

High-Level Expression and Secretion of Methyl Parathion Hydrolase in *Bacillus subtilis* WB800

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The methyl parathion hydrolase (MPH)-encoding gene *mpd* was placed under the control of the P43 promoter and *Bacillus subtilis* *nprB* signal peptide-encoding sequence. High-level expression and secretion of mature, authentic, and stable MPH were achieved using the protease-deficient strain *B. subtilis* WB800 as the host.

Organophosphate compounds are used extensively in agricultural and domestic pest control and as chemical warfare agents; these compounds inhibit the acetylcholinesterase of animals (8). Mass application of these compounds in the environment causes serious problems. Organophosphate hydrolase (OPH) isolated from soil microorganisms detoxified organophosphates effectively (14, 18), and bioremediation of organophosphate compounds in the environment by using bacterial enzymes may provide an efficient, convenient, and economical method for detoxification. We previously isolated *Plesiomonas* sp. strain M6, which is capable of hydrolyzing methyl parathion at high efficiency (5), and the gene (*mpd*) encoding a novel methyl parathion hydrolase (MPH) was cloned (5) and expressed in *Escherichia coli* (9). However, the previous work showed that *E. coli* could process only a small proportion of the inactive precursor polypeptide, comprising the signal peptide and the mature enzyme, to produce the active MPH (9).

Most microorganisms that produce OPH are gram-negative bacteria, and the OPH is located intracellularly or secreted into the periplasm. The outer membrane acts as a permeability barrier and limits interaction between the pesticides and OPH residing within the cells (12). This bottleneck reduces the application efficacy. *Bacillus subtilis* can serve as an efficient and safe host for recombinant protein secretion (3, 4, 7, 16, 17). Secreted proteins usually remain in biologically active forms (13, 15), and downstream purification is greatly simplified. In this work, the *mpd* gene was fused with the *nprB* signal peptide-encoding sequence, and the organophosphate hydrolase was expressed and secreted in *B. subtilis* under the control of the P43 promoter (20).

The methyl parathion hydrolase-encoding gene *mpd* (GenBank accession no. AF338729) cannot be expressed in *B. subtilis* by using its own promoter. To achieve expression and secretion of MPH in *B. subtilis*, the secretion signal peptide-encoding sequence of the *nprB* gene was fused with the mature

mpd gene. A typical cleavage site (ASA-A) (19) for signal peptidase I was designed for the release from *B. subtilis* of MPH with an authentic N terminus (Fig. 1B). The mature *mpd* gene sequence (9) was cloned using the primer pair P1 (5'-GCGCTGCAGCACCGCAGGTG-3') and P2 (5'-CGCAAGCTTTCATCATCACTTGGGGTTGACGACCGA-3') from plasmid pMT1 (5). To introduce the PstI site (underlined) upstream, the codon GCC encoding the first amino acid of mature MPH was modified to GCA (Fig. 1B). Two additional stop codons (TGATGA) and the HindIII site (underlined) were introduced with primer P2. Primers P3 (5'-CGCGGATCCTGATAGGTGGTATGTTTTTCGC-3') and P4 (5'-CTTG GTCAAGTTGCGCATGTGTACATTCCTCTCTT-3') were used to clone the P43 promoter sequence from *B. subtilis* 168 (2) chromosomal DNA, and the BamHI site (underlined) was introduced in the forward primer P3. Primers P5 (5'-AAGAGAGGAATGTACACATGCGCAACTTGACCAAG-3') and P6 (5'-GCGCTGCAGCTGAGGCATG-3') were used to clone the signal peptide-encoding sequence of the *nprB* gene from *B. subtilis* 168 chromosomal DNA, and the PstI site (underlined) was introduced in the reverse primer P6. The initial codon (TTG) of the *nprB* signal peptide-encoding sequence was modified to ATG in primer P5. The two PCR fragments of the P43 promoter and the *nprB* signal peptide-encoding sequence were spliced by overlap extension (11). The spliced fragment and the mature *mpd* gene PCR fragment were then digested with PstI and ligated together. The ligation product was purified and digested with BamHI/HindIII. The *mpd* gene with the P43 promoter and *nprB* signal peptide-encoding sequence was cloned into pUBC19, and the recombinant plasmid was designated pP43NMK (Fig. 1A).

Competent cells of *B. subtilis* WB800 (21) were transformed with pP43NMK. Transformants able to hydrolyze methyl parathion were screened on super-rich medium (10) agar plates containing 10 µg/ml kanamycin and 100 µg/ml methyl parathion, where they form yellow halos of *p*-nitrophenol around the colonies. A preculture of WB800(pP43NMK) was grown overnight at 37°C in super-rich medium with kanamycin (10 µg/ml), and 1% (vol/vol) of this preculture was used to inoculate 500 ml of the same culture medium in a 1-liter flask. The culture was maintained at 37°C, and 20-µl aliquots were taken

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strain M6. The biological activity of the recombinant MPH band was confirmed by zymogram analysis as a single band with a yellow halo (Fig. 3, lane 7). Using bovine serum albumin as the standard, the amount of MPH in the SDS-polyacrylamide gel was estimated with the densitometry analysis software BandScan (Glyko). This indicated, on extrapolation, that 53 mg/liter of enzyme was present in the culture medium after 96 h of cultivation, which was equivalent to an activity of 27.1 U/ml. MPH activities in cell culture lysates (treated with sonication) and in the culture supernatant of the original *Plesiomonas* strain M6 were only 0.96 U/ml and 0.029 U/ml, respectively. Therefore, a 28.3-fold increase in recombinant enzyme was achieved, and the activity of extracellular MPH produced by the engineered *B. subtilis* strain was 945.4-fold higher than that found in the original producer bacterium.

For purification of the recombinant MPH from *B. subtilis* WB800(pP43NMK), 50 ml super-rich medium in a 150-ml flask was inoculated with 0.5 ml preculture and incubated at 37°C for 96 h. The culture supernatant was concentrated 10-fold by freeze-drying and dialyzed overnight against 20 mM HEPES buffer (pH 7.5) with two changes of buffer. The supernatant was then applied to a DEAE-Sephadex A-50 column (2.5 by 10 cm), which had been preequilibrated with 20 mM HEPES buffer (pH 7.5) at 4°C. By adjusting the pH of the loading and elution buffers to pH 7.5, most of the contaminating proteins were bound to the column. MPH was eluted with the same buffer at a flow rate of 0.5 ml/min, and only four distinct bands could be detected by SDS-PAGE after this purification step. Total protein in the fractions was detected by the Bradford method (1). The yield of the partially purified MPH was 86.3% (the deviation between different batches was less than 10%), and samples were purified fivefold to a specific activity of 155.6 U/mg. Active fractions were concentrated and further purified by preparative SDS-PAGE and electroelution. The electroeluted enzyme produced a single band following SDS-PAGE.

The calculated molecular mass of MPH is 31,397 Da, but because of the relatively low migration rate, MPH had an apparent molecular mass of 34 kDa as determined by SDS-PAGE. The exact molecular mass of recombinant MPH was determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry to be 31,384 Da. The N-terminal sequence of purified MPH was determined by high-performance liquid chromatography-electrospray ionization mass spectrometry as AAPQVR, demonstrating that the *B. subtilis* *nprB* signal peptide sequence had been processed correctly.

For the first time, we have achieved a high level of expression and secretion of active MPH in *B. subtilis* WB800. The expression level of MPH was markedly higher than the 20.5 U/ml obtained using an *E. coli* expression system (9) that produced no detectable extracellular activity. This will greatly improve the application efficiency of the engineered strain to degrade organophosphorus pesticides in farm products and in contaminated soil and water. The new expression/secretion vector, containing the *nprB* typical signal peptide I cleavage site, that we have constructed should also facilitate the expression and secretion of other useful proteins in forms that retain their authentic N-terminal sequences. However, enzyme production efficiency is not yet high enough for practical industrial applications, e.g., for enzyme-based products, and research to further increase MPH yields is in progress.

We thank Daniel Zeigler from the *Bacillus* Genetic Stock Center and Sui-Lam Wong from the University of Calgary for providing bacterial strains and plasmid vectors. We are also grateful to John Buswell from the Edible Fungi Institute, Shanghai Academy of Agricultural Sciences, for the helpful comments and linguistic revision of the manuscript.

This work was supported by grants from the Jiangsu Province Natural Science Foundation of the People's Republic of China (no. BK200171) and the National Natural Science Foundation (no. 30300005) and by the 863 Hi-Tech Research and Development Program of the People's Republic of China (project no. 2004AA214102 and 2004AA246070).

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