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Exploring the Biosynthesis of Unsaturated Fatty Acids in *Bacillus cereus* ATCC 14579 and Functional Characterization of Novel Acyl-Lipid Desaturases

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At low temperatures, *Bacillus cereus* synthesizes large amounts of unsaturated fatty acids (UFAs) with double bonds in positions Δ5 and Δ10, as well as Δ5,10 diunsaturated fatty acids. Through sequence homology searches, we identified two open reading frames (ORFs) encoding a putative Δ5 desaturase and a fatty acid acyl-lipid desaturase in the *B. cereus* ATCC 14579 genome, and these were named BC2983 and BC0400, respectively. Functional characterization of ORFs BC2983 and BC0400 by means of heterologous expression in *Bacillus subtilis* confirmed that they both encode acyl-lipid desaturases that use phospholipids as the substrates and have Δ5 and Δ10 desaturase activities. Thus, these ORFs were correspondingly named *desA* (Δ5 desaturase) and *desB* (Δ10 desaturase). We established that DesA utilizes ferredoxin and flavodoxins (Flds) as electron donors for the desaturation reaction, while DesB preferably employs Flds. In addition, increased amounts of UFAs were found when *B. subtilis* expressing *B. cereus* desaturases was subjected to a cold shock treatment, indicating that the activity or the expression of these enzymes is upregulated in response to a decrease in growth temperature. This represents the first work reporting the functional characterization of fatty acid desaturases from *B. cereus*.

Bacillus cereus is widespread in nature, being frequently isolated from soil and growing plants. This microorganism is also well adapted to growth in the intestinal tract of insects and mammals. From these habitats, it easily spreads to food, where it may cause an emetic or a diarrheal type of food-associated illness that is becoming increasingly important in the industrialized world. The *B. cereus* group is constituted by Gram-positive, spore-forming, facultative anaerobic bacteria that have the ability to grow at temperatures between 4°C and 50°C, depending on the strain (1). Temperature is one of the most important environmental factors to which microorganisms have to respond. Cold adaptation in bacteria requires several changes in cellular components, in particular, membrane modifications, mainly in the fatty acyl moieties (2). These modifications are known to decrease the melting point of fatty acids (FAs) and to improve bacterial adaptation to lower growth temperatures (3). Of these adjustments, the best characterized is the biosynthesis of unsaturated fatty acids (UFAs), which is carried out by FA desaturases, a special type of oxygenase that can remove two hydrogens from a fatty acyl chain, catalyzing the formation of a double bond in the substrate. Desaturases use activated molecular oxygen and two reducing equivalents for catalysis (4–6).

The biosynthesis of UFAs in bacilli was extensively investigated by Fulco (7), who described that most species have a negligible amount of UFAs in their membrane lipids. In some *Bacillus* species that synthesize Δ5 UFAs, such as *Bacillus megaterium* and *Bacillus subtilis*, the proportion of UFAs has been observed to dramatically increase when the bacteria are grown at low temperatures (7). Some psychrophilic species of *Bacillus* as well as *B. cereus* have an unusually high proportion of UFAs (8, 9). Two recent studies on *B. cereus* strains showed that the adaptation of the membrane lipid composition of cells grown aerobically and isothermally at low temperatures was principally associated with a large increase in the proportion of UFAs (10, 11). *B. cereus* species contain a high proportion of Δ10 UFAs in their membrane lipids at 37°C, while at low temperatures they synthesize both Δ10 and Δ5 isomers (12). These findings suggest that this organism possesses a Δ10 desaturase (Des) that is active regardless of the growth temperature, while at lower temperatures a Δ5 desaturation system is induced. However, direct experimental evidence supporting the idea that *B. cereus* contains two desaturases with different regioselectivities has not yet been reported. Moreover, the molecular details of the regulation of the biosynthesis of UFAs by temperature in *B. cereus* are completely unknown.

The analysis of *B. cereus* ATCC 14579 genome projects revealed the presence of two acyl-lipid desaturase genes, BC2983 and BC0400, annotated on the basis of sequence similarity as a Δ5 desaturase and a putative FA desaturase, respectively (13). As functional predictions are never conclusive for desaturases, the aim of this study was to characterize these two open reading frames (ORFs) from *B. cereus* ATCC 14579 by heterologous expression in *B. subtilis*. This work allowed us to assign the correct enzymatic activity to each desaturase as well as to establish the nature of the substrate and electron donors involved in the desaturation process. We also describe the impact of growth temperature on the FA composition of *B. cereus* membrane lipids.

At present, most of the work concerning desaturases and their relationship with cold adaptation in *Bacillus* has been extensively and exclusively described for *B. subtilis*. To our knowledge, this is the first report on the isolation of a desaturase from *B. cereus* and

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Bacillus subtilis		
JH642	*trpC2 pheA1*	Laboratory stock
LC5	JH642 *des*::Km^r	22
MA1154	LC5 *amyE*::pSG1154 Km^r Sp^r	This study
MA2983	LC5 *amyE*::P$_{xyl}$-BC2983 Km^r Sp^r	This study
MA0400	LC5 *amyE*::P$_{xyl}$-BC0400 Km^r Sp^r	This study
LSC2983	LC5 *amyE*::pBC2983 Km^r Cm^r	This study
LSC0400	LC5 *amyE*::pBC0400 Km^r Cm^r	This study
L43	JH642 *fer*::Km^r	6
LSC28	L43 *ykuNOP*::P$_{spac}$-*ykuNOP* P$_{ykuNOP}$-*lacZ* Km^r Erm^r Lm^r	6
LSA43	L43 *des*::Sp^r Km^r	This study
LSA28	LSC28 *des*::Sp^r Km^r Erm^r Lm^r	This study
LSA4383	LSA43 *amyE*::pBC2983 Sp^r Km^r Cm^r	This study
LSA4300	LSA43 *amyE*::pBC0400 Sp^r Km^r Cm^r	This study
LSA2883	LSA28 *amyE*::pBC2983 Sp^r Km^r Cm^r Erm^r Lm^r	This study
LSA2800	LSA28 *amyE*::pBC0400 Sp^r Km^r Cm^r Erm^r Lm^r	This study
LSC16	MA2983 *plsC*::P$_{spac}$-*plsC* Km^r Sp^r Cm^r	This study
LSC17	MA0400 *plsC*::P$_{spac}$-*plsC* Km^r Sp^r Cm^r	This study
Escherichia coli DH5α	*supE44 thi-1* Δ*lacU169* ϕ80*lacZ*ΔM15 *endA1 recA1 hsdR17 gyrA96 relA1 trp6 cysT329*::*lac inm*$^{\lambda pI(209)}$	Laboratory stock
Plasmids		
pCR-Blunt II-Topo	*E. coli* cloning vector, Km^r	Invitrogene
pGEM-T Easy	*E. coli* cloning vector, Amp^r	Promega
pSG1154	Expression vector that integrates at the *amyE* locus of *B. subtilis*, Sp^r	18
pJM116	Integrative vector to construct transcriptional fusions to *lacZ* that integrates at the *amyE* locus of *B. subtilis*, Cm^r	5
pDesSp	*Bacillus subtilis des*::Sp^r cloned into pBluescript	M. C. Mansilla, personal communication
pLP6	P*spac*::*plsC* cloned into pDH88, Cm^r	20
pBC2983	pGEM-T Easy containing a 1,032-bp fragment corresponding to the BC2983 gene	This study
pBC0400	pGEM-T Easy containing a 1,086-bp fragment corresponding to the BC0400 gene	This study
pLC2983	pCR-Blunt II-Topo containing a 1,479-bp fragment corresponding to the BC2983 gene and the 5′ upstream region	This study
pLC0400	pCR-Blunt II-Topo containing a 1,433-bp fragment corresponding to the BC0400 gene and the 5′ upstream region	This study
pSG2983	pSG1154 containing a 1,032-bp fragment corresponding to the BC2983 gene	This study
pSG0400	pSG1154 containing a 1,086-bp fragment corresponding to the BC0400 gene	This study
pJM2983	pJM116 containing a 1,479-bp fragment corresponding to the BC2983 gene and the 5′ upstream region	This study
pJM0400	pJM116 containing a 1,433-bp fragment corresponding to the BC0400 gene and the 5′ upstream region	This study

[a] Cm^r, Km^r, Erm^r, Lm^r, Sp^r, and Amp^r, resistance to chloramphenicol, kanamycin, erythromycin, lincomycin, spectinomycin, and ampicillin, respectively.

one of the few reports of such enzymes in bacteria. *B. cereus* food-borne poisonings are the result of the ingestion of foods supporting a high rate of multiplication of these bacteria and, in the case of food with refrigerated storage, adaptation to low temperatures. Understanding the ability of *B. cereus* to grow at low temperatures will help control multiplication in refrigerated food and prevent outbreaks of food-borne poisoning.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The bacterial strains used in the present study are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were routinely grown in Luria-Bertani (LB) broth at 37°C. Spizizen salts supplemented with 0.5% glucose and trace elements (14) were used as minimal medium (MM) for *B. cereus*; 0.01% each tryptophan and phenylalanine was added to the same medium to grow *B. subtilis*. MM-MIV is MM with a concentration of 0.01% of methionine, isoleucine, and valine (6). Antibiotics were added to the media at the following concentrations: ampicillin (Amp), 100 μg ml^{-1}; chloramphenicol (Cm), 5 μg ml^{-1}; kanamycin (Km), 5 μg ml^{-1} for the experiments with *B. subtilis* and 50 μg ml^{-1} for the experiments with *E. coli*; and spectinomycin (Sp), 50 μg ml^{-1}. For the macrolides-lincosamides-streptogramin B, 0.5 μg erythromycin ml^{-1} and 12.5 μg lincomycin ml^{-1} were used.

General molecular techniques. Chromosomal DNA was isolated using standard techniques (15). In all cases, DNA fragments were obtained by PCR using the oligonucleotides described in Table 2. Oligonucleotides were purchased from Genbiotech SRL, Argentina. PCR products of the expected sizes were purified from gels using an AxyPrep DNA gel extraction kit (Axygen Bioscience), ligated into the pGEM-T Easy vector or pCR-Blunt II-Topo vector (Promega, Madison, WI), and transformed into *E. coli* DH5α (15). Plasmid DNA was prepared using a Wizard DNA purification system (Promega Life Science) and sequenced. Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (16). The *amy* phenotype was assayed for colonies grown for 48 h in LB-starch plates by flooding the plates with 1% I_2-KI solution (17).

TABLE 2 Oligonucleotide primers

Primer name	Sequence (5′–3′)[a]
BC2983XhoI	GGCTCGAGGAAAGGAATTAGAACAATG
BC2983EcoRI	CCGAATTCGCAATTATAGTTCAGATTTCAC
BC0400XhoI	CGCAAGGCTCGAGATAAGAAAGGAG
BC0400EcoRI	TCATTGCAAGAATTCATTAATTTTTACTG
BC2983upEcoRI	GAATTCAGCAATACATCAACAGCAT
BC0400upEcoRI	GAATTCAATCCTTATGGAATCTATACA

[a] Restriction sites are underlined.

amy-positive colonies produced a clear halo, while *amy* mutant colonies gave no halo at all. All plasmids and primers used in this study are listed in Tables 1 and 2, respectively.

Construction of *B. subtilis* strains MA2983 and MA0400. The sequences of the BC2983 and BC0400 genes of *B. cereus* ATCC 14579 were amplified using primers BC2983XhoI and BC2983EcoRI and primers BC0400XhoI and BC0400EcoRI, respectively (Table 2). The 1,032-bp product corresponding to the BC2983 gene and the 1,086-bp product corresponding to the BC0400 gene were purified and cloned in the pGEM-T Easy vector (Promega Life Science), yielding plasmids pBC2983 and pBC0400, respectively. Following restriction and ligation to the pSG1154 expression vector (18), the recombinant plasmids pSG2983 and pSG0400 carrying the individual genes under the transcriptional control of the inducible promoter (P_{xyl}) were obtained. The constructs were used to transform the *B. subtilis* LC5 strain. After selection on LB agar supplemented with Km at 5 μg ml^{-1} and Sp at 100 μg ml^{-1}, recombinant strains MA2983 and MA0400, respectively, were isolated. P_{xyl} was induced by adding xylose to the bacterial cultures at a final concentration of 0.8%.

Construction of *B. subtilis* strains LSC2983 and LSC0400. The sequences of the BC2983 and BC0400 genes of *B. cereus* ATCC 14579 carrying the 5′ upstream regions (435 bp and 352 bp long, respectively) were amplified using primers BC2983upEcoRI and BC2983EcoRI in the case of the BC2983 gene and BC0400upEcoRI and BC0400EcoRI in the case of the BC0400 gene (Table 2). The 1,479-bp product corresponding to the BC2983 gene and the 1,433-bp product corresponding to the BC0400 gene were purified and cloned in the pCR-Blunt II-Topo vector (Promega Life Science), yielding plasmids pLC2983 and pLC0400, respectively, which were subsequently sequenced. The fragments were excised from these plasmids with the EcoRI restriction enzyme and ligated into the EcoRI site of vector pJM116 (19). This plasmid integrates into the *amyE* locus of the chromosome of *B. subtilis* by a double homologous recombination process. The recombinant plasmids pJM2983 and pJM0400 carrying both genes under the transcriptional control of their own promoters were used to transform the *B. subtilis* LC5 strain. After selection on LB plates with Km at 5 μg ml^{-1} and Cm at 5 μg ml^{-1}, recombinant strains LSC2983 and LSC0400, respectively, were isolated. Successful integration into the *amyE* locus was confirmed by the appearance of the amylase-negative phenotype in LB-starch plates.

Construction of electron donor-deficient strains. To characterize the electron donors for *B. cereus* desaturases, we used *B. subtilis* strain L43 (which is deficient in ferredoxin [Fer]) and strain LSC28 (which lacks Fer and conditionally expresses the *ykuNOP* operon) (6). Strains L43 and LSC28 were first transformed with plasmid pDesSp in order to disrupt the *B. subtilis des* gene. After selection in LB medium supplemented with the appropriate antibiotic, we obtained *B. subtilis* strains LSA43 and LSA28, respectively. These strains were then transformed with plasmids pJM2983 and pJM0400 to express *B. cereus des* genes. After selection with Cm at 5 μg ml^{-1}, recombinant strains LSA4383, LSA4300, LSA2883, and LSA2800 were isolated (Table 1). The P_{spac} promoter was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to the bacterial cultures at a final concentration of 0.5 mM, and the experiments were conducted as described before (6).

Construction of *B. subtilis* strains LSC16 and LSC17. PlsC is an acyl-ACP-1-acylglycerol-phosphate acyltransferase involved in the synthesis of phosphatidic acid, the precursor of membrane phospholipids (PLs). The recombinant plasmid pLP6 (20) carrying the ribosome binding site and the 5′ portion of *plsC* under the transcriptional control of the inducible *spac* promoter (P_{spac}) was used to transform the *B. subtilis* MA2983 and *B. subtilis* MA0400 strains. This plasmid was integrated into the *plsC* gene by a single-crossover event, generating *B. subtilis* strains LSC16 and LSC17, respectively (Table 1). This approach resulted in the conditional inactivation of the target gene, whose expression can be controlled by the IPTG-induced P_{spac} promoter. Desaturases were expressed by adding xylose to the bacterial cultures at a final concentration of 0.8%, and the *plsC* gene was expressed by adding IPTG to the bacterial cultures at a final concentration of 0.5 mM.

Analysis of fatty acids by GC-MS. To determine the FA composition, the strains were grown to an optical density at 600 nm (OD_{600}) of 0.4 at 37°C and split into two aliquots; one of these cultures was maintained at the same temperature, and the other one was transferred to 25°C. The cultures were harvested in stationary phase. Total cellular FAs were prepared by the method of Bligh and Dyer (21). The fatty acid methyl esters (FAMEs) were prepared by transesterification of glycerolipids with 0.5 M sodium methoxide in methanol (22) and then analyzed in a PerkinElmer Turbo Mass gas chromatograph-mass spectrometer on a capillary column (30 mm by 0.25 mm in diameter) of 100% dimethylpolysiloxane (PE-1; PerkinElmer). Helium at 1 ml min^{-1} was used as the carrier gas, and the column temperature was programmed to rise by 4°C min^{-1} from 140°C to 240°C. Branched-chain fatty acids (BCFAs), straight-chain FAs, and UFAs used as reference compounds were obtained from Sigma Chemical Co. The positions of the double bonds in UFAs were determined by gas chromatography (GC)-mass spectrometry (MS). FAMEs were converted to dimethyl disulfide (DMDS) adducts, as previously described (22), and then separated on a PE-1 column ramped from 140 to 280°C at 4°C min^{-1}. The spectra were recorded in the electron impact mode at 70 eV, using 1-s scans of *m/z* 40 to 400. Alternatively, dimethyloxazoline derivatives were prepared by adding 0.25 g of 2-amino-2-methyl-1-propanol to up to 2 mg of lipid sample, as described by Christie (23).

Growth and metabolic labeling of mutant strains. The *B. subtilis* LSC16 and LSC17 strains were grown overnight at 37°C in MM with 0.5 mM IPTG, 0.8% xylose, and the corresponding antibiotics. Cells were resuspended in MM and grown either in the presence or in the absence of IPTG. After 5 to 6 h, having reached OD_{600} values of 0.4 to 0.5, cells without IPTG stopped growing because of PlsC depletion. At this point, both cultures were labeled with 1 μCi ml^{-1} [^{14}C]acetate for 3 h at 37°C (20). Following incubation, cells were collected and lipids were prepared by the method of Bligh and Dyer (21). Labeled lipids were analyzed using silica gel layers (Analtech) developed with petroleum ether-diethylether-acetic acid (70/30/2, vol/vol/vol) to separate the neutral lipids. The radioactivity on the plates was visualized using a PhosphorImager screen. The spots corresponding to PLs and free fatty acids (FFAs) were removed from the layers and converted to methyl esters by adding 1.5 ml of methanol and 0.5 ml of concentrated sulfuric acid, followed by incubation at 80°C for 2 h. Labeled FAMEs were applied to 10% silver nitrate-impregnated plates with Silica Gel G (thickness, 0.5 mm; Analtech). Chromatographic separation was achieved in a toluene solvent system at −20°C and detected using a PhosphorImager screen (Typhoon 9200).

B. subtilis strains LSA4383 and LSA4300 were grown overnight in MM supplemented with methionine, isoleucine, and valine (MIV) in order to avoid the prolonged lag phase observed in the *B. subtilis* L43 strain. On the following day, cells were diluted 1:10 in MM-MIV. Cells were grown to mid-exponential phase and labeled for 2 h with 0.2 μCi of [^{14}C]palmitate (58 mCi/mM) at 25°C.

B. subtilis strains LSA2883 and LSA2800 were grown overnight in MM-MIV supplemented with 0.5 mM IPTG. On the following day, fresh cultures were started by washing twice and diluting the cultures grown overnight at a 1:10 dilution in the same medium without inducer. Two-milliliter samples were taken and labeled with 0.2 μCi [^{14}C]palmitate for 2 h at 25°C. After a 4-h period of arrested growth, the inducer was added

TABLE 3 FA composition of total membrane lipid extract from *B. cereus* ATCC 14579[a]

Fatty acid(s) or ratio	% of total fatty acids at indicated temp	
	37°C	25°C
Iso-$C_{12:0}$	1.6 ± 0.3	0.5 ± 0.1
n-$C_{12:0}$	0.5 ± 0.1	0.8 ± 0.1
Iso-$C_{13:0}$	1.6 ± 0.1	1.0 ± 0.1
Anteiso-$C_{13:0}$	0.6 ± 0.1	0.7 ± 0.1
n-$C_{13:0}$	0.1 ± 0.0	0.4 ± 0.1
Iso-$C_{14:0}$	13.0 ± 1.0	10.0 ± 1.0
n-$C_{14:0}$	7.0 ± 1.0	13.1 ± 0.7
Iso-$C_{15:0}$	6.1 ± 0.5	6.0 ± 1.0
Anteiso-$C_{15:0}$	3.5 ± 0.3	5.0 ± 2.0
Iso-$C_{15:1}$ Δ10	0.5 ± 0.1	0.8 ± 0.3
n-$C_{15:0}$	1.1 ± 0.1	1.7 ± 0.3
Iso-$C_{16:2}$	0.5 ± 0.1	1.4 ± 0.3
Iso-$C_{16:1}$ Δ5	1.2 ± 0.1	3.4 ± 0.3
Iso-$C_{16:1}$ Δ10	6.0 ± 0.1	4.0 ± 1.0
n-$C_{16:2}$	1.5 ± 0.3	10.4 ± 0.8
Iso-$C_{16:0}$	19.0 ± 1.0	4.8 ± 0.1
n-$C_{16:1}$ Δ5	0.8 ± 0.3	2.6 ± 0.8
n-$C_{16:1}$ Δ10	15.0 ± 2.0	18.4 ± 0.3
n-$C_{16:0}$	14.5 ± 0.6	7.0 ± 1.0
Iso-$C_{17:1}$ Δ10	0.4 ± 0.1	0.7 ± 0.2
Anteiso-$C_{17:1}$ Δ10	0.3 ± 0.1	0.6 ± 0.2
Iso-C17:0	1.5 ± 0.4	2.4 ± 0.6
n-$C_{17:1}$ Δ5	0.1 ± 0.4	0.2 ± 0.0
Anteiso-$C_{17:0}$	1.0 ± 0.2	0.6 ± 0.2
n-$C_{17:1}$ Δ10	0.1 ± 0.0	0.1 ± 0.0
n-$C_{17:0}$	0.3 ± 0.0	0.2 ± 0.0
Iso-$C_{18:1}$ Δ5	0.1 ± 0.0	0.2 ± 0.0
Iso-18:0	0.6 ± 0.1	0.2 ± 0.0
n-$C_{18:1}$ Δ5	0.5 ± 0.1	1.7 ± 0.3
n-$C_{18:0}$	1.6 ± 0.1	1.7 ± 0.4
Total UFAs	27 ± 4	45 ± 4
Δ5 UFAs	2.7 ± 0.9	8 ± 1
Δ10 UFAs	22 ± 2	25 ± 2
Δ5,10 UFAs	2.0 ± 0.0	12 ± 1
Total BCFAs	58 ± 5	42 ± 7
Iso-BCFAs	52 ± 4	35 ± 5
Anteiso-BCFAs	5 ± 1	7 ± 2
Iso/anteiso ratio	9.6 ± 0.8	5.1 ± 0.6

[a] Cells were grown at 37°C in Spizizen salts MM supplemented with glucose to exponential phase and then shifted to 25°C. Total lipids were extracted and transesterified to yield FAMEs, and the products were identified by GC-MS. Values are the means of the results of three experiments and are expressed as the percentage of total FAs. n, normal FAs.

at a final concentration of 0.5 mM. Cells were incubated for 2 h, and a 2-ml sample was processed as described before (6). The spots of the different fatty acids were quantified by use of ImageQuant software (version 5.2).

RESULTS

Fatty acid biosynthesis in *Bacillus cereus* ATCC 14579 and its relation to growth temperature. The influence of a temperature downshift on the FA composition in *B. cereus* ATCC 14579 was determined as described in Materials and Methods. This analysis indicates that at 37°C the FA composition of *B. cereus* is comprised by even- and odd-numbered iso-FAs (C_{12} to C_{17}), odd-numbered anteiso-FAs (C_{15} and C_{17}), and even- and odd-numbered normal FAs (C_{14} to C_{18}) (Table 3). In addition to these saturated fatty acids (SFAs), we also identified UFAs differing in chain length and branching pattern (Table 3; Fig. 1A). As shown in Fig. 1B and C, we found that these UFAs contain double bonds at positions Δ5 and Δ10. Also, as shown in the region between 8 and 11 min (see the enlargement at the bottom of Fig. 1A), we found two C_{16} acyl chains that were identified to be Δ5,10 diunsaturated FAs (Fig. 1D). Furthermore, in cells subjected to cold shock stress, there was an increase in FA desaturation from 27% to 45% (Table 3). Specifically, the monounsaturated Δ5 isomers were augmented from 2.7% to 8%, while the Δ5,10 diunsaturated FAs increased from 2% to 12%. However, the content of Δ10 UFAs observed at 37°C was not significantly influenced by the temperature downshift. Nevertheless, we cannot exclude the possibility that the synthesis of Δ10 isomers increases at low temperatures and that the products are used by the Δ5 desaturase to give Δ5,10 diunsaturated FAs. We also noted that the ratio of iso-BCFAs/anteiso-BCFAs was significantly changed after cold shock, from 9.6 ± 0.8 to 5.1 ± 0.6. However, this modification in the branching pattern was mainly due to a decrease in the proportion of iso-BCFAs, instead of an increase in the proportion of anteiso-BCFAs, as was reported for other *Bacillus* species subjected to cold shock stress (3, 24). Thus, these data suggest that the main mechanism used by *B. cereus* to increase lipid disorder is an increase in the synthesis of UFAs, rather than the synthesis of a higher proportion of anteiso-BCFAs.

Identification of putative acyl-lipid desaturase genes in *Bacillus cereus* ATCC 14579. A BLAST search of the *B. cereus* ATCC 14579 database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the sequence of the previously characterized *B. subtilis* Δ5 Des as the query sequence allowed us to identify *B. cereus* gene BC2983. This gene encodes a polypeptide of 343 amino acid residues with a molecular mass of 39,805 Da and is classified as a possible membrane-bound Δ5 desaturase. Comparison of the deduced amino acid sequence of the *B. cereus* BC2983 gene with the sequence of *B. subtilis* Δ5 Des by the Clustal W program rendered values of 66.2% identity (ID) and 77.6% similarity (S).

A second gene, BC0400, identified as a putative FA desaturase, was found when searching for genes annotated as desaturases in the *B. cereus* ATCC 14579 genome at the JCVI-CMR site (Comprehensive Microbial Resources; http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). The protein encoded by the BC0400 gene is 361 amino acids in length and showed an ID of 18.9% and an S of 34.1% compared to the amino acid sequence of the Δ5 Des of *B. subtilis* and 17.4% ID and 32.4% S compared to the amino acid sequence of the protein encoded by the *B. cereus* BC2983 gene. The hydropathy profile of the primary sequence (from the version 2.0 server at http://www.cbs.dtu.dk/services/TMHMM) is similar to the profiles of the sequences of all known membrane-associated desaturases. They possess several transmembrane domains and three appropriately spaced histidine-rich clusters characteristic of membrane-bound desaturases essential for catalysis. These motifs are presumed to compose the Fe-binding active centers of the enzymes (25). Conserved motifs I, II, and III, the first, second, and third histidine (His) boxes from the protein encoded by *B. cereus* BC2983 gene, respectively, are 80%, 100%, and 100% identical to the respective tracks from the *B. subtilis* desaturase. The sequences of His boxes I, II, and III of the protein encoded by the *B. cereus* BC0400 gene showed IDs of 40%, 50%, and 60%, respectively, to the sequences of His boxes I, II, and III of *B. subtilis* desaturase. These characteristics imply that the BC2983 and BC0400 genes encode fatty acyl desaturases.

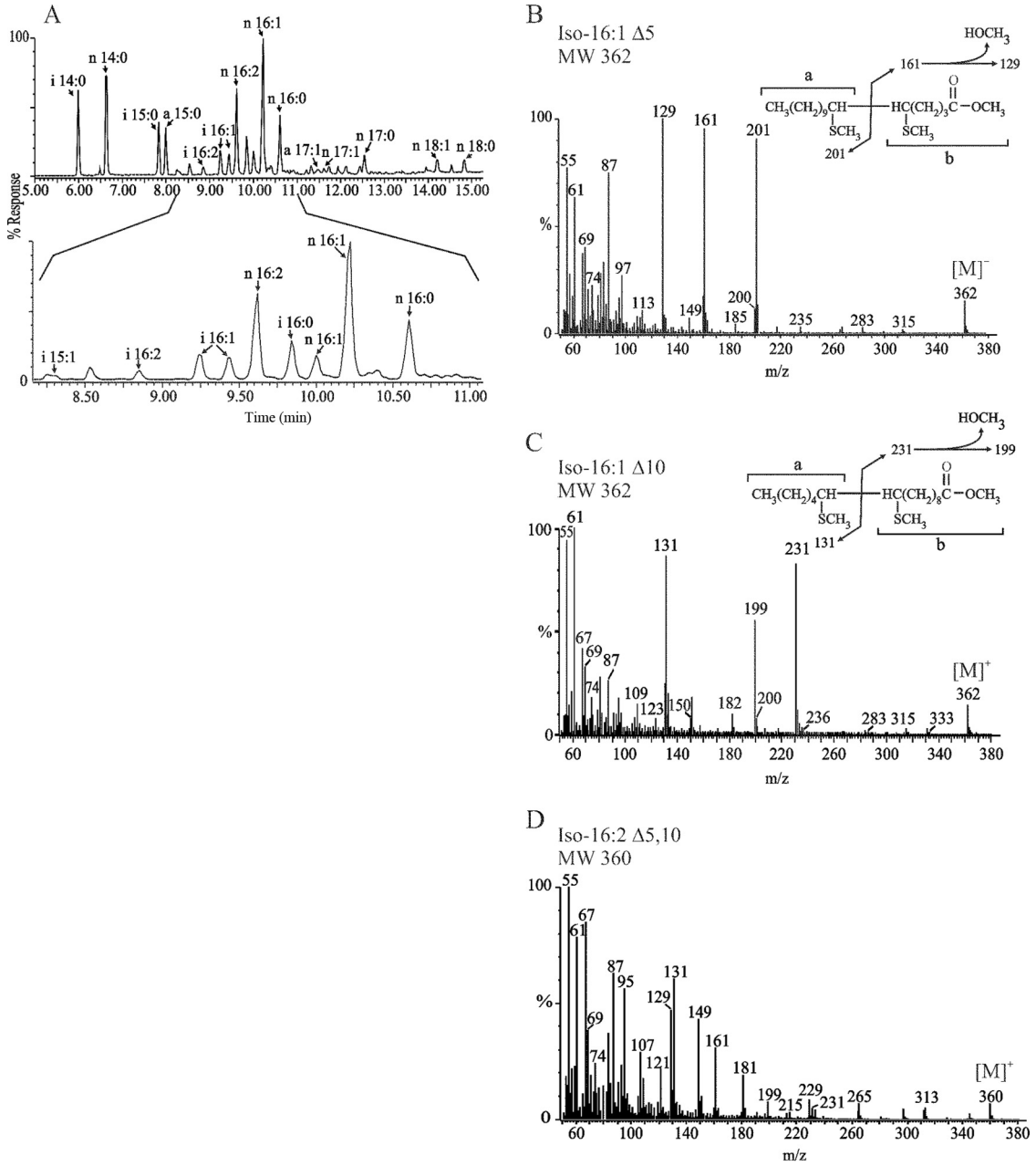

FIG 1 FAs synthesized by *B. cereus* ATCC 14579. (A) GC-MS of FAMEs. The peaks corresponding to the identified FAs are indicated by arrows. The numbers on the *x* axis represent times (in minutes). i, iso-BCFAs; a, anteiso-BCFAs; n, normal FAs. (B to D) Mass spectra of DMDS derivatives separated by GC of iso-$C_{16:1}$ Δ5, iso-$C_{16:1}$ Δ10, and iso-$C_{16:1}$ Δ5,10, respectively. Aliphatic fragments a and b containing the carboxyl group are indicated by brackets. $[M]^+$, molecular ion; MW, molecular weight.

Cloning and functional characterization of fatty acid desaturases from *Bacillus cereus* ATCC 14579. The functional characterization of the putative *B. cereus* desaturases was carried out in *B. subtilis des*-negative strain LC5, which is unable to synthesize UFAs due to the lack of the Δ5 desaturase (22). To this end, *B. cereus* genes coding for the putative ORFs BC2983 and BC0400 were cloned under the control of the P_{xyl} promoter in vector pSG1154 and integrated ectopically at the *amyE* locus of strain LC5. The resultant strains were named MA2983 and MA0400, respectively, and their FAs were analyzed by GC-MS, as described in Materials and Methods. As shown in Fig. 2A, the MA1154 control strain (containing the empty vector) does not contain UFAs, while the *B. subtilis* MA2983 strain grown in the presence of xylose showed additional peaks compared with the FA profile of the control strain (Fig. 2B). These peaks were identified as Δ5 UFAs, and the nature of these isomers was confirmed by unequivocal determination of the double bond positions through the analysis of the mass spectrum of the DMDS derivatives (Fig. 2D). Using the same approach, we determined that *B. subtilis* MA0400 grown in the presence of xylose synthesized only Δ10 UFAs, as shown in Fig. 2C

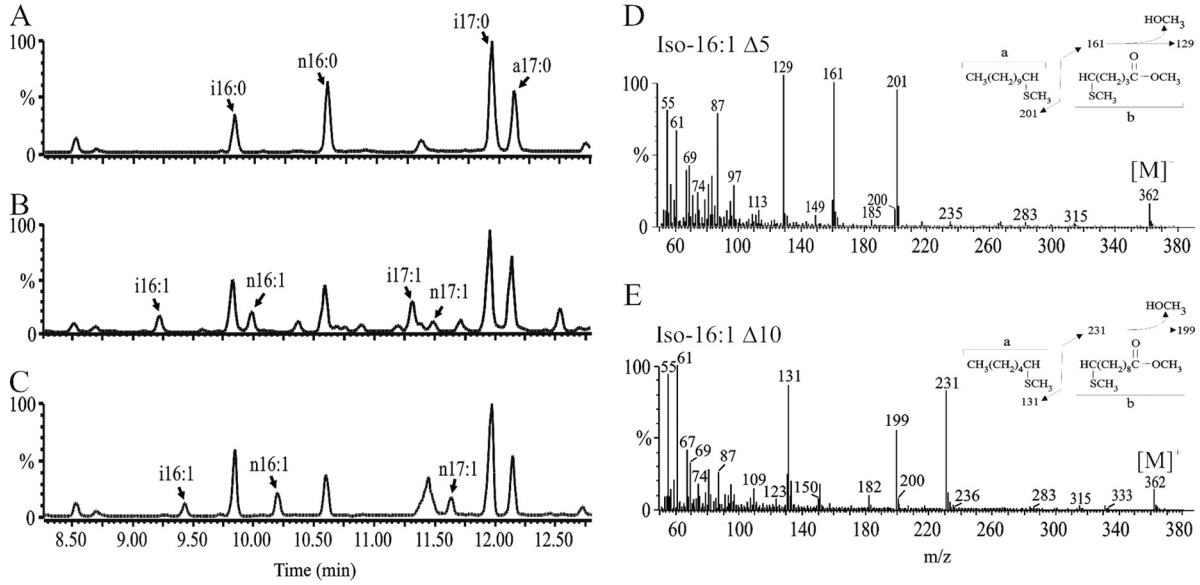

FIG 2 Fatty acids synthesized by *B. subtilis* strains expressing *B. cereus* desaturases. (A to C) GC-MS of the FAMEs of strains MA1154, MA2983, and MA0400, respectively, at 25°C. The peaks corresponding to the identified fatty acids are indicated by arrows. i, iso-BCFAs; a, anteiso-BCFAs; n, normal fatty acids. The numbers on the *x* axes represent times (in minutes). (D and E) Mass spectra of DMDS derivatives separated by GC of iso-C16:1 Δ5 and iso-C16:1 Δ10, respectively. Aliphatic fragments a and b containing the carboxyl group are indicated by brackets. The numbers on the *x* axes represent *m*/*z*. $[M]^+$, molecular ion.

and E. These data confirm our previous bioinformatic predictions that the BC2983 and BC0400 ORFs code for proteins with desaturase activity and allowed us to correctly assign the regioselectivities for the proteins as Δ5 and Δ10 desaturases, respectively. Thus, the gene encoding the Δ5 desaturase of *B. cereus* was named *desA*, and the gene encoding Δ10 desaturase was named *desB*.

To further study the expression of the *desA* and *desB* genes from their own promoters, their sequences were cloned into the pJM116 vector, including the 5′ upstream regions preceding the start codons (the sequences were 435 bp and 352 bp long, respectively). The resulting plasmids were integrated at the *amyE* locus of *B. subtilis* LC5 strains, yielding *B. subtilis* strains LSC2983 (for *desA*) and LSC0400 (for *desB*). These strains were grown in MM and assayed for lipid desaturation by GC-MS, as described in Materials and Methods. As shown in Table 4, strains expressing either the *desA* or the *desB* gene contained larger amounts of Δ5 and Δ10 UFAs, respectively, when shifted to 25°C. These results show that either the expression or the activities of DesA and DesB are upregulated by a downshift in growth temperature.

Substrate specificity of membrane-bound *Bacillus cereus* ATCC 14579 DesA and DesB desaturases. The consensus motifs found in both the DesA and DesB desaturases strongly indicate that they are membrane-bound associated proteins. The membrane-bound FA desaturases were subdivided into two groups, on the basis of the differences in substrate specificities that they exhibit: acyl coenzyme A (acyl-CoA) desaturases and lipid desaturases. Acyl-CoA desaturases introduce double bonds into FAs esterified to CoA, while lipid desaturases utilize fatty acyl moieties of membrane lipids as the substrates (4). In order to determine the substrate specificities of *B. cereus* desaturases, we used an *in vivo* system in which FA synthesis was uncoupled from PL synthesis by specific inhibition of the latter process.

PlsC is an acyl-ACP-1-acylglycerol-phosphate acyltransferase involved in the synthesis of phosphatidic acid, the precursor of membrane PLs. In *B. subtilis*, PL synthesis ceases following depletion of the PlsC acyltransferase, but FA synthesis continues at a high rate, leading to the accumulation of FFAs arising from dephosphorylation of 1-acyl glycerol phosphate, followed by deacylation of monoacylglycerol (20). These FFAs could, in principle, be converted to acyl-CoAs by the *B. subtilis* acyl-CoA synthetases LcfA and YhfL (20). Thus, to test if either acyl-CoA thioesters or complex lipids can be desaturated by *B. cereus* desaturases, we introduced the *plsC* gene under the control of an IPTG-regulated promoter in *B. subtilis* strains expressing either *desA* or *desB*. The resultant strains were named LSC16 and LSC17, respectively. When these strains are deprived of IPTG, PL synthesis ceases, leading to the accumulation of FFAs (20). Strains LSC16 and LSC17 were labeled with [^{14}C]acetate in the presence or in the absence of IPTG, and FFAs were separated from PLs in silica gel plates (Fig. 3). The analysis of these fractions with silver nitrate-impregnated plates showed that about 26% and 70% of the total label present in the PL fractions of strains LSC16 and LSC17, respectively, corresponded to UFAs. However, no UFAs were found in the FFA fraction, indicating that desaturases cannot act on FAs which are not bound to PLs (Fig. 3B). Our results strongly suggest

TABLE 4 UFA composition of *B. subtilis* strains expressing *B. cereus* desaturases[a]

B. subtilis strain[b]	% of UFAs at indicated temp	
	37°C	25°C
LSC2983	12 ± 1	39.7 ± 0.4
LSC0400	15 ± 2	30 ± 3

[a] Cells were grown at 37°C in Spizizen salts MM supplemented with glucose to exponential phase and then shifted to 25°C. Total lipids were extracted and transesterified to FAMEs, and the products were identified by GC-MS. Values are the means of the results of three experiments and are expressed as percentage of total FAs.
[b] *B. subtilis* strains LCS2953 and LSC0400 express DesA (Δ5 desaturase) and DesB (Δ10 desaturase), respectively.

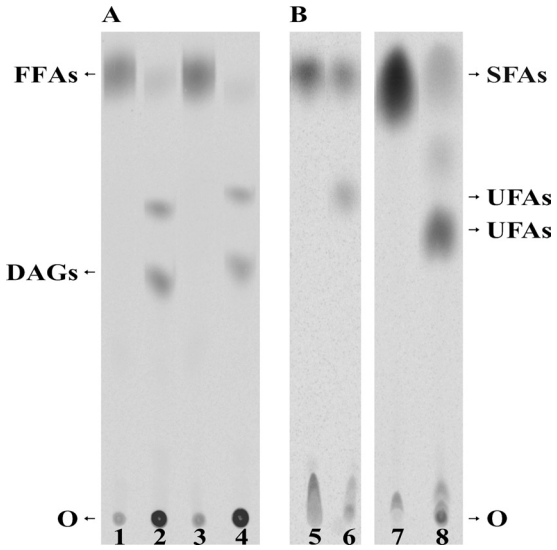

FIG 3 Autoradiogram of the products of [^{14}C]acetate labeling of *B. subtilis* strains LSC16 and LSC17. Cells were grown overnight at 37°C in MM with 0.5 mM IPTG. After they were washed, cells were resuspended in fresh MM, grown either in the presence or in the absence of IPTG, and labeled with 1 μCi ml^{-1} [^{14}C]acetate for 3 h at 37°C. (A) Labeled lipids were analyzed using silica gel. The spots corresponding to PLs and FFAs were removed from the layers and converted to methyl esters. (B) Labeled FAMEs were applied to 10% silver nitrate-impregnated Silica Gel G plates. The radioactivity on the plates was visualized using a PhosphorImager screen. Lanes 1 and 5, LSC16 without IPTG; lanes 2 and 6, LSC16 with IPTG; lanes 3 and 7, LSC17 without IPTG; lanes 4 and 8, LSC17 with IPTG. DAG, diacylglycerol; O, origin.

that DesA and DesB recognize the acyl chains of PLs as the substrates to introduce double bonds at positions Δ5 and Δ10, respectively. Thus, we conclude that both desaturases are in fact acyl-lipid desaturases.

Electron transport donors involved in *Bacillus cereus* ATCC 14579 DesA and DesB desaturase activity. The complex process of introducing a double bond into FAs requires iron cofactors, molecular oxygen, and two reducing equivalents for catalysis (4–6). Electrons are supplied from NAD(P)H by two different but functionally equivalent electron transport systems that are specific for the subcellular compartments rather than for the class of the desaturase under study. In the case of plant endoplasmic reticulum desaturases and animal and fungal acyl-CoA desaturases, the donor is cytochrome b_5, either in the form of the cytochrome b_5 fused domain or in its free form. For the soluble acyl-ACP desaturase and the integral membrane acyl-lipid desaturases from plastids and cyanobacteria, electrons are delivered by ferredoxins, which are ubiquitous soluble iron-sulfur proteins involved in a variety of redox reactions (26). In a previous report, we demonstrated that the *fer* gene encoding a 4Fe-4S Fer (26), as well as the *ykuN* and *ykuP* genes encoding two flavodoxins (Flds) (27), which are mobile electron carriers containing flavin mononucleotide as the prosthetic group, is able to transfer electrons to the *B. subtilis* Δ5 Des to catalyze the O_2-dependent desaturation of the acyl chains of membrane PLs (6). To determine the influence of these proteins on *B. cereus* desaturation processes, we constructed *B. subtilis* strains LSA4383 and LSA4300 that lack Fer and express *desA* and *desB*, respectively, under the control of their native promoters. To test whether Fer is required for DesA and DesB activity, the *fer*-deficient strains LSA4383 and LSA4300 and *fer*-positive isogenic strains (LSC2983 and LSC0400) were labeled in MM-MIV with radioactive palmitate and assayed for the conversion of this FA to *cis*-hexadecenoic acid. Using silver nitrate-impregnated thin-layer chromatography, the synthesis of *cis*-hexadecenoic acid was detected in all strains. However, the desaturation of palmitic acid in Fer-deficient strain LSC4383, expressing DesA, was reproducibly found to be about 50% of the lipid desaturation activity observed for parental strain LSC2983 (data not shown). On the other hand, the Δ10 desaturase activity was almost not influenced by the absence of Fer (data not shown). As mentioned above, Flds could function physiologically in the biosynthesis of UFAs in *B. subtilis* (6). Therefore, to test if the YkuN and YkuP *B. subtilis* Flds could also mediate electron transfer to *B. cereus* desaturases, we constructed strains *B. subtilis* LSA2883 and LSA2800 (Table 1). These strains lack Fer, conditionally express the *ykuNOP* operon under the control of the P_{spac} IPTG-inducible promoter, and express *desA* and *desB*, respectively. Lipid desaturation in these strains was assessed by labeling cells with [^{14}C]palmitate either in the presence or in the absence of IPTG, as described before (6). In the absence of the inducer, in both strains, FA desaturation decreased, reaching radioactivity values between 3 and 7% of the total radioactivity incorporated into FAs. When IPTG was added, UFA synthesis was reestablished (Table 5). Thus, we conclude that DesA uses Fer and the Flds as electron donors, while Flds are the main electron donors for DesB desaturation.

DISCUSSION

In this work, we describe the biosynthesis of UFAs in *B. cereus* strain ATCC 14579 grown under defined conditions at 37°C and after cold shock at 25°C. We also report the functional characterization of the enzymes involved in this process. It was previously shown that *B. cereus* grown in different media and at different temperatures synthesized UFAs with double bonds in different positions in membrane lipids (11, 12, 28). In the present study, we established that in a minimal defined medium devoid of FAs, *B. cereus* synthesizes Δ5, Δ10, and Δ5,10 UFAs. In addition, we show that *B. cereus* responds to a decrease in the ambient temperature by increasing the proportion of UFAs, especially Δ5 isomers, in the membrane lipids. Interestingly, this adaptive response is markedly different from what is observed in other bacilli, which mainly stimulate anteiso-BCFA biosynthesis to increase membrane disorder (29). Prompted by this finding and using analytical, physiological, and functional assays, we demonstrated that *B. cereus* contains two desaturases that insert double bonds at positions Δ5 and Δ10 of the acyl chain, and these were named DesA

TABLE 5 Desaturase activity in *B. subtilis* strains depleted of Fer and Flds[a]

	Desaturase activity			
	LSC2883		LSC2800	
FA	Without IPTG	With IPTG	Without IPTG	With IPTG
SFAs	97 ± 2	65 ± 3	93 ± 3	37 ± 3
UFAs	3 ± 2	35 ± 3	7 ± 3	63 ± 3

[a] Cells were grown to log phase at 37°C in MM-MIV with or without IPTG. Two-milliliter samples were labeled with 0.2 μCi of [^{14}C]palmitate at 25°C for 2 h. Labeled FAs were extracted and fractionated by argentation–thin-layer chromatography. The spots of the different FAs were quantified by ImageQuant (version 5.2). Values are the means of five independent experiments and are expressed as the percentage of total fatty acids.

and DesB. As bioinformatic predictions strongly indicated that the DesA and DesB desaturases are membrane-bound associated proteins, we attempted to elucidate the nature of the *in vivo* substrate(s) of these enzymes using a *B. subtilis plsC* mutant, which allows uncoupling of FA synthesis from PL synthesis. The results obtained using this mutant strain are compatible with a lipid-linked desaturation mechanism in which the FAs remain esterified to membrane phospholipids during the desaturation reaction. Thus, our data strongly suggest that DesA and DesB are acyl-lipid desaturases.

We also report that Fer and the two Flds YkuN and YkuP are able to transfer the reducing equivalents to *B. cereus* DesA and DesB desaturases, in order to catalyze the desaturation reaction, when expressed in *B. subtilis* strains. Although the characterization of the electron donor of *B. cereus* desaturases was performed in a heterologous host, a BLAST search of *B. cereus* ATCC 14579 databases identified protein BC1483, whose sequence shares 90% identity with that of *B. subtilis* Fer. In addition, using *B. subtilis* YkuN and YkuP Flds as queries, we identified two orthologous sequences in *B. cereus*, BC3541 and BC1376, which share 55% and 47% identities, respectively, with the sequences of the proteins from *B. subtilis*. These findings indicate that it is highly probable that both Fer and Flds are the physiological electron donors of *B. cereus* desaturases.

When *desA* and *desB* were expressed under the control of their own promoters in the heterologous host *B. subtilis*, we determined that both desaturases produce a greater proportion of UFAs when the cultures grown at 37°C were transferred to 25°C. This observation contrasts with the data shown in Table 3 indicating that in its natural host, *B. cereus*, the Δ10 isomer content is not influenced by variations in growth temperature. This might suggest that in *B. cereus* the Δ10 UFAs synthesized by DesB could be further desaturated by DesA, yielding larger amounts of Δ5,10 UFAs at lower temperatures. The cold shock induction of UFA synthesis could be due to an increase in the expression or in the desaturation activities of both desaturases at lower temperatures. Interestingly, the DesK-DesR pathway (29) described in *B. subtilis* is absent in *B. cereus*, suggesting that the thermal regulatory mechanisms controlling the expression of *B. cereus* desaturase genes are different from those described for *B. subtilis* Δ5 Des.

UFAs play a key role in maintaining proper membrane lipid fluidity in many poikilothermic organisms, which appears to be necessary for normal cell function. As we show in the present work, the UFAs comprise more than 40% of the total acyl chains of membrane phospholipids in *B. cereus* subjected to cold shock. This suggests that, under cold shock conditions, acyl-lipid desaturases are essential for *B. cereus*. Thus, it would be interesting to evaluate the impact of disrupting *desA* and *desB* genes in order to uncover the role of the encoded proteins in maintaining appropriate membrane fluidity in *B. cereus*. Clearly, further studies are necessary to understand the role of these two acyl-lipid desaturases in *B. cereus* physiology and the molecular basis of the mechanisms underlying temperature-regulated UFA synthesis in this organism.

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