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Enhanced Expression of Insecticidal Crystal Proteins in Wild *Bacillus thuringiensis* Strains by a Heterogeneous Protein P20

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Abstract. P20 is a small chaperone-like protein encoded by a *cry11A* operon in *Bacillus thuringiensis* subsp. *israelensis* (Bti); it is essential to Cyt1Aa expression. In this report, the gene *P20* was transformed into wild strains of subsp. *kurstaki* to raise the yield of crystal proteins. As a result, larger crystals were produced by the transformant than by the wild control, and most were in the form of a big bipyramid (average 2.4 μm long); some were irregular because of too high expression, while the spores turned out to be small spheroids unlike the long rods in the wild strains. SDS-PAGE analysis confirmed that Cry1A protoxin production was doubled by P20, but no increase of Cry2A production was observed. Besides, P20 caused obvious changes not only in bacterial morphology, but in the sporulation process as well. Further investigation discovered that a serious degradation happened to Cry1A in vivo of the wild strains, and reconfirmed that P20 was effective in preventing the degradation. Our results suggest that P20 is useful in engineered strain construction with enhanced protein expression.

Bacillus thuringiensis (Bt) is the most widely used biological control agent up to now. Over one hundred insecticidal crystal proteins have been identified from various strains [7, 11, 18]. The high expression and crystallization of these proteins distinguish Bt from other, closely genetically related species such as *Bacillus cereus*, and this becomes an interesting issue of theoretical and practical senses. Several factors are proved to be involved in high production: effective promoters, stable mRNA, coexpression, and the assistance of other proteins [2–4]. Attempts to change at both the transcriptional and post-transcriptional levels are successful in enhancing the yield of some Cry proteins, such as knocking out the protease genes encoding alkaline protease A and neutral protease increased Cry1Bb production [9, 22]; switching to a robust promoter can result in greater production or start the expression at the proper time for crystallization [4, 5, 13–15, 19].

In addition, some small proteins are beneficial to Cry protein expression and even crystallization [1, 6, 10, 26]. For instance, a 252-amino-acid protein named ORF2 in the cry2Aa operon in the *kurstaki* subspecies (Btk) is necessary for Cry2Aa crystal formation, supposed to act as a scaffold or a matrix during Cry2Aa crystallization [10]. Helper proteins P20 (20 kDa) and P19 (19 kDa), the two accessory proteins found in subsp. *israelensis* (Bti), are encoded by *cry11A* operon (*cryIVD* in the former nomenclature system) [8, 12] and are proved to have the effect of promoting Cry11A production [25, 26]. A Cry11A coexpressed protein, Cyt1Aa (24 kDa), is a cytolytic toxin and is toxic to host cells such as Bt and *Escherichia coli*, while P20 can fold the expressed Cyt1Aa peptides into crystals, thereby avoiding its attack on the host cells [1, 23, 24]. In Bti, therefore, P20 is crucial for securing cell survival with Cyt1Aa expression.

Recently, P20 was proved effective in enhancing the expression of other *cry* genes in plasmid-cured acrystalliferous mutants [10, 16]. In crystal minus $Cry^{-}B$ mutant, P20 increased the production of an N-half truncated Cry1C which was disabled in crystallization and normal expression [13, 16]. In an acrystalliferous strain 4Q7, the yield of Cry2A was enhanced by P20 to about 30% [10]. Cry2A is thought naturally truncated, without the crystal-forming do-

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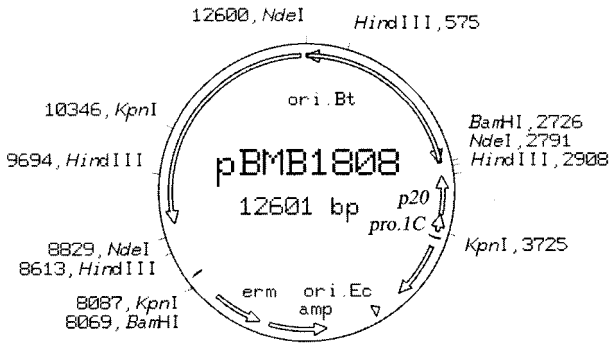

Fig. 1. Construction of Bt/*E. coli* shuttle plasmid pBMB1808 containing the *p20* gene. In this plasmid: *P20*, gene of helper protein p20; *pro.1C*, *cry1C* promoter; erm, amp are erythromycin and penicillin resistance gene; ori. Bt and ori. Ec are replica origins of Bt and *E. coli*, respectively.

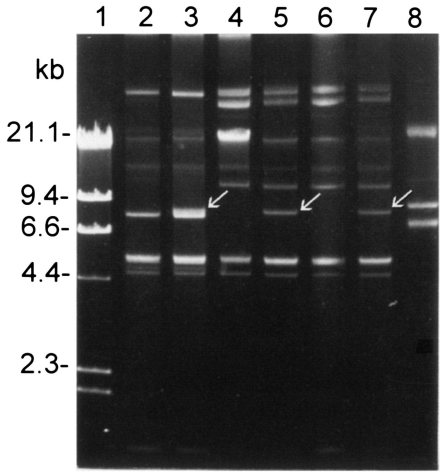

Fig. 2. Plasmid pattern of the Bt transformants with pBMB1808. Lane 1, λ/*Hin*dIII; lanes 2, 4, 6 are the recipient strains YBT1520, YBT1535, and YBT833, respectively; lanes 3, 5, 7 are their corresponding transformants with pBMB1808; lane 8, the donor plasmid pBMB1808. It showed that all the transformants obtained the new band at the same position.

main of large Cry1 protoxins. Both cases are common in the failure of expression.

In a previous report, we found that P20 was also effective in enhancing the expression of Cry1Ac in an acrystalliferous strain BMB171 [20]. Different from other reports, we used a 130-kDa full-length protoxin to test the effect of P20, which was believed no problem in protein expression. Actually, we found Cry1Ac suffered serious degradation during its synthesis. As a result, we obtained a doubled yield of Cry1Ac in the presence of P20 in that report. Is it also effective when transformed into wild Btk strains, which usually have multiple *cry* genes and are very well coexpressed? If so, we might obtain an engineered strain with a high yield of toxic proteins. That would be meaningful in insecticidal agent development. This intrigues us to test P20's effect in wild Btk strains. As expected, P20 doubled the production of Cry1A protoxins deposited into super bipyramids in wild Btk strains.

Materials and Methods

Gene and bacterium. The gene *p20* was originally cloned from Bti [21]. To transfer *p20* into wild Bt strains, a plasmid pBMB1808 was constructed based on the Bt/*E. coli* shuttle vector pHT304 according to Sanchis et al. [17] (Fig. 1). *E. coli* mutant strain DH-5α was used for plasmid amplification. Three wild Bt strains YBT-1520 (also called 218), YBT833, and YBT 1535 were isolated and preserved in our lab; all harbor *cry1Aa*, *b*, *c*, and *cry2A* genes, but they varied in toxicity against larvae of *Heliothis armigera*, *plutella xylostella* [21].

Cell transformation and recombinant strain screening. The plasmid pBMB1808 was transformed into three wild Bt strains by electroporation in an SG (sucrose–glycerol) solution at 1.25 kV, 200 Ω, 25 μF in a 1-mm cuvette. After 1 h recovery incubation, the transformants were plated on antibiotic solid LB containing 25 μg/mL erythromycin (Em) for the first screening, then further confirmed by plasmid pattern comparisons. Then they were reconfirmed by PCR amplification with a pair of *p20* specific primers (5′: GGAGG ATCCA TGGGG ACAGA AAATG GAGTG; 3′: GAACT TTCGA ATTTG CAAGGC).

SDS-PAGE and protein quantification. SDS-PAGE samples were processed and loaded in a strict comparable parallel to examine the expression of crystal proteins in Bt transformants. After being activated in LB medium overnight, all strains were inoculated into 50 mL PM liquid medium by 1% inoculum (for transformants, add 25 mg/mL Em), cultivated at 28°C with constant shaking until the cells were lysed. After thorough mixing, 1 mL culture was pipetted into 1.5-mL Eppendorf tube, and the crystal and spore mixture was collected by centrifugation. Then the mixture was washed three times with 1 M NaCl and ddw, respectively. Finally, the crystal spore mixture was resuspended in 0.5 mL ddw. After that a 100-μL mixture was processed with 2 × sample buffer at 99°C for 4 min. Then, a 20-μL treated sample was loaded per lane. The gels were stained with Coomassie Brilliant Blue R-250, and the protein concentration of 130-kDa bands was quantified by the Dense-Scanning Quantification program of the Bioimage System as described by the manufacturer.

Microscopy. For microscopic observation of the crystals, all Bt strains were cultivated in PM medium at 28°C until cell lysis. The culture was plated directly on a glass slide or stained with carbolic acid/azaleine for light microscopic observation. For the scanning electron microscope, spores and crystals were washed with 1 M NaCl as above, plated on a piece of glass slide cover, and placed on a specimen holder; then electric conduction was processed in an ion coater (IB 5, Eiko). Dimensions of 30 crystals were measured at 10,000× magnification, and the volume of crystals was calculated according to the formula, W^2 L/3 (W, width; L, length), as shown in a previous paper [9].

Determination of the role of P20 during Cry1 protein expression. The role of P20 in protein expression was determined by monitoring Cry1A concentration variation from the start to the end of sporulation. Two strains, YBT-1520 and its *p20* transformant BMB21882, were used in this test, both of them processed in parallel, cultivated in PM medium under constant shaking at 28°C, 200 rpm. At an interval from

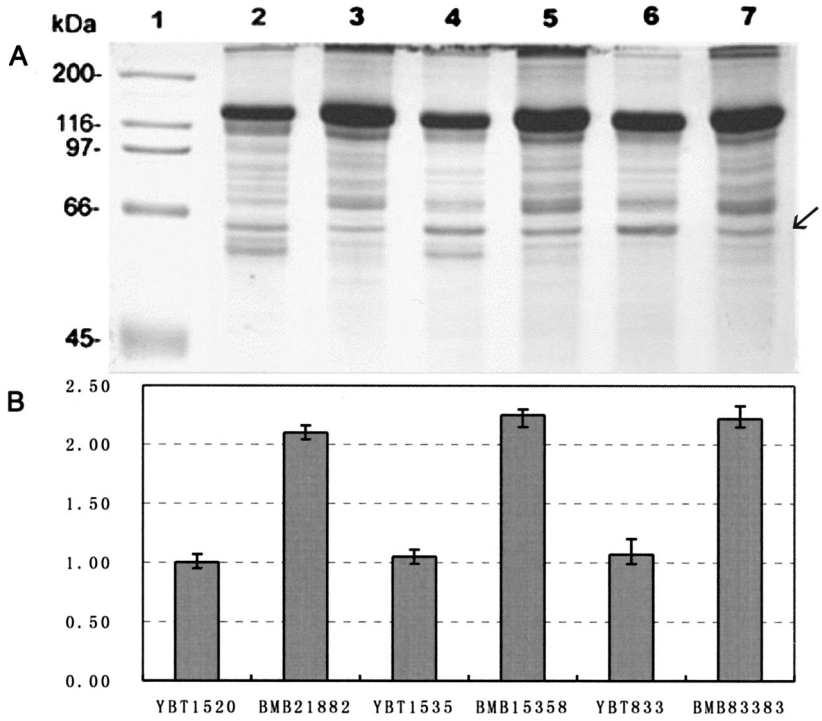

Fig. 3. SDS-PAGE analysis of ICPs expression in the natural strains and their pBMB1808 transformants. (A) SDS-PAGE results. Lane 1, standard protein markers; lanes 2, 4, 6 are natural isolates YBT1520, YBT1535, and YBT833, respectively; lanes 3, 5, 7 are the corresponding transformants of pBMB1808. (B) Quantification of 130-kDa proteins with dense-scanning program; on the vertical axis, the relative concentrations of protoxin proteins indicated by the absorptive values.

13 h to 25 h, a 1 mL culture was removed and processed for SDS-PAGE analysis as described above.

Results

The *B. thuringiensis* transformants obtained. In contrast with the wild-type strains, the obtained Bt transformants of pBMB1808, BMB21882, BMB15358, and BMB83383 showed identical plasmid patterns except for the donor plasmid, so all three transformants harbored pBMB1808 (Fig. 2). PCR amplification with a pair of *p20* specific primers showed that all of the transformants gave the anticipated 583-bp band of *p20* gene, while the recipient strain gave a negative result (data not shown). This indicated that the transforming process was successful.

Enhanced expression of Cry1A protein. The productivity of the Cry1A protein in the obtained transformants was first detected by SDS-PAGE; this analysis was strictly parallel with the wild strains. The SDS-PAGE results demonstrated that the 130-kDa Cry1A proteins were more highly expressed in all three transformants (Fig. 3). Quantification analysis showed that the amount of Cry1A in BMB21882, BMB15358, and BMB83383 was 2.14, 2.15, and 2.08 times that of their recipient strains, respectively. However, the Cry2A protein had no increase in concentration (about 60 kDa; arrow). In contrast, it decreased in BMB83383 (Fig. 3, lanes 6 and 7).

Crystal and spore morphological changes. The process of crystal and spore formation was inspected under a phase contrast microscope. The transformants showed obvious differences in morphology and sporulation time compared with their recipients. It was found that the wild strain had a short sporulation process, starting earlier and ending earlier. The spores and crystal were in typical shapes and shared equally the intracellular space of the mother cells (Fig. 4A); the sporulated cells of the wild strains were completely auto-lysed within 36 h in NSM medium, and after cell lysis, the spores and crystals were free from each other, showing similar size (Fig. 4B). However, in the transformants two aspects were different; one is the sporulation time, and the other is the shape of the sporulated cells, spores, and crystals. We found that the appearance of spores in the transformant cell was several hours later (Fig. 4C), and the sporulation process was extensively elongated; even after 36 h of shaking incubation there were still many cells in the state of "sporulation" (Fig. 4D); later on, the sporulated cells became short and condensed. The resultant crystals were so large that most of the mother cell was occupied and the spore was small and round and pushed to the very corner of the sporulated cells (Fig. 4D–E). Even when kept at 4°C for 3 days after fermentation, the cells still could not be totally lysed, and spores and crystals seemed difficult to be detached

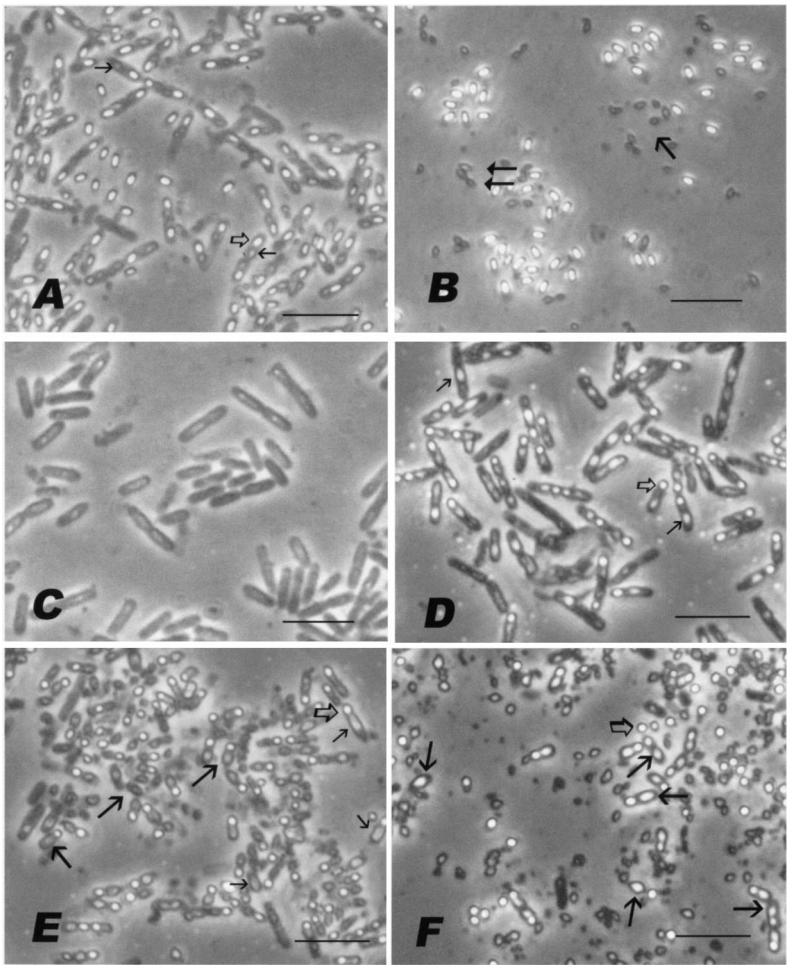

Fig. 4. Comparing crystal and spore development of YBT1520 (218) and BMB21882 by phase-contrast LM (bar 50 μm). "→" points to crystals, "⇨" points to spores. (A) YBT1520 at 18 h cultivation, showing spore and crystal in the sporulated cells; (B) YBT1520 at 36 h cultivation, showing the released spores and crystals; (C) BMB21882 at 18 h cultivation, cells are just at the start of sporulation; (D) BMB21882 at 36 h cultivation, most cells at the end of sporulation; (E) BMB21882 at 48 h cultivation, cells are in lysis, crystals are bigger than those in Fig. 4B; (F) BMB21882, 3 days after fermentation kept at 4°C, some big crystals still attached with spores.

Table 1. Crystal size of three wild Bt strains and their pBMB1808 transformants[a]

Strains	YBT1520	BMB21882	YBT1535	BMB15358	YBT833	BMB83383
Length (μm)	1.85	2.45	1.76	2.34	1.87	2.39
Width (μm)	0.8	1.06	0.75	1.12	0.80	0.95
Volume (μm^3)	0.39	0.92	0.33	0.98	0.40	0.72

[a]The values are the averages of 30 crystals each treatment; the volume was calculated according to the values of length and width.

from each other (Fig. 4F). This was different from the wild strains (Fig. 4B).

Further, the exact dimensions of the resultant crystals were examined with SEM. The crystal of the transformants averaged 2.40 μm long and 1.06 μm wide, its calculated volume was about 1.85 times of their recipient control (Table 1), and some crystals were even 3.8 μm long (Fig. 5B); this may be the largest crystal ever reported, but the spore was small and round, only 1.10 μm in diameter, while in the wild strains, the crystal averaged 1.82 μm long and 0.78 μm wide and the spore was about 1.8 μm long in the shape of a rod. Here, we present the pictures of only two pairs of recipient/transformant, YBT1520 and YBT1535 (Fig. 5) because the other is very similar.

Crystal protein degradation and the role of P20 in wild Bt strain. To detect the effects of P20 on the expression of crystal protein, the concentration of Cry1 protein was monitored throughout its expression process; an up-and-down variation and a steady increase were observed without and with P20 presence (Fig. 6). After 13 h cultivation in PM medium at 28°C,

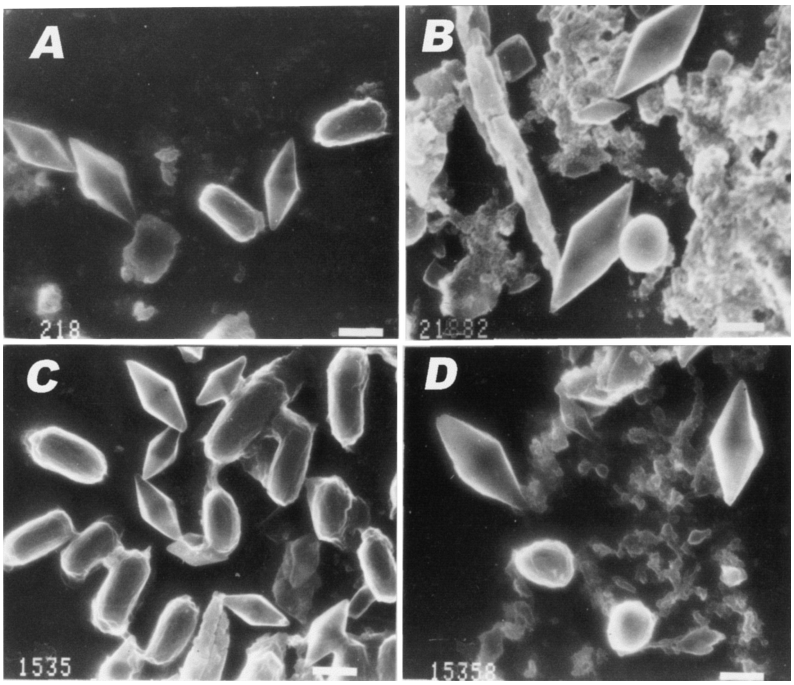

Fig. 5. SEM observation of parasporal crystals and spores of the P20 transformants and their natural isolate recipients. (A) YBT1520; (B) BMB21882; (C) YBT1535; (D) BMB15358 (bar, 1 μm). The natural isolates showed long rod spores and regular bipyramidal crystals (in A, C); irregular spores (round) and large crystals (3.8 μm long) of P20 transformants (in B, D); a tiny crystal also present in B, which might be one of multiple crystals in a cell.

the recipient strain YBT1520 had started to express Cry1 proteins (lane 2) and accumulated to the maximum within about 3 h. Afterwards, however, Cry1 concentration decreased continuously (from 16 h to 22 h, lanes 3–4). At the end of sporulation (25 h, lane 5), more than one-third of the expressed Cry1 protein degraded in the absence of P20. This reflected that the expressed protoxin was degraded in vivo before crystallization. In contrast, in BMB21882, the start of Cry1 protein expression was delayed about 3 h, at 13 h no protein expression was detected (Fig. 6, lane 6), at about 16 h expression started (Fig. 6, lane 7), and protoxin concentration rose constantly afterwards. At the end of sporulation, crystal protein concentration was 2.03 times that of the recipient control, YBT1520 (Fig. 6, lanes 5 and 10).

Discussion

Cry1 is a major group of insecticidal proteins produced by *B. thuringiensis* [11, 18]; raising their production is an important task in Bt agent development. In this report, the expression of Cry1A of wild Btk strains was doubled by Helper Protein P20 as detected by SDS-PAGE (Fig. 3). By comparing the P20 present with the wild strains, no difference during logarithmic growth phase was observed, but later on changes happened to the former, its sporulation process seemed to be delayed, but at last more proteins and bigger crystals resulted (Figs. 4 and 5), the volume of a single crystal was twice that of the control (Table 1). This corroborated that each cell produced two times as much proteins as the control, and the high production is the result of high expression (not the result of the fermentation process). The results of protein quantification (Figs. 3 and 6) and the morphology observations (Figs. 4 and 5) were consistent with each other.

In the tested wild strains, Cry1A had been discovered suffering a serious degradation in vivo during expression (Fig. 6); this was also observed in an acrystalliferous mutant strain BMB171 when transformed with the *cry1Ac* gene [20]. The degradation may be common in endotoxin expression; this is the result of hydrolysis of intracellular proteases. Thus, the method to prevent proteolysis would result in more protein at last.

Actually, degradation happened both to peptides in synthesizing and the protoxins synthesized; full-length protoxins were heavily degraded after expression (Fig. 6, lanes 3–5). It is known that P20 possibly works as a molecular chaperone [10, 27], and it is effective to protein only in synthesizing (neonate protein). But after being synthesized, P20 would be futile. However, at this point co-expressing a proper protease inhibitor might save this part from degradation. Therefore, *p20* combined with a protease inhibitor gene would further enhance the crystal protein production.

In summary, a serious degradation was discovered occurring to 130-kDa protoxins in wild Bt strains, and P20 was effective in enhancing the production of these proteins, suggesting that P20 is useful in engineered strain construction.

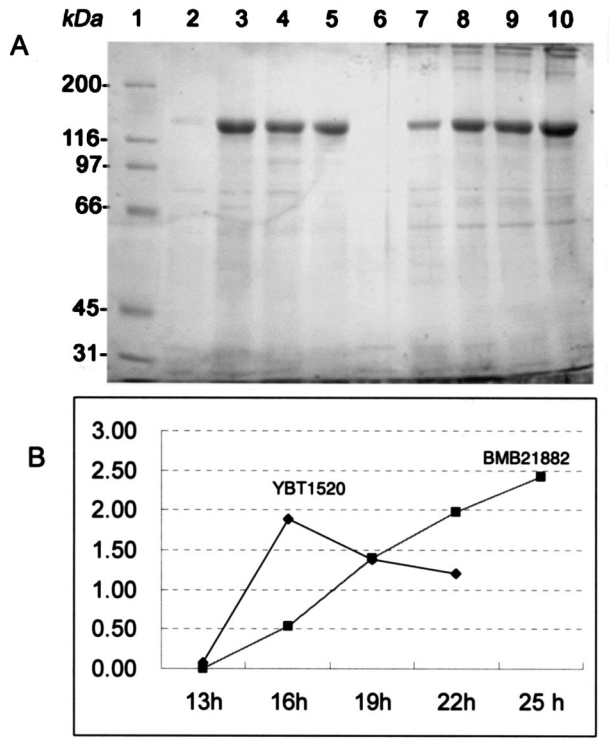

Fig. 6. Protoxin concentration variation during the sporulation phase in the absence and presence of P20. (A) SDS-PAGE analysis. Lane 1, standard protein markers; 2–5 and 6–9 are the results of ICPs expression at 13th, 16th, 19th, and 22nd h of cultivation in YBT1520 and BMB21882, respectively. Lane 10, the final concentration of ICPs in BMB21882 after 25 h of cultivation and 2 days at 4°C for cell lysis. (B) Protein quantification of 130 kDa protoxin on SDS-PAGE gels. X-axis is incubation time; Y-axis is relative concentrations of protoxin proteins.

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