The \textit{yjoB} gene of \textit{Bacillus subtilis} encodes a protein that is a novel member of the AAA family

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Received 1 October 2003; received in revised form 27 November 2003; accepted 27 November 2003

First published online 16 December 2003

Abstract

The \textit{yjoB} gene of \textit{Bacillus subtilis} encodes a 48.8-kDa protein belonging to the AAA family. Members of this family contain a 200–250-amino acid residues AAA domain carrying a Walker A and B ATP-binding site assumed to be part of a molecular chaperone. The \textit{yjoB} gene belongs to the \textit{\sigma^W} regulon, and members of this regulon have been reported to be transiently induced when cells enter the stationary growth phase. This assumption was confirmed here for \textit{yjoB} by Western blot experiments and by analysis of a transcriptional fusion. Purified \textit{YjoB} protein exhibited ATPase activity but was unable to prevent aggregation of denatured citrate synthase. An alignment of \textit{YjoB} with a subgroup of AAA proteins present in Archaea suggests that \textit{YjoB} might be involved in the modulation of the activity of one or more proteases.

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Keywords: \textit{yjoB}; \textit{ftsH}; ATPase; AAA family; Citrate synthase

1. Introduction

The AAA ATPases (\textit{\Delta}TPases associated with diverse cellular activities) constitute a large family of ATPases involved in a wide array of cellular processes and occurring in all organisms [1–4]. The hallmark of these proteins is a highly conserved domain of approximately 250 residues, which contains the Walker A and B motifs [5] typical for P-loop ATPases as well as a second region of high sequence conservation, C-terminal to the ATP-binding motifs [6]. The activities carried out by these well-conserved AAA proteins are numerous and include controlling protein folding and unfolding, the assembly and disassembly of protein complexes, protein transport through membrane fusion and protein degradation. The mechanisms of these proteins, and the function of ATP in each of them, are topics of current interest.

In prokaryotes, three different classes of AAA proteins have been described so far, the membrane-anchored metalloprotease \textit{FtsH} [7], the PAN (for proteasome-activating nucleotidase) and the ARC proteins (for AAA ATPase forming ring-shaped complexes) [8]. All three proteins have in common that they form ring-like structures consisting of six (in the case of \textit{FtsH} [9,10] and ARC [8]) or seven (PAN) [11] subunits. While \textit{FtsH} proteins have been reported only in eubacteria, PAN occurs in most but not all archaeal genomes, and ARC has been reported in those eubacteria possessing a genuine 20S proteasome [12]. The PAN AAA protein of \textit{Methanococcus jannaschii} exhibits high sequence similarity to the ATPases of the eukaryotic 19S regulator [13], and this is assumed to be also true for those present in other archaeal species. Recombinant PAN of \textit{M. jannaschii} stimulated degradation of substrate proteins up to 25-fold when added to 20S proteasomes from \textit{Thermoplasma acidophilum} [11]. Such a role has not yet been demonstrated for the ARC proteins, but it is likely that they play a role in ATP-dependent protein degradation.

In \textit{Bacillus subtilis} and a few other eubacterial species a second protein with a AAA domain is present. The gene encoding this protein has been denoted \textit{yjoB} in \textit{B. subtilis} [14]. While the function of this 48.8-kDa protein is unknown, the presence of an AAA domain suggests that it might act as a molecular chaperone. This assumption is derived from the observations that the \textit{Escherichia coli}
Thefts protein is able to interact with denatured alkaline phosphatase [15] and the purified AAA domain of the *Saccharomyces cerevisiae* Yme1 metalloprotease binds unfolded polypeptides and suppresses their aggregation [16]. It has already been published that *yjoB* is a member of the σ^W^ regulon [17]. This regulon is induced when cells enter stationary phase [17] and after challenge of the cells to an alkaline shock [18] or to the antibiotic vancomycin [19]. We have been interested in elucidating the function of YjoB, where we assume that it acts as a molecular chaperone. In the present communication, we present data on the expression of the *yjoB* gene and demonstrate that purified YjoB exhibits ATPase activity.

2. Materials and methods

2.1. Bacterial strains, plasmid and DNA manipulations

All bacterial strains and plasmids used in this study are compiled in Table 1. Bacteria were routinely grown in Luria–Bertani (LB) broth and in some experiments in Spizizen minimal medium [20] supplemented with ampicillin (100 μg ml\(^{-1}\)), chloramphenicol (5 μg ml\(^{-1}\)) or erythromycin (1 μg ml\(^{-1}\)) as appropriate. CaCl\(_2\)-competent *E. coli* cells [21] of strain XL1-Blue and competent cells of *B. subtilis* [22] were used in transformation experiments.

2.2. Construction of strains and transcriptional fusions

The *yjoB* gene was inactivated by the integration of pMUTIN4 into the gene essentially as described [23]. This was accomplished by amplification of an about 300-bp fragment from *yjoB* using the two primers YJOBK05 (5'-GGCCATGAATTCTGCGGTTAACGATGAAAAGATGCCG-3', *Eco*RI site underlined) and YJOBK03 (5'-GGCCATGGATCCCTGCGATCGACTTCACTAACG-3'; *Bam*HI site underlined), inserting it into *Eco*RI/*Bam*HI-cleaved pMUTIN4, transformation of the recombinant plasmid into *B. subtilis* strain 1012 and selection for erythromycin resistance. Integration at the correct location was verified by Southern blotting (strain RCM2).

Construction of a transcriptional fusion between the promoter region of *yjoB* and the *bgaB* reporter gene was accomplished by amplification of the complete promoter region using the two primers PYOB5 (5'-GGCCATGAATTCTGCGGTTAACGATGAAAAGATGCCG-3', *Eco*RI site underlined) and PYOB3 (5'-GGCCATGGATCCCTGCGGTTAACGATGAAAAGATGCCG-3'; *Bam*HI site underlined) followed by ligation of the *Bam*HI-cut fragment into pBgaB and integration of the operon fusion at the *amyE* locus by a double crossover event using *B. subtilis* strain AM01 resulting in strain SD01. Strain MK01 was obtained by transformation of RCM2 with chromosomal DNA of the MW01 strain.

2.3. Transcriptional analysis

Preparation of total *B. subtilis* RNA, hybridization and Northern blot analysis were performed as described [24]. Hybridizations specific for *htpG* were carried out with digoxigenin (DIG)-labeled riboprobe RNA synthesized in vitro with T3 RNA polymerase using an about 300-bp polymerase chain reaction (PCR) fragment generated with plasmid pSD07 as template. In vitro RNA labeling was accomplished according to the manufacturer's instructions (DIG RNA Labeling Kit; Roche Diagnostics, Mannheim, Germany).

2.4. β-Galactosidase assay

Cultures of *B. subtilis* strains were grown in minimal medium to an OD\(_{578}\) of 0.7 at 37°C, and aliquots were taken at the times indicated in the experiments. The activity

<table>
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<th>Bacterial strains and plasmids</th>
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<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 (rK mK) supE44 relA1 Δ[lacZYA-argF]U169 F' proAB lacF' lacZΔM15 Tn10</td>
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<td>Stratagene</td>
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<td>1012</td>
<td>leuA8 metB5 trpC2 hsdR1</td>
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<td>pMUTIN4</td>
<td>integrative vector allowing fusion of chromosomal promoters to <em>lacZ</em></td>
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<td>pBgaB</td>
<td>integrative vector allowing in vitro fusion of promoter fragments to <em>bgaB</em></td>
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<td>pSD07</td>
<td>pQE-30 containing <em>yjoB</em></td>
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ity of the β-galactosidase was assayed according to Mogk and coworkers [25].

2.5. Overexpression and purification of YjoB and antibody production

To facilitate the overproduction and purification of YjoB, the gene was first amplified by PCR using chromosomal DNA of strain 1012 as a template using the two primers YJOB5 (5'-GGCCATGCGTATCCGA-CATTATGCTCCT-3'; BamHI site underlined) and YJOB3 (5'-GGCCATGCGTATCCGACATGAAATA-CACCGTATT-3'; SalI site underlined). The amplicon was digested with BamHI and SalI and cloned into BamHI/SalI-linearized pQE-30, resulting in pSD07. This plasmid was introduced into E. coli strain XL1-Blue for overproduction of His6-YjoB. This His6-tagged protein was purified from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cells (1 mM IPTG for 4 h) as described [26] and was used to raise polyclonal antibodies in a rabbit.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis

Sample preparation for SDS-PAGE and immunoblot analysis was performed as described previously [27]. 5 µg total cellular protein was applied per lane. Polyclonal antibodies raised against YjoB were used for visualization of cross-reacting material.

2.7. ATPase activity

ATPase activity was measured in buffer A (50 mM Tris–HCl, pH 7, 30 mM KCl, 30 mM NH₄Cl, 0.5 mM Mg-acetate) as described [28,29]. A typical YjoB ATPase reaction mixture (50 µl) contained 4 mM ATP and different amounts of His6-tagged YjoB. The reactions were stopped by addition of 800 µl of color reagent (0.034% malachite green (Sigma) and 10.5 g 31 ammonium molybdate in 1 N HCl and 0.1% Triton X-100) and 100 µl of 34% citric acid. After 40 min at room temperature, absorption was measured at 660 nm. Construction of a recombinant pQE30 vector directing the production of His-tagged Pbp4* has been described [30].

2.8. Citrate synthase aggregation assay

Suppression of aggregation of denatured citrate synthase was essentially tested as described [31]. Citrate synthase (Roche Diagnostics) was unfolded at 20°C in 6.0 M GdmCl, 20 mM DTE, 50 mM Tris–HCl, pH 8.0 for 1 h and then diluted 100-fold to a final concentration of 0.15 µM in 60 mM GdmCl, 50 mM NaCl, 2 mM DTE, 50 mM Tris–HCl, pH 8.0 at 25°C. Aggregation was monitored by the increase in light scattering at 360 nm in the absence and presence of YjoB or trigger factor using a Hitachi F-4010 fluorescence spectrometer equipped with a temperature-controlled cell holder and a magnetic stirrer. The trigger factor used was the mutant (W151F) form [32] and was received as a gift from the group of Prof. F.X. Schmid, University of Bayreuth. It was used as a positive control since this chaperone binds with high affinity to unfolded protein substrates [33].

3. Results

3.1. The YjoB protein exhibits a AAA domain

An alignment of the amino acid sequences of FtsH and YjoB is presented in Fig. 1. As can be seen, both proteins exhibit increased similarity within the region of the AAA module shown in bold letters (28% identical and 34% conserved amino acid residues totaling up to 62% similarity). The YjoB does not carry transmembrane domains (it is present within the soluble fraction, see below) suggesting that the location of this protein is within the cytoplasm.

The alignment further shows that YjoB ends with the first

Fig. 1. Amino acid sequence alignment of the two B. subtilis proteins FtsH and YjoB using the CLUSTAL program. The amino acid residues of the AAA domain are in bold letters, ATP-binding site Walker A and B motifs and are marked by a dark background, and the metal-binding HEGGH motif in FtsH by a light background. The metal-binding HEGGH motif in FtsH is underlined. Asterisks, double dots and single dots indicate completely, highly and moderately conserved residues, respectively.
histidine residue of the Zn$^{2+}$-binding domain. We conclude from this alignment that yjoB encodes a soluble protein with an assumed molecular chaperone activity.

### 3.2. A yjoB knockout is viable

We first asked whether yjoB is an essential gene. To answer this question, yjoB was successfully inactivated by insertion of the pMUTIN4 plasmid as described in Section 2 resulting in strain RCM2. Since this strain turned out to be fully viable, yjoB is non-essential under the conditions tested. Next, we compared the growth behavior of the mutant strain to that of its isogenic wild-type. Both strains exhibited comparable growth curves and reached the same OD in LB and in minimal medium (data not shown). We also tested growth of both strains under conditions of heat shock, alkali stress and after addition of vancomycin. No significant differences in the growth of both strains were measured (data not shown).

Then, we asked whether a yjoB ftsH double knockout is viable, which turned out to be the case, too (data not shown). We also analyzed the growth behavior of the double knockout in LB and in minimal medium. No significant differences in the growth of both strains were measured (data not shown). In summary, we conclude that yjoB is not essential for growth under the conditions tested here and even a yjoB ftsH double knockout turned out to be viable.

### 3.3. Induction of yjoB occurs during the transition phase and is $\sigma^W$-dependent

It has been reported that yjoB is induced at the level of transcription when cells enter stationary phase and upon increasing the external pH [17,18]. We asked whether this induction is also reflected at the level of the protein. To specifically detect YjoB in cellular extracts, the gene was cloned, fused to a His-tag at its 5' end, overproduced in E. coli, purified and used to raise polyclonal antibodies (for details, see Section 2).

To check whether the amount of YjoB protein increases in B. subtilis 1012 cells during growth in LB medium, aliquots were taken at different times during growth and analyzed for the amount of YjoB by Western blotting. As can be seen from Fig. 2, no YjoB protein could be detected up to 1 h before cells stopped growth, and its amount increased up to $t_0$ (defined by the end of exponential growth) followed by a decrease. The same experiment was carried out in a sigW::neo knockout strain and failed to detect any YjoB as expected (data not shown) thereby confirming our DNA macroarray data obtained with the sigW::neo strain [18]. These experiments confirm that yjoB is indeed induced at the end of the exponential growth phase and that its expression is completely dependent on the alternative sigma factor $\sigma^W$. To quantify induction of yjoB, its expression was followed by measuring the $\beta$-galactosidase activity using strain SD01. In this strain, a transcriptional fusion between the promoter region of yjoB and the bgaB gene was constructed and integrated ectopically at the amyE locus. Strain SD01 was grown in minimal medium to follow more precisely the induction which is delayed under these conditions. Aliquots were withdrawn at different time points in 1-h intervals starting in the mid-exponential growth phase for $\beta$-galactosidase determinations. While the reporter enzyme activity was...
rather low during mid-exponential growth, it started to increase during late exponential growth, reached its maximum when cells entered the stationary growth phase and thereafter declined (Fig. 3). Again, these results clearly demonstrate the induction of \( yjoB \) at the end of exponential growth in more detail pointing to a specific role for the protein during the transition phase.

3.4. Purified \( yjoB \) exhibits ATPase activity

As already mentioned, \( yjoB \), being classified as a member of the AAA family, contains an AAA module with Walker motifs A and B (see Fig. 1). To find out whether \( yjoB \) is able to hydrolyze ATP, His-tagged \( yjoB \) was purified and assayed for its ATPase activity. While heat-denatured \( yjoB \) was completely inactive (data not shown), native protein was able to hydrolyze ATP in a time-dependent way (Fig. 4) thereby confirming the prediction that \( yjoB \) encodes an ATPase. To exclude the possibility that a contaminating protein was responsible for the ATPase activity, we purified His-tagged PbpE4* by the same protocol. This protein belongs to the group of penicillin-binding proteins, and these proteins have never been reported to exhibit ATPase activity [34]. When Pbp4* was tested it turned out to be completely devoid of ATPase activity (Fig. 4). Therefore, an ATPase activity contaminating the \( yjoB \) preparation can be largely excluded.

Fig. 4. \( yjoB \) exhibits ATPase activity. ATPase reactions were performed as described in Section 2. At the indicated times ATP hydrolysis was analyzed by measuring the amount of released inorganic phosphate (P) as detailed in Section 2. 21 nmol \( yjoB \) (•) and Pbp4* (□) were incubated with 4.2 mM ATP each. The background value resulting from spontaneous cleavage of ATP has been subtracted from all given values.

Fig. 5. \( yjoB \) does not prevent aggregation of citrate synthase. Unfolded citrate synthase (solubilized in 6 M GdmCl) was diluted to a final concentration of 0.15 mM in 10 mM Tris–HCl, pH 8.0, 100 mM Na-phosphate buffer, 0.5 M urea, equilibrated at 15°C in the absence (●) or presence of 1.13 mM \( yjoB \) (□) without and (●) with 7.7 mM ATP) or 6 mM trigger factor (W151F) (×). When chemically denatured citrate synthase was mixed with \( yjoB \), the initial turbidity was increased about threefold. The increase in light scattering at 500 nm is shown as a function of the incubation time.
3.5. Purified YjoB is unable to prevent aggregation of denatured citrate synthase

Molecular chaperones are able to bind non-native proteins. We asked whether YjoB might act as a molecular chaperone. Therefore, we first checked for chaperone activity using a classical chaperone test. Citrate synthase was chemically denatured in 6 M GdmCl and then diluted 100-fold in the same buffer without the chaotropic agent. Aggregation of citrate synthase was then monitored by the increase in turbidity. While aggregation of citrate synthase occurred readily in the absence of any protein and reached a saturation level after about 200 s, addition of trigger factor completely prevented the formation of aggregates (Fig. 5). On the contrary, added YjoB either in the presence or in the absence of ATP did not prevent aggregation of citrate synthase though in the presence of ATP this process was retarded (Fig. 5). These results suggested to us that YjoB is not active as a general chaperone.

4. Discussion

More than 1000 proteins carrying the AAA module have been described so far [35] and these proteins are involved in many different cellular activities in all three kingdoms of life. In many cases, these proteins perform chaperone-like functions that assist in the assembly, operation or disassembly of protein complexes. In eubacteria two classes of AAA proteins have been described so far, the FtsH metalloproteases and the ARC proteins, while an additional class named PAN is present only in Archaea [7,12]. Here, we report on the genetic and biochemical analysis of a new member of the AAA family designated yjoB [14]. Deletion of yjoB from the B. subtilis chromosome did not lead to a discernible phenotype under standard laboratory conditions, and even a yjoBftsH double knockout turned out to be viable. Since it has been published that the yjoB gene is a member of the W regulon [17,18], we decided to test this observation at the level of both translation and transcription. Western blot experiments and analysis of a transcriptional fusion clearly indicated that yjoB is strongly induced when cells enter the stationary phase. These data suggest that yjoB exerts its physiological function at the end of exponential growth.

All members of the AAA family tested so far exhibit ATPase activity. To find out whether this also holds true for YjoB, the purified protein was shown to cleave ATP in a concentration-dependent manner, e.g., 82 nmol YjoB released about 14 nmol phosphate within 80 min whereas the same amount of heat-denatured YjoB was completely inactive (Fig. 4). This finding prompted us to ask whether YjoB acts as a general molecular chaperone. When purified YjoB was incubated with chemically denatured citrate synthase in the presence or absence of ATP, it was unable to prevent its aggregation suggesting that it does not act as a general chaperone. Alternatively, it might act as a specific chaperone as has been described for, e.g., SecB and PapD of E. coli. The SecB chaperone is a cytosolic protein involved in the export of several proteins to keep them in an unfolded, translocation-competent state [36]. The PapD chaperone acts in the periplasm where it binds to the P-pili components as they emerge into the periplasmic space [37]. This binding prevents premature assembly and subsequent degradation of the pili subunits.

Most interestingly, when the AAA signature of YjoB was used as a query, it exhibits similarity to the PAN proteins (Fig. 6) which are present in those archaeal species containing a 20S proteasome. In eukaryotic cells, most proteins in the cytosol and nucleus are degraded via the ubiquitin-proteasome pathway [38]. The proteasome consists of a proteolytic core complex (the 20S proteasome) and the 19S regulatory complexes which cap the 20S particle at one or both ends. Besides in Archaea, a 20S proteasome-like particle has also been found in some Archaea and in eubacterial actinomycetes [12], but not in other eubacteria such as B. subtilis. Unlike the actinomycetes, other bacteria have a more distantly related complex called ClpQY. This ATP-dependent protease is composed of the proteolytic component ClpQ, whose primary sequence is highly similar to β-type proteasome subunits, and ClpY, a member of the Hsp100/Clp family of ATPases. In vivo, ClpQ and ClpY form a complex, which is induced, along with the cell’s other ATP-dependent proteases, by heat shock [39]. Under these conditions, ClpQY appears to play a role in the degradation of abnormal, heat-damaged proteins. Therefore, it is tempting to speculate that the YjoB AAA protein (i) forms a ring-like structure, and (ii) is involved in the degradation of substrate proteins thereby constituting a regulatory subunit.

Acknowledgements

We thank R.C. Mancini for the construction of strain RCM2, R. Mayer for his help in carrying out the molecu-
ular chaperone assay and the Deutsche Forschungsgemeinschaft, EU Project QLRT-1999-00413 and the Fonds der Chemischen Industrie for financial support.

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