Phosphatidylinositol as a Component of the Ice Nucleating Site of *Pseudomonas syringae* and *Erwinia herbicola*

Abstract. *Phosphatidylinositol has been identified as a major component of the ice nucleating site on the outer surface of two bacteria,* Pseudomonas syringae *and* Erwinia herbicola. *Plant lectins binding to inositol and a highly purified phosphatidylinositol-specific hydrolase (a C_{II} lipase) inhibited or decreased the efficiency of the ice nucleating activity (INA) of both bacteria. Extracts of these two INA^+ bacteria had phosphatidylinositol synthase activity while extracts from related INA^-* Pseudomonas *or* Erwinia *strains had no detectable synthase activity. An* Escherichia coli *strain acquired phosphatidylinositol synthase activity when transformed to the INA^+ phenotype with recombinant plasmids containing fragments of* P. syringae *DNA.*

The discovery that ice nuclei can have a biogenic origin has been of great interest to investigators concerned with frost damage and plant pathology (*1*). The initial discoveries of Vali (*2*) and Schnell and Vali (*3*) led to the identification of two closely related naturally occurring plant bacteria, *Pseudomonas syringae* and *Pseudomonas fluorescens* as major terrestrial sources of active ice nuclei. *Erwinia herbicola*, an unrelated Gram-negative bacterium, was then shown to be an additional biogenic source of ice nuclei (*4*). Bacterial strains with ice nucleating activity (INA) rapidly convert supercooled water into ice at temperatures of −2° to −4°C while the well-known ice nucleating activity of silver iodide is only effective at temperatures of −8°C or lower. The INA^+ strains have been implicated in causing frost damage of plants and in forming ice nuclei from supercooled water in clouds.

The ice nucleating site is on the bacterial cell wall and probably consists of two components (*5, 6*). One of these components is a protein since it contains a sulfhydryl group (*6*), is heat labile, and is susceptible to proteases (*1*). The nucleating site has a carbohydrate-like component, possibly a glucoside- or mannoside-like element, at or near the ice nucleating site (*6*). There is evidence that *E. herbicola* may have only two nucleating sites per cell while *P. syringae* may have four to six sites per cell (*6*). Orser *et al.* (*7*) have isolated molecular clones from both *P. syringae* and *E. herbicola* genomes that can transform *E. coli* from an INA^- to an INA^+ phenotype.

Our investigations resulted from the observation of Warner (*8*) that mesoinositol, which is formed from glucose, has hydroxyl groups that form a structure highly compatible with the hexagonal lattice structure of ice (*8*) and thus could be the sugar component of the nucleating site. Inositol, in phosphatidylinositol, is a relatively rare component of the cell walls of Gram-negative bacteria (*9*), and we have not yet been able to identify phosphatidylinositol in chloroform extracts of INA^+ cells by direct analysis. This might be expected if there are only a few ice nucleating sites per cell containing phosphatidylinositol.

We reported earlier (*6*) that five plant lectins, all with a binding specificity mainly for mannosides or glucosides, had varying abilities to inhibit the INA of both *P. syringae* and *E. herbicola*. In view of the possibility that phosphatidylinositol might be a surface component of bacterial cells involved in ice nucleation, the binding of these lectins to phosphatidylinositol isolated from two different sources was examined. A hemagglutination binding assay for lectins was carried out with washed human red blood cells; the various lectins caused hemagglutination within 1 hour. Different amounts of phosphatidylinositol prepared as vesicles from either soybeans or from yeast (*10*) were added to the assay system as hemagglutination inhibitors (Table 1). Both types of phosphatidylinositols showed inhibition of lectin-induced hemagglutination and the minimum concentration of phospholipid necessary to completely inhibit visible hemagglutination was related to the degree of inhibition of INA by lectins. This suggested that phosphatidylinositol could be at the site of the ice nucleating activity.

The presence of phosphatidylinositol on the surface of these cells was further examined by treating the cells with a phosphatidylinositol-specific hydrolase (a C_{II} lipase). This enzyme was purified from the supernatants of broth cultures of *Bacillus cereus* (ATCC 10876) with a modification of the methods used by Ikejawa and Taguchi (*11*) and by Sundler *et al.* (*10*). The radioactive enzyme assay was adapted from that described by Sundler *et al.* (*10*), except that [^{3}H]inositol (water-soluble) was incorporated into phosphatidylinositol (chloroform-soluble) with the use of CDP-diacylglycerol (CDP, cytidine diphosphate) and chicken liver phosphatidylinositol synthase (E.C. 2.7.8.) to produce a radioactive substrate (*12*). Only one protein peak was apparent on the final hydroxyapatite column and only one protein band with a molecular size of 30 kD was detected by polyacrylamide gel electrophoresis (PAGE). Phosphatidylinositol hydrolase has been reported to have a molecular size of 30 kD (*10, 11*). The purified enzyme fractions had no hydrolytic activity on phosphatidylcholine even though the original *B. cereus* culture fluids contained a high concentration of this enzyme. However, it is possible that there were minor protein contaminants (1 to 2 percent) in the preparation that could not be detected in the 50-μg protein sample analyzed.

Fractions from the Sephadex G-75 column used early in the purification procedure (legend to Fig. 1) were assayed for C_{II} lipase activity and for their effect on the INA of *P. syringae*. The C_{II} lipase activity and the activity that reduced the efficiency of the INA of *P. syringae* cochromatographed. In the final preparation, the C_{II} lipase activity and an activity which reduced the *P. syringae* INA copurified (Fig. 1).

The INA efficiency of *Erwina herbicola* cells decreased approximately 40 percent when treated with fraction 5 from Fig. 1. The INA of *E. herbicola* was more resistant to the lipase than was INA of *P. syringae* (which decreased by ≥90 percent) and this result agrees with the greater resistance of *E. herbicola* to lectins, sulfhydryl reagents, and other reagents (*6*).

The final step in the biosynthesis of phosphatidylinositol involves a synthase

Table 1. Lectin inhibition of bacterial INA and inhibition of lectin-induced hemagglutination by phosphatidylinositol . For hemagglutination, type A human red blood cells were used at a final concentration of 1.25 percent. Lectins were added to a final concentration of 125 μg/ml. The inhibition of the INA was done earlier (*6*).

Lectins	Inhibition of INA (percent)		Minimum phosphatidylinositol concentration (*M*)	
	P. syringae	*E. herbicola*	Soy bean	Yeast
Fava bean	95	60	1.4×10^{-4}	1.4×10^{-4}
Lentil	90	80	2.3×10^{-4}	1.4×10^{-4}
Concanavalin A	10	5	4.7×10^{-4}	2.3×10^{-4}
Garden pea	None	None	$>4.7 \times 10^{-4}$	2.3×10^{-4}
Wheat germ	None	None	$>4.7 \times 10^{-4}$	4.7×10^{-4}

catalyzed reaction. Sonicated extracts of the INA$^+$ bacteria *P. syringae* and *E. herbicola* had phosphatidylinositol synthase activity whereas extracts from INA$^-$ bacteria had no synthase activity (Table 2) (*13*). Extracts of the INA$^+$ *P. syringae* strain 31 rif-1 had synthase activity while extracts of an INA$^-$ *P. syringae* strain 31 rif-31 had no synthase activity. Finally, extracts of *E. herbicola* var. *ananas* strain B/7, which is INA$^-$, also had no detectable synthase activity. A K12 strain, *E. coli* HB101, and *E. coli* B have no phosphatidylinositol (*9*) and their extracts had no synthase activity. Like many membrane-bound enzymes, the synthase activity varied in different preparations (Table 3), and the exact conditions for its maximal release from the cell wall or membrane remain to be determined.

Table 2. Phosphatidylinositol synthase activity in bacterial extracts. The cells from 2-day-old bacterial cultures were collected, sonicated, treated with ribonuclease (20 μg/ml) and deoxyribonuclease (30 μg/ml), and dialyzed against tris, *p*H 8.0. The other components were as described (*15*). The synthase activity is expressed as picomoles per hour per milligram of protein.

Source of extract	INA	Synthase activity
E. herbicola	+	83
E. herbicola var. *ananas* strain B/7	−	<0.1
P. syringae strain C-9	+	51
P. syringae strain 31 rif-1	+	4
P. syringae strain 31 rif 1-28	−	<0.1

Table 3. Phosphatidylinositol synthase activity in extracts of *E. coli* containing plasmid pUC8 plus *P. syringae* DNA. The assay procedure was as described in (*15*). The synthase activity is expressed as picomoles per hour per milligram protein.

Bacteria	INA	Synthase activity	
		Exp. 1	Exp. 2
Untransformed *E. coli* HB101	−	<.05	<0.1
Transformed *E. coli* HB101 with pUC8	−	<.05	<0.1
Transformed *E. coli* HB101 with pUC8 + *P. syringae* DNA strain 1	+	0.31	19.
Transformed *E. coli* HB101 with pUC8 + *P. syringae* DNA strain 2	+	0.42	8.8
Pseudomonas syringae strain C-9	+	3.9	1.1

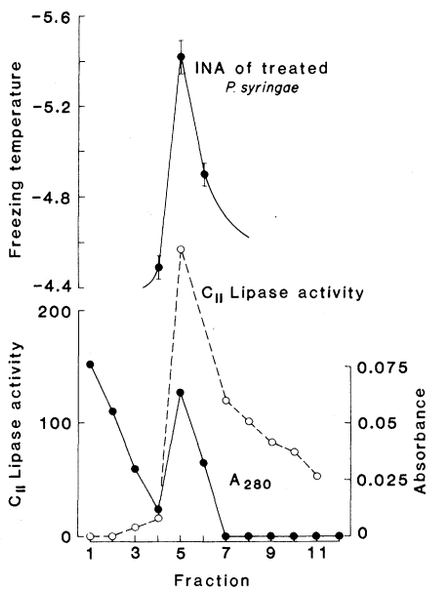

Fig. 1. Purification of phosphatidylinositol hydrolase (a C_{II} lipase) and the decrease in the INA of *P. syringae*. The enzyme from the supernatant of the culture was precipitated with ammonium sulfate, chromatographed on Sephadex G-100 and Sephadex G-75 and adsorbed and eluted from two hydroxyapatite columns. A 5-ml sample of C_{II} lipase (containing 0.3 mg of protein), purified by column chromatography on a 20-ml hydroxyapatite column, was added to a 6.0-ml column of hydroxyapatite, coupled to agarose (HA-Ultrogel; LKB) and eluted with a linear gradient of 0.13 to 0.24*M* phosphate, *p*H 6.8. Fractions (1.5 ml) were collected and the peak activity was eluted at 0.185*M* phosphate. For the C_{II} lipase activity assay, 0.77 μmol of soybean phosphatidylinositol (Sigma), 1.6 μmol of Triton X-100 (*10*), 0.1*M* tris, *p*H 8.0, and approximately 300,000 count/min of ^{3}H-labeled phosphatidylinositol were mixed in 125 μl total volume. Then, 25 μl of the column fraction was added to each tube and the reaction mixture was incubated for 25 minutes at room temperature. The reaction was stopped by the addition of a mixture of 600 μl of chloroform, methanol, and HCl (10:5::0.1). A portion (100 μl) of the methanol aqueous phase was taken for liquid-scintillation counting. The effect of the enzyme column fractions on the INA of *P. syringae* was determined by incubating 50 μl of the column fraction with 150 μl of *P. syringae* at 1.0×10^8 per milliliter in 0.1*M* tris, *p*H 7.5, containing 7 m*M* EDTA. The cells had been grown on Tryptone–yeast extract agar slants and washed three times in tris buffer. After 30 minutes at room temperature the enzyme-cell mixture was diluted 1:50 in tris buffer containing 0.02*M* Mg_2SO_4. The INA, defined as the temperature at which 50 percent of a diluted bacterial sample froze, was determined as described (*6*).

Using the general procedures outlined by Orser *et al.* (*7*), we have cloned the ice nucleating activity from *P. syringae* and transferred it to *E. coli* HB101. In our experiments, high molecular weight *P. syringae* DNA was digested to completion with the restriction enzyme BAM HI, ligated to a plasmid pUC8 (*14*), and the resultant recombinant plasmids used to transform *E. coli* HB101. Two transformed *E. coli* strains have been obtained (from approximately 500 ampicillin-resistant transformants) which were INA$^+$ and both were at least as active in ice nucleation as the DNA-donating *P. syringae* strain. In two separate experiments there was low and variable but definite phosphatidylinositol synthase activity in the extracts of both these transformed *E. coli* strains (Table 3) and none was detected in the control INA$^-$ transformants.

While phosphatidylinositol appears to be one component of the ice nucleating site on both *P. syringae* and *E. herbicola*, the identity of the sulfhydryl protein at or near the site is not known. One attractive candidate is the phosphatidylinositol synthase itself since (i) it only occurs in INA$^+$ bacteria and not in closely related but INA$^-$ strains, (ii) it is a cell wall or cell membrane component (*12, 15*), and (iii) it is known to be a sulfhydryl protein (*15*).

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