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A Phosphate Starvation-Inducible Ribonuclease of *Bacillus licheniformis*

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The BLi03719 protein of *Bacillus licheniformis* DSM13 belongs to the most abundant extracellular proteins under phosphate starvation conditions. In this study, the function of this phosphate starvation inducible protein was determined. An amino-acid sequence analysis of the BLi03719-encoding gene showed a high similarity with genes encoding the barnase of *Bacillus amyloliquefaciens* FZB42 and binase-like RNase of *Bacillus pumilus* SARF-032. The comparison of the control strain and a BLi03719-deficient strain revealed a strongly reduced extracellular ribonuclease activity of the mutant. Furthermore, this knockout mutant exhibited delayed growth with yeast RNA as an alternative phosphate and carbon source. These results suggest that BLi03719 is an extracellular ribonuclease expressed in *B. licheniformis* under phosphate starvation conditions. Finally, a BLi03719 mutant showed an advantageous effect on the overexpression of the heterologous *amyE* gene under phosphate-limited growth conditions.

Keywords: *Bacillus licheniformis*, barnase, binase, phosphate starvation, ribonuclease

Introduction

Recently, the phosphate starvation response of the industrially relevant bacterium *Bacillus licheniformis* has been determined in-depth at the transcriptional and translational levels [11]. It was shown that genes encoding intracellular and extracellular nucleases such as *yhcR* (similar to 5′-nucleotidase), *yfkN* (similar to 2′,3′-cyclic-nucleotide 2′-phosphodiesterase), *nucB* (extracellular deoxyribonuclease), *yurI* (ribonuclease), and *rph* (ribonuclease PH) are significantly induced by the consumption of the essential nutrient phosphate [11]. It is supposed that these enzymes support the acquisition of alternative phosphate resources under nutrient-limited environmental conditions. A secretome analysis revealed that besides the predicted deoxyribonucleases (DNases) NucB and YfkN and the phytase Phy, the BLi03719 protein also belongs to the most dominant protein spots in the extracellular proteome of *B. licheniformis* under phosphate starvation conditions [11].

At the beginning of this work, little information was available concerning the activity of the ribonucleases expressed in *B. licheniformis* under phosphate starvation conditions. Preliminary sequence analyses indicated that this phosphate-starvation-inducible *Bli03719* gene of *B. licheniformis* DSM13 (GenBank Accession No. NC_006270.3) encodes a small pre-protein of 151 amino acids with potential ribonuclease activity. For other members of the genus *Bacillus*, two small secreted ribonucleases have been reported; the barnase from *Bacillus amyloliquefaciens* and the binase from *Bacillus intermedius* (renamed *Bacillus pumilus* [21]) [1, 5, 16]. The barnase and binase ribonucleases are small extracellular proteins consisting of 110 and 109 amino acids with guanyl-specific RNase activity, respectively [9, 17, 21]. Ulyanova *et al*. [21] suggested that the barnase and the

Table 1. Bacterial strains used in this study.

Strain	Relevant genotype	Reference
B. licheniformis DSM13	Wild type	DSMZ, Germany
B. licheniformis MW3	Δ*hsdR1*, Δ*hsdR2*	[24]
B. licheniformis ΔBLi03719	Δ*hsdR1*, Δ*hsdR2*, Δ*BLi03719*	This study
B. licheniformis TL2A	Δ*hsdR1*, Δ*hsdR2*, pKUC3	This study
B. licheniformis TL2B	Δ*hsdR1*, Δ*hsdR2*, Δ*BLi03719*, pKUC3	This study
B. licheniformis TL3A	Δ*hsdR1*, Δ*hsdR2*, pKUC4	This study
B. licheniformis TL3B	Δ*hsdR1*, Δ*hsdR2*, Δ*BLi03719*, pKUC4	This study

binase ribonucleases (RNases) can be regarded as molecular twins according to their high similarities in structure, physical-chemical, and catalytic properties.

In order to investigate the function of the putative RNase-encoding gene *BLi03719* of *B. licheniformis* DSM13, the corresponding gene was deleted and the extracellular and intracellular ribonuclease activity was analyzed in comparison with the control strain. The effect of the mutant on growth behavior and the overexpression of heterologous genes under phosphate-limited growth conditions were investigated.

Materials and Methods

Strains and Cultivation

All bacterial strains used in this study are listed in Table 1. *B. licheniformis* MW3, which ensures high transformation efficiencies due to the deletion of two type I restriction modification systems *hsdR1* and *hsdR2*, was used as the control strain [24]. Belitzky Minimal Medium (BMM) [19] was used in all growth experiments. The cells were cultivated at 37°C under vigorous agitation in BMM containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4 \cdot 7H_2O$, 27 mM KCl, 7 mM sodium citrate dihydrate, and 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2 \cdot 2H_2O$, 1 µM $FeSO_4 \cdot 7H_2O$, 10 µM $MnSO_4 \cdot 4H_2O$, and 11 mM glucose. For the phosphate starvation experiments, the concentration of phosphate was reduced to 0.15 mM. For the analysis of ribonucleic acid (RNA) utilization, the *B. licheniformis* control strain MW3 and mutant strains were cultivated in phosphate-limited BMM with the addition of 5 mg/ml of yeast RNA or isolated total RNA when cells enter the transient phase.

Mutagenesis Procedure

The deletion of the *BLi03719* gene was performed by a homologous recombination-based method. The upstream (FA) and downstream (FB) regions of the *BLi03719* gene from *B. licheniformis* DSM13, each 700 bp in length, were amplified with the primer pairs 1/2 and 3/4, respectively (Table 2). The sequence of the *erm* gene encoding the erythromycin resistance methylase (900 bp) from the plasmid pAX01 [6] was amplified with the primer pairs 5/6 (Table 2). The FA-*erm*-FB fusion sequence was constructed by means of the precise gene fusion polymerase chain reaction (PCR) strategy described by Yon and Fried [26] with the primer pairs 1/4 (Table 2). The PCR amplicon of the fusion sequence was then transformed into *B. licheniformis* MW3 cells that carry the plasmid pMMcomK. The role of pMMcomK is to support the natural competence of *B. licheniformis* cells by overproducing ComK, the key regulator responsible for the transcription of competence genes [10]. The pMMcomK plasmid is lost by the host strain if the antibiotic tetracycline is not present in the growth medium.

The transformation procedure for *B. licheniformis* MW3 is described in detail by Hoffmann *et al*. [10]. The chromosomal DNA of the colonies obtained from transformation was isolated and used as the template for screening the mutants of the ribonuclease gene by PCR with the primer pair 1/7 (Table 2). Primer 7 is located on chromosome at position 750 bp downstream from the end of the *BLi03719* gene. To verify the sequence, the PCR products were sent for sequencing (Agowa, Germany). The *B. licheniformis* strain with the deletion of the *BLi03719* gene was named *B. licheniformis* ΔBLi03719.

Growth Analysis of Control and Mutant Strains with Yeast RNA

The *B. licheniformis* control and mutant strains were grown in 500 ml Erlenmeyer flasks containing 200 ml of phosphate-limited BMM. The growth condition was set at 37°C, 250 rpm for 20 h. After 8 h of cultivation, 5 mg/ml of Yeast RNA (Sigma-Aldrich Co, USA) was added to the growth medium. The growth abilities of both strains were compared by measuring the optical density of the growth medium every two hours at a wavelength of 500 nm.

RNA Isolation and Northern Blot Analysis

Cell disruption was performed by using the RiboLyser Cell Disrupter (Thermo Electron Corporation, Germany). Total RNA was isolated and purified by using the KingFisher mL pipetting robot (Thermo Electron Corporation, Germany) and the MagNA Pure LC RNA isolation Kit I (Roche Diagnostics, Germany) as described in detail by Jürgen *et al*. [13]. The quality of the isolated total RNA was analyzed by means of the Bioanalyzer 2100 from Agilent (Germany).

The effect of the putative ribonuclease BLi03719 on the stability

Table 2. Sequences of primers used in this study.

Primer number	Sequence 5′-3′
1	TGCTGGATCCCGGAAAGATTGCACAATC
2	GATATGGTGCAAGTCAGTCACGGATAAAGTCCTC
3	CTATGAGTCGCTTTTGTGTCAGACCGGATCGTC
4	TGCTGAATTCATTTTCGCTGACGCCAATC
5	GAGGACTTTATCCGTGACTGACTTGCACCATATC
6	GACGATCCGGTCTGACACAAAAGCGACTCATAG
7	GATGAAACCTATCTGCTGGACACTGGATTC
8	GAGTATCTAGAATCCATCCTGCTCGGGATC
9	GAATCGTTTTGCAAACATATATTAACCTCC
10	GGAGGTTAATATATGTTTGCAAAACGATTC
11	GATGTAAGGCGGTGGATACATGTTTG
12	GTTTTTTTTAAATTTAAACATATATTAACCTCCTTTTG
13	CAAAAGGAGGTTAATATATGTTTAAATTTAAAAAAAAC
14	GTCGAGGTACCATAGAAAAAGAGCATTTTTTG
15	GATGCAGGATATACAGCCATTCAG
16	CTAATACGACTCACTATAGGGAGACGTTTCAGATAGGACTGTAC

of the heterologous *amyE* mRNA was analyzed by northern blot analyses. Northern blot analyses were performed as described by Wetzstein *et al*. [25]. The specific hybridization reaction for *amyE* was performed with appropriate digoxigenin-labeled RNA probes. The probes were synthesized with the T7 RNA polymerase from the T7 promoter-containing internal PCR products of the *amyE* gene using the primer pairs 15/16 (Table 2).

Analysis of the Extracellular Ribonuclease Activity

The extracellular ribonuclease activity was analyzed by using a method described by Mossakowska *et al*. [14], in which yeast RNA (Roche, Germany) was used as the substrate for the RNase enzyme assay. A volume of 100 µl of growth medium supernatant was mixed with 900 µl of prewarmed 0.1 M Tris/HCl, pH 8.5, containing 2 mg/ml of yeast RNA. The mixture was then incubated at 37°C for 10 min. RNA hydrolysis was observed by the decrease in the absorbance at 298.5 nm. The ribonuclease enzyme activity was determined by comparing the rates of RNA hydrolysis against those obtained by known quantities of ribonuclease T1 ranging between 1 and 50 ng/ml (Sigma-Aldrich Co, USA).

Furthermore, the RNA degradation activity of extracellular ribonucleases was determined by using total RNA as the substrate isolated from exponentially growing cells of *B. licheniformis* MW3. For the assay, the supernatant of *B. licheniformis* cultures was diluted 20 times in distilled water. Afterwards, 9 µl of diluted solution was mixed with 1 µl of the total RNA sample and incubated at 37°C for 10 min. The mixtures were then denatured at 70°C for 2 min. One microliter of each reaction solution was loaded onto the RNA chip, and the RNA-degradation pattern was determined by using the RNA 6000 nano Kit and the Bioanalyzer 2100 (Agilent, Germany) according to the manufacturer's protocol.

The *B. licheniformis* control and mutant strains were grown under phosphate-limited growth conditions as described by Hoi *et al*. [11]. Samples of the supernatant were taken at the exponential growth phase, the transient phase, and 1, 2, 3, and 4 h after onset of the stationary growth phase (which corresponds to 4, 5, 6, 7, 8, and 9 h after inoculation, respectively) and used for RNase enzyme assays as described above.

Construction of Strains Carrying Heterologous α-Amylase and Xylanase Reporter Genes

In order to determine the effect of the putative ribonuclease BLi03719 on the intracellular RNA degradation activity, the expression of heterologous α-amylase and xylanase mRNA was investigated in the control and mutant strains. For this purpose, the 300 bp sequence containing the *phy* promoter (regulating the expression of the phytase gene, a strong inducible gene under phosphate starvation conditions [11]) from *B. licheniformis* DSM13 was cloned in front of the α-amylase and xylanase genes using the primer pairs 8/9 and 8/12, respectively. The *amyE* and *xynA* genes of *B. subtilis* 168 were amplified with the appropriate primer pairs, 10/11 and 13/14 (Table 2). The *phy*′-′*amyE* and *phy*′-′*xynA* fusions were obtained by means of the precise gene fusion polymerase chain reaction strategy described by Yon and Fried [26] using the primer pairs 8/11 and 8/14. The insertion of the PCR fusion fragments into the XbaI and KpnI sites of the multicopy plasmid pKUC (the combination of pUC18 and pKTH290 [20]) resulted in the correspondent vectors pKUC3 carrying the *amyE*

gene and pKUC4 carrying the *xynA* gene. Subsequently, the vector pKUC3 was transformed into the control strain *B. licheniformis* MW3 and into the mutant strain *B. licheniformis* Δ*BLi03719*, resulting in strains TL2A and TL2B. The vector pKUC4 was transformed into the strain *B. licheniformis* MW3 and in the *B. licheniformis* Δ*BLi03719* strain, resulting in the strains TL3A and TL3B, respectively. The obtained strains were then cultivated in the phosphate-limited BMM as described above. Culture supernatants for enzyme assays were taken at the exponential growth phase, the transient phase, and 1, 2, 3, and 4 h after onset of the stationary growth phase.

Analysis of Amylase and Xylanase Enzyme Activity

The amylase activity was determined with the Ceralpha kit (Megazyme International Ireland Ltd, Ireland). Amylase activity was calculated in "international units" (IU) by the equation IU/ml = 4.6 × (ΔE_{400} × 4.7 × Dilution), where ΔE_{400} = Absorbance at 400 nm (reaction) – Absorbance at 400 nm (blank). One international unit of activity is defined as the amount of enzyme required to release 1 µmol of glucose-reducing sugar equivalents per minute under defined conditions of temperature and pH (40°C, pH 6.5).

The xylanase activity was measured using the modified dinitrosalicylic acid (DNSA) method [2]. Xylan from beechwood (Sigma-Aldrich Co., USA) was used as a substrate at a concentration of 1% (w/v) in 0.05 M $NaPO_4$ buffer (pH 6.0). The calibration curve was prepared from a concentration of xylose ranging from 0.5 to 50 mM. A volume of 60 µl of sample was added to 540 µl of 1% xylan (prewarmed at 50°C) and incubated for exactly 10 min at 50°C. The reaction was terminated by adding 600 µl of DNSA solution and incubated for 10 min at 100°C. The reaction mixture was cooled on ice for 5 min and the absorbance was measured at 540 nm against the reaction blank. One unit of xylanase activity was defined as the amount of enzyme that liberates one micromole of reducing-sugar equivalent to xylose per minute under the assay conditions described.

Results

Analysis of the Genomic Context of the *BLi03719* Gene

A basic local alignment search tool (BLAST) analysis of the 453-bp-long nucleotide sequence of the *BLi03719* gene from *B. licheniformis* DSM13 (NC_006322.1) revealed 65% sequence similarities to a ribonuclease-encoding gene of *Bacillus pumilus* SAFR-032 (binase-like RNase) and 71% similarity to the barnase-encoding gene of *Bacillus amyloliquefaciens* FZB42 (data not shown). The *BLi03719* gene encodes a small pre-protein with 151 amino acids as reported for binase-like RNase and barnase. However, a BLAST analysis of the mature protein (residues 29–151)

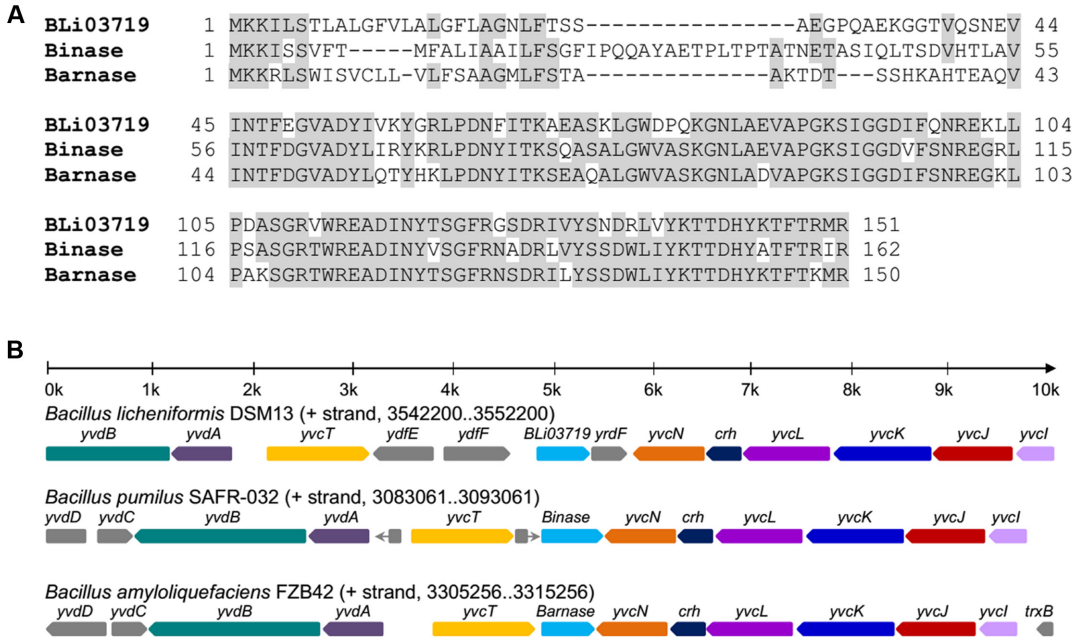

Fig. 1. Comparison of amino acid sequences and gene neighborhoods of selected ribonucleases.
(**A**) Multiple alignment of the amino acid sequences of BLi03719, binase, and barnase. The alignment was performed using the Clustal Omega algorithm at EMBL-EBI Web Services with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalo/). The proteins with the following UniProtKB accession numbers were used for comparison: Q65EH6 for BLi03719 in *B. licheniformis* DSM13; A8FHP7 for binase-like RNase in *B. pumilus* SAFR-032; and A7Z948 for barnase in *B. amyloliquefaciens* FZB42. (**B**) Gene neighborhood comparison. Data were generated from the MicrobesOnline Database (http://www.microbesonline.org).

revealed 75% sequence similarity with the barnase of *B. amyloliquefaciens* FZB42 and 73% sequence similarity with the binase-like RNase of *B. pumilus* SAFR-032 (Fig. 1A).

The genomic organization of the chromosomal loci of *BLi03719* of *B. licheniformis* DSM13 is similar to the binase-like RNase and barnase genomic environments. The ribonuclease genes are imbedded in a region that is flanked upstream by the *yvcT* gene and downstream by the *yvcI-yvcN* operon (Fig. 1B). Furthermore, the *BLi03719* gene of *B. licheniformis* DSM13 is flanked upstream by two more genes, named *ydfE* and *ydfF*, and downstream by the *yrdf* gene (Fig. 1B). The *ydfE* and *ydfF* genes were predicted to encode a putative flavoprotein and a putative transcriptional regulator, respectively. The *yrdf* gene from *B. licheniformis* DSM13 codes for a putative ribonuclease inhibitor. Its deduced amino acid sequence shows 62% similarity to the amino acid sequence of the barstar ribonuclease inhibitor protein of *B. amyloliquefaciens* FZB42 (data not shown).

The promoter analysis of the *BLi03719* gene revealed four TT(T/C/A)ACA-like motifs located between positions -80 and -140, which share strong similarity to so-called "Pho boxes" [11] (data not shown). These sequence motifs most likely play a role for the binding of the transcription factor PhoP. A putative PhoP box, which could explain the specific induction of the corresponding gene during phosphate limitation, was also predicted for the promoter region of the binase-like RNase gene of *B. pumilus* [22].

Analysis of the Extracellular Ribonuclease Activity

In order to investigate the physiological role of BLi03719, a *B. licheniformis* mutant strain was constructed in which the 453 bp sequence containing the *BLi03719* gene was deleted. The growth and the ribonuclease activity of this mutant strain were compared with the control strain under phosphate-limited growth conditions (Fig. 2). The growth experiments revealed no differences in the growth rate of the control and the mutant strains under these conditions (Fig. 2A).

The extracellular ribonuclease activity of both strains was tested by an incubation assay of culture supernatants with yeast RNA. The control strain *B. licheniformis* MW3 showed a maximal total RNase activity of about 6 ng/ml. In contrast, the *BLi03719*-deficient strain revealed a significant lower total RNase activity of about 1 ng/ml (Fig. 2A).

Furthermore, the activity of extracellular ribonucleases was tested by adding total RNA isolated from exponentially growing *B. licheniformis* MW3 cells as the substrate. Samples were taken and the integrity of RNA was determined with the Bioanalyzer. The results of the RNA degradation experiments

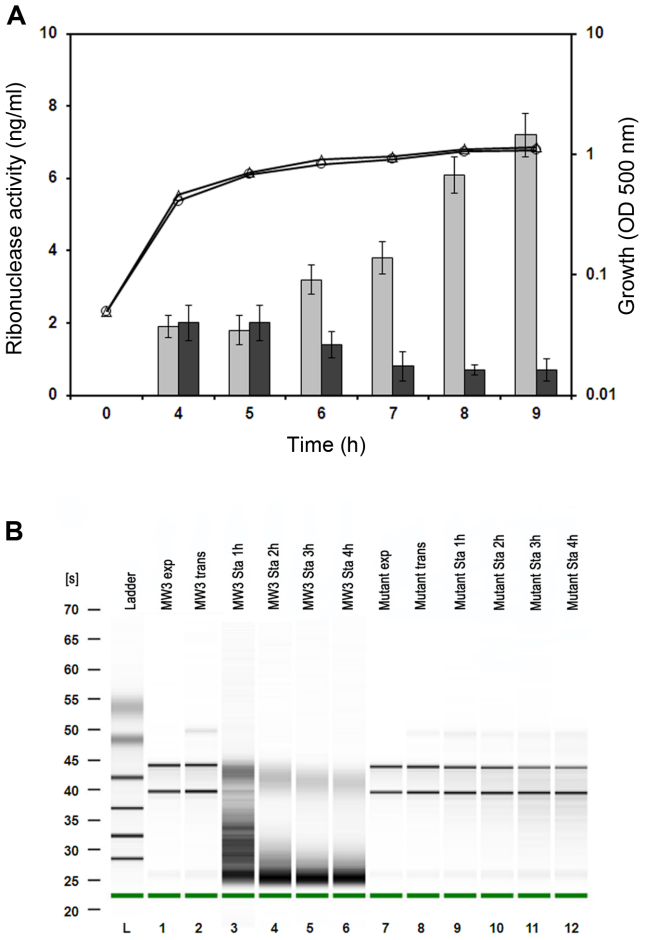

Fig. 2. Analysis of the extracellular ribonuclease activities.
(**A**) Extracellular ribonuclease activity determined by a method described by Mossakowska *et al.* [14]. Standard deviation based on $n = 3$, independent cultivations. *Lines* indicate cell growth, while *bars* indicate ribonuclease activity. Growth: *triangles* MW3, *circles* Δ*BLi03719*; Ribonuclease activity: *grey bars* MW3, *black bars* Δ*BLi03719*. (**B**) Analysis of RNA quality visualized with the Bioanalyzer 2100. A total RNA sample, isolated from *B. licheniformis* MW3 at the exponential growth phase, was used as substrate for the determination of ribonuclease activity of the control strain MW3 and mutant strain Δ*BLi03719*. Samples were taken during the exponential phase, transient phase, and 1, 2, 3, and 4 h after entry into the stationary phase, respectively. Control strain *B. licheniformis* MW3 lines 1–6; mutant strain lines 7–12.

revealed a strong degradation of the supplemented total RNA in the case of the control strain *B. licheniformis* MW3, whereas the *BLi03719*-deficient mutant strain was characterized by a strongly reduced extracellular RNase activity (Fig. 2B).

In addition, the effect of the BLi03719 protein on the growth of the control strain *B. licheniformis* MW3 and the *BLi03719* mutant strain with yeast RNA as substrate in the

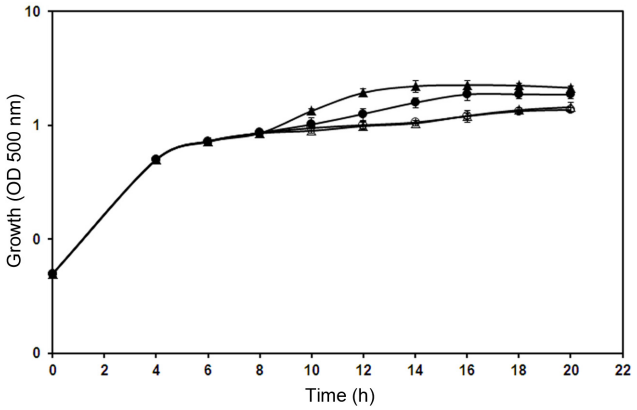

Fig. 3. Growth of *B. licheniformis* MW3 control and the mutant strain Δ*BLi03719* with and without addition of yeast RNA ($n = 3$, independent cultivations).
Yeast RNA was added to the growth medium after 8 h of cultivation. *Open symbols* without addition of Yeast RNA; *filled symbols* with addition of Yeast RNA; *triangles* MW3, *circles* Δ*BLi03719*.

medium was analyzed (Fig. 3). The results revealed that *B. licheniformis* MW3 cells can use yeast RNA as an alternative phosphate source for their growth when the inorganic phosphate source in the medium is depleted. However, there was a significantly postponed lag phase in the growth of the *BLi03719*-deficient strain compared with the control *B. licheniformis* MW3 under these conditions (Fig. 3).

Analysis of the Intracellular Ribonuclease Activity

In order to verify whether the extracellular ribonuclease BLi03719 might also have an effect intracellularly, the integrity of total RNA was determined in the *BLi03719*-deficient strain in comparison with the control strain. In both strains, the highest cellular RNA concentration was observed during the exponential growth phase (approximate 3 µg/µl) (Fig. 4A). Interestingly, when cells of the control strain *B. licheniformis* MW3 entered the stationary growth phase, the total cellular RNA was quickly degraded, ending in a final concentration of about 1 µg/µl. In contrast, the amount of total cellular RNA in the Δ*BLi03719* mutant strain still remained at higher concentrations of about 2 µg/µl until the late stationary phase (Fig. 4A).

Northern blot analysis of samples of the strains *B. licheniformis* TL2A and TL2B revealed that the *amyE* gene was strongly expressed both in the control (*B. licheniformis* TL2A) and the mutant strain (*B. licheniformis* TL2B) under phosphate starvation conditions. However, the *amyE* transcripts in the control strain TL2A were slightly degraded in

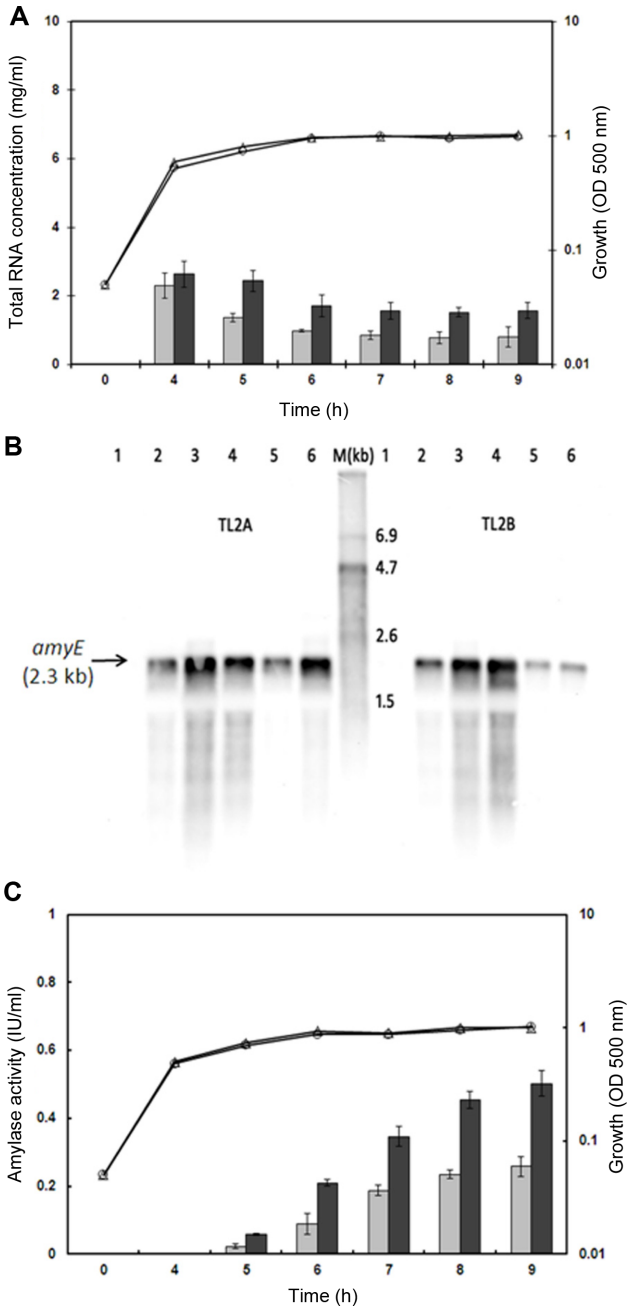

Fig. 4. Analysis of the intracellular ribonuclease activities.
(**A**) Total RNA concentrations isolated from *B. licheniformis* MW3 control and the mutant strain ($n = 3$, independent cultivations). *Lines* indicate cell growth, while *bars* indicate total RNA concentration. Growth: *triangles* MW3, *circles* Δ*BLi03719*; Total RNA concentration: *grey bars* MW3, *black bars* Δ*BLi03719*. (**B**) Northern blot analysis of the *amyE* gene expression in the control and mutant strains under phosphate starvation conditions. (**C**) Recombinant amylase activity in *B. licheniformis* control and mutant strains ($n = 3$, independent cultivations). *Lines* indicate cell growth, while *bars* indicate amylase activity (IU). Growth: *triangles* TL2A, *circles* TL2B; Amylase activity: *grey bars* TL2A, *black bars* TL2B.

comparison with the mutant strain TL2B (Fig. 4B). Since an increased degradation of the *amyE*-transcript was observed in the control strain in contrast to the mutant strain, the impact of BLi03719 on the α-amylase activity of strains TL2A and TL2B was determined. The model protein AmyE showed a maximal amylase activity of about 0.2 IU in the control strain TL2A and an at least 2-fold higher activity of about 0.5 IU in the BLi03719 deficient strain TL2B (Fig. 4C). These data are consistent with the results of the northern blot and showed that the higher degradation of the *amyE* transcript resulted in a decreased enzyme activity.

In order to investigate whether the BLi03719 ribonuclease has a general effect on the expression of heterologous genes in *B. licheniformis* cells under phosphate starvation conditions, the expression of the *xynA* gene was analyzed.

As shown in Fig. 5, there were no differences in the xylanase activity (Fig. 5A) comparing the control strain TL3A and the *BLi03719*-deficient strain TL3B, indicating that BLi03719 has no effect on the expression of *xynA* and hence no effect on the xylanase enzyme activity. In addition, these data were verified at the protein level (Fig. 5B). In both strains, XynA remained on the same level throughout the whole cultivation.

Discussion

The similarities of the nucleotide and amino acid sequences of BLi03719 from *B. licheniformis* to the barnase gene from *B. amyloliquefaciens* and binase-like RNase gene from *B. pumilus* suggest that the *BLi03719* gene encodes a ribonuclease. In addition, the similar genomic pattern of adjacent genes indicates that the BLi03719 enzyme might also be involved in the acquisition of alternative phosphorous or carbon resources under nutrient-limitation conditions as reported for the binase and the barnase enzymes [21]. The presence of potential PhoP-binding sites in the promoter region of the *BLi03719* gene suggests that the expression of this gene is regulated in a PhoPR-dependent manner under phosphate limitation conditions. This is in agreement with the strong expression of the *BLi03719* gene at the transcriptional level and the high abundance of this protein in the secretome under phosphate starvation conditions in *B. licheniformis* [11].

Prevoius studies described that binase-like and barnase-

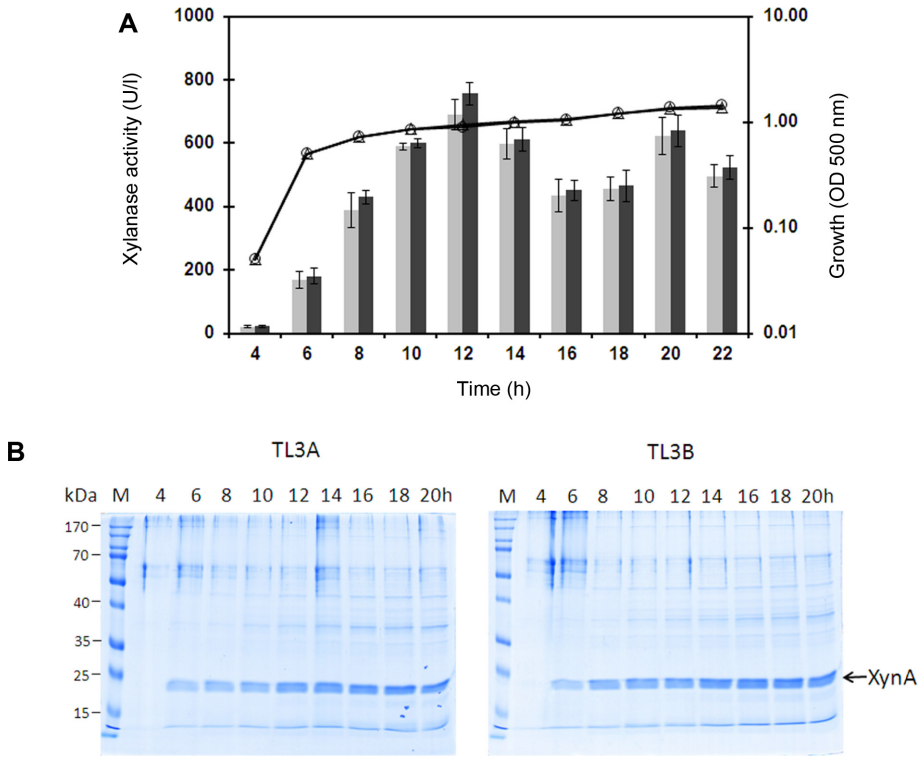

Fig. 5. Expression of the xylanase gene in *B. licheniformis* MW3 control and mutant strains.
(**A**) Recombinant xylanase activity in the control and mutant strains ($n = 3$, independent cultivations). *Lines* indicate cell growth, while *bars* indicate xylanase activity. Growth: *triangles* TL3A, *circles* TL3B; Xylanase activity: *grey bars* TL3A, *black bars* TL3B. (**B**) One-dimensional polyacrylamide gel electrophoresis separation of extracellular proteins in 20 μl of culture medium after removal of the cells by centrifugation.

like RNases were expressed differently to phosphate concentrations in the medium [22, 27, 28]. Whereas barnase-like RNases respond less sensitively to the concentration of phosphate in the medium, binase-like RNases are significantly induced at low phosphate concentrations. Ulyanova *et al*. [22] have shown that PhoP regulates the transcription of binase-like RNases under phosphate limitation conditions and was therefore described as a new member of the *Bacillus* PhoP regulon. In contrast, barnase-like RNases were regulated PhoP independently in a so-far unknown manner [22].

Data of this study underline that the *BLi03719* gene in *B. licheniformis* DSM13 encodes an extracellular ribonuclease that is regulated by the phosphate concentration in the medium. The expression of this gene is only induced when inorganic phosphate in the medium is limited. Previous studies have shown that this gene is not detectable under nitrogen or glucose limitation conditions in *B. licheniformis* DSM13 [23]. Our results suggest that the BLi03719 ribonuclease could be one of the major extracellular RNases in *B. licheniformis* DSM13 under these growth conditions. This is in agreement with the high accumulation of the BLi03719 protein in the extracellular proteome of phosphate-starving *B. licheniformis* cells [11].

Although BLi03719 seems to be one of the major extracellular RNases in *B. licheniformis* DSM13, the lower total cellular RNA concentrations and the degradation of the *amyE* transcripts in the control strain *B. licheniformis* MW3 compared with the *BLi03719*-deficient strain indicate that the intracellular BLi03719 ribonuclease activity in *B. licheniformis* is not completely inhibited during phosphate starvation conditions. This could be due to a lack of a specific inhibitor for this RNase activity. Bacterial species have developed a number of strategies to control the activities of their ribonucleases, such as by post-transcriptional regulation, post-translational modification, *trans*-acting inhibitors, and the secretion of such enzymes [4]. For *B. amyloliquefaciens*, the intracellular activity of the barnase ribonuclease is specifically inhibited by barstar, a small protein of 89 amino acids [7, 8]. It has been demonstrated that 30–40% of the barnase enzyme can be stored intracellularly for up to 90 min in a complex with the barstar inhibitor protein [3]. It is interesting to note that according to Ulyanova *et al*. [21], the binase-expressing *B. pumilus* does not possess a barstar-encoding gene. Instead, this bacterium encodes a paralogous putative RNase inhibitor (YrdF), which shares 49% amino acid sequence identity with the barstar protein. A similar putative ribonuclease inhibitor-encoding gene (*yrdf*) is located immediate downstream of the *BLi03719* gene in *B. licheniformis* DSM13. The sequence analysis also suggests that there is no transcriptional terminator structure between *BLi03719* and the *yrdf* gene. A terminator structure can only be found at the end of the *yrdf* gene. Therefore, the *BLi03719* and *yrdf* genes seem to belong to one operon. However, a specific inhibitory effect of YrdF for the RNase activity of BLi03719 remains questionable. Ulyanova *et al*. [21] stressed that all attempts to purify a physiological inhibitor of binase have failed so far. In this respect, it is worth mentioning that Nijland *et al*. [15] observed that the overexpression of a barnase-encoding gene from *B. licheniformis* EI-34-6, which is homologous to *BLi03719*, requires the co-expression of the *yrdF* homologous gene from *B. licheniformis* in *Bacillus subtilis* NZ8900.

Moreover, the protein BLi03719 of *B. licheniformis* DSM13 is not detectable in the soluble cytoplasmic proteome, but it belongs to the most dominant protein spots in the extracellular proteome under phosphate starvation conditions [11, 23]. Sharipova *et al*. [18] described that about 1% of the binase activity could be associated with the cell wall, but no significant binase activity is detectable in the cytoplasm. In this respect, the observed advantageous effect of the *BLi03719*-deficient strain for the overexpression of the heterologous *amyE* gene under phosphate starvation conditions is remarkable. However, no difference was observed for the overexpression of the heterologous model protein xylanase between the *BLi03719*-deficient strain and the control strain. These data suggest that the *xynA* transcript is somehow more stable against ribonuclease activity. As reported for *Bacillus stearothermophilus* No. 236, an ORF located immediately downstream of the *xynA* gene is proven to encode a protein involved in the stabilization of the *xynA* mRNA [12]. A similar protein-encoding gene (DEAD-box RNA helicase) is located immediately downstream of the *xynA* gene in *B. licheniformis* DSM13.

This study has shown that BLi03719 seems to be one of the major extracellular RNases in *B. licheniformis* DSM13 under phosphate starvation conditions. The results of the expression analysis of heterologous genes *amyE* and *xynA* indicate that such ribonuclease-encoding genes could be potential targets for the optimization of the expression of selected heterologous genes under specified nutrient-limited growth conditions.

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