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Construction of recombinant *Bacillus subtilis* strains for efficient pimelic acid synthesis

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Abstract As a precursor, pimelic acid plays an important role in biotin biosynthesis pathway of *Bacillus subtilis*. Fermentations supplemented with pimelic acid could improve the production of biotin, however, with a disadvantage-high cost. So it is necessary to improve the biosynthesis of pimelic acid via genetic engineering in *B. subtilis*. In this study, we constructed a recombinant *B. subtilis* strain for improving the synthesis of pimelic acid, in which a maltose-inducible P_{glv} promoter was inserted into the upstream of the cistron *bioI-orf2-orf3* and, meanwhile, flanked by the tandem cistrons via a single crossover event. The copy number of the integrant was amplified by high-concentration resistance screen and increased to 4-5 copies. The production of pimelic acid from multiple copies integrant was about 4 times higher than that from single copy (1017.13 μg/ml VS. 198.89 μg/ml). And when induced by maltose the production of pimelic acid was about 2 times of that under non-induction conditions (2360.73 μg/ml VS. 991.59 μg/ml). Thus, these results demonstrated that the production of pimelic acid was improved obviously through reconstructed *B. subtilis*. It also suggested that our expression system provided a convenient source of pimelic acid that would potentially lower the cost of production of biotin from engineered *B. subtilis*.

Keywords: *Bacillus subtilis*, biosynthesis, integration, P_{glv} promoter, pimelic acid, single crossover event

INTRODUCTION

Biotin is a sulfur-containing vitamin as a cofactor involved in central pathways in prokaryotic and eukaryotic metabolism, *i.e*. as the CO_2-carring prosthetic group of carboxylases, decarboxylases and trans carboxylases (Bower et al. 1996; Streit and Entcheva, 2003; Pirner and Stolz, 2006). The biotin biosynthesis in many prokaryotic microorganisms has been discovered and the genes involved in the biosynthesis pathway had been identified and cloned (Otsuka and Abelson, 1978; Gloeckler et al. 1990; Kiyasu et al. 2001). The genes involved in the conversion of pimelic acid to biotin are arranged in a single operon in the order *bioWAFDB* in *B. subtilis*. The pimelic acid is the known precursor of biotin biosynthesis in *B. subtilis* (Streit and Entcheva, 2003). The amount of its synthesis in natural strains could not meet the demand for industrial production of biotin. In the absence of added pimelic acid feeding, levels of biotin and biotin vitamins were greatly reduced. Adding pimelic acid in the fermentations of biotin can remarkably improve the production of the direct precursor dethiobiotin (Berkovitch et al. 2004).

Intriguing world market of biotin, in which humans and animals require several hundred micrograms of biotin per day, has been mainly supported by the chemically synthesized biotin so far (Streit and Entcheva, 2003). Since the chemical synthesis usually caused environmental burden, the biosynthesis of biotin became an attracted strategy. The well-characterized biotin biosynthesis pathway of most

microbes and the modern DNA recombinant techniques make it available to develop genetic engineering bacterium for biotin-overproduction (Streit and Entcheva, 2003; Lin et al. 2010; Cronan and Lin, 2011; Lin and Cronan, 2011). Thereby, much effort has been made and the recombinant strains over-producing biotin were obtained in *B. subtilis*, *Serratia marcescens* and *E. coli* (Sakurai et al. 1995; Van Arsdell et al. 2005). Although the biotin yields achieved are already close to an almost profitable amount, none of the genetic engineering recombinant strains really produces enough biotin to allow cost-effective production yet (Streit and Entcheva, 2003). Because the high cost raising by the addition of biotin precursors during the biotin fermentation processes limited the commercial application.

The pimelic acid was considered to be one of the preferred additives. The production of dethiobiotin in the feeding added the pimelic acid is about ten-fold higher than in the fermentation with no addition (Berkovitch et al. 2004). However, the addition of pimelic acid was linked with high cost and environmental burden. So constructing an efficient system to improve the biosynthesis of pimelic acid is one of the necessary strategies and pre conditions for biotin over-producing recombinant strains. Cytochrome P450BioI (CYP107H1) is believed to supply pimelic acid equivalents for biotin biosynthesis in *Bacillus subtilis* (Cryle and De Voss, 2004; Cryle and Schlichting, 2008). There are some reports showed that the pimelic acid was formed most likely by $P450_{BioI}$ from acyl ACPs by oxidative cleavage (Stok and De Voss 2000; Green et al. 2001). Further research indicated that in-chain cleavage of fatty acids or fatty acyl ACPs is the likely *in vivo* role of $P450_{BioI}$ (Cryle et al. 2003; Cryle and De Voss, 2004). Moreover, the crystal structure and some other details of the acyl-ACP BioI were investigated (Cryle and Schlichting, 2008; Cryle, 2010). Therefore, it is an economical and efficient method that improve the production of pimelic acid by high-expressed the BioI *in vivo*.

In this study, a resultant recombinant was obtained, in which the *bioI, orf2* and *orf3* were created a repeated copy and the P_{glv} was flanked by the three cistrons. The copy number of expression cassette inserted into the chromosome was amplified via selection stress of antibiotic, and the pimelic acid was produced in high level. Hence, we constructed an efficient system for improving the biosynthesis of pimelic acid in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonuclotides and DNA manipulation techniques

The bacterial strains and plasmids used in this study were listed in Table 1, and specific primers used in this study were listed in Table 2. The isolation and manipulation of recombinant DNA was performed with standard techniques (Sambrook and Russell, 2001). *B. subtilis* electrotransformation was performed as previously described (Yang et al. 2006).

Construction of integration vector and recombinant *Bacillus subtilis* strain

To construct the delivery vector, the chloramphenicol-resistance gene (Cm^R, 1.0 kb) and the cistrons *bioI-orf2-orf3* (2.8 kb) were PCR amplified from the pGJ103 (Yang et al. 2006; Zhang et al. 2007) and *B. subtilis* 168 chromosome DNA, using the primer pairs Cm-up/Cm-down and Cobio1-up/ Cobio2-down, respectively. The amplified Cm^R gene and the cistrons were cloned into pGEM-T vector respectively, resulting in pLHX1 and pLHX2. And then, the kpnI-ApaI-treated Cm^R gene was cloned into the corresponding sites of pE3 vector, yielding pLHX5. The ApaI-XbaI-treated P_{glv} promoter was cloned into corresponding sites of pLHX2, resulting in pYG57. The expression cassette of the cistrons, excised from pYG57 with ApaI and XbaI, was cloned into the corresponding sites of pLHX5, yielding pLHX8.

Construction of recombinant *Bacillus subtilis* strains

To facilitate single crossover event between the P_{glv}-*bioI-orf2-orf3* cassette and chromosomal DNA, *B. subtilis* PY79 was electro-transformed with 0.5 μg pLHX8 and the recombinants were selected on TBAB agar plate with 5 μg/ml chloramphenicol. Since the delivery vector could not self-replicate in *B. subtilis*, the colonies appeared on the screen plate should be integrants (Figure 1). The obtained recombinant was designed as BpLHX8.

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Description[a]	Source
E. coli		
DH5α	*F⁻φ80dlacZΔM15Δ* (*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *DeoR thi-1 phoA supE44 λ⁻ gyrA96 relA1*	Stored in this lab
B. subtilis		
PY79	*B. subtilis* 168 prototroph *SPβ⁻*	BGSC
BpLHX8	*B. subtilis* 168 (Δ*bioI-orf2-orf3*:: Amp-Cm-P*glv*)	This work
Plasmids		
pE3	Amp^R, *B. subtilis-E. coli* shuttle expression vector	Yang et al. 2006
pGJ103	Cm^R, *B. subtilis-E. coli* shuttle expression vector	Yang et al. 2006
pGJ113	Cm^R, P_{glv} promoter	Yang et al. 2006
pYG57	Amp^R, Plasmid containing P_{glv}-*bioI-orf2-orf3*	This work
pLHX1	PGEM-T vector with 1 kb PCR fragment containing Cm^R gene	This work
pLHX2	PGEM-T vector with a PCR fragment containing *bioI-orf3-orf3*	This work
pLHX5	Cm^R, pE3 vector containing a Cm^R gene	This work
pLHX8	Amp^R, Cm^R, *B. subtilis* P_{glv}-*bioI-orf3-orf3* integration vector	This work

[a]Amp^R: Ampicillin-resistance; Cm^R: chloramphenicol-resistance; BGSC:Bacillus Genetic Stock Center.

To examine the single crossover event between pLHX8 and chromosomal DNA, the BpLHX8 chromosomal DNA digested with EcoRI and KpnI was subjected to southern blot analysis by using the DIG-labeled Cm^R gene as probe. For further confirming that the single crossover event occurred as expected in BpLHX8, PCR assays were carried out by using of four primer pairs.

Because the repetition of *bioI-orf2-orf3* was created by the single crossover event in the recombinant *B. subtilis* BpLHX8, the copy-number amplification of the region flanked by the *bioI-orf2-orf3* in the BpLHX8 chromosome can be performed. For amplifying the P_{glv}-*bioI-orf2-orf3* cassette, BpLHX8 was incubated and screened in the culture medium with high concentration of chloramphenicol. The *B. subtilis* BpLHX8 was streaked repeatedly in TBAB medium with Cm^R concentrations up to 50 μg/ml, and the isolated resistance colon was, designed as BpLHX8-Cm_{50} (Van Arsdell et al. 2005). As a result, we obtained recombinant strain BpLHX8-Cm_{50}.

To examine the efficiency of pimelic acid synthesis in the reconstructed strains, the production of pimelic acid from BpLHX8 and BpLHX8-Cm_{50} were determined, respectively. The two strains were cultured in 2 x MSR medium without antibiotics respectively, while *B. subtilis* PY79 was used as negative control.

Table 2. Primers used in this study.

Primer	Sequences (5'→3')[a]	Restriction site
Cobio1-up	**TT**GAATTCGTGACAATTGCATCGTCAACTGC	*Eco*RI
Cobio2-down	**TT**TCTAGACCAATGGTTTGCTCAGGCGG	XbaI
Cm-up	**TT**GGGCCCTTATAAAAGCCAGTC	*Apa*I
Cm-down	**TT**GGTACCTTTGACAGCTTATCATC	*Kpn*I
Int1-up	**GG**ATTTTTCGCGTAGCGTCA	
Int1-down	CTGAAATGGGGTCGGGCA	
Int2-up	TGCCCGACCCCATTTCAG	
Int2-down	TTTTTGACAGCTTATCATC	
Int3-up	**TT**GGGCCCGGCATGTATCCGAATC	*Apa*I
Int3-down	**TT**TCTAGACCAATGGTTTGCTCAGGCGG	XbaI

[a] The additional sequence (lower case) and restriction site (underlined) are indicated.

Medium and cultivation

B. subtilis PY79, BpLHX8 and BpLHX8-Cm_{50} were cultured in 5 ml of TBAB (Sambrook and Russell, 2001) at 37ºC, respectively, with the appropriate concentration of Cm^R or not. Then 600 μl overnight culture was inoculated in 30 ml fresh 2 x MSR medium (5.0% yeast extract, 3.0% bactotrypton, 0.6% K_2HPO_4, 1.0% glucose) without antibiotics (Ye et al. 1999). For maltose induction, cells were grown to the cell density of 100 klett units and maltose was added to a final concentration of 5% (Yang et al. 2006). For different culture time after induction, the cells were harvested by centrifugation at 10000 g for 30 min, and the supernatants were filtrated with 0.45 μm MF-Millipore^{TM} Filters (Whatwan) for chromatography analysis.

Southern blot analysis

The chromosomal DNA was digested with *EcoR*I-*Xba*I and separated on a 0.8% BIOWEST agarose gel. The DNA was transferred with 0.4 M NaOH to a nylon filter (Hybond-N^+, Amersham) and fixed with UV light (Stratalinker UV cross-linker, Stratagene). Southern blot was performed with DIG DNA labeling and Detection Kit (Roche Diagnostics Corporation) according to the instructions provided by the suppliers. The copy number of cistrons of BpLHX8-Cm_{50} was determined using a DIG-labeled Cm resistance gene probe. In this experiment, 5x, 3x, 1x samples (the EcoRI-XbaI-treated BpLHX8 chromosomal DNA) were designed, respectively.

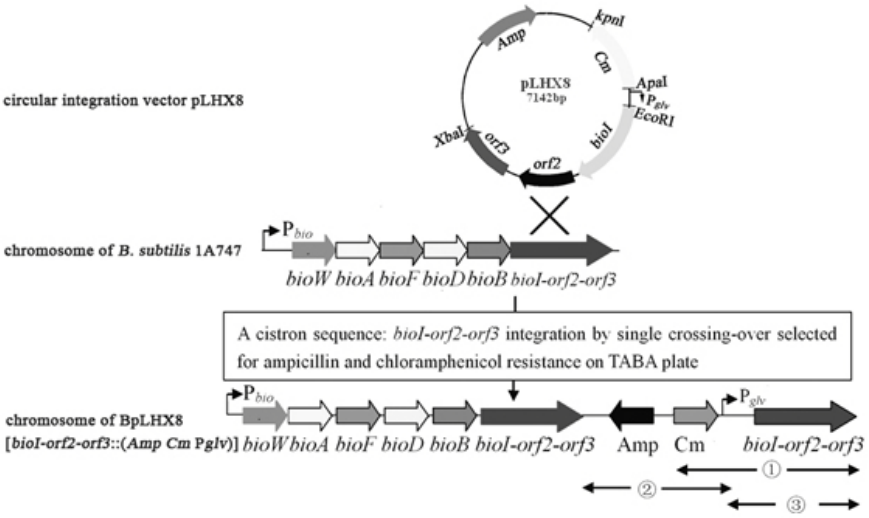

Fig. 1 Construction of BpLHX8 through a single crossover event between the delivery vector pLHX8 and *B. subtilis* PY79 chromosome. X indicates one crossover event. The numbers 1, 2 and 3 represent the integrons amplified by primers int1, int2, and int3, respectively.

RP-HPLC analysis

Reverse phase-high performance liquid chromatography (RP-HPLC) was used to determinate the production of pimelic acid in recombinant strains. RP-HPLC system was comprised of a LC-10A^{VP} pump (SHIMADZU), a SPD-10A^{VP} UV/VIS detector (SHIMADZU), a ValueChrom data acquisition system (Bio-Rad). The Zorbax SB-C18 column (5 μm, 4.6 x 250 mm, Agilent) was maintained at 45ºC with an injection volume of 10 μl, wavelength 210 nm. The mobile phase for determination of pimelic acid consisted of a mixture of 0.02M K_2HPO_4 (pH 3.0)-methanol (90:10, v/v) at a flow-rate of 1.3 ml/min.

RESULTS

Construction of integration vector

To introduce the strong promoter P_{glv} into the upstream of *bioI* and create a repeat of *bioI-orf2-orf3 in B. subtilis* chromosome DNA, the delivery vector pLHX8 was constructed, in which the *bioI-orf2-orf3* was under the control of P_{glv}. The chloromycetin resistance gene was employed as a selection marker in the delivery vector for the recombinant selection and copy number of gene amplification.

When the single crossover event between the delivery vector constructed in this study and the *B. subtilis* chromosome occurred using the *bioI-orf2-orf3* as homogenous arms, the entire delivery vector would be introduced into the *B. subtilis* chromosome and the repeated *bioI-orf2-orf3* would be generated.

Construction of recombinant strains

To reconstruct *B. subtilis* strains, the resultant delivery vector pLHX8 was used to transform the *B. subtilis* PY79 through a single crossover event, resulting in the Cm^R recombinant strain BpLHX8 (Figure 1). Then BpLHX8 chromosomal DNA was subjected to southern blot analysis. The results indicated that a 1 kb-size band generated from the *B. subtilis* BpLHX8 chromosomal DNA, whereas no band generated from *B. subtilis* PY79 chromosomal DNA (Figure 2). This confirmed that the pLHX8 was inserted into the *B. subtilis* PY79 chromosome. Further PCR assay showed that the single crossover event between the delivery vector and *B. subtilis* PY79 chromosomal DNA occurred in

accordance with our strategy and the P_{glv} promoter located upstream the *bioI-orf2-orf3*. As expected, the reconstructed strain was obtained (Figure 3).

The gradient of Cm^R (50 μg/ml) was used to increase the copy-number of cistrons in BpLHX8. To estimate the copy number of P_{glv}-*bioI-orf2-orf3* cassette, southern blot was preformed and the result (Figure 4a) suggested approximate 4-5 copies of P_{glv}-*bioI-orf2-orf3* cassette in BpLHX8-Cm_{50}. Additionally, the PCR determination also confirmed that the estimation of copy-number of target cassette in BpLHX8-Cm_{50} chromosome.

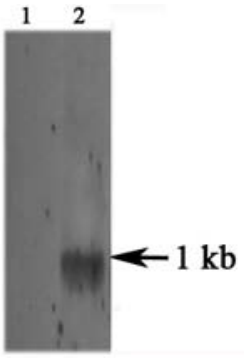

Fig. 2 Southern blot analysis of the chromosomal integration of pLHX8 using a DIG-labeled Cmresistance gene probe. The chromosomal DNA of the integrants and *B. subtilis* PY79 were digested with *Xba*I-*Eco*RI. The digested DNAs were then hybridized to a positively charged Nylon membrane and determinated using Digoxin (lane1, *B. subtilis* PY79; lanes 2, BpLHX8).

And the production of pimelic acid from BpLHX8-Cm_{50} was maximal (1017.13 μg/ml) at 24 hrs that was about 5 times higher than that from BpLHX8 (Figure 4b). Again, this result demonstrated the amplification of copy number of P_{glv}-*bioI-orf2-orf3* cassette contributed to the accumulation of pimelic acid. Whereas, no detectable-levels pimelic acid was shown in *B. subtilis* PY79. This probably resulted from the production of pimelic acid in *B. subtilis* PY79 is too low to detect for it beyond the sensibility of HPLC. The results mentioned above demonstrated that the production of pimelic acid from the reconstructed strains was increased significantly compared to the wild type.

In the recombined strains, BpLHX8 and BpLHX8-Cm_{50}, the *bioI-orf2-orf3* was under the control of maltose utilization operon promoter P_{glv}, of which the P_{glv} is an inducible promoter and is positive regulated by maltose. Thereby, to improve the production pimelic acid production from the BpLHX8-Cm_{50}, the effect of the maltose on pimelic acid production was investigated. The production of pimelic acid from BpLHX8- Cm_{50} in medium supplemented with maltose was higher than without it. The production of pimelic acid from BpLHX8-Cm_{50} with maltose-induction reached maximal at 24 hrs (Figure 4c) and was about 2.4 times higher than no induction (2360.73 μg/ml VS. 991.59 μg/ml). This suggested that maltose induction obviously improve the pimelic acid biosynthesis in the reconstructed strains.

DISCUSSION

Increasing the expression level of genes that involved in metabolite pathway is common approach to improve the accumulation of secondary metabolites. The usual strategy to achieve this aim is employing the strong promoter driven the target genes or increasing the copy-number of gene. The pimelic acid is an important precursor in biosynthesis pathway of biotin. Its accumulation can remarkably increase the production of the biotin. The *bioI*, *orf2* and *orf3* genes of *B. subtilis* were involved in pimelic acid biosynthesis pathway. Thus, we employed the strong promoter P_{glv} driven the *BioI-orf2-orf3* to enhance the gene expression level, and meanwhile, created a repetition of *BioI-orf2-orf3* via a single crossover event in *B. subtilis* chromosome that make the copy-number amplification of *BioI-orf2-orf3* available. The resultant recombinant demonstrated the accumulation of pimelic acid and again, increasing copy number enhanced the pimelic acid production significantly. Furthermore,

maltose induction doubled the production of pimelic acid. Thus, we reconstructed *B. subtilis* strains in this study for efficient production of pimelic acid through integration approach and high level production of pimelic acid was obtained. By using of the reconstructed *B. subtilis* strains, the pimelic acid will not need to be added in the biotin ferment. Moreover, the inducer of this system, maltose, is cheap and economical. Therefore, this method can reduce the industrial cost and environmental pollution significantly. Hence, this work supplies a potential and practical system which will play an important role in the biotin ferment.

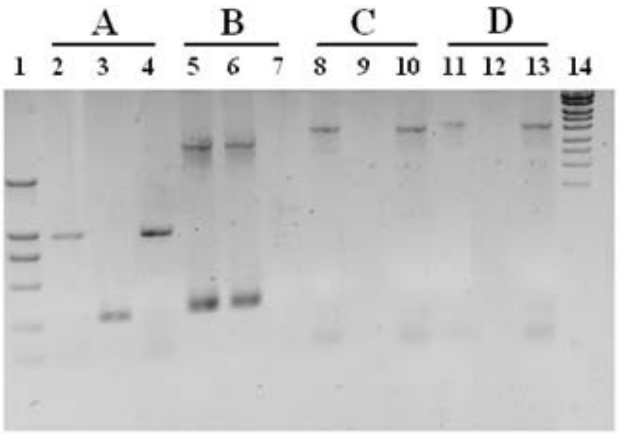

Fig. 3 PCR assay of the juxtaposition of the P_{glv} promoter and the [*biol-orf2-orf3*] cassette. Plasmids or *B. subtilis* chromosomal DNA was used as the template. Lane 1, DNA Marker (2.0, 1.5, 0.75, 0.5, 0.25, 0.1 kb, from the top to down) and lane 14, DNA Marker (10.0, 8.0, 6.0, 4.0, 3.0, 2.5, 2.0, 1.5 kb, from the top to down). (A) PCR amplified using the primer pair Cm-up/Cm-down (1.0 kb). Lane 2, pLHX1 (the positive control); lane 3, PY79 chromosome (the negative control); lane 4, BpLHX8. (B) PCR amplified using the primer pair int3-up/int3-down (3.0 kb). Lane 5, pLHX8 (the positive control); lane 6, BpLHX8; lane 7, PY79 chromosome (the negative control); (C) The primer pair int1-up/int1-down (4.0 kb). Lane 8, pLHX8 (the positive control); lane 9, PY79 chromosome (the negative control); lane 10, BpLHX8; (D) PCR amplified using PCR fragments with the primer pair int2-up/int2-down (4.2 kb). Lane 11, pLHX8 (the positive control); lane 12, PY79 chromosome (the negative control); lane 13, BpLHX8. The lands at about 100bp are primer dimmers.

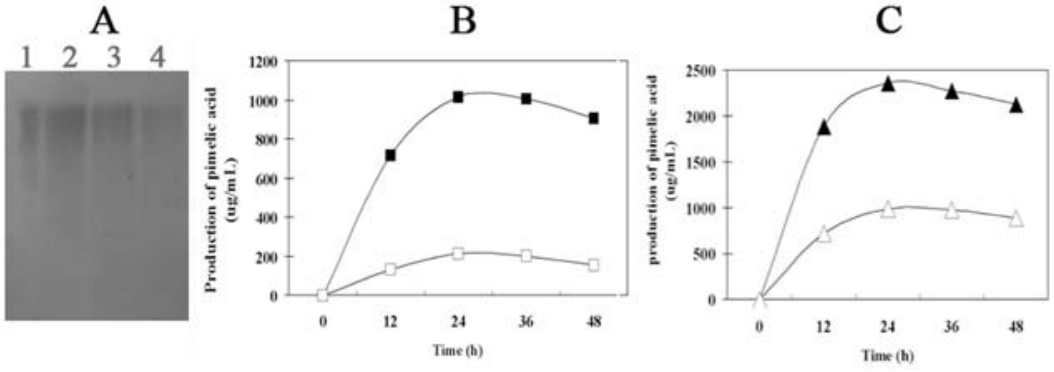

Fig. 4 (A) Southern blot analysis of the copy number of cistrons in integrant BpLHX8-Cm_{50} using a DIG-labeled Cm resistance gene probe. And the panels 2, 3, 4 represent 5x, 3x, 1x control samples (BpLHX8 chromosome DNA digested with *Xba*I-*Eco*RI). The panel 1 represents BpLHX8-Cm_{50}. Inclusion of the copy standards allowed us to calculate the approximate copy number of transgenes integrated for BpLHX8-Cm_{50} is increased to 4-5 copies. (B) The production of pimelic acid from BpLHX8 and BpLHX8-Cm_{50}. Solid square (■) and hollow square (□) represent pimelic acid production from BpLHX8-Cm_{50} and BpLHX8, respectively. (C) The production of pimelic acid from BpLHX8-Cm_{50} with maltose-induction. Solid triangle (▲) and hollow triangle (Δ) represent pimelic acid production from BpLHX8-Cm_{50} with maltose-induction and without maltose-induction, respectively.

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