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Preparation, crystallization and preliminary X-ray analysis of protein YtlP from *Bacillus subtilis*

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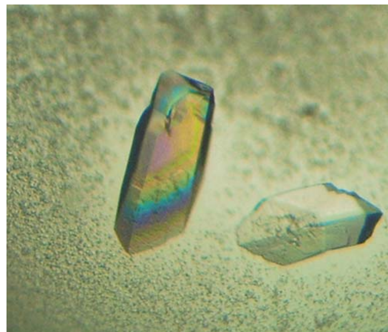
Bacillus subtilis YtlP is a protein that is predicted to belong to the bacterial and archael 2′-5′ RNA-ligase family. It contains 183 residues and two copies of the H*X*T*X* sequence motif conserved among proteins belonging to this family. In order to determine the structure of YtlP and to compare it with the paralogue YjcG and identified 2′-5′ RNA ligases, the gene *ytlP* was amplified from *B. subtilis* genomic DNA and cloned into expression vector pET-21a. The soluble protein was produced in *Escherichia coli*, purified to homogeneity and crystals suitable for X-ray analysis were obtained. The crystal diffracted to 2.0 Å and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 34.16, b = 48.54, c = 105.75 Å.

1. Introduction

The *Bacillus subtilis ytlP* gene encodes a 183-amino-acid residue protein of so far unknown physiological function. The primary sequence of YtlP is similar to that of proteins belonging to the 2′-5′ RNA-ligase family (Fig. 1). The 2′-5′ RNA-ligase activity, capable of joining *Saccharomyces cerevisiae* tRNA-splicing intermediates in the absence of ATP to form a 2′-5′ phosphodiester linkage, was first detected in extracts from *Escherichia coli* (Greer *et al.*, 1983). The *E. coli* enzyme was subsequently identified and similar enzymes were detected in *Methanococcus jannaschii* and *B. stearothermophilus* (Arn & Abelson, 1996). The primary sequence identity among members of the 2′-5′ RNA-ligase family is relatively low, but they all contain two copies of the H*X*T*X* motif (where *X* is a hydrophobic residue). These highly conserved motifs are characteristic of the bacterial and archeal 2′-5′ RNA-ligase family (Kato *et al.*, 2003). The first structure of this family was that from *Thermus thermophilus* HB8, which showed a high structural similarity to *Arabidopsis thaliana* cyclic phophodiesterase (Kato *et al.*, 2003). The crystal structure of 2′-5′ RNA ligase from another archeal species, *Pyrococcus horikoshii*, was determined and appeared to have a substrate preference that probably differed from that of *T. thermophilus* (Rehse & Tahirov, 2005). A putative integral membrane-transport protein from *P. furiosus* also seems to belong to the 2′-5′ RNA-ligase family. The ^{1}H, ^{13}C and ^{15}N resonance assignments of a soluble part of this protein have been reported and the structure has been deposited in the PDB (Okada *et al.*, 2006). The putative 2′-5′ RNA ligase YjcG from *B. subtilis* is a member of the YjcG-like subgroup of the 2H phosphoesterase superfamily (Mazumder *et al.*, 2002). Proteins belonging to this group have been suggested to react on unknown molecules with 2′-3′ cyclic phosphoester linkages. To date, the structures of the enzymes *A. thaliana* CPDase (Hofmann *et al.*, 2000), rat CNPase (Kozlov *et al.*, 2003) and human CNPase (Sakamoto *et al.*, 2005) from this family have been reported. All the structures showed conserved bilobal arrangement of two $\alpha\alpha\beta$ modules. In *B. subtilis*, only YjcG and YtlP have two copies of the conserved H*X*T*X* motif. The preliminary X-ray analysis of YjcG has been published and the structure is to be released in the PDB (Li *et al.*, 2005). The structures of YtlP and YjcG should help us to understand the function of YjcG-like proteins in bacteria.



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2. Materials and methods

2.1. Gene cloning and expression

The *ytlP* gene was amplified by polymerase chain reaction (PCR) from *B. subtilis* strain 168 genomic DNA. The primers used were 5′-CGCGGATCCATGCCAGATATACGGCCTCATT-3′ and 5′-CCGCCTGAGAGATAATTGAAATTTGAATATTTCC-3′, which contain sequences for a *Bam*HI and an *Xho*I restriction site, respectively. The PCR product was cloned into the corresponding sites in pET-21a (Novagen). The resulting plasmid, pLPL4, was transformed into *E. coli* strain BL21(DE3). Cells were grown at 310 K in Luria–Bertani medium containing 50 μg ml^{-1} kanamycin until mid-exponential growth phase. At this point, *ytlP* expression was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 m*M* and the culture was grown at 291 K for 20 h. Cells were harvested by centrifuguation at 6700*g* for 10 min at 277 K and were then suspended in lysis buffer containing 20 m*M* Tris–HCl, 0.5 *M* NaCl, 1 m*M* PMSF, 1 μg ml^{-1} DNAse, 10 μg ml^{-1} RNAse pH 7.5.

2.2. Protein purification

The cells were lysed by sonication. The lysate was centrifuged at 34 700*g* for 50 min at 277 K. The supernatant was applied onto a 1 ml HiTrap Ni column (GE Healthcare) equilibrated with buffer *A* containing 20 m*M* Tris–HCl, 0.5 *M* NaCl pH 7.5. Unbound proteins were eluted using buffer *A* and loosely bound proteins were eluted using 5% buffer *B* (20 m*M* Tris–HCl, 0.5 *M* NaCl, 0.5 *M* imidazole pH 7.5) in buffer *A*. Tightly bound proteins were then eluted with buffer *B*. Fractions containing YtlP were further purified using a HiLoad Superdex 75 column (GE Healthcare) with 150 m*M* NaCl in 20 m*M* Tris–HCl pH 7.5 as eluant. The purity of the protein was examined by SDS–PAGE.

2.3. Crystallization

Crystallization was performed at 289 K using the hanging-drop vapour-diffusion method. Purified YtlP was concentrated to 16.6 mg ml^{-1} by ultrafiltration (Millipore Amicon) and Crystal Screen I, Crystal Screen II and Index kits (Hampton Research) were used for initial screening. A mixture of 1 μl protein solution and 1 μl reservoir solution was equilibrated against 500 μl reservoir solution.

2.4. Data collection

X-ray diffraction data were collected on a Bruker–Nonius FR591 rotating-anode generator with Cu *K*α radiation and a Bruker SMART 6000 CCD detector. The crystal was flash-cooled in the presence of glycerol and maintained at 100 K in a nitrogen-gas stream during data collection. The data were processed with the Bruker *PROTEUM* online software.

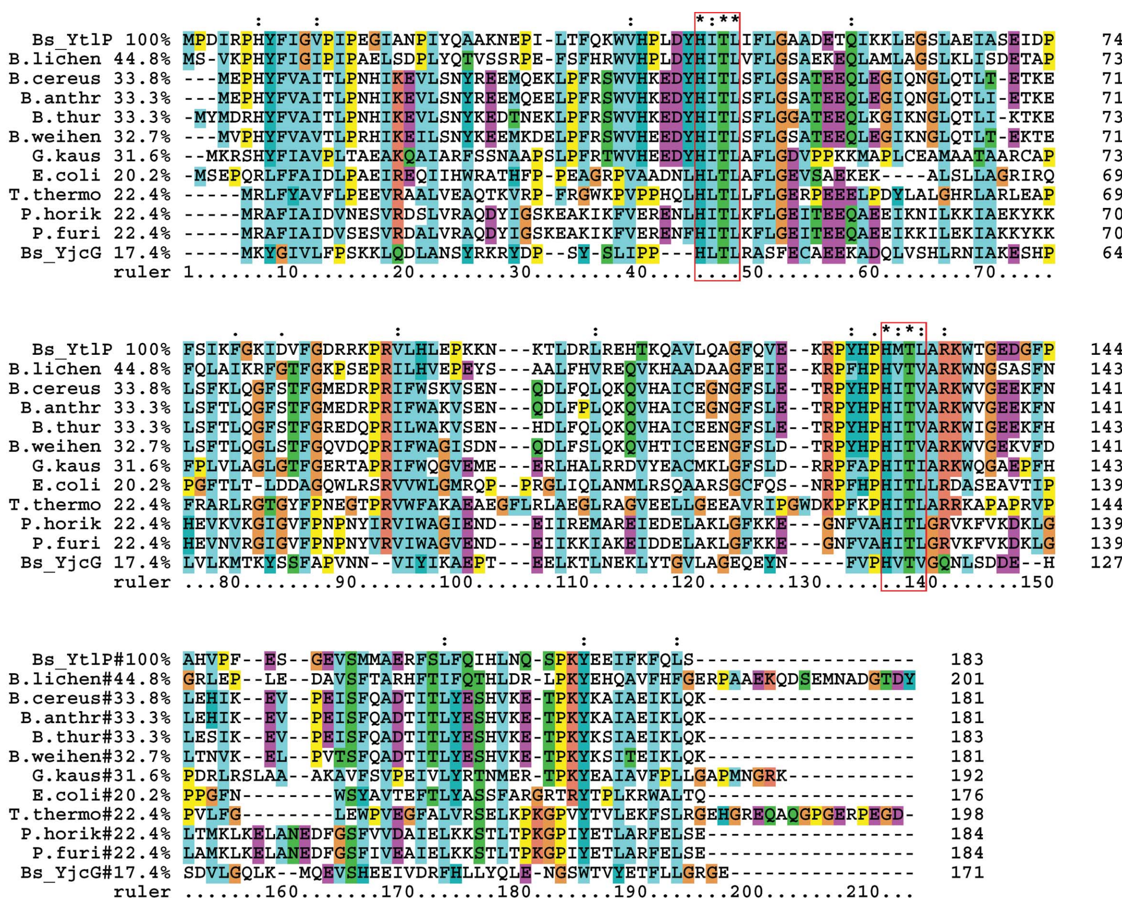

Figure 1
Multiple alignment of amino-acid sequences related to YtlP. The two conserved H*X*T*X* motifs in the polypeptides are highlighted by red boxes. The percentages indicate the sequence identity of the paralogues to YtlP. The alignment was performed using the program *ClustalX* (Thompson *et al.*, 1997). Bs_YtlP, *B. subtilis* YtlP; B.lichen, *B. licheniformis*; B.cereus, *B. cereus*; B.anthr, *B. anthracis*; B.thur, *B. thuringiensis*; B.weihen, *B. weihenstephanensis*; G.kaus, *Geobacillus kaustophilus*; E.coli, *E. coli*; T.thermo, *T. thermophilus*; P.horik, *Pyrococcus horikoshii*; P.furi, *P. furiosus*; Bs_YjcG, *B. subtilis* YjcG.

Figure 2
Crystals of *B. subtilis* YtlP. The dimensions of the crystal are approximately 0.1 × 0.2 × 0.05 mm.

3. Results

The sequence of the cloned *ytlP* gene in plasmid pLPL4 was confirmed by DNA sequencing (AuGCT Biotechnology). YtlP produced in *E. coli* strain BL21 (DE3) containing pLPL4 was water-soluble and could be purified to homogeneity in two steps. SDS–PAGE showed that at least 95% of the protein in the preparations was YtlP, with an apparent molecular weight of about 25.0 kDa. This weight matches the theoretical molecular weight of the recombinant YtlP (21.0 kDa) plus about 4.0 kDa fusion peptide including the hexahistidyl tag.

Microcrystals were obtained under several conditions. After optimizing the concentration of the precipitant and the pH value of the buffer of the initial crystallization conditions, large single crystals were obtained in 0.1 *M* HEPES pH 8.0, 30%(*v*/*v*) Jeffamine ED-2001 pH 7.0. The crystals grew to dimensions of 0.1 × 0.2 × 0.05 mm in 3 d (Fig. 2). The final crystallization was carried out by the hanging-drop vapour-diffusion method; the volumes of precipitant, protein solution and reservoir solution were kept the same as in the initial screens. The crystal diffracted to a resolution of 2.0 Å and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 34.16, b = 48.54, c = 105.75 Å. For one molecule per asymmetric unit, the Matthews coefficient is 2.1 Å^3 Da^{-1} and the solvent content is 40.6% (Matthews, 1968). Data statistics are listed in Table 1. The completeness of the diffraction data is relatively low, which is a consequence of the square shape of the detector. Although the similarities between 2′-5′ RNA ligases are low, several initial phasing trials using molecular replacement were carried out. However, no positive results were obtained. Since there are four methionine residues in the 183-amino-acid YtlP, phase determination using the anomalous signal from SeMet is in progress.

Table 1
Data-collection statistics for YtlP.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	50–2.0 (2.11–2.0)
Completeness (%)	92.3 (79.0)
R_{sym}† (%)	7.7 (15.8)
Mean $I/\sigma(I)$	9.1 (4.4)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 34.16, b = 48.54, c = 105.75
No. of observed reflections	89925
No. of unique reflections	11565
Molecules per ASU	1
V_M (Å^3 Da^{-1})	2.1

† $R_{sym} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$, where the summation is over all reflections.

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