Ann Microbiol (2011) 61:371–374
DOI 10.1007/s13213-010-0121-5

SHORT COMMUNICATION

Bacillus subtilis fadB (*ysiB*) gene encodes an enoyl-CoA hydratase

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Received: 30 April 2010 / Accepted: 3 August 2010 / Published online: 29 August 2010

Abstract Fatty acids are essential components of membranes and are an important source of metabolic energy. In bacteria, the β-oxidation pathway is well known in *Escherichia coli*. *Bacillus subtilis* possesses a considerable number of genes, organized in five operons, that are most likely involved in the β-oxidation of fatty acids. Among these genes, only one product, $FadR_{Bs}$ (YsiA), has been recently characterized as a transcriptional regulatory protein which negatively regulates the expression of β-oxidation genes including those belonging to the *lcfA* operon, including $fadR_{Bs}$ (*ysiA*). The probable involvement of the $FadR_{Bs}$ (YsiA) regulon members in β-oxidation is inferred from data based on BLASTP similarity of their gene products. In this work, we report the cloning and the expression of *B. subtilis* $fadB_{Bs}$(*ysiB*), belonging to the *lcfA* operon, and the functional characterization of its product as an enoyl-CoA hydratase, demonstrating the actual involvement of these genes in fatty acid β-oxidation.

Keywords β-oxidation · *Bacillus subtilis* · $fadB_{Bs}$ · enoyl-CoA hydratase · *lcfA* operon

Introduction

Fatty acids are essential components of membranes and are an important source of metabolic energy. Fatty acid degradation and biosynthesis pathways have been mainly studied in the model prokaryote *Escherichia coli*. Fatty acids that are intracellularly formed or extracellularly supplied are degraded through β-oxidation when cells are starved of a carbon source. In *E. coli*, the degradation pathway is catalyzed by the enzymes encoded by the *fad* regulon which is responsible for the transport and activation of long-chain fatty acids and their β-oxidative cleavage into acetyl-CoAs (Cronan and Rock 1996; Campbell and Cronan 2001a, b). *Bacillus*

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subtilis possesses a considerable number of genes, organized in five operons, that are possibly involved in the β-oxidation of fatty acids due to their similarity with corresponding *E. coli* genes; these are: *lcfA, lcfB(yhfL), fadB*$_{Bs}$*(ysiB), acdA, fadNAE(yusLKJ)* (Matsuoka et al. 2007) and the *mmgABC* genes transcribed by the σ^E-RNA polymerase (Bryan et al. 1996). The conservation of protein sequences among different species suggested that β-oxidation plays indispensable functions under certain physiological conditions in *B. subtilis*, such as sporulation (González-Pastor et al. 2003) as well as calcium carbonate biomineralization (Barabesi et al. 2007). Among the genes products involved in fatty acids β-oxidation in *B. subtilis*, only FadR$_{Bs}$ (YsiA) has been recently characterized as a transcriptional regulatory protein, belonging to the TetR family, which negatively regulates the expression of majority of β-oxidation genes including those belonging to the *lcfA* operon (*fadR*$_{Bs}$, *fadB*$_{Bs}$, *etfB, etfA, fadNAE, fadHG*$_{Bs}$*(ykuFG), lcfB*, and *fadF*$_{Bs}$*(ywjF)-acdA-rpoE*) (Fujita et al. 2007; Matsuoka et al. 2007). However, the involvement of the FadR$_{Bs}$ (YsiA) regulon members in β-oxidation is only inferred from BLASTP results of their gene products (Matsuoka et al. 2007), therefore some compelling evidence is much needed to demonstrate what these genes actually do. The *B. subtilis* FadB$_{Bs}$ protein has previously been found to be homologous to eukaryotic and prokaryotic proteins belonging to the crotonase superfamily. The crotonase superfamily is comprised of mechanistically different proteins that share a conserved quaternary structure. Some enzymes in the superfamily have been shown to display hydratase and isomerase activity as well as the hydrolysis of thioesthers. Bacterial members of this superfamily have been found to be involved in different metabolic pathways such as fatty acids beta oxidation FadB$_{Ec}$ (DiRusso 1990), polyhydroxyalkanoate PHA (Tamao et al. 2003; Sato et al. 2007) and butanol biosynthesis CRT in *Clostridium acetobutilycum* (Inui et al. 2008). In this work, we report the cloning and the expression of *B. subtilis* FadB$_{Bs}$(YsiB) and its functional characterization as an enoyl-CoA hydratase.

Materials and Methods

Bacterial strains, plasmids Bacterial strains used in this study were *B. subtilis* 168 (*trpC2*, lab. stock) and *E. coli* BL 21 DE3 Gold (*tet*^{R}, Novagen); plasmid pET21b (*amp*^{R}, Novagen) was used to clone the *fadB*$_{Bs}$(*ysiB*) gene of *B. subtilis*. It carries an N-terminal T7•Tag sequence plus an optional C-Terminal His•Tag sequence.

DNA manipulation Isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonucleases, and transformation of *E. coli* were carried out by standard procedures. The *fadB*$_{Bs}$(*ysiB*) gene was amplified by PCR using the forward primer 5′-CCCTCGAGTTCGCCTTTGAACTGAGG-3′ and the reverse oligonucleotide primer 5′-GGAATTCCATATGAATGCAATTTCACTT-3′ (restriction sites used in cloning are underlined), and DNA of strain 168 as template. The amplified DNA was purified with Wizard Promega.

Purified *fadB*$_{Bs}$ and pET21b were digested with *Nde*I, *Ava*I (New England BioLabs), the resulting fragments were ligated and then used for transformation of *E. coli* BL21 DE3 Gold. Correct cloning of *fadB*$_{Bs}$ in plasmid pET21b-*fadB*$_{Bs}$ was confirmed by nucleotide sequencing.

Production and analysis of FadB$_{Bs}$ Recombinant *E. coli* BL21 DE3 (pET21b-*fadB*$_{Bs}$) cells were inoculated in 10 ml of LB medium containing Ampicillin 100 mg l^{-1} and Tetracyclin 12.5 mg l^{-1}. After an overnight growth at 37°C in a reciprocal shaker, cells were diluted in 50 ml of fresh LB medium (Amp 100 mg l^{-1}, Tet 12.5 mg l^{-1}) in a 500-ml flask (OD$_{600}$ about 0.2–0.3) and then cultivated at 37°C with vigorous aeration for 3 h, followed by 1 mM IPTG addition and an additional incubation for 2 h to achieve His$_6$-Tagged FadB production. After IPTG induction, cells were harvested (8,000 *g*, 20 min at 4°C) and resuspended in 50 mM MOPS pH 7.00 and 300 mM NaCl. The suspension was incubated on ice for 30 min with 1 mg/ml of lysozyme (Sigma) and then mechanically homogenized. After centrifugation (20,000 *g*, 30 min at 4°C), the resultant soluble fraction (crude extract) was loaded directly onto a Protino® 150 Column (Macherey-Nagel) and eluted with 50 mM MOPS pH 7.00, 300 mM NaCl, 250 mM imidazole. All purification procedures were carried out at 4°C to prevent protein denaturation and loss of activity.

The enoyl-CoA hydratase activity was measured by assaying the hydration of crotonyl-CoA (Sigma) at 263 nm (UltroSpec 2100 pro; Amersham Bioscience, Milan, Italy) through the decrease in absorbance of the coniugated double-bond band at 263 nm. In a final volume of 3 mL, 0.1 mL of enzyme solution were added to 44 mM Tris HCl buffer (pH 7.5), 0.0044% bovine serum albumin, 0.67 mM EDTA and 0.107 mM crotonyl-CoA. Decrease in absorbance at 263 nm was measured at 25°C. One unit (EU) of enoyl-CoA hydratase was defined as the amount of enzyme able to hydratate 1 μmol of crotonyl-CoA per min (using $\varepsilon_{263}=6.7\times10^3$ M^{-1}cm^{-1}). The specific activity of enoyl-CoA hydratase was defined as the activity of enoyl-CoA hydratase per milligram of protein. The kinetic constants, maximum reaction rate (V_{max}), and Michaelis constant (K_m), and their standard deviations, were determined using R 2.5.1 software (R Foundation for Statistical Computing, Vienna). Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis 12% was performed according to Laemmli protocol (Laemmli 1970) and stained with Blue

Brilliant Coomassie R250. Total proteins were assayed using Coomassie Blue Dye staining protocol (Bradford 1976)

Results and discussion

In order to assign a function to *fadB*$_{Bs}$ gene product, database search was carried out using default BLASTP parameters (http://blast.ncbi.nlm.nih.gov), results of which showed that FadB$_{Bs}$ has the highest identity with enoyl-CoA hydratase of *Bacillus amyloliquefaciens* FZB42 (77%, ABS74918) and with putative enoyl-CoA hydratase from *Bacillus licheniformis* DSM13 (73%, AAU41868) and ATCC14580 (73%, AAU24508), both only putatively attributed to their function. We found an enoyl-CoA hydratase/isomerase (ECH) domain in FadB$_{Bs}$ through SWISS-PROT and TrEMBL database search with the Expasy ScanProsite tool. The ECH domain is also shared with the crotonase of *C. acetobutylicum*, encoded by the *crt* gene belonging to a gene cluster showing a similar organization to that of the *lcfA* operon (Barabesi et al. 2007). Moreover, sequence alignement of the ECH domain found in FadB$_{Bs}$ with those of known enoyl-CoA hydratases from different sources displays significant identities with the residues known to be important for catalysis (Fig. 1; Angnihotri and Hung-wen 2003). These findings suggested that FadB$_{Bs}$ could be active towards crotonyl-CoA. In order to test this hypothesis, FadB$_{Bs}$ was modified to have a N-Terminal His-Tag, produced in *E. coli* and purified by a Ni-column (Macherey-Nagel). The *E. coli* crude extract and the eluate after purification showed an intense band of approximately 30.5 kDa (theoretical MW= 28.3 kDa) after SDS-PAGE separation (Fig. 2). Both crude extract and eluate were tested for proteins content, and enoyl-CoA hydratase activity using crotonyl-CoA as substrate by following absorbance decrease at 263 nm, according to described protocols (Hartmanis and Gatenbeck 1984). The activity of the crude extracts was 231 EU/mg of proteins [1 EU of enoyl-CoA hydratase was defined as the amount of enzyme able to hydratate 1 μmol of crotonyl-CoA per min (using $\varepsilon_{263}=6.7\times10^3\ M^{-1}cm^{-1}$)] and the first FadB$_{Bs}$ eluate showed enoyl-CoA hydratase activity of 1,603 EU/mg of proteins (yield 15% and purification 7-fold). We performed a kinetic analysis on the purified enzyme measuring both kinetic and specific constant parameters in the direction of the hydratation towards crotonyl-CoA. K_m value was 46.9 μM, very similar to other reported values for bacterial and mammals crotonases, e.g., *C. acetobutylicum* 30 μM (Bang et al. 2001), *E. coli* 50 μM (Binstock and Schulz 1981), *Homo sapiens* 30 μM (Jiang et al. 1996), and *Sus scrofa* 13 μM (Fong and Schulz 1977), confirming an analogue behavior for this ubiquitous enzyme. This evidence further suggests the identification of a real crotonyl-CoA activity. The values of FadB$_{Bs}$ specific constants were: k_{cat} 32±7 min^{-1}, k_{cat}/K_m 690± 190 $min^{-1}\,\mu M^{-1}$.

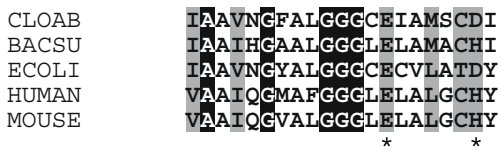

Fig. 1 Partial sequence alignment of proteins assigned as ECHs (enoyl-CoA hydratases) from bacterial and mammalian sources indicating the conserved residues present near the active site. Highlighted residues in *gray* indicate 75% identity, in *black* 100% identity. Residues marked with an *asterisk* are those important for catalysis. CLOAB *Clostridium acetobutylicum*, BACSU *Bacillus subtilis*, ECOLI *Escherichia coli*, HUMAN *Homo sapiens sapiens*, MOUSE *Rattus norvegicus*

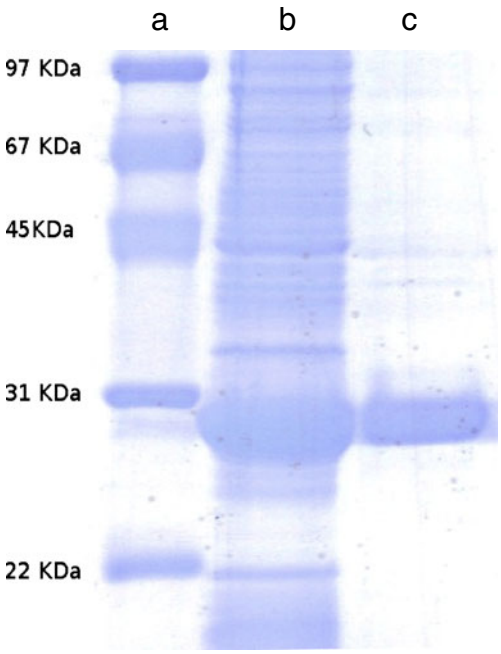

Fig. 2 SDS-PAGE analysis of FadB$_{Bs}$ (YsiB)-His$_6$-Tag from recombinant *E. coli* BL21 Gold DE3 cells. Lane **a** molecular weight standard, Bio-Rad low MW-range standard (cat. n. 161-0304); lane **b** total proteins obtained by *E. coli* lysis (crude extract); lane **c** first elution from Protino columns

B. subtilis FadB$_{Bs}$, as well as FadN, has been proposed to be involved in the fatty acid β-oxidation pathway (Fujita et al. 2007). Data reported in this work revealed that FadB$_{Bs}$ is active in the hydratation of crotonyl-CoA, supporting the possibility of its direct involvement in the β-oxidation pathway. The findings here reported contribute to a new and relevant piece of information to the poorly characterized β-oxidation pathway in *B. subtilis*.

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