Random mutagenesis by insertion of error-prone PCR products to the chromosome of Bacillus subtilis

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Abstract

Bacillus subtilis is an attractive host for directed evolution of the enzymes whose substrates cannot be transported across the cell membrane. However, generation of mutant library in B. subtilis still suffers problems of small library size, plasmid instability and heterozygosity. Here, large library of random mutant was created through inserting error-prone PCR (epPCR) product to the chromosome of B. subtilis. Specifically, epPCR product was fused with flanking regions and antibiotic resistant marker using a PCR-based multimerization method, generating insertion construct. epPCR product was integrated into chromosome via homologous recombination after insertion construct was transformed into the supercompetent cells of B. subtilis strain SCK6. The transformation efficiency of insertion construct was improved though increasing the number of competent cell and the length of flanking regions. A library containing 3.5×10^5 random mutant was construction using per μg insertion construct, which is sufficient for directed evolution. Moreover, the library generation process could be accomplished within one day. The effectiveness of this method was confirmed by improving the activity of Methyl Parathion Hydrolase (MPH) toward chlorpyrifos and to enhance the secretion level of MPH in B. subtilis. Taken together, present work provides a fast and efficient method to integrate epPCR product into the chromosome of B. subtilis, facilitating directed evolution and expression optimization of target protein.

Keywords

epPCR, Chromosomal integration, Large library, Directed evolution,

Bacillus subtilis

1 INTRODUCTION

Directed evolution has proved to be a powerful tool to improve the activity, stability and substrate specificity of enzymes (Goldsmith et al. 2017; Romero et al. 2009; Roodveldt et al. 2005; Wong et al. 2004; Wong et al. 2007; Zeymer et al. 2018). This tool involves two crucial steps: generation of a library containing sufficient gene variants and high-throughput screening of library members with desired properties (Packer et al. 2015; Wong et al. 2006). Because of its high transformation efficiencies, rapid growth rates, well-established manipulation approaches (Crameri et al. 1996), *Escherichia coli* is generally employed as host organism for library creation. However, proteins are usually expressed in cytoplasm in *E. coli* , making the screening of library more difficult if the substrate of the protein cannot be transported into cell. Therefore, the organisms such as *Bacillus subtilis* and *Pichia pastoris* that can secret proteins into medium have been developed as alternative hosts for library generation (Jiang et al. 2019; Liu et al. 2017; Reetz et al. 2007; Wang et al. 2012).

B. subtilis is an important industrial host for production of various recombinant proteins due to its GRAS (generally recognized as safe) status, excellent protein secretion ability and mature fermentation processes (Commichau et al. 2014; Marcus et al. 2004; Schumann et al. 2007; Terpe et al. 2006; Yang et al.

2011). Although *B. subtilis* has well-developed genetic manipulation tools, its transformation frequency is still much lower than that of *E. coli* . Thus, the library of random mutants generated through digestion and ligation is too small to fulfill the need of direct evolution in *B. subtilis* . Several strategies have been adopted to address this limitation. A routine strategy is first constructing the library of variants in *E. coli* and then transferring the library into *B. subtilis* , which is time-consuming and of low efficiency (Caspers et al. 2010; You et al. 1996; Zhao et al. 1999). Melnikov et al. (1999) attempted to clone error-prone PCR (epPCR) product via marker-replacement recombination with a structurally similar helper plasmid resident in the transformation recipient. But they found that it was difficult to recover $>10^3$ transformants/µg of epPCR product. Given the phenomenon that multimeric plasmid has much higher (approximately three orders of magnitude) transformation frequency than that of monomeric plasmid in *B. subtilis* (Canosi et al. 1978), Shafikhani et al. (1997) generated large libraries of random mutants (3×10^6) in *B. subtilis* by PCR-based plasmid multimerization method. In this method, epPCR product was fused with linearized vector through PCR extension to generate linear plasmid multimer which could be converted to circular form through homologous recombination after entering cell; however, each cell may take several variants, decreasing screening efficiency. Increasing the number of competent cells is an effective method to create larger library. To this end, Zhang and Zhang et al. (2011) constructed a *B. subtilis* strain SCK6 whose competence can be induced by artificially controlling the expression of master regulator ComK. The procedure for preparing supercompetent cell of strain SCK6 was further improved by Li et al. (2017). The transformation frequencies of multimeric plasmid, monomeric plasmid and integration plasmid into strain SCK6 could reach 10^7, 10^4 and 10^5 transformants/µg DNA.

This study aims to construct large mutant library through inserting epPCR product into the chromosome of *B. subtilis* , which will solve the problems of plasmid instability and heterozygosity faced by multi-copy plasmid mediated strategies (Melnikov et al. 1999; You et al. 2012; Zhang and Zhang 2011). We accomplished the library construction within one day and the generated library had sufficient mutants ($>10^5$) for the need of direct evolution.

2 MATERIALS AND METHODS

2.1 Bacterial strains, primers and growth conditions

The *B. subtilis* SCK6 strain was provided by Daniel R. Zeigler from the Bacillus Genetic Stock Center. Oligonucleotide synthesis (Table 1) and DNA sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). *B. subtilis* strains were cultivated in Lysogeny Broth (LB) (Bertani et al. 2004) medium, YN medium (Li et al. 2017) or 2×Super-Rich (2×SR) (Song et al. 2017) medium. LB medium consists of 1% tryptone, 0.5% yeast extract and 0.5% NaCl. YN medium is composed of 0.7% yeast extract and 1.8% nutrient broth. 2×SR medium (3% tryptone, 5% yeast extract and 0.6% K_2HPO_4, pH 7.2) was used for fermentation. Solid medium was obtained by adding 15 g/L agar to the liquid medium. Unless otherwise indicated, the final concentrations of antibiotics were as follows (mg/L): zeocin (Zeo), 20; erythromycin (Em), 5. The inoculums (1%) were transferred into 250 mL flasks containing 30 mL 2×SR medium, and incubated at 37 with shaking at 200 rpm. Chlorpyrifos ($>99\%$) was purchased from the Macklin Biochemical Technology Co., Ltd. (Shanghai, China).

2.2 Construction of Methyl Parathion Hydrolase (MPH) secretion strain

Four DNA fragments of P_{cry3A} promoter (Adams et al. 1994), the coding region of signal peptide (SP_{aprE}), MPH-encoding gene *mpd* (Zhang et al. 2005), and T1T2 transcription terminator (Hartl et al. 2001) were fused by overlap PCR (Shevchuk et al. 2004) using the primers listed in Table 1, generating MPH expression cassette P_{cry3A} -*mpd* . The expression cassette was then fused with flanking regions of *amyE* and Zeo resistant marker (Zeo^R), producing insertion construct. Insertion construct was transformed into the competent cells of strain SCK6 and selected by Zeo. P_{cry3A} -*mpd* was finally integrated at the locus of *amyE* of strain SCK6, resulting in strain BPC1.

2.3 Generation of MPH mutant library

The right flanking region (RF) of *amyE* locus was amplified from strain BPC1 using primer pair P1/P5. Another fragment containing left flanking region (LF), Zeo^R and P_{cry3A} was amplified from strain BPC1 using primer pair P6/P2. These two fragments were fused by overlap PCR (Shevchuk et al. 2004) using primer pair P1/P2, resulting in fragment RF-LF-Zeo^R-P_{cry3A} . The cleavage site Sac I was introduced between RF and LF.

The coding region of SP$_{aprE}$ and MPH was amplified by error-prone PCR with primer pair P3/P4 using the Starmut Random Mutagenesis Kit (GenStar, Beijing, China). The PCR reaction solution with a total volume of 50 μL contained 0.02 ng/μL template, 25 μL 2×buffer, 3 μL StarMut Enhancer, 0.4 mM primer pair P3/P4. The PCR was conducted as follows: initial denaturation at 95 °C for 2 min and subsequent steps of 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min for 30 cycles total. The overlap of 47 bp between the left end of the epPCR product and the right end of RF-LF-Zeo^R-P_{cry3A} was introduced through primers P4 and P1, and the overlap of 45 bp between the right end of the epPCR product and the left end of RF-LF-Zeo^R-P_{cry3A} was introduced through primers P3 and P2.

To generate the multimer of fragment LF-Zeo^R-P_{cry3A} -*mpd* -RF, each 50 μL reaction system contained 0.2 mM dNTP, equal molar amount of RF-LF-Zeo^R-P_{cry3A} and epPCR product (~30 pmol for each fragment), and 0.04 U/μL Phusion polymerase without the addition of primers. The prolonged overlap extension PCR was conducted as follows: denaturation at 98 °C for 30 s; 30 cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C at a rate of 2 kb/min; followed by 72 °C extension for 10 min. The multimer product was digested with Sac I to release the monomer of fragment LF-Zeo^R-P_{cry3A} -*mpd* -RF which was transformed into the competent cells of strain SCK6 according to following procedure.

2.4 Transformation of *B. subtilis*

The *B. subtilis* SCK6 strain was inoculated into 4 ml of YN medium with 5 mg/L erythromycin in a test tube. The cells were cultivated at 37 °C with shaking at 220 rpm overnight (~12 h). The culture was diluted to an optical density at 600 nm (OD600) of 1.0 in a fresh YN medium containing 1.5 % (w/v) xylose and then was grown for 2 h. The resulting cell culture was ready to be transformed. 100 ng of DNA was mixed with different volumes of the supercompetent cells in a 1.5-ml eppendorf tube and cultivated at 37 °C with shaking at 220 rpm for 90 min.

2.5 Screening of the mutant library

Transformants were selected on LB plates containing Zeo and colonies were then transferred to LB plates containing 50 mg/L chlorpyrifos using sterile toothpick. Strain BPC1was used as control. After incubation at 37 for 12 h, the MPH activity of each colony was determined by the size of transparent halos. Mutants with larger halo were selected for further verification.

2.6 MPH purification and chlorpyrifos-hydrolyzing

activity assay

MPH purification was performed following the method described by Zhao et al. (2019). Briefly, the cells harboring MPH expression cassette were grown in 2xSR medium for 36 h at 37 . The supernatant of the cultures was used to purify C-terminal His-tagged MPH through Ni-NTA affinity chromatography according to the manufacturer's instructions (Sangon Biotech Co., Ltd., Shanghai, China). Protein concentration was measured by the BCA Kit (Shanghai, Sangon Biotech Co., Ltd., China). Target proteins were analyzed by SDS-PAGE and the gel was stained with Coomassie brilliant blue R250. The activity of MPH toward chlorpyrifos was measured as described previously (Xie et al. 2014).

2.7 Site-directed mutagenesis

Each mutation was introduced into wildtypeP_{cry3A} -*mpd* expression cassette through overlap PCR (Shevchuk et al. 2004). The genomic DNA of strain BPC1 was used as template and the primers were listed in Table 2. The PCR product was directly transformed into strain SCK6, generating the desired mutants.

3 RESULTS

3.1 Scheme for random mutagenesis by insertion of PCR products to the chromosome

As shown in Figure 1, error-prone PCR (epPCR) product is firstly assembled into an insertion construct consisting of left flanking region (LF), antibiotic resistant marker (Ab^R), epPCR product and right flanking region (RF); then, after the insertion construct is transformed into *B. subtilis* competent cells, epPCR product is inserted into the chromosome of *B. subtilis* through homologous recombination. Efficient assembly of insertion construct is the crucial step of this method. The plasmid multimerization method (Shafikhani et al. 1997; You et al. 2012) was employed to assemble insertion construct. First, LF, RF and Ab^R are fused together through overlap PCR (Shevchuk et al. 2004) generating fragment RF-LF-Ab$^{R.}$; notably, RF is put ahead of LF and a cleavage site of restriction enzyme is introduced between RF and LF. Gene of interest (GOI) is amplified by epPCR. The left end of GOI overlaps (40-50 bp) with the right end of RF-LF-Ab^R, while the right end of the GOI overlaps (40-50 bp) with the left end of RF-LF-Ab^R. Then, equal molar amount of GOI and RF-LF-Ab^R are mixed, the multimer of insertion construct (LF-Ab^R-GOI-RF) is generated by prolonged overlap extension PCR. Finally, the multimer is cut at the cleavage site between RF and LF, releasing the monomer of insertion construct.

3.2 Development of the random mutagenesis system

The above scheme was tested by direct evolution of Methyl Parathion Hydrolase (MPH) to improve its activity toward chlorpyrifos, a pesticide that often contaminates food and environment (Lu et al. 2013). To begin with, secretion expression of MPH was realized through inserting MPH expression cassette at the *amyE* locus of *B. subtilis* ; the transcription of *mpd* is driven by promoterP_{cry3A} and the secretion of MPH is mediated by signal-peptide of AprE (SP$_{AprE}$) (Figure 2a). Then, the coding region of SP$_{AprE}$ and MPH was amplified by epPCR, and another fragment of RF-cleavage site (Sac I)-LF-Zeo^R-P_{cry3A} was generated via overlap PCR. After multimerization, the product was converted to monomer of insertion construct (LF-Zeo^R-Pcry3A-mpd-RF) by Sac I digestion, which was confirmed by agarose gel electrophoresis. As shown in Figure 2b, neither of the two fragments (RF-LF-Zeo^R-P_{cry3A} and epPCR product) could be detected on gel after multimerization, indicating most of the two fragments were transformed to multimers. Sac I digestion product matched the size of insertion construct, which was further verified by DNA sequencing. To enhance the transformation efficiency of insertion construct, the amount of competent cell was increased for each transformation reaction. As shown in Figure 2c, when 400 μL competent cells were mixed with 100 ng DNA, the transformation efficiency was highest reaching 2.71×10^5 CFU/μg DNA. Additionally, the transformation efficiency was positive correlated with the length of flanking region. The flanking region of 0.5, 1 and 1.5-kb at each side led to the transformation efficiency of 1.24×10^5, 2.59×10^5 and 3.53×10^5 CFU/μg DNA (Figure 2d), respectively. The flanking region of 1 kb at each side of insertion construct was used to generate a library of *mpd*variants. Ten clones were randomly selected, and DNA sequencing result of SP$_{AprE}$ and MPH coding region shows that each mutant harbored 1-5 mutations, with an overall mutation rate of 0.31%.

3.3 Screening of the mutant library of MPH variants

As shown in Figure 3a, the mutants were grown on LB plate containing 50 mg/L chlorpyrifos. A transparent halo could form around the colony when chlorpyrifos is hydrolyzed by MPH (Xie et al. 2014). Enlarged halo indicated enhanced MPH activity, which may be caused by improved catalytic efficiency or increased MPH number. Two mutants, named MT1 and MT2, which formed significantly bigger transparent halos were screened from about 12000 colonies. When grown in liquid medium, the growth curves of both mutants were similar with that of strain BPC1. (data not shown), but the maximum extracellular MPH activity of mutants MT1 and MT2 were 2.47 and 2.77 fold that of strain BPC1 (Figure 3b), respectively.

3.4 Identification of the effect of each mutation on MPH

The SP$_{AprE}$ and MPH coding region of mutants MT1 and MT2 was sequenced. The mutation sites were shown in Figure 4a. Mutant MT1 contained three mutations, with two (T47C and G81T) located in SP$_{AprE}$ coding region and one (G938A) located in the MPH coding region. Among these three mutations, two led

to amino acid change (Leu16Ser and Arg313His), while G81T (Ala27Ala) is a synonymous mutation. All three mutations of mutant MT2 located in MPH coding region (T806A, C821T and C892T), resulting in the amino acid substitutions of Ile269Ser, Ala274Val and Pro298Ser.

To determine the contribution of each mutation to the enhanced extracellular MPH activity of mutants MT1 and MT2, each mutation was individually introduced into strain BPC1, generating mutants MT-16, MT-27, MT-269, MT-274, MT-298 and MT-313. All six mutations did not affect the growth of their hosts compared with strain BPC1 (data not shown). The extracellular MPH activity of mutants MT-16, MT-27, MT-269, MT-298 and MT-313 were 1.73, 1.40, 1.75, 1.48 and 2.03 fold that of strain BPC1, while mutant MT-274 showed negative effect on the extracellular MPH activity (Figure 4b). The extracellular MPH of mutants MT-16, MT-27, MT-269, MT-298 and MT-313 were purified through Ni-NTA affinity chromatography. The MPH protein yield of MT-269, MT-298 and MT-313 were close to that of strain BPC1, while the MPH protein yield of MT-16 and MT-27 were 1.70 and 1.46 fold that of strain BPC1 (Figure 4d). Moreover, the specific activity of variants T806A, C892T and G938A were 1.53, 1.43 and 1.52 fold of wild-type MPH, while the variants T47C and G81T were comparable with that of wild-type MPH (Figure 4c). Taken together, mutations T47C and G81T in the SP$_{AprE}$ coding region enhanced extracellular MPH activity through increasing MPH protein yield, while the contribution of mutations T806A, C892T and G938A was enhancing the catalytic activity of MPH against chlorpyrifos. Additionally, to further enhance the extracellular MPH activity, two new mutants were created through recombining the mutations. Mutant MT-C2 harbors mutations T47C and T806A, and mutant MT-C3 contains mutations of T47C, T806A and G938A. The extracellular MPH activities of mutants MT-C2 and MT-C3 were 2.78 and 3.32 fold that of strain BPC1 (Figure 4e and 4f), respectively, indicating that the contribution of these mutations could be accumulated when combined together.

4 DISCUSSION

In present work, to efficiently insert epPCR product into the chromosome of B. subtilis , we first employed a PCR based multimerization method to fuse epPCR product with flanking region and Ab^{R}; then, the insertion construct was transformed into supercompetent cells using a modified protocol, generating 3.53×10^5 transformations/μg DNA. The library could be created within one day and the library is large enough for the need of directed evolution (10^4-10^5 mutants are usually screened in directed evolution). Although the library generated here is smaller than those constructed by multimeric plasmid method (Li et al. 2017; Zhang and Sathitsuksanoh et al. 2011), our method solves the problems of plasmid instability and heterozygosity encountered in plasmid based method.

A crucial step in present method is the assembly of the insertion construct (LF-Ab^{R}-GOI-RF). We had attempted to fuse the three fragments (LF-Ab^{R}, GOI and RF) through PCR extension and Gibson assembly, but both methods resulted in low yield of insertion construct. On contrary, PCR based multimerization method (Shafikhani et al. 1997; You et al. 2012) realized efficient assembly of insertion construct (Figure 2d). Another key step is competent cell preparation and transformation. Strain SCK6 was grown in YN medium and induced by 1.5% xylose, which remarkably improves transformation efficiency as described by Li et al. (2017). We further demonstrated that increasing the amount of competent cell and the length of flanking region can enhance the transformation efficiency of the insertion construct (Figure 2c).

Plasmid or chromosome is used to carry expression cassette when producing a protein in B. subtilis . Plasmid mediated protein expression has the limitations of plasmid instability and safety concern (the use of Ab^{R}) (Bron et al. 1991). Chromosomal integration manner can overcome the limitations of plasmid manner, but low gene dose usually leads to low protein yield. Therefore, transcription and translation level of GOI need to be dramatically enhanced to achieve high protein yield in integration manner. Mutant MT1 harbors two mutations in SP$_{AprE}$ coding region, both of which could improve the yield of MPH (Figure 4b and 4d). Nijland et al. (2007) reported that a single amino acid change remarkably increased the secretion level of β-toxin. Therefore, the random mutagenesis strategy established here can find its application in improving protein secretion level in B. subtilis .

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

XY developed the project idea and revised the manuscript. BY performed most of the experiments, analyzed the data, and prepared the manuscript. YL, QT and XY did some data analysis, performed some experiments. MC provided consultation for the work and contributed significantly to the preparation of the manuscript. All authors reviewed the manuscript and agreed with the content.

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TABLE 1 Primers used in this study

Primers	Sequence (5' to 3')	Purpose
P1	tgaactttatctgagaatagtcaatcttcggaaatcccaggtggc	For the construction of multimer of insertion constru
P2	cattttcttcctccctttcttatcataatacataattttcaaactg	
P3	cagtttgaaaattatgtattatgataagaaagggaggaagaaaatg	
P4	gccacctgggatttccgaagattgactattctcagataaagttca	
P5	cagcgcaaatgctcccgctatcatcgagctccagcatccttgcagtcttcatatg	
P6	catatgaagactgcaaggatgctggagctcgatgatagcgggagcatttgcgctg	
P7	tttggaaagcgaggga	For the construction of variant T47C

Primers	Sequence (5' to 3')	Purpose
P8	ctgaacgccatcgtaaagattgacgttaacgcaaacaacaaacttatc	
P9	gataagtttgttgtttgcgttaacgtcaatctttacgatggcgttcag	
P10	cgttggttgtatccgtgt	
P11	cgcacctgcggtgctgcagcctgagcagacatgttgctgaacgcc	For the construction of variant G81T
P12	ggcgttcagcaacatgtctgctcaggctgcagcaccgcaggtgcg	
P13	gcggcggacttgccgtcgatgtcgagctgggtcgtgacgctggggtcgtc	For the construction of variant T806A
P14	gacgaccccagcgtcacgacccagctcgacatcgacggcaagtccgccgc	
P15	gccttcttgcgctccaccgcgacggacttgccgtcgatgtcgagc	For the construction of variant C821T
P16	gctcgacatcgacggcaagtccgtcgcggtggagcgcaagaaggc	
P17	gatgtggccgatgccggggaacgacaggtggctcgccgcgatcag	For the construction of variant C892T
P18	ctgatcgcggcgagccacctgtcgttccccggcatcggccacatc	
P19	gagtagttcaccggcacgaaatggtagcccttgccttcggcgc	For the construction of variant G938A
P20	gcgccgaaggcaagggctaccatttcgtgccggtgaactactc	

FIGURES LEGENDS

FIGUER 1 The scheme for insertion of epPCR products to the chromosome of *B. subtilis.* Firstly, fragment RF-LF-Ab^R is generated by overlap PCR and fragment GOI is amplified by error-prone PCR (epPCR). Secondly, the DNA multimer is formed by prolonged overlap extension PCR using fragments RF-LF-Ab^R and GOI. Thirdly, the DNA multimers is digested to monomer (LF-Ab^R-GOI-RF) at the cleavage site introduced between RF and LF. Finally, the insertion construct is transformed into competent cells. LF, left flanking region; Ab^R, antibiotic resistant marker; GOI, gene of interest; RF, right flanking region; scissors, restriction endonuclease digestion.

FIGUER 2 Development of the random mutagenesis system.(a) Construction of MPH secretion strain BPC1. PromoterP_{cry3A} controls transcription of *mpd* and the signal-peptide of AprE (SP$_{AprE}$) mediates the secretion of the Methyl Parathion Hydrolase (MPH). (b) Analysis of insertion construct assembly process by agarose gel electrophoresis. The arrow indicates the position of the target DNA. (c) The effect of competent cell number on transformation efficiency of insertion construct. The amount of the insertion construct was 100 ng and contained 1-kb flanking region. (d) The effect of flanking region size on transformation efficiency of insertion construct. The amount of the insertion construct was 100 ng and the volume of competent cells is 400 μL. All data were collected from at least three biological replicates and are shown as the mean ± SD. Bars indicated by the same letter are not significantly different (P > 0.05, evaluated by Duncan's test).

FIGUER 3 Screening of the library of MPH variants. (a) Screening of the library on LB plate containing 50 mg/L chlorpyrifos. Frist, transformants were selected on LB plates containing Zeocin. Then, the colonies were transferred to LB plates containing 50 mg/L chlorpyrifos using sterile toothpick. When chlorpyrifos are hydrolyzed by MPH, a transparent halo forms around the colony. (b) The extracellular activities of MPH in the supernatant of strain BPC1 and its mutants. All data were collected from at least three biological replicates and are shown as the mean ± SD.

FIGUER 4 Identification of the effect of each mutation on MPH . (a) Base mutations in *mpd* variants. The vertical line indicates the base mutations in the variants. (b) The extracellular activities of MPH in the supernatant of strain BPC1 and six mutants with single mutation. (c) Specific activity of wild-type MPH and the five variants with single mutation. (d) SDS-PAGE analysis of extracellular MPH in the supernatant of the strain BPC1 and its mutants. (e) SDS-PAGE analysis of extracellular MPH in the supernatant of the mutants MT-C2 and MT-C3. Mutant MT-C2 harbors mutations T47C and T806A, and mutant MT-C3 contains mutations of T47C, T806A and G938A. The strains were grown on 2×SR medium and incubated at 37°C with shaking at 200 rpm for 36 h. Equal amounts (20 μl) of culture supernatant were loaded into each lane. (f) The extracellular activities of MPH in the supernatant of strain BPC1, MT-C2 and

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MT-C3. M, protein markers. The arrow indicates the position of the target band. All data were collected from at least three biological replicates and are shown as the mean ± SD. Bars indicated by the same letter are not significantly different (P > 0.05, evaluated by Duncan's test).

(a)

(b)

(c)

(d)

(a)

(b)

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