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Full Length Research Paper

Deletion of the *yhdT* gene of *Bacillus subtilis* and its influence on hemolysis

Jie Liu*, Qiong Zhang and Chunhong Fang

College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi Province, China.

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***Bacillus subtilis* is commonly used as a probiotic. Recently, some metabolites of *B. subtilis* were reported to cause hemolysis; however, the mechanism by which these metabolites cause hemolysis remains to be clarified. In this study, we cloned the hemolysis-associated gene *yhdT* and constructed a *yhdT* gene-deletion mutant of the *B. subtilis* 224 strain. Further, we determined the hemolytic activity of the culture supernatant of the *yhdT* gene-deletion mutant and found that the hemolytic activity of the *yhdT* gene-deletion mutant was lower than that of wild-type *B. subtilis* 224. Thus, our results indicate that the *yhdT* gene contributes to the hemolytic activity of *B. subtilis* 224.**

Key words: *Bacillus subtilis*, *yhdT* gene, hemolysis.

INTRODUCTION

Bacillus subtilis has a wide range of applications as a probiotic in industry (Swain and Ray, 2007; Deepak et al., 2008) , agriculture (Paulitz and Belanger, 2001), graziery (Leser et al., 2008), and medicine (Peng and Xie, 2002; Wang et al., 2002). However, recent reports (Hofemeister et al., 2004; Liu et al., 2007, 2009; Monteiro et al., 2005) have indicated that some metabolites of *B. subtilis* may be hemolytic. The hemolytic activity of the strain is often associated with its pathogenicity, and hemolysins have been considered to contribute to virulence (Hughes et al., 1983; Waalwlk et al., 1982; Wekh et al., 1981). For example, staphylococcal α-hemolysin (Phillips et al., 2006), streptolysin-O (Kehoe et al., 1987), and *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) (Chiang and Chou, 2008) are all major pathogenicity factors that can cause a variety of diseases in humans. Currently, very few studies have investigated hemolysin production in *B. subtilis*, and the hemolytic mechanism of *B. subtilis* remains to be elucidated.

To clarify the hemolytic mechanism of *B. subtilis*, hemolytic strain *B. subtilis* 224 was chosen as the subject in this paper. *B. subtilis* 224 was separated from human skin and has strong antagonism against many pathogenic bacteria and fungi, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and so on (Zhou et al., 2003). Therefore some *B. subtilis* 224 micro-ecological preparations were manufactured, including Subtilobiogen (Qi et al., 1991) and *B. Subtilis* Viable-Preparation (Peng and Xie, 2002; Wang et al., 2002), which can diminish inflammation to cure burn and scald. However, because *B. Subtilis* 224 could cause hemolysis (Liu et al., 2007, 2009), their use was much limited, even forbidden.

The completion of sequencing and annotation of the *B. subtilis* 168 genome (Kunst et al., 1997) has provided comprehensive information about the genetic background of *B. subtilis* and revealed the following 8 hemolysis-associated genes: *yhdP*, *yhdT*, *yugS*, *yrkA*, *yqhB*, *yqxC*, *yplQ*, and *ytjA*. These annotations were obtained only by bioinformatics analysis, and there wasn't any experimental prove. Therefore, the identification of the hemolysis-associated genes is a prerequisite for elucidating the hemolytic mechanism of *B. subtilis*.

In this study, we constructed a *yhdT* gene-deletion mutant *B. subtilis* 224 and determined the hemolytic activity of the culture supernatant of this mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

B. subtilis 224 was used as the source of chromosomal DNA and the target gene, and *Escherichia coli* JM109 was used as the host

*Corresponding author. E-mail: liujie2003@hotmail.com. Tel: +8613484549186. Fax: +86 29 87092262.

in the cloning procedures. These bacterial strains were routinely cultured at 37°C in Luria-Bertani (LB) medium (0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7.4). The plasmids pMD18-T (TaKaRa) and pMD18-T-Neo^r (pMD18-TN) constructed by ourselves (Liu et al., 2007), were used as the cloning and integration vectors, respectively.

Characterization of YhdT

First, we determined the physicochemical properties of YhdT on the basis of its amino acid sequence by using the ProtParam tool at ExPASy (http://www.expasy.org). Subsequently, we predicted the protein domain structure on the basis of Pfam analysis (http://pfam.sanger.ac.uk/). Finally, we analyzed the amino acid sequence using basic local alignment search tool for proteins (BLASTP) and compared this sequence with non-redundant databases (NCBI) to obtain the proteins that were homologous to YhdT; the sequences of these proteins were further compared using the ClustalW (1.8) multialign software.

Cloning of the *yhdT* gene

Polymerase chain reaction (PCR)-amplification of the *yhdT* gene was performed using the genomic DNA of *B. subtilis* 224, which was extracted by performing a previously described method, Liu and Zhai (2004), with Ex-Taq DNA polymerase (TaKaRa) and the following primers: 5′-ATGGACGATATTGACAGTCT-3′ (T_1) and 5′-TTATTTCTCGCTCAAGGTTG-3′ (T_2). The amplified fragments were ligated to pMD18-T vectors (TaKaRa) and used to transform *E. coli* JM109 cells. The recombinant plasmids obtained using this method was verified by restriction enzyme digestion and DNA sequencing.

Construction of the *yhdT* gene-deletion mutant

The *yhdT* mutator plasmid was constructed as previously mentioned (Huang et al., 2004; Sun et al., 2004). The upstream region (UR) of the *yhdT* gene was amplified by PCR using the genomic DNA of *B. subtilis* 224 and the following primers: 5′-ATCGGTACCGCGTGCTCATTGCTTTAAC-3′ (UR_1) and 5′-TTATCTAGACGGTATCATAATTTCTTTGGC-3′ (UR_2), which contained *Kpn*I and *Xba*I restriction sites (underlined), respectively. The PCR products were digested with *Kpn*I and *Xba*I and then ligated with the recombinant plasmid pMD18-TN that was treated using the abovementioned double restriction digestion approach. Similarly, the downstream region (DR) of the *yhdT* gene was amplified by PCR with the primers 5′-CCTGTCGACATGACAGAAGAAGAACGAATG-3′ (DR_1) and 5′-TTACTGCAGTATGAACCGATGCGAATGGA-3′ (DR_2), which contained *Sal*I and *Pst*I restriction sites, respectively. The PCR products were digested with *Sal*I and *Pst*I and then ligated with the plasmid pMD18-TN-UR, which was treated by using the same double restriction digestion approach.

The *yhdT* mutator plasmid was linearized with *Kpn*I and *Pst*I and then used in the electrotransformation of competent cells of *B. subtilis* 224. The positive transformants were screened from an agar plate supplemented with neomycin (100 mg/L).

PCR identification of the *yhdT* gene-deletion mutant

The *yhdT* gene-deletion mutant was identified by PCR with the primers: 5′-CTGGATTCATCGACTGTG-3′ (P_{neo}) and 5′-TCTTCAGCACCTTTATCG-3′ ($P_{DR\text{-}out}$), which were designed according to the sequence downstream of the neomycin resistance gene and the genomic DNA sequence adjacent to the 3' end of DR of the *yhdT* gene, respectively. The amplified fragments obtained from the genomic DNA of positive transformants were ligated into pMD18-T and sequenced by the Shanghai Sangon Company.

Detection of the hemolytic activity of the culture supernatant of the *yhdT* gene-deletion mutant

Firstly standard growth curves for wild-type *B. subtilis* 224 and its *yhdT* gene-deletion mutant were made, then we took the samples during the stationary phase of growth to detect the hemolytic activity of their culture supernatants as previously described (Baida and Kuzmin, 1996; Morán et al., 2002), respectively. The reaction mixtures contained: 200 µl of the culture supernatant diluted in phosphate buffered saline (PBS, pH 8.0) and 200 µl of erythrocyte suspension adjusted to a concentration of 2% (v/v) with PBS. The mixtures were gently mixed in 1.5 ml microcentrifuge tubes and incubated at 37°C. After centrifugation at 790 × *g* for 3 min, the intact erythrocytes formed a pellet and the supernatants were collected; the absorbance values for the released hemoglobin in the supernatant were measured at 540 nm using a spectrophotometer. One hemolytic unit (HU) was defined as the dose causing 50% hemolysis. To obtain controls showing 100% hemolysis, we mixed 0.2 ml of the erythrocyte suspension with 0.2 ml of distilled water containing saponin.

RESULTS

In silico structural analysis of YhdT

Amino acid sequence analysis of the YhdT protein indicated that it is composed of 461 amino acids with a predicted molecular weight of 51,519.8 Da and a theoretical isoelectric point (pI) of 4.94. The sequence analysis revealed the presence of a domain of unknown function (DUF21) from 5 to 202, which is usually associated with hemolytic activity; 2 cystathionine-β-synthase (CBS) domains from 217 - 275 and from 284 - 342; and a CorC_HlyC domain from 357 - 433, which may be involved in modulating the transport of ion substrates. The sequence of this protein exhibits 59% identity and 79% similarity with the hemolysin from *Bacillus sp.* NRRL B-14911 (accession no. ZP_01170636), 60% identity and 78% similarity with the hemolysin from *Geobacillus kaustophilus* HTA426 (accession no. YP_146417), 61% identity and 80% similarity with the TlyC family hemolysin from *Bacillus pumilus* ATCC 7061 (accession no. ZP_03052845), and 58% identity and 76% similarity with the TlyC family hemolysin from *Listeria grayi* DSM 20601 (accession no. ZP_04443578). The sequence alignment of YhdT with the 4 homologous proteins is shown in Figure 1.

PCR amplification of *yhdT* and identification of the recombinant plasmid

The PCR products were electrophoresed on a 1.0% (w/v) agarose gel containing ethidium bromide (0.5 mg/L). The length of the amplified fragment was consistent with the theoretical length of the *yhdT* gene (1,386 bp). The am-

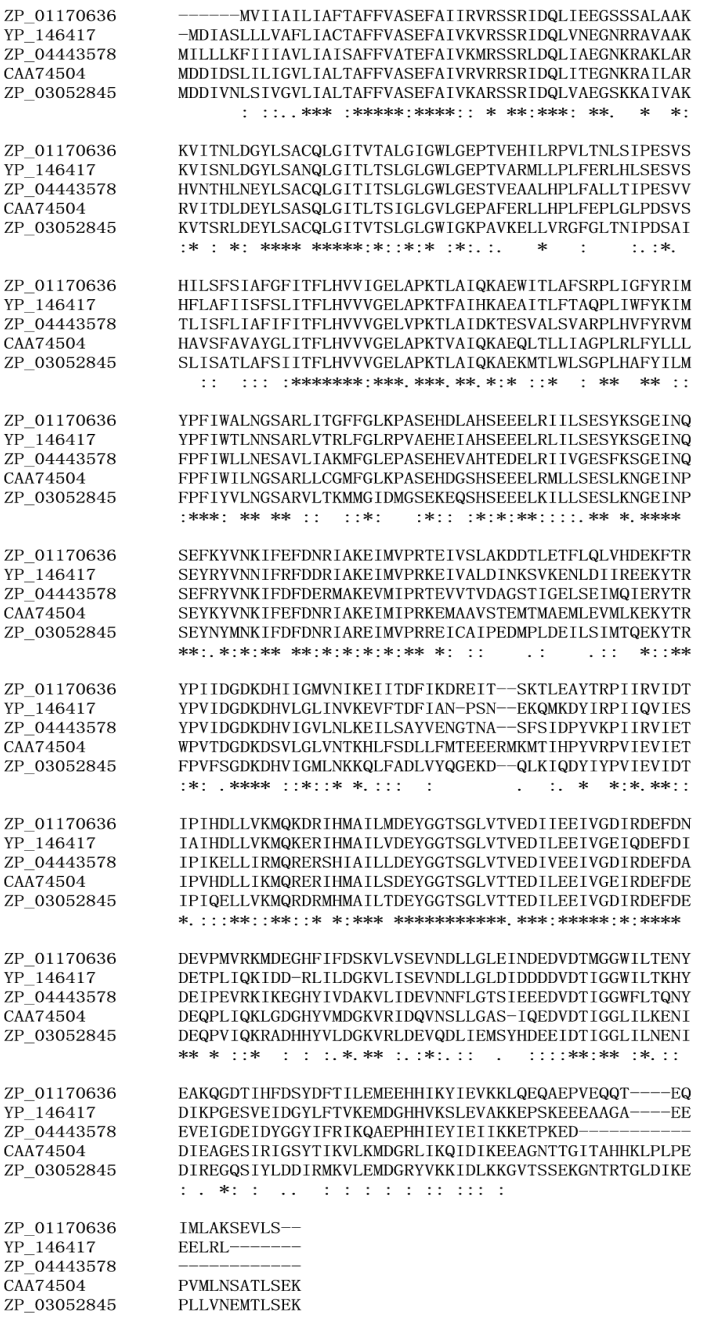

Figure 1. Alignment of the amino acid sequences of *B. subtilis* 224 YhdT with other bacterial hemolysins.

plified fragment was then ligated to a pMD18-T vector, and the recombinant plasmid was identified by digestion with the restriction enzymes *EcoR*I and *Sal*I. Agarose gel electrophoresis revealed 2 distinct fragments belonging to the pMD18-T vector (2,692 bp) and the *yhdT* gene (1,386 bp). Finally, the recombinant plasmid was sequenced, and the obtained sequence was identical to that reported in GenBank (accession no. AL009126).

Alignment was maximized by introducing gaps, which have been indicated by dashes. Identical (*), highly similar (:), and similar (.) amino acids have also been indicated. Abbreviations: ZP_01170636, the hemolysin from *Bacillus sp.* NRRL B-14911 (accession no. ZP_01170636); YP_146417, the hemolysin from *Geobacillus kaustophilus* HTA426 (accession no. YP_146417); ZP_04443578, the TlyC family hemolysin from *Listeria grayi* DSM 20601 (accession no. ZP_04443578); CAA74504, YhdT from *B. subtilis* 224 (accession no. CAA74504); ZP_03052845, the TlyC family hemolysin from *Bacillus pumilus* ATCC 7061 (accession no.

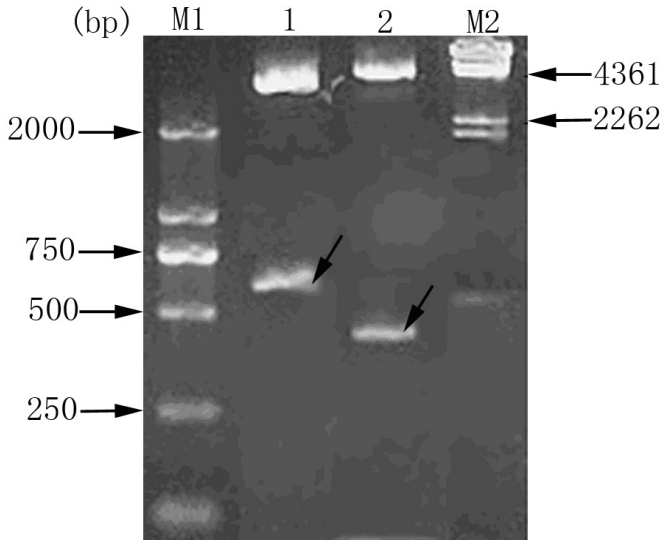

Figure 2. Identification of the *yhdT* mutator plasmid by double restriction digestion.

Lane M_1: DL2000 DNA marker; lane 1: the *yhdT* mutator plasmid digested with the restriction enzymes *Kpn*I and *Xba*I; lane 2: the *yhdT* mutator plasmid digested with the restriction enzymes *Sal*I and *Pst*I; lane M_2: λ-Hind, digest DNA Marker; the arrows on lane 1 and lane 2 indicates the 638bp UR and the 407 bp DR of the *yhdT* gene, respectively.

ZP_03052845).

Construction of the *yhdT* gene-deletion mutant

The *yhdT* mutator plasmid was verified by digestion with the restriction enzymes *Kpn*I and *Xba*I. Agarose gel electrophoresis of the digestion products revealed 2 distinct fragments belonging to pMD18-TN-DR (~4.5 kb) and UR (638 bp) (Figure 2, lane 1). Similarly, the *yhdT* mutator plasmid was digested with *Sal*I and *Pst*I, and agarose gel electrophoresis of the digestion products revealed 2 fragments for pMD18-TN-UR (~4.7 kb) and DR (407 bp) (Figure 2, lane 2).

Then, the linearized *yhdT* mutator plasmid was introduced into *B. subtilis* 224 by electroporation, and the positive transformants were selected on LB agar plates containing neomycin (100 mg/L). These transformants were inoculated in selective LB medium and their genomic DNA was extracted and used in PCR to achieve a double-crossover homologous recombination between the wild-type *yhdT* and the mutator plasmid. A total of 105 colonies were screened, and a specific fragment was found to be amplified in the 102nd colony; no fragments appeared in the other colonies (Figure 3). The putative mutant was designated as *yhdT*102.

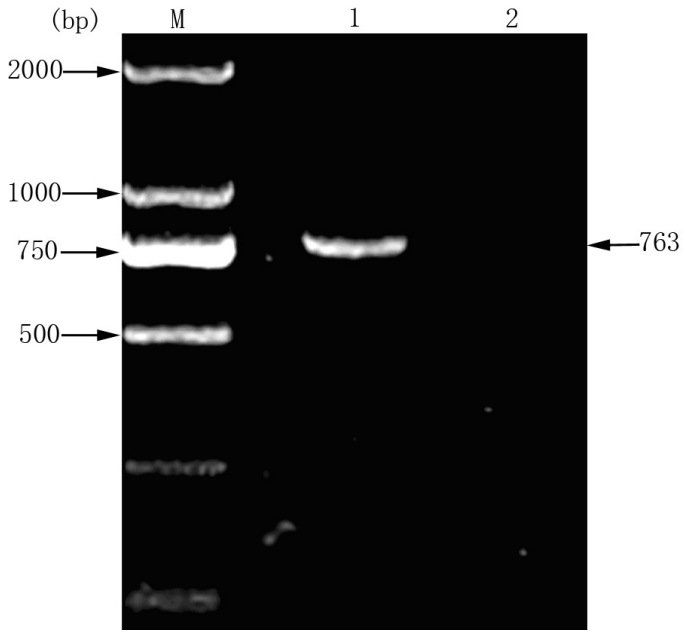

Figure 3. PCR identification of the *yhdT* gene-deletion mutant.

Lane M: DL2000 DNA marker; lane 1: PCR-amplified fragments by using the genomic DNA of the *yhdT* gene-deletion mutant as the template; lane 2: PCR amplification by using the genomic DNA of wild-type *B. subtilis* 224 as the template (negative controls). The specific amplified band on lane 1 indicates that the *yhdT* gene is deleted in *B. subtilis* 224.

Molecular analysis of the *yhdT* gene-deletion mutant

PCR analysis of the genomic DNA isolated from the mutant revealed inactivation of the *yhdT* gene. The *yhdT*-specific primers P_{neo} and $P_{DR\text{-}out}$ yielded a 763 bp fragment; no fragment appeared in the other 104 colonies. The specific fragment was purified using the Agarose Gel DNA Extraction Kit and ligated to the pMD18-T vector. The recombinant plasmid was identified by digestion with the restriction enzymes *EcoR*I and *Sal*I. Agarose gel electrophoresis revealed 2 fragments of the pMD18-T simple vector (2,692 bp) and the objective band (763 bp). Finally, we sequenced the confirmed recombinant plasmid.

In the event of double-crossover homologous recombination between the wild-type *yhdT* and the mutator plasmid, the 160 bp nucleotide sequence of the *yhdT* gene is displaced by the neomycin-resistance gene expression cassette; therefore, the resultant nucleotide sequence will contain part of the neomycin resistance gene sequence, the entire sequence of DR, and the 131 bp genomic DNA sequence adjacent to the 3' end of DR of the *yhdT* gene. The sequencing results were identical to the predicted results (Figure 4).

Figure 4. Nucleocide sequence evidence of the *yhdT* gene-deletion mutant.

P_{neo} and $P_{DR\text{-}out}$ indicate the location of forward and reverse primers, respectively, used to amplify the 763bp PCR product from the *yhdT* gene-deletion mutant for sequencing; the box indicates the location of the restriction enzyme *Sal*I between the neomycin resistance gene and the DR of the *yhdT* gene; DR_1 and DR_2 indicate the location of forward and reverse primers, respectively, used to amplify the 407 bp DR of the *yhdT* gene; the nucleicide sequence between DR_2 and $P_{DR\text{-}out}$ indicates the genomic DNA sequence adjacent to the 3' end of DR of the *yhdT* gene. The sequencing result suggests that there is a double crossover event of homologous recombination between the wild-type *yhdT* and the mutator plasmid.

Detection of hemolytic activity

To compare the hemolytic activity of the *yhdT* gene-deletion mutant with its wild type, their growth curves were determined, respectively. The result indicated that their growth curves were completely same (data not shown) and the deletion of the *yhdT* gene produced no influence on the growth of *B. subtilis* 224. Therefore, we sampled the culture supernatants of wild-type *B. subtilis* 224 and its *yhdT* gene-deletion mutant to detect their hemolytic activity, which were cultured in LB medium for 24 h, just in the stationary phase of growth. The hemolytic activity was determined by measuring the absorbance values at 540 nm using a spectrophotometer. The results indicated that the hemolytic activity of the *yhdT* gene deletion mutant was lower than that of the wild-type *B. subtilis* 224 (Figure 5).

Each datum point in Figure 5 represents mean of 3 - 6 experimental determinations. Although the decrease in the hemolytic activity was not significant, it was sufficient to indicate that the *yhdT* gene contributed to the hemolytic activity of *B. subtilis* 224.

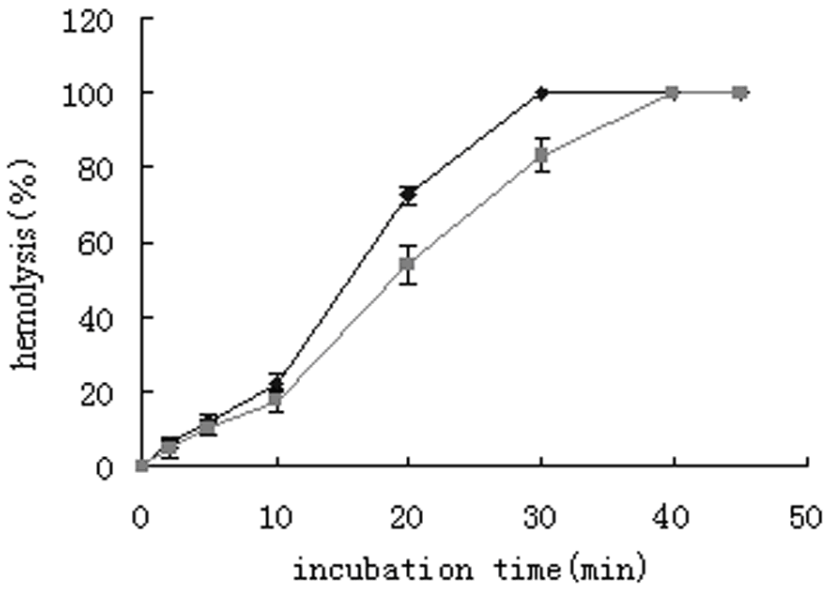

Figure 5: Detection of the hemolytic activity of the *yhdT* gene-deletion mutant.

Erythrocyte suspensions (2%) mixed with equal volumes (0.2 ml) of the culture supernatant of the *yhdT* gene-deletion mutant were incubated at 37℃ (■), and (□) for the hemolysis caused by the culture supernatant of the wild-type *B. subtilis* 224 at 37℃. Compared with the wild-type B. subtilis 224, the decrease of the hemolytic activity of the *yhdT* gene-deletion mutant indicated that the *yhdT* gene contributed to the hemolytic activity of *B. subtilis* 224.

DISCUSSION

Although recent studies have indicated that some metabolites of *B. subtilis* can cause hemolysis, few reports have been published on the hemolysin genes in *B. subtilis* and their hemolytic mechanism. In this study, we cloned the hemolysis-associated gene *yhdT* and constructed a *yhdT* gene-deletion mutant of *B. subtilis* 224 for the first time. Then, we assessed the hemolytic activity in the culture supernatant of the *yhdT* gene-deletion mutant. Our results indicated that the hemolytic activity of the *yhdT* gene-deletion mutant was lower than that of the wild-type *B. subtilis* 224. Thus, the *yhdT* gene contributed to the hemolytic activity of *B. subtilis* 224.

Bioinformatics analysis of the amino acid sequence of *yhdT* from *B. subtilis* 224 revealed that this sequence shared high identity with the TlyC family hemolysin, which have been found in many microorganisms and identified to have strong hemolytic activity in *Serpulina hyodysenteriae* (Huurne et al., 1994) and *Rickettsia* (Radulovic et al., 1999). However, YhdT, one of the TlyC homologs, was reported in *B. subtilis* for the first time and the identification of the *yhdT* gene helped to elucidate the hemolytic mechanism of *B. subtilis*.

We assessed the hemolytic activity in the culture supernatant of the *yhdT* gene-deletion mutant constructed in the study. We found that the hemolytic activity of the *yhdT* gene-deletion mutant was lower than that of the wild-type *B. subtilis* 224, thereby suggesting that the *yhdT* gene contributes to the hemolytic activity of *B. subtilis* 224. Thus, the protein encoded by *yhdT* may be a virulence factor associated with a variety of diseases caused by *B. subtilis*, such as septicemia (Matsumoto et al., 2000) and bacteremia (Galanos et al., 2003). However, the deletion of the *yhdT* gene could interrupt hemolysis of *B. subtilis*, thereby implying that *yhdT* is not the only gene responsible for hemolysis of *B. subtilis*.

For the purpose of further characterization of the *yhdT* gene, we are attempting to construct a expression system to obtain the YhdT protein and detect it hemolytic activity. In addition, we will prepare an anti-YhdT antibody to confirm the presence of YhdT protein in the culture supernatant of *B. subtilis* 224.

In summary, our results confirm that the *yhdT* gene contributes to the hemolytic activity of *B. subtilis* 224. We have not only determined the functions of the *yhdT* gene, but our results can be used to design further studies to elucidate the hemolytic mechanism in *B. subtilis.* In addition, our results provide a foundation for future studies on achieving the desired improvements in the genetic makeup of *B. subtilis* and assessing the safety of *B. subtilis* as a probiotic.

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REFERENCES

Baida GE, Kuzmin NP (1996). Mechanism of action of hemolysin III from *Bacillus cereus.* Biochimica. et Biophysica. Acta. 1284: 122-124.

Chiang ML, Chou CC (2008). Expression of superoxide dismutase, catalase and thermostable direct hemolysin by, and growth in the presence of various nitrogen and carbon sources of heat-shocked and ethanol-shocked *Vibrio parahaemolyticus.* Int. J. Food. Microbiol. 121: 268-274.

Deepak V, Kalishwaralal K, Ramkumarpandian S, Babu SV, Senthilkumar SR, Sangiliyandi G (2008). Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. Bioresour. Technol. 99: 8170-8174.

Galanos J, Perera S, Smith H, O'Neal D, Sheorey H, Waters MJ (2003). Bacteremia due to three *Bacillus* species in a case of Munchausen's syndrome. J. Clin. Microbiol. 41: 2247-2248.

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryava N, Steinborn G, Franke P, Grammel N, Zwintscher A, Leenders F, Hitzeroth G, Vater J (2004). Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Mol. Genet. Genomics, 272: 363-378.

Huang IH, Waters M, Grau RR, Sarker MR (2004). Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. FEMS. Microbiol. Lett. 233: 233-240.

Hughes C, Hacker J, Roberts A, Goebel W (1983). Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. Infect. Immun. 39: 546-551.

Huurne AA, Muir S, van Houten M, van der Zeijst BA, Gaastra W, Kusters JG (1994). Characterization of three putative *Serpulina hyodysenteriae* hemolysins. Microb. Pathog. 16: 269-282.

Kehoe MA, Miller L, Walker JA, Boulnois GJ (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging thiol-activated toxins. Infect. Immun. 55: 3228-3232.

Kunst F, Ogasawara N, Moszer I (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis.* Nature, 390: 249-256.

Leser TD, Knarreborg A, Worm J (2008). Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. J. Appl. Microbiol. 104: 1025-1033.

Liu G, Zhai C (2004). A current and speedy extraction method for bacterial chromosomal DNA from a trifle of broth. Med. J. West. China. 16: 111-113.

Liu J, Fang C, Jiang Y, Yan R (2009). Characterization of a Hemolysin Gene *ytjA* from *Bacillus subtilis*. Curr. Microbiol. 58: 642-647.

Liu J, Fang C, Li W, Jiao H, Yu J, Li J (2007). Construction of *Bacillus subtilis* 224 *yugS* mutant by homologous recombination method. Biotechnol. Bull. 4: 148-151.

Matsumoto S, Suenaga H, Naito K, Sawazaki M, Hiramatsu T, Agata N (2000). Management of suspected nosocomial infection: an audit of 19 hospitalized patients with septicemia caused by *Bacillus species*. Jpn. J. infect. Dis. 53: 196-202.

Monteiro L, Mariano RLR, Souto-Maior AM (2005). Antagonism of *Bacillus spp.* Against *Xanthomonas campestris* pv. *Campestris*. Braz. Arch. Boil. Technol. 48: 23-29.

Morán AC, Martínez MA, Siñeriz F (2002). Quantification of surfactin in culture supernatants by hemolytic activity. Biotechnol. Lett. 24: 177-180.

Paulitz TC, Belanger RR (2001). Biological control in green house systems. Annu. Rer. Phythopathol. 39: 103-133.

Peng W, Xie J (2002). Effect of *Bacillus subtilis* viable-preparation for second-degree burn wound healing. Qinghai. Med. J. 32: 5-6.

Phillips JR, Tripp TJ, Regelmann WE, Schlievert PM, Wangensteen OD (2006). Staphylococcal alpha-toxin causes increased tracheal epithelial permeability. Pediatr. Pulmonol. 41: 1146-1152.

Qi H, Ma Z, Wang S, Wang Z, Di W, Yu Y, He Y, Hu L, Li Z, Liu J, Shen BJ (1991). Research summary report on Subtilobiogen. Chin. J. Microecol. 3: 1-11.

Radulovic S, Troyer JM, Beier MS, Lau AOT, Azad AF (1999). Identification and molecular analysis of the gene encoding *Rickettsia typhi* Hemolysin. Infect. Immun. 67: 6104-6108.

Sun J, Li J, Wang A, Tang G, Wang H (2004). Construction and functional analysis of the *pepB* gene disruptant in *Aspergillus niger*. Acta Microbiologica Sinica, 44: 766-770.

Swain MR, Ray RC (2007). Alpha-amylase production by *Bacillus subtilis* CM3 in solid state fermentation using cassava fibrous residue. J. Basic. Microbiol. 47: 513-517.

Waalwlk C, van den Bosch JF, MacLaren DM, de Graaff J (1982). Hemolysin plasmid encoding for the virulence of a nephropathogenic *Escherichia coli* strain. Infect. Immun. 35: 32-37.

Wang W, Gao Q, He S (2002). Observation of curative effect of *Bacillus subtilis* viable preparation for second-degree burn in the face and neck. J. Ningxia. Med. Coll. 24: 363 -364.

Wekh RA, Dellinger EP, Minsbew B, Falkow S (1981). Hemolysin contributes to virulence of extraintestinal *E. coli* infections. Nature, (London) 294: 665-667.

Zhou S, Li X, Lu L (2003). The study of *Bacillus Subtilis* inhibition action. Heilongjiang Med. J. 16: 205-206.