Deletion of the novel gene mother cell lysis X results in Cry1Ac encapsulation in the *Bacillus thuringiensis* HD73

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The novel protein McIX (mother cell lysis X) in *Bacillus thuringiensis* subsp. *kurstaki* strain HD73 (*B. thuringiensis* HD73) was characterized in this work. McIX has no known domain and its gene deletion in HD73 resulted in Cry1Ac encapsulation in the mother cell and did not influence Cry1Ac protein production or insecticidal activity. In vitro cell wall hydrolysis experiments showed that McIX cannot hydrolyze the cell wall. In mclX deletion mutants, the expression of cwlC (which encodes a key cell wall hydrolase) was significantly decreased, as shown by the β-galactosidase activity assay. McIX cannot directly bind to the cwlC promoter, based on the electrophoretic mobility shift assay (EMSA). The cwlC was reported to be regulated by σK and GerE. However, the transcriptional activities of sigK and gerE showed no difference between HD73 and the mclX deletion mutant. It is indicated that McIX influenced cwlC expression independently of σK or GerE, through a new pathway to regulate cwlC expression. mclX deletion could be a new approach for insecticidal protein encapsulation in *Bacillus thuringiensis*.

**KEYWORDS**

*Bacillus thuringiensis*, mother cell lysis, cell wall lytic enzyme C, Cry1Ac encapsulation, transcriptional regulation

**Introduction**

*Bacillus thuringiensis* is a microbial insecticide used worldwide in agriculture (Jouzani et al., 2017). The most important feature of *B. thuringiensis* is the formation of spores and the production of insecticidal parasporal crystals. When *B. thuringiensis* grown to mature, the spore and crystal protein was released from mother cell. The crystal protein is toxic to Lepidoptera, Coleoptera, Diptera and other agricultural pests...
(Beegle and Yamamoto, 1992; Jouzani et al., 2017). However, the insecticidal activity of crystal protein was frequently reduced by environmental factors such as sunlight exposure (Myasnik et al., 2001; Zogo et al., 2019). Encapsulation of crystal proteins by physicochemical, mechanical, or molecular biological techniques can protect their activity from sunlight exposure (Lerelus et al., 1995; Manasherob et al., 2002; Jallouli et al., 2014; Chen et al., 2018; de Oliveira et al., 2021).

Encapsulation of crystal protein by physicochemical and mechanical techniques has some advantages and disadvantages. Encapsulation by extrusion is simple to use but low productivity, encapsulation by fluidized bed is high productivity but difficult to control, encapsulation by electrospinning is easy to expand production but the media may be toxic (de Oliveira et al., 2021). Crystal protein encapsulation in mother cell by molecular biological techniques was proved to be a good strategy to improve Cry protein stability under ultraviolet (UV) radiation (Sanchis et al., 1999; Zhou et al., 2014). The mother cell wall can protect Cry protein from inactivation by UV radiation. Deletion of sigK led to blocking mother cell lysis, encapsulating crystal proteins within the mother cell wall. This technology not only constructed spore-free strain, which had no pollution to the environment and no competition with wild B. thuringiensis strains, but also improved the UV resistance of crystal proteins in the field condition (Sanchis et al., 1999). Encapsulation Cry1Ba in the mother cell of sigK mutant also increasing UV radiation resistance (Zhou et al., 2014). However, some cty genes are regulated by σK, and cty gene expressions were reduced in the sigK deletion mutant (Adams et al., 1991; Bravo et al., 1996).

Cell wall hydrolases are important in cell wall metabolism, they are classified as glycosidases and peptidases. Glycosidases (glucosaminidase, muramidase, and lytic transglycosylase) and peptidases (amidase, endopeptidase, and carboxypeptidase) cleave different sites of cell wall peptidoglycan (Do et al., 2020a). In B. subtilis, cell wall hydrolases involved in mother cell lysis (CwlB, CwlH, CwlC) are amidases with functional redundancy. Single amidase gene deletion did not affect mother cell lysis, while cwlB cwlH cwlC deletion mutant significantly reduced mother cell lysis (Nagroho et al., 1999). In B. thuringiensis, CwlB and CwlC show low sequence identities with B. subtilis hydrolases. Deletion of the cwlB gene can delay lysis of mother cells (Yang et al., 2013). Deletion of the cwlC can block the lysis of mother cells without influence on Cry1Ac production (Chen et al., 2018). As the essential hydrolase in mother cell lysis, CwlC was reported to be regulated by σK and GerE in B. thuringiensis (Chen et al., 2018). No other genes have been reported to be deleted for encapsulation crystal protein except sigK and cwlC.

In this work, we identified a hypothetical gene whose deletion resulted in Cry1Ac encapsulation in the mother cell. The mother cell did not lysis because deletion of this hypothetical gene reduced the cwlC expression. This discovery of the regulatory mechanism of cwlC is independent from the regulatory pathway of σK or GerE. Studies on this new gene may contribute to a deeply understanding of cwlC expression. The deletion of this new gene encapsulated Cry1Ac in the mother cell with no effect on sporulation frequency, Cry1Ac protein production, or insecticidal activity. This could be a potential new approach for Cry protein encapsulation by molecular biology techniques.

**Materials and methods**

**Bacterial strains, growth conditions, and plasmids**

Tables showed the strains (Supplementary Table 1), primers (Supplementary Table 2), and plasmids (Supplementary Table 3) used in this work. The B. thuringiensis subsp. kurstaki strain HD73 (GenBank Accession Number: NC_020238.1) was used for transformations (Du and Nickerson, 1996; Liu et al., 2017). All B. thuringiensis strains were cultured in SSM (Schaeffer’s sporulation medium) (Schaeffer et al., 1965) or LB (Luria-Bertani) medium under 30°C, with 100 µg/ml Kan (Kanamycin) or 5 µg/ml Ery (Erythromycin) if needed. The E. coli strain TG1 was used for constructing the vector (Hoffmann et al., 2015). The E. coli ET12567 was used for extracting non-methylated vectors on a large-scale and transforming into B. thuringiensis. All E. coli strains were grown in LB medium at 37°C, with 100 µg/ml Amp (Ampicillin) if needed.

**DNA manipulation and transformation**

The EasyPure Plasmid MiniPrep Kit (Transgen, Beijing, China) was used to extract vectors from E. coli cells listed in Supplementary Table 3. The PrimeSTAR Max DNA Polymerase (Takara, Beijing, China) was used to perform PCR, using a Mastercycler X50 (Eppendorf). Primers were synthesized in a company (Sangon, China). The EasyPure Quick Gel Extraction Kit (Transgen, Beijing, China) was used to purified DNA fragments, the Seamless Assembly Cloning Kit (Clonesmarter, United States) was used to connect DNA fragment and the linearized vector. Plasmid was introduced into the strain HD73 by electroporation (GenePulser Xcell, Bio-Rad).

**Construction of the mother cell lysis X deletion mutant strain HD (ΔmclX)**

To delete the mclX gene in the HD73 genome, pMAD (Arnaud et al., 2004) (a temperature-sensitive suicide plasmid) with the mclX mutation box was constructed. The mclX mutation box was amplified as follows. A 725-bp fragment
upstream of mclX (mclX fragment A) which contains a 12-bp overlap with the mclX 5′ end, was amplified by PCR, and mclX-1 and mclX-2 were used as primers. A 677-bp fragment downstream of mclX (mclX fragment B) which starts 4-bp away from mclX 3′ end, was amplified by PCR, and mclX-5 and mclX-6 were used as primers. A 1,473-bp fragment of the kan resistance gene was amplified by PCR, and mclX-3 and mclX-4 were used as primers. The overlapping PCR was used in amplifying the mclX mutation box (mclX fragment A, kan fragment, and mclX fragment B), using mclX-1 and mclX-6 as primers, and the resulting fragment of the mclX mutation box was inserted into the pMAD plasmid. Then, the recombinant plasmid was introduced into HD73. The mclX mutation box allelic replacement was performed as reported (Yang et al., 2013). The mclX ORF (12–663) was replaced by kan fragment which detected by PCR with primers mclX-1 and mclX-6.

Expression and purification of mother cell lysis X-His protein

The method of protein expression and purification was previously described (Zhang et al., 2020). A BL21(pETmclX) strain was grown in LB liquid medium until reaching an OD600 of 0.7. Then, 1 mM isopropyl-beta-D-thiogalactoside (Solarbio, Beijing, China) was added to induce MclX-His expression, and bacteria were harvested after overnight growth at 18°C and sonicated (CP750, Cole-Parmer). The supernatant was loaded onto a 2-ml suspension of Ni Sepharose 6 Fast Flow (GE Healthcare, Sweden) with wash buffer (25 mM Tris-HCl, 50 mM imidazole, 0.5 M NaCl, pH 8.0) and elution buffer (25 mM Tris-HCl, 250 mM imidazole, 0.5 M NaCl, pH 8.0). The purified MclX-His protein was analyzed by 10% SDS–PAGE (Mini protein III, Bio-rad).

β-galactosidase assays

The method of the β-galactosidase activity assay was previously described (Miller, 1972). The error bars represent three independent experiment results.

Sporulation frequency analysis

Liquid samples (50-ml) of HD73 and HD (ΔmclX) were harvested at T24 (24 h after the exponential phase ended). Heated samples (under 65°C for 20 min) and unheated samples were diluted in a gradient, coated on the LB agar plates. Then the number of grown colonies in heated and unheated sample plates was counted. The sporulation frequency is the clone number ratio of the heated sample to the unheated sample. The error bars represent three independent experiment results.

Cry1Ac protein production

The method was performed as previous described (Zhang et al., 2018). The wild-type strain HD73 and the strain HD (ΔmclX) were harvested at T24 and lyophilized into powder, same quality of samples was taken and determined by 10% SDS–PAGE (Bio-rad). The results of three independent experiments are consistent.

Cell wall preparation and hydrolysis

For cell wall preparation and hydrolysis, method was previously described (Yang et al., 2013; Chen et al., 2018). CwlC added to cell wall was used as a positive control. The cell wall itself was used as a negative control, and the weight of MclX

Construction of the complemented strain

To construct the complemented strain HD (ΔmclX:mclX), the mclX promoter and open reading frame (PmclX-mclX, 998-bp) were amplified by PCR, using HFlmclX-F and HFlmclX-R as primers. The PmclX-mclX fragment was inserted into the pH315 plasmid (Arantes and Lereclus, 1991) to generate the lacZ gene. The recombinant plasmid pHFlmclX was introduced into HD73. The mclX mutation box allelic replacement was performed as reported (Yang et al., 2013). The mclX ORF (12–663) was replaced by kan fragment which detected by PCR with primers mclX-1 and mclX-6.

Construction of a PmclX-lacZ fusion strain

To analyze the transcriptional activity of the mclX promoter, a 335-bp fragment upstream of the mclX ATG start codon was amplified by PCR, using PmclX-F and PmclX-R as primers. The PmclX fragment was inserted into the pH304-18Z plasmid (Arantes and Lereclus, 1991) carrying the lacZ gene. The recombinant plasmid pHFlmclX was introduced into the HD73 strain, mutant strain HD (ΔsigK) or HD (ΔgerE).

Construction of a mother cell lysis X-His fusion protein

To express the MclX protein in an E. coli strain, an mclX gene fragment without a termination codon was amplified by PCR, using MclX-F and MclX-R as primers. The resulting fragment was inserted into the pET21b plasmid to generate pETmclX with a C-terminal His tag. The plasmid pETmclX was transformed into the BL21 strain for expressing the MclX-His protein.
was the same as that of CwlC. The results of three independent experiments are consistent.

**Bioassay of insecticidal activity**

A bioassay was carried out as previously described (Chen et al., 2018). Each test was conducted with three independent cultures replicated.

**Western blot analysis**

Cell lysates (the equivalent of 2 ml at the time point) were suspended in 600 µl of Tris buffer (pH 8.0) and analyzed by 10% SDS–PAGE (Bio-rad). Anti-CwlC was generated by a company (Beijing Protein Innovation Inc., China), and the second antibody (HRP-conjugated goat anti-mouse IgG) was bought (CWBiotech, Beijing, China). The results of three independent experiments are consistent.

**Electrophoretic mobility shift assays**

EMSA (Electrophoretic mobility shift assay) was carried out as previous described (Shen et al., 2021). The cwlC promoter was labeled with 6-carboxyfluorescein (FAM) (Supplementary Table 3). The results of three independent experiments are consistent.

**Results**

**Deletion of the hypothetical gene HD73_RS12920 can encapsulate Cry1Ac in mother cell**

To search for key genes involved in mother cell lysis, T7 transcriptome data (Tn stands for n hours after the exponential phase ended) of the strain HD73 was analyzed (Peng et al., 2015). T7 stands for the late sporulation stage, at which the exosporium genes of the spore coat begin to be transcribed (Peng et al., 2016). Transcription of cwlC had not started at T7, which means that mother cell lysis also had not started. Highly expressed hypothetical genes were screened (Supplementary Table 4) and genetic deletion mutants were constructed.

Fortunately, deletion of a hypothetical gene showed a phenotype in which the mother cell did not lyse. This hypothetical gene is HD73_RS12920 (which encodes a protein in NCBI with the reference sequence WP_000101499.1). HD73_RS12920 is 663-bp in length and has no known domains. According to the deletion mutant phenotype, which displayed no mother cell lysis, HD73_RS12920 was named mclX (mother cell lysis X).

In the mclX deletion mutant HD (ΔmclX) (method described in “Materials and Methods”), the mother cell did not lyse, while the mother cells of wild-type strain HD73 lysed at 1, 3, and 5 days after the bacterial growth reached the end of the exponential phase (Figure 1A). There was no difference in vegetative growth between the wild-type strain HD73 and HD (ΔmclX) (Supplementary Figure 1). To further demonstrate the role of MclX in mother cell lysis, we constructed a genetically complemented strain HD (ΔmclX:ΔmclX), as described in section “Materials and methods.” In the mother cell lysis stage, most wild-type and HD (ΔmclX:ΔmclX) mother cells have lysed (Supplementary Figure 2). The results showed that MclX played an important role in mother cell lysis.

There were no differences between the wild-type strain HD73 and HD (ΔmclX) in sporulation frequency or Cry1Ac protein production at T24 (Figures 1B,C). The second instar larva of Plutella xylostella was used to determine the insecticidal activity of the wild-type strain HD73 and HD (ΔmclX). P. xylostella larvae were fed prepared cabbages treated with a mixture of spores and crystals of the wild-type strain or the HD
(ΔmclX) strain. The LC$_{50}$ of the wild-type strain HD73 was 8.89 µg/ml and the LC$_{50}$ of the HD (ΔmclX) strain was 10.2 µg/ml, displaying no significant differences (Table 1). Therefore, the deletion of the mclX encapsulated Cry1Ac in HD73 mother cells, without effect on sporulation frequency, Cry1Ac protein production, or insecticidal activity.

Transcriptional regulation of the mother cell lysis X gene

MclX has no known domain and a molecular size of 25.6 kDa. Secondary structure alignment (PSIPRED)$^1$ revealed the presence of several $\alpha$-helices and $\beta$-strands in MclX (Supplementary Figure 3). In the B. thuringiensis HD73 genome, mclX has an upstream gene, RS12915, which encodes a YozE family protein, and a downstream gene, RS12925, which encodes a hypothetical protein (Figure 2A). In the B. cereus group, MclX is very conserved (Supplementary Figure 4). Streptococcus pneumoniae, Bacillus subtilis, and Rhodococcus qingshengii, have a MclX homologous protein with 100, 62, and 65% similarity, respectively.

The promoter region of the mclX gene (335-bp upstream of the mclX ATG start codon) was fused to the lacZ gene, and transformed into HD (ΔsigK), HD (ΔgerE), and wild-type HD73. In HD73, the transcription of P$_{mclX}$ initiated at T6 and reached a peak at T12 (Figure 2B, circles). In HD (ΔsigK), the transcriptional activity of P$_{mclX}$ was completely abolished (Figure 2B, triangles). In HD (ΔgerE), P$_{mclX}$ transcriptional activity was increased compared to that in wild-type strain HD73 (Figure 2B, squares). All above results demonstrate that mclX was highly expressed in the late sporulation stage, controlled by $\sigma^K$, and negatively regulated by GerE.

Mother cell lysis X cannot hydrolyze the cell wall in vitro

HD (ΔmclX) displayed no mother cell lysis. MclX has no known domains, including DNA binding domains or any functional domains. Regarding its role in mother cell lysis, MclX may be an essential cell wall hydrolase or it may affect the function of the cell wall hydrolase CwlC.

To test whether MclX could be a cell wall hydrolase, the MclX protein hydrolyzed cell wall was analyzed in vitro. The MclX-His protein was expressed and analyzed by SDS–PAGE, its molecular weight was approximately 30 kDa (Figure 3A). The MclX-His protein and the wide-type strain HD73 cell wall were incubated together. The optical density at 540 nm (OD$_{540}$) showed cell wall turbidity. CwlC is a known hydrolase that can reduce the cell wall turbidity (Figure 3B, squares). However, there was no reduction in the OD$_{540}$ values of MclX.

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1 http://bioinf.cs.ucl.ac.uk/psipred/

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**TABLE 1 Insecticidal activities of B. thuringiensis strains against Plutella xylostella.**

| Strain       | LC$_{50}$ (µg of protein/ml) | 95% confidence interval |
|--------------|------------------------------|-------------------------|
| HD73         | 8.89                         | 6.95–13.96              |
| HD(ΔmclX)    | 10.2                         | 7.81–16.01              |
protein-containing cell wall (Figure 3B), suggesting that MclX cannot hydrolyze the cell wall in vitro.

Mother cell lysis X gene deletion decreases cwlC expression.

Previous research indicated that cwlC is controlled by σK and positively regulated by GerE (Chen et al., 2018). Since HD (ΔmclX) exhibited a similar phenotype to HD (ΔcwlC), whether the deletion of the mclX affected the transcriptional activity or protein expression of CwlC was analyzed.

The transcriptional activity of cwlC was investigated in HD (ΔmclX). β-galactosidase activity assays indicated that cwlC expression significantly decreased in HD (ΔmclX), compared to that in wild-type strain (Figure 4A). Whether MclX could directly bind to the cwlC promoter was tested by EMSA. MclX was found to be unable to directly bind to the cwlC promoter...
(Figure 4B). It is speculated that mclX deletion might affect cwlC transcription levels by affecting transcriptional activities or protein functions of σK and GerE. To investigate this, the transcriptional activities of sigK and gerE in HD (ΔmclX) and the wild-type strain were analyzed, showing no differences (Figures 4C,D). The protein functions of σK and GerE in HD (ΔmclX) were detected. hxpB, which was found to be regulated by σK and GerE (Peng et al., 2016), showed no differences in transcription between the wild-type strain and HD (ΔmclX) (Supplementary Figure 5). Thus, McIX indirectly affected cwlC transcriptional activity, not by affecting σK or GerE.

Moreover, CwlC protein production was investigated in HD (ΔmclX). In HD (ΔmclX) cell lysate, CwlC protein was barely visible in the anti-CwlC immunoblot, whereas significant amounts of CwlC protein were produced in the wild-type strain cell lysate (Figure 5), with the same bacterial volumes taken at the same time point and using the same loading volumes.

Altogether, CwlC expression was dramatically decreased and barely detectable in HD (ΔmclX).

Discussion

In this work, the novel gene mclX was characterized, whose deletion resulted in Cry1Ac encapsulation in the mother cell of the HD73 strain (Figure 1A). McIX was unable to hydrolyze the cell wall in vitro (Figure 3B), and it is a newly identified mother cell lysis-associated protein which is not an amidase. McIX can be found not only in the B. cereus group but also in some S. pneumoniae strains (Supplementary Figure 4). S. pneumoniae is a Gram-positive human pathogen leading to global health problems (Engholm et al., 2017). Its McIX homologous protein shares 100% similarity with that of HD73, and the role of McIX in S. pneumoniae cell lysis maybe very interesting and warrants further studies.

cwlC encodes a key cell wall hydrolase, and its deletion resulted in Cry1Ac encapsulation in the mother cell of the HD73 strain (Figure 1A). McIX was unable to hydrolyze the cell wall in vitro (Figure 3B), and it is a newly identified mother cell lysis-associated protein which is not an amidase. McIX can be found not only in the B. cereus group but also in some S. pneumoniae strains (Supplementary Figure 4). S. pneumoniae is a Gram-positive human pathogen leading to global health problems (Engholm et al., 2017). Its McIX homologous protein shares 100% similarity with that of HD73, and the role of McIX in S. pneumoniae cell lysis maybe very interesting and warrants further studies.

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Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material
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