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Molecular evolution of the ATPase subunit of three archaeal sugar ABC transporters☆

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Abstract

The sequence of genes encoding extracellular amylopullulanase and putative maltodextrin ATP binding cassette (ABC) transporter of a hyperthermophilic archaeon, *Thermococcus litoralis*, was determined. The *mdxK* gene, which encodes an ATPase subunit of the putative maltodextrin ABC transporter, has extraordinarily high similarity with the *malK* gene, which encodes an ATPase subunit of trehalose/maltose ABC transporter of the same organism. DNA sequence comparison revealed that the *malK* gene was generated through the duplication of the *mdxK* gene before lateral gene transfer of the *mal* gene cluster from *T. litoralis* to *Pyrococcus furiosus*.

Keywords: ABC transporter; Amylopullulanase; *Thermococcus litoralis*; Glycoside hydrolase family 57; Molecular evolution

It is known that hyperthermophilic archaea *Thermococcus litoralis* and *Pyrococcus furiosus* utilize the same novel modified Embden–Meyerhof glycolytic pathway, involving ADP-dependent glucokinase, ADP-dependent phosphofructokinase, and glyceraldehyde-3-phosphate:ferredoxin oxidoreductase [1,2]. However, the pathway from starch or long maltodextrins outside of the cell to the modified EM pathway seems to differ between the two organisms. In *P. furiosus*, two extracellular enzymes; amylopullulanase [3] and extracellular α-amylase [4] seem to be the first enzymes in the pathway, hydrolyzing extracellular starch or long maltodextrins to produce short maltodextrins. The short maltodextrins produced are transported into cells via two ATP binding cassette (ABC) transport systems, trehalose/maltose ABC transporter, and maltodextrin ABC transporter [5,6]. On the other hand, in *T. litoralis*, extracellular α-amylase activity has not been detected [7], nor has maltodextrin ABC transporter been reported. In addition, neither the nucleotide nor the amino acid sequence of amylopullulanase has been reported yet, although it has been purified from the medium of *T. litoralis* and partially characterized [7].

Here, we report sequencing of the genes encoding amylopullulanase and putative maltodextrin ABC transporter of *T. litoralis*. Amylopullulanase and putative maltodextrin-binding protein were expressed in *Escherichia coli*, purified and characterized. Interestingly, nucleotide sequence of the ATPase subunit gene of *T. litoralis* maltodextrin ABC transporter is extraordinarily homologous to those of trehalose/maltose transporter of *T. litoralis* and *P. furiosus*, despite the low sequence similarity of other subunits. Sequence comparison of these genes provided a new insight into molecular evolution of ATPase subunit of these sugar transporters, which is responsible for energization of transport.

☆ *Abbreviations:* ABC, ATP binding cassette; ORF, open reading frame; TLAPU, amylopullulanase from *Thermococcus litoralis*; *Eco*MBP, *Escherichia coli* maltose binding protein; *Tli*TMBP, *T. litoralis* trehalose/maltose binding protein; *Pfu*MBP, *Pyrococcus furiosus* maltodextrin binding protein.

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Materials and methods

Bacterial strains. For DNA manipulation, *E. coli* strains JM109 and XL10-Gold (Stratagene) were used. *E. coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene) was used for heterologous expression of the target genes.

Chemicals. The DNA Ligation Kit and ExTaq DNA polymerase were from Takara (Kyoto, Japan). Oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). Soluble starch was purchased from Merck. Pullulan was from Hayashibara Biochemical Laboratories (Okayama, Japan). Maltooligosaccharides and other reagents were from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise noted.

Preparation of a DIG-labeled probe. The DNA probe for gene cloning was prepared by means of PCR. Two amino acid sequences, EPKPLNV and TLDGENP, that are conserved in the amylopullulanases from *P. furiosus* and *Thermococcus hydrothermalis* were chosen, and a sense primer (5′-GA(A/G)CC(T/C/A/G)AA(A/G)CC(T/C/A/G)CT(T/C/A/G)AA(T/C)GT-3′) and an anti-sense one (5′-AC(T/C/A/G)CT(T/C/A/G)GA(T/C)GG(T/C/A/G)GA(A/G)AA(T/C)CC-3′) for PCR were constructed based on the amino acid sequences. Chromosomal DNA from *T. litoralis* (DSM5473) was prepared as described previously [8]. PCR was performed in a 50 μL reaction mixture with 500 ng *T. litoralis* chromosomal DNA as the template and 100 pmol each of two primers with 1 nmol of DIG-dUTP (Roche Diagnostics).

Southern hybridization. *Thermococcus litoralis* chromosomal DNA was digested with restriction endonucleases. The resultant fragments were separated on a 0.8% agarose gel, transferred to a Hybond N^+ membrane (Amersham Biosciences), and then hybridized with the DIG-labeled PCR product. The hybridized fragments were detected with the DIG Nucleic Acid Detection Kit (Roche Diagnostics).

Sequencing strategy. Upstream and downstream of the cloned fragment were sequenced as follows. *T. litoralis* chromosomal DNA was digested with a restriction enzyme, followed by self-ligation of the resultant fragments, by which a circular DNA library was generated. Using two primers, which were designed to anneal back to back, and ExTaq DNA polymerase, PCR was performed with the circular DNA library as a template, the flanked regions of the cloned region being amplified. Then, the PCR product was sequenced directly. By repeating this procedure, a total of 8920-bp, including the cloned 2539-bp region, was sequenced.

Construction of an expression plasmid for amylopullulanase. DNA fragment encoding residues 25–848 of amylopullulanase was amplified by PCR, and then ligated between the *Nde*I and *Xho*I sites of pET-21a to obtain pTLAPU2.

Expression and purification of recombinant amylopullulanase. *E. coli* cells expressing amylopullulanase were suspended in 20 mM Mes–NaOH buffer, pH 6.5 (buffer A). The suspension was sonicated and centrifuged. The supernatant was incubated for 30 min at 85 °C and then centrifuged to remove denatured *E. coli* proteins. The resultant supernatant was mixed with a 0.1 volume of 5 M NaCl, and then applied to a Ni^{2+} charged HiTrap-chelating column (Amersham Biosciences) equilibrated with buffer A containing 0.5 M NaCl. After washing with buffer A containing 0.5 M NaCl and 0.05 M imidazole, pH 6.5, the enzyme was eluted with buffer A containing 0.5 M NaCl and 0.5 M imidazole, pH 6.5.

Construction of an expression plasmid for maltodextrin binding protein. DNA fragment encoding residues 35–436 of maltodextrin binding protein was amplified by PCR, and then ligated between the *Bam*HI and *Eco*RI sites of pRSETB (Invitrogen) to obtain pHisMdxE.

Expression and purification of recombinant maltodextrin binding protein. *Escherichia coli* cells expressing maltodextrin binding protein were suspended in 20 mM Tris–HCl, pH 7.4. The suspension was sonicated and centrifuged. The supernatant was incubated for 30 min at 80 °C and then centrifuged to remove denatured *E. coli* proteins. The resultant supernatant was mixed with a 0.1 volume of 5 M NaCl, and then applied to a Ni^{2+} charged HiTrap chelating column equilibrated with 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl (buffer B). Nonspecifically bound proteins were washed out with buffer B containing 0.05 M imidazole, pH 7.4. The enzyme was subsequently eluted with buffer B containing 0.5 M imidazole, pH 7.4.

Maltodextrin binding assay. Fifty microliters of 20 mM Tris–HCl, pH 7.4, containing 24 μg of maltodextrin binding protein was added to 50 μL of amylose resin (New England Biolabs) equilibrated with 20 mM Tris–HCl, pH 7.4, followed by gentle shaking for 10 min. The resin was washed twice with 100 μL of 20 mM Tris–HCl, pH 7.4. Then MBP was eluted with 50 μL of 20 mM Tris–HCl, pH 7.4, containing maltose, maltotriose, or maltohexaose. The amount of MBP eluted was determined by SDS–PAGE. The sample proteins were mixed with an equal volume of sample buffer and then boiled for 5 min prior to electrophoresis. Twenty microliters was applied to the gel. The gels were stained with Coomassie brilliant blue.

Results and discussion

Gene structure

Although the gene encoding amylopullulanase of *T. litoralis* has not been cloned yet, DNA sequences encoding amylopullulanase have been reported for closely related organisms, *T. hydrothermalis* [9], *Pyrococcus abyssi* (EMBL accession code, AJ248283.1), and *P. furiosus* [3]. Based on the two consensus regions in these amylopullulanases, a DIG-labeled probe was generated by PCR. A 2.5-kb *Eco*RI fragment of *T. litoralis* chromosomal DNA was most clearly detected by Southern blotting (data not shown). The chromosomal DNA digested with *Eco*RI was size fractionated on a 0.8% agarose gel, and then a library with an insert size of approximately 2–3 kb was constructed at the *Eco*RI site of pUC18. After screening of the recombinant plasmid by Southern hybridization, a positive clone, carrying a 2.5-kb *Eco*RI fragment, was obtained. Then, we sequenced the flanking regions of the cloned fragment. Finally, we sequenced an 8.9-kb *T. litoralis* DNA fragment containing seven ORFs (Fig. 1). ORF 6 encoded a hypothetical protein of 138 amino acids. The protein encoded by ORF 7 exhibits similarity to phospho-sugar mutase, although its 3′ region was not sequenced.

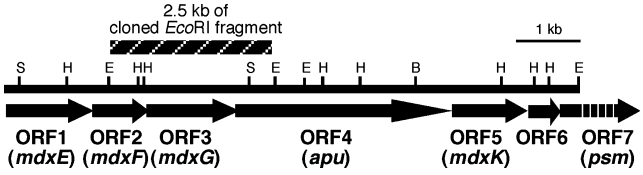

Fig. 1. Structure of the gene cluster for maltodextrin uptake. The solid line indicates the region sequenced in this study. Arrows indicate the locations and directions of the ORFs. Restriction sites are also shown: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and S, *Sph*I. *mdxE*, gene encoding maltodextrin binding protein; *mdxF* and *mdxG*, genes encoding transmembrane permease subunits of maltodextrin transporter; *apu*, gene encoding amylopullulanase; *mdxK*, gene encoding ATPase subunit of maltodextrin transporter; and *psm*, gene encoding putative phospho-sugar mutase.

ORF 4 encodes amylopullulanase

ORF 4 encodes a putative amylopullulanase. The deduced amino acid sequence indicates a putative amylopullulanase consisting of 1089 amino acid residues with a calculated molecular mass of 124 kDa, which is consistent with the reported molecular mass (119 kDa) of amylopullulanase purified from a culture of *T. litoralis* [7]. As expected, the putative amylopullulanase of *T. litoralis* was homologous to three previously reported amylopullulanases that belong to glycoside hydrolase family 57 (according to Henrissat's classification [10,11]); 77%, 75%, and 74% identity with a putative amylopullulanase of *P. abyssi*, amylopullulanase of *P. furiosus*, and type II pullulanase of *T. hydrothermalis*, respectively. Residues 25–848 of the *T. litoralis* putative amylopullulanase, which was assumed to be a minimum component of amylopullulanase on sequence comparison with *P. furiosus* amylopullulanase, were expressed in *E. coli*.

The purified recombinant enzyme hydrolyzed pullulan, producing only maltotriose. No other oligosaccharides were produced in the course of the reaction (Fig. 2A), indicating that the recombinant enzyme only hydrolyzes the α-1,6-glucosidic linkage of pullulan in an exo manner. The recombinant enzyme also degraded starch into glucose and a series of maltodextrins, mainly ranging from maltotriose to maltoheptoase (Fig. 2B). The amount of maltose produced was significantly low as compared to that of glucose or other maltodextrins. When maltodextrins were used as substrates, the recombinant enzyme hydrolyzed the α-1,4 linkages of maltotetraose or longer maltodextrins in an exo manner, producing shorter maltodextrins and glucose (Fig. 2C), although maltotetraose and maltopentaose were poor substrates. These results indicated that the protein encoded by ORF4 is amylopullulanase. Hereafter, we will call this enzyme TLAPU. The pullulanase/amylase activity ratio of the recombinant TLAPU was about 16 (data not shown), suggesting its preference for the α-1,6 linkage compared to the α-1,4 linkage. It exhibited a pH optimum of 6.5, and a temperature optimum of above 100 °C (data not shown).

Protein encoded by ORF 1 has affinity for long maltodextrins

The amylopullulanase gene was flanked by ORF 1, 2, 3, and 5, which encode components of a binding protein-dependent sugar-transporting ABC transporter. The protein encoded by ORF 1 is homologous to *E. coli* maltose binding protein (*Eco*MBP), a periplasmic ligand-binding subunit of well-characterized *E. coli* maltose/maltodextrin ABC transporter. *E. coli* maltose/maltodextrin ABC transporter consists of two intramembrane permease subunits, MalF and MalG, and two copies of ATPase subunit, MalK, in addition to *Eco*MBP [12]. ORF 2, 3, and 5 encode homologs of the *E. coli* MalF, MalG, and MalK proteins, respectively. It has been reported that *T. litoralis* has another homolog of *E. coli* maltose/maltodextrin transporter, the trehalose/maltose ABC transporter, whose components are encoded by the *malE*, *malF*, *malG*, and *malK* genes. *T. litoralis* trehalose/maltose ABC transporter takes up trehalose and maltose, but cannot transport maltotriose or longer maltodextrins [13,14]. *P. furiosus* also has two homologs of *E. coli* maltose/maltodextrin transporter; the trehalose/maltose ABC transporter and the maltodextrin ABC transporter [5,6]. To determine the ligand specificity of the protein encoded by ORF1, we performed an amylose binding assay [15]. The purified protein encoded by ORF1 was bound to amylose resin, indicating that this protein has the ability to bind an α-1,4-linked glucose polymer. This characteristic is similar to *Eco*MBP and *Pfu* MBP rather than *Tli*TMBP, which was not bound to amylose resin [13]. The binding protein was eluted with a lower concentration of maltotriose and maltohexaose from amylose resin than that of maltose (Fig. 3), indicating that the protein

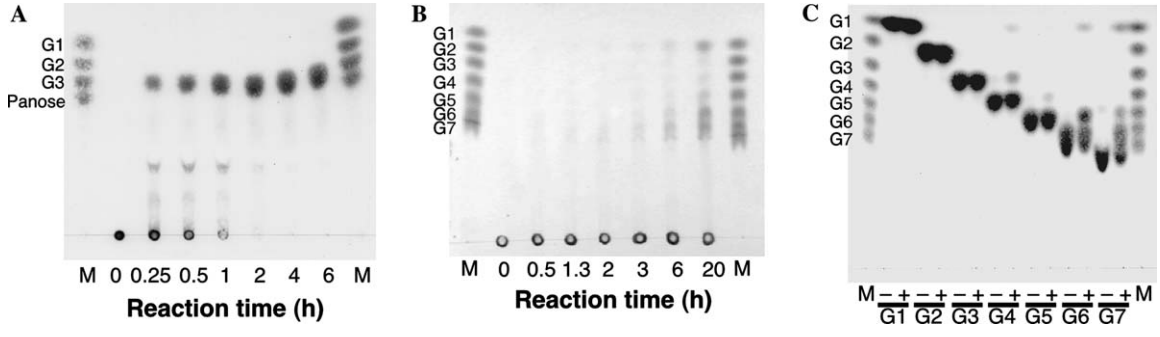

Fig. 2. Characterization of recombinant TLAPU. TLAPU was incubated with pullulan (A), starch (B), and maltodextrins (C) at 90 °C in 20 mM Hepes–NaOH (pH 7.0) for the indicated times (24 h for maltodextrins). The reaction products were identified by thin layer chromatography on Kiselgel 60 (Merck). The products were developed by multiple ascents with a solvent system of 1-butanol/ethanol/water (5:5:3) and detected by charring with H_2SO_4.

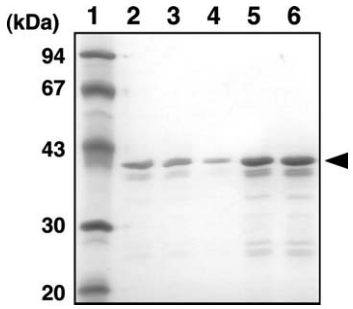

Fig. 3. *Thermococcus litoralis* maltodextrin binding protein exhibits higher affinity to maltotriose and maltohexaose and than to maltose. Maltodextrin binding protein bound to amylose resin was eluted with specific concentrations of linear or cyclic maltodextrins and then analyzed on SDS–PAGE (see Materials and methods). Lane 1, molecular weight markers; lane 2, 1 mM maltose; lane 3, 0.3 mM maltose; lane 4, 0.1 mM maltose; lane 5, 0.1 mM maltotriose; and lane 6, 0.1 mM maltohexaose. The numbers on the left are the estimated molecular masses of the marker proteins.

encoded by ORF 1 exhibits higher affinity to maltotriose or longer maltodextrins compared to maltose. This characteristic indicates that ORF 1 encodes a maltodextrin binding protein. Based on the specificity of the binding protein in addition to the result that TLAPU mainly produces maltotriose and longer maltodextrins, it is reasonable to consider that proteins encoded by ORF 1, 2, 3, and 5 constitute maltodextrin ABC transporter. Hence, ORF 1, 2, 3, and 5 were designated as the *mdxE*, *mdxF*, *mdxG*, and *mdxK* genes, respectively. Above results suggest that *T. litoralis* also has a maltodextrin uptake system, like *P. furiosus*. However, our experiments do not exclude the possibility that this transporter takes up other substrates.

Insight into molecular evolution of mdxK and malK genes

To our surprise, there are only 3 nucleotide changes, all of which do not affect amino acid sequence, between *mdxK* and one of the two *T. litoralis malK* sequences reported by Greller et al. [16]. There are 30 nucleotide changes, which cause 4 amino acid residue substitutions, in the two *malK* sequences from the same *T. litoralis* strains, maintained in different laboratories [16]. This difference was attributed to mutations of the same gene accumulated during multiple cell generations. Given that the *malK* gene has been cloned by PCR [16], however, it is much more likely that one of the two *malK* sequences is of the *mdxK* gene reported here.

Table 1
Amino acid identity between *T. litoralis* maltodextrin transporter components and other related ABC transporter components

T. litoralis maltodextrin transporter	*T. litoralis* trehalose/maltose transporter	*P. furiosus* maltodextrin transporter
MdxE	*Tli*TMBP (27%)	*Pfu*MBP (59%)
MdxF	MalF (26%)	MalF2 (74%)
MdxG	MalG (33%)	MalG2 (51%)
MdxK	MalK (98%)	MalK2 (83%)

Despite the very high similarity between the MdxK and MalK proteins of *T. litoralis*, the MdxE, MdxF, and MdxG proteins in the *T. litoralis* maltodextrin transporter are more similar to the counterparts in the *P. furiosus* maltodextrin transporter than to those in the *T. litoralis* trehalose/maltose transporter (Table 1). This suggests that *malK* gene of *T. litoralis* was recently generated from the *mdxK* gene by gene duplication, or vice versa. Interestingly, the trehalose/maltose ABC transporters of *T. litoralis* and *P. furiosus* are almost identical even at the nucleotide level. It has been proposed that this is the result of recent lateral gene transfer of 16-kb region containing the transporter genes [5]. There are 36 nucleotide differences between the *mdxK* gene of *T. litoralis* and the *malK* gene of *P. furiosus*. Because these 36 nucleotide changes include all the 31 substitutions observed between the *mdxK* and *malK* genes of *T. litoralis*, indicating that the *malK* gene was generated through the duplication of the *mdxK* gene. Taken together, we concluded that the 16-kb region was transferred from *T. litoralis* to *P. furiosus* after the *malK* gene generation from the *mdxK* gene as shown in Fig. 4. In the whole genome of *P. horikoshii*, more than 25 ORFs have been assigned as ATPase sub-

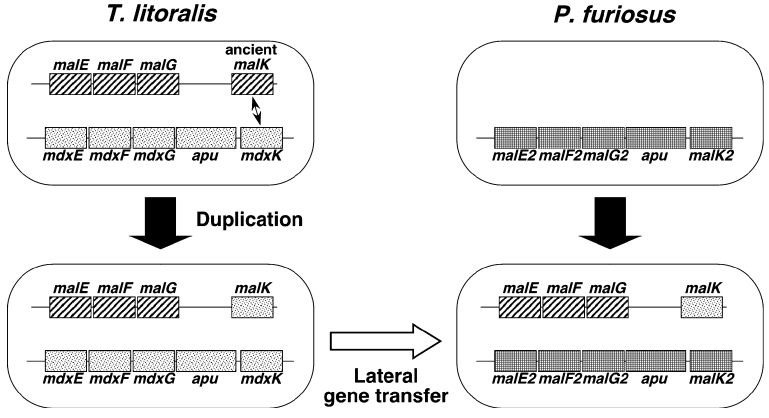

Fig. 4. Possible model for duplication of the *mdxK* gene and lateral 16-kb region transfer.

unit of ABC transporter. The more cognates of ABC transporters, the more kinds of solute cells are able to utilize or secrete. Gene duplication and lateral gene transfer might be a potent force in the evolution of ABC transporters in archaea.

Nucleotide sequence

The 8.9-kb nucleotide sequence reported in this paper has been submitted to the DDBJ under Accession No. AB054186.

Acknowledgments

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