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Involvement of the CasK/R two-component system in optimal unsaturation of the *Bacillus cereus* fatty acids during low-temperature growth

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ABSTRACT

Bacillus cereus sensu lato is composed of a set of ubiquitous strains including human pathogens that can survive a range of food processing conditions, grow in refrigerated food, and sometimes cause food poisoning. We previously identified the two-component system CasK/R that plays a key role in cold adaptation. To better understand the CasK/R-controlled mechanisms that support low-temperature adaptation, we performed a transcriptomic analysis on the ATCC 14579 strain and its isogenic Δ*casK/R* mutant grown at 12 °C. Several genes involved in fatty acid (FA) metabolism were downregulated in the mutant, including *desA* and *desB* encoding FA acyl-lipid desaturases that catalyze the formation of a double-bond on the FA chain in positions Δ5 and Δ10, respectively. A lower proportion of FAs presumably unsaturated by DesA was observed in the Δ*casK/R* strain compared to the parental strain while no difference was found for FAs presumably unsaturated by DesB. Addition of phospholipids from egg yolk lecithin rich in unsaturated FAs, to growth medium, abolished the cold-growth impairment of Δ*casK/R* suggesting that exogenous unsaturated FAs can support membrane-level modifications and thus compensate for the decreased production of these FAs in the *B. cereus* Δ*casK/R* mutant during growth at low temperature. Our findings indicate that CasK/R is involved in the regulation of FA metabolism, and is necessary for cold adaptation of *B. cereus* unless an exogenous source of unsaturated FAs is available.

1. Introduction

Bacillus cereus sensu lato (sl) is composed of a set of human foodborne pathogens with seven ubiquitous species able to adapt to a wide range of environmental conditions (Graumann and Marahiel, 1996; Stenfors Arnesen et al., 2008). Altogether these bacteria are able to grow from 5 °C to 50 °C (Lechner et al., 1998; Zhou et al., 2010) and include psychrotolerant, mesophilic and thermotolerant strains (Guinebretière et al., 2008). Moreover, because of their ability to form spores (Kim et al., 2014), *B. cereus* strains are able to survive food processing and may develop in the chill chain or during food refrigeration up to consumption (Stenfors Arnesen et al., 2008). The upshot is that *B. cereus* is one of the major pathogens commonly encountered in foodborne illness outbreaks (Markland et al., 2013).

Several studies describing the way *B. cereus* adapts to low temperature (Brillard et al., 2010; Broussolle et al., 2010; de Sarrau et al., 2013a; Diomandé et al., 2014) have shown that cold adaptation by *B. cereus* is multifactorial (translational factors, membrane modifications, etc.). One key strategy, crucial to the cold survival of bacteria, is the ability to modulate membrane fluidity (Russell, 1984). In addition to physically protecting the cell, the membrane facilitates component exchanges with the environment. Higher membrane fluidity could enable improved transport, thus ensuring osmotic balance and nutrient supply. As low temperatures alter membrane fluidity, bacteria have to adapt membrane composition in order to decrease the melting point of their phospholipids.

Bacterial growth at low temperature causes changes in FA profile, such as decreased FA chain length, and increase of the anteiso/iso-branched-chain FA and unsaturated FA (UFAs) (Haque and Russell, 2004; Kaneda, 1977). Membrane components can usually sense the physical stress corresponding to a decrease in temperature and mount a bacterial adaptive response (Chintalapati et al., 2004; Mansilla et al., 2003). *Bacillus subtilis* DesK/R was the first two-component system (TCS) described as involved in cold adaptation. The sensor protein DesK was shown to sense an increase in membrane thickness in response to a decrease in temperature (Aguilar et al., 2001; Cybulski et al., 2010). The cognate response regulator DesR thus regulates the expression of a desaturase gene. Desaturases are oxygenases that can remove two hydrogen atoms from a fatty acyl chain and then catalyze

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the formation of a double bond in the substrate (Sperling et al., 2003). These enzymes use activated molecular oxygen and two reducing equivalents for catalysis (Chazarreta-Cifre et al., 2011; Shanklin and Cahoon, 1998). In *B. subtilis*, the Des protein was found to be responsible for maintaining membrane fluidity during low-temperature growth (Cybulski et al., 2002). In *B. cereus*, two desaturases DesA and DesB were described (Chazarreta Cifre et al., 2013), but no mechanism similar to *B. subtilis* DesK/R has yet been described for members of the *B. cereus* group.

We previously identified a TCS named CasK/R, widespread among *B. cereus sensu lato* (*B. cereus sl*), including *B. anthracis*, *B. thuringiensis* and *B. weihenstephanensis* (Diomandé et al., 2014). The isogenic *casK/R* mutants constructed in several mesophilic and cold-tolerant strains of *B. cereus sl* showed impaired growth and survival abilities in addition to morphological changes at low temperatures, revealing a major role of this TCS for *B. cereus sl* cold adaptation (Diomandé et al., 2014). The aim of the present study was to identify the regulon of CasK/R and the mechanism involving CasK/R during *B. cereus* low temperature adaptation. Here, we show that CasK/R plays a role in regulating FA composition at low temperature and that lecithin, an exogenous UFAs source sometimes found in food, can be used by *B. cereus* to restore its growth ability at low temperature in the absence of CasK/R.

2. Materials and methods

2.1. Strains and growth conditions

B. cereus strains used in this study were ATCC 14579 and Δ*casKR*, its isogenic *casK/R* mutant (Diomandé et al., 2014). They were regularly grown in Luria broth (LB) medium with vigorous agitation at 37 °C, 13 °C or 12 °C. As required, kanamycin concentration used for bacterial selection was 150 μg/mL. For growth with FAs supplementation, the Difco™ Synthetic Broth AOAC was used as previously described (Song et al., 2012).

2.2. Transcriptome analysis

Cultures of *B. cereus* ATCC 14579 WT and Δ*casK/R* strains were grown in LB medium at 12 °C with shaking. As WT and Δ*casK/R* strains displayed different growths at 12 °C, we standardized the culture samples by harvesting at entry into exponential phase (EP) when cultures rose from an initial $OD_{600nm} = 0.001$ to reach $OD_{600nm} = 0.006$ (monitored on the spectrophotometer Helios Epsilon, Thermo scientific) and at entry into stationary phase (SP) when the cultures reached $OD_{600nm} = 3.4$.

Total RNAs were extracted as follows: cells from 100 mL (EP) or 2 mL (SP) of culture were centrifuged at 8000 × *g* for 3 min at 4 °C and the pellets were immediately frozen at −80 °C in TRI-Reagent (Ambion, Huntingdon, United Kingdom) as described before (Brillard et al., 2010).

Cell disruption was performed by adding 0.3 g of sterile 0.1 mm zirconium beads (VWR) followed by 3 runs of 30 s at a speed 6 on a FastPrep Instrument (FP120). The RNAs were then phenol-chloroform -extracted.

cDNAs were generated on 20 μg of RNA isolated from the WT and the Δ*casK/R* strains as described before (van Schaik et al., 2007). After purification, cDNAs were labeled with Cy3 and Cy5. The combined cDNAs of WT and Δ*casK/R* strains were hybridized onto a 70-mer oligonucleotide micro-array covering all 5352 open reading frames of the *B. cereus* ATCC 14579 genome. The microarrays were hybridized with 300 ng labeled cDNA for each sample. After hybridization at 60 °C for 17 h, the microarrays were washed with 6× SSC-0.005% Triton X-102 (10 min, 20 °C), 0.1× SSC-0.005% Triton X-102 (10 min, 20 °C) and 0.1× SSC-0.005% Triton X-102 (10 min, 37 °C). Slides were scanned with an Agilent microarray scanner (G2565BA) (extended dynamic range scan mode) and data were processed as described before (van Schaik et al., 2007) using the web-based VAMPIRE platform (*P*-value threshold < 0.05) (Hsiao et al., 2005). All experiments were performed with two biological duplicates (including Cy3/Cy5 dye-swaps).

2.3. Fatty acid (FA) composition

FAs methyl esters were prepared as previously described (Brillard et al., 2010; de Sarrau et al., 2013b). Culture samples were harvested in stationary phase (as described above). A volume of 50 mL of cultures was collected. Samples were washed once with a saline solution (0.9% NaCl w/v), except for cultures with exogenous-source FA which were washed three times. The FA obtained from 60 mg of cells (fresh weight) were transesterified by the ester link method (Schutter and Dick, 2000). The reaction consisted of alkaline methanolysis breaking the ester link in the lipid and producing FA methyl esters by reaction with 5 mL of 0.2 M KOH in methanol at 37 °C for 1 h. One milliliter of 1 M acetic acid was then added to lower pH to 7.0. pH was checked with pH test strips. FA methyl esters were then extracted by adding 3 mL of hexane. The supernatant (apolar phase) was transferred into clean tubes and concentrated by evaporation at room temperature under a continuous nitrogen flow to obtain approximately 200 μL of extract. The extracts were injected into a gas chromatography-mass spectrometry (GC-MS) system (Shimadzu QP 2010-01) under the conditions described before (de Sarrau et al., 2013b) and processed by real-time GC-MS analysis.

2.4. Growth with fatty acids sources

Three independent cultures of *B. cereus* ATCC 14579 WT and Δ*casK/R* strains were carried out in an automated turbidimeter (Microbiology Bioscreen C Reader, Labsystems, Uxbridge, UK) as previously described (Diomandé et al., 2014). A fresh colony was used to inoculate 10 mL of LB and incubated at 30 °C with shaking for 9 h. Ten microliters was then used to inoculate 10 mL fresh LB, and incubated at 30 °C with shaking for 17 h. This second culture was centrifuged at 4000 rpm, 5 min and the cells were washed once in 10 mL of AOAC medium. The pellet was then resuspended in 10 mL of AOAC medium and this sample was diluted to a concentration of 10^5 CFU/mL in AOAC medium supplemented with 25 μg/mL, 125 μg/mL or 375 μg/mL of purified lecithin (Sigma) as a source of UFAs or supplemented with 375 μg/mL of hydrogenated lecithin (Sigma) as a source of saturated FA. Purified lecithin and hydrogenated lecithin contain FA of the same chain length. Negative controls consisted of the WT and Δ*casK/R* strains grown in AOAC without supplementation. Three replicate microplate wells were filled with the dilutions of each inoculated medium to a final volume of 300 μL per well. Cultures were incubated under vigorous constant shaking, and OD_{600} was measured at 1-h intervals at 12 °C over a 10-day incubation period.

3. Results

3.1. The casK/R mutation affects genes involved in the fatty acids metabolism

A transcriptome analysis was performed on cells that were harvested at two determined growth phase corresponding to the entry into exponential phase (EP) and the entry into stationary phase (SP) (see Materials and methods), taking into account the growth delay of the Δ*casK/R* strain at 12 °C when compared to the WT strain (Fig. 1).

The comparative transcriptome analysis revealed 95 genes downregulated and 53 upregulated in the Δ*casK/R* strain during both the EP and SP growth phase at low temperature compared to the WT strain (Table S1). Among the genes differentially expressed in both EP and SP, 15 were involved in FA metabolism (Table 1).

Six downregulated genes were putatively involved in the FA biosynthesis pathway. Among them, *fabH*, *fabF* and *fabI* are involved in saturated FA synthesis according to the KEGG pathway classification. Moreover, the *desA* and *desB* genes, respectively encoding a Δ5 and a

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