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Seasonal Population Changes and Characterization of Ice-Nucleating Bacteria in Farm Fields of Central Alberta†

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During the summer of 1983 in central Alberta, changes in the bacterial population inhabiting the leaves of field beans (*Phaseolus vulgaris* L.) and canola (*Brassica napus* L. Altex) were studied to determine if ice-nucleating bacteria were present on these plants. Three colony types (white, yellow, and peach-colored) were found on field beans and canola leaves. Approximately 25% of the isolates from the white colony group, which dominated the population, were ice-nucleating bacteria. No ice-nucleating bacteria were present on canola leaves. Out of a total of 76 ice-nucleating bacteria isolated, 5 representative cultures were characterized in detail and identified as *Pseudomonas fluorescens*. The fatty acid composition of these cultures was essentially identical to that of typical *P. fluorescens* cultures and was altered by varying the growth temperature from 10 to 30°C.

Frost damage to crops is a serious problem which may cause major reductions in yield. For example, an early frost in August 1983 reduced the grain yield in parts of the Canadian central prairie provinces by 15 to 25%. Such damage may occur at temperatures only slightly below 0°C because of the presence of ice-nucleating bacteria.

Although the freezing point of water is 0°C, absolutely pure liquid water may be super-cooled to near −40°C and will still remain liquid. The presence of dust or other particles may serve as a nucleus for ice formation in liquid water cooled to about −10 to −14°C. Ice-nucleating bacteria are very efficient initiators of ice crystal formation, which can begin when water is cooled to only −2 to −6°C. Thus, the presence of ice-nucleating bacteria may determine whether plants are severely damaged by periods of low temperature (−2 to −6°C) which otherwise would not be harmful (14).

Three species of ice-nucleating bacteria have been found: *Pseudomonas fluorescens* Migula, *Pseudomonas syringae* van Hall, and *Erwinia herbicola* (Löhnis) Dye (11–13, 16).

Only about 50% of *P. syringae* isolates and even much lower proportions of *E. herbicola* and *P. fluorescens* are capable of ice nucleating (11). Ice-nucleating strains of *P. syringae* and *E. herbicola* are commonly found on plants in the United States, but those of *P. fluorescens* are rather rare and found only in water (17).

It was suspected that ice-nucleating bacteria common to central Alberta may be different from those in the United States because of significant differences in seasonal temperatures between the two regions. If so, the bacteria in Alberta should be well adapted to a cool climate. The fatty acid composition of the membrane lipids of microorganisms is closely related to their growth temperature and is altered to maintain the proper fluidity of membrane lipids for growth at the given temperature. A detailed study in this area should contribute to a better understanding of the physiology of such bacteria.

This study reports a seasonal population study of ice-nucleating bacteria in central Alberta fields and the isolation and characterization of 76 strains of ice-nucleating bacteria, all of which were *P. fluorescens*. Five isolates with high ice-nucleating activity levels were further characterized to determine the effect of temperature on growth and cellular fatty acid composition.

MATERIALS AND METHODS

Bacterial strains. Two reference strains, *P. fluorescens* ATCC 13525 (non-ice-nucleating) and *P. syringae* (RSB) (ice-nucleating), were obtained from J. N. Campbell, University of Alberta, Edmonton, Alberta, Canada, and from R. C. Schnell, Environment Research Laboratories, Boulder, Colo., respectively. A total of 76 ice-nucleating bacteria were isolated and purified as described below and were assigned strain numbers.

Growth and maintenance of bacteria. Reference cultures and ice-nucleating isolates were routinely cultured on Trypticase soy broth or agar (BBL Microbiology Systems, Cockeysville, Md.). They were maintained on nutrient agar (BBL) slants and transferred every 3 months. Cultures were normally grown at 30°C.

Sampling and counting of leaf surface bacteria. Field bean (*Phaseolus vulgaris* L.) and canola (*Brassica napus* L. Altex) seeds were planted in eight plots on the Ellerslie farm, University of Alberta, Edmonton, Alberta, Canada, on 24 June 1983. The sampling of leaves for bacterial counts began on 3 August 1983. Leaf samples (1 to 8 g [fresh weight]) were washed with 100 ml of 10 mM phosphate buffer (pH 7.0), and the washings were used for plate counts on Trypticase soy agar within 2 h. After overnight incubation at 33°C, colonies growing on the plates were counted.

Measurement of ice-nucleating activity. The initial screening for ice-nucleating bacteria was carried out as follows. Single colonies from the Trypticase soy plates were restreaked to single colonies, transferred to tubes containing 5 ml of Trypticase soy broth, and incubated at 30°C overnight on a rotary shaker. The cultures were then diluted 1:10 in water, and their freezing points were measured as described (16, 23).

For more precise measurement of ice-nucleating activity, each culture was initially diluted with high-pressure liquid chromatography-grade water (J. T. Baker, Phillipsburg, N.J.) to 1.15×10^9 cells per ml and was further diluted by serial 10-fold dilutions. A thermoelectric cold plate model

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TCP-12 (Thermoelectric Unlimited Inc., Wilmington, Del.) was used to test the freezing points of 10 individual droplets (10 μl each) from each dilution. As the temperature of the cold plate was reduced, the freezing temperatures of individual drops were recorded. The numbers of ice-nucleating cells in a dilution were determined by the most-probable-number method (7). For these calculations, a droplet with a freezing temperature of −10°C or above was considered to contain ice-nucleating cells.

Morphology and Gram reaction. Optical measurement of cell size was made by using a calibrated eyepiece graticule. A Phillips EM300 electron microscope was used to observe flagella and cell sections. A fresh culture from Trypticase soy agar was stained negatively with sodium phosphotungstate at pH 7.0 for the observation of flagella. An overnight culture on Trypticase soy broth was fixed, embedded, and sectioned for examination of the cell envelope and for confirmation of the Gram reaction of isolates. Air-dried films of the same Trypticase soy culture were stained by the Hucker method for gram reactions.

Characterization of ice-nucleating isolates. The ice-nucleating isolates were purified by plating and were characterized. Urease, poly-β-hydroxybutyrate production, starch hydrolysis, gelatin hydrolysis, and nitrate reduction were tested (21). Cyanide inhibition, H_2S production, arginine dehydrolase and lysine decarboxylase activity, fluorescein production (on King's medium B), and citrate utilization were also determined (4). Acetoin production was estimated with an α-naphthol creatine reagent (9). The nonfermenter system for the rapid identification of nonfermentative and oxidase-positive gram-negative rods (Flow Laboratories, Inc., McLean, Va.) was also used (2).

Optimal temperature for growth. A Toyo model CGI gradient temperature incubator (Scientific Industry Inc., Mineola, N.Y.) was used to provide a linear temperature gradient from 5 to 37°C. The incubator accommodated 40 L-shaped culture tubes containing 10 ml each of Trypticase soy broth. Each tube was inoculated with 10 μl of an overnight culture grown on the same medium at room temperature. Growth was estimated by measuring optical density on a Klett-Summerson colorimeter with a no. 66 filter.

Fatty acid analysis. Trypticase soy broth (1 liter in 2-liter flasks) was inoculated with isolate W-11 and incubated at 10, 15, 20, 25, or 30°C until the mid-logarithmic phase began. These cultures were harvested by centrifugation, washed with an 0.85% NaCl solution, and freeze-dried. The freeze-dried cells (50 mg) were methylated at 50°C for 2 h with 3 ml of methanol containing 0.3 ml of acetyl chloride. The fatty acid methyl esters were extracted with *n*-hexane and analyzed by a 5830 A gas chromatograph with a Grob injection system (Hewlett-Packard Co., Palo Alto, Calif.). The bacterial fatty acids were quantified by comparison with a methyl tridecanoate internal standard.

The tentative identification of bacterial fatty acids was done by comparing their retention times with those of authentic fatty acid samples on a polar and nonpolar column. The polar column was a capillary DX-4 column (10 m by 0.259 mm; J & W Scientific Inc., Rancho Cordova, Calif.), which operated at an initial temperature of 65°C and rose 10°C/min for 7 min and then 3°C/min up to a final temperature of 200°C. The nonpolar column was a capillary SP-2100 column (10 m by 0.25 mm; Supelco Inc., Bellefonte, Pa.), which operated under the same conditions except that the column temperature during the second part of the temperature program rose 2°C/min.

Mass spectrometry was used to confirm the tentative identification of bacterial fatty acid methyl esters by gas-liquid chromatography. Two gas chromatograph-mass spectrometer systems were used: a Finnigan-Mat 4500 quadrapole GC/MS and a Finnigan-Mat 212 magnetic sector medium-resolution mass spectrometer coupled with a Varian 3700 gas chromatograph. Electron impact spectra were used routinely. The location of unsaturation along the methylene chain of fatty acids was determined by mass spectrometry of their pyrollidine derivatives (1). Chemical ionization spectra were used only for the determination of the molecular weights of hydroxy fatty acid methyl esters.

Infrared spectra were obtained with a no. 283 infrared spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). Samples were prepared by placing a hexane solution of fatty acids on an NaCl plate and by letting the solvent evaporate. The characteristic adsorption at 10.35 μm was used to determine the presence of *trans* isomers (24).

A column chromatograph with silicic acid impregnated with silver nitrate was used to separate saturated from monounsaturated fatty acid methyl esters (5). Thin-layer chromatography was used to separate hydroxy fatty acid methyl esters from a mixture of saturated and unsaturated fatty acid methyl esters (8).

RESULTS

Changes in bacterial populations on field beans and canola during the 1983 growing season. The total number of bacteria on bean leaves remained fairly constant (3×10^5 to 1.3×10^6 CFU/g of fresh leaf) for the first 5 weeks, until the first frost struck in week 6 (Fig. 1A). Then, the number of bacteria increased rapidly by approximately 10-fold in 1 week and 200-fold in 2 weeks. This suggests that the total number of bacteria had been limited by available nutrients. Once frost damaged the bean cells and released more nutrients, this was no longer true.

The bacteria isolated from bean leaves were divided into three groups based on colony color: white, yellow, and peach. The proportions of these three groups changed during the growing season. Yellow colonies were initially dominant, accounting for approximately 55% of the total population. However, they did not remain dominant, and after the first frost decreased from 55 to 20% of the total population (Fig. 1B). White colonies formed the next largest group initially and slowly increased for 8 weeks until they made up approximately 80% of the total. Peach-colored colonies were about nil in week 1 but appeared to amount to 40 to 50% of the total colonies during the next 3 weeks; thereafter, they decreased to almost nil again.

The changes in the populations of the three color groups of bacteria isolated from canola leaves were very similar to those from field beans, except that the total number of cells after the first frost did not greatly increase (data not shown).

Isolation of ice-nucleating bacteria. *E. herbicola*, the most common ice-nucleating bacterium in the United States, forms yellow colonies. None of the yellow colonies from bean leaves, however, showed ice-nucleating activity, nor did any of the peach-colored colonies. Only white colonies had ice-nucleating activity; approximately 25% of them were positive. A total of 76 ice-nucleating cultures were isolated from bean leaves in this study. No ice-nucleating bacteria were isolated from canola plants, even though they were cultivated in the same general area as the bean plants.

Characterization of ice-nucleating isolates. From the 76 cultures of ice-nucleating isolates, 5 (W-11, W-20, W-31,

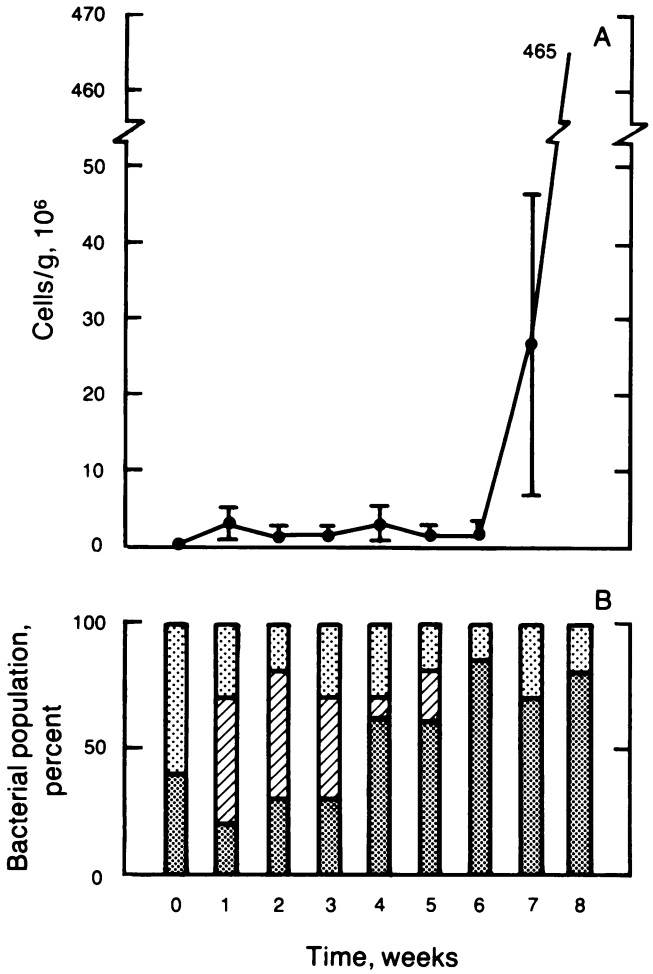

FIG. 1. Total bacterial counts on field bean (*P. vulgaris* L.) leaves during the growing season. Leaf samples were washed with buffer, and viable cell numbers were determined in the washings by plate count (A). The relative proportion of each of the three colony color groups in the population was also recorded: white (▩), yellow (▩), and peach (▩) (B).

W-51, and W-66) were chosen for detailed study because of their high ice-nucleating activity levels. They were all motile, gram-negative rods (0.5 to 0.9 μm wide by 2 to 4 μm long. Each of the five isolates had one to three polar flagella and produced fluorescent pigment during growth on King's medium B but only a trace amount of pigment on Trypticase soy agar. Neither pyocyanin nor blue pigment was produced by the isolates. They were positive for oxidase, arginine dehydrolase, lipase, and lysine decarboxylase, as well as for gelatin hydrolysis and acid production on MacConkey agar, and were inhibited by KCN. They were negative for nitrate reduction and starch hydrolysis and did not produce H_2S, poly-β-hydroxybutyrate, or acetoin. The isolates grew well at 30 but not at 42°C. They utilized glucose, fructose, sucrose, inositol, α-sorbitol, citrate, and lactate for growth as sole carbon sources but did not utilize adonitol, ethanol, propylene glycol, L-valine, β-alanine, butyrate, and propionate. From these tests the isolates were identified as *P. fluorescens* (10, 15, 20). This identification was supported by a test with the N/F (nonfermenter) system (Corning Medical Products, Roslyn, N.Y.).

The guanine plus cytosine content of the DNA from isolate W-11 was 62.8 mol%, which is similar to the values reported (59.4 to 61.3 mol%) for *P. fluorescens* ATCC 13525 DNA (18).

Ice-nucleating activity of isolates. The freezing points of isolate W-11 in a series of 10-fold dilutions of a cell suspension are shown in Fig. 2. The freezing points of all 10 sample drops for each of the dilutions were in close agreement, with the exception of those for the 10^4 dilution. The freezing temperatures of the 10 sample drops of this dilution were scattered widely (−7 to −27°C). This indicates that some but not all the cells in a suspension have ice-nucleating activity.

The minimum number of cells required to yield a positive result for ice-nucleating activity (a 10-μl drop freezes at −10°C) was determined by the most-probable-number method. Values varied widely by isolate: 3,400 (W-11), 20 (W-20), 1,100 (W-31), 100 (W-51), 5,500 (W-66) and 700 (*E. herbicola*) cells. This is interpreted to mean that only one cell in the calculated number of cells for each isolate had an active nucleus.

Optimal temperature for growth. The response of strain W-11 to temperature was typical of the other ice-nucleating isolates (Fig. 3).

Good growth was observed between 12 to 35°C, with the maximum growth rate of 1 doubling/h found between 30 to 35°C. The minimum temperature for growth was 7°C, with a doubling time of 5.3 h. All five isolates showed essentially identical growth responses to temperature.

Identification of cellular fatty acids of isolate W-11. The saturated fatty acid fraction of strain W-11 contained lauric, myristic, palmitic, and stearic acids. Their equivalent chain lengths measured on a polar column were identical to those measured on a nonpolar column (Table 1).

The monounsaturated fraction included two fatty acids, *cis*-9-hexadecenoic and *cis*-11-octadecenoic. The equivalent chain lengths of the respective fatty acids measured on the two columns differed by 0.35. The position and geometry of

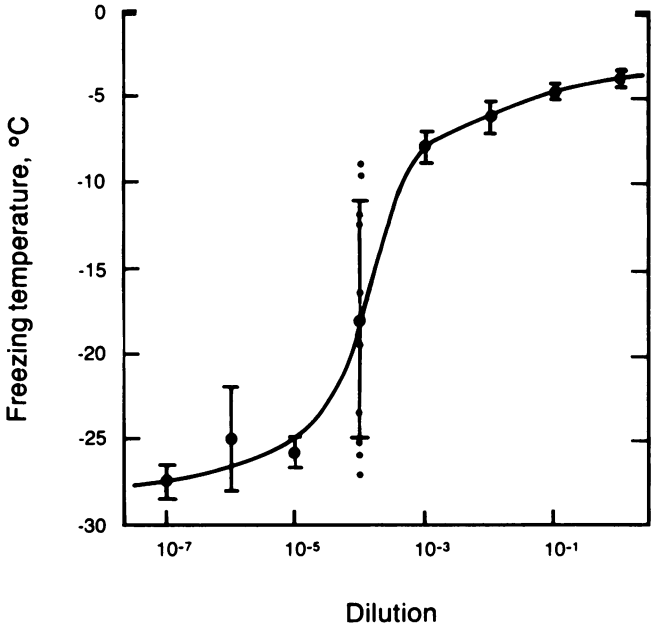

FIG. 2. Freezing temperatures of isolate W-11 culture. The culture was diluted in a 10-fold series, and the freezing point of each dilution was determined on a cold plate. Bars show ranges of 10 sample drops for each dilution.

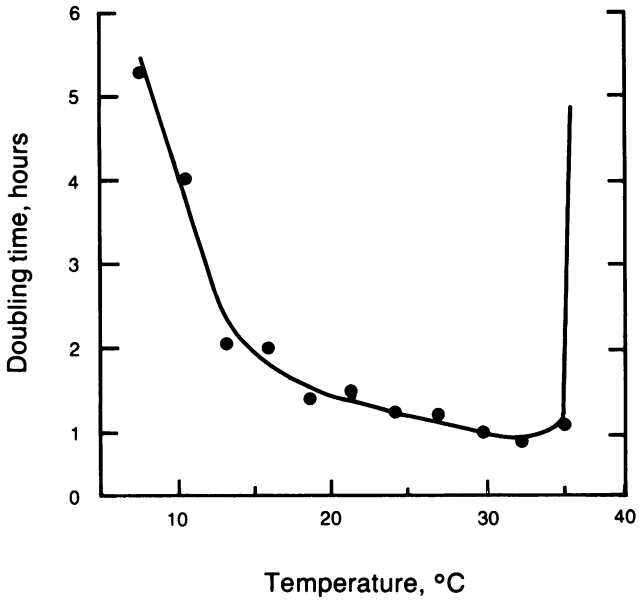

FIG. 3. Doubling times of isolate W-11 grown on Trypticase soy broth at varying temperatures. The isolate was grown in culture tubes incubated in a gradient temperature incubator, and its growth was followed photometrically. Growth in the logarithmic phase was used to calculate doubling time at varying temperatures.

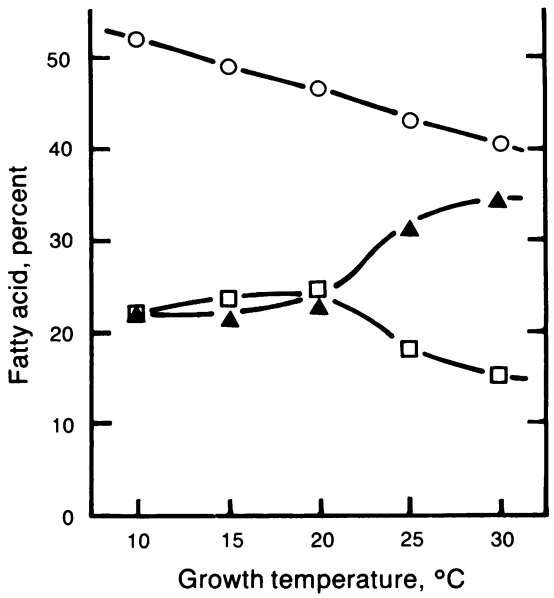

FIG. 4. Effects of growth temperature on composition of cellular fatty acids of isolate W-11. The isolate was grown in culture medium at five different temperatures, washed, and freeze-dried. Palmitic (▲), *cis*-9-hexadecenoic (○), and *cis*-11-octadecenoic (□) cellular fatty acids in the five samples were analyzed by gas-liquid chromatography.

unsaturation of these fatty acids were determined by mass spectrometry and infrared spectroscopy.

The hydroxy fraction was composed of three fatty acids: 3-hydroxydecanoic, 2-hydroxydodecanoic, and 3-hydroxydodecanoic. The equivalent chain lengths of the respective hydroxy fatty acids measured on the two columns differed widely between 2.1 to 2.8. The molecular weights and positions of the hydroxy group of these fatty acids were established by mass spectrometry.

Fatty acid composition and its relation to growth temperature. Major fatty acids of isolate W-11 grown at 30°C were palmitic, *cis*-9-hexadecenoic, and *cis*-11-octadecenoic, which amounted to 90% of the total fatty acids; the minor fatty acids were lauric, myristic, stearic, 3-hydroxydecanoic, 2-hydroxydodecanoic, and 3-hydroxydodecanoic. The amount of total fatty acids linearly decreased from 72 mg/g at 10°C to 59 mg/g of dry cells at 30°C (Fig. 4).

At lower growth temperatures, the amount of palmitic acid was reduced, whereas both major unsaturated fatty acids, *cis*-9-hexadecenoic and *cis*-11-octadecenoic, increased in quantity. These changes are similar to what has been reported for many other microorganisms.

DISCUSSION

The characterization of five ice-nucleating bacterial strains isolated from the Ellerslie farm shows that they are *P. fluorescens*. According to the classification of Stanier et al. (22), the five isolates are most closely related to biotype A or F, although they do not meet some of the required criteria. Ice-nucleating strains of *P. fluorescens* are not usually found on plants, but strains of biotype G have been isolated from water (17).

It was surprising that ice-nucleating strains of *E. herbicola* were not isolated in our study of farm fields in central Alberta. These strains have been found in the leaf washings of plants collected throughout the United States. The lack of

TABLE 1. Identification of cellular fatty acids of ice-nucleating isolate W-11

Peak no.	Fatty acid			Equivalent chain length on column:		
	Chemical name	Abbreviation	% of culture[a]	DX-4 (polar)	SP-2100 (nonpolar)	ΔECL[b]
1	Lauric	$C_{12:0}$	4.0	11.98	12.00	−0.02
2	Myristic	$C_{14:0}$	0.4	13.96	14.00	−0.04
3	3-Hydroxydecanoic	$C_{10:0}$ (3-OH)	1.4	14.18	11.38	2.80
4	2-Hydroxydodecanoic	$C_{12:0}$ (2-OH)	0.9	15.30	13.16	2.14
5	Palmitic	$C_{16:0}$	34.5	16.01	16.00	0.01
6	*cis*-9-Hexadecenoic	$C_{16:1}$ (*cis*)	40.3	16.10	15.75	0.35
7	3-Hydroxydodecanoic	$C_{12:0}$ (3-OH)	0.6	16.16	13.50	2.66
8	Not defined	$C_{17:0}$ (Cy)	2.4	17.10	16.80	0.33
9	Stearic	$C_{18:0}$	0.8	18.00	18.02	−0.02
10	*cis*-11-Octadecenoic	$C_{18:1}$ (*cis*)	15.3	18.09	17.71	0.38

[a] Compostion of culture grown at 30°C on Trypticase soy broth.
[b] Difference in equivalent lengths of fatty acids measured on polar and nonpolar columns.

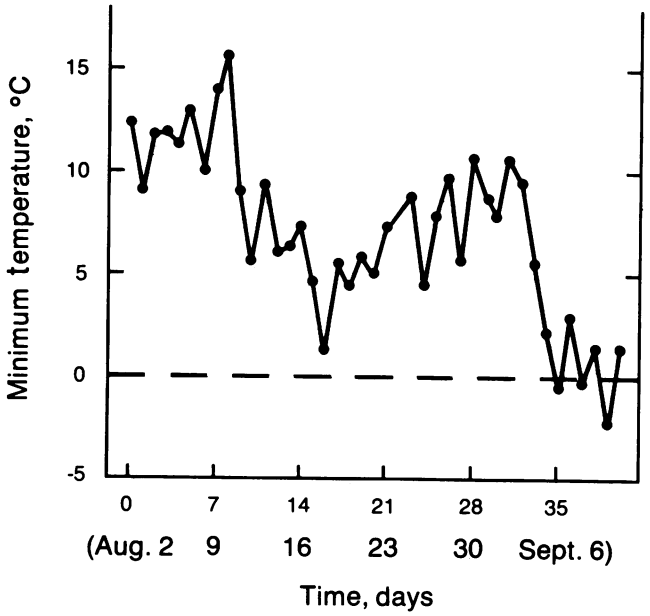

FIG. 5. Minimum daily temperatures during growth of field beans (*P. vulgaris* L.). Data were provided by the Atmospheric Environment Service of Environment Canada and were recorded at Edmonton International Airport, which is located near the farm field monitored.

E. herbicola in our samples may have been due to the cool summer climate of central Alberta (Fig. 5). However, if this was the sole reason, one would question why ice-nucleating *P. syringae* was not found in this study. *P. syringae* is known to be a psychrophilic bacterium (25), and there are many reports demonstrating the occurrence of ice-nucleating *P. syringae* on crop and fruit plants in the northern United States (6). Additional factors, such as the survival of the bacteria on leaf surfaces and interactions with the host plant, must be considered, and in this respect the absence of ice-nucleating bacteria on canola plants is noteworthy.

The fatty acid composition of strain W-11 is very similar to that found by Oyaizu and Komagata (19) for *P. fluorescens*. A small (3% of the total fatty acids) but significant amount of three hydroxy acids were present in strain W-11 that are also present in similar amounts in their strains. This is further evidence that strain W-11 is probably *P. fluorescens* or very closely related to it.

The growth characteristics of strain W-11 with respect to temperature are affected by its fatty acid composition. The fatty acid composition was not affected significantly when isolate W-11 was grown at 20°C or lower, but it changed at 25°C or higher (Fig. 4). This suggests that the organism is a faculative psychrophile capable of growth at low temperature but with the ability to adjust its cellular fatty acid composition for growth at higher temperatures. Data for doubling times support this possibility (Fig. 3).

Unsaturated fatty acids play an important role in controlling the fluidity of membrane lipids at the proper level for growth at a given temperature. In bacteria there are two alternate pathways for synthesizing unsaturated fatty acids. One operates under aerobic conditions, and the other functions under anaerobic conditions (3). The occurrence of *cis*-9-hexadecenoic and *cis*-11-octadecenoic acids indicates that strain W-11 synthesizes these fatty acids from an unsaturated C_{10} intermediate anaerobically in a way similar to that of *Escherichia coli*.

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