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Identification and characterization of WhiB-like family proteins of the *Bifidobacterium* genus

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ABSTRACT

Bifidobacteria are strictly anaerobic bacteria, that are an important component of human microbiote due to their probiotic characteristics. They are frequently exposed to a variety of stresses, therefore, identification of genes implicated in stress responses in bifidobacteria is critical for biomedicine and maintenance of industrial strains. The WhiB-like family proteins unique for *Actinobacteria* are transcriptional regulators involved in major cellular processes, including stress responses. The aim of this study was the identification of WhiB-like family proteins of the *Bifidobacterium* genus of the *Actinobacteria* class and functional characterization of conservative *whiB*-like genes. The DNA sequence database of 36 strains revealed a family of WhiB-encoding genes. It were identified the *wblE* orthologs in all *Bifidobacteria* species and the *whiB2* orthologs in all bifidobacterial strains except of all strains of *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium gallicum*. Some strains, in particular, those of the *Bifidobacterium longum* group, contain additional *whiB*-like genes of different length and a low degree of similarity in sequences. The *wblE* and *whiB2* genes of the *Bifidobacterium* genus are evolutionary conservative and ancient genes. The real-time PCR analysis showed that transcription of *wblE* is induced by a variety of stress conditions such as heat shock, osmotic, oxidative stresses, by antibiotic tetracycline and bile salt treatment, the nutrient starvation and entry into late stationary phase. The *wblE* gene may play a significant role in general stress responses in bifidobacteria.

1. Introduction

Bifidobacteria are an important component of human gastrointestinal microbiota [1]. In recent years bifidobacteria have been subject of growing interest due to their potential health-promoting or probiotic properties [2]. Some bifidobacterial strains with probiotic activities are used in so-called functional food [3]. Probiotic bifidobacteria protect host intestine against pathogens via competitive exclusion, positively influence the intestinal cell physiology, contribute to host nutrition and participate in development of the immune system [4,5].

The *Bifidobacterium* genus belongs to the *Actinobacteria* class and is represented by high G + C Gram-positive, non-motile, non-sporulating, non-gas producing, strictly anaerobic bacteria [6]. The *Bifidobacterium* genus is comprised of 37 species with four taxa (*Bifidobacterium longum*, *Bifidobacterium pseudolongum*, *Bifidobacterium animalis*, *Bifidobacterium thermacidophilum*) subdivided into subspecies [5]. The species that reside in the human gastrointestinal tract, that is, *Bifidobacterium breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium bifidum*, *B. adolescentis*, *B. animalis* subsp. *lactis* are being extensively studied due to their probiotic properties [7].

The probiotic bifidobacteria can be exposed to potentially stressful conditions in industrial processes, in the course of passage through gastrointestinal tract and in their natural environments. These bacteria have to be capable of rapid response to a variety of stress that is essential for survival [8]. Therefore, it is important to identify the genes implicated in stress response in bifidobaceria. We focused on the genes encoding the WhiB-like family proteins. This family is unique for actinobacteria and have not been found in other prokaryotic or eukaryotic organisms [9]. The WhiB-like proteins are transcriptional regulators involved in major cellular processes such as cell division [10], pathogenicity [11,12], antibiotic resistance [13], response to oxidative stress [14] and to a wide variety of other stresses [15]. Initially the *whiB* gene has been identified in *Streptomyces coelicolor* as essential for sporulation of aerial hyphae. The genes involved in sporulation have been identified in mutants that gained a 'white' (whi) phenotype and were unable to form normal gray spore pigment [16]. The WhiB family proteins are small <200 amino acid residues. They contain four

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conserved near-invariant cysteine residues C(×n)C(×2)–C(×5)C, including common CXXC motif. Proteins with this motif have been implicated in manifold functions such as disulfide reductase activity [17], redox sensing [18] and coordination of metal cofactors [19]. Some members of the WhiB family bind to the redox-sensitive [4Fe–4S] cluster [20,21], similarly to metal-coordinating DNA-binding proteins Fnr of *Escherichia coli* [22] that bind [4Fe–4S] cluster under anaerobic conditions. The Fe–S cluster proteins play essential roles in sensing external signals as well as the intracellular redox state [23]. It was reported the data consistent with the function of WhiB-like (Wbl) proteins as disulfide reductases [20,24]. The presence of a putative helix–turn–helix motif in the C-termini of WhiB implies that these proteins may bind to DNA [25]. A one-hybrid system in *E. coli* demonstrated the *in vivo* interaction between WhiB3 of *Micobacterium tuberculosis* and the promoters of several genes whose expression is known to be influenced by *whiB3* [26].

The *whiB-like (wbl)* genes are present in the majority of sequenced actinobacterial genomes including 76 non-spore-forming intracellular pathogens. Most actinobacterial species contain multiple copies of the *wbl* gene. Genome sequencing revealed over 270 Wbl homologues [9]. Genes related to *whiB* are also present in some actinophages that had likely acquired them from actinobacteria [26]. Including *whiB* and *whiD*, there are 14 *wbl* family genes in *S. coelicolor*, 11 on the chromosome and 3 localized on the giant linear plasmid [27]. The homologues of these genes are present also in 4 other streptomycetes [28]. In *M. tuberculosis* genome 7 *whiB*-like genes (*whiB1* to *whiB7*) have been discovered [29]. Using quantitative real-time reverse transcription-PCR (RT-PCR), Geman and colleagues [15] analyzed the expression of 7 *whiB*-like genes in *M. tuberculosis* under various growth conditions and in response to stresses such as the exposure to antibiotics, nutrient deprivation, acid, ethanol, detergent, oxidative, heat, and low iron. That study showed that WhiB family members were highly responsive to a wide variety of stresses and therefore may play a key role in general stress responses in mycobacteria. Here we report the identification and characterization of *whiB* **-** like genes of *Bifidobacterium* genus and analyze their role in sensing of the environmental stress signals.

2. Materials and methods

2.1. Bacterial strains, media, culture condition and reagents

The industrial probiotic *B. longum* subsp. *longum* strain B 379M obtained from the culture collection of Gabrichevsky Epidemiology and Microbiology Research Institute, Moscow. The *E. coli* DH5 (F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) (Stratagene) was used for general subcloning procedures. The expression of recombinant proteins was carried out in *E. coli* BL21(DE3) (F^-, *ompT*, *gal*, *dcm*, *lon*, *hsd*, *SB* (*rB– mB–*) (DE3) (Novagen, Germany). The Luria-Bertani (LB) broth and LB agar were used to culture *E. coli*. Cells were grown at 37 °C with shaking (250 r.p.m.). Bifidobacterial strain was cultured in Mann Rogosa Sharp (MRS) agar supplemented with 0.05% L-cysteine–HCl and in Bifidobacterium Broth (BB) from HiMedia (India) at 37 °C under anaerobic conditions. The concentrations of antibiotics were: ampicillin 100 μg/ml, tetracycline 3 μg/ml. The pET32a plasmid was purchased from Novagen. T4 DNA ligase, restriction enzymes and prestained protein molecular weight marker were from Fermentas (Lithuania). PCR kits with *Taq* polymerase from Dialat Ltd (Moscow, Russia), oligonucleotide primers were synthesized by Syntol (Moscow, Russia). Plasmid purification kits and RNeasy Mini kits were from Qiagen (Germany).

2.2. DNA techniques

The manipulations with DNA, including restriction endonuclease digestion, isolation and ligation of DNA fragments, *E. coli* transformation, agarose and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described by Sambrook et al. [30]. Preparation of genomic DNA from *B. longum* subsp. *longum* strain B 379M was performed as described by Regnault et al. [31].

2.3. Protein expression and purification

The gene-specific primers were used to amplify the complete ORFs of *whiB2* and *wblE* from genomic DNA of *B. longum* subsp. *longum* strain B 379M as a template. The nucleotide sequences were designed based on the open reading frame of BL1008/*whiB2* and BL1011/*wblE* from chromosomal DNA of *B. longum* subsp. *longum* NCC 2705 (GenBank accession number NP 696180 and NP 696183, respectively) with additional nucleotides at the N-terminus for cloning into *Eco*R1/*Hind*III sites of *pET32a* vector under an isopropyl thio-β-D-galactoside inducible promoter. Primer sequences were to *whiB2* gene: F 5′-TCCGAATTCATGTGGGGTG TGGTCGACG-3′ and R 5′-CCGCAAGCTTCTACGCCCGCTCCCGGT-3′; to *wblE* gene F 5′-ATCCGAATTCATGAGCAGCGCTTTTGATTGG-3′, and R5′-CCGCAAGCTTTCAGATTTG CATCTGCATGGC-3′. PCR amplifications were carried out as follows: each of 30 cycles 94 °C 40 s, 58 °C 40 s, 72 °C 1 min, then the final extension at 72 °C for 10 min. The PCR products, used for cloning and sequencing, were purified with the QIAGEN gel extraction kit according to the manufacturer's instructions. The PCR product was determined by sequencing both the strands of DNA in Applied Biosystem Genetic Analysator 3100 using primers to *whiB2* and *wblE* genes. The amplified ORFs were cloned into the pET32a vector. Recombinant plasmids were subcloned into *E. coli* DH5 strain. The colonies of transformants were analyzed by PCR using the standard primers T7prom:5′-TTAA TACGACTCACTATAGG-3′; T7term:5′-CTAGT TATTGCTCAGCGG-3` and S.Tag: 5′-CGAACGCCAGCACATGGACAG-3′. PCR products were carried out as follows: each of 25 cycles 94 °C 40 s, 52 °C 40 s, 72 °C 1 min, then the final extension at 72 °C for 10 min. To expression of recombinant protein *E. coli* BL21 (DE3) strain was used. The recombinant plasmids pET32-whiB and pET32-wblE were transformed on competent cells of *E. coli* BL21(DE3). 2 ml of LB broth containing 100 μg/ml ampicillin was inoculated with 50 ml of transformed cells and incubated overnight at 37 °C and 250 r.p.m. Fifty microlitres of the overnight cultures was used to reinoculate 2 ml of fresh LB broth containing 100 μg/ml ampicillin and was then incubated at 37 °C and 250 r.p.m. until A_{600} 0.6. After this, the cultures were induced by the addiction of 1 mM isopropyl thio-beta-D-galactoside (IPTG) for 3 h at 28 °C. Pellet of cells from 4 ml culture were resuspended in 500 μl lysis buffer PMSF (20 mM/1 KH_2PO_4, 200 mM/1 NaCl, 0.1 mM/1 PMSF, 5 mM/1 EDTA, pH 7.2). After one cycle of freezing-thawing cells were lysed by sonication. Proteins were resolved by 12% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

2.4. Total RNA isolation and RT-PCR

To study stress responses, the cultures of bifidobacteria were grown in BB to an A_{600} 0.8, divided into 50 ml aliquots and treated with 1 mM H_2O_2, 3 μg/ml tetracycline (subinhibitory concentrations), NaCl (4%), bile salt (0.5%). One 50 ml aliquot of broth culture was centrifuged (15 min 5000 × g), the pellet was washed in PBS, centrifuged and then the pellet resuspended in PBS (same volume). One 50 ml aliquot of broth culture was incubated at 45 °C. The cultures were treated with stress stimuli for 2 h at 37 °C under anaerobic conditions. To study the level of expression at