Exopolyphosphatase of *Lysinibacillus sphaericus* is Essential for Stresses Resistance and Production of Binary Toxins

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Abstract

The Exopolyphosphatase (PPX) of *Lysinibacillus sphaericus* is encoded by the Bsph_1303 gene (ppx), being responsible for the hydrolysis of inorganic polyphosphates to orthophosphate (Pi). In the present work, we identified and characterized the ppx from *L. sphaericus*. We found that ppx disruption resulted in massive polyP accumulation, compromised the resistance to high osmotic, high pH and high temperature, and impaired the motility and biofilm formation. Additionally, results obtained from the ppx null mutant demonstrated that PPX was involved in the production of binary toxins and growth in MBS medium, a medium for toxicity production. These results indicated that PPX of *L. sphaericus* appeared to be essential for the resistance to stresses and binary toxins production.

Keywords: Polyphosphate; Binary toxins; *Lysinibacillus sphaericus*; Exopolyphosphatase

Abbreviations

PolyP: Polyphosphate; PPX: Exopolyphosphatase

Introduction

Inorganic Polyphosphates (polyPs) are linear polymers consisting of tens to hundreds of Orthophosphate (Pi) residues linked by energy-rich phosphoanhydride bonds. Numerous investigations indicate that polyP is essential for the growth of microorganisms, their responses to various environmental stresses, and the virulence of pathogens [1]. PolyP is synthesized by Polyphosphate Kinase (PPK) that catalyses the reversible transfer of terminal phosphate of ATP to polyP chain [2]. PolyP can be degraded by the Exopolyphosphatase (PPX) that releases Pi processively from the termini of the polyP chain [3].

Although polyP has been shown to have important functions in many microorganisms, there are few studies on the role of this polymer in *Bacillus*. Feasible reason is that the important model organism, *Bacillus subtilis* does not harbor the ppx gene [1]. The first study about polyP in *Bacillus cereus* demonstrated that polyP is involved in motility, biofilm formation, and sporulation

[4]. Other investigations reported that the exogenous long-chain polyP inhibited the cereulide toxin synthesis [5] and septum formation, and caused cell lysis [6]. Additionally, in *Bacillus thuringiensis*, overexpression of ppx gene increases bioinsecticide production [7].

L. sphaericus is mesophilic, aerobic rod-shaped bacteria, and some of them produce parasporal crystal proteins specifically toxic to *Culex* and *Anopheles* mosquito larvae and have been widely used as biocontrol agents. The larvicidal activity of *L. sphaericus* is mainly due to the production of two crystalline mosquito-larvicidal proteins of 42 kDa (BinA) and 51 kDa (BinB) during sporulation [8,9]. *L. sphaericus* is considered one of the most successful microbial larvicide and has been commercialized over the past decade [10]. Besides the binary toxins, some other toxins such as sphaericolysin, vegetative mosquitocidal toxins, Cry48/ Cry49 toxin are also responsible for its larvicidal activity [10]. To date, the mechanism by which Bin kills mosquito larvae remains largely unsolved. One hypothesis is that pore formation by binary toxins may be sensed by p38 mitogen-activated protein kinase, whose activity might induce autophagy [10,11]. Moreover, to our knowledge there was nearly no study on resistance of *L. sphaericus* to environmental stresses, which was essential for its recycling in mosquito breeding sites. Recently, we identified the ppx gene of *L. sphaericus* C3-41 and found that ppx gene affected polyP accumulation and bacterial growth [12]. Various stresses were encountered when *L. sphaericus* accessed to the haemocoel of mosquito and recycled in mosquito cadaver [13,14]. So, responses to all stresses were essential for growth and toxin production of *L. sphaericus*. Recent genetic and molecular analyses have revealed how several strategies enable bacteria to persist and overcome host immune defences [15]. PolyP is essential for growth, survival and the virulence of pathogens [1,16].

Here, we have provided evidence that PPX of *L. sphaericus* is required for stress resistance such as osmotic pressure, high pH value and high temperature, and for motility and biofilm

formation. We also demonstrated that binary toxins production is affected by PPX.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used for this study are listed in Table 1. *Escherichia coli* DH5α and BL21 (DE3) were used as hosts for cloning and expression, respectively. The ppx was amplified from *L. sphaericus* C3-41 genomic DNA with the primer pairs: Fppx and Rppx. The PCR product was excised and cloned between the *Bam*HI/*Sac*I sites of pET28a+ to generate the plasmid pET28a-BsPPX.

Null mutant allele of ppx was obtained by using a two-step PCR strategy [12]. The resulting plasmid for allelic displacement

and reverse mutation of ppx were named as pNR5101-PPX and pHT304-ppx, respectively. The primers were listed in Table 2.

Media and growth conditions

Unless otherwise specified, *E. coli* strain was grown at 37°C in Luria-Bertani (LB) medium. *L. sphaericus* were grown at 30°C in LB or MBS medium (pH 7.4) [17]. Antibiotics were added to growth media at the following final concentrations: Ampicillin (100 µg/ ml), Erythromycin (20 µg/ ml), Kanamycin (50 µg/ ml for *E. coli*, 10 µg/ ml for *L. sphaericus*) and tetracycline (10 µg/ ml). For solid medium, agar was added to a final concentration of 1.5% (w/ v).

Cloning and expression of ppx genes

Recombinant plasmid pET28a-PPX was transformed into *E.*

Table 1: Strains and plasmids used for this study.

Strains or plasmids	Characteristics	Source or reference
<i>Escherichia coli</i>		
JM109	Cloning host	Lab stock
BL21(DE3)	Expression host	Lab stock
<i>B. sphaericus</i>		
C3-41	Wild type strain bearing native pBsph plasmid	Lab stock
Δppx	C3-41 mutant with deletion of the ppx gene	This study
Δppx (pHT304-ppx)	Δppx with plamid pHT304-ppx	This study
Plasmids		
pET28a	Expression vector in <i>E. coli</i>	Lab stock
pET28a-PPX	pET28a with ppx gene	This study
pRN5101	Recombinant vector used in gene disruption experiments, Amp ^r , Erm ^r	[26]
pRN5101-PPX	pRN5101 carrying partial ppx deletion gene	This study
pHT304	Plasmid designed for gene expression in Bacillus.	[27]
pHT304-spc	pHT304 with spc promoter region	This study
pHT304-ppx	pHT304 with ppx gene fused to the downstream of spc promoter region	This study
pMarC333	Source of the spectinomycin promoter region	[28]

Table 2: Oligonucleotide primers designed to generate DNA fragment for plasmid construction.

Primers	Sequences (5' to 3')	Restriction sites
Primers used in plasmid construction		
Fppx	CGCGGATCCATGCTTGAAGGATGTGATTATC	<i>Bam</i> HI
Rppx	TA CGAGCTCGGCTCATTATTCGTTATTT	<i>Sac</i> I
Fppx-up-ko	ACGCGTCTGACTGTTCCATGTGGATAACGTGACA	<i>Sall</i>
Rppx-up-ko	GCCCCTCTTCATTGTTATACTGATA	
Fppx-down-ko	AAGGATGGAATAAGGATGACAAC	
Rppx-down-ko	CTAGCTAGCCCTCCAGTAGTACAACGACTGTT	<i>Nhe</i> I
Fkan-ppx	TATCAGTATAACAATGAAGAGGGGCTAAACCCAGCGAACCA	
Rkan-ppx	GTTGTCATCCTTATTCATCCTTTCTAGGTACTAAAACA	
Fppx-up	TGTGAACAAAATAAAC	
Rppx-up	TGGTTCGCTGGGTTTA	
Fppx-down	GTCATTGGGATTTTTA	
Rppx-down	TGTTTTAGTACCTAGA	
Fppx-com	CGCGGATCCATGCTTGAAGGATGTGATTATC	<i>Bam</i> HI
Rppx-com	TA CGAGCTCGGCTCATTATTCGTTATTT	<i>Sac</i> I
Fspc-promoter	CCCAAGCTTAGAGTTGGTAGCTCTTGATCC	<i>Hind</i> III
Rspc-promoter	CGCGGATCCGATTTCACCTCGTTGATTATGT	<i>Bam</i> HI

Restriction sites engineered into the primers are shown in underlined letters.

coli BL21 (DE3). Cells were grown at 37°C to an OD₆₀₀ of 0.6. For expression of PPX, IPTG was added to a final concentration of 0.1 mM and growth was continued for 16h at 20°C. Cells were harvested, re-suspended in 10 mM Tris-HCl (pH 7.5) with 1 mM MgCl₂, 1 mM PMSF and disrupted by sonication. Recombinant and His6-tagged PPX was purified from the cell-free supernatant by chromatography on Ni²⁺-NTA agarose.

PPX activity assay

The activity of PPX was measured by the detection of Pi product [3]. Briefly, the reaction mixture (100 µL) contained 50 mM Tricine/ KOH (pH 8.0), 1 mM MgCl₂, 175 mM KCl, 22 µg PPX, and polyP as indicated (provided kindly by Shinichi Kato [Regene Tiss Inc.]). MgCl₂ was added last to avoid precipitation of the polyP. The Pi generated by PPX was continuously assayed for 45 min using Molecular Probes' EnzChek® Phosphate Assay Kit according to the manufacturer's protocol. One unit of enzyme is defined as the amount releasing 1pmol of phosphate from polyP per min.

Construction of ppx mutants pNR5101-PPX plasmid for knockout of ppx genes was transformed into *L. sphaericus* C3-41 by electroporation [12]. The resulting mutation of ppx gene was named as Δppx and the reverse mutation of ppx gene was named as ΔppxC. All primers for knockout and validation of Δppx and construction reverse mutation of ppx were presented in Table 2.

Extraction and quantification of polyP

The PolyP was extracted from *L. sphaericus* cells with Glass milk as described previously [18]. Briefly, 2 ml of *L. sphaericus* WT, Δppx and ΔppxC cultures at time points indicated were pelleted in a 1.5 ml tube for 2 min, respectively. To the pellet were added 0.5 ml of 4 M Guanidine Isothiocyanate (GITC)-50 mM Tris-HCl, pH 7.0 (GITC lysis buffer) and sonicated briefly; a 10 µL sample was removed for protein estimation. To each tube were added 30 µL of 10% sodium dodecyl sulphate, 0.5 ml of 95% ethanol, and 5 µL of Glass milk (Bio 101). After being centrifuged briefly, the pellet was then suspended in 0.5 ml of cold New Wash buffer (5 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5mM EDTA and 50% ethanol). The washed pellet was then resuspended in 50 µL of 50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂-20 µg each of DNase I and RNase A per ml and incubated at 37°C for 10 min to remove DNA and RNA. The pellet was washed first with 150 µL of 4 M GITC lysis buffer and 150 µL of 95% ethanol, and then twice in New Wash buffer. PolyP was eluted from the pellet with 50 µL of 50 mM Tris-HCl (pH 8.0) at 95°C for 2 min.

The amount of PolyP in *L. sphaericus* cells was quantitated as described previously [12]. The amount of polyP in *L. sphaericus* cells was measured based on the character that polyP can be transformed to ATP by PPK reverse activity. The ATP concentration was assayed with Bioluminescence Assay Kit CLSII (Roche), and concentration of polyP is given in terms of Pi residues.

Resistance to stress conditions

The resistance to stress conditions of wild type, Δppx and ΔppxC were performed as described [19]. To test resistance to

oxidative stress, 100 µL overnight cultures were spread on LB agar medium in 90-mm-diameter Petri dishes. Subsequently, 3 MM filter paper disc (5 mm in diameter) containing 5µl of a solution of 50 mM menadione (sigma) were layered on the plates. After 2 days of incubation at 30°C, the halos of growth inhibition were measured.

Biofilm and motility assays

Biofilm formation and motility assays were performed as described [4,20].

Western blot analysis

L. sphaericus cultures were grown in MBS medium at 30°C. Cells were harvested at indicated times and washed twice with sterile water and then centrifuged. The soluble proteins were electrophoresed in 12% SDS-PAGE gels and subsequently transferred to PVDF membranes. After blocking, the membranes were incubated for 2 hours with primary antibody (anti-BinA, 1:3000 and anti-BinB 1:1000) followed by incubation with goat anti-mice HRP (horseradish peroxidase, 1:3000 dilution) secondary antibody (Millipore). Finally, membranes were developed using the luminal (ECL; Amersham Biosciences).

Bioassays

The toxicities of *L. sphaericus* C3-41, Δppx and ΔppxC against fourth instar larvae of a susceptible *Culex quinquefasciatus* colony were assayed by bioassay, performed as described by Yang et al. [21]. Lethal concentrations of 50% and 90% were determined by probit analysis with a program indicating mean and Standard Error (SE).

Statistical analysis

Results are indicated as means and the standard deviation (n = 3-8). The significances of the difference of the means in experiments carried out with wild type, Δppx and ΔppxC were calculated using a Student t test.

Results

Expression of ppx and activity assays

The gene encoding putative counterparts of PPX has been identified in *L. sphaericus* (Bsph_1303). The putative PPX of *L. sphaericus* C3-41 was of 524 amino acid residues, with a calculated molecular masses of 57.64 kDa, and 44% identical to those of *B. cereus* E33L. The gene products were expressed as N-terminal His-tagged proteins in *E. coli* and purified by affinity chromatography on Ni²⁺-NTA agarose (Figure 1A). As shown in Figure 1B, recombinant PPX demonstrated polyP type-dependent activity. The polyP₁₄, polyP₆₀ and polyP₁₃₀ were hydrolyzed by PPX with a specific activity of 6.48 × 10³, 6.83 × 10³ and 14.24 × 10³ units/ mg protein, respectively (Figure 1D). Furthermore, recombinant protein PPX also demonstrated a polyP concentration-dependent activity (Figure 1C), hydrolyzing 3 µM polyP₁₃₀ and 10 µM polyP₁₃₀ with specific activity of 6.75 × 10³ and 23.3 × 10³ units/ mg protein, respectively (Figure 1D). These results confirmed the function of PPX in polyP metabolism.

Figure 1: Enzymatic activity of PPX. (A) SDS-PAGE of purified PPX. Lane 1: standard protein marker; lane 2: purified PPX; (B) Time course of different length polyP (5 µM) degradation by PPX (22 µg); (C) Time course of different concentration polyP130 degradation by PPX (22 µg); (D) The specific activity of PPX when hydrolyzing different polyP length and concentration. The specific activity of PPX was measured according to (B) and (C).

Inactivation of ppx resulting in polyP accumulation

In order to prove the effect of the ppx gene on accumulation of polyP, the Δppx was obtained by homologous recombination. As shown in Figure 2, there was significantly more abundant polyP in Δppx compared with that in WT and the polyP levels in Δppx were reduced significantly by expressing ppx gene.

Effect of a mutation in ppx on the resistance to stresses in *L. sphaericus*

L. sphaericus C3-41 WT and the Δppx were grown in LB medium under different conditions (high salt, high temperature, low pH, or high pH) in order to evaluate their ability to grow under stress conditions (Figure 3). Growth under no stress conditions (LB medium) was not affected by PPX disruption (Figure 3A). The extent of the effect of the Δppx varied under the different stress conditions. The effect of PPX disruption seems different with stress. High salt seems little effect on initial growth rate but final OD. High pH seems to affect initial growth rate. High temperature seems not affect growing rate but decrease of OD seems earlier in Δppx mutant (Figure 3B, C and D). The defect could be partially restored by plasmid expression of ppx in the mutant strain. Growth in the presence of menadione (a superoxide radical generator) was not affected by the Δppx under our experimental conditions (data not shown).

Additionally, the biofilm formation and swimming on semisolid agarose plates of mutants were dramatically decreased (Figure 4). It is clear that the decrease was not attributable to the impairment of growth, because the growth rates of mutants were indistinguishable from that of WT in LB medium (Figure 3A).

Binary toxins production by mutant and control strains

Growth of Δppx was impaired compared with WT when grown in MBS medium (Figure 5A), a medium for toxicity production [17]. In order to compare binary toxins production by Δppx with that by WT, we analyzed protein levels of binary toxins in the bacterial cells with the same wet-weight through SDS-PAGE and

Figure 2: Measurement of polyP extracted from *L. sphaericus* WT, Δppx and ΔppxC grown in LB to log-phase. The results are from three measurements. ***, p < 0.01.

Figure 3: Motility and biofilm formation of WT and mutant. (A) Motility. Cells were inoculated on swimming plates with 12-h incubation at 30°C and they were photographed. Each is representative of the strain; (B) Biofilm formation. An overnight LB culture (2µl) was inoculated into 200 µL of LB in 96-well plates and incubated at 30°C without shaking. After 12-h incubation, the media were removed; each well was rinsed with sterile water, and were stained with crystal violet, extracted and measured. Data represent mean values and standard deviations for eight replicates. ***, p < 0.01.

western blot. Results suggested that binary toxins production by the *Appx* was significant lower compared with WT at different times (Figure 5B and C). Bioassay results against fourth instar larvae showed that the toxicity of *Appx* was significant lower than that of wild type (Table 3). The binary toxins production and toxicity could be partially restored by expression of *ppx* gene in the mutant strain.

Discussion

In the present study, we identified the PPX function of *L. sphaericus* C3-41 and disrupted the *ppx* gene with homologous recombination. PolyP extraction and quantitation showed that polyP were significantly more abundant in *Δppx* than in WT. Moreover, our data indicated that a high intracellular concentration of polyP in the *Appx* compromised the resistance of *L. sphaericus* to high osmotic stress, high pH and high temperature,

Table 3: Larvicidal activity of <i>L. sphaericus</i> C3-41, <i>Appx</i> and <i>ΔppxC</i> .		
Strains	LC ₅₀ *(95% CL) [†]	LC ₉₀ *(95% CL) [†]
C3-41	0.320 (1.154 < CL < 2.043) [†]	5.218 (3.797 < CL < 8.548) [†]
<i>Appx</i>	9.544 (16.021 < CL < 28.601) [†]	36.459 (25.364 < CL < 71.270)
<i>ΔppxC</i>	2.339 (3.251 < CL < 4.438) [†]	7.224 (9.111 < CL < 12.602) [†]

*LC50 and LC90, volume (in μL) of cultures of *L. sphaericus* C3-41, *Appx* and *ΔppxC*.
[†]95% CL, 95% confidence limits.

and impaired mobility and biofilm formation. Biofilm formation and motility is one of ways to adapt to stresses and stringencies by concentrating nutrients and escaping environmental stresses [22]. Previous study showed that *ppk* gene knockout resulted in polyP deficiency and affected the growth of *L. sphaericus* C3-41[12]. These results suggested that maintenance of the polyP levels within a normal range were essential for growth and its responses to stresses.

The Binary toxins comprises two proteins, BinA and BinB that are co-transcribed from a single operon just before the end of exponential growth and into sporulation, and strains blocked in early stage sporulation do not accumulate toxin crystals [10]. Presently, information about the expression control of toxin was rare. Notably, El-Bendary et al. [23] identified two genes involved in sporulation, *spo0A* and *spoilAC*, which might control expression of the binary toxin genes. Our lab identified several sporulation-associated genes by transposon-mediated insertional mutagenesis, which affected expression of binary toxin genes [24]. These studies indicated that crystal protein synthesis is dependent on initiation of sporulation in *L. sphaericus*. In this study, we found that *ppx* disruption compromised the production of binary toxins. Although the *B. cereus* *Appx* impaired sporulation efficiency [4], the impairment of sporulation in *L. sphaericus* *Appx* was not observed (result not shown). Possibly, polyP affected binary toxins production by other unknown reasons, which remain to be investigated.

In summary, we provide evidence that *ppx* mutation accumulated massive polyP, compromised resistance to high osmotic stress, high pH and high temperature, and impaired motility and biofilm formation, and toxin production.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Figure 4: Effect of a mutant in *ppx* from *L. sphaericus* on growth under different stress conditions. Growth curves of *L. sphaericus* strains (WT, *Δppx* and *ΔppxC*) are shown. (A) Growth in LB medium; (B) growth in LB medium with 0.6 M NaCl; (C) growth in LB medium adjusted to pH 9; (D) growth in LB medium at 42°C. The results are the means of at least three experiments.

Figure 5: Effect of a mutant in *ppx* from *L. sphaericus* on growth and binary toxins production grown in MBS medium. (A) Growth curves of WT, *Appx* and *ΔppxC*; (B) western blot analysis of binary toxins proteins synthesis WT, *Appx* and *ΔppxC*.

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