Involvement of *etfA* gene during $CaCO_3$ precipitation in *Bacillus subtilis* biofilm

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Abstract

The *eftA* gene in *Bacillus subtilis* has been suggested to be involved in the oxidation/reduction reactions during fatty acid metabolism. Interestingly *etfA* deletion in *B. subtilis* results in impairment in $CaCO_3$ precipitation on the biofilm. Comparisons between the wild type *B. subtilis* 168 and its *etfA* mutant during *in vitro* $CaCO_3$ crystal precipitation (calcite) revealed changes in phospholipids membrane composition with accumulation of up to 10% of *anteiso*-$C_{17:0}$ and 11% *iso*-$C_{17:0}$ long fatty acids. Ca^{2+} nucleation sites such as dipicolinic acid and teichoic acids seem to contribute to the $CaCO_3$ precipitation. *etfA* mutant strain showed up to 40% less dipicolinic acid accumulation compared with *B. subtilis* 168, while a *B. subtilis* mutant impaired in teichoic acids synthesis was unable to precipitate $CaCO_3$. In addition, *B. subtilis etfA* mutant exhibited acidity production leading to atypical flagella formation and inducing extensive lateral growth on the biofilm when grown on 1.4% agar. From the ecological point of view, this study shows a number of physiological aspects that are involved in $CaCO_3$ organomineralization on biofilms.

Introduction

Electron transfer flavoproteins (ETFs) are *alpha beta*-heterodimers involved in the electron transfer during the oxidation/reduction reactions that takes place in fatty acid metabolism of eukaryotic mitochondria as well as bacteria (Tsai and Saier, 1995). Comparison of the amino acid sequences among all available ETFs and ETF-like proteins revealed the existence of different groups of ETFs. The groups are related to acyl-CoA dehydrogenases or enclose ETF-likes proteins that are involved in growth under anaerobic condition (Weidenhaupt et al. 1996). In obligate anaerobic bacteria such as *Clostridium acetobutylicum*, the co-expression of *etfA* and *etfB* was essential for the butyryl-CoA dehydrogenase (BCD) activity of fatty acids biosynthesis (Boynton et al. 2006; Inui et al. 2008). EtfA-B proteins of *Bacillus subtilis* show 64% and 57% similarity with EtfA and EtfB proteins from *C. acetobutylicum*, respectively. However in *B. subtilis* 168 *etfA-B* functions are only putative, the genes are not essential for growth and do not results in differential cell morphology (Kobayashi et al. 2003; Barabesi et al. 2007). In *B. subtilis, etfA* is regulated by the central regulator *fadR*. The *fadR* regulon is involved in in the β-oxidation cycle comprises five operons: *lcfA-fadR-fadB-etfB-etfA*, *lcfB*, *fadN-fadA-fadE*, *fadH-fadG*, and *fadF-acdA-rpoE* (Matsuoka 2007; Tojo et al 2011).

Interesting, when *B. subtilis* 168 is grown on B4 precipitation medium supplemented with calcium acetate (0.25% w/v) it produces $CaCO_3$ calcite crystals on biofilm after 6-7 day of incubation at 39°C (Barabesi et al. 2007). Barabesi and collaborators (2007) generated a mutant strain in *etfA* gene which cannot precipitate $CaCO_3$ crystals on its biofilm. In summary, *eft*A mutation is linked to fatty acid metabolism but its overall effects in cell physiology during $CaCO_3$ precipitation are still unknown. The main goal of this study is the elucidation of the physiological responses altered by the *eftA* mutation in *B. subtilis* during $CaCO_3$ precipitation *in*

vitro.

Materials and Methods

Bacterial strains and growth conditions. Strains used in this study were *Bacillus subtilis* 168 (Anagnostopoulos and Spizizen, 1961), *B. subtilis* 168 mutated in *etfA* gene (Barabesi et al. 2007), and *B. subtilis* EB1451 [*hisA1 argC4 metC3 tagO*::Erm^r] impaired in teichoic acid synthesis. *B. subtilis* cultures were routinely grown on Nutrient Agar (Oxoid) and standard B4 precipitation medium (0.4% yeast extract, 0.5% dextrose, 0.25% calcium acetate and 1.4% agar when solid medium was prepared) (Boquet et al. 1973). For studies related to flagella formation, modified B4 medium lacking calcium acetate was used to avoid calcium interferences during chemotaxis (Ordal et al. 1983; Herbaud et al. 1998). Modified buffered B4 medium was prepared by addition of 1.2% TRIS-HCl stabilized at pH 7.3 with 2N HCl.

Unless specified otherwise, biofilms were grown on plates incubated at 39° C inside a plastic bag to prevent dehydration.

Membrane Fatty Acid Methyl Esters (FAME) analysis (Eder, 1995)**.** Cultures from *B. subtilis* 168 strain and *etfA* mutant were normalized to OD_{600}=0.5 and 200 µl were inoculated on B4 plates. Plates were incubated 8 hours, 7 and 30 days at 37°C and biofilms were scraped and subjected to whole cell fatty acid methyl esters analysis. All the experiments were conducted in triplicates. FAME analysis samples were initially homogenized and subjected to saponification at 100ºC with 1 ml of methanolic NaOH (15% NaOH in 50% methanol) followed by esterification of the fatty acids at 80°C with 2 ml of 3.25 N HCl in 46% (vol/vol) methanol. FAMEs were extracted into 1.25 ml of 1:1 (vol/vol) methyl-tert-butyl etherhexane followed by

an aqueous washing of the organic extract with 3 ml of 1.2% (wt/vol) NaOH methylation and extraction as described by Kidd Haack et al. (1994). Profiles for total fatty acids methyl esters (FAME) in each sample were generated after gas chromatography using a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column. The chromatography was conducted using an HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, California) with the oven temperature ramping up from 170 ºC to 270 ºC at 5 ºC per minute and a final step of 300 ºC for 2 min. Individual compounds were quantified by flame ionization detector (FID) in response to internal standards added prior to CG analyses. Fatty acids between 9 and 20 carbons in length were analyzed and identified using the Sherlock MIS software (MIDI, Inc., Newark, Delaware). Final (%) of total fatty acids resulted from an automatic comparison of the composition of the unknown strain to a stored database using a covariance matrix, principal component analysis and pattern recognition software. The covariance matrix takes into account the mole-for-mole relationship of the conversion of one fatty acid to another (e.g. 16:0 to 16:1 due to action of a desaturase), which might occur in relation to a temperature shift or age difference. The pattern recognition software uses calculations of cross terms (e.g. ratios between fatty acid amounts) in addition to the principal component base. Experiments were conducted in triplicate.

Dipicolinic acid (DPA) assays. B. subtilis biofilms were grown in B4 solid medium and scraped from the plates after 12 hours, 1, 2, 3, 4 days of incubation at 39 °C until 30 mg of wet weight was achieved. Due to the formation of $CaCO_3$ crystals on *B. subtilis* 168, biofilms samples were centrifuged at 10,000xg for 10 min and the upper phases were collected into a new pre-weighted tube. These samples were resuspended in 1 ml of distilled water and measurements of total DPA were performed according to Nicholson and Setlow, 1990. Experiments were conducted in triplicate.

Microscopic analysis. To monitor crystal formation in the biofilms we used a stereo microscope Leica ES2. An Optical Nikon Eclipse E400 microscope was used for microscopic analysis of flagella content. Samples from the *B. subtilis* biofilms were stained using the BD Flagella Stain Dropper kit according to the manufacture's manual (Becton, Dickinson and Company). To record images microscopes were integrated into a Nikon Spot Insight digital camera.

Experiments were conducted from three different sections of the biofilm and at least three different plates were tested.

Results

B. subtilis eftA mutant accumulates up to 21% more long fatty acids ($C_{17:0}$) when compared to B. subtilis 168 during in vitro $CaCO_3$ precipitation

B. subtilis 168, as well as other soil bacteria, is capable of $CaCO_3$ (calcite) formation if grown in B4 precipitating medium starting from day 7th at 39°C (Figure 1, A). On the opposite, mutation in the *eftA* gene prevented calcite crystal formation in *eftA B. subitlis* mutants grow under similar conditions. (Figure 1, B) (Barabesi et al. 2007). To measure the variation of total lipids within the plasma membrane during $CaCO_3$ precipitation, *B. subtilis* 168 and *eft*A mutant were analysed via Fatty Acid Methyl Ester (FAME) analysis (Table I). During the first 8 hours of growth on B4 medium no differences were reported among the *B. subtilis* 168 and *etfA* mutant. However, after one week and one month of incubation, differences were reported for $C_{15:0}$ and $C_{17:0}$ (Table I, Figure 2). *B. subtilis* 168 accumulated up to 13 % more of *anteiso*-$C_{15:0}$ when compared with *etfA* mutant. On the contrary, longer fatty acids *anteiso*-$C_{17:0}$ and *iso*-$C_{17:0}$ accumulated in *etfA* mutant, up to 10% and 11%, respectively, after one month of incubation (Figure 2).

Ca^{2+} chelants such as dipicolinic acid and teichoic acid contribute to $CaCO_3$ precipitation in B. subtilis.

In *Clostridium perfringens*, EftA catalyses the formation of pyridine-2,6-dicarboxylic acid (commonly named dipicolinic acid or DPA) (Osburn et al. 2010). Interestingly, dipicolinic acid forms a complex with calcium ions within the endospore core (Hintze and Nicholson 2010). Consequently, we compared the levels of DPA of *B. subtilis* 168 and the *B. subtilis eftA* mutant biofilms during growth on B4 medium. Although initially DPA accumulation is similar for both strains, after 42 hours dipicolinic acid concentration was 40% lower in the *B. subtilis eft*A mutant when compared with the wild type (Figure 3). Considering another Ca^{2+} chelator in addition to dipicolinic acid, we also wondered whether the external charge of teichoic acids could have a role in $CaCO_3$ precipitation. *B. subtilis* EB1451 strain mutated in *tagO* gene and unable to produce any wall teichoic acid was tested (D'Elia et al. 2006). When *B. subtilis* EB1451 biofilm was formed on B4 solid medium and incubated at 37°C for 21 days, $CaCO_3$ precipitation was impaired.

B. subtilis eftA mutant is capable of flagella formation when compared to B. subtilis 168

Mutation of *eftA* is known to cause increasing of acidity of the *B. subtilis* biofilm due to an excess of proton (H^+) extrusion from the cell (Marvasi et al. 2010). Release of H^+ across the cytoplasmic membrane is coupled with rotation of most bacterial flagella including those from *B. subtilis* (Matsuura et al. 1979; Ito et al. 2005). To analyse phenotypic changes in flagella formation in *B. subtilis* caused by *eftA* mutation, calcium acetate was eliminated from B4 medium as calcium tunes *Bacillus* sp. chemotaxis (Ordal et al. 1983; Herbaud et al. 1998).

Interestingly, the biofilm morphology of *B. subtilis eftA* mutant showed extensive lateral growth (Figure 4, panel A) indicative of the presence of highly motile cells. Lateral growth was not observed for *B. subtilis* 168 strain (Figure 4, panel B). Further microscopic observations on the bacteria growing in the edges of the lateral branches formed by *B. sutbilis etf*A mutant revealed the presence of flagella (Figure 3, panel A). To our knowledge, flagella formation at 1.4% of agar has not been previously reported for any *Bacillus subtilis* strain (Senesi et al. 2004).

To determine if extensive lateral growth was associated with an excess of protons (H^+) extrusion previously reported in the *B. subtilis etfA* mutant, strains were grown on buffered B4 medium to sequestrate H^+ from the medium. Lateral growth and flagella formation were not observed when the *B. subtilis* strains were grown on B4 pH 7.3 buffered medium (Figure 4, panel C, D).

Discussion

A number of bacteria are capable to form minerals by biologically induced mineralization (BIM) processes in which the minerals generally nucleate and grow extracellularly as a result of bacterial metabolic activities (Visscher et al. 2010). Calcium carbonate precipitation in *B. subtilis* is one of these BIM processes that is poorly understood at the physiological and genetic levels. Recently, Barabesi et al. 2007 isolated a mutant strain in *etfA* gene impaired in $CaCO_3$ crystals formation on its biofilm. Further analysis showed that the impairment was originated due to an excess of production of protons from the biofilm (Marvasi et al. 2010).

eftA participate in fatty acid *β-oxidation* cycle, affecting the utilization of palmitic acid as representative of long chain fatty acids and the accumulation of branched and straight long chain acyl-CoAs in *B. subtilis* (Matsuoka et al. 2007). The FAME profiles during $CaCO_3$ precipitation

of *B. subtilis* 168 and *etfA* mutant biofilms (Table I) indicated that mutations in *eftA* caused an accumulation of *anteiso*-$C_{17:0}$ and *iso*-$C_{17:0}$, compared to the shortest *anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$ after the fist week of incubation. The percentage of fatty acids accumulation in both *B. subtilis* 168 and *etfA* mutant does not change after 8 hours, while the difference are clearly visible after 1 week where *anteiso*-$C_{15:0}$ decreases and *iso*-$C_{17:0}$ and *anteiso*-$C_{17:0}$ increase in *B. subtilis eftA* (Figure 2). Accumulation of long chain fatty acids may be explained due to *etfA* deletion, which inhibits YsiA, a central regulator in fatty acid degradation, causing the accumulation of branched and straight long chain acyl-CoAs (Matsuoka et al. 2007). The link between plasma membrane fatty acids composition and $CaCO_3$ precipitation may be ascribed due to changes of membrane fluidity which is one of the responses that the bacteria uses to cope with environmental stress (Beranová et la 2008). We can speculate that such changes may lead to the change of number of calcium nucleation sites. Indeed, membrane fluidity has been linked with the control of important calcium nucleation sites such as DPA and teichoic acids (Cowan et al. 2004, Zhang and Rock 2008). As previously mentioned, DPA and teichoic acids are able to bind calcium. DPA is a main key player of spore resistance to many environmental stresses during long periods of dormancy and in *Clostridium perfringens*, EtfA protein catalyses the formation of DPA (Huang et al. 2007; Orsburn et al. 2010). DPA analysis showed that *B. subtilis etfA* biofilm exhibits a 40% less DPA when compared with the *B. subtilis* 168 (Figure 3). Dipicolinic acid forms a complex with calcium ions within the endospore core and mutants in dipicolinic acid fail in accumulate calcium on the cell surface (Hintze and Nicholson 2010, Hanson et al 1978). The reduction of DPA in the mutant could contribute to the impairment of $CaCO_3$ precipitation at neutral pH.

Teichoic acids could also be responsible of mineral precipitation, serving as nucleation site for

calcium. To confirm this hypothesis, *B. subtilis* mutant EB1451 strain (D'Elia et al. 2006) unable to produce any wall teichoic acid was totally impaired in $CaCO_3$ precipitation. Current research has shown the role of teichoic acids as nucleation sites or Ca^{2+}-carriers. It is well known that the cell wall of Gram positive bacteria presents teichoic acids covalently bound to the cell wall or anchored in the cytoplasmic membrane as lipoteichoic acid (Thomas et al. 2014). The polyphosphate groups of teichoic acid provide one-half of the metal binding sites for calcium (Thomas et al. 2014), while the other Ca^{2+} bind the carboxyl units of peptidoglycan. According with Thomas and collaborators (2014) curvature of Scatchard plots showed two regions of binding affinity: Region I $K_A = (1.0 \pm 0.2) \times 10^6$ M^{-1} and Region II $K_A = (0.075 \pm 0.058) \times 10^6$ M^{-1}. Binding capacity for both regions (η_2) is 0.70 ± 0.04 μmol/mg for Ca^{2+}. Calcium binding constants are highly dependent from the calcium concentration and cell wall type (Thomas et al. 2014). Experiments on crystal formation *in vitro* show controversial data: Adsorption of hydroxyapatite to [3H]-lipoteichoic acid effects positively crystal growth (Damen et al. 1994) while in oral streptococci, lipoteichoic acids inhibited Ca^{2+}-phosphate precipitation in a distinct pH-range just above the acid solubility of the mineral by complexing Ca^{2+}-ions (Bergmann et al 1991).

With reference to the flagella formation experiment, it was carried out mainly to confirm the acidic phenotype of *etfA*. Prior studies revealed that the *etfA* mutant extruded 0.7 moles H^+L^{-1} more with respect to *B. subtilis* 168 strain on B4 medium and that the extrusion of protons was contributing to the impairment of $CaCO_3$ (Marvasi et al. 2010). Interestingly, *B. subtilis eftA* mutant exhibits flagella formation when grown on B4 media plates with agar concentration 1.4% with extensive lateral growth (Figure 4). However, if the *B. subtilis etfA* mutant was plated on B4 buffered medium pH 7.3 such lateral grow and flagella formation was arrested. Protons extrusion

across the cytoplasmic membrane is coupled with rotation of most bacterial flagella including *B. subtilis* (Matsuura et al. 1979; Ito et al. 2005). These data seem to suggest that in *B. subtilis etfA* mutant the proton excess could induce flagella formation through an indirect system. Once the protons are sequestered (such as it is the case in the buffered B4 medium), *B. subtilis* flagella formation is arrested. In *B. subtilis* strain PB1831 strain, Senesi and collaborators (2004) reported lack of flagella formation during growth at agar concentrations ranging from 0.5 to 2%. All these data, show how *etfA* mutation, which has been previously characterized as the main responsible for the impairment of $CaCO_3$ precipitation, leads to several different metabolic divergences when compared with the wild type that ultimately contribute in the prevention of $CaCO_3$ organomineralization in *B. subtilis* biofilm. DPA and teichoic acids could contribute to organomineralization in many other bacterial species (Marvasi et al. 2011).

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CAPTIONS

Table I. Fatty Acid Methyl Ester (FAME) analysis for *B. subtilis* 168 and its *eft*A mutant biofilms.

Fig. 1 *In vitro* $CaCO_3$ precipitation on *B. subtilis* 168 and *etfA* mutant biofilms during growth in B4 medium. Strains magnification 10X. Arrows show examples of calcium carbonate crystals. *B. subtilis etfA* mutant biofilm, notice the complete absence of crystals after 8 days of incubation (Barabesi et al. 2007).

Fig. 2 Comparison of the percentage of the total amount of fatty acids of *anteiso*-$C_{15:0}$, *iso*-$C_{17:0}$ and *anteiso*-$C_{17:0}$ fatty acids produced by *B. subtilis* 168 (Δ) and its *eft*A mutant (○) during growth on B4 medium. Errors bars represent the standard deviation. Arrows indicate when $CaCO_3$ precipitations occur.

Fig. 3 Dipicolinic acid quantification during *B. subtilis* 168 (Δ) and its *eft*A mutant (○) biofilm formation after 7 days (168 hours) of incubation on B4 medium. Error bars represent standard deviations of three replicated samples. Arrow shows when crystals formation occurs on *B. subtilis* 168 biofilm.

Fig. 4 Comparison of lateral growth and flagella formation of *B. subtilis eftA* mutant and *B. subtilis* 168. The lower section of each figure represents the cells on the top square after flagella staining (magnification 1000X). Biofilms were produced after incubation for 5 days at 37°C

(1.4% agar, without calcium acetate). A) *B. subtilis etfA* mutant biofilm developed lateral growth and flagella. B) No lateral growth and flagella was developed by *B. subtilis* 168. C and D) *B. subtilis etfA* mutant and 168 growth under buffered condition (TRIS 1.2% pH 7.3). No lateral growth and flagella were reported for both strains.