

Constitutive expression of phosphoketolase, a key enzyme for metabolic shift from homo- to heterolactic fermentation in *Enterococcus mundtii* QU 25

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Phosphoketolase (PK) is responsible for heterolactic fermentation; however, the PK gene of *Enterococcus mundtii* QU 25, *xfpA*, is transcribed constitutively, even under homolactic fermentation conditions. In order to deduce the regulatory mechanisms of PK activity in QU 25, XfpA levels in QU 25 cells under hetero- and homolactic fermentation conditions were tested using western blotting. The results showed that the XfpA protein expression was similar under both conditions and that the expression products formed complexes, most likely homodimers, indicating that the regulation of PK activity is downstream of translation.

Key words: heterolactic fermentation, metabolic shift, phosphoketolase activation, homodimer, *Enterococcus mundtii*, xylose concentration, constitutive expression

Phosphoketolases (PKs) catalyse the cleavage of D-xylulose 5-phosphate (X5P) and inorganic phosphate to form acetyl phosphate and D-glyceraldehyde 3-phosphate. Based on substrate preference, the enzymes are classified into two groups: X5P phosphoketolase (XPK, EC4.1.2.9), whose substrate is only X5P, and X5P/D-fructose 6-phosphate (F6P) phosphoketolase (XFPK, EC4.1.2.22), which is able to cleave F6P to D-erythrose 4-phosphate and acetyl phosphate as well as X5P. Genes for XPK-type PK have been found mainly in lactic acid bacteria of the phylum *Firmicutes*, with XPK comprising the first step in the PK pathway of heterolactic fermentation from pentoses. Genes for XFPK-type PK reside in the genomes of bifidobacteria and some eukaryotes, such as fungi. XPK is a major member of the PK pathway for heterolactic fermentation in *Firmicutes*, and XFPK is a member of both the F6P shunt and PK pathways in bifidobacteria [1, 2]. The non-rooted phylogenetic tree of PK exhibits a dumbbell shape with two distinct groups, XPK and XFPK, and the amino acid sequence homologies between them were shown to be more than 40%. Furthermore, the crystal structure of XFPK from *Bifidobacterium breve* 203

revealed that the minimal functional unit of this enzyme is a homodimer [3].

Lactic acid bacteria have been proposed for use in the bioconversion of non-exhaustive and plant-derived biomasses to produce optically pure L-lactic acid, as a feedstock for green plastic poly-L-(+)-lactic acid [4]. *Enterococcus mundtii* QU 25 is a non-dairy lactic acid bacterium, isolated from ovine faeces, that ferments xylose [5, 6]. This strain produces high levels of L-lactic acid with high optical purity when cultured under optimal conditions, using not only glucose but also xylose as the sole carbon source, and is a likely candidate for this purpose, as cellulose and xylan are major ingredients of various biomasses. In a previous study, QU 25 produced L-lactic acid via homolactic fermentation using a pentose-phosphate pathway, and no PK activity was detected when cells were cultured in media containing xylose as the sole carbon source (100 g/L). Conversely, a metabolic shift to heterolactic fermentation was observed when cells were cultured in media with xylose concentrations of less than 25 g/L, with by-products such as acetic acid, formic acid, and ethanol emitted in addition to lactic acid, and PK activity was detected at substantial levels [6]. Therefore, PK is likely to be a key enzyme in the metabolic shift between homo- and heterolactic fermentation in QU 25.

The complete 3.02 Mb genome sequence of QU 25 has been analysed, and a coding sequence, EMQU_1837, was annotated as D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase and was denoted as *xfpA*. Other coding

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sequences did not exhibit similarities to those of PKs [7]. Next, we found that *xfpA* was transcribed constitutively by RNA sequencing and northern analyses under both the homo- and heterolactic fermentation conditions [8], which means that the metabolic shift in QU 25 was regulated downstream of transcription.

To increase the yield of pure L-lactate produced by *Enterococcus mundtii* QU 25 from biomasses, it is crucial to investigate the metabolic shift between homo- and heterolactic fermentation employed by this strain. In this study, we detected essentially the same amount of XfpA protein in the QU 25 cells, regardless of the fermentation type, though the PK activities were detected only in the cells obtained under heterolactic fermentation conditions.

The QU 25 cells were obtained by batch cultures at 43°C and pH 7.0 in modified Man, Rogosa, and Sharpe (mMRS) media, including the indicated amount of xylose as the sole carbon source under the culture conditions described previously [6]. Cultures were monitored by measuring optical density at 562 nm, consumption of xylose, and production of byproducts. PK activities were measured at 43°C and pH 6.5 using the hydroxamate assay to detect the production of acetyl phosphate, as reported by Meile *et al.* [1].

To confirm that *xfpA* is a PK gene in QU 25 and to obtain antibodies against XfpA for the detection of the protein in QU 25 cells under various conditions by western blotting, a recombinant XfpA (rXfpA)-overexpressing *Escherichia coli* strain was constructed as follows. Two primers, the forward primer (*Hind*III) 5'-CCCAAGCTTgaAAGGAGgtgtcgatATGACAAACGTAGACAAAC-3' and the reverse primer (*Xho*I) 5'-ccgctcgagTTCCTTACCCGAGCTAACTCC-3', were used for amplification of the *xfpA* sequence in the QU 25 genome, and the resulting polymerase chain reaction product was inserted between the *Hind*III and *Xho*I sites of the modified pETNH vector. In this construct, a 6xHis tag was added to the C-terminal region of XfpA. The *Escherichia coli* Rosetta II system was used for the overproduction of rXfpA, and the obtained recombinant protein was purified as described previously [9]. Rabbit serum sensitized with the obtained rXfpA was prepared by ordering it from Eurofins Genomics Inc. (Tokyo, Japan) and used as polyclonal anti-rXfpA antibodies, after affinity purification on blots to polyvinylidene difluoride membranes [10]. A polyclonal antibody against Sigma factor A (SigA) of *Bacillus subtilis* 168, which consists of 371 amino acid residues, was gifted by F. Kawamura [11]. SigA, known as one of the major sigma factors, is a component of RNA polymerase holoenzyme, and orthologues of its gene are widespread in bacteria. The amino acid sequence identity between SigAs of *B. subtilis* 168 and of QU 25 is 92.8% in the 277 amino acid residues of their C-terminal parts.

After sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), the total protein obtained from QU 25 cells was subjected to western blotting as described previously [12], and for complex analyses of XfpA, cross-linking was performed with 1% formaldehyde for 15 min at room temperature prior to disruption of the cells, to

form covalent bonds between components of complexes as described elsewhere [13]. For complex analyses, native PAGE in the absence of SDS and 2-mercaptoethanol was also performed according to the protocol of Onda (<http://www.pssj.jp/archives/files/articles/011.pdf>) prior to western blotting.

The deduced XfpA protein of *Enterococcus mundtii* QU 25 consisted of 791 amino acids, and its average molecular mass was calculated to be 89,514.2 daltons. The rXfpA obtained exhibited weak but distinct activities when using X5P as a substrate (Supplementary Fig. 1) but did not when F6P was used as a substrate (data not shown). In addition, a non-rooted phylogenetic tree of PKs was constructed by adding the data of XfpA from QU 25 to that reported by Suzuki *et al.* [3] with the software FigTree ver. 1.4.1 (<http://tree.bio.ed.ac.uk/software/figtree/>). Supplementary Fig. 2 displays the position of XfpA in the XPK group. These results indicate that *xfpA* of QU 25 is a PK gene and that XfpA belongs to the XPK group, despite the annotation, D-Xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase.

XfpA proteins in QU 25 cells obtained under homo- and heterolactic fermentation conditions were examined by western blotting using anti-rXfpA antibodies. When QU 25 was grown in mMRS medium supplemented with 25 g/L of xylose, lactic acid, the by-products of acetic and formic acids, and ethanol were detected in the growth medium, showing heterolactic fermentation (Supplementary Table 1), similar to the results reported previously [6]. This indicates that PK in the QU 25 cells was active under these conditions. When QU 25 was grown in the same medium supplemented with 100 g/L of xylose, acetic and formic acids and ethanol were scarcely detected in the medium, indicating that homolactic fermentation occurred (Supplementary Table 2), as reported previously [6], and that PK was not functional in these cells. However, as the results of western blotting show in Fig. 1a, anti-rXfpA antibodies detected proteins with molecular masses of approximately 90 kilodaltons (kDa) in all the samples from QU 25 cells, under all culture conditions, those were most likely XfpA, since their molecular masses were consistent with that calculated for the XfpA of QU 25 (89,514.2 daltons). The ratio of the amount of XfpA in the cells grown for 12 hr (semi-stationary phase, see Supplementary Table 1) of homolactic fermentation to that of heterolactic fermentation was calculated to be 0.96 ± 0.18 , after their band intensities were standardized using SigA, which is known to be a protein expressed constitutively. Therefore, these results indicate that an almost equal amount of XfpA resides in QU 25 cells under both hetero- and homolactic fermentation, although PK activity was not detected in the cells obtained under homolactic fermentation conditions. A slightly larger band than those in QU 25 cells (Fig. 1a, lane 7) was also detected in the *Escherichia coli* cells producing rXfpA with the 6xHis tag, whose molecular mass was calculated to be 90,227.0 daltons.

As the bifidus PKs have been reported to act as a homodimer [3], attempts were made to examine whether or

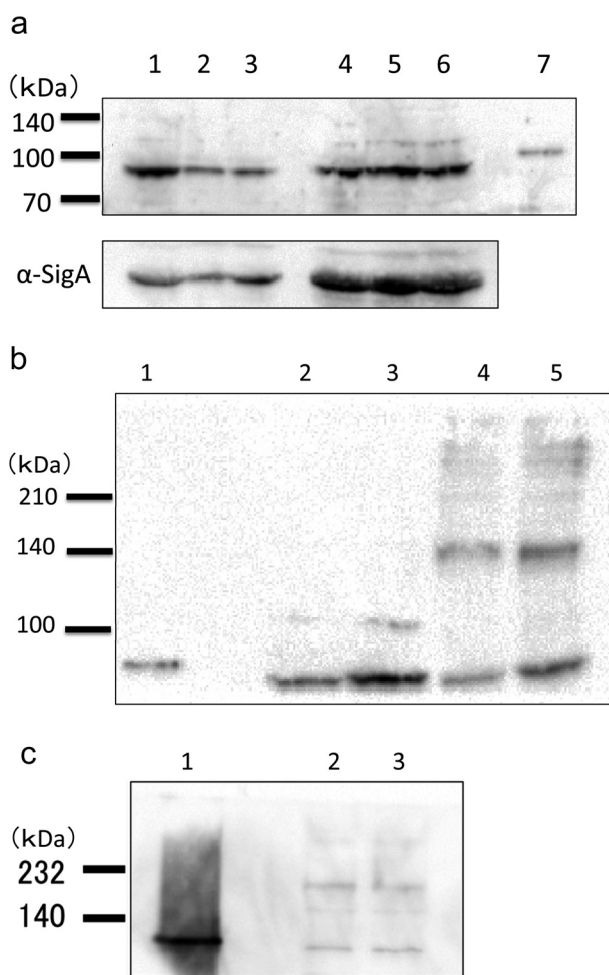


Fig. 1. Western blot analysis of XfpA in QU 25 cells using anti-rXfpA antibodies. (a) Cells grown at 43°C and pH 7.0 were obtained at 6 hr (lanes 1, 4), 12 hr (lanes 2, 5), and 24 hr (lanes 3, 6) after inoculation in mMRS media containing 25 g/L xylose (lanes 1–3) and 100 g/L xylose (lanes 4–6). In lane 7, purified rXfpA was applied. SigA, which was detected by anti-SigA antibodies, was used as a control for loading. (b) Complex analyses of XfpA I. Cross-linking was performed with 1% formaldehyde to form covalent bonds between components of complexes before cell disruption. (1) rXfpA, (2) cells grown in mMRS containing 25 g/L xylose without cross-linking, (3) cells grown in mMRS containing 100 g/L xylose without cross-linking, (4) cells grown in mMRS containing 25 g/L xylose with cross-linking, (5) cells grown in mMRS containing 100 g/L xylose with cross-linking. (c) Complex analyses of XfpA II. Native-PAGE was performed. (1) rXfpA, (2) cells grown in mMRS containing 25 g/L xylose, (3) cells grown in mMRS containing 100 g/L xylose. kDa, kilodalton(s).

not XfpA forms a homodimer to gain its enzymic activity in heterolactic QU 25 cells. Then, after cross-linking with formaldehyde, proteins of QU 25 cells grown for 12 hr under both fermentation conditions were subjected to western blotting using anti-rXfpA antibodies. As shown in Fig. 1b,

the antibodies detected complexes, including XfpA, with molecular masses of approximately 180 kDa under both homo- and heterolactic fermentation. In addition, western blotting with native-PAGE (Fig. 1c) showed that two major bands were detected in both samples, with sizes of less than 100 kDa and less than 200 kDa. These results indicate that XfpA forms complexes in QU 25 cells, regardless of its activities, and it is likely that the complexes are homodimers of XfpA because of their molecular masses.

To date, some regulatory mechanisms underlying PK activation in bacteria have been elucidated, such as transcriptional induction and carbon catabolite repression [14], along with post-translational allosteric-effects on their substrates and downstream metabolites [15]. Abdel-Rahman [6] reported that PK was responsible for heterolactic fermentation, because only QU 25 cells obtained under heterolactic fermentation conditions had PK activities. We have investigated the regulatory mechanisms of PK activity with the metabolic shift between homo- and heterolactic fermentation. However, RNA sequencing and northern blotting did not show quantitative differences between transcripts of *xfpA* (the PK gene of QU 25) in both fermentation types of cells [8]. In this paper, the gene product XfpA was examined by western blotting, and it was shown that XfpA resides in the cells obtained under heterolactic fermentation conditions in essentially the same amount as in those obtained under homolactic fermentation conditions and that the proteins form complexes, most likely homodimers. These results indicate that the regulation of PK activity is downstream of the translational level. However, it is unlikely that the allosteric effect reported above leads to the activation of PK in the QU 25 cells, because it is not gradual. Therefore, other possibilities for the regulatory mechanisms of PK activation in QU 25 at the post-translational level should be discussed, such as protein modification and redox regulation. For protein modification, the possibility of acetylation and phosphorylation by one of the products of PK, acetyl phosphate, has been previously mentioned by Glenn and Smith [15] in the *Lactobacillus plantarum* PK. In addition, there are a number of examples for activation by phosphorylation of other bacterial enzymes, such as bacterial two-component signal transduction, in which histidine protein kinases are activated by autophosphorylation depending on the environmental stimuli, following which the phosphoryl groups are transferred to response regulators to form active transcriptional regulators [16]. For redox regulation, reduced thioredoxins activate some enzymes by forming –SH groups from disulphide bonds present in the enzymes [17]. Further investigation is needed.

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