cells in a specific manner. It would be possible to speculate that at the early stages of the infection, the bacteriocin might provoke some structural alteration of the cell envelope, or disturbance of enzyme systems in the cell envelope.

We describe in this report some properties of phospholipase A of *E. carotovora* 645ArT, a strain sensitive to carotovoricin Er, which was able to be activated by treatment with the bacteriocin, and isolation of mutants deficient in phospholipase A activity. Phospholipid degradation in a phospholipase A-deficient mutant was considerably reduced compared to the wild strain when both strains were treated with carotovoricin Er. This result suggests that phospholipid degradation in carotovoricin Er-treated cells was caused mainly by the action of phospholipase A and that treatment of the sensitive cells by carotovoricin Er caused an activation of phospholipase A which exists in a latent form in the normal cells.

MATERIALS AND METHODS

Bacterial strains. *E. carotovora* Er (AMS 6082) was used as a bacteriocinogenic strain and *E. carotovora* 645Ar, which was kindly supplied by Prof. H. Tsuyama of the Faculty of Agriculture, Iwate University, Morioka, was used as a sensitive strain to carotovoricin Er (3-5). *E. carotovora* 645ArT (5) was a thymine-requiring mutant derived from *E. carotovora* 645Ar. *E. coli* K1061, a mutant requiring unsaturated fatty acid for growth, was kindly supplied from Dr. H. R. Kaback of the Roche Institute for Molecular Biology, Nutley, New Jersey, U.S.A.

Medium and growth conditions. The organisms were usually grown in M9 minimal medium supplemented with 0.2% of glucose and casein acid hydrolysate respectively, or nutrient broth at 30° with reciprocal shaking (120 cycles/min) as described previously (3). Nutrient agar was made by solidifying the nutrient broth

with 1.5% agar.

Preparation of carotovoricin Er. Carotovoricin Er was purified from the culture of *E. carotovora* Er induced with 0.2 μg per ml of mitomycin-C (Kyowa Hakko Kogyo Co., Ltd., Tokyo) and prepared for use as described previously (3, 4).

Preparation of ^{14}C -labeled phospholipids. ^{14}C -Phospholipids were extracted from the cells of *Escherichia coli* K12 W2252 grown in the presence of 0.1 μCi per ml of [$1\text{-}^{14}\text{C}$] acetate (Specific activity 58 mCi/mmol, The Radiochemical Centre, Amersham, England) according to the procedure of BLIGH and DYER (6). ^{14}C -Phosphatidylethanolamine and ^{14}C -cardiolipin were purified by silicic acid column chromatography (7). ^{14}C -Phosphatidylglycerol was further purified by DEAE-cellulose column chromatography (8), because the fraction of phosphatidylglycerol from silicic acid column chromatography contained a significant amount of phosphatidylethanolamine. Specific radioactivities and purities of ^{14}C -phosphatidylethanolamine, ^{14}C -phosphatidylglycerol, and ^{14}C -cardiolipin were 352 dpm/nmol (98%), 382 dpm/nmol (95%), and 780 dpm/nmol (97%), respectively. [$1\text{-}^{14}\text{C}$]-acyl-glycerolphosphorylethanolamine was prepared from ^{14}C -phosphatidylethanolamine (48 μmol) by treatment with phospholipase A_2 from *Vipera russelli* (Sigma Chemical Co., St. Louis, U.S.A.) and purified on Silica gel thin-layer (Silica gel 60, E. Merck, Darmstadt, West Germany) chromatography with chloroform-methanol-water (65:25:4, v/v) as a solvent. Purity of [$1\text{-}^{14}\text{C}$]-acyl-glycerolphosphorylethanolamine was 90%, and its specific radioactivity 170 dpm/nmol. Purity of ^{14}C -phospholipids was determined by counting radioactivities on the Silica gel plate developed with appropriate solvent systems. Chemical amount of phospholipid was determined by colorimetric analysis of phosphate as described by BARTLETT (9) using α -glycerophosphate as a standard.

Preparation of enzyme. Mid-log phase cells grown in M9-glucose-casamino acid medium (200 ml) were harvested and washed twice with 20 ml of ice-cold 0.02 M tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer containing 0.15 M NaCl, pH 7.2, and suspended in 20 ml of 0.05 M Tris-HCl buffer, pH 7.2. The cells were disrupted by a Kubota sonicator at 10 kHz for 3 min. After centrifugation at $5,000\times g$ for 10 min to remove intact cells and cell debris, the supernatant was used for enzyme preparation. Protein concentration was determined by the method of LOWRY *et al.* (10) using bovine serum albumin as standard.

Assay of phospholipase A and lysophospholipase. Unless otherwise noted, phospholipase A was assayed in the reaction mixture containing 0.025 M Tris-HCl buffer, pH 7.2, ^{14}C -phosphatidylethanolamine (100 nmol), 5 mM CaCl_2 , 50% methanol and 0.1 ml of enzyme preparation in a total volume of 2 ml. The mixture was incubated at 30° for 30 min, and the reaction was terminated by adding 1 ml of methanol and 4 ml of chloroform. After vigorous mixing for 30 sec, the mixture was centrifuged at $2,000\times g$ for 5 min to separate the two layers. The lower chloroform phase was evaporated to dryness under reduced pressure at 30° , and the residue was dissolved in a small volume of chloroform-methanol (2:1,

v/v) and spotted on the Silica gel thin-layer plate. Chromatography was carried out with chloroform-methanol-acetic acid (65:25:10, v/v) as a solvent. The reaction mixture for lysophospholipase assay contained 0.05 M glycine-NaOH buffer, pH 10.0, [1-¹⁴C]-acyl-glycerolphosphorylethanolamine (100 nmol) and 0.1 ml of enzyme preparation in a total volume of 1 ml. Incubation was done at 30° for 30 min, and the reaction was terminated by adding 6 ml of chloroform-methanol (2:1, v/v). Lipids were extracted as described above and separated on the Silica gel thin-layer plate with petroleum ether-diethyl ether-formic acid (55:45:1.5, v/v) as a solvent. Each area on the thin-layer plates corresponding to free fatty acid, lysophosphatidylethanolamine or phosphatidylethanolamine was scraped off and their radioactivities were measured with a Packard Tri-Carb 3385 liquid scintillation spectrometer in a toluene scintillation fluid. One unit was defined as the amount of the enzyme releasing 1 nmol free fatty acid per min under these conditions.

Isolation of outer membrane. Middle log-phase cells of *E. carotovora* 645ArT grown in 1 liter of M9-glucose-casamino acid medium were harvested and washed once with 100 ml of ice-cold 10 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid buffer, pH 7.4, and disrupted in a French pressure cell as described by KAMIO and NIKAI (11). The membrane fraction was subjected to sucrose density gradient centrifugation (11) to separate the outer membrane from the inner. Adenosinetriphosphatase (ATPase), a marker enzyme of the inner membrane, was assayed in 1 ml of the reaction mixture containing 20 mM Tris-HCl buffer, pH 9.0, 2.5 mM ATP-Na₂ and 1 mM MgCl₂. The reaction was carried out at 30° for 20 min and terminated by adding 0.1 ml of 0.5 N HCl. Inorganic phosphate liberated was determined by the method of TAUSKY and SHORR (12).

Isolation of a mutant deficient in phospholipase A. Properties of a major phospholipase A of *E. carotovora* 645ArT were quite similar to those of *E. coli* detergent-resistant (dr)-phospholipase A (13). Therefore, the procedures developed for the isolation of mutants defective in dr-phospholipase A activity of *E. coli* by OHKI *et al.* (13) were applied to isolate a mutant deficient in phospholipase A of *E. carotovora* 645ArT, with a slight modification.

The mutagenized cells of *E. carotovora* 645ArT with N-methyl-N'-nitro-N-nitrosoguanidine according to the method of ADELBERG *et al.* (14) were spread on nutrient agar medium (about 80 colonies/plate), and the plates were incubated at 30° for 36 hr. The colonies were transferred to fresh nutrient agar plates by the replica method, and these plates were then incubated at 30° for 30 hr. Soft agar (3 ml) was carefully poured over the colonies on the replicated plates, which were incubated at 30° overnight. Soft agar contained M9-minimal medium, 0.5% sodium dodecylsulfate (SDS), 5% ethanol, 2 × 10⁸ cells of *E. coli* K1061 per ml and 0.5% agar. The colonies on the original plates corresponding to the colonies which could not support growth of the unsaturated fatty acid auxotroph *E. coli* were isolated and purified. Among 10⁴ colonies tested, 3 colonies were isolated and confirmed to be mutants deficient in phospholipase A.

RESULTS

Properties of phospholipase A and lysophospholipase

No phospholipase A activity could be detected on assay in the absence of detergent or methanol in the reaction mixture using ^{14}C -phosphatidylethanolamine as a substrate. As shown in Fig. 1 and Table 1, however, considerable activity was found if the reaction mixture was supplemented with an adequate concentration of

Table 1. Effects of Ca^{2+} , methanol and EDTA on phospholipase A activity.

Assay system	Additions or omissions	Enzyme activity	
		unit/mg protein	(%)
A	None	26.6	(100)
	+ CaCl_2 (5 mM)	42.1	(158)
	+ EDTA (5 mM)	1.4	(5)
B	Complete	11.9	(100)
	— Methanol	0.3	(2.5)
	— CaCl_2 , + EDTA (1 mM)	0.9	(7.6)

The reaction mixture of assay system A contained 50 mM Tris-HCl buffer, pH 7.2, 0.5% sodium cholate, 100 nmol of ^{14}C -phosphatidylethanolamine and enzyme preparation (50 μg protein) in a total volume of 1 ml. The complete composition of assay system B was described in MATERIALS AND METHODS. These mixtures were incubated at 30° for 30 min. Phospholipase A activity was determined as described in MATERIALS AND METHODS. EDTA, ethylenediaminetetraacetic acid.

cholate (0.5%) or methanol (50%). Similar activation of phospholipase A was also found with the addition of Triton X-100 (0.2%), deoxycholate (0.5%) or SDS (0.1%) instead of methanol or cholate. Ca^{2+} stimulated phospholipase A about 1.5-fold, and ethylenediaminetetraacetic acid (EDTA) reduced the activity to 5–8% of the control (Table 1). In contrast to phospholipase A activity, lysophospholipase of *E. carotovora* 645ArT was inhibited by various detergents such as cholate, deoxycholate, Triton X-100 and SDS, and methanol. EDTA partially inhibited the enzyme activity (about 60%). The results are shown in Table 2. pH optima for phospholipase A and lysophospholipase were pH 7.2 and pH 10.0, respectively. In our assay conditions for phospholipase A, nearly equimolar amounts of fatty acid and lysophosphatidylethanolamine were formed from ^{14}C -phosphatidylethanolamine, as shown in Fig. 2. Similar time courses of fatty acid and lysophosphatidylglycerol formation were obtained using ^{14}C -phosphatidylglycerol as a substrate. These results indicate that lysophospholipid formed by phospholipase A would not be appreciably hydrolyzed due to the inhibitory action of detergents and methanol on lysophospholipase activity.

In carotovoricin Er-treated cells, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were deacylated specifically, while no deacylation of cardiolipin (CL) was observed (5). If deacylation of PE and PG was caused by phospholipase

Fig. 1.

Fig. 2.

Fig. 1. Activation of phospholipase A by cholate.

The reaction mixture (1 ml) contained 0.05 M Tris-HCl buffer, pH 7.2, 100 nmol of ^{14}C -phosphatidylethanolamine, sodium cholate (final concentrations of zero to 1.0%) and enzyme preparation (0.1 mg protein) in a total volume of 1 ml. After incubation at 30° for 30 min, 6 ml of chloroform-methanol (2:1, v/v) was added to the mixtures and lipids were extracted. The amounts of free fatty acids were determined as described in MATERIALS AND METHODS.

Fig. 2. Time course of formation of free fatty acid and lysophosphatidylethanolamine by phospholipase A.

The standard reaction mixture with enzyme preparation (50 μg protein) was used. After incubation at 30° for a desired time, the reaction was terminated. The radioactivities of free fatty acid and lysophosphatidylethanolamine were determined as described in MATERIALS AND METHODS. $\circ$, free fatty acid; $\bullet$, lysophosphatidylethanolamine.

Fig. 3. Substrate specificity of phospholipase A from *E. carotovora* 645ArT.

The enzyme activity (26 μg protein) was assayed using ^{14}C -phosphatidylethanolamine ($\circ$), ^{14}C -phosphatidylglycerol ($\bullet$), or ^{14}C -cardiolipin ($\times$) as substrate.

A in the cells treated with carotovoricin Er, the enzyme should hydrolyze only PE and PG. Figure 3 shows that phospholipase A, indeed, hydrolyzed only PE and PG, but not CL.

Phospholipase A of *E. carotovora* 645ArT was resistant to heat-treatment and

only 8% of original activity was lost by heating at 100° for 10 min, and even a slight activation was observed by heat-treatment at 40° to 80° for 10 min. On the other hand, about 30% of original lysophospholipase activity was lost by heat-treatment at 100° for 10 min. These properties of the phospholipase A of this organism are quite similar to those of dr-phospholipase A of *E. coli* with respect to resistance to detergent and heat, and Ca²⁺ requirement (15, 16). Dr-phospholipase A of *E. coli* has been found to be located in the outer membrane (16–18). We hence examined the location of phospholipase A of *E. carotovora* 645ArT. Most of the phospholipase A activity of this organism was found in the membrane fraction obtained by centrifugation at 100,000×*g* for 45 min (Table 3), and 82% of activity in the membrane fraction was recovered in the outer membrane fraction which was free from ATPase (Table 4). Lysophospholipase was also mainly recovered in the membrane fraction. Significant activity (32%), however, was found in the cytoplasmic fraction (Table 3).

Table 2. Inhibitory effect of detergents, EDTA and methanol on lysophospholipase.

Additions	Lysophospholipase	
	unit/mg protein	(%)
None	25.5	(100)
Cholate (0.2%)	16.7	(65)
Deoxycholate (0.2%)	1.7	(7)
Triton X-100 (0.2%)	4.1	(16)
SDS (0.2%)	1.1	(4)
EDTA (5 mM)	9.4	(38)
Methanol (50%)	0.7	(3)

Lysophospholipase activity was measured in the reaction mixture described in MATERIALS AND METHODS with the additions noted.

Table 3. Location of phospholipase A and lysophospholipase.

Enzyme source	Phospholipase A		Lysophospholipase	
	unit/mg protein	total activity (unit)	unit/mg protein	total activity (unit)
Whole sonicate	14.5	181	27.1	423
Supernatant after centrifugation	0.4	4	12.4	136
Precipitate after centrifugation	37.9	125	92.2	203

Exponentially growing cells of *E. carotovora* 645ArT were disrupted by sonication. The sonicate was centrifuged at 100,000×*g* for 45 min. The precipitate obtained was dispersed in 0.05 M Tris-HCl buffer, pH 7.2. Phospholipase A and lysophospholipase activities in each preparation were assayed.

Table 4. Location of phospholipase A.

Fraction	Phospholipase A (unit)	ATPase (unit) ^a
Total membrane	80.4	2.3
Outer membrane	65.9	0
Inner membrane	8.9	1.8

^a One unit of ATPase was the amount of the enzyme liberating 1 μ mol of inorganic phosphate per min under our assay conditions.

Table 5. Phospholipase A and lysophospholipase activities of *E. carotovora* 645-ArT and its phospholipase A-deficient mutants.

Strain	Phospholipase A		Lysophospholipase	
	unit/mg protein	(%)	unit/mg protein	(%)
<i>E. carotovora</i> 645ArT	11.1	(100)	29.0	(100)
Mutant No. 1	0.1	(1.0)	10.0	(34)
No. 2	0.2	(1.8)	10.4	(38)
No. 3	0	(0)	7.0	(24)

The organisms were grown to middle log-phase in M9-glucose-casamino acid medium. Preparation of enzyme and enzyme assay were done as described in MATERIALS AND METHODS.

Isolation of a mutant deficient in phospholipase A

In order to ascertain that phospholipase A of *E. carotovora* 645 ArT is involved in the degradation of phospholipids induced by carotovoricin Er, we isolated mutants deficient in phospholipase A activity. The isolation of the mutant was carried out at 30°, the optimal growth temperature for this organism. The colonies which did not support the indicator strain were detected after growth at 30°. They showed a defect in phospholipase A activity as described below. There was no difference however, at least in growth rates, either as regards nutrient broth or minimal medium at 30° between these mutants and the parent strains. As shown in Fig. 4, the enzyme from the parent cells hydrolyzed PE to liberate nearly the same amounts of free fatty acid and lyso-PE. On the other hand, the enzyme from the mutant cells failed to hydrolyze PE, and no free fatty acid was formed. Phospholipase A activities of these mutants were less than 2% of that of the parent strain (Table 5). In addition to the lack of phospholipase A activity, these mutants showed a partial defect in lysophospholipase activity (Table 5).

Action of carotovoricin Er on the mutant

Marked degradation of phospholipid was found when the cells of the parent strain were treated with the carotovoricin Er in 0.05 M phosphate buffer, pH 7.2. Degradation of phospholipid was decreased when the cells were treated with the bacteriocin in the presence of 5 mM MgCl₂ and the rate of degradation was about

Fig. 4. Analysis of reaction products from phosphatidylethanolamine resulting from phospholipase A activity of *E. carotovora* 645ArT and its phospholipase A-deficient mutant (No. 1) by thin-layer chromatography.

The enzyme preparations (50 μ g protein, each) from the parent and mutant strains were added to the phospholipase A assay mixture, and the reaction mixtures were incubated at 30° for 30 min. After the reaction, lipids were extracted and developed on a Silica gel thin-layer plate. Distribution of radioactivity was analyzed with an Aloka thin-layer chromatogram scanner. Reaction products resulting from phospholipase A activity from the parent strain (upper line) and phospholipase A-deficient mutant strain No. 1 (lower line) were indicated.

1/7 as much as that of the cells treated in the absence of MgCl_2 (5). As shown in Fig. 5, degradation of phospholipid in mutant No. 1, which is deficient in phospholipase A, was less than 1/10 that of the parent cells even in the absence of Mg^{2+} . On the other hand, when the mutant cells were treated with carotovoricin Er in the presence of 5 mM Mg^{2+} , no detectable amount of free fatty acid was released, at least within the 20 min following the addition of the bacteriocin (Fig. 5). The

Fig. 5. Release of fatty acid in the cells of *E. carotovora* 645ArT and phospholipase A-deficient mutant treated with carotovoricin Er.

E. carotovora 645ArT and its phospholipase A-deficient mutant (No. 1) were grown in M9-glucose-casamino acid medium in the presence of [^{14}C] acetate (0.4 $\mu\text{Ci/ml}$). The log-phase cells washed once with M9-buffer were suspended in 0.05 M Na-K phosphate buffer, pH 7.2 containing 0.15 M NaCl (buffer A) or 0.01 M Na-K phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM MgCl_2 (buffer B) at the cell concentration of 2×10^8 cells per ml. Mixtures (3 ml) containing 2.5 ml of the cell suspension and 0.5 ml of the bacteriocin solution (40 units/ml) or 0.5% of bovine serum albumin (control) were incubated at 30° . One-half ml of the mixture was withdrawn at various periods of incubation, and lipids were extracted by the method of BLIGH and DYER (6). Free fatty acid was separated from phospholipids by thin-layer chromatography with petroleum ether-diethyl ether-formic acid (55:40:1.5, v/v). The percentage radioactivities in the fatty acid fraction to the total radioactivity of phospholipids were plotted against incubation time with carotovoricin Er.

$\times$, *E. carotovora* 645ArT, control in the buffer A; $\circ$, *E. carotovora* 645ArT treated with carotovoricin Er in the buffer A; $\bullet$, *E. carotovora* 645ArT treated with carotovoricin Er in the buffer B; Δ , the mutant treated with carotovoricin Er in the buffer A; $\blacktriangle$, the mutant treated with carotovoricin Er in the buffer B.

cell lysis of the sensitive strain induced by carotovoricin Er was found to be enhanced by phosphate and repressed by Mg^{2+} (4). The lysis caused by carotovoricin Er was also decreased in the mutant strain when the cells were treated with the bacteriocin in M9-medium in which the parent cells were lysed by the bacteriocin (Fig. 6-A). As shown in Fig. 6-B, a modified M9-medium containing a low concentration of phosphate (10 mM) and 5 mM MgCl_2 partially protected the parent strain from the lysis caused by the bacteriocin, while the modified M9-medium prevented the lysis of the mutant strains completely. Even in the modified M9-medium, growth of the mutant was inhibited by the bacteriocin, suggesting the mutant still retained the sensitivity to the bacteriocin. As shown in Fig. 7, the mutant strain showed the same sensitivity to carotovoricin Er as the parent strain. These results clearly indicate that phospholipase A of carotovoricin Er-sensitive cells is responsible for degradation of phospholipids and cell lysis caused by carotovoricin Er.

Fig. 6. Lysis of phospholipase A-deficient mutant after treatment with carotovoricin Er.

One-fifth ml of carotovoricin solution (100 units/ml) or 0.5% of bovine serum albumin was added to the early log-phase culture of *E. carotovora* 645ArT and phospholipase A-deficient mutant No. 1 as indicated by the arrows. These cultures were incubated at 30° with shaking, and their optical density was measured with a Hitachi photoelectric colorimeter (FPW-4). A, M9-glucose-casamino acid medium; B, a modified M9-glucose-casamino acid medium in which phosphate was supplemented as 10 mM Na-K phosphate buffer, pH 7.2, and 5 mM MgCl₂ was supplemented.

○, *E. carotovora* 645ArT control; ●, *E. carotovora* 645ArT treated with carotovoricin Er; Δ, the mutant control; ▲, the mutant treated with carotovoricin Er.

Fig. 7. Susceptibility of *E. carotovora* 645ArT and phospholipase A-deficient mutant to carotovoricin Er.

log-phase cells grown in M9-glucose-casamino acid medium were washed once with M9 buffer, and suspended in 0.05 M Na-K phosphate buffer, pH 7.2 containing 0.15 M NaCl and 1.5×10^8 cells per ml. Carotovoricin Er solution (0.1 ml) was added to the cell suspensions (0.4 ml) at final concentrations of zero to 4 units per ml. The mixtures were incubated at 30° for 20 min, and viable cells were counted on nutrient agar medium.

○, *E. carotovora* 645ArT; ●, phospholipase A-deficient mutant No. 1.

DISCUSSION

As demonstrated in this report, various properties of a major phospholipase A of *E. carotovora* 645ArT such as detergent- and heat-resistances, requirement of Ca²⁺ for optimal activity and location in the outer membrane, were similar to those of dr-phospholipase A of *E. coli* (15, 16). Dr-phospholipase A of *E. coli* has been demonstrated to have phospholipase A₁, A₂, lysophospholipase L₁, and L₂ activi-

ties (15, 16). The specific site cleaved by phospholipase A of *E. carotovora* 645ArT has not been examined in detail. It was, however, suggested that the phospholipase A might also have lysophospholipase L₁ activity, because the mutants deficient in phospholipase A showed an additional defect in lysophospholipase L₁ activity as shown in Table 5. About 60 to 70% of lysophospholipase L₁ activity of the parental strain which was resistant to heating at 100° for 10 min but sensitive to EDTA, was found in the membrane fraction. This lysophospholipase L₁ activity would be attributed to the phospholipase A, since the activity was not detected in the mutant deficient in the phospholipase A.

The mutant of *E. carotovora* 645ArT deficient in the phospholipase A isolated in this laboratory had no phospholipase A activity *in vitro* (Table 5). In spite of complete loss of the phospholipase A activity, this mutant could grow in nutrient broth and minimal medium as the parental strain (Fig. 6). A mutant of *E. coli* deficient both in dr-phospholipase A and ds (detergent sensitive)-phospholipase A has been reported to have no defect in growth, no alteration in turnover of polyglycerophosphatides and propagation of bacteriophages (13, 19, 20). Hence, physiological functions of bacterial phospholipases remain unknown at present (21, 22). Our results with mutants deficient in phospholipase A demonstrate that hydrolysis of phospholipids in the cells affected by carotovoricin Er was catalyzed by phospholipase A in the sensitive cells. LUSK and PARK (23) have reported that the degradation product of PE in colicin K-treated *E. coli* was not detected in the dr-phospholipase A- and ds-phospholipase A-negative mutant although the sensitivity of the mutant to colicin K was the same as the parental strain. In phospholipase A-deficient mutant, free fatty acid was not detected at least within 20 min after challenge of carotovoricin Er (Fig. 5). A small amount of free fatty acid was found after further incubation. This is possibly due to the residual activity of the phospholipase A *in vivo* or other minor phospholipase A. We found another phospholipase A activity resembling that of ds-phospholipase A of *E. coli* (24) using ¹⁴C-phosphatidylglycerol as a substrate in the absence of detergent or methanol, although its activity was low (2 units/mg protein). Cell lysis induced by carotovoricin Er was also prevented in the phospholipase A-deficient mutant although growth of the cells ceased immediately after addition of carotovoricin Er to the culture medium (Fig. 6). This result indicates that the phospholipase A-deficient mutant still retained the sensitivity to the bacteriocin. The sensitivity of the mutant to carotovoricin Er was the same as that of the parent strain (Fig. 7). Our results clearly indicate that carotovoricin Er activates the latent phospholipase A to degrade the membrane phospholipids and leads the cells to lysis. We concluded that the ability of carotovoricin Er to induce the activation of phospholipase A might not be a primary bactericidal action. The phospholipase A-deficient mutant will be useful for study of the true bactericidal effect of carotovoricin Er on sensitive cells, because virtually no decomposition of the membrane occurs in the mutants infected by the bacteriocin.

We are grateful to Prof. H. Tsuyama and Dr. H. R. Kaback for their kind donation of bacterial strains. We also thank Miss A. Sato of this faculty for her help in the operation of the liquid scintillation spectrometer and Dr. S. Okuda of this laboratory for his useful discussions. This investigation was supported in part by a Grant-in-Aid for Scientific Research (to H. T., 443026) from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

- 1) P. REEVES, *The Bacteriocins: Molecular Biology, Biochemistry, and Biophysics*, Vol. 11, Springer-Verlag, New York (1972).
- 2) D. E. BRADLEY, *Bacteriol. Rev.*, **31**, 230 (1967).
- 3) Y. ITOH, K. IZAKI, and H. TAKAHASHI, *J. Gen. Appl. Microbiol.*, **24**, 27 (1978).
- 4) Y. ITOH, K. IZAKI, and H. TAKAHASHI, *J. Gen. Appl. Microbiol.*, **26**, 51 (1980).
- 5) Y. ITOH, K. IZAKI, and H. TAKAHASHI, *J. Gen. Appl. Microbiol.*, **26**, 85 (1980).
- 6) E. G. BLIGH and W. J. DYER, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
- 7) Y. KANEMASA, Y. AKAMASTU, and S. NOJIMA, *Biochim. Biophys. Acta*, **144**, 382 (1967).
- 8) J. H. LAW and B. ESSEN, *In Methods in Enzymology*, Vol. 14, ed. by S. P. COLOWICK and N. O. KAPLAN, Academic Press, New York (1969), p. 665.
- 9) G. R. BARTLETT, *J. Biol. Chem.*, **234**, 466 (1959).
- 10) O. H. LOWRY, N. J. ROSEBROUGH, H. L. FARR, and R. J. RANDALL, *J. Biol. Chem.*, **193**, 265 (1951).
- 11) Y. KAMIO and H. NIKAIDO, *Biochemistry*, **15**, 2561 (1976).
- 12) H. H. TAUSKY and E. SHORR, *J. Biol. Chem.*, **202**, 675 (1953).
- 13) M. OHKI, O. DOI, and S. NOJIMA, *J. Bacteriol.*, **110**, 864 (1972).
- 14) E. A. ADELBERG, M. MANDEL, and G. C. C. CHEN, *Biochem. Biophys. Res. Commun.*, **18**, 788 (1965).
- 15) M. NISHIMURA, S. NAKAIKE, Y. TAMORI, and S. NOJIMA, *Eur. J. Biochem.*, **73**, 115 (1977).
- 16) C. J. SCANDELLA and A. KORNBERG, *Biochemistry*, **10**, 4447 (1971).
- 17) D. A. WHITE, F. A. ALBRIGHT, W. J. LENNARZ, and C. A. SCHNAITMAN, *Biochim. Biophys. Acta*, **249**, 636 (1971).
- 18) R. M. BELL, R. D. MAVIS, M. J. OSBORN, and P. R. VAGELOS, *Biochim. Biophys. Acta*, **249**, 628 (1971).
- 19) M. ABE, N. OKAMOTO, O. DOI, and S. NOJIMA, *J. Bacteriol.*, **119**, 543 (1974).
- 20) O. DOI and S. NOJIMA, *J. Biochem.*, **80**, 1247 (1976).
- 21) C. R. H. RAETZ, *Microbiol. Rev.*, **42**, 614 (1978).
- 22) H. V. D. BOSCH, *Biochim. Biophys. Acta*, **604**, 191 (1980).
- 23) J. E. LUSK and M. H. PARK, *Biochim. Biophys. Acta*, **394**, 129 (1975).
- 24) O. DOI and S. NOJIMA, *Biochim. Biophys. Acta*, **260**, 244 (1972).