Microbiology (2008), **154**, 1333–1340 DOI 10.1099/mic.0.2007/014399-0

The role of a purine-specific nucleoside hydrolase in spore germination of *Bacillus thuringiensis*

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Received 30 October 2007
Revised 4 February 2008
Accepted 6 February 2008

A homologous gene (*iunH*) of a putative nucleoside hydrolase (NH), which had been identified from the exosporia of *Bacillus cereus* and *Bacillus anthracis* spores, was cloned from *Bacillus thuringiensis* subsp. *kurstaki*. Disruption of *iunH* did not affect the vegetative growth and sporulation of *Bacillus thuringiensis*, but promoted both inosine- and adenosine-induced spore germination. The inosine- or adenosine-induced germination rate decreased when the wild-type *iunH* gene was overexpressed in *Bacillus thuringiensis*. The *iunH* gene product was characterized as a purine-specific NH. The kinetic parameters of IunH with inosine as substrate were K_m=399±115 μM, k_{cat}=48.9±8.5 s^{-1} and k_{cat}/K_m=1.23×10^5 M^{-1} s^{-1}. The optimal pH and temperature for IunH were found to be pH 6 and 80 °C. Meanwhile, the specific activity of inosine hydrolase in intact spores of the wild-type strain with inosine as substrate was 2.89±0.23×10^{-2} μmol min^{-1} (mg dry wt)$^{-1}$. These results indicate that IunH is important in moderating inosine- or adenosine-induced germination of *Bacillus thuringiensis* spores.

INTRODUCTION

Enzymes with nucleoside hydrolase (NH) activity are widely distributed in both prokaryotes and eukaryotes (Giabbai & Degano, 2004). Non-specific NHs, the most well-known of which is the inosine/uridine-preferring NH (IU-NH) from *Crithidia fasciculata*, hydrolyse both purine and pyrimidine nucleosides (Parkin *et al.*, 1991). Purine-specific NHs (inosine/adenosine/guanosine-preferring NHs] display strict specificity (Parkin, 1996; Pelle *et al.*, 1998; Versees *et al.*, 2001). NHs are crucial in the salvage pathway of protozoan parasites which are deficient in the pathway for *de novo* synthesis of purine nucleotides (Gopaul *et al.*, 1996). Non-parasitic NHs, for example the salivary purine nucleosidase of the mosquito *Aedes aegypti*, may be involved in the degradation of modified nucleosides found in tRNA (Giabbai & Degano, 2004; Ribeiro & Valenzuela, 2003), but apart from this, no clear role for non-parasitic NHs, including the NH identified from the exosporia of *Bacillus anthracis* and *Bacillus cereus*, has been reported so far (Giabbai & Degano, 2004; Redmond *et al.*, 2004; Ribeiro & Valenzuela, 2003; Todd *et al.*, 2003).

Bacillus thuringiensis, *B. anthracis* and *B. cereus* are members of the Gram-positive endospore-forming *B. cereus* group. They could be classified as one species on the basis of genetic evidence, even though they demonstrate widely different phenotypes and pathological effects (Helgason *et al.*, 2000). As a natural pesticide, commercial formulations of *B. thuringiensis* containing separate entities of crystals and spores has become the leading biological insecticide used to control agricultural pests (Crickmore, 2006; Liu *et al.*, 1998). *B. anthracis* is the cause of the acute and often lethal disease anthrax, and *B. cereus* is a ubiquitous soil bacterium and opportunistic human pathogen (Helgason *et al.*, 2000).

Under conditions of nutrient depletion at high cell density, vegetative cells of *Bacillus* species can transform into spores by a process called sporulation. The spore is metabolically dormant, and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals. The dormant spore also monitors its environment, and when conditions become favourable again, the spore germinates and is converted back into a new vegetative cell (Setlow, 2003). In nature, spores probably germinate in response to nutrient germinants which are generally single amino acids, sugars or purine nucleosides (Setlow, 2003). Among nutrient germinants, the combination of L-alanine and inosine is the best germinant for most *Bacillus* spores. Inosine is an independent germinant for *B. cereus* and *B. thuringiensis* spores, and an important co-germinant in *B. thuringiensis*, *B. cereus* and *B. anthracis* spore germination (Foerster & Foster, 1966; Hornstra *et al.*, 2006).

The first event in nutrient-induced spore germination is probably the activation of the germinant receptors, which

Abbreviation: NH, nucleoside hydrolase.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of *iunH* is EU072023.

are located in the inner membrane of the spore (Hornstra *et al.*, 2005). To activate the receptors, germinants must first penetrate the outer spore layers and access their corresponding receptors. The outermost layer of the *B. cereus* group spore is a loose-fitting, balloon-like structure known as an exosporium, which consists mainly of protein, polysaccharides, lipids and ash (Matz *et al.*, 1970). Dozens of proteins, including a putative NH, were identified from the exosporia of *B. cereus* and *B. anthracis* spores (Redmond *et al.*, 2004; Steichen *et al.*, 2003; Todd *et al.*, 2003). Although the role of some proteins has been studied, most functions remain to be elucidated (Boydston *et al.*, 2006; Ramarao & Lereclus, 2005; Steichen *et al.*, 2005; Yan *et al.*, 2007).

Widespread distribution of NH raised the question of whether these genes indeed encode NH enzymes, and most importantly, what their role is, especially in non-parasitic organisms which could recycle nitrogenous bases via NP-catalysed phosphorolysis (Giabbai & Degano, 2004). In this paper, we have characterized one non-parasitic, purine-specific NH from *B. thuringiensis* and demonstrated its role in moderating inosine- or adenosine-induced spore germination.

METHODS

Bacterial strains, plasmids and culture conditions. All the strains and plasmids used in this study are listed in Table 1. *B. thuringiensis* was routinely grown in Luria–Bertani (LB) medium at 30 °C containing appropriate antibiotics (25 μg erythromycin ml^{-1} or 10 μg chloramphenicol ml^{-1}). Casein hydrolysate/yeast-containing medium (CCY) was used for spore preparation (Stewart & Halvorson, 1953). For subcloning, *Escherichia coli* DH5α was grown at 37 °C in LB medium, containing ampicillin, erythromycin or chloramphenicol when necessary, for propagating plasmids.

DNA manipulations and transformation. *B. thuringiensis* genomic DNA was isolated as described previously (Gonzalez *et al.*, 1981). Primers P1 (5′-GCTCTAGAGAACCGATAATACCAGC-3′, *Xba*I site is underlined), P2 (5′- GGGGTACCGAAGTCGCCAATAAATAG-3′, *Kpn*I site is underlined), P4 (5′-GGGGTACCGTCATAATAATCGTC-TTCTTG-3′, *Kpn*I site is underlined) and P5 (5′-GCTCTAGA-CTAAATGATGAATTGGACC-3′, *Xba*I site is underlined) were designed according to the conserved sequence of the *B. cereus* group. The DNA fragment containing *iunH* was amplified from genomic DNA of *B. thuringiensis* subsp. *kurstaki* CGMCC 1.1752 with primers P1 and P2. After digestion with *Xba*I and *Kpn*I, the amplified DNA fragment was ligated into the corresponding sites of pKSV7 to generate pIC. Sequencing of the fragment was carried out by the SunBiotech Company (Beijing, PR China). Southern hybridization was carried out with probes labelled with a digoxigenin DNA labelling kit (Roche Biochemicals), according to the manufacturer's instructions. All PCRs were performed with *Pfu* DNA polymerase (TaKaRa) using standard conditions.

For *B. thuringiensis* electroporation, 80 μl cell suspensions in 40 % PEG were used per cuvette plus 1 μl (about 1 μg) plasmid DNA (dissolved in distilled water) at 11 kV cm^{-1}, 1000 Ω and 25 μF.

Construction of the *iunH* disruption mutant. To construct an *iunH* disruption mutant, a DNA fragment corresponding to the upstream region of *iunH* (extending from positions −1003 to +223 with respect to the *iunH* translation initiation site) was amplified with primers P3 (5′-ACGCGTCGACGCTGTCATCGGTCTACTC-3′, *Sal*I site is underlined) and P4, and a DNA fragment corresponding to the downstream region of *iunH* (extending from positions −306 to +846 with respect to the *iunH* stop codon) was amplified with primers P5 and P6 (5′-CGGGATCCAATGATTGATTTTTATTATGAG-3′, *Bam*HI site is underlined) from *B. thuringiensis*. These PCR products were digested with *Sal*I and *Bam*HI, respectively. A 1256 bp DNA fragment containing the erythromycin resistance gene (*erm*) was amplified from pHT3101 with primers P7 (5′-ATAGGATCCAATAAGGGCGACACG-3′, *Bam*HI site is underlined) and P8 (5′-ACGCGTCGACCCCTTAG-AAGCAAACT-3′, *Sal*I site is underlined), and digested with *Sal*I and *Bam*HI. The digested PCR fragments were purified, mixed in equal amounts and ligated with T4 DNA ligase. The ligation mixture was used as a template to amplify the complete tripartite DNA fragment

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Source or reference
B. thuringiensis subsp. *kurstaki* 1.1752	Wild-type strain for this study	CGMCC†
E. coli		
DH5	*supE44* Δ*lacU169*(*φ*80 *lacZ*Δ*M15*)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al.* (1989)
BL21 (DE3)	*hsdS gal* (*λcIst857 indl sam7 nin5 lacUV5*-T7 *gene1*)	Novagen
Plasmids		
pBluescript KS	Cloning and subcloning vector	Stratagene
pET28a	Expression vector in *E. coli*, Km^r	Novagen
pKSV7	Integrative vector, Ap^r Cm^r	Smith & Youngman (1992)
pIC	pKSV7 carrying *iunH*	This work
pIE	pET28a carrying *iunH*	This work
pID	pKSV7::*iunH*::Em^r	This work
pHT3101	Expression vector, Ap^r Em^r	Lereclus *et al.* (1989)

*Ap, Ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin.
†CGMCC, China General Microbiological Culture Collection Centre, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, PR China.

with primers P4 and P5. Then, the amplified 3.6 kb DNA fragment was digested with *Xba*I and *Kpn*I, and inserted into the corresponding sites of pKSV7 to give pID. Subsequently, pID was introduced into *B. thuringiensis* by electroporation, and cultured on LB plates containing erythromycin at 30 °C for 2 days. Transformants containing pID were selected and confirmed by plasmid isolation and digestion. One of the confirmed transformants was randomly selected and cultured overnight at 30 °C in LB broth without any antibiotic; then the culture was diluted and spread onto an LB plate containing erythromycin. After growing for about 10 h at 42 °C, colonies were replicated on LB plates containing chloramphenicol. Then, the replicated plates and the original plates were cultured at 30 °C. Chloramphenicol-sensitive and erythromycin-resistant strains were selected. Subsequently, the disruption mutant was confirmed by PCR analysis and Southern hybridization.

For complementation experiments, plasmid pIC, containing *iunH* and its putative promoter region, was transformed into the *iunH* disruption mutant by electroporation.

Spore preparation and germination analysis. To prepare spores, *B. thuringiensis* was grown in CCY medium at 30 °C for 48 h. Spores were harvested and washed 5–10 times with cold distilled water. All spore preparations were free (>99 %) of vegetative and sporulating cells. For the germination assay, the heat-activated spores were diluted in germination buffer (10 mM NaCl, 10 mM Tris/HCl, pH 7.4). Small aliquots (1.2 ml) of the heat-activated spores at an OD_{600} of 1 (about 1.5×10^8 c.f.u. ml^{-1}) were supplemented with inosine (ranging from 0.01 mM to 10 mM), adenosine (0.1–2 mM) or guanosine (0.1–2 mM) separately, or 0.01 mM inosine with 1 mM L-alanine, and incubated at 37 °C. The decrease in OD_{600} was monitored for up to 60 min for inosine-induced germination and 90 min for adenosine-induced germination. Phase darkening of germinated spores was observed by phase-contrast microscopy. The data obtained are means from triplicate experiments performed with three independent spore preparations.

Expression and purification of IunH. To study the properties of IunH *in vitro*, it was necessary to obtain an adequate amount of IunH protein. Therefore, *iunH* was amplified from the genomic DNA of *B. thuringiensis* with the primers P9 (5′-GGACTTCCATATGAGAAT-AGTTAATAAGAAAA-3′, *Nde*I site is underlined) and P10 (5′-CGGAATTCTTAAGGACAATCTGGCT-3′, *Eco*RI site is underlined). The start codon (GTG) was replaced by ATG. The amplified fragment was digested with *Nde*I and *Eco*RI, and inserted into the corresponding sites of pET28a to generate a recombinant plasmid, pIE. Then, pIE was transformed into *E. coli* BL21(DE3) for high-level expression of *iunH* under the control of the T7 promoter. As the N terminus of IunH was designed to contain six consecutive histidines, the His6-tagged IunH was purified to homogeneity by Ni-NTA affinity chromatography. The concentration of the purified protein was determined by the method of Bradford using BSA as standard (Smith *et al.*, 1985).

Enzyme assays. Since IunH contains one consensus N-terminal {D, N}XDXXXDD aspartate cluster which is a fingerprint for NH enzymes, the enzymic activity of His6-tagged IunH was measured using inosine, adenosine, guanosine, uridine or cytidine as substrate. A volume (200 µl) of the reaction mixture, containing 50 mM HEPES (pH 7.3), the purified His6-tagged IunH and substrate, was incubated for 5 min at room temperature. Hydrolysis of the substrate (inosine, uridine or cytidine) was followed by continuous reading of A_{280} on a Beckman DU-800 UV spectrophotometer. The conversion of a 1 mM solution of inosine, uridine or cytidine to products resulted in a change in A_{280} of 0.92, 2.04 and 3.42, respectively, at pH 7.3 (Parkin *et al.*, 1991). Hydrolysis of adenosine or guanosine was determined with the reducing sugar assay described by Parkin (1996).

The kinetic parameters K_m and V_{max} were determined at room temperature using a $1/v$-$1/[S]$ plot, where $[S]$ is the concentration of inosine (between 0.2 and 2.5 mM). k_{cat} was derived from the equation $k_{cat}=V_{max}/[E]$, where $[E]$ is the concentration of IunH in the reaction mixture.

To check the effect of pH, IunH activity was measured in the following buffers: 100 mM potassium phosphate, 50 mM HEPES, 30 mM CHES and 30 mM MES, covering the pH range from 4 to 10. To assess the optimal temperature of the enzyme, the inosine hydrolase activity of IunH was assayed at temperatures ranging from 40 to 100 °C.

Inosine hydrolase activity of the intact spores and the vegetative cells. Inosine hydrolase activity in the intact spores or the vegetative cells was determined spectrophotometrically using the difference in absorption between the nucleoside and the purine base (Parkin, 1996). All measurements were carried out at room temperature. Intact spores or vegetative cells at an OD_{600} of about 1 were incubated with 0.5 mM inosine in a total volume of 900 µl of 50 mM HEPES (pH 7.3) for 0, 10, 20, 30 and 40 min, respectively. Spores or vegetative cells were removed by centrifugation. The supernatant was assayed for a change in absorbance at 280 nm, and the resultant pellets were dried at 42 °C for at least 24 h to determine their dry weight.

RESULTS

Cloning of an *iunH*-homologous gene from *B. thuringiensis*

BLAST searches of the *B. cereus* ATCC 14579 genomic sequence released by the DOE Joint Genome Institute (www.jgi.doe.gov) revealed five predicted proteins (GenBank accession numbers BC2331, BC2683, BC2889, BC3552 and BC5134) that are homologous to NH. The amino acid sequence of BC2889 is almost identical to the amino acid sequence of the putative NH purified from exosporia of *B. cereus* and *B. anthracis* (Redmond *et al.*, 2004; Steichen *et al.*, 2003; Todd *et al.*, 2003). Based on the sequence of BC2889 and its homologues found in members of the *B. cereus* group, a 1.4 kb DNA fragment was amplified from *B. thuringiensis* genomic DNA by PCR. Sequencing showed that the DNA fragment contains an ORF, which was designated *iunH* (Fig. 1). *iunH* consists of 966 nt and encodes a protein containing 321 aa with a predicted molecular mass of 36.2 kDa. IunH contains one N-terminal {D, N}XDXXXDD aspartate cluster, which is a fingerprint for NH enzymes, and one conserved {V,I,L,M}HD{P,A,L} tetrapeptide sequence approximately 230 aa downstream from the N-terminal aspartate cluster (Giabbai & Degano, 2004) (Fig. 2).

Disruption of *iunH* increases the rate of spore germination initiated by inosine or adenosine

To investigate the physiological role of IunH in *B. thuringiensis*, an *iunH* disruption mutant of *B. thuringiensis* was constructed by homologous recombination (Fig. 1). The *iunH* disruption mutant showed normal colony morphology, growth and sporulation. Spore germination was measured by monitoring the decrease in OD_{600} of a spore suspension at 37 °C after the addition of either

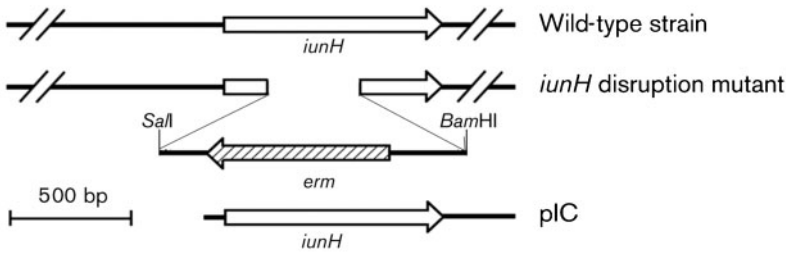

Fig. 1. Disruption of the *iunH* gene in *B. thuringiensis*. The arrow represents *iunH* and its orientation, and the figure is to scale. The *iunH* gene was interrupted by an erythromycin-resistant cassette (*erm*) in the disruption mutant. The entire *iunH* gene with its promoter was inserted into pKSV7 to generate pIC.

inosine or adenosine at the desired concentrations to the heat-activated spores. The dependence of spore germination on inosine concentration was measured in the wild-type strain, the *iunH* disruption mutant and the complemented strain. The germination rates of all spores were increased when the concentration of inosine was increased from 0.01 to 1 mM. As the germination rate was low when spores were induced by 0.01 mM inosine, no significant difference could be observed among the three strains (Fig. 3a). When 0.1 mM inosine was used, the OD_{600} of the spore suspension of the *iunH* disruption mutant decreased by about 45 % during germination in contrast to a decrease of about 20 % in the wild-type spore suspension. As expected, the OD_{600} of the complemented strain spores

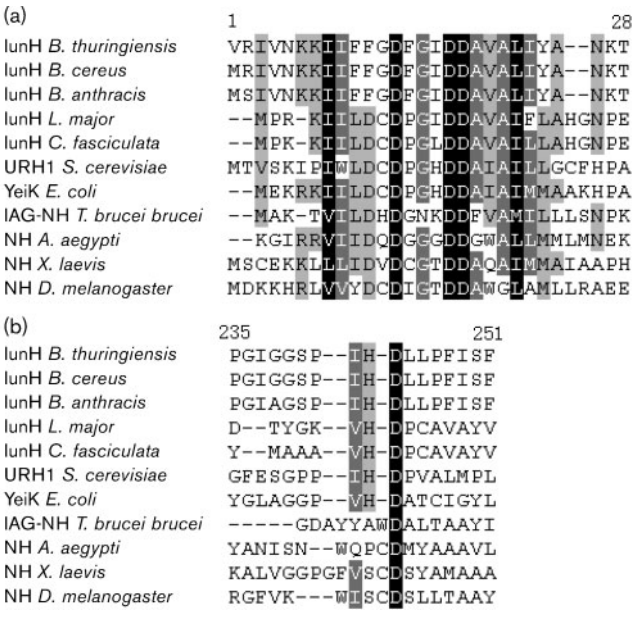

Fig. 2. Amino acid sequence alignment of *B. thuringiensis* IunH with other NH proteins. Residue numbers are taken from the IunH sequence. (a) N-terminal region with the {D, N}XDXXXDD aspartate cluster conserved in all NH proteins. (b) Alignment of the region containing the {V,I,L,M}HD{P,A,L} tetrapeptide sequence approximately 230 aa downstream from the N-terminal aspartate cluster. These sequences were sourced as follows: IunH, *B. thuringiensis* (EU072023); IunH, *B. cereus* (YP084206); IunH, *B. anthracis* (NP845228); IunH, *Leishmania major* (P83851); IunH, *Crithidia fasciculata* (Q27546); URH1, *Saccharomyces cerevisiae* (AAG44107); YeiK, *Escherichia coli* (AAA60514); inosine/adenosine/guanosine-preferring NH (IAG-NH), *Trypanosoma brucei brucei* (XP843889); NH, *Aedes aegypti* (XP001651977); NH, *Xenopus laevis* (NP001079470); NH, *Drosophila melanogaster* (NP572912).

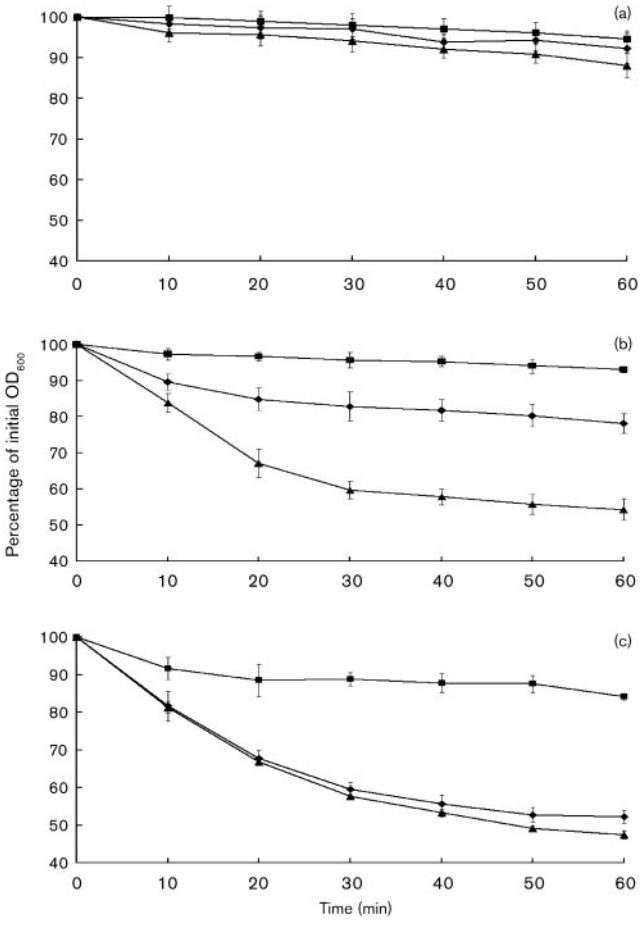

Fig. 3. Effect of *iunH* on spore germination initiated by inosine in *B. thuringiensis*. Spores of the wild-type (◆), the *iunH* disruption mutant (▲) or the complemented strain (■) were heat-activated and subsequently incubated in germination buffer supplemented with 0.01 (a), 0.1 (b) or 1 mM (c) inosine. The decrease in OD_{600} was measured periodically and plotted as a percentage of the initial OD_{600} versus time.

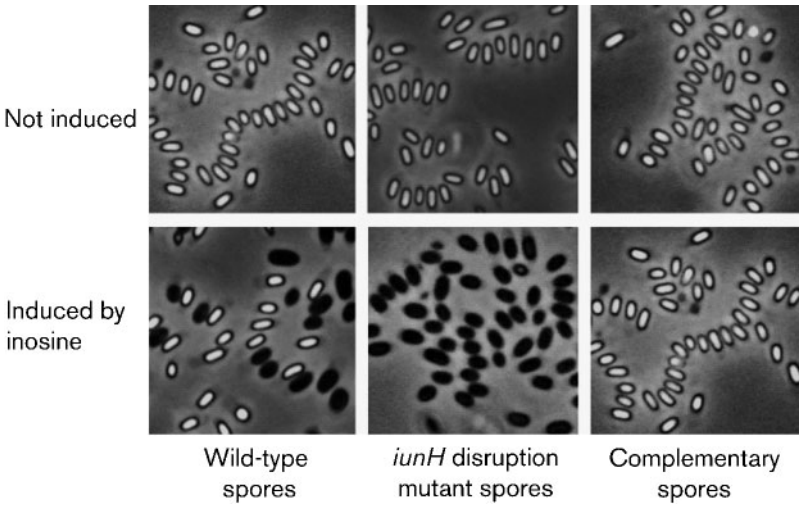

Fig. 4. Nucleoside-induced, germination-associated, phase bright–phase dark transition efficiency in *B. thuringiensis*. All the *iunH* mutant spores became completely phase-dark after incubation with 0.1 mM inosine for 30 min, while only some of the wild-type spores and almost none of the complemented strain spores turned phase-dark under these conditions.

decreased by less than 10 %, which is even less than that of the wild-type spores (Fig. 3b). Phase-contrast microscopy showed that all the *iunH* mutant spores became completely phase-dark after incubation with 0.1 mM inosine for 30 min, while only some of the wild-type spores and almost none of the complemented strain spores turned phase-dark under these conditions (Fig. 4). When the concentration of inosine was increased to 1 mM, the germination rates of both the *iunH* disruption mutant and the wild-type spores increased significantly. However, the germination rate of complemented strain spores increased only slightly (Fig. 3c) and complemented strain spores could only germinate completely at a final concentration of 10 mM inosine (data not shown). L-Alanine (1 mM) could not induce spore germination, but concomitant addition of 0.01 mM inosine could stimulate the germination in the wild-type strain and in the *iunH* disruption mutant, while the germination rate of complemented strain spores did not increase (data not shown).

In addition to inosine, adenosine has been found to be required for spore germination of several *Bacillus* species (Hills, 1949; Lawrence, 1955; Hornstra *et al.*, 2006). Adenosine-induced germination of wild-type spores, the *iunH* mutant spores and the complemented strain spores was assayed in *B. thuringiensis*. As with inosine, the germination rate increased with increasing concentrations of adenosine. When 0.5 mM adenosine was used, the OD_{600} of the *iunH* disruption mutant spore suspension decreased by about 50 % during germination in contrast to a decrease of about 30 % in the wild-type spore suspension. However, the OD_{600} of the complemented strain spore suspension decreased by only about 10 % (Fig. 5). Only a small percentage of complemented strain spores could germinate (date not shown) even at the highest concentration of adenosine used (about 3 mM, due to the low solubility of adenosine). In addition, guanosine, which is also a substrate of IunH, barely induced spore germination. Therefore, *iunH* can moderate the inosine- or adenosine-induced spore germination rate in *B. thuringiensis*.

Substrate specificity of IunH

To characterize its function and substrate specificity, *iunH* was overexpressed in *E. coli* BL21(DE3). Although the bulk of the expressed protein was in inclusion bodies, a clear band of IunH was observed in the supernatant of the extracts of *E. coli* BL21(DE3)/pIE (Fig. 6). His6-tagged IunH was purified to homogeneity by Ni-NTA affinity chromatography and the enzyme activity of IunH was measured with inosine, adenosine, guanosine, uridine or cytidine as substrate. As shown in Table 2, IunH can hydrolyse inosine, adenosine and guanosine with the following activity order: inosine >adenosine >guanosine. The K_m and K_{cat} values with adenosine or guanosine as substrate were not calculated due to the low sensitivity of the method employed for assaying reducing sugars. Hydrolase activity could not be detected when uridine or cytidine was used as a substrate. Thus, IunH belongs to a

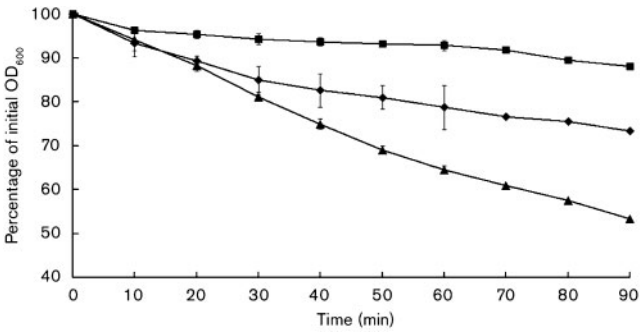

Fig. 5. Effect of *iunH* on spore germination initiated by 0.5 mM adenosine in *B. thuringiensis*. Spores of the wild-type (◆), the *iunH* disruption mutant (▲) or the complemented strain (■) were heat-activated and subsequently incubated in germination buffer supplemented with 0.5 mM adenosine. The decrease in OD_{600} was measured periodically and plotted as a percentage of the initial OD_{600} versus time.

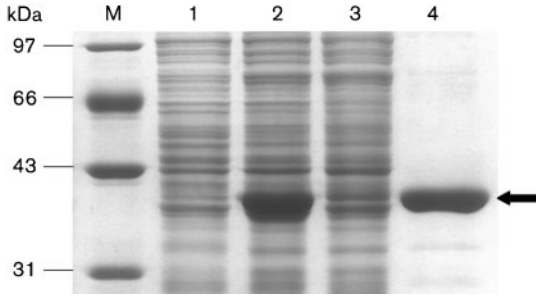

Fig. 6. SDS-PAGE of IunH expressed in *E. coli* and its purification. Lanes: M, standard molecular mass markers; 1, total protein of IPTG-uninduced BL21(DE3) containing pIE; 2, total protein of IPTG-induced BL21(DE3) containing pIE; 3, supernatant of IPTG-induced BL21(DE3) containing pIE; 4, purified IunH protein from Ni-NTA affinity chromatography. Samples were separated on an 8% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. The arrow indicates the position of IunH.

class of purine-specific NHs (inosine/adenosine/guanosine-preferring NH), based on its substrate specificity.

Kinetic characterization of the recombinant IunH

Kinetic studies were used to substantiate the catalytic properties of the expressed recombinant IunH. In standard reaction buffer (50 mM HEPES, pH 7.3) with inosine as substrate, the specific activity of the inosine hydrolase of IunH was 57.2 ± 2.56 μmol min^{-1} (mg protein)$^{-1}$. The kinetic parameters of IunH were $K_m = 399 \pm 115$ μM, $k_{cat} = 48.9 \pm 8.5$ s^{-1} and $k_{cat}/K_m = 1.23 \times 10^5$ M^{-1} s^{-1}.

The optimal pH and temperature for IunH

The effect of pH on the inosine hydrolase activity of IunH was examined at room temperature with 3 mM inosine as substrate. Inosine hydrolase activity of the purified recombinant IunH could be detected over a wide range of pH values, the highest activity being obtained at pH 6 (Fig. 7a). The activity was stable over a wide temperature range, the highest activity being at 80 °C with high activity being maintained up to 100 °C (Fig. 7b). This result indicates that IunH is a highly heat-stable enzyme.

Table 2. Specific activity of IunH with different substrates

Substrate	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]
Inosine	57.2 ± 2.56
Adenosine	11.76 ± 3.02
Guanosine	1.32 ± 0.44
Uridine	<0.2
Cytidine	<0.1

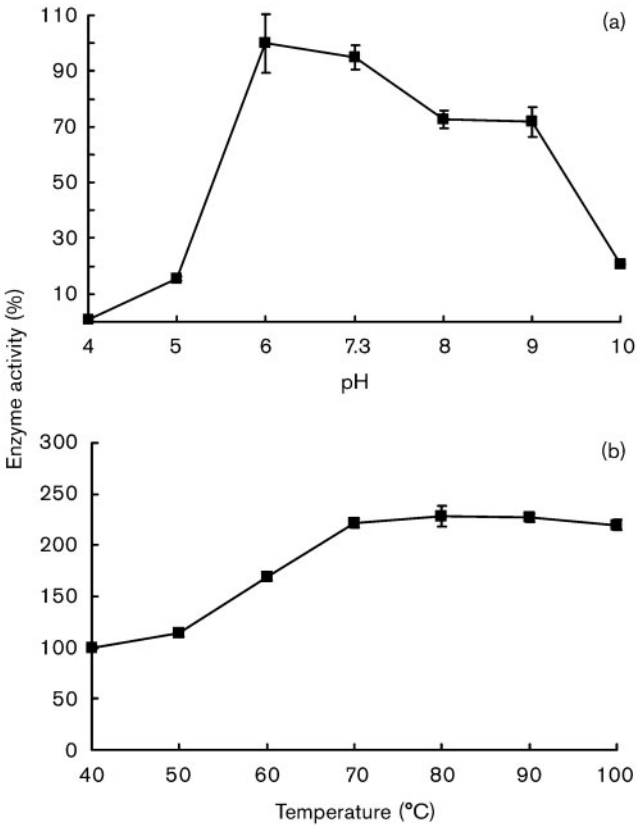

Fig. 7. Activity of purified IunH as a function of pH (a) and temperature (b). The inosine hydrolase activity of IunH was measured at room temperature in 100 mM potassium phosphate, 50 mM HEPES, 30 mM CHES or 30 mM MES, covering the pH range from 4 to 10. To assess the optimal temperature of the enzyme, the inosine hydrolase activity of IunH was assayed at temperatures ranging from 40 to 100 °C. The activity of IunH at pH 6.0 or at 40 °C was defined as 100%.

Activity of inosine hydrolase in dormant spores

Sequence comparisons have revealed that *B. thuringiensis* IunH has a high level of similarity with its homologues purified from the exosporia of *B. cereus* and *B. anthracis* (Redmond *et al.*, 2004; Steichen *et al.*, 2003; Todd *et al.*, 2003). IunH is probably located in the exosporium of *B. thuringiensis* spores and, therefore, inosine hydrolase activity in intact spores was measured. The specific activity of inosine hydrolase in wild-type spores of *B. thuringiensis* was $2.89 \pm 0.23 \times 10^{-2}$ μmol min^{-1} (mg dry wt)$^{-1}$, while the *iunH* mutant spores exhibited no detectable activity. The specific activity in the complemented strain spores was $8.40 \pm 0.69 \times 10^{-2}$ μmol min^{-1} (mg dry wt)$^{-1}$, which is almost threefold higher than observed in the wild-type spores (Fig. 8). The higher activity of IunH in complemented strain spores may be due to the introduction of multiple copies of *iunH* by pKSV7 (Smith & Youngman, 1992). Although there was considerable IunH activity in the spores, no activity was detected in intact vegetative cells

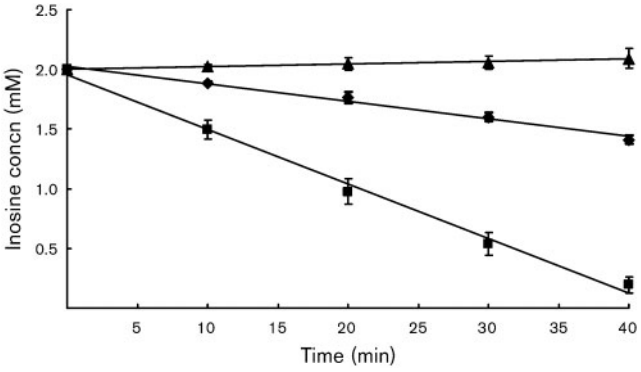

Fig. 8. Activity of inosine hydrolase in whole, dormant spores. Whole spores of the wild-type (◆), the *iunH* disruption mutant (●) or the complemented strain (■) were incubated with 0.5 mM inosine in a total volume of 900 µl 50 mM HEPES (pH 7.3) for 0, 10, 20, 30 and 40 min, respectively. Spores were removed by centrifugation and the supernatant was assayed for the change in OD_{280}. Spore preparations whose optical density was examined in advance, were centrifuged and the deposits were dried at 42 °C for at least 24 h to determine their dry weight.

from the wild-type, *iunH* disruption mutant and complemented strains (data not shown).

DISCUSSION

Inosine-initiated spore germination starts with the interaction of inosine with its corresponding receptors, such as GerI, GerQ and GerR. Then the spores undergo the release of dipicolinic acid and cations, hydrolysis of the peptidoglycan cortex, germ-cell-wall expansion and finally resumption of vegetative growth (Hornstra *et al.*, 2006; Setlow, 2003). This process has been described in detail, but how it is regulated is still poorly understood. Here, we provide evidence that the amount of inosine or adenosine acting as germinant can be modulated by the activity of the inosine hydrolase IunH, which converts inosine to hypoxanthine and ribose, and adenosine to adenine and ribose. This allows for modulation of the inosine- or adenosine-induced germination efficiency. Disruption of *iunH* indeed resulted in an increase in the inosine- or adenosine-induced germination rate, whereas overexpression in the complemented strain resulted in a significant decrease of its germination-triggering capacity.

Unlike the NH from *C. fasciculata*, whose activity decreases rapidly at pH values below 7 (Parkin *et al.*, 1991), *B. thuringiensis* IunH showed highest inosine hydrolase activity at pH 6. Given the fact that the spores can germinate in the alkaline environment of the insect gut (Schnepf *et al.*, 1998), the relatively low activity of IunH at alkaline pH values may increase the sensitivity of spores to the germinant. In addition, the heat stability of IunH enables it to function even after the spores have been exposed to high temperatures, which is especially important when dealing with spores in feeds and foods.

The *in vivo* inosine hydrolase activity indicates that the spores are able to monitor inosine levels in their environment. The difference in inosine hydrolase activity of wild-type, disruption mutant and complemented strain spores has confirmed the role of the inosine hydrolase function of IunH in modulating inosine-induced germination. Five ORFs encoding putative inosine-preferring NHs have been found in the genomes of *B. cereus* strain ATCC 14579 and four ORFs have been found in *B. cereus* strain ATCC 10987 (Ivanova *et al.*, 2003; Rasko *et al.*, 2004). This number is similar to that found in the genome of *B. thuringiensis* (Challacombe *et al.*, 2007). The disruption of *iunH* eliminates inosine hydrolase activity in spores and promotes inosine-initiated germination of *B. thuringiensis*. This indicates that IunH is the most prominent NH in the exosporium, moderating nucleoside-induced germination capacity in *B. thuringiensis*.

ACKNOWLEDGEMENTS

We are grateful to Professor Dafang Huang (Biotech Institute, CAAS, China) for the gift of plasmid pHT3101. We are also grateful to Dr Paul Babitzke (Penn State University, PA, USA) for plasmid pKSV7. This work was supported by grants from the National Natural Science Foundation of China (Grant Nos. 30430010) and the National Basic research Program of China.

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Edited by: T. Abee