

## Ice Nucleating Activity of *Pseudomonas syringae* and *Erwinia herbicola*

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Received 29 June 1982/Accepted 10 September 1982

Chemical and biological properties of the ice nucleating sites of *Pseudomonas syringae*, strain C-9, and *Erwinia herbicola* have been characterized. The ice nucleating activity (INA) for both bacteria was unchanged in buffers ranging from pH 5.0 to 9.2, suggesting that there were no essential groups for which a change in charge in this range was critical. The INA of both bacteria was also unaffected by the addition of metal chelating compounds. Borate compounds and certain lectins markedly inhibited the INA of both types of bacterial cells. Butyl borate was not an inhibitor, but borate, phenyl borate, and *m*-nitrophenyl borate were, in order, increasingly potent inhibitors. These compounds have a similar order of affinity for *cis* hydroxyls, particularly for those found on sugars. Lentil lectin and fava bean lectin, which have binding sites for mannose or glucose, inhibited the INA of both bacteria. All other lectins examined had no effect. The inhibition of INA by these two types of reagents indicate that sugar-like groups are at or near the ice nucleating site. Sulfhydryl reagents were potent inhibitors of the INA of both bacteria. When treated with *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, or iodoacetamide, the INA was irreversibly inhibited by 99%. The kinetics of inactivation with *N*-ethylmaleimide suggested that *E. herbicola* cells have at least two separate ice nucleating sites, whereas *P. syringae* cells have possibly four or more separate sites. The effect of infection with a virulent phage (Erh 1) on the INA of *E. herbicola* was examined. After multiple infection of a bacterial culture the INA was unchanged until 40 to 45 min, which was midway through the 95-min latent period. At that time, the INA activity began falling and 99% of the INA was lost by 55 min after infection, well before any cells had lysed. This decrease in INA before lysis is attributed to phage-induced changes in the cell wall.

The findings that ice nuclei can have a biogenic origin and, more particularly, that certain bacteria can initiate freezing at relatively warm temperatures are relatively recent discoveries. Over 40 years ago, it was established that water could be supercooled to quite low temperatures without freezing, and it was thought that various inorganic minerals, analogous to the well-known nucleating activity of silver iodide, were responsible for initiating freezing of supercooled water in the atmosphere and on trees and agricultural crops. In 1971, Vali (20) reported that the top layers of natural soils which were rich in organic material were a better source of freezing nuclei than deeper layers, although both contained the same minerals. This was followed by the demonstration by Schnell and Vali, in 1972 (16), that large numbers of freezing nuclei, active at temperatures of  $-4^{\circ}\text{C}$  or warmer, were formed as

natural vegetation decayed. Further, when the decayed vegetation was autoclaved, ice nucleation at temperatures above  $-10^{\circ}\text{C}$  was lost. This was the first report of the autocatalytic nature of the formation of "biogenic" ice nuclei and of its chemical nature. Subsequent studies by Maki and his colleagues in 1975 (11, 12), by Fresh in 1976 in Vali's laboratory (21), and by Army et al. (3), led to the identification of two closely related bacteria, namely, *Pseudomonas syringae* van Hall and *Pseudomonas fluorescens* biotype G Migula, as major terrestrial sources of active ice nuclei. This was followed almost immediately by the determination by Lindow et al. (9) that *Erwinia herbicola*, also a gram-negative bacteria but quite unrelated to *P. syringae* or *P. fluorescens*, was also active in forming ice nuclei. These two types of bacteria, the *Pseudomonas* and *Erwinia* species, have been found on plants and in the atmosphere throughout North America, Europe, and Asia (8, 12, 13, 15, 17, 21), and a related but not identified

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bacteria with ice nucleating activity (INA) was found in marine aerosols (17). It seems apparent that bacterial strains play a critical role in causing frost damage on plants and in forming ice nuclei from supercooled water in clouds.

The nature of the ice nucleation site on these cells, beyond the heat lability, was studied initially by Maki and his colleagues (11–13). They found that the INA of harvested *P. syringae*, *P. fluorescens*, and *E. herbicola* was not stable to storage at 4°C for even 24 h. The activity was partially stabilized by treatment with 0.3% Formalin, but this treatment lowered the freezing temperature of a *Pseudomonas* culture from –3 to –8°C. They also reported that the INA of *P. fluorescens* was not reduced by treatment with specific antibody. Upon sonic disruptions, whereas much of the INA was lost, the remaining activity was associated with the cell wall (13). Not all bacterial cells in a culture have an ice nucleating site. Maki et al. (11, 12) found that as few as 1 in 1,000 *P. syringae* cells had an active ice nucleating site.

Maki et al. (11) have described a number of characteristic properties of the ice nucleating site of these bacteria. The INA was reported to be sensitive to heating for 5 min at 65°C, to heavy metal ions such as Hg<sup>2+</sup>, and to a variety of positively charged organic molecules, including cetyl-pyridinium chloride, and dyes such as methylene blue and crystal violet. Although the addition of antibiotics such as penicillin, streptomycin, tetracycline, or polymixin inhibited cell growth, they did not directly affect the INA of harvested cells.

These results suggested that a protein(s) may be involved in ice nucleation but they offered little specific evidence of the chemical nature or the location of the ice nucleating site on these bacteria. This paper reports some chemical characteristics of the ice nucleating sites of *P. syringae* and *E. herbicola*. Since the antifreeze proteins of some fishes contain carbohydrates (14), attention was paid to the possibility that carbohydrate-like compounds may be involved. Two quite different kinds of reagents, lectins and substituted borates, which react with carbohydrates were found to inhibit the INA of both types of bacteria. Additional evidence supporting the role of a protein at the site was obtained by studying the effects of well-known protein sulfhydryl reagents. Finally, the effect of virulent phage infection on the INA of *E. herbicola* was examined.

#### MATERIALS AND METHODS

**Bacteria and bacteriophage strains.** The ice nucleating active strains of *P. syringae* C-9 and *E. herbicola* were sent to us from the laboratory of Leroy Maki and Karen White at the University of Wyoming, Laramie. A number of *Erwinia* species were obtained from the

American Type Culture Collection, including *E. amylovora* ATCC 15880, *E. herbicola* subsp. *anas* ATCC 11530, *E. carotovora* subsp. *carotovora* ATCC 15713, *E. uredoovora* ATCC 19321, and *E. stewartii* ATCC 8199. All bacterial strains were routinely cultured on tryptone-yeast extract-glycerol slants. Normally the cultures were incubated at 18 to 19°C. A virulent double-stranded DNA phage, Erh 1 (for *E. herbicola*), was isolated, purified, and characterized as described elsewhere (6). Standard techniques for virulent phages (1) were used to grow and assay the phage stocks.

For testing various reagents on bacteria, the cells were washed off 1- to 2-day-old agar slants with either 0.05 M phosphate buffer, pH 7.5, or saline. The cell concentration was adjusted to a Klett reading of 100, which corresponds to about  $2 \times 10^9$  bacteria per ml. These suspensions were used immediately, and INA was reasonably stable at room temperatures for 3 to 4 h. However, upon storage overnight in the cold, the INA of both bacteria greatly decreased.

**Chemicals and other materials.** The following lectins were obtained from Sigma Chemical Co.: asparagus pea lectin, concanavalin A, garden pea type III lectin, gorse lectin, horseshoe crab lectin, lentil lectin, and wheat germ agglutinin. Fava bean lectin was isolated by the procedure of Allen et al. (2). Most of the sugars were obtained from Pfanstiehl Chemical Co. Phenyl borate was obtained from Sigma Chemical Co., *m*-nitrophenyl borate was from K and K Laboratories,

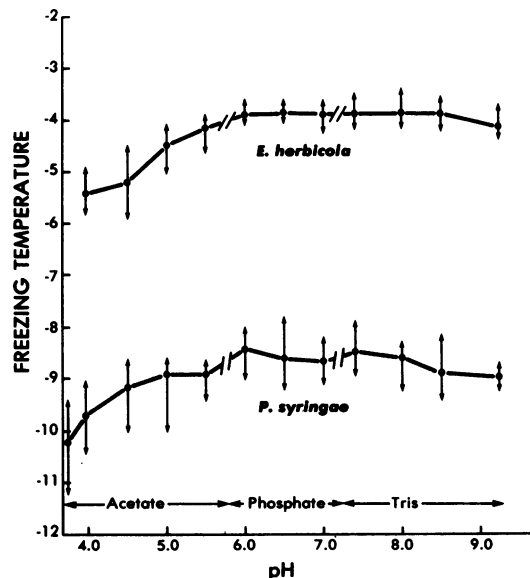


FIG. 1. Effects of pH on the INA of *E. herbicola* and *P. syringae*. The bacterial cells were grown overnight at 19°C, washed off agar slants with saline, and then diluted with the various buffers at 0.05 M. The *E. herbicola* was diluted to about  $10^8$  cells per ml, whereas the *P. syringae* was diluted to a final concentration of about  $10^5$ /ml. The freezing temperature plotted (see text) is that at which 50% of the drops froze; the arrows indicate the freezing temperatures for 10 and 90% of the drops.

and *m*-butyl borate was from Applied Science Laboratory. Most of the sulfhydryl reagents were obtained from Aldrich Chemical Co. Other reagents were obtained from regular commercial sources.

**Determination of INA.** The INA was determined with a freezing nucleus spectrometer (Thermoelectronics cold plate TCP-2), essentially as described by Vali (20). Twenty drops, each of 10  $\mu$ l, were placed on a paraffin-coated piece of aluminum foil held tightly on a 1-cm-thick, 7.6-cm-diameter copper block on the cold plate. The temperature was measured with a 9-cm thermistor probe inserted in the copper block and connected to a digital thermometer. The temperature was slowly lowered, usually at a rate of 1°C/min. The temperatures at which 10%, 50%, and 90% of the drops were frozen were recorded. The 50% point was normally used as the criterion for the freezing temperature, and, when graphically appropriate, the freezing points for 10 and 90% of the drops are also given as distribution arrows. A quantitative measure of the degree of inhibition of INA by various reagents was obtained by comparing the freezing temperature in the presence of an inhibitor to the dilution of the untreated bacteria that would give the same freezing temperature. For example, 0.04 M phenyl borate (see below) lowered the freezing temperatures of a given concentration of bacteria to that of a bacterial concentration approximately 2 logs more dilute. This change in freezing temperature as compared with the standard curve of freezing temperatures for dilutions of untreated bacteria was used to calculate percent inhibitions. For the example used, the inhibition would be 99%.

## RESULTS

**Effect of pH and metal chelating compounds on INA.** Figure 1 shows that the INA of both *P. syringae* and *E. herbicola* was largely unaffected by the pH of the various buffers from about pH 5.0 to 9.2. Below pH 5.0 both types of cells showed a decrease in INA as the pH was lowered. These results show that this ice nucleating site is not sensitive to change in charges which many molecules, especially proteins, exhibit over this neutral pH range. The decrease in the INA below pH 5.0 suggests that a carboxyl group may be involved.

Since metal ions are capable of ordering water molecules and thus can affect ice nucleation, the effect of reagents which bind metal ions was examined. These included 0.05 M EDTA, 1 mM 8-hydroxyquinoline, *o*-phenanthroline, and 1 mM dithiothreitol. EDTA complexes a variety of loosely bound metal ions such as Mg<sup>2+</sup> and Mn<sup>2+</sup>, whereas *o*-phenanthroline and 8-OH-quinoline can react with tightly complexed divalent metal ions such as Fe<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, and Ni<sup>2+</sup>, as well as with some trivalent metal ions. Dithiothreitol can react with any metal ion capable of forming a mercaptide. None of these reagents affected the INA of either type of bacteria after incubation for 1 to 2 h at 18 to 19°C. Therefore, it appears unlikely that metal ions play a significant role in ice nucleation.

**Effect of borate compounds on the INA of *E.***

*herbicola* and *P. syringae*. The interaction of carbohydrates with water is of some interest especially since the work of Feeney and Yeh (4) has shown that the antifreeze compounds in the blood of certain cold water fishes are usually, but not exclusively, glycoproteins. Borate and various derivatives of borate were examined for their ability to inhibit the INA of bacteria since these compounds can form tight complexes with the *cis* hydroxyls found in many sugars (18, 22). The effect of 0.04 M phenyl borate addition on the INA of *E. herbicola* and *P. syringae* is shown in Fig. 2. The INA of both bacteria was markedly inhibited by the addition of the phenyl borate. The effect of various other borate derivatives on the INA of the two types of bacteria is shown in Fig. 3 and 4. In these experiments, the initial bacterial concentration was adjusted to  $2 \times 10^8$ /ml and the concentration of borate compounds was varied. At this relatively high concentration of cells, low concentrations of all borate compounds, such as 0.01 M, had little or no effect on the INA of the cells. At higher concentrations, borate itself was a good inhibitor, but phenyl borate and especially *m*-nitro-

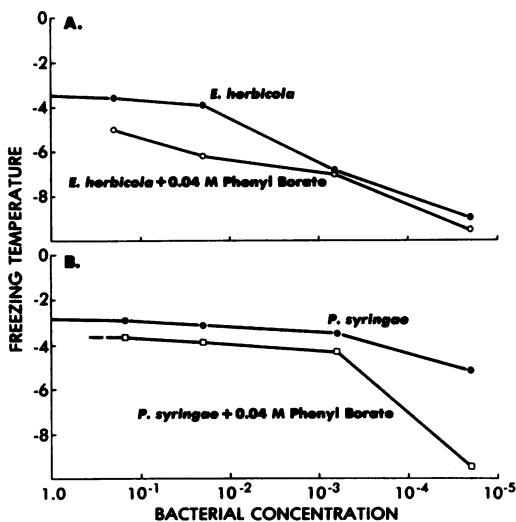


FIG. 2. Effect of phenyl borate on the INA of *E. herbicola* (A) and *P. syringae* (B). The freezing temperature plotted is that at which 50% of the drops were frozen. The bacteria were washed off agar slants with 0.05 M phosphate buffer, pH 7.5. The original concentration was adjusted to about  $2 \times 10^8$ /ml, and the freezing temperature of various dilutions of the cells was measured. A 0.1 M solution of phenyl borate at pH 7.5 was added to various separate dilutions of the cells to a final concentration 0.04 M, and the cells were incubated with the phenyl borate for 40 min. The freezing temperature then was measured in the presence of the phenyl borate. The freezing temperature of the buffer, with or without phenyl borate, was about  $-16^{\circ}\text{C}$ .

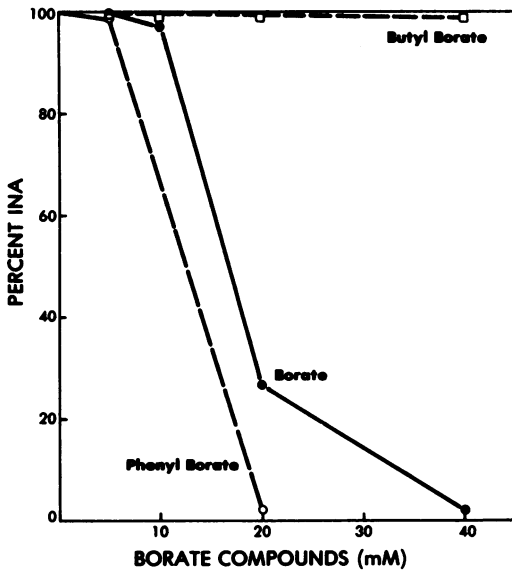


FIG. 3. Effect of various borate compounds on the INA of *P. syringae*. These compounds were added to bacteria which had a final concentration of  $2 \times 10^8$ /ml. The percent INA was calculated by using as a standard a freezing temperature curve for dilutions of the bacteria. When the freezing temperature in the presence of the borate compound lowered the freezing temperature to that a 1:10 dilution of the bacterial cells, this is plotted as 10% of the INA. The other experimental conditions are given in the legend to Fig. 2.

phenyl borate (18) were still more effective inhibitors. Butyl borate had almost no effect on the INA. The relative effectiveness of these compounds are what is predicted from the relative ability of these compounds to form complexes with sugar-like hydroxyl groups. For example, *m*-nitrophenyl borate has the highest affinity for hydroxyl groups and butyl borate has the lowest. It should also be noted (Fig. 4) that *P. syringae* was more sensitive to these borate compounds than was *E. herbicola*. For example, when mixed with 0.04 M phenyl borate, *P. syringae* retained only 0.2 to 0.3% of its INA, whereas *E. herbicola* had 4 to 5% of its INA remaining. It should be pointed out that *P. syringae* was also considerably more sensitive to sulfhydryl reagents than was *E. herbicola* (see below).

The reversibility of the phenyl borate inhibition was examined briefly. Dialysis could not be used to remove the borate compounds since the INA of the cells was not stable to storage. Therefore, after incubation, the bacteria-borate solution was diluted 1:10. In typical experiments some, but not complete, reversibility was observed. For example, when phenyl borate (40 mM) was added to inhibit the *E. herbicola* INA to only 4% that of the untreated cells, the 10-fold

dilution (reducing the phenyl borate to 0.004 M) restored the INA to 20% of that found in a similar dilution of untreated cells. This increase of relative INA from 4 to 20% upon dilution was observed also with *P. syringae*, and the relative increases in INA upon dilution of the borate-bacteria mixtures in different experiments ranged from 5-fold (4 to 20%) to 10-fold (1 to 10%).

INA of various purified lectins and effect of lectins on INA of bacteria. Before examining the action of various lectins (10) on the INA of *P. syringae* and *E. herbicola* it was necessary to determine the INA of the individual lectins. Figure 5 shows the INA of the lectins used in these experiments. Surprisingly, garden pea (*Pisum sativum*) lectin had a significant INA which was proportional to the concentration tested. Similarly, asparagus pea (*Lotus tetragonobubus*) lectin and gorse (*Ulex europaeus*) lectin also had some activity. On the other hand, lentil lectin and fava bean lectin, both of which inhibited the bacterial INA, had no detectable INA at concentrations up to 2 mg/ml. It should be noted that

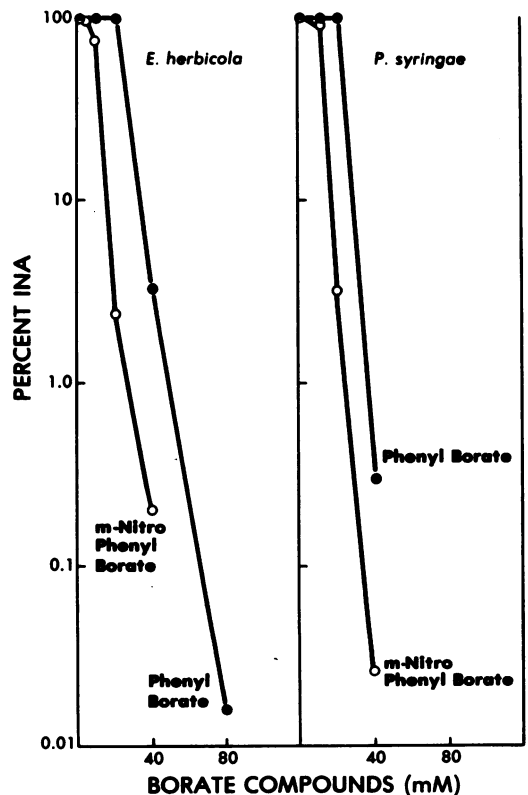


FIG. 4. Effect of phenyl borate and *m*-nitrophenyl borate on the INA of *E. herbicola* and *P. syringae*. The experiment and calculations are similar to those given for Fig. 3.

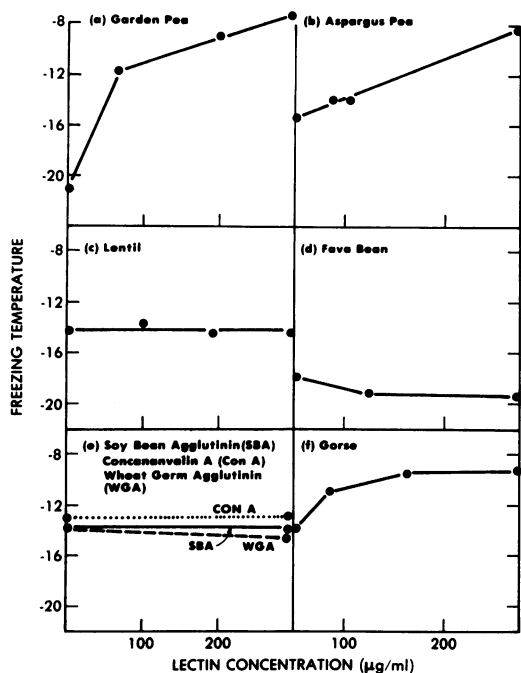


FIG. 5. INA of various lectins. The freezing temperatures are given as the temperatures at which 50% of the sample drops were frozen. Only one concentration of soy bean agglutinin, concanavalin A, and wheat germ agglutinin was tested. The suspending medium was 0.05 M phosphate buffer, pH 7.0.

most of the lectins are glycoproteins, with concanavalin A being a notable exception (10). However, there appears to be no evident correlation between the sugar content and their nature and the INA of the lectins. Garden pea lectin, which has INA, contains about 0.3% glucose, whereas lentil lectin contains 2 to 3% *N*-acetylglucosamine and glucose and fava bean lectin contains a small percentage of glucosamine and mannose (2, 5, 10).

Inhibition by highly purified lentil lectin at  $2.7 \times 10^{-6}$  M after 30 min of incubation of the INA of *P. syringae* is shown in Fig. 6. At high concentrations of bacterial cells the change in freezing temperatures was small, but at a dilution of  $10^{-3}$  equal to  $2 \times 10^5$  cells per ml, lentil lectin lowered the freezing temperature from  $-8$  to  $-14^\circ\text{C}$ . The inhibition at all bacterial concentrations was about 90%. When the lectin was preincubated for 1 h with a blocking sugar (in this case, *N*-acetylglucosamine) before it was added to the bacterial cells, the inhibition of the INA was partially relieved to about 30%. The INA of *E. herbicola* was also inhibited 80 to 90% by lentil lectin at the same concentration. The only other lectin which inhibited the INA of these bacteria was that obtained from fava beans (2). At 167  $\mu\text{g/ml}$  (equal to  $3.5 \times 10^{-6}$  M), this

lectin inhibited the INA of *P. syringae* 95%. The INA of *E. herbicola* was somewhat less sensitive and was inhibited 60% after incubation with 167  $\mu\text{g}$  of the fava bean lectin per ml. A summary of the effects of the lectins examined on the INA of these cells is shown in Table 1. At concentrations of usually  $2 \times 10^{-6}$  to  $4 \times 10^{-6}$  M, only lentil lectin and fava bean lectin were good inhibitors. Concanavalin A was a marginal inhibitor (5 to 10%), whereas the five other lectins tested had no effect. Both lentil lectin and fava bean lectin have a binding specificity for either free or bound mannose or glucose or glucosamine residues or both. Concanavalin A, garden pea lectin, and wheat germ agglutinin are able to bind to mannose or glucose residues or both like lentil lectin and fava bean lectin, but they do not inhibit the INA of these cells. The specificity of the other lectins (group II) was directed toward galactose, fucose, or glucuronic acid. Negative results on INA with any one lectin do not completely rule out the presence of certain sugars at or near the INA site. Nevertheless, it seems likely that the INA site of both *P. syringae* and *E. herbicola* contains a mannose- or glucose-like structure rather than another sugar residue.

**Inhibition of the INA of *P. syringae* and *E. herbicola* by sulfhydryl reagents.** Several observations suggested that a protein(s) could be at the INA site of both bacteria: (i) heat lability at  $65^\circ\text{C}$ , (ii) inhibition of  $\text{Hg}^{2+}$ , (iii) inhibition by Formalin, and (iv) the well-known ability of proteins to interact with water (4). We found that treatment of these cells with mild oxidizing

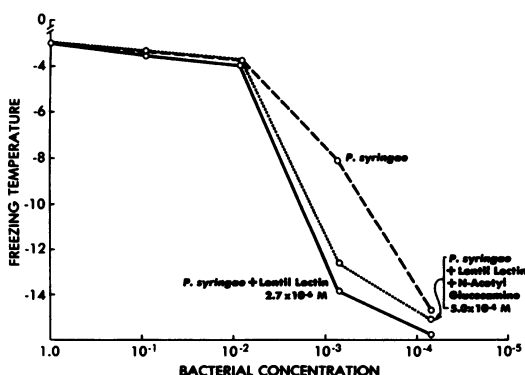


FIG. 6. Effect of lentil lectin on the INA of *P. syringae*. The freezing measurements were carried out by procedures similar to those given in the legend to Fig. 2. The bacterial cells, initially at  $2 \times 10^8/\text{ml}$ , were incubated with the lectin for 1 h at room temperature before the freezing temperature was measured. When the blocking sugar was present, the lectin plus sugar were incubated for 30 min before the bacteria were added.

TABLE 1. Effect of lectins on INA of *P. syringae* and *E. herbicola*<sup>a</sup>

Lectins	Main sugar specificity	% Inhibition of INA of:	
		<i>P. syringae</i>	<i>E. herbicola</i>
Group I (specific for mannose and glucose)			
Lentil	Mannoside/glucosides	90	80
Fava bean	Mannosides/glucosides	95	60
Concanavalin A	Mannosides/glucosides	10	5
Garden pea	Mannosides/glucosides	None	None
Wheat germ agglutinin	N-Acetylglucosamine	None	None
Group II (specific for various sugars)			
Soy bean agglutinin	N-Acetylgalactosamine	None	Very slight
Asparagus pea	L-Fucose	None	None
Gorse	L-Fucose	None	None
Horseshoe crab	Endotoxin/glucuronic acid	None	None

<sup>a</sup> The conditions used in these experiments are given in the text.

reagents such as 1 to 2% H<sub>2</sub>O<sub>2</sub> or low concentrations of I<sub>2</sub> rapidly and irreversibly inactivated the INA of both kinds of cells. These results suggest that a -SH group on a protein at the INA site might be critical. The marked inhibition of the INA by three sulfhydryl reagents is shown in Table 2. The inhibition of INA by these compounds was completely blocked by the prior addition of dithiothreitol. The inhibition of INA by *p*-hydroxymercuribenzoate was not surprising. However, this compound is much more specific in reacting with sulfhydryl groups than is the mercuric ion. Iodoacetamide and especially *N*-ethylmaleimide (NEM) have an extremely

high specificity in reacting with sulfhydryl groups. It should be noted that these last two reagents were considerably more effective in inhibiting the INA of *P. syringae* than in inhibiting that of *E. herbicola*. This is in agreement with the relative sensitivity of the INA of these two kinds of bacteria to borate compounds. This pattern of sensitivity supports the conclusion that the -SH group of the INA site may be near the active carbohydrate moiety.

The relationship between the viability of these cells and their INA after exposure to these sulfhydryl reagents was examined. It was found that 1 mM NEM irreversibly killed about 99% of both bacteria within 30 s. It was possible that cell death as measured by loss of colony-forming ability led to a secondary change in the cell surface which interfered with the INA. Therefore, both types of cells were suspended in buffer and exposed to a dose of UV light from a standard germicidal lamp (in the presence of dithiothreitol) so that only 0.001% of the cells were still viable. The killing by UV light caused no detectable change in the INA of either kind of bacterial cell.

The properties of the reaction of these bacteria with NEM, especially the rapid inactivation rate and the ability to stop the reaction by dilution into dithiothreitol, made it possible to study the kinetics of NEM inactivation of the INA (Fig. 7). In four separate experiments on the inactivation of the INA of *E. herbicola* by 1

TABLE 2. Effect of sulfhydryl reagents on the INA of bacteria

Bacteria	% INA after treatment with given Sulfhydryl reagent <sup>a</sup>		
	NEM (1 mM)	<i>p</i> -OH-mercuribenzoate (1 mM)	Iodoacetamide (50 mM)
<i>E. herbicola</i>	5.0	0.01	0.2
<i>P. syringae</i>	0.2	0.2	0.01

<sup>a</sup> The bacterial cells were incubated with these reagents for 1 h at 25°C in 0.05 M Tris buffer, pH 7.2.

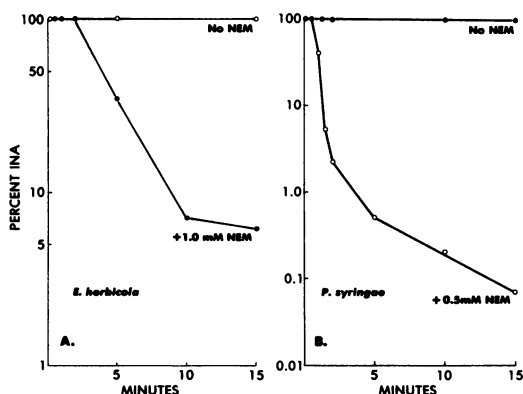


FIG. 7. Effect of NEM on the INA of *E. herbicola* (A) and *P. syringae* (B). (A) NEM was added to a suspension of *E. herbicola* at  $2 \times 10^8$ /ml to a final concentration of 1.0 mM. After incubation at 23°C, samples were removed at various times and added to 10 volumes of a solution containing 0.010 M Tris (pH 7.2) and 1 mM dithiothreitol. The percent INA of the diluted samples was calculated as described in the legend to Fig. 3. (B) NEM was added to a suspension of *P. syringae* to a final concentration of 0.5 mM, and the experiment was carried out as described in (A).

mM NEM at 23°C, an initial lag or delay in the action of the NEM was observed. The reaction rate was then logarithmic until it leveled off after 10 min. If the linear part of the curve is extrapolated to the start of the reaction, zero time, it intercepts the ordinate at an INA equivalent to twice that of the original untreated cells. This extrapolation implies that there are two independent sites on the *Erwinia* cells which can initiate ice formation.

The inactivation kinetics of the INA of *P. syringae* by NEM (three experiments) also exhibited an initial delay or shoulder. In these experiments, the greater sensitivity of the *P. syringae* to NEM made an extrapolation to the ordinate at zero time less precise, but it appears that the active *Pseudomonas* cells have four or more independent sites.

The possibility that the INA sulfhydryl group was near the carbohydrate moieties was examined. Both kinds of cells were first incubated with 0.04 M phenyl borate for 30 min at 25°C to allow the borate to complex to the hydroxyl groups, and then 1 mM NEM was added and the mixture was incubated for an additional 5 min. After a 10-fold dilution into dithiothreitol, the INA of the cells was determined. The preincubation with phenyl borate had no measurable effect on the subsequent inactivation of the INA by NEM.

**Phage susceptibility and *Erwinia* strain specificity: inhibition of the INA of *E. herbicola* by infection with a virulent phage.** The effect of virulent phage infection of the INA of *E. herbicola* was examined. The properties of this phage, called Erh 1, have been described elsewhere (6). It is a double-stranded DNA phage which readily kills its host cells within 5 to 10 min after adsorption. It has a latent period of about 95 min and a burst size of 140 per cell (6). The host range of this virulent phage, originally isolated on *E. herbicola* subsp. *herbicola*, was examined by plating it on various other *Enterobacteriaceae* and other strains of *Erwinia*. This phage did not form plaques on any non-*Erwinia* bacteria. Surprisingly, it formed plaques with 100% efficiency on the distantly related *E. stewartii* but not on the closely related *E. herbicola* subsp. *ananas*, *E. amylovora*, *E. caratovora*, or *E. uredovora*. The INA of all of these bacterial strains was measured and the only strain with INA, in addition to *E. herbicola* subsp. *herbicola*, was the closely related *E. herbicola* subsp. *ananas*. For this strain, the freezing temperature of a concentration of  $4 \times 10^8$  cells per ml was  $-3.8^\circ\text{C}$ , whereas at similar concentrations the freezing temperatures were  $-20.5^\circ\text{C}$  for *E. stewartii*,  $-19^\circ\text{C}$  for *E. uredovora*,  $-18.9^\circ\text{C}$  for *E. amylovora*, and  $-19.9^\circ\text{C}$  for *E. caratovora*. Since bacteriophage Erh 1

plated on *E. stewartii* (in addition to *E. herbicola*), an *Erwinia* strain which has no INA, but did not plate on *E. herbicola* subsp. *ananas*, which has INA, it seems likely that there is no direct relationship between the INA site on the surface of these bacteria and the site which permits successful phage infection.

Figure 8 shows the INA of a culture of multiply infected *E. herbicola* 120 min after infection. At this time, the INA of the culture has been reduced to <1% of the control uninfected cells. Figure 8 also shows that the purified phage at  $10^{10}$  particles per ml had a freezing temperature of about  $-14.5^\circ\text{C}$ , whereas the 0.05 M phosphate buffer (pH 7.2) used for diluting the culture had a freezing temperature of about  $-20^\circ\text{C}$ . The low INA of the purified phage is not significant and may well be due to traces of *E. herbicola* cell wall in the phage preparation.

The kinetics of the decrease in the INA of *E. herbicola* after infection (Fig. 9) was compared with the course of phage growth in a one-step growth experiment. The phage titer representing infected bacteria was unchanged until the end of the latent period at 95 min. At this point, the cells began to lyse, liberating new phage. Lysis was largely complete at 150 min. The change in the INA of the infected culture is plotted on the left side of Fig. 9. There was no effect on the INA of the infected cells (zero change in freezing temperature) until about 42 to 45 min, which is midway through the latent period. At this time, the INA began to decrease, and by 60 min the freezing temperature of the culture had fallen  $2.5^\circ\text{C}$ . No cells had lysed at 60 min, and by the time lysis began, the freezing temperature decreased a total of  $3.5^\circ\text{C}$ . There was no further decrease in INA of this culture even as all the cells lysed.

## DISCUSSION

The remarkable discovery only a few years ago that two gram-negative but otherwise unrelated types of bacteria, *Pseudomonas* and *Erwinia* species, can convert supercooled water into ice has drawn attention to a phenomenon of considerable theoretical and practical importance. The fundamental questions about the INA of *P. syringae* and *E. herbicola* (and a few closely related other strains) include (i) the size and chemical nature of the nucleating site, (ii) the mechanism of the interaction with water molecules, (iii) the number of such sites per bacterial cell and (iv) their location on the bacterial surface, (v) the factors which control the production of these sites on the cell surface, and (vi) the similarities and differences between the INA sites of *P. syringae* and *E. herbicola* on the cell surface. This study has been concerned mainly with the chemical nature of the ice nucle-

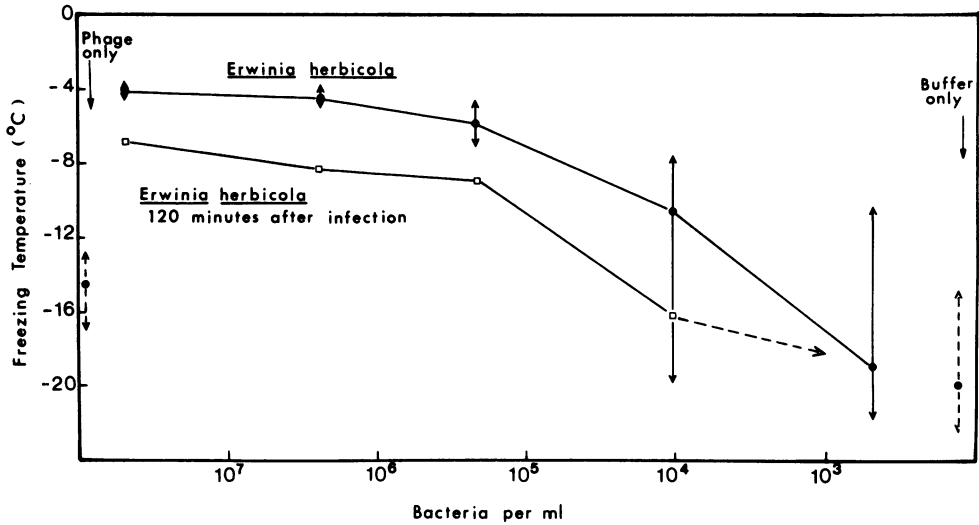


FIG. 8. Effect of infection of *E. herbicola* with a virulent bacteriophage, Erh 1, on the INA of the host cells. The bacterial cells were grown at 23°C in the yeast extract-tryptone broth to a concentration of  $2 \times 10^8$ /ml. One portion of the culture was infected with purified Erh 1 phage particles so that there were eight phage particles per bacterial cell. Freezing temperature measurements were made on dilutions of the uninfected cells and, 120 min later, on dilutions of the infected culture. The freezing temperatures for the diluting buffer and for purified phage (at a higher concentration than used in these experiments, i.e., at  $10^{10}$ /ml) are also given.

ating site. In addition, we have compared some biological features of the site on the different cells.

In some respects, the INA can be compared to the activity of an enzyme which changes the rate at which  $\text{H}_2\text{O}_1 \rightleftharpoons \text{H}_2\text{O}_s$ . This nucleating activity, of course, does not affect the final thermodynamic state, but only the rate at a given temperature at which the structure of supercooled liquid water is converted to the lattice structure of ice. It should be noted that the melting temperature of ice containing these active bacteria is still 0°C. Further, the bacterial ice nucleating site is chemically unchanged during the reaction and the bacteria can repeat the nucleation event innumerable times. In the absence of nucleating agents, small ice structures form spontaneously in supercooled water which, if the temperature is not too low, will readily melt. In the presence of the active ice nucleating bacteria, freezing of one 10- $\mu\text{l}$  drop can be observed to occur in a fraction of a second. It appears that the bacteria must organize the water oxygen and hydrogen atoms to fit an icelike lattice. This is not a typical enzyme-catalyzed reaction in which it is obvious that some chemical bonds are broken and that new ones are formed, but the changes during the freezing of water are clearly analogous.

The finding of a sulfhydryl group at the INA site of both *P. syringae* and *E. herbicola* was not anticipated since sulfhydryl groups on the surface of cells growing with vigorous aeration

(pseudomonads being obligate aerobes) would be expected to be readily oxidized. It must be assumed that the ice nucleating site offers some protection against oxidation. It is likely that the sulfhydryl group is from a cysteine residue on a protein since (other than reduced glutathione and a few other small molecules) this is the source of practically all sulfhydryl groups in cells. Therefore, the sensitivity of the INA to sulfhydryl reagents confirms the presence of a protein at the ice nucleating site. The nature of the interaction of -SH groups with liquid water or with ice has never been studied. In our experiments, 1 mM dithiothreitol, which has a highly active -SH group, had no INA. Although -SH groups can be assumed to behave analogously to -OH groups, any differences in ease of bond formation, especially as a function of temperature, may be critical in forming the ice lattice.

The presence of mannose or glucose, like -OH groups, at the ice nucleating site of both bacteria suggests that there may be a glycoprotein at the active site. On the other hand, the extensive work on the fish antifreeze proteins showed that not all of them contain carbohydrates. It is possible that the -OH groups recognized by both borate and the lectins are on a unique modified carbohydrate residue attached to a nonprotein component of the cell wall.

The kinetics of the reaction of NEM with these bacteria suggests strongly that *E. herbi-*



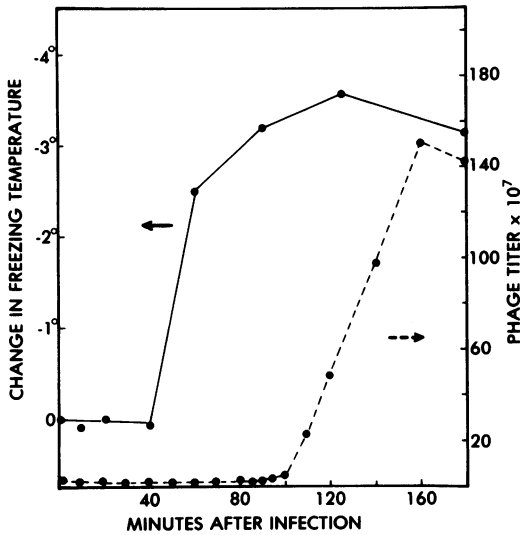


FIG. 9. Changes in the INA of *E. herbicola* in the course of virulent phage infection. A culture of *E. herbicola* in broth at 23°C was multiply infected with phage and then diluted 1:100 in fresh broth to stop phage reinfection and maintain conditions for one-step growth (1). The diluted culture was assayed at various times for plaque-forming ability, and this result is plotted (broken lines) on the ordinate on the right side of the graph. Simultaneously, samples of the diluted infected culture were diluted an additional 10-fold in cold buffer to stop all growth. The change in freezing temperatures of these samples as infection proceeded is plotted (solid lines) on the left side of the graph. The decrease in the freezing temperatures is shown on the ordinate as minus degrees Celsius.

*cola* has only two independent ice nucleating sites per cell and that *P. syringae* has possibly four to eight such sites. No previous experiments have been reported dealing with the question of number of sites per cell, and although additional confirmation is still needed, one can narrow the investigation to possible structures on the cell surface which are present in only a few copies per cell. It is appropriate to emphasize here the similarities and differences in the ice nucleating sites of *P. syringae* and *E. herbicola*. Both types of cells are sensitive to the same lectins and both are insensitive to other lectins. Similarly, both are sensitive to the same borate compounds and to the same sulfhydryl reagents. Further, the *P. syringae* cells are more sensitive than *E. herbicola* cells to borate compounds, fava bean lectin, and NEM. It can be concluded that both types of bacteria have similar, but not necessarily identical, structures at the active site but that the *P. syringae* cells have a more exposed site and more of them per cell than do the *E. herbicola* cells.

A critical question concerns the size of the ice

nucleating site and the number of molecularly separate components or domains at the site. All attempts so far to break up the cells and isolate a component as active as the whole cell have been unsuccessful. Isolated cell walls do have some INA but the activity is quite low (11), suggesting that the site has several components with a critical spatial arrangement and that procedures which disrupt such a structure will not permit the recovery of the original activity.

The possibility that the ice nucleating site has separate domains and is multicomponent is supported by the observation that even prolonged treatment with Formalin or NEM (Fig. 7) or even phage lysis does not completely abolish the INA of either type of bacteria. The remaining activity, usually with a freezing temperatures at  $-8^{\circ}\text{C}$ , is similar to that found in isolated cell walls. Also, Vali (20) has calculated that at  $-2^{\circ}\text{C}$  the ice nucleating site would require the equivalent of a drop surface with a curvature distance of about 20 nm. At  $-20^{\circ}\text{C}$  a curvature distance of only 2 nm would suffice to order sufficient water molecules into a stable ice structure. At temperatures at which both bacteria have INA, such as  $-2$  to  $-3^{\circ}\text{C}$ , the curvature distance required might be about 18 nm. No single proteins are known which provide an idealized surface with a curvature of this magnitude; the largest proteins with individual domains might offer only a 4- to 5-nm idealized curvature (14). However, if the surface on the protein available to organize liquid water was highly irregular, and further, if all of the irregular surface had nucleating activity, then it is possible, but unlikely, that a single protein molecule could initiate ice formation. These constraints suggest that at least at temperatures at or above  $-3^{\circ}\text{C}$  there may well be several individual components at the ice nucleating site.

It is perhaps obvious to emphasize that it is the cell surface of these bacteria where the ice nucleating site must be, but it is not clear whether the site must be on the outer membrane or the inner membrane. One possibility which cannot be excluded is that the active site is a specialized juncture of the inner and outer membranes. In this connection, the change in the INA during *Erwinia* phage growth is relevant. The sharp decrease in INA midway through the latent period corresponds to the time when the first phage particles are being assembled from their components. It is well known from the work of Simon (19) that viral assembly reactions occur in close proximity to the inner cell membrane. These reactions are known to cause changes in the inner cell membrane, to change transport mechanisms (7), and even to perturb the outer surface of the bacterial cell. These changes in cell surfaces usually involve the cell

lipid elements, especially the phospholipids. Because of their hydrophobic properties, phospholipids must also be considered as likely components, in addition to the protein and carbohydrate moieties, of the ice nucleating site, all of which participate in converting liquid water into ice.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI 06336 and, later, AI 28370 from the National Institute of Allergy and Infectious Diseases.

The experiments on phage infection and with some of the lectins was carried out at the University of Colorado; the rest of the study was carried out later at the University of California, San Francisco.

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