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ORIGINAL ARTICLE

Monounsaturated fatty acid aerobic synthesis in *Bradyrhizobium* TAL1000 peanut-nodulating is affected by temperature

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Abstract

Aims: The aim of this work was to clarify the mechanism of monounsaturated fatty acid (MUFA) synthesis in *Bradyrhizobium* TAL1000 and the effect of high temperature on this process.

Methods and Results: *Bradyrhizobium* TAL1000 was exposed to a high growth temperature and heat shock, and fatty acid composition and synthesis were tested. To determine the presence of a possible desaturase, a gene was identify and overexpressed in *Escherichia coli*. The desaturase expression was detected by RT-PCR and Western blotting. In *B.* TAL1000, an aerobic mechanism for MUFA synthesis was detected. Desaturation was decreased by high growth temperature and by heat shock. Two hours of exposure to 37°C were required for the change in MUFA levels. A potential Δ9 desaturase gene was identified and successfully expressed in *E. coli*. A high growth temperature and not heat shock reduced transcript and protein desaturase levels in rhizobial strain.

Conclusions: In *B.* TAL1000, the anaerobic MUFA biosynthetic pathway is supplemented by an aerobic mechanism mediated by desaturase and is down-regulated by temperature to maintain membrane fluidity under stressful conditions.

Significance and Impact of the Study: This knowledge will be useful for developing strategies to improve a sustainable practice of this bacterium under stress and to enhance the bioprocess for the inoculants' manufacture.

Introduction

Several organisms such as bacteria can encounter a wide range of environments and must adapt to new conditions to survive. Temperature is one of the main environmental factors that bacteria must withstand. High-temperature stresses are known to cause protein aggregation and denaturation, and to cope with these stresses, a cellular response occurs (Hongsthong *et al.* 2009). The plasma membrane plays a key role in cell viability as a selective barrier between living cells and their environment. The barrier function of the cytoplasmic membrane is known to depend critically on the physical state of lipid bilayers (Cronan *et al.* 1987), making it susceptible to changes in environmental temperature. In fact, normal cell function requires membrane lipid bilayers that are largely fluid; indeed, the bilayers of most organisms are entirely or mostly fluid at physiological temperatures.

Unsaturated fatty acids (UFA) have an important effect on the fluidity and function of biological membranes (Aguilar and de Mendoza 2006; Li *et al.* 2009). In addition to their biophysical characteristics, UFA play critical roles in many biological systems. For example, monounsaturated fatty acids (MUFA), which contain one double bond in an acyl chain, play a significant role in the control of metabolism and can serve as mediators in signal transduction (Dobrzyn and Ntambi 2005).

Temperature-dependent changes in the ratio of unsaturated to saturated fatty acids (FA) in membrane lipids are considered one of the regulatory mechanisms that

maintain membrane fluidity (Shivaji and Prakash 2010). Increased incorporation of UFA decreases the melting temperature of membrane phospholipids, whereas increased incorporation of saturated FA has the opposite effect (Cronan and Rock 1996).

In bacteria, two mechanisms may be responsible for the synthesis of UFA, one anaerobic and one aerobic. The anaerobic pathway, elucidated in detail for *Escherichia coli*, produces *cis*-vaccenic (18:1Δ11) FA by a specific 2,3-dehydratase acting on C-10 synthesized by FA synthase (FAS) type II (Magnuson *et al.* 1993). In addition, in certain bacteria, the introduction of double bonds into the FA may occur by a different mechanism that is mediated by desaturases. This reaction is catalysed by oxygen-dependent desaturation of the full-length FA chain and requires a specific electron transport chain (Shanklin and Cahoon 1998). FA desaturases play an important role in maintaining UFA homoeostasis in many organisms during temperature changes via feedback regulation (Aguilar and de Mendoza 2006). Two desaturases have been described in *Pseudomonas aeruginosa*. These enzymes modify existing membrane phospholipid-associated FA and produce UFA from exogenous saturated FA, supplementing the anaerobic mechanism. One of these enzymes, acyl-CoA desaturase, is regulated by growth temperature (Zhu *et al.* 2006). In addition, *Bacillus subtilis* controls membrane fluidity through the induction of an acyl-lipid desaturase that is regulated by temperature changes (Aguilar *et al.* 1998).

During the summer season, the soils often experience a high temperature, which influences crop yields. Peanut (*Arachis hypogaea* L.) is an important legume in Córdoba Province that provides food for direct human consumption and is used in several food products. Peanut plays a significant role in the economies of many countries, including Argentina. Legumes, as peanut, are usually nodulated by indigenous root-nodule bacteria (rhizobia), and this process is usually assumed to be adequate. However, high (not extreme) soil temperatures will affect the survival of rhizobial populations; will delay nodulation, affects root hair infection, bacteroid differentiation, nodule structure and the functioning (Zahran 1999). All of the environmental factors that influence rhizobial survival and possibly growth will affect the size of the indigenous population as well as the adaptation and viability of the inoculant cells and consequently also determine nodule occupancy (Vlassak and Vanderleyden 1997).

The survival of rhizobia under stressful conditions is often determined by their capacity to adapt by altering the composition of the lipid bilayer of cell membranes, which regulate or integrate many vital processes. The primary lipid components of the bilayer in rhizobia peanut-nodulating are phospholipids (PL), of which FA are major components and play key roles. Recently, we reported the important role of FA in the adaptive response to high growth temperature in two strains of peanut-nodulating rhizobia, which mainly synthesize unsaturated FA 18 : 1 (Paulucci *et al.* 2011). Furthermore, the anaerobic pathway is the only mechanism of FA synthesis that has been described to date in rhizobia (Lopez-Lara and Geiger 2000).

The FA composition profiles of several rhizobia have been determined but have mainly been used for chemotaxonomic purposes (Tighe *et al.* 2000). Some studies describe the participation of FA in the adaptive response to changing environmental conditions (Théberge *et al.* 1996; Boumahdi *et al.* 1999; Drouin *et al.* 2000). However, little is known about the presence of an aerobic mechanism of FA synthesis and how both growth and shock temperature influence FA synthesis. In addition, rhizobial nucleotide sequences with similarity to different putative FA desaturase genes have been submitted to public databases, but their functions have not been demonstrated experimentally.

Therefore, the aim of the present work was to clarify the mechanism of MUFA synthesis in *Bradyrhizobium* TAL1000 peanut nodulating using exogenous substrates and to examine the effect of high temperature on this process. Furthermore, we sought to determine the presence of a desaturase gene in this bacterium.

The possibility that desaturation may be used as a complementary pathway for MUFA synthesis by rhizobial strains would be of relevance to the study of adaptations to temperature change. Because this micro-organism would be used in major inoculant industry in Argentina and must be stable at room temperature for long periods to obtain a high-quality production for export, such a finding would furthermore be of great practical importance.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and oligonucleotide sequences used in this study are listed in Table 1.

Bradyrhizobium TAL1000 was cultured at 28°C or at 37°C in B^- medium for 24 h for growth studies (Spaink *et al.* 1992). For heat shock experiments, *B.* TAL1000 was grown for 16 h at 28°C and shifted to 37°C for 2 h.

Escherichia coli DH5α was used for all routine DNA manipulations and cloning procedures. *Escherichia coli* BL21(DE3)pLysS was used as a host for the pET17b plasmid containing the putative desaturase gene from *B.* TAL1000. The recombinant *E. coli* strains were grown in Luria–Bertani (LB) broth containing the required antibiotics at 37°C.

Table 1 Strains, bacterial plasmids and oligonucleotide sequences used in this study

Strains, plasmids and oligonucleotides	Description	Source or reference
Plasmids		
pET	pET17b vector expression	EMD Biosciences
pET-*des*	pET17b harbouring a putative desaturase gene from *B.* TAL1000	This study
Rhizobial strain		
Bradyrhizobium sp. TAL1000	Rhizobial symbiont strain of *Arachis hypogaea*	NifTAL Microbiological Resource Center (USA)
E. coli strains		
E. coli BL21(DE3)pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) pLysS(cm^R)	(Davanloo *et al.* 1984)
E. coli pET	*E. coli* BL21(DE3)pLysS harbouring pET plasmid	This study
E. coli pET-des	*E. coli* BL21(DE3)pLysS harbouring pET-*des* plasmid	This study
Oligonucleotide primers		
DES1 Forward	TGCGGCCACGGCTCGTTTTTC	This study
DES1 Reverse	AGTACTTCCGGCAGACGGTAG	This study
DES2 Forward	ATGAGCGCACATGTCTATCCA	This study
DES2 Reverse	TTACTTCGTCTTTCGTCCACC	This study
DES3 Forward	GCGGCCGCATGAGCGCACA NotI	This study
DES3 Reverse	CTCGCGTTACTTCGTCTTTCGT XhoI	This study

Incorporation of radioactive substrates

A total of 0·5 μCi of [1-^{14}C]palmitic (16 : 0) or [1-^{14}C] stearic (18 : 0) was added to 25 ml of culture at the time of inoculation. The cultures of rhizobial and transformed *E. coli* strains were incubated at the appropriate temperature with shaking for the indicated length of time (24 h for *B.* TAL1000). The cells were then harvested by centrifugation at 6000 ***g*** for 10 min at 4°C. Pellets were washed twice with 0·9% NaCl and used for further studies.

Lipid extraction

Lipids were extracted from washed bacteria with chloroform/methanol/water (Bligh and Dyer 1959). The lower phase containing lipids was dried under N_2 and dissolved in an appropriate volume of chloroform/methanol (2: 1, by vol).

Separation and quantification of radioactive fatty acids based on the degree of unsaturation

Fatty acid methyl esters (FAME) were prepared from total lipid extracts with 10% BF_3 in methanol (Morrison and Smith 1964) and resolved according to the number of double bonds on TLC plates impregnated with $AgNO_3$ (10%, w/v) using hexane/ethyl ether/acetic acid (94: 4: 2, by vol) as the solvent. FAME bands were detected under UV light after spraying the plates with dichlorofluorescein, elution (Henderson and Tocher 1992) and drying in counting vials. A volume of 3 ml of Optiphase Hisafe 2 (PerkinElmer, Waltham, MA, USA) was added to each vial, and radioactivity was measured using a liquid scintillation counter (Beckman LS 60001 C, Brea, CA, USA) (Kates 1972).

Analysis of fatty acids by GLC

Fatty acid methyl esters from *B.* TAL1000, recombinant *E. coli* cells and recombinant *E. coli* supplemented with 18 : 0 prepared as above were analysed using a Hewlett Packard 5890 II gas chromatograph equipped with a column as described in Paulucci *et al.* (2011). Alternatively, a highly polar column, HP 88, of cyanopropyl (length, 60 m; inner diameter, 0·25 mm; film thickness, 0·2 μm) and a flame ionization detector was used. Gas chromatograph conditions were as follows: injector temperature, 250°C; detector temperature, 300°C; and nitrogen as carrier gas. The temperature was programmed at 120°C for 1 min and then increased by 10°C min^{-1} to 175°C for 10 min, 5°C min^{-1} to 210°C for 5 min and 5°C min^{-1} to 230°C for 5 min. The peak areas of carboxylic acids in total ions were used to determine relative quantities. Fatty acids were identified by comparing retention times to commercial standards (Sigma Chemical Co., St. Louis, MO, USA).

Amplification and sequencing of a putative desaturase gene of *B.* TAL1000

Genomic DNA from *B.* TAL1000 was obtained using the JETFLEX Genomic DNA Purification Kit. Two primers, DES1 forward and DES1 reverse (Table 1), were designed

according to the sequences of putative desaturase genes of diverse rhizobial strains (GenBank accession Nº AAK64726 (*E. meliloti* 1021), YP467975 (*Rhizobium etli* CFN) and BAB50163 (*Mesorhizobium loti*).

PCR amplification was performed in a total volume of 25 μl containing 1 μl of genomic DNA, 1 μl of Taq polymerase (Promega), 2·5 μl of 10× Taq buffer, 1·5 μl of $MgCl_2$ (50 mmol l^{-1}), 2 μl of dNTP (10 mmol l^{-1} each) and 5 μl of primers (5 μmol l^{-1} each) using the following procedure: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 59°C for 40 s and 72°C for 1 min and a final extension at 72°C for 10 min.

The amplified 750-bp PCR fragment was sequenced after agarose gel purification.

Subsequently, two primers, DES2 forward and DES2 reverse (Table 1), were designed based on the sequence of the PCR fragment according to the sequence of the putative desaturase gene of *E. meliloti* 1021 (GenBank accession Nº AAK64726). The PCR mixture and amplification conditions were the same as described above with the exception of the annealing temperature (62°C). The amplified PCR fragment was purified by gel extraction and sequenced. A nucleotide sequence containing 1023 bp of a putative desaturase gene from *B.* TAL1000 was obtained and submitted to the GenBank database (accession number: JF966782).

Cloning and expression of a putative desaturase gene of *B.* TAL1000 in *Escherichia coli*

Two PCR primers, DES3 forward and DES3 reverse (Table 1), were designed to amplify the nucleotide sequences of the gene encoding the putative desaturase protein of *B.* TAL1000 (DES). Restriction sites (NotI and XhoI) were added at the 5′ end of each primer for cloning purposes (underlined). A gene was amplified by PCR. PCR amplification was performed in a total volume of 25 μl containing 1 μl of template DNA, 0·2 μl of *Pfu* DNA polymerase (3 U μl^{-1}) Promega), 2·5 μl of *Pfu* DNA polymerase 10× buffer, 0·5 μl of dNTP (10 mmol l^{-1} each) and 2·5 μl of primers (5 μmol l^{-1}) using the following procedure: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 62°C for 30 s and 72°C for 4 min and a final extension at 72°C for 5 min.

The resulting products were cloned into the pET17b expression vector. The expression construct for the putative gene (listed in Table 1) was used to transform *E. coli* BL21(DE3)pLysS using the method of Chung *et al.* (1989). Positive, ampicillin-resistant transformants screened on LB agar plates were further validated by PCR and subsequent sequencing.

Escherichia coli BL21(DE3)pLysS clone was subsequently used in overexpression experiments. Cells were grown overnight at 37°C with shaking in LB medium (20 ml) supplemented with ampicillin (100 mg ml^{-1}). Culture aliquots were used to inoculate fresh media and were grown at 37°C to an OD_{600} of 0·3–0·5. To induce gene expression, 0·1 mmol l^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures, which were grown for a further 12 h prior to harvesting. Control experiments were performed under the same experimental conditions using *E. coli* transformants containing the empty plasmid pET17b. For FA composition experiments and to test the activity of the overexpressed product on 18 : 0, cultures were supplemented with 400 μmol l^{-1} 18 : 0 at inoculation and collected after 12 h of induction.

Gel electrophoresis of recombinant protein of *B.* TAL1000

Recombinant *E. coli* cells were grown as described above. Cells pelleted from 50 ml cultures were resuspended in 15 ml of lysis buffer containing 20 mmol l^{-1} Tris-HCl pH 8, 300 mmol l^{-1} NaCl, 20 mmol l^{-1} imidazole, 0·1% Tween, 10% glycerol and 20 mmol l^{-1} β-mercaptoethanol. The suspension was supplemented with 1 μg ml^{-1} DNase and 0·5% PMSF, and cells were disrupted by ultrasonication. The homogenate was centrifuged at 29 000 ***g*** for 30 min at 4°C. The proteins contained in the supernatant were analysed by SDS-PAGE on a 10% polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

RT-PCR

Total RNA (1 μg) from cells *B.* TAL1000 grown at 28°C, grown at 16 h and transferred at 2 h at 37 and 37°C for 24 h was prepared with Nucleospin RNA kit (Promega, Madison, WI, USA) and used in a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) according to the manufacturer protocol (Access RT-PCR System kit of Promega). The conditions were 45°C for 45 min followed by 1 cycle at 94°C for 2 min, 40 cycles at 94°C for 30 s, 60°C for 1 min and 68°C for 2 min and a final cycle at 68°C for 7 min.

The specific primers for desaturase gene used were CTGGCAATATCGGCATCCATC and TTACTTCGTCT TTCGTCCACC. PCR products were analysed on a 2% agarose gel and visualized with ethidium bromide.

Western blotting

The expression of the putative desaturase protein of *B.* TAL1000 was estimated by Western blotting. Membrane protein fractions from cells of *B.* TAL1000 growing at 28 and 37°C were obtained using the method of Mizuno and Kageyama (1979) and analysed on a 12% SDS-PAGE. The bands were transferred to a nitrocellulose membrane

using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) at a constant voltage of 100 V for 1 h at 4°C. Blotting and detection were performed with a primary antibody against rat Δ9 desaturase, which was kindly provided by Dr. Omar Rimoldi, INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata-Argentina) and the Immun-Blot Assay Kit (Bio-Rad).

Statistical analyses

Data were compared by a one-way analysis of variance (ANOVA) test.

Results

Exogenous fatty acid desaturation and effect of high growth temperature on *B.* TAL1000

Figure 1 shows the distribution of radioactivity among different FA fractions after incorporation of radioactive FA into *B.* TAL1000 cells grown at 28°C (Fig. 1a) and 37°C (Fig. 1b). When [1-^{14}C]16 : 0 was added to *B.* TAL1000 cultures grown at 28°C, a considerable amount of radioactivity was incorporated into the monounsaturated FA fraction (67%). When [1-^{14}C]18 : 0 was added to the cell cultures grown at 28°C, less radioactivity was recovered in the monounsaturated FA fraction (47·2%).

When *B.* TAL1000 was grown at 37°C and [1-^{14}C] 16 : 0 was used as a substrate, monounsaturated FA fraction labelling decreased 56% relative to 28°C. When [1-^{14}C]18 : 0 was used as a substrate, a 21% lower level of radioactivity was recovered from the monounsaturated FA fraction at 37°C compared with 28°C.

Effect of heat shock on fatty acid composition and on the desaturation of exogenous substrates in *B.* TAL1000

A heat shock study was performed to determine the time required by the cells to alter both their FA composition and the desaturation of exogenous substrates.

Table 2 demonstrates that 18:1Δ11 FA decreased from 54·30 to 30·06% and 18:1Δ9 from 4·55 to 2·10, whereas 18 : 0 FA increased from 16·63 to 23·53%. Moreover, 19 : 0 cyclopropane and eicosatrienoic (20 : 3) FA increased from 3·92 to 11·99% and from 8·72 to 21·62%, respectively, as a result of heat shock.

These changes mainly affected the degree of unsaturation, which modified the U/S (sum of unsaturated to sum of saturated FA) ratio from 2·77 to 1·27. This difference represents 63% of the change that occurs when cells grow at 37°C for 24 h.

Figure 2 shows that heat shock (37°C) for 2 h provoked changes in the desaturation of radioactive FA 16 : 0 (Fig. 2a) and 18 : 0 (Fig. 2b). A decrease of 15 and 32% in radioactive recuperation in the monounsaturated fraction was observed when 18 : 0 or 16 : 0 was the substrate, respectively.

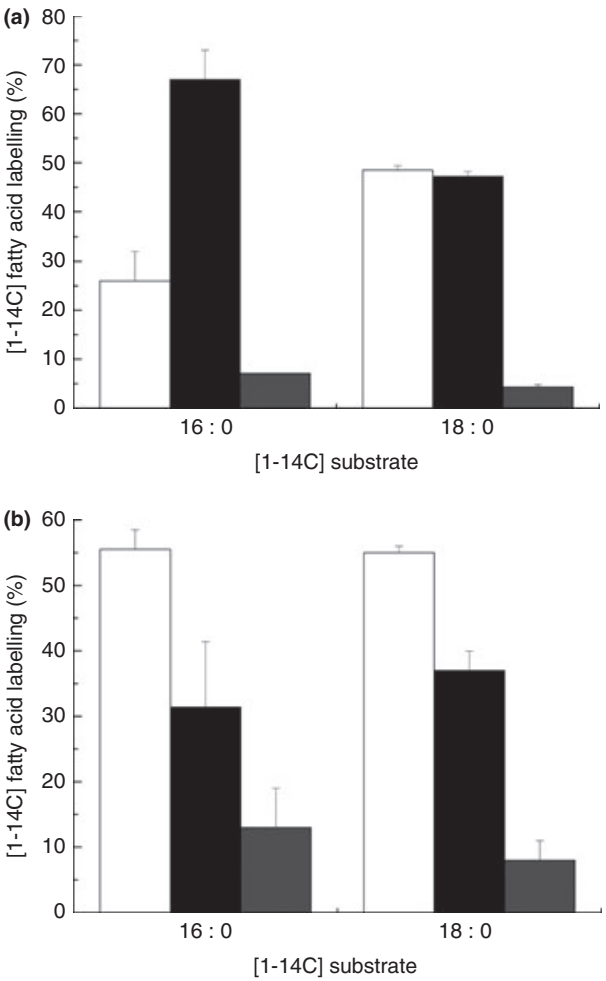

Figure 1 Desaturation of fatty acids measured in *Bradyrhizobium* TAL1000. [1-^{14}C]16 : 0 or [1-^{14}C]18 : 0 FA were added to *B.* TAL1000 cell cultures and incubated at 28°C (a) or 37°C (b) until the late exponential phase. Total lipids were extracted, methanolysed and separated according to the degree of unsaturation using TLC plates impregnated with 10% $AgNO_3$. The results are expressed as percentage of total radioactivity incorporated into each FA fraction. Values represent means ± SEM from three independent experiments. (▭) Saturated; (▬) Monounsaturated and (▬) Polyunsaturated.

Isolation and sequence analysis of the *B.* TAL1000 desaturase gene

A DNA fragment of approximately 750 bp in length was amplified from *B.* TAL1000 using the DES1 pair of primers (Table 1). The amplified DNA fragment showed a very high sequence identity (99%) to a putative desaturase gene from *E. meliloti* 1021.

Table 2 Effects of heat shock on fatty acid composition of *Bradyrhizobium* TAL1000

Fatty acid (%)		
	0 h	2 h
16 : 0	6·01 ± 0·4	8·56 ± 0·8
16 : 1	5·93 ± 0·5	2·14 ± 0·2
18 : 0	16·63 ± 0·7	23·53 ± 3·5
18:1 Δ11	54·30 ± 4·0	30·06 ± 6·0
18:1 Δ9	4·55 ± 0·6	2·10 ± 0·2
19 : 0	3·92 ± 0·1	11·99 ± 1·2
20 : 3	8·72 ± 1·1	21·62 ± 1·3
U/S*	2·77	1·27

Bradyrhizobium TAL1000 cells were cultivated 16 h at 28°C, and the cultures were transferred to 37°C (0 h) for 2 h for heat shock (2 h). FAME were obtained from total lipids and analysed by GC. The percentage of each FA is relative to total FA, defined as 100%.
*Ratio between sum of unsaturated and sum of saturated FA.
Values represent means ± SEM of three independent experiments.

Therefore, another pair of primers (DES2) was designed from the full-length gene sequence of the putative desaturase gene of *E. meliloti* 1021. The nucleotide sequence that was acquired contained a 1023 bp. A BLAST search revealed that the primary structure of this putative desaturase was highly similar to that of *E. meliloti* 1021 (GenBank accession no. AAK64726). This sequence, encoding a putative Δ12 FA desaturase-like protein, was designated *PhFAD12* [National Center for Biotechnology Information (NCBI)]. The obtained sequence of *B.* TAL1000 was aligned with different desaturase sequences and showed the histidine-rich motifs characteristics, which are highly conserved among membrane bond desaturases and proposed to form the potential diiron active site (Fig. 3).

Functional expression of *des* TAL in *Escherichia coli*

To determine the function of the putative desaturase proteins of *B.* TAL1000, the gene amplified with oligonucleotides DES3 (*des*) was cloned into the pET17b vector and expressed in *E. coli* BL21(DE3)pLysS.

Figure 4 shows the results of SDS-PAGE analysis of the total proteins from *E. coli* cells transformed with the pET-*des* plasmid compared with cells transformed with empty pET. Induction of the *des* gene with IPTG resulted in the synthesis of a polypeptide with an apparent molecular mass of 50 kDa as determined by SDS-PAGE.

In Fig. 5, the distribution of radioactivity among different FA fractions after the incorporation of radioactive FA [1-^{14}C]16 : 0 (a) and [1-^{14}C]18 : 0 (b) into *E. coli* pET and *E. coli* pET-des cells is shown. When [1-^{14}C] 16 : 0 was added to the cultures (Fig. 5a) of strains transformed with the putative desaturase, a considerable quantity of radioactivity was recovered from the monounsaturated FA fraction (18·3%) compared with the *E. coli* strain harbouring empty plasmid (1·3%). When [1-^{14}C]18 : 0 was added to the cultures (Fig. 5b), we also found a significant amount of radioactivity in the monounsaturated FA fraction (12·7%) compared with the control strain (1·6%). A lower level of radioactivity was recovered from the monounsaturated FA fraction when [1-^{14}C]18 : 0 was used as substrate compared with [1-^{14}C]16 : 0.

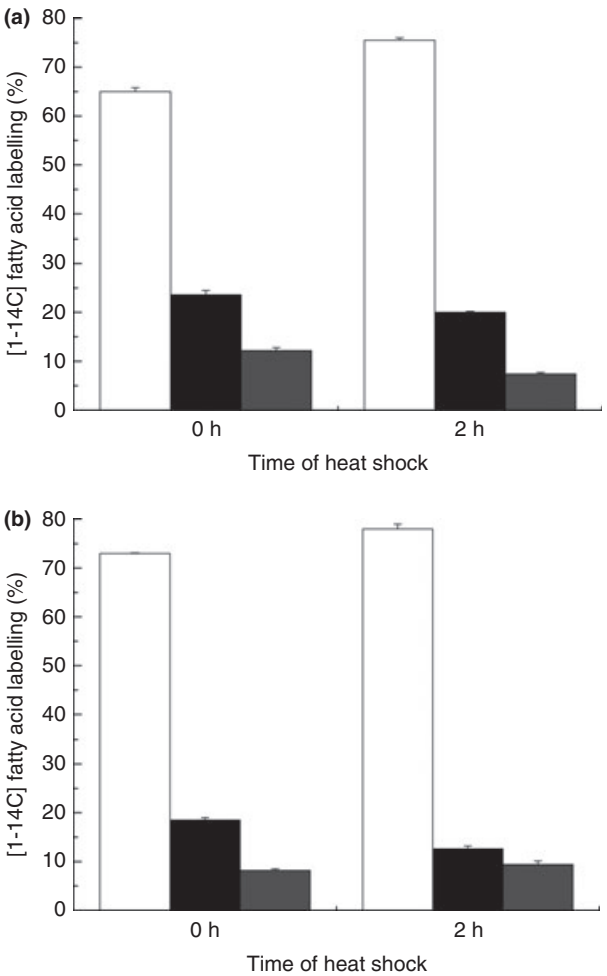

Figure 2 Effect of heat shock on the desaturation of exogenous substrates in *Bradyrhizobium* TAL1000. *B.* TAL1000 cells were cultivated 16 h at 28°C, and the cultures were transferred to 37°C (0 h) for 2 h for heat shock (2 h). Radioactive substrates 16 : 0 (a) or 18 : 0 (b) were added. Fatty acid methyl esters were obtained from total lipids and by TLC plates impregnated with 10% $AgNO_3$. The results are expressed as the percentage of total radioactivity incorporated into each FA fraction. [a]Ratio between sum of unsaturated and sum of saturated fatty acids. Values represent means ± SEM of three independent experiments. (▭) Saturated; (▬) Monounsaturated and (▬) Polyunsaturated.

Figure 3 Multiple alignments of amino acid sequences of putative desaturase from *Bradyrhizobium* TAL 1000 (GenBank accession no AEQ32060) with different putative desaturase. *Bradyrhizobium japonicum* USDA 110 GenBank accession no. NP768887, *Sinorhizobium meliloti* 1021 GenBank accession no. AAK64726, *Bradyrhizobium elkanii* GenBank accession no. BAB55901 and *Bacillus subtilis* GenBank accession no. AAB84436, using ClustalW and Boxshade. Three conserved histidine clusters (one of HXXXH and two HXXHH) are indicated by the boxes numbered 1, 2 and 3.

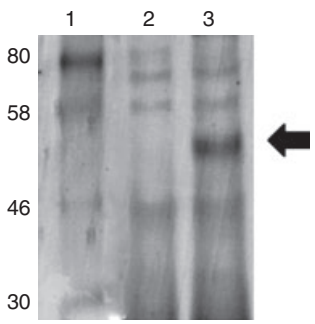

Figure 4 SDS-PAGE analysis of recombinant protein expressed in *Escherichia coli* after induction with IPTG. IPTG (0·1 mmol l^{-1}) was added to the growth medium for the induction of *des,* and the culture was incubated at 37°C for 12 h prior to analysis of cellular proteins. Lane 1, prestained protein molecular mass marker; lane 2, total protein from *E. coli* pET and lane 3, total protein from *E. coli* pET-*des*. The arrow indicates recombinant protein.

Because the radioactive substrates 16 : 0 and 18 : 0 were desaturated by the transformed *E. coli* strains, we analysed the FA composition. After 12 h of IPTG induction, the cultures of transformed *E. coli* strains were collected and their FA compositions were analysed by GC (Table 3). Myristic acid (14 : 0), 16 : 0, palmitoleic (16:1Δ9) and 18:1Δ11 were the main FA constituents found in *E. coli* pET. The changes in the pattern of 16 : 0 content between the pET-des transformant and the control strain indicate that the desaturase can use 16 : 0 as substrate. In the recombinant strain, 16 : 0 levels decreased from 56·9 to 37·4%, whereas the 16:1Δ9 level increased from 9·4 to 19·2%. An increase of 40% in the 18:1Δ11 content was observed in the pET-*des* strain. However, we were not able to detect 18:1Δ9. The lack of 18:1Δ9 production in the recombinant strain may have been a result of the lower level of the substrate, 18 : 0, for the enzyme expressed. Therefore, the cultures were supplemented with 18 : 0 and collected, and their FA compositions were analysed by GC (Table 3). Under these conditions, the 18 : 0 content was reduced (68·3%), and we were able to detect 18:1Δ9 (7·3%) in cells transformed with the desaturase gene. The retention time of this fatty acid methyl derivative demonstrates that the novel peak is 18:1 Δ9 methyl ester.

Effects of high growth temperature and heat shock on the expression of the *B.* TAL1000 desaturase gene and enzyme

Because temperature is one of the main environmental factors that regulate desaturase activity, we evaluated the effect of high growth temperature and heat shock on the expression of Δ9 desaturase from *B.* TAL1000 using RT-PCR (Fig. 6a) and Western blot (Fig. 6b). The thermal shock applied for 2 h did not result in a change in neither of the transcript level nor of the desaturase protein level. However, a change in band intensity of primary transcript and desaturase protein was observed after 24 h of growth at 37°C.

Discussion

The presence of an aerobic mechanism for MUFA synthesis in *B.* TAL1000 was demonstrated in this study by *in vivo* desaturation of radioactive 16 : 0 and 18 : 0 substrates, indicating the first biochemical evidence of an FA desaturase in rhizobia. In 1989, Wada *et al.* (1989) demonstrated

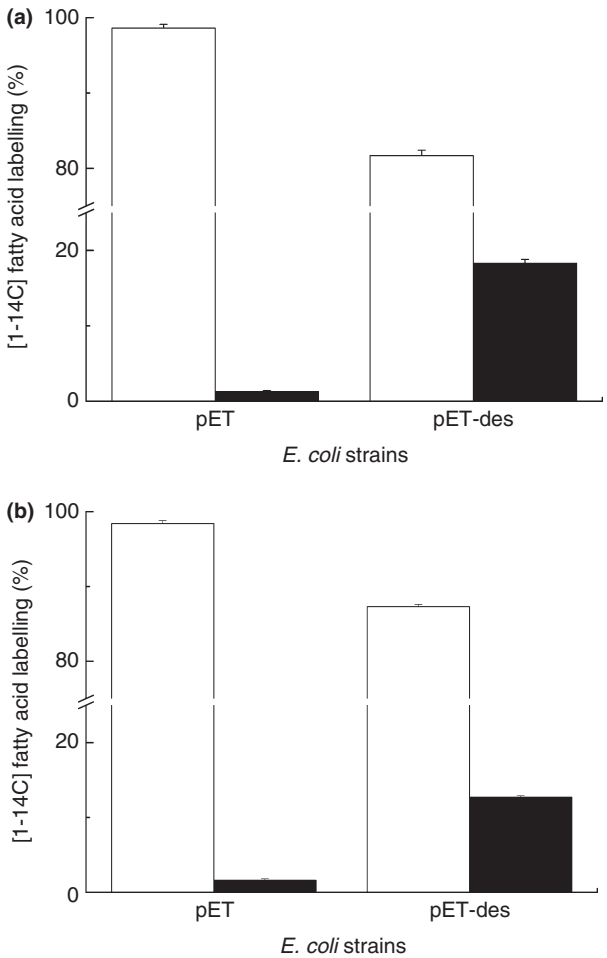

Figure 5 Desaturation of fatty acids measured in transformed strains of *Escherichia coli*. [1-^{14}C]16 : 0 (a) or [1-^{14}C]18 : 0 (b) FA were added to the cell culture of the transformed strain of *E. coli* and incubated at 37°C to an OD_{600} of 0·3–0·5, and the expression of recombinant protein was induced by IPTG for 12 h. Total lipids were extracted, methanolysed and separated according to the degree of unsaturation using TLC plates impregnated with 10% $AgNO_3$. The results are expressed as percentage of total radioactivity incorporated into each FA fraction. Values represent means ± SEM from three independent experiments. (▭) Saturated and (▬) Monounsaturated.

the existence of an aerobic pathway for MUFA synthesis in *Pseudomonas* strain E-3 using radioactive FA as substrate. In addition, these authors also reported that in *Pseudomonas* strain E-3, both mechanisms of MUFA synthesis, anaerobic and aerobic, were present.

The expression of desaturase genes is important because it provides the molecular basis for the acclimatization of organisms to changing environmental temperatures (Nishida and Murata 1996). Wild-type *E. coli* does not encode an FA desaturase but does contain all of the complementary systems needed for the measurement of desaturase activity (Cao *et al.* 2010). These features have led to the

Table 3 Fatty acid composition of *Escherichia coli* transformed with empty or recombinant plasmids

	Fatty acids (%)											
	14 : 0		16 : 0		16:1Δ9		18 : 0		18:1Δ11		18:1Δ9	
E. coli strain	−18 : 0	+18 : 0	−18 : 0	+18 : 0	−18 : 0	+18 : 0	−18 : 0	+18 : 0	−18 : 0	+18 : 0	−18 : 0	+18 : 0
pET	6·6 ± 0·8	4·7 ± 0·5	56·9 ± 0·6	49·7 ± 1·0	9·4 ± 0·8	8·3 ± 0·9	1·4 ± 0·5	12·0 ± 0·9	25·7 ± 1·0	25·3 ± 1·2	ND†	ND†
pET-des	6·4 ± 0·7	4·8 ± 0·6	37·4 ± 1·7*	41·9 ± 1·3*	19·2 ± 1·5*	16·8 ± 1·2*	0·9 ± 0·3	3·8 ± 0·3*	36·1 ± 1·0*	25·3 ± 1·4	ND†	7·3 ± 0·8*

Strains were grown at 37 °C on LB medium without (−) or with (+) 18 : 0 FA (400 μmol l^{-1}) to an OD_{600} of 0·3–0·5, and the expression of recombinant protein was induced by the addition of IPTG. After an overnight induction (12 h), total lipids were extracted, and total lipid FA were converted to methyl esters and analysed by GC as described in the text. The percentage of each fatty acid is relative to total FA (defined as 100%). Values represent means ± SEM of three independent experiments.

*Difference from control (pET) value statistically significant at $P < 0·05$ level.

†ND, not detected.

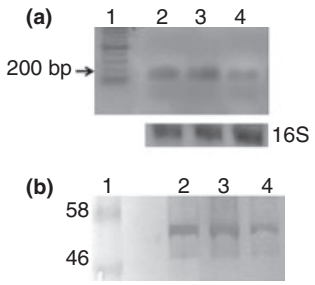

Figure 6 Effects of high growth temperature and heat shock on the expression of the *B*. TAL1000 desaturase gene and enzyme. The level of transcript and protein desaturase was examined by RT-PCR (a) and Western blotting (b) analysis. (a) Lane 1, nucleic acid marker (cienmarker Promega); lane 2, RNA of *B.* TAL1000 cells incubated for 24 h at 28°C; lane 3, RNA of *B.* TAL1000 cells incubated for 16 h at 28°C and transferred to 37°C for 2 h; lane 4, RNA of *B.* TAL1000 cells incubated for 24 h at 37°C. To control RNA integrity and that equal amounts of RNA were used in each reaction of RT-PCR, RNA (10 μg) from each condition was loaded on a 1·2% (w/v) agarose gel containing formaldehyde, visualized with ethidium bromide and the band correspond to 16S, photography. Equal RNA loading was also confirmed measuring RNA concentration spectroscopically at 260 nm. (b) Lane 1, molecular mass marker; lane 2, desaturase in *B.* TAL1000 cells incubated for 24 h at 28°C; lane 3, desaturase in *B.* TAL1000 cells incubated for 16 h at 28°C and transferred to 37°C for 2 h; lane 4, desaturase in *B.* TAL1000 cells incubated for 24 h at 37°C.

wide use of *E. coli* for the expression of these enzymes. In *B.* TAL1000, molecular studies helped to identify and sequence a possible desaturase gene, which shares high similarity with the *E. meliloti* 1021 desaturase gene present in the GenBank database. The *des* gene was successfully expressed in *E. coli* BL21(DE3)pLysS using the pET expression system, resulting in an approximately 50-kDa protein. The desaturase activity of the recombinant protein could be readily detected when assayed *in vivo* with radioactive 16 : 0 or 18 : 0 FA as substrates, which were both converted to the corresponding MUFA. This activity was also reflected in the FA composition of the recombinant strain, which exhibited important decreases in 16 : 0 and 18 : 0 and significantly enhanced proportions of 16:1Δ9 and 18:1Δ11. The appearance of 18:1Δ9 FA in transformed *E. coli* incubated with 18 : 0 FA indicates the presence of a Δ9 desaturase (Fig. 5b). These results are consistent with those of Cao *et al.* (2010).

The desaturation of FA that was measured *in vivo* in strains of *E. coli* transformed with this gene matches that found for the rhizobial strain *B.* TAL1000. Therefore, in the transformed strain of *E. coli,* the amount of radioactivity incorporated into the MUFA fraction was also higher when 16 : 0 was added to the culture medium as a substrate compared with 18 : 0.

The tolerance of organisms to their changing environment determines their choice of natural habitat. MUFA play a key role in the maintenance of the fluidity and thereby in the correct function of biological membranes.

Previously, we demonstrated that in *B.* TAL1000, the quantity of MUFA was decreased by high growth temperature (Paulucci *et al.* 2011). The present study shows that a 2-h exposure to 37°C is required for a change in the quantity of MUFA to occur.

In this study, we demonstrated that the desaturation of saturated FA was also decreased both at high growth temperature and by heat shock, causing a reduction in MUFA synthesis. The decreased desaturation at high growth temperature appears to be associated with the reduced transcript and protein desaturase levels detected; it would implicate a transcriptional regulation. In contrast, during heat shock, the FA desaturation decreased in *B.* TAL1000 would be associated with a different regulation mechanism that not involves neither transcript nor protein level changes. This last type of regulation would allow *B.* TAL1000 to quickly modify its FA composition during temperature changes and thereby maintain the biophysical properties of its membranes.

Although the molecular mechanism by which temperature regulates FA desaturation is not well known, different hypothesis have been suggested. In bacteria, temperature may regulate desaturase activity at a transcriptional level as has been observed for the *Bacillus subtilis* (Aguilar *et al.* 1998), *Synechocystis* (Los *et al.* 1993) and *Pseudomonas aeruginosa* (Zhu *et al.* 2006).

However, other regulation type have been described for desaturases of different organisms such as wheat roots (Horiguchi *et al.* 2000), *Arabidopsis* leaves (Matsuda *et al.* 2005) and for the seed-specific soybeans (Tang *et al.* 2005).

Therefore, we conclude that in *B.* TAL1000, the anaerobic MUFA biosynthetic pathway is supplemented by an aerobic mechanism mediated by desaturase, specifically, a Δ9 desaturase. The FA desaturation is downregulated by heat shock and a high growth temperature, although different mechanisms are involved. This system can be understood as a means for maintaining the correct membrane fluidity and composition and the correct functionality of the embedded proteins when these micro-organisms are exposed to stressful conditions.

This knowledge may be useful for developing strategies to improve the survival of rhizobia during the production and storage of inoculants, upon release into the soil and during the establishment of symbiosis.

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