ELSEVIER

FEMS Microbiology Ecology 32 (2000) 111–120

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Physiological adaptation to low temperatures of strains of *Rhizobium leguminosarum* bv. viciae associated with *Lathyrus* spp.[1]

Pascal Drouin [a], Danielle Prévost [a,*], Hani Antoun [b]

[a] *Soils and Crops Research and Development Centre, Agriculture and Agri-Food Canada, 2560 Hochelaga Blvd., Sainte-Foy, Que., Canada G1V 2J3*

[b] *Département des Sols et de Génie Agro-Alimentaire, Faculté des Sciences de l'Agriculture et de l'Alimentation, Pavillon Charles-Eugène Marchand, Université Laval, Sainte-Foy, Qué., Canada G1K 7P4*

Received 19 July 1999; received in revised form 6 March 2000; accepted 6 March 2000

Abstract

Strains of *Rhizobium leguminosarum* bv. viciae, isolated from the legume species *Lathyrus japonicus* and *Lathyrus pratensis* in northern Quebec (Canada), showed different capacities for growing at low temperature. In the present study, we investigated some mechanisms related to cold adaptation. Two cold-adapted strains (psychrotrophs) were compared to a poorly adapted strain and to a cold-sensitive strain (reference strain) for freezing survival, protein induction and fatty acid composition under low temperature. Following cold shocks (25°C to 10, 5 and 0°C), a common 6.1-kDa CSP (cold shock protein) was induced in all strains, but the total number of CSPs synthesized at 0°C was higher in cold-adapted strains than in the cold-sensitive strain. The synthesis of CAPs (cold acclimation proteins) was observed under continuous growth at 5°C in all three strains capable of growth at this temperature. Levels of survival after 24 h at −80°C where higher in cold- (79%) and poorly adapted (64%) strains than in the cold-sensitive strain (33%), but a 2-h acclimation period at 5°C before freezing doubled the survival of the cold-sensitive strain. Low temperature conditions affected similarly the fatty acid composition of all strains, regardless of their cold adaptation level. The proportion of unsaturated fatty acids increased significantly with the lowering of growth temperature from 25 to 5°C, but showed a tendency to decrease after a cold shock from 25 to 5°C. A specific unsaturated fatty acid, *cis*-12 octadecanoic acid, was produced during growth at 5°C. The unsaturated *cis*-vaccenic acid was the principal component under all conditions. The cold adaptation trait was weakly reflected in symbiosis with the agronomic legume, *Lathyrus sativus*, with which one cold-adapted strain showed a slightly higher nitrogenase activity and shoot dry matter yield than a commercial strain under a sub-optimal temperature regime. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Temperate climates are characterized by short growing seasons, which are subjected to temperatures below the optimal for symbiotic nitrogen fixation. Many studies have shown that sub-optimal temperatures affect the competitiveness of rhizobia for nodulation [1], delay root infection and inhibit nodule development and nitrogenase activity [2–5]. In general, rhizobia have a poor growth at temperatures below 10°C, but they are tolerant to 4°C [6]. The production and excretion of Nod metabolites by *Rhizobium leguminosarum* bv. trifolii are reduced by lowering the temperature [7]. Consequently, growth of legumes can be significantly reduced as reported with alfalfa [8] and soybean [9] under cold conditions in Canada. In many studies, cold-adapted rhizobia isolated from arctic or sub-arctic regions showed the capacity to improve symbiotic nitrogen fixation and yield of legumes under low temperature conditions [10,11]. Furthermore, some rhizobia isolated from the arctic legumes *Astragalus* and *Oxytropis* spp. were classified as psychrotrophs or psychrotolerant [11].

The mechanisms related to the capacity to grow at low temperature have been investigated with many species of psychrophilic, psychrotrophic and mesophilic bacteria [12]. The cold shock response is one of the well characterized mechanisms which describes the expression of specific genes producing cold shock proteins (CSPs) after an abrupt shift to low temperature. In *Escherichia coli*, the CspA (7.4 kDa) [13] has been reported to be similar (61% similarity) to the CspB (7.36 kDa) found in *Bacillus subtilis* [14]. These two proteins appear to function as a transcription enhancement factor for the expression of cold inducible genes. Many Antarctic psychrotrophic bacteria

* Corresponding author. Tel.: +1 (418) 657 7980 ext. 217; Fax: +1 (418) 648 2402; E-mail: prevostd@em.agr.ca

[1] Contribution No. 658 of Agriculture and Agri-Food Canada Research Centre at Sainte-Foy.

0168-6496/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-6496(00)00020-9

have a *csp* gene similar to *cspA* from *E. coli* [15]. Typically, other psychrotrophs such as *Lactococcus lactis* [16], *Listeria monocytogenes* [17,18], *Bacillus psychrophilus* [19] and *Aquaspirillus arcticum* [20] produced several CSPs with a range of different molecular masses. For rhizobia, a major CSP of 11.1 kDa was expressed in arctic (psychrotrophs) and temperate (mesophiles) strains belonging to different species [21]. In *Sinorhizobium meliloti*, a homologue of the gene *cspA* has been shown to encode for a novel 10.6-kDa polypeptide [22]. Bacteria can also produce cold acclimation proteins (CAPs) which are more strongly expressed during continuous growth at low temperatures than at high temperatures. In the psychrotrophic bacteria *A. arcticum* [20] and *B. psychrophilus* [19], some CAPs produced during growth at 0°C were also CSPs. The CAPs may be involved in the maintenance of metabolic functions at low temperature by replacing cold-denatured proteins, conserving membrane fluidity, acting as antifreeze proteins or as anabolic enzymes involved in their synthesis [23].

The ability of bacteria for growth at low temperature also depends on membrane fluidity and permeability which are maintained by the lipid composition and function. Low temperature induces an increase in the proportion of unsaturated fatty acids or in the degree of fatty acid unsaturation, a decrease in chain length, an increase in methyl branching, an increase in the ratio of *anteiso*-branching relative to *iso*-branching, or a combination of these changes [24]. A recent study with cyanobacteria demonstrated that cold tolerance was dependent on the unsaturation of membrane lipids by desaturase enzymes [25]. As observed with many bacterial species, the proportion of *cis*-vaccenic acid, a major unsaturated fatty acid, increased 30% by lowering the growth temperature of two strains of *R. leguminosarum* bv. viciae [26]. In rhizobia, a membrane functioning under low temperatures could favor nodulation since several symbiotic proteins (p*Sym* Nod) are membrane-associated [27]. The induction of *nodFE* gene in *R. leguminosarum* bv. viciae resulted in de novo synthesis of phospholipids with specific polyunsaturated fatty acids [28]. However, differences in unsaturated fatty acids proportions at low temperatures between psychrophiles and mesophiles have not been clearly demonstrated because studies compared different bacterial species which could have shown interspecies variations [12].

In a previous study, strains of rhizobia isolated from *Lathyrus japonicus* and *Lathyrus pratensis* indigenous to northern Quebec (Canada) were identified as *R. leguminosarum* bv. viciae and many of them showed very good growth at 5°C in comparison to temperate strains [29]. In order to elucidate the observed cold adaptation, in the present study we report on protein synthesis (CSPs and CAPs), survival to freezing, fatty acid composition and symbiotic effectiveness of strains of *R. leguminosarum* having different thermoadaptation levels.

2. Materials and methods

2.1. Bacterial strains

Strains Lj3 and Lj8 of *R. leguminosarum*, isolated from *L. japonicus* (Hudson's Bay arctic zone of Quebec, Canada) and strain Lp0610 isolated from *L. pratensis* (Val d'Or, temperate zone, Quebec, Canada) were used [29]. These strains were compared to the type strain *R. leguminosarum* bv. viciae ATCC 10004. The commercial strain 175P1 (LiphaTech) isolated from *Vicia dasycarpa* was used as a control in the symbiotic effectiveness test. Strains were maintained on yeast extract mannitol (YM) agar slants [30] at 4°C and stored at −70°C in YM containing 10% (v/v) glycerol.

2.2. Growth rates

The growth rate of each strain was monitored by measuring the total protein content [31] of the cells growing at 0, 2.5, 5, 10, 15 and 25°C. Cells were cultivated in 250-ml Erlenmeyer flasks containing 100 ml of YM broth and incubated at appropriate temperatures in water-bath shakers at 200 rpm. The mean generation times were calculated during the exponential phase.

2.3. Survival to freezing

Cells were grown in YM medium at 25°C to mid-exponential phase (optical density at 650 nm = 0.6). Aliquots of the cell cultures were diluted in 0.85% NaCl, plated onto YM agar in triplicate and incubated at 25°C for 72 h before colony forming units (cfu) were counted. One-ml aliquots of the 25°C cultures were either immediately frozen to −80°C for 24 h or incubated for 2 h at 5°C before freezing. Cells were thawed at room temperature for 10 min and the number of cfu were determined. The percentage of cells surviving freezing was calculated by dividing the number of cfu recovered after each freezing treatment by the number of cfu found at 25°C or after the 5°C incubation.

2.4. Cold shock and cold acclimation treatments

Cells were grown at 25 and 5°C (for cold acclimation) in 250-ml Erlenmeyer flasks containing 100 ml of YM broth until the mid-exponential phase (optical density at 650 nm = 0.6). For cold shock, an aliquot of 1 ml of the cell cultures grown at 25°C was transferred in a microtube and subjected to the appropriate cold shock temperature in water-bath shakers. The incubation times were 10 min for the shock at 15°C, 20 min for the shock at 10°C, 40 min for the shock at 5°C and 24 h for the shock at 0°C.

Table 1
Growth characteristics of five strains of *R. leguminosarum* bv. viciae at different temperatures

Growth temperature (°C)	Mean generation time (h)				
	Lp0610	Lj3	Lj8	ATCC 10004	175P1
25	3.3	2.5	2.1	1.8	3.0
15	4.0	3.9	4.7	4.2	10.0
5	23.4	21.5	29.2	NG[a]	30.1
2.5	39.5	38.9	48.4	NG	55.0
0	57.5	52.8	NG	NG	NG

[a]NG = no growth.

2.5. Radiolabelling of proteins

Cell samples of 200 μl were placed in microcentrifuge tubes and equilibrated for 1 min at the desired temperature. Radiolabelling was initiated by the addition of L-[^{35}S]methionine (Amersham, Canada) to a level of 370 μBq (10 μCi). Radiolabelling times were 5 min at 25°C, 10 min at 5°C and as indicated above for the shock treatments. The radiolabelling was stopped by centrifugation for 5 min at 13 800 × *g* at 4°C, after which the radioactive medium was removed and proteins from cell pellets were extracted as described by Berg et al. [32], except for the lysis buffer used [19]. Radioactivity was determined with 1 μl of the SDS-solubilized protein extract dropped on a glass-fiber filter (Whatman GF/C) and rinsed with 3 ml of cold 25% TCA (trichloroacetic acid) and 10 ml of cold 8% TCA. Filters were placed in liquid scintillation cocktail (Ready protein, Beckman) and analyzed using an LKB Rack Beta 1217 liquid scintillation spectrometer.

2.6. Polyacrylamide gel electrophoresis (PAGE) of proteins

Two-dimensional PAGE was performed as described by O'Farrell [33] using a Bio-Rad Protean IIxi system. Equal amounts of samples equivalent in protein content and TCA-precipitable radioactivity (10^5 cpm) were loaded onto the electrofocusing (2.5-mm diameter) gels prepared with 5 g of urea, 1.33 ml of acrylamide/bis acrylamide-filtered solution (28.4/1.62 g in 100 ml of distilled water), 2 ml of 10% detergent Nonidet P-40, 0.4 ml of 5–7 ampholytes (Bio-Rad), 0.1 ml of 3–10 ampholytes (Bio-Rad), 1.97 ml of distilled water, 15 μl of ammonium persulfate and 10 μl of TEMED. Gels were run in non-equilibrium mode [34] for 4 h at 350 V. Focalization gels were loaded on a linear 10–20% gradient sodium dodecyl sulfate–PAGE (SDS–PAGE) gels and electrophoresed at 10 mA for 15 h. The second dimension gels were treated for fluorography using dimethyl sulfoxide [34]. Autoradiography of the fluorograms was performed with Kodak X-OMAT-AR film exposed during 2–4 days at −80°C. Fluorograms were digitized by scanning with a densitometer (Bio-Rad Densitometer model 620) and compared with the program 2D-Analyst (Bio-Rad).

To determine the apparent isoelectric point of proteins, the first dimension electrofocusing gels were run under equilibrium conditions for 15 h at 400 V followed by 1 h at 600 V. The isofocalization gels were then loaded on a linear 10–20% SDS–PAGE as described above.

2.7. Fatty acid composition

Cells grown at 25°C, shocked from 25 to 5°C, or grown at 5°C were analyzed to determine their fatty acid composition. The extraction of lipids was carried out according to Bligh and Dyer [35] and the preparation of methyl esters of fatty acids (FAME) was carried out according to Metcalfe et al. [36]. FAME were analyzed on a Hewlett Packard 5890 II gas chromatograph equipped with a DB-225 column (30-m length, 50% cyanopropylphenyl, 50% methyl, J&W Scientific) and a flame ionization detector. The elution column temperature was 35°C followed by a linear gradient of 20°C min^{-1} up to 180°C followed by a gradient of 3.0°C min^{-1} up to 220°C. Injector and detector temperatures were 215 and 230°C, respectively. The carrier gas used was hydrogen at a flow rate of 20 ml min^{-1}. Fatty acids were identified by the comparison of retention times with commercial standards (Sigma, St. Louis, MO, USA). The identity of fatty acids was confirmed by gas chromatography–mass spectrometry using a Varian 3600 gas chromatograph coupled with a Varian VG 7070E mass spectrometer. Statistical analyses were conducted using the GLM (General Linear Models) procedure of SAS (Statistical Analysis System Institute, 1985). Homogeneity of variance was confirmed by a Bartlett test [37] and a comparison of means was done according to an LSD multiple range test [37]. The ratios of total unsaturated (*cis* type) to saturated fatty acids was determined.

2.8. Symbiotic effectiveness

Lathyrus sativus plants were grown and inoculated as described by Prévost et al. [38]. The experimental design was a randomized complete block with four replicates.

Table 2
Effect of freezing and acclimation before freezing on the viability of four strains of *R. leguminosarum* bv. viciae grown at 25°C

Strain	Viability (% of cells surviving)	
	24 h at −80°C	2 h at 5°C, 24 h at −80°C
Cold-adapted		
Lp0610	78.9 ± 4.1[a]	82.5 ± 3.5
Lj3	79.8 ± 1.6	85.8 ± 2.3
Poorly adapted		
Lj8	63.7 ± 7.4	72.6 ± 9.3
Cold-sensitive		
ATCC 10004	32.7 ± 4.5	60.3 ± 8.6

[a]Means (± S.D.) of triplicate experiments.

Plants were harvested at mid-flowering, after 34 days at 20/15°C (day/night) and after 43 days at 15/7°C. Just before harvest, intact plants were tested for nitrogenase activity by the acetylene reduction assay [39]. For each assay (1 h after the beginning of the photoperiod), the pots of inoculated treatments were each placed in a sealed Plexiglas chamber in the presence of 10% (v/v) acetylene and incubated in the growth cabinet. Gas samples (0.2 ml) were taken after 1- and 2-h incubation and analyzed for ethylene content on a Perkin-Elmer gas chromatograph equipped with a Porapak-R-column and a flame ionization detector according to the previously described procedure [39]. Plant shoots were dried at 60°C in a forced air oven for 48 h for the determination of dry matter.

Statistical analysis was performed for each temperature and means were compared by using the Duncan multiple range test [37].

3. Results

3.1. Growth characteristics

At 25°C, strains Lj8 and ATCC 10004 grew faster than the other three strains (Table 1). However, at temperatures of 15°C and lower, strains Lj3 and Lp0610 showed shorter mean generation times and they were capable of growth at 0°C. Accordingly for the purpose of this study, strains Lj3 and Lp0610 were classified as cold-adapted. Strain Lj8 and strain 175P1 (used only in the symbiotic effectiveness study) showed slower growth rates at 5°C and were capable of growth at 2.5°C. They were, thus, considered as poorly adapted. The reference strain ATCC 10004 could not grow at 5°C or below and was classified as cold-sensitive.

3.2. Survival to freezing

The viability of cells after a 24-h freezing period at −80°C was greater for the two cold-adapted strains Lj3 and Lp0610 (79%) and the poorly adapted strain Lj8 (64%) than for the cold-sensitive strain ATCC 10004 (33%) (Table 2). For all strains, an acclimation period of 2 h at 5°C before freezing increased survival to the freezing treatment. This increase varied from to 4 to 9% for the three strains Lj3, Lj8 and Lp0610. The acclimation period had the greatest effect on the cold-sensitive strain ATCC 10004 in which survival almost doubled.

3.3. CSPs and CAPs

A typical autoradiography pattern of proteins separated by 2D gel electrophoresis is presented in Fig. 1 for the cold-adapted strain Lp0610. Patterns of cells grown at 25°C (Fig. 1A) were similar to those grown at 5°C (Fig. 1C), but more proteins were observed at 25°C. Few pro-

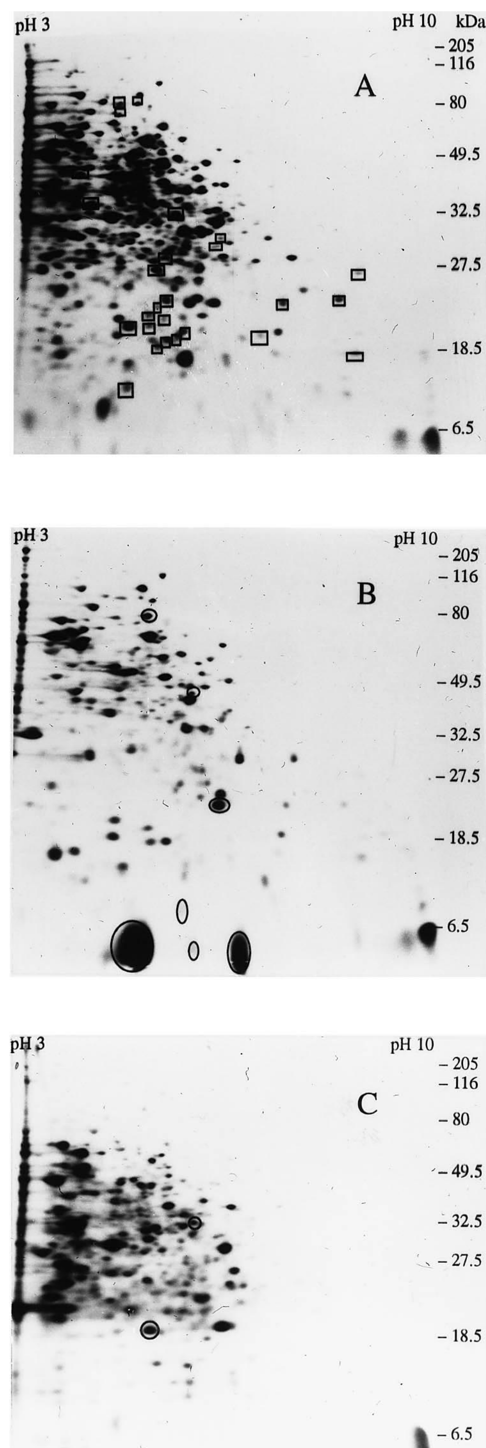

Fig. 1. Autoradiograms of the 2D polyacrylamide gels (10–20% gradient) after isoelectric focussing of the cytosolic extracts from [^{35}S]methionine-labeled cells of *R. leguminosarum* bv. viciae strain Lp0610 grown under three different treatments: (A) continuous growth at 25°C, (B) cold shock from 25 to 0°C and (C) continuous growth at 5°C. □ in (A) represents proteins which are not visible after continuous growth at 5°C, ○ in (B) and (C) represents major newly synthesized proteins not visible under continuous growth at 25°C.

Table 3
CSPs synthesized by four strains of *R. leguminosarum* bv. viciae under different shock temperatures

CSP (kDa)	Presence of CSPs														
	Lp0610				Lj3				Lj8				ATCC 10004		
	15°C	10°C	5°C	0°C	15°C	10°C	5°C	0°C	15°C	10°C	5°C	0°C	15°C	10°C	5°C
115							+	+							
90								+							
77				+			+	+		+	+	+			
58								+							
41				+								+			
23				+								+			
6.5				+				+							
6.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.9				+				+							
5.5				+		+	+	+							

teins were specifically synthesized under growth at 5°C and are likely to be CAPs. A shock from 25 to 0°C (Fig. 1B) allowed the synthesis of CSPs which were not present at 25°C.

Only one CSP (6.1 kDa) was common to all strains and at every shock temperature varying from 15 to 0°C (Table 3). The synthesis of this protein increased with the decrease of shock temperatures (results not shown). The three strains capable of growth at 5°C (Lp0610, Lj3 and Lj8) produced other CSPs from 10 to 0°C with a greater number at 0°C; beside the 6.1-kDa CSP, only one other CSP (77 kDa) was common to these strains. The two cold-adapted strains Lp0610 and Lj3 showed a higher number of proteins (seven and eight, respectively) than the poorly adapted strain Lj8 (three proteins). The cold-sensitive strain ATCC 10004 showed only the 6.1-kDa protein under all shock temperatures. This strain did not show high levels of ^{35}S incorporation at 0°C under our experimental conditions and, thus, was not further evaluated at this temperature.

The synthesis of CAPs at 5°C was observed in all three strains capable of growth at this temperature (Table 4). Their number varied from two to five depending on the strain, and only a 30-kDa protein was common to all three strains.

3.4. Fatty acid composition under cold conditions

The fatty acid profiles obtained at optimal temperature were similar among all strains, except for the presence of the minor component nonadecanoic acid (C19:0) in strains Lj8 and ATCC 10004 (Table 5). For all strains and under all tested temperatures, *cis*-vaccenic acid ($C18{:}1^{\Delta 11}$) was the major fatty acid component.

The proportions of saturated fatty acids palmitic (C16:0), stearic (C18:0) and nonadecanoic did not change after a cold shock at 5°C (Table 5) in comparison to those found at 25°C, but palmitic acid significantly decreased in strain Lj8, while stearic acid in strain Lj3 and nonadecanoic acid significantly increased in strains Lp0610 and ATCC 10004. No general trends could be observed for the changes in proportions of unsaturated fatty acids. *cis*-Vaccenic acid, the major unsaturated component, decreased in all four strains after cold shock. Oleic acid ($C18{:}1^{\Delta 9}$) and octadecenoic 11-methoxy acid ($C18{:}1^{\Delta 11}$) were found in higher proportions in strains Lp0610 and Lj3 than in strains Lj8 and ATCC1004 at 25°C but did not show any specific tendency under shock conditions.

For the three strains Lp0610, Lj3 and Lj8 grown at 5°C (Table 5), the proportions of saturated fatty acids palmitic and stearic significantly decreased. In contrast, the proportions of unsaturated fatty acids increased, except for oleic and octadecenoic 11-methoxy acids. The proportions of the major unsaturated fatty acid component, *cis*-vaccenic acid, increased by a factor of 1.5 (mean of the three strains), representing 80% of total fatty acids. A fatty acid not detected at 25°C and under cold shock condition, *cis*-12 octadecenoic acid ($C18{:}1^{\Delta 12}$), was specifically synthesized during growth at 5°C representing 8–12% of total fatty acids present in the three tested strains.

The determination of the ratio of *cis*-unsaturated over saturated fatty acid percentages is a valuable approach to compare strains. At 25°C, the ratios varied from 2.2 to 4.8 among the four strains independently of their cold adaptation characteristics. These ratios were slightly lower after a shock at 5°C (varying from 2.1 to 3.3), reflecting the decrease of *cis*-unsaturated and the increase of some saturated fatty acids. Under continuous growth at 5°C, the

Table 4
CAPs synthesized by three strains of *R. leguminosarum* bv. viciae under continuous growth at 5°C

CAP (kDa)	Presence of CAPs in strain		
	Lp0610	Lj3	Lj8
54	–	+	+
30	+	+	+
29	–	–	+
19	+	+	–
7.0	–	+	+

Table 5
Fatty acid composition (% of total fatty acids) of four *R. leguminosarum* bv. viciae strains tested under different temperature conditions

Fatty acid type	Rhizobial strain	Temperature conditions					
		continuous growth (25°C)		cold shock (25 to 5°C)		continuous growth (5°C)	
Saturated							
Palmitic acid (C16:0)	Lp0610[a]	9.74 ± 3.05	a[b]	7.60 ± 1.49	a	1.42 ± 0.87	b
	Lj3	4.47 ± 0.21	a	7.10 ± 2.05	a	0.73 ± 0.14	b
	Lj8	11.36 ± 2.58	a	7.98 ± 2.07	b	2.00 ± 0.80	c
	ATCC 10004	4.05 ± 0.67	a	4.99 ± 0.90	a		
Stearic acid (C18:0)	Lp0610	13.53 ± 2.13	a	13.56 ± 0.38	a	3.55 ± 1.21	b
	Lj3	9.94 ± 0.31	b	16.55 ± 1.33	a	1.62 ± 0.56	c
	Lj8	16.71 ± 2.22	a	18.27 ± 6.08	a	3.50 ± 1.35	b
	ATCC 10004	12.67 ± 1.99	a	12.94 ± 1.97	a		
Nonadecanoic acid (C19:0)	Lp0610	nd[c]		1.69 ± 0.41		nd	
	Lj3	nd		nd		nd	
	Lj8	0.31 ± 0.06	a	1.59 ± 1.19	a	nd	
	ATCC 10004	0.31 ± 0.08	b	1.55 ± 0.24			
Unsaturated							
trans-Elaidic acid ($C18{:}1^{\Delta 9}$)	Lp0610	0.40 ± 0.14	c	1.06 ± 0.10	b	5.07 ± 0.40	a
	Lj3	0.82 ± 0.65	a	nd		2.82 ± 0.97	a
	Lj8	0.26 ± 0.10	b	2.84 ± 0.27	a	2.50 ± 0.63	a
	ATCC 10004	0.66 ± 0.72	a	1.66 ± 0.42	a		
Oleic acid ($C18{:}1^{\Delta 9}$)	Lp0610	10.70 ± 0.84	b	14.27 ± 1.43	a	2.13 ± 1.28	c
	Lj3	10.09 ± 0.25	b	17.74 ± 2.01	a	1.04 ± 0.40	c
	Lj8	14.57 ± 1.67	a	13.70 ± 3.72	a	2.57 ± 0.96	b
	ATCC 10004	7.96 ± 1.18	a	10.23 ± 1.12	b		
cis-Vaccenic acid ($C18{:}1^{\Delta 11}$)	Lp0610	56.54 ± 2.01	b	50.03 ± 3.03	c	75.25 ± 3.15	a
	Lj3	59.30 ± 4.27	ab	49.58 ± 8.35	b	85.56 ± 13.01	a
	Lj8	48.98 ± 17.05	b	46.39 ± 8.75	b	79.27 ± 8.12	a
	ATCC 10004	64.30 ± 1.11	a	54.67 ± 3.56	b		
Octadecenoic acid, 11-methoxy ($C18{:}1^{\Delta 11}$)	Lp0610	9.09 ± 0.57	a	11.79 ± 0.82	a	nd	
	Lj3	15.39 ± 3.52	a	9.03 ± 2.17	b	nd	
	Lj8	7.82 ± 1.44	b	9.23 ± 1.97	b	nd	
	ATCC 10004	10.05 ± 0.63	b	13.96 ± 0.73	a		
cis-12 Octadecenoic acid ($C18{:}1^{\Delta 12}$)	Lp0610	nd		nd		12.58 ± 0.73	
	Lj3	nd		nd		8.23 ± 1.36	
	Lj8	nd		nd		10.17 ± 1.22	
	ATCC 10004	nd		nd			

[a]Lp0610, Lj3: cold-adapted, Lj8: poorly adapted, ATCC 10004: cold-sensitive.
[b]Means on a same line followed by the same letter are not significantly different at $P > 0.05$ according to a LSD test. ±: S.E.M.
[c]nd = not detected.

ratios varied from 17 to 40. However, these increases in ratios at 5°C were similar for the three strains (8.4, 7.5 and 6.3 times greater than ratios found at 25°C).

3.5. Symbiotic efficiency

The symbiotic efficiency of rhizobial strains was evaluated with *L. sativus* (chickling vetch), because this legume species is of agronomic value (green manure) and seeds are commercially available. In preliminary experiments, strain Lp0610 was efficient with *L. sativus* while the reference strain ATCC 10004 and the two strains Lj3 and Lj8 were not efficient. Consequently, only strain Lp0610 was tested and compared to the commercial strain 175P1.

Under both temperature regimes (Table 6), the two strains 175P1 (poorly adapted, commercial) and Lp0610 (cold-adapted) were symbiotically efficient, as observed by the red pigmentation of nodules, indicating an active nitrogenase system. At the optimal temperature regime, strain 175P1 was significantly more efficient than strain Lp0610 by producing 49% higher nitrogenase activity and 38% more shoot dry weight. At the sub-optimal temperature regime, the symbiotic efficiency of the two strains did not differ significantly, but strain Lp0610 produced 7% more shoot dry weight and 8% more nitrogenase activity than strain 175P1. *Lathyrus* plants nodulated by strain Lp0610 and those grown with nitrogen showed a higher yield at sub-optimal than at optimal temperatures while those nodulated by strain 175P1 showed a lower yield. This result may be explained by the low capacity of strain 175P1 to perform at low temperatures.

4. Discussion

From this study, the strains Lj3 (*L. japonicus*) from an

Table 6
Nitrogenase activity (ARA[a]) and shoot dry matter yield of whole plants of *L. sativus* cultivated under two temperature regimes and inoculated by two strains of *R. leguminosarum* bv. viciae

Strain	Temperature regimes			
	sub-optimal (7–15°C), 43 days growth		optimal (18–22°C), 34 days growth	
	ARA (μM C_2H_2/h/pot)	shoot dry weight (g/pot)	ARA (μM C_2H_2/h/pot)	shoot dry weight (g/pot)
175P1	39.57 a[b]	5.87 a	53.95 a	6.86 a
Lp0610	42.91 a	6.32 a	27.46 b	4.32 b
N-control		14.76		11.50

[a]ARA = acetylene reduction assay.
[b]Means within a column followed by the same letter are not significantly different at $P > 0.05$ according to the Waller–Duncan multiple range test (Bayes' LSD).

arctic zone and Lp0610 (*L. pratensis*) from a temperate northern zone of the province of Quebec (Canada) can be classified as psychrotrophs on the basis of the definition of Morita [40] because they can grow at 0°C and their optimal growth temperature is around 28°C (< 40°C for psychrotrophs). Strain Lj8 (*L. japonicus*), also originating from the arctic region cannot be classified as a psychrotroph since it does not grow at 0°C. However, this strain shows a better adaptation to cold than the two temperate strains used for comparison, ATCC 10004 and 175P1. We have observed variations in the capacity to grow at 5°C in a previous study where most isolates (29 out of 30) from *L. japonicus* showed a very good growth yield, while only a few strains (9 out of 49) from *L. pratensis* had this capacity [29]. Rhizobia isolated from the arctic legumes *Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia* indigenous to the Melville Peninsula (Canada) also showed differences in their cold adaptation on the basis of their growth yield at 5°C [38] and some isolates were classified as psychrotrophs according to their temperature growth range [21]. Strains of *R. leguminosarum* bv. trifolii isolated from clovers in different regions of Finland showed a better growth adaptation at 10°C but could not be classified as psychrotrophs since they were unable to grow at 5°C [41].

Survival after freezing at −80°C varied among strains of *R. leguminosarum* bv. viciae used in this work. The two psychrotrophs Lj3 and Lp0610 and the cold-adapted strain Lj8 showed high viability (79 and 64%, respectively) after freezing at −80°C in comparison to the cold-sensitive strain ATCC 10004 (33%). These results differ from those obtained with three strains of psychrotrophic arctic rhizobia which showed a lower survival (∼56%) after 3 h freezing at −10°C than three mesophilic temperate strains (∼70%) used for comparison [21]. In the present study, the fact that an incubation at 5°C before freezing only slightly increased the survival of cold-adapted strains and doubled that of the cold-sensitive strain suggests the presence of some intrinsic properties in cold-adapted strains which reduce freezing damage. Similarly with a cold-sensitive strain of *B. subtilis*, the 27% survival rate after freezing at −80°C was increased to 96% after a 2-h incubation at 10°C [14].

All cold shock temperatures (from 15 to 0°C) induced, in the four strains tested, a common major CSP of 6.1 kDa. Similarly, a common major CSP of 11.1 kDa was induced in three strains of arctic rhizobia and three strains of temperate rhizobia (from *Medicago*, *Onobrychis* and *Oxytropis*) having different thermoadaptation levels [21]. Other major CSPs for different bacterial species showed molecular masses varying between 7.0 kDa in *Streptomyces* [42] and 29 kDa in *Lactococcus lactis* ssp. lactis [16]. The apparent size of the 6.1-kDa CSP in the present study is comparable to that of the major CSPs, CspA (7.40 kDa), found in *E. coli*, CspA (10.6 kDa) recently characterized in *S. meliloti* [22] and CspB (7.36 kDa) reported in *B. subtilis* [14,43].

The higher number of CSPs at 0°C observed in the cold-adapted strains of this study is similar to the higher number of CSPs observed at −10°C with cold-adapted arctic rhizobia when compared to temperate rhizobia [21]. In each of these two studies, the major CSP component (6.1 kDa for *R. leguminosarum* strains and 11.1 kDa for arctic and temperate rhizobia) was common to all cold-adapted and non-adapted strains. Other CSP components common to the cold-adapted strains of *R. leguminosarum* were absent in the cold-sensitive strain while, in the study with arctic and temperate rhizobia, some CSPs were common and other were specific to each of the arctic and temperate strains [21]. The different taxonomic status of arctic strains (*Mesorhizobium* sp.) [44] and temperate strains (*Rhizobium* sp. and *S. meliloti*) may be responsible for these differences. Comparison between closely phylogenetically related rhizobia-like strains of *R. leguminosarum* used in the present study allowed the identification of CSPs that are more related to cold tolerance than to interspecies variations. In comparison to the CSPs, the CAPs are strongly synthesized during growth at low temperatures. The three strains Lp0610, Lj3 and Lj8 that could grow at 5°C synthesized two to four CAPs of low molecular mass (7.0 to 54 kDa), which differed from CSPs and were not found at 25°C. These CAPs have molecular masses identical to some CAPs observed in *Enterococcus faecalis* [45]. In previous studies with psychrophilic bacteria *A. arcticum*, *B. psychrophilus* and *Arthrobacter globiformis* some CAPs were reported to be similar to CSPs

[19,20,23], but other CAPs were specific to growth at low temperatures.

The lowering of growth temperature from 25 to 5°C affected similarly the fatty acid proportion and composition of the three strains able to grow at 5°C. The increase in the proportion of *cis*-vaccenic acid and the decrease of palmitic acid corroborate previous observations made with Gram-negative bacteria [46]. The major component for all strains, the unsaturated fatty acid *cis*-vaccenic, increased from 57% at 25°C to 80% at 5°C. Stearic and oleic acids were the other important components at 25°C. *cis*-Vaccenic and stearic acids constitute the major components in many species of *Rhizobium*, *Bradyrhizobium* [47] and other Gram-negative bacteria [46]. Furthermore, *cis*-vaccenic acid was also the major component in two strains of *R. leguminosarum* bv. viciae, increasing from 57% at 22°C to 76% at 10°C [26] and in a strain of *R. leguminosarum* bv. trifolii grown at 30°C [48]. The significant increase in the proportion of total unsaturated fatty acids with the lowering of temperature (from 80 to 96%) is also similar to the results obtained with *E. coli* [49]. These changes in fatty acid proportions are necessary for the regulation of membrane fluidity and permeability, to insure substrate uptake for growth at low temperature [50]. The unsaturated fatty acid *cis*-12 octadecenoic acid produced specifically during growth at 5°C and not detected at 25°C was also reported in two cold-sensitive (not able to grow at 7°C) strains of *R. leguminosarum* bv. viciae during growth at 15 and 10°C, but not at higher temperatures [26]. Thus, these similarities between cold-adapted and cold-sensitive strains in fatty acid profiles at low temperatures suggest that other mechanisms such as a higher activity of desaturation allow the cold-adapted *R. leguminosarum* bv. viciae strains to grow at 2.5 or 0°C. The fatty acid composition after a cold shock from 25 to 5°C differed slightly from that observed under continuous growth at 5°C. The proportions of *cis*-vaccenic acid, the major constituent in all strains, decreased from 57% at 25°C to 50% after a cold shock at 5°C, in contrast to an increase to 80% during growth at 5°C. This reduction after a cold shock may be due to the oxidative decomposition of fatty acids [51] and to the lack of de novo synthesis of fatty acids due to the short exposure of cells under cold conditions. It would be worthwhile to follow modifications in fatty acid profiles over time at 5°C in order to determine differences between cold-adapted and non-adapted strains.

It is well established that the strain of *Rhizobium* plays an important role in determining the efficiency of nitrogen fixation at low temperature [52,53]. Many studies with rhizobia from temperate regions showed a relation between cold adaptation for growth in pure cultures and symbiotic effectiveness (nodulation and nitrogenase activity) under cold conditions [41,54]. Psychrophilic arctic rhizobia in symbiosis with the temperate legume sainfoin expressed higher N_2-fixing activity and produced up to three times more shoot dry matter yield than temperate rhizobia at low temperatures and under field conditions, but were similar under optimal growth conditions [55,56]. However, the present study does not allow a clear conclusion to be drawn about the efficiency at low temperature of the cold-adapted strain of *R. leguminosarum* Lp0610 in symbiosis with *L. sativus* because its higher values for nitrogenase activity and shoot dry weight were not significantly different from those obtained with the commercial strain 175P1. This suggests that the cold adaptation of rhizobia is not always reflected in their symbiotic efficiency at low temperatures since the nitrogenase activity in plant nodules depend on properties of both partners and their interaction [11].

In conclusion, the cold adaptation mechanisms investigated in strains of *R. leguminosarum* confirm results obtained with other bacteria: low temperatures induce the synthesis of CSPs, CAPs and an increase in unsaturated fatty acids. Differences between cold-adapted and cold-sensitive *R. leguminosarum* strains were observed in the number of CSPs, but a common CSP of 6.1 kDa was induced in all strains. The fatty acid composition did not differ between strains, but the unsaturated fatty acid component detected specifically at low temperatures may be involved in the modification of the membrane fluidity. Further investigation should focus on the isolation, characterization and comparison of the CSPs and CAPs produced by psychrotrophic and mesophilic rhizobia. Identification of cold-induced genes could be performed by differential hybridization of cDNA library using cDNA probes synthesized from adapted and non-adapted rhizobial strains growing at low and optimal temperature.

Acknowledgements

We thank Dr. Paul Nadeau for critical reading of the manuscript and Léon Lambert for technical help. Financial support for this research was provided by a research operating grant from the National Sciences and Engineering Research Council of Canada (NSERC) and by the Fonds pour la formation des chercheurs et l'aide à la recherche (FCAR) Québec.

References

[1] Hardarson, G. and Jones, D.G. (1979) Effect of temperature on competition amongst strains of *Rhizobium trifolii* for nodulation of two white clover varieties. Ann. Appl. Biol. 92, 229–236.

[2] Roughley, R.J. and Date, R.A. (1986) The effect of strain of *Rhizobium* and of temperature on nodulation and early growth of *Trifolium semipilosum*. Exp. Agric. 22, 123–131.

[3] Lynch, D.H. and Smith, D.L. (1994) The effects of low root-zone temperature stress on two soybean (*Glycine max*) genotypes when combined with *Bradyrhizobium* strains of varying geographic origin. Physiol. Plant. 90, 105–113.

[4] Fyson, A. and Sprent, J.I. (1982) The development of primary root

nodules on *Vicia faba* L. grown at two temperatures. Ann. Bot. 50, 681–692.

[5] Waughman, G.J. (1977) The effect of temperature on nitrogenase activity. J. Exp. Bot. 28, 949–960.

[6] Graham, P.H. (1992) Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can. J. Microbiol. 38, 475–484.

[7] McKay, I.A. and Djordjevic, M.A. (1993) Production and excretion of nod metabolites by *Rhizobium leguminosarum* bv. trifolii are disrupted by the same environmental factors that reduce nodulation in the field. Appl. Environ. Microbiol. 59, 3385–3392.

[8] Rice, W.A. and Olsen, P.E. (1988) Root-temperature effects on competition for nodule occupancy between two *Rhizobium meliloti* strains. Biol. Fertil. Soils 6, 137–140.

[9] Lynch, D.H. and Smith, D.L. (1993) Early seedling and seasonal N_2-fixing symbiotic activity of two soybean [*Glycine max* (L.) Merr.] cultivars inoculated with *Bradyrhizobium* strains of diverse origin. Plant Soil 157, 289–303.

[10] Prévost, D. and Bromfield, E.S.P. (1991) Effect of low root temperature on symbiotic nitrogen fixation and competitive nodulation of *Onobrychis viciifolia* (sainfoin) by strains of arctic and temperate rhizobia. Biol. Fertil. Soils 12, 161–164.

[11] Prévost, D., Drouin, P. and Antoun, H. (1999) The potential use of cold-adapted rhizobia to improve symbiotic nitrogen fixation in legumes cultivated in temperate regions. In: Fundamentals and Application of Cold-adapted Organisms (Margesin, R. and Schninner, F., Eds.) pp. 161–176. Springer, Heidelberg.

[12] Russell, N.J. (1990) Cold adaptation of microorganisms. Phil. Trans. R. Soc. Lond. 326, 595–611.

[13] Goldstein, J., Pollitt, S.N. and Inouye, M. (1990) Major cold shock protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87, 283–287.

[14] Willimsky, G., Bang, H., Fischer, G. and Marahiel, M.A. (1992) Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. J. Bacteriol. 174, 6326–6335.

[15] Ray, M.K., Sitaramamma, T., Ghandhi, S. and Shivaji, S. (1994) Occurrence and expression of *cspA*, a cold shock gene, in Antarctic psychrotrophic bacteria. FEMS Microbiol. Lett. 116, 55–60.

[16] Panoff, J.-M., Legrand, S., Thammavongs, B. and Boutibonnes, P. (1994) The cold shock response in *Lactococcus lactis* subsp. *lactis*. Curr. Microbiol. 29, 213–216.

[17] Bayles, D.O., Annous, B.A. and Wilkinson, B.J. (1996) Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. 62, 1116–1119.

[18] Phan-Thanh, L. and Gormon, T. (1995) Analysis of heat and cold shock proteins in *Listeria* by two dimensional electrophoresis. Electrophoresis 16, 444–450.

[19] Whyte, L.G. and Inniss, W.E. (1992) Cold shock proteins and cold acclimation proteins in a psychrotrophic bacterium. Can. J. Microbiol. 38, 1281–1285.

[20] Roberts, M.E. and Inniss, W.E. (1992) The synthesis of cold shock proteins and cold acclimation proteins in the psychrophilic bacterium *Aquaspirillum arcticum*. Curr. Microbiol. 25, 275–278.

[21] Cloutier, J., Prévost, D., Nadeau, P. and Antoun, H. (1992) Heat and cold shock protein synthesis in arctic and temperate strains of rhizobia. Appl. Environ. Microbiol. 58, 2846–2853.

[22] O'Connell, K.P. and Thomashow, M.F. (2000) Transcriptional organization and regulation of a polycistronic cold shock operon in *Sinorhizobium meliloti* RM1021 encoding homologs of the *Escherichia coli* major cold shock gene *cspA* and ribosomal protein gene *rpsU*. Appl. Environ. Microbiol. 66, 392–400.

[23] Berger, F., Morellet, N., Menu, F. and Potier, P. (1996) Cold shock and cold acclimation proteins in the psychrotrophic bacterium *Arthrobacter globiformis* SI55. J. Bacteriol. 178, 2999–3007.

[24] Russell, N.J. (1984) Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends Biochem. Sci. 9, 108–112.

[25] Murata, N. and Wada, H. (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. Biochem. J. 308, 1–8.

[26] Théberge, M.-C., Prévost, D. and Chalifour, F.-P. (1996) The effect of different temperatures on the fatty acid composition of *Rhizobium leguminosarum* bv. viciae in the faba bean symbiosis. New Phytol. 134, 657–664.

[27] Dénarié, J., Debellé, F. and Rosenberg, C. (1992) Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46, 497–531.

[28] Geiger, O., Spaink, H.P. and Lugtenberg, B.J.J. (1993) Biosynthesis of lipo-oligosaccharides: phospholipids of *Rhizobium* contain *nodE*-determined highly unsaturated fatty acid moieties. In: New Horizons in Nitrogen Fixation (Palacios R., Mora, J. and Newton, W.E.), p. 233. Kluwer Academic Publishers, Dordrecht.

[29] Drouin, P., Prévost, D. and Antoun, H. (1996) Classification of bacteria nodulating *Lathyrus japonicus* and *Lathyrus pratensis* in northern Quebec as strains of *Rhizobium leguminosarum* biovar viciae. Int. J. Syst. Bacteriol. 46, 1016–1024.

[30] Vincent, J.M. (1970) A manual for the practical study of root nodules. IBP Handbook 15, p. 164. Blackwell Scientific Publications, Oxford.

[31] Daniels, L., Hanson, R.S. and Phillips, J.A. (1994) Chemicals analysis. In: Methods for General and Molecular Bacteriology (Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R.), p. 542. American Society for Microbiology, Washington, DC.

[32] Berg, G.R., Inniss, W.E. and Heikkila, J.J. (1987) Stress proteins and thermotolerance in psychrotrophic yeast from arctic environments. Can. J. Microbiol. 33, 383–389.

[33] O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007–4021.

[34] Dunbar, B.S. (1987) Two-dimensional Electrophoresis and Immunological Techniques, p. 372. Plenum Press, New York.

[35] Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37, 911–917.

[36] Metcalfe, L.D., Schimitz, A.A. and Pelka, J.R. (1966) Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38, 415–515.

[37] Steel, R.G.D. and Torrie, J.H. (1980) Principles and Procedures of Statistics: a Biometrical Approach, 2nd edn. McGraw-Hill, New York.

[38] Prévost, D., Bordeleau, L.M., Caudry-Reznick, S., Schulman, H.M. and Antoun, H. (1987) Characteristics of rhizobia isolated from three legumes indigenous to the Canadian high arctic: *Astragalus alpinus, Oxytropis maydelliana*, and *Oxytropis arctobia*. Plant Soil 98, 313–324.

[39] Turner, G.L. and Gibson, J.A. (1981) Measurement of nitrogen fixation by indirect means. In: Method for Evaluating Biological Nitrogen Fixation (Bergerson, F.J.), pp. 111–139. Wiley, New York.

[40] Morita, R.Y. (1975) Psychrophilic bacteria. Bacteriol. Rev. 29, 144–167.

[41] Lipsanen, P. and Lindström, K. (1986) Adaptation of red clover rhizobia to low temperatures. Plant Soil 92, 55–62.

[42] Av-Gay, Y., Aharoniwitz, Y. and Cohen, G. (1992) *Streptomyces* contain a 7.0 kDa cold shock like protein. Nucleic Acid Res. 20, 5478.

[43] Jones, P.G., Van Bogelen, R.A. and Neidhardt, F.C. (1987) Induction of proteins in response to low temperature in *Escherichia coli*. J. Bacteriol. 169, 2092–2095.

[44] Laguerre, G., van Berkum, P., Amarger, N. and Prévost, D. (1997) Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis* and *Onobrychis*. Appl. Environ. Microbiol. 63, 4748–4758.

[45] Panoff, J.-M., Corroler, D., Thammavongs, B. and Boutibonnes, P. (1997) Differentiation between cold shock proteins and cold acclimation proteins in a mesophilic Gram-positive bacterium, *Enterococcus faecalis* JH2-2. J. Bacteriol. 179, 4451–4454.

[46] Suutari, M. and Laakso, S. (1994) Microbial fatty acids and thermal adaptation. Crit. Rev. Microbiol. 20, 285–328.

[47] MacKenzie, S.L., Lapp, M.S. and Child, J.J. (1979) Fatty acid composition of *Rhizobium* spp. Can. J. Microbiol. 25, 68–74.

[48] Orgambide, G.G., Huang, Z.-H., Douglas, A.G. and Dazzo, F.B. (1993) Phospholipid and fatty acid compositions of *Rhizobium leguminosarum* biovar *trifolii* ANU843 in relation to flavone-activated pSym gene expression. Lipids 28, 975–979.

[49] Marr, A.G. and Ingraham, J.L. (1962) Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. 84, 1260–1267.

[50] Fukunaga, N. and Russell, N.J. (1990) Membrane lipid composition and glucose uptake in two psychrotolerant bacteria from Antarctica. J. Gen. Microbiol. 136, 1669–1673.

[51] Wada, M., Fukunaga, N. and Sasaki, S. (1989) Mechanism of biosynthesis of unsaturated fatty acids in *Pseudomonas* sp. strain E-3, a psychrotrophic bacterium. J. Bacteriol. 171, 4267–4271.

[52] Pankhurst, C.E. and Layzell, D.B. (1984) The effect of bacterial strain and temperature changes on the nitrogenase activity of *Lotus pedunculatus* root nodules. Physiol. Plant. 62, 404–409.

[53] Layzell, D.B., Rochman, P. and Canvin, D.T. (1984) Low root temperature and nitrogenase activity in soybean. Can. J. Bot. 62, 965–971.

[54] Ek-Jandér, J. and Fåhraeus, G. (1971) Adaptation of *Rhizobium* to sub-arctic environment in Scandinavia. Plant Soil special volume, 139–137.

[55] Prévost, D., Antoun, H. and Bordeleau, L.M. (1987) Effects of low temperatures on nitrogenase activity in sainfoin (*Onobrychis viciifolia*) nodulated by arctic rhizobia. FEMS Microbiol. Ecol. 45, 205–210.

[56] Prévost, D., Bordeleau, L.M., Michaud, R., Lafrenière, C., Waddington, J. and Biederbeck, V. (1994) Nitrogen fixation efficiency of cold-adapted rhizobia in sainfoin (*Onobrychis viciifolia*): laboratory and field evaluation. In: Symbiotic Nitrogen Fixation (Graham, P. H., Sadowsky, M.J. and Vance, C.P.), pp. 171–176. Kluwer Academic Publication, Dordrecht.