
**RECOMBINANT PLASMID pZQ12, RECOMBINANT BACTERIUM FOR
SYNTHESIZING ODD AND EVEN CARBON CHAIN MONOMER
COPOLYMERIZED MCL-PHA AND APPLICATION THEREOF**

5 **TECHNICAL FIELD**

The present invention relates to the technical field of genetic engineering, and in particular, to a recombinant plasmid pZQ12, a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA and an application thereof.

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BACKGROUND

With the continuous consumption of petroleum fossil fuels and the increasing environmental pollution caused by petrochemical plastics, the development and synthesis of bio-based materials have attracted much attention. Polyhydroxyalkanoate (PHA), as a storage material for carbon and energy sources, is a kind of biopolyester synthesized by microorganisms. The PHA has been increasingly used as an environment-friendly material to replace conventional petroleum-based plastics due to its excellent properties such as biodegradability and biocompatibility. 3-hydroxyalkanoate (3HA), a composition monomer of PHA, is of a wide variety of types and has various properties. Because of the diversity of monomer structures of the PHA, the properties of materials produced thereby are also diversified (i.e., from rigid and brittle hard plastics to soft elastomers), and therefore, the PHA has broader application prospects than other biodegradable materials.

In general, medium-chain-length polyhydroxyalkanoate (mcl-PHA) contains 6-14 carbon atoms. The monomer structure and composition of conventional mcl-PHA are difficult to control, high in production cost, and low in industrial yield, and the existing mcl-PHA is single in type and limited in application range. Therefore, novel mcl-PHA applicable in the fields such as biomaterials, fine chemicals, medicine and nutrition need to be developed and synthesized urgently. In addition, mcl-PHA containing only even carbon chain monomers has shown ideal physical and mechanical properties, and if a component containing odd carbon chain monomers is added thereto, the biomaterial can be endowed with higher strength and flexibility, and thus becoming more novel and practical. However, currently synthesized mcl-PHA monomers containing odd carbon chain monomers include only 3HHp, 3HN, etc., which are short in carbon chain length, single in monomer type, expensive in carbon source for

synthesis, toxic to microbial cells, and unfavorable to cell growth and mcl-PHA synthesis and accumulation.

SUMMARY

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In view of the above, the present invention provides a recombinant plasmid pZQ12, and a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA. The recombinant bacterium can be fermented under the condition of using only glucose as a carbon source, to synthesize mcl-PHA in which odd and even carbon chain
10 monomers are copolymerized.

To achieve the above purpose, the present invention provides the following technical solutions.

In one aspect, the present invention provides a recombinant plasmid pZQ12, where a *cimA* gene is inserted into NcoI and BamHI multiple cloning sites of a plasmid pACYCDuet-1; a
15 *leuBCD* gene is inserted into BamHI and SacI multiple cloning sites of the plasmid pACYCDuet-1; an *ilvA* gene is inserted into SacI and AscI multiple cloning sites of the plasmid pACYCDuet-1; and a *thrA*BC* gene is inserted into the AscI multiple cloning site of the plasmid pACYCDuet-1;

the *cimA* gene has a nucleotide sequence represented by SEQ ID No. 1 in the sequence
20 table;

the *leuBCD* gene has a nucleotide sequence represented by SEQ ID No. 2 in the sequence table;

the *ilvA* gene has a nucleotide sequence represented by SEQ ID No. 3 in the sequence table.

25 the *thrA*BC* gene has a nucleotide sequence represented by SEQ ID No. 4 in the sequence table.

In another aspect, the present invention provides a preparation method for the above recombinant plasmid pZQ12, including the following steps:

1) performing double enzyme digestion on the plasmid pACYCDuet-1 and the *cimA* gene
30 by using NcoI and BamHI respectively, to obtain enzyme-digested pACYCDuet-1 and *cimA* gene;

2) ligating the enzyme-digested pACYCDuet-1 and *cimA* gene of step 1) to obtain recombinant plasmid pACYCDuet-1-*cimA*;

3) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-*cimA*

of step 2) and the *leuBCD* gene by using BamHI and SacI respectively, to obtain enzyme-digested pACYCDuet-1-cimA and *leuBCD* gene;

4) ligating the enzyme-digested pACYCDuet-1-cimA and *leuBCD* gene of step 3) to obtain recombinant plasmid pACYCDuet-1-cimA-leuBCD;

5) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-cimA-leuBCD of step 4) and the *ilvA* gene by using SacI and AscI respectively, to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD and *ilvA* gene;

6) ligating the enzyme-digested pACYCDuet-1-cimA-leuBCD and *ilvA* gene of step 5) to obtain recombinant plasmid pACYCDuet-1-cimA-leuBCD-ilvA;

7) performing single enzyme digestion on the recombinant plasmid pACYCDuet-1-cimA-leuBCD-ilvA of step 6) and the *thrA*BC* gene by using AscI respectively, to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD-ilvA and *thrA*BC* gene; and

8) ligating the enzyme-digested pACYCDuet-1-cimA-leuBCD-ilvA and *thrA*BC* gene of step 7) to obtain the recombinant plasmid pZQ12.

Preferably, the preparation method for the *cimA* gene in step 1) includes the following steps:

a. extracting total DNA of *Methanobacterium* sp. MB1; and

b. by using the total DNA of step a as a template, amplifying the *cimA* gene with primers cimA-F and cimA-R to obtain the *cimA* gene; where

the primer cimA-F has a nucleotide sequence represented by SEQ ID No. 5 in the sequence table; and

the primer cimA-R has a nucleotide sequence represented by SEQ ID No. 6 in the sequence table.

Preferably, the preparation method for the *leuBCD* gene in step 3) includes the following steps:

(1) extracting total DNA of *Escherichia coli* MG1655; and

(2) by using the total DNA of step (1) as a template, amplifying the *leuBCD* gene with primers leuBCD-F and leuBCD-R to obtain the *leuBCD* gene; where

the primer leuBCD-F has a nucleotide sequence represented by SEQ ID No. 7 in the sequence table; and

the primer leuBCD-R has a nucleotide sequence represented by SEQ ID No. 8 in the sequence table.

Preferably, the preparation method for the *ilvA* gene in step 5) includes the following steps:

[1] extracting total DNA of *Pseudomonas fluorescens* PICF7; and

[2] by using the total DNA of step [1] as a template, amplifying the *ilvA* gene with primers *ilvA*-F and *ilvA*-R to obtain the *ilvA* gene; where

the primer *ilvA*-F has a nucleotide sequence represented by SEQ ID No. 9 in the sequence table; and

5 the primer *ilvA*-R has a nucleotide sequence represented by SEQ ID No. 10 in the sequence table.

Preferably, amplification procedures for the amplification in steps b, (2), and [2] are independently: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all
10 experience 30 cycles; and final extension: 72°C, 10 min.

Preferably, the preparation method for the *thrA*BC* gene in step 7) includes the following steps:

(S1) extracting total DNA of *Escherichia coli* MDS42; and

(S2) by using the total DNA of step S1 as a template, performing amplification with
15 primers *thrA*-F1/*thrA*-R1 and *thrA*-F2/*thrA*-R2 respectively to obtain two fragments;

(S3) after mixing the two fragments obtained in step S2, by using the obtained mixed fragment as a template, performing amplification with primers *thrA*-F1 and *thrA*-R2 to obtain a *thrA** gene subjected to site-directed mutagenesis;

(S4) by using the total DNA of step S1 as a template, performing amplification with a
20 primer *thrBC*-F/*thrBC*-R to obtain a *thrBC* gene; and

(S5) by using the *thrA** gene of step S3 and the *thrBC* gene of step S4 as templates, performing amplification with a primer *thrA*BC*-F/*thrA*BC*-R to obtain the *thrA*BC* gene; where

the primer *thrA*-F1 has a nucleotide sequence represented by SEQ ID No. 11 in the
25 sequence table;

the primer *thrA*-R1 has a nucleotide sequence represented by SEQ ID No. 12 in the sequence table;

the primer *thrA*-F2 has a nucleotide sequence represented by SEQ ID No. 13 in the sequence table;

30 the primer *thrA*-R2 has a nucleotide sequence represented by SEQ ID No. 14 in the sequence table;

the primer *thrBC*-F has a nucleotide sequence represented by SEQ ID No. 15 in the sequence table;

the primer thrBC-R has a nucleotide sequence represented by SEQ ID No. 16 in the sequence table;

the primer thrA*BC-F has a nucleotide sequence represented by SEQ ID No. 17 in the sequence table; and

5 the primer thrA*BC-R has a nucleotide sequence represented by SEQ ID No. 18 in the sequence table.

In still another aspect, the present invention provides a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA, where the recombinant bacterium is prepared using the following method: transferring the above
10 recombinant plasmid pZQ12 and a recombinant plasmid pQQ05 into *Escherichia coli* LZ09;

the *Escherichia coli* LZ09 is obtained by knocking out acetic acid synthesis genes *ackA* and *poxB*, and a lactate synthesis gene *ldhA* from *Escherichia coli* LZ05.

In still yet another aspect, the present invention provides an application of the above recombinant bacterium in the synthesis of odd and even carbon chain monomer copolymerized
15 mcl-PHA.

The present invention provides a recombinant plasmid pZQ12, a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA and an application thereof. In the present invention, the recombinant plasmid pZQ12 includes: a *cimA* gene inserted into NcoI and BamHI multiple cloning sites of a plasmid pACYCDuet-1; a
20 *leuBCD* gene inserted into BamHI and SacI multiple cloning sites of the plasmid pACYCDuet-1; an *ilvA* gene inserted into SacI and AscI multiple cloning sites of the plasmid pACYCDuet-1; and a *thrA*BC* gene inserted into the AscI multiple cloning site of the plasmid pACYCDuet-1. By inserting the *cimA*, *leuBCD*, *ilvA*, and *thrA*BC* genes into particular multiple cloning sites of the plasmid pACYCDuet-1, the recombinant plasmid can overexpress
25 related enzyme genes and provide more precursor-propionyl coenzyme A for the synthesis of odd carbon chain monomers, thereby implementing the synthesis of the odd carbon chain monomers described in the present invention.

Further, the present invention provides a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA, where the recombinant bacterium is
30 obtained by transferring the recombinant plasmid pZQ12 described in the aforementioned solution and a recombinant plasmid pQQ05 into *Escherichia coli* LZ09. By knocking out acetic acid synthesis gene *ackA* and *poxB*, and a lactic acid synthesis gene *ldhA* in *Escherichia coli* LZ05, an LZ09 strain is constructed so as to inhibit the synthesis of by-products such as acetic acid and lactic acid, and the recombinant plasmid pQQ05 capable of synthesizing even

carbon chain monomers and extending carbon chain and the recombinant plasmid pZQ12 capable of synthesizing odd carbon chain monomers are transferred into the *Escherichia coli* LZ09, so that the recombinant bacterium can better use a carbon source to synthesize mcl-PHA in which odd and even carbon chain monomers are copolymerized. The results of the
5 embodiments show that the synthesis amount of the odd and even carbon chain monomer copolymerized mcl-PHA accounts for about 3.9% of the dry cell weight.

In addition, the recombinant bacterium provided by the present invention uses inexpensive and widely-sourced glucose as a sole carbon source, thus effectively avoiding the disadvantages, such as cytotoxicity and high price, of the carbon source applied in the
10 conventional synthesis method, and the newly synthesized mcl-PHA has a wide variety of carbon chain monomers, and covers the number of odd and even carbon atoms from 6 to 14.

BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 is an electrophoretogram of a *cimA* gene verified by PCR (where M represents Marker);

FIG. 2 is an electrophoretogram of a *leuBCD* gene verified by PCR (where M represents Marker);

20 FIG. 3 is an electrophoretogram of an *ilvA* gene verified by PCR (where M represents Marker);

FIG. 4 is an electrophoretogram of a knockout gene verified by PCR (where M represents Marker; FIG. 4-a is the verification of *ackA* gene knockout; FIG. 4-b is the verification of *poxB* gene knockout; and FIG. 4-c is the verification of *ldhA* gene knockout);

25 FIG. 5 is an electrophoretogram of a *thrA** gene verified by PCR (where M represents Marker); and

FIG. 6 is an electrophoretogram of a *thrBC* gene verified by PCR (where M represents Marker).

DETAILED DESCRIPTION

30 The present invention provides a recombinant plasmid pZQ12, where a *cimA* gene is inserted into NcoI and BamHI multiple cloning sites of a plasmid pACYCDuet-1; a *leuBCD* gene is inserted into BamHI and SacI multiple cloning sites of the plasmid pACYCDuet-1; an *ilvA* gene is inserted into SacI and AscI multiple cloning sites of the plasmid pACYCDuet-1;

and a *thrA*BC* gene is inserted into the *AscI* multiple cloning site of the plasmid pACYCDuet-1.

The *cimA* gene has a nucleotide sequence represented by SEQ ID No. 1 in the sequence table.

5 The *leuBCD* gene has a nucleotide sequence represented by SEQ ID No. 2 in the sequence table.

The *ilvA* gene has a nucleotide sequence represented by SEQ ID No. 3 in the sequence table.

10 The *thrA*BC* gene has a nucleotide sequence represented by SEQ ID No. 4 in the sequence table.

In the present invention, the *cimA* gene is a citramalate synthase gene, and the enzyme is used for synthesizing citramalic acid. In the present invention, the *cimA* gene is derived from *Methanobacterium* sp. MB1.

15 In the present invention, the *leuBCD* gene is used for synthesizing leucine. In the present invention, the *leuBCD* gene is derived from *Escherichia coli* (*Escherichia coli* MG1655).

In the present invention, the *ilvA* gene is a threonine dehydratase gene, and the function thereof is to perform threonine dehydration. In the present invention, the *ilvA* gene is derived from *Pseudomonas fluorescens* (*Pseudomonas fluorescens* PICF7).

In the present invention, the *thrA*BC* gene is used for synthesizing threonine.

20 The recombinant plasmid pZQ12 provided by the present invention can provide a precursor for synthesizing odd carbon chain monomers, thereby laying a foundation for further synthesis of medium-chain-length polyhydroxyalkanoate (mcl-PHA) in which odd and even carbon chain monomers are copolymerized.

25 The present invention provides a preparation method for the recombinant plasmid pZQ12 described in the aforementioned solution, including the following steps:

1) performing double enzyme digestion on the plasmid pACYCDuet-1 and the *cimA* gene by using *NcoI* and *BamHI* respectively, to obtain enzyme-digested pACYCDuet-1 and *cimA* gene;

30 2) ligating the enzyme-digested pACYCDuet-1 and *cimA* gene of step 1) to obtain recombinant plasmid pACYCDuet-1-*cimA*;

3) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-*cimA* of step 2) and the *leuBCD* gene by using *BamHI* and *SacI* respectively, to obtain enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene;

4) ligating the enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene of step 3) to

obtain recombinant plasmid pACYCDuet-1-cimA-leuBCD;

5) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-cimA-leuBCD of step 4) and the *ilvA* gene by using SacI and AscI respectively, to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD and *ilvA* gene;

6) ligating the enzyme-digested pACYCDuet-1-cimA-leuBCD and *ilvA* gene of step 5) to obtain recombinant plasmid pACYCDuet-1-cimA-leuBCD-*ilvA*;

7) performing single enzyme digestion on the recombinant plasmid pACYCDuet-1-cimA-leuBCD-*ilvA* of step 6) and the *thrA*BC* gene by using AscI respectively, to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD-*ilvA* and *thrA*BC* gene; and

8) ligating the enzyme-digested pACYCDuet-1-cimA-leuBCD-*ilvA* and *thrA*BC* gene of step 7) to obtain the recombinant plasmid pZQ12.

Double enzyme digestion is performed on the plasmid pACYCDuet-1 and the *cimA* gene by using NcoI and BamHI respectively, to obtain enzyme-digested pACYCDuet-1 and *cimA* gene. In the present invention, the preparation method for the *cimA* gene preferably includes the following steps:

a. extracting total DNA of *Methanobacterium sp.* MB1; and

b. by using the total DNA of step a as a template, amplifying the *cimA* gene with primers *cimA-F* and *cimA-R* to obtain the *cimA* gene; where

the primer *cimA-F* has a nucleotide sequence represented by SEQ ID No. 5 in the sequence table; and

the primer *cimA-R* has a nucleotide sequence represented by SEQ ID No. 6 in the sequence table.

In the present invention, the total DNA of the *Methanobacterium sp.* MB1 is preferably extracted. The present invention has no special limitation to the method for extracting the total DNA of the *Methanobacterium sp.* MB1, and a conventional DNA extraction method in the art can be used. In the embodiments of the present invention, a kit produced by TIANGEN is used for extraction.

In the present invention, after the total DNA of the *Methanobacterium sp.* MB1 is obtained, by using the total DNA of the *Methanobacterium sp.* MB1 as a template, the *cimA* gene is preferably amplified with primers *cimA-F* and *cimA-R* to obtain the *cimA* gene; the primer *cimA-F* has a nucleotide sequence represented by SEQ ID No. 5 in the sequence table; and the primer *cimA-R* has a nucleotide sequence represented by SEQ ID No. 6 in the sequence table. In the present invention, the amplification system for the amplification of the *cimA* gene is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL,

template: 1 μ L, cimA-F: 1 μ L, cimA-R: 1 μ L, HiFi DNA polymerase: 0.5 μ L, and ddH₂O: 37.5 μ L; and the total system is 50 μ L.

In the present invention, the amplification procedures for the amplification of the *cimA* gene are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

The present invention has no special limitation to the method for performing double enzyme digestion on the plasmid pACYCDuet-1 and the *cimA* gene by using NcoI and BamHI respectively, and a conventional enzyme digestion method in the art can be used.

In the present invention, after the enzyme-digested pACYCDuet-1 and *cimA* gene are obtained, the enzyme-digested pACYCDuet-1 and *cimA* gene are preferably ligated to obtain recombinant plasmid pACYCDuet-1-cimA. In the present invention, the ligation is preferably performed using T4 DNA ligase. In the present invention, the temperature of the ligation is preferably 25°C. The ligation system is preferably as follows: 10 $\times$ T4 DNA Ligase Buffer: 1 μ L, *cimA* gene fragment: 7 μ L, pACYCDuet-1: 1 μ L, T4 DNA Ligase: 0.5 μ L, and ddH₂O: 0.5 μ L; and the total system is 10 μ L.

In the present invention, after the recombinant plasmid pACYCDuet-1-cimA is obtained, double enzyme digestion is preferably performed on the recombinant plasmid pACYCDuet-1-cimA and the *leuBCD* gene by using BamHI and SacI respectively, to obtain enzyme-digested pACYCDuet-1-cimA and *leuBCD* gene. In the present invention, the preparation method for the *leuBCD* gene includes the following steps:

(1) extracting total DNA of *Escherichia coli* MG1655; and

(2) by using the total DNA of step (1) as a template, amplifying the *leuBCD* gene with primers leuBCD-F and leuBCD-R to obtain the *leuBCD* gene; where

the primer leuBCD-F has a nucleotide sequence represented by SEQ ID No. 7 in the sequence table; and

the primer leuBCD-R has a nucleotide sequence represented by SEQ ID No. 8 in the sequence table.

In the present invention, the total DNA of the *Escherichia coli* MG1655 is preferably extracted. The present invention has no special limitation to the method for extracting the total DNA of the *Escherichia coli* MG1655, and a conventional DNA extraction method in the art can be used. In the embodiments of the present invention, a kit produced by TIANGEN is used for extraction.

In the present invention, after the total DNA of the *Escherichia coli* MG1655 is obtained,

by using the total DNA of the *Escherichia coli* MG1655 as a template, the *leuBCD* gene is preferably amplified with primers leuBCD-F and leuBCD-R to obtain the *leuBCD* gene; the primer leuBCD-F has a nucleotide sequence represented by SEQ ID No. 7 in the sequence table; and the primer leuBCD-R has a nucleotide sequence represented by SEQ ID No. 8 in the sequence table. In the present invention, the amplification system for the amplification of the *leuBCD* gene is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL, leuBCD-F: 1 μL, leuBCD-R: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL.

In the present invention, the amplification procedures for the amplification of the *leuBCD* gene are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

The present invention has no special limitation to the method for performing double enzyme digestion on the plasmid pACYCDuet-1-cimA and the *leuBCD* gene by using BamHI and SacI respectively, and a conventional enzyme digestion method in the art can be used.

In the present invention, after the enzyme-digested pACYCDuet-1-cimA and *leuBCD* gene are obtained, the enzyme-digested pACYCDuet-1-cimA and *leuBCD* gene are preferably ligated to obtain recombinant plasmid pACYCDuet-1-cimA-leuBCD. In the present invention, the ligation is preferably performed using T4 DNA ligase. In the present invention, the temperature of the ligation is preferably 25°C. The ligation system is preferably as follows: 10×T4 DNA Ligase Buffer: 1 μL, *leuBCD* gene fragment: 7 μL, pACYCDuet-1-cimA: 1 μL, T4 DNA Ligase: 0.5 μL, and ddH₂O: 0.5 μL; and the total system is 10 μL.

In the present invention, after the recombinant plasmid pACYCDuet-1-cimA-leuBCD is obtained, double enzyme digestion is preferably performed on the recombinant plasmid pACYCDuet-1-cimA-leuBCD and the *ilvA* gene by using SacI and AscI respectively, to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD and *ilvA* gene. In the present invention, the preparation method for the *ilvA* gene preferably includes the following steps:

[1] extracting total DNA of *Pseudomonas fluorescens* PICF7; and

[2] by using the total DNA of step [1] as a template, amplifying the *ilvA* gene with primers *ilvA*-F and *ilvA*-R to obtain the *ilvA* gene; where

the primer *ilvA*-F has a nucleotide sequence represented by SEQ ID No. 9 in the sequence table; and

the primer *ilvA*-R has a nucleotide sequence represented by SEQ ID No. 10 in the sequence table.

In the present invention, the total DNA of the *Pseudomonas fluorescens* PICF7 is preferably extracted. The present invention has no special limitation to the method for extracting the total DNA of the *Pseudomonas fluorescens* PICF7, and a conventional DNA extraction method in the art can be used. In the embodiments of the present invention, a kit
5 produced by TIANGEN is used for extraction.

In the present invention, after the total DNA of the *Pseudomonas fluorescens* PICF7 is obtained, by using the total DNA of the *Pseudomonas fluorescens* PICF7 as a template, the *ilvA* gene is preferably amplified with primers *ilvA*-F and *ilvA*-R to obtain the *ilvA* gene; the primer *ilvA*-F has a nucleotide sequence represented by SEQ ID No. 9 in the sequence table; and the
10 primer *ilvA*-R has a nucleotide sequence represented by SEQ ID No. 10 in the sequence table.

In the present invention, the amplification system for the amplification of the *ilvA* gene is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL, *ilvA*-F: 1 μL, *ilvA*-R: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL. In the present invention, the amplification procedures for the
15 amplification of the *ilvA* gene are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

The present invention has no special limitation to the method for performing double
20 enzyme digestion on the plasmid pACYCDuet-1-*cimA*-*leuBCD* and the *ilvA* gene by using *Sac*I and *Asc*I respectively, and a conventional enzyme digestion method in the art can be used.

In the present invention, after the enzyme-digested pACYCDuet-1-*cimA*-*leuBCD* and *ilvA* gene are obtained, the enzyme-digested pACYCDuet-1-*cimA*-*leuBCD* and *ilvA* gene are preferably ligated to obtain recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD*-*ilvA*. In the
25 present invention, the ligation is preferably performed using T4 DNA ligase. In the present invention, the temperature of the ligation is preferably 25°C. The ligation system is preferably as follows: 10×T4 DNA Ligase Buffer: 1 μL, *ilvA* gene fragment: 7 μL, pACYCDuet-1-*cimA*-*leuBCD*: 1 μL, T4 DNA Ligase: 0.5 μL, and ddH₂O: 0.5 μL; and the total system is 10 μL.

In the present invention, after the recombinant plasmid
30 pACYCDuet-1-*cimA*-*leuBCD*-*ilvA* is obtained, single enzyme digestion is performed on the recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD*-*ilvA* and the *thrA***BC* gene by using *Asc*I respectively, to obtain enzyme-digested pACYCDuet-1-*cimA*-*leuBCD*-*ilvA* and *thrA***BC* gene.

In the present invention, the preparation method for the *thrA***BC* gene preferably includes

the following steps:

(S1) extracting total DNA of *Escherichia coli* MDS42; and

(S2) by using the total DNA of step S1 as a template, performing amplification with primers thrA-F1/thrA-R1 and thrA-F2/thrA-R2 respectively to obtain two fragments;

5 (S3) after mixing the two fragments obtained in step S2, by using the obtained mixed fragment as a template, performing amplification with primers thrA-F1 and thrA-R2 to obtain a *thrA** gene subjected to site-directed mutagenesis;

(S4) by using the total DNA of step S1 as a template, performing amplification with a primer thrBC-F/thrBC-R to obtain a *thrBC* gene; and

10 (S5) by using the *thrA** gene of step S3 and the *thrBC* gene of step S4 as templates, performing amplification with a primer thrA*BC-F/thrA*BC-R to obtain the *thrA*BC* gene; where

the primer thrA-F1 has a nucleotide sequence represented by SEQ ID No. 11 in the sequence table;

15 the primer thrA-R1 has a nucleotide sequence represented by SEQ ID No. 12 in the sequence table;

the primer thrA-F2 has a nucleotide sequence represented by SEQ ID No. 13 in the sequence table;

20 the primer thrA-R2 has a nucleotide sequence represented by SEQ ID No. 14 in the sequence table;

the primer thrBC-F has a nucleotide sequence represented by SEQ ID No. 15 in the sequence table;

the primer thrBC-R has a nucleotide sequence represented by SEQ ID No. 16 in the sequence table;

25 the primer thrA*BC-F has a nucleotide sequence represented by SEQ ID No. 17 in the sequence table; and

the primer thrA*BC-R has a nucleotide sequence represented by SEQ ID No. 18 in the sequence table.

30 In the present invention, the total DNA of the *Escherichia coli* MDS42 is preferably extracted. In the present invention, the *Escherichia coli* is preferably *Escherichia coli* MDS42. The present invention has no special limitation to the method for extracting the total DNA of the *Escherichia coli* MDS42, and a conventional DNA extraction method in the art can be used. In the embodiments of the present invention, a kit produced by TIANGEN is used for extraction.

In the present invention, after the total DNA of the *Escherichia coli* MDS42 is obtained, by using the total DNA of the *Escherichia coli* MDS42 as a template, amplification is preferably performed with primers thrA-F1/thrA-R1 and thrA-F2/thrA-R2 respectively to obtain two fragments; the primer thrA-F1 has a nucleotide sequence represented by SEQ ID No. 11 in the sequence table; the primer thrA-R1 has a nucleotide sequence represented by SEQ ID No. 12 in the sequence table; the primer thrA-F2 has a nucleotide sequence represented by SEQ ID No. 13 in the sequence table; and the primer thrA-R2 has a nucleotide sequence represented by SEQ ID No. 14 in the sequence table.

In the present invention, the amplification system for the amplification using the primers thrA-F1/thrA-R1 and thrA-F2/thrA-R2 is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL, thrA-F1 or thrA-F2: 1 μL, thrA-R1 or thrA-R2: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL.

In the present invention, the amplification procedures for the amplification using the primers thrA-F1/thrA-R1 and thrA-F2/thrA-R2 are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

In the present invention, after the two fragments are obtained, the two fragments are mixed, and by using the obtained mixed fragment as a template, amplification is performed with primers thrA-F1 and thrA-R2 to obtain a *thrA** gene subjected to site-directed mutagenesis. The site-directed mutagenesis is to perform site-directed mutagenesis at position 799 of the *thrA* gene of the *Escherichia coli* MDS42, and replaces C with T.

In the present invention, by using the total DNA of the *Escherichia coli* MDS42 as a template, amplification is preferably performed with a primer thrBC-F/thrBC-R to obtain the *thrBC* gene. The primer thrBC-F has a nucleotide sequence represented by SEQ ID No. 15 in the sequence table; and the primer thrBC-R has a nucleotide sequence represented by SEQ ID No. 16 in the sequence table.

In the present invention, the amplification system for the amplification of the *thrBC* gene is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL, thrBC-F: 1 μL, thrBC-R: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL. In the present invention, the amplification procedures for the amplification of the *thrBC* gene are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min,

where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

In the present invention, after the *thrA** gene and the *thrBC* gene are obtained, by using the *thrA** gene and the *thrBC* gene as templates, amplification is preferably performed with a primer *thrA*BC-F/thrA*BC-R* to obtain the *thrA*BC* gene. The primer *thrA*BC-F* has a nucleotide sequence represented by SEQ ID No. 17 in the sequence table; and the primer *thrA*BC-R* has a nucleotide sequence represented by SEQ ID No. 18 in the sequence table.

In the present invention, the amplification system for the amplification of the *thrA*BC* gene is preferably as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL, *thrA*BC-F*: 1 μL, *thrA*BC-R*: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL. In the present invention, the amplification procedures for the amplification of the *cimA* gene are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min.

The present invention has no special limitation to the method for performing single enzyme digestion on the plasmid pACYCDuet-1-*cimA-leuBCD-ilvA* and the *thrA*BC* gene by using *AscI* respectively, and a conventional enzyme digestion method in the art can be used.

In the present invention, after the enzyme-digested pACYCDuet-1-*cimA-leuBCD-ilvA* and *thrA*BC* gene are obtained, the enzyme-digested pACYCDuet-1-*cimA-leuBCD-ilvA* and *thrA*BC* gene are ligated to obtain the recombinant plasmid pZQ12. In the present invention, the ligation is preferably performed using T4 DNA ligase. In the present invention, the temperature of the ligation is preferably 25°C. The ligation system is preferably as follows: 10×T4 DNA Ligase Buffer: 1 μL, *thrA*BC* gene fragment: 6 μL, pACYCDuet-1-*cimA-leuBCD-ilvA*: 2 μL, T4 DNA Ligase: 0.5 μL, and ddH₂O: 0.5 μL; and the total system is 10 μL.

The present invention provides a recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA, where the recombinant bacterium is prepared using the following method: transferring the recombinant plasmid pZQ12 described in the aforementioned solution and a recombinant plasmid pQQ05 into *Escherichia coli* LZ09; the *Escherichia coli* LZ09 is obtained by knocking out acetic acid synthesis genes *ackA* and *poxB*, and a lactate synthesis gene *ldhA* from *Escherichia coli* LZ05.

In the present invention, the *Escherichia coli* LZ05 and the recombinant plasmid pQQ05 are preferably prepared using a method (Synthesis of polyhydroxyalkanoates from glucose that

contain medium-chain-length monomers via the reversed fatty acid β -oxidation cycle in *Escherichia coli*, Zhuang, et al., 2014(24): 78-86).

The present invention has no special limitation to the specific method for knocking out the acetic acid synthesis genes *ackA* and *poxB*, and the lactate synthesis gene *ldhA*, and a conventional method in the art can be used. In the present invention, the specific process of knocking out the acetic acid synthesis genes *ackA* and *poxB*, and the lactate synthesis gene *ldhA* includes: designing upstream and downstream primers of the acetic acid synthesis genes *ackA* and *poxB*, and the lactate synthesis gene *ldhA*, and amplifying homologous recombinant fragments with corresponding resistant genes by using plasmid pKD3 or pKD4 as the template. Electrotransformed competent cells are prepared, and the homologous recombinant fragments with corresponding resistance genes are transferred into the prepared electrotransformed competent cells through an electrotransformation process, the knocked-out strains are then screened and identified, and the electrotransformed helper plasmids and corresponding resistance genes are knocked out.

In the present invention, the primers for knocking out the acetic acid synthesis gene *ackA* are preferably *ackA*-pKD4-F and *ackA*-pKD4-R; the primer *ackA*-pKD4-F has a nucleotide sequence represented by SEQ ID No. 19 in the sequence table; and the primer *ackA*-pKD4-R has a nucleotide sequence represented by SEQ ID No. 20 in the sequence table.

In the present invention, the primers for knocking out *poxB* are preferably *poxB*-pKD3-F and *poxB*-pKD3-R; the primer *poxB*-pKD3-F has a nucleotide sequence represented by SEQ ID No. 21 in the sequence table; and the primer *poxB*-pKD3-R has a nucleotide sequence represented by SEQ ID No. 22 in the sequence table.

In the present invention, the primers for knocking out the acetic acid synthesis gene *ldhA* are preferably *ldhA*-pKD4-F and *ldhA*-pKD4-R; the primer *ldhA*-pKD4-F has a nucleotide sequence represented by SEQ ID No. 23 in the sequence table; and the primer *ldhA*-pKD4-R has a nucleotide sequence represented by SEQ ID No. 24 in the sequence table. In the present invention, the amplification system for the amplification of the homologous recombinant fragments with corresponding resistance genes is preferably as follows: 10 $\times$ HiFi DNA polymerase buffer: 5 μ L, dNTPs (2.5 mM): 4 μ L, template: 1 μ L, upstream primer: 1 μ L, downstream primer: 1 μ L, HiFi DNA polymerase: 0.5 μ L, and ddH₂O: 37.5 μ L; and the total system is 50 μ L. In the present invention, the amplification procedures for the amplification of the homologous recombinant fragments with corresponding resistance genes are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience

30 cycles; and final extension: 72°C, 10 min.

In the present invention, the knockout of the acetic acid synthesis genes *ackA* and *poxB*, and the lactic acid synthesis gene *ldhA* can inhibit the synthesis of by-products such as acetic acid and lactic acid, so that the carbon source is maximally used in the synthesis of the odd and even carbon chain monomer copolymerized mcl-PHA.

In the present invention, after knocking out the acetic acid synthetic genes *ackA* and *poxB*, and the lactic acid synthetic gene *ldhA*, PCR is preferably used to verify whether the genes are knocked out; the primers for the PCR identification are preferably *ackA*-F, *ackA*-R, *poxB*-F, *poxB*-R, *ldhA*-F, and *ldhA*-R; the *ackA*-F has a nucleotide sequence represented by SEQ ID No. 25 in the sequence table; the *ackA*-R has a nucleotide sequence represented by SEQ ID No. 26 in the sequence table; the *poxB*-F has a nucleotide sequence represented by SEQ ID No. 27 in the sequence table; the *poxB*-R has a nucleotide sequence represented by SEQ ID No. 28 in the sequence table; the *ldhA*-F has a nucleotide sequence represented by SEQ ID No. 29 in the sequence table; and the *ldhA*-R has a nucleotide sequence represented by SEQ ID No. 30 in the sequence table.

The present invention provides an application of the recombinant bacterium described in the aforementioned solution in the synthesis of odd and even carbon chain monomer copolymerized mcl-PHA. In the present invention, the specific method for synthesizing the odd and even carbon chain monomer copolymerized mcl-PHA preferably includes: streaking the recombinant bacterium on a plate, picking up single clones, and inoculating the single clones into LB media (the specific components are: Tryptone: 10 g, Yeast Extract: 5 g, and NaCl: 10 g; distilled water is added to 1000 mL; the pH value is 7.0; the LB media are sterilized at 121°C for 20 min before use) for culture at 36-38°C to a logarithmic growth phase to obtain a seed solution, then inoculating the seed solution at an inoculation amount of 5% into a fermentation medium (the LB medium) with a liquid volume of 100 mL/500 mL for shake flask culture at 28-31°C and 240-260 rpm for 70-75 h; after the fermentation, centrifuging the fermentation broth to obtain bacteria for synthesizing the odd and even carbon chain monomer copolymerized mcl-PHA, and after freeze-drying the bacteria, taking 15-30 mg of dry powder of the bacteria for monomer composition and content detection of the subsequent product.

The technical solutions in the present invention are clearly and completely described below with reference to the embodiments of the present invention. Apparently, the described embodiments are merely some rather than all of the embodiments of the present invention. All other embodiments obtained by a person of ordinary skill in the art based on the embodiments of the present invention without creative efforts shall fall within the scope of projection of the

present invention.

Embodiment 1

a. Total DNA of *Methanobacterium* sp. MB1 is extracted;

b. by using the total DNA of step a as a template, a *cimA* gene is amplified with primers
5 *cimA*-F and *cimA*-R (the amplification system is as follows: 10×HiFi DNA polymerase buffer:
5 µL, dNTPs (2.5 mM): 4 µL, template: 1 µL, *cimA*-F: 1 µL, *cimA*-R: 1 µL, HiFi DNA
polymerase: 0.5 µL, and ddH₂O: 37.5 µL; and the total system is 50 µL). The amplification
procedures are as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s;
annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and
10 extension all experience 30 cycles; and final extension: 72°C, 10 min, so that a *cimA* gene is
obtained. PCR is used for verification, and the specific result is shown in FIG. 1. As can be
seen from FIG. 1, a *cimA* gene band appears, i.e., the *cimA* gene is obtained.

(1) Total DNA of *Escherichia coli* (*Escherichia coli* MG1655) is extracted;

(2) by using the total DNA of step (1) as a template, a *leuBCD* gene is amplified with
15 primers *leuBCD*-F and *leuBCD*-R (the amplification system is as follows: 10×HiFi DNA
polymerase buffer: 5 µL, dNTPs (2.5 mM): 4 µL, template: 1 µL, *leuBCD*-F: 1 µL, *leuBCD*-R:
1 µL, HiFi DNA polymerase: 0.5 µL, and ddH₂O: 37.5 µL; and the total system is 50 µL). The
amplification procedures are as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation:
94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation,
20 annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min, so that a
leuBCD gene is obtained. PCR is used for verification, and the specific result is shown in FIG.
2. As can be seen from FIG. 2, a *leuBCD* gene band appears, i.e., the *leuBCD* gene is obtained.

[1] Total DNA of *Pseudomonas fluorescens* (*Pseudomonas fluorescens* PICF7) is
extracted;

[2] by using the total DNA of step [1] as a template, an *ilvA* gene is amplified with primers
25 *ilvA*-F and *ilvA*-R. The amplification system is as follows: 10×HiFi DNA polymerase buffer: 5
µL, dNTPs (2.5 mM): 4 µL, template: 1 µL, *ilvA*-F: 1 µL, *ilvA*-R: 1 µL, HiFi DNA polymerase:
0.5 µL, and ddH₂O: 37.5 µL; and the total system is 50 µL. The amplification procedures are as
follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C,
30 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience
30 cycles; and final extension: 72°C, 10 min, so that an *ilvA* gene is obtained. PCR is used for
verification, and the specific result is shown in FIG. 3. As can be seen from FIG. 3, an *ilvA* gene
band appears, i.e., the *ilvA* gene is obtained.

1) Double enzyme digestion is performed on a plasmid pACYCDuet-1 and the *cimA* gene

by using NcoI and BamHI respectively, to obtain enzyme-digested pACYCDuet-1 and *cimA* gene;

2) the enzyme-digested pACYCDuet-1 and *cimA* gene are ligated using T4 DNA Ligase to obtain recombinant plasmid pACYCDuet-1-*cimA*;

5 3) double enzyme digestion is performed on the recombinant plasmid pACYCDuet-1-*cimA* and the *leuBCD* gene by using BamHI and SacI respectively, to obtain enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene;

4) the enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene are ligated using the T4 DNA Ligase to obtain recombinant plasmid pACYCDpACYCDuet-1-*cimA-leuBCD*;

10 5) double enzyme digestion is performed on the recombinant plasmid pACYCDuet-1-*cimA-leuBCD* and the *ilvA* gene by using SacI and AscI respectively, to obtain enzyme-digested pACYCDuet-1-*cimA-leuBCD* and *ilvA* gene;

6) the enzyme-digested pACYCDuet-1-*cimA-leuBCD* and *ilvA* gene are ligated using the T4 DNA Ligase to obtain recombinant plasmid pACYCDuet-1-*cimA-leuBCD-ilvA*.

15 **Embodiment 2**

By using plasmid pKD3 or pKD4 as a template, homologous recombinant fragments with corresponding resistance genes are amplified with primers *ackA*-pKD4-F, *ackA*-pKD4-R, *poxB*-pKD3-F, *poxB*-pKD3-R, *ldhA*-pKD4-F, and *ldhA*-pKD4-R. Electrotransformed competent cells are prepared, an electrotransformation process is performed, and strains from
20 which the genes are knocked out are screened to obtain an LZ09 strain. The obtained LZ09 strain is verified by PCR (the primers for the PCR verification are *ackA*-F, *ackA*-R, *poxB*-F, *poxB*-R, *ldhA*-F, and *ldhA*-R). The specific result is shown in FIG. 4. As can be seen from FIG. 4, there is no electrophoresis band in a, b, and c, indicating that the genes have been knocked out.

25 **Embodiment 3**

(S1) Total DNA of *Escherichia coli* (*Escherichia coli* MDS42) is extracted;

(S2) by using the total DNA of step S1 as a template, amplification is performed with primers *thrA*-F1/*thrA*-R1 and *thrA*-F2/*thrA*-R2 respectively. The amplification system is as follows: 10×HiFi DNA polymerase buffer: 5 μL, dNTPs (2.5 mM): 4 μL, template: 1 μL,
30 *thrA*-F1 or *thrA*-F2: 1 μL, *thrA*-R1 or *thrA*-R2: 1 μL, HiFi DNA polymerase: 0.5 μL, and ddH₂O: 37.5 μL; and the total system is 50 μL.

The amplification procedures are preferably as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10

min, so that two fragments are obtained;

(S3) after mixing the two fragments obtained in step S2, by using the obtained mixed fragment as a template, amplification is performed with primers thrA-F1 and thrA-R2 to obtain a *thrA** gene subjected to site-directed mutagenesis (PCR is used for verification, and the specific result is shown in FIG. 5. As can be seen from FIG. 5, a *thrA** gene band appears, i.e., the *thrA** gene is obtained);

(S4) by using the total DNA of step S1 as a template, amplification is performed with a primer thrBC-F/thrBC-R to obtain a *thrBC* gene (the amplification system is as follows: 10×HiFi DNA polymerase buffer: 5 µL, dNTPs (2.5 mM): 4 µL, template: 1 µL, thrBC-F: 1 µL, thrBC-R: 1 µL, HiFi DNA polymerase: 0.5 µL, and ddH₂O: 37.5 µL; and the total system is 50 µL). The amplification procedures for the amplification of the *thrBC* gene are as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min. PCR is used for verification, and the specific result is shown in FIG. 6. As can be seen from FIG. 6, a *thrBC* gene band appears, i.e., the *thrBC* gene is obtained;

(S5) by using the *thrA** gene of step S3 and the *thrBC* gene of step S4 as templates, amplification is performed with a primer thrA*BC-F/thrA*BC-R (the amplification system is preferably as follows: 10×HiFi DNA polymerase buffer: 5 µL, dNTPs (2.5 mM): 4 µL, template: 1 µL, thrA*BC-F: 1 µL, thrA*BC-R: 1 µL, HiFi DNA polymerase: 0.5 µL, and ddH₂O: 37.5 µL; and the total system is 50 µL. The amplification procedures are as follows: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, where the denaturation, annealing, and extension all experience 30 cycles; and final extension: 72°C, 10 min, so that a *thrA*BC* gene is obtained.

The recombinant plasmid pACYCDuet-1-cimA-leuBCD-ilvA of Embodiment 1 and the *thrA*BC* gene obtained above are enzyme-digested using AscI respectively, and the enzyme-digested pACYCDuet-1-cimA-leuBCD-ilvA and *thrA*BC* gene are ligated using T4 DNA ligase to obtain the recombinant plasmid pZQ12.

Embodiment 4

The recombinant plasmid pZQ12 obtained in Embodiment 3 and a recombinant plasmid pQQ05 are transferred into *Escherichia coli* LZ09 to obtain a recombinant bacterium. The recombinant bacterium is streaked on a plate, a single clone is picked up and added to a flask containing an LB medium for culture at 37°C to a logarithmic growth phase to obtain a seed solution, the seed liquid is then inoculated at an inoculation amount of 5% into a

fermentation medium (the LB medium) with the liquid volume of 100 mL/500 mL for fermentation at 30°C and 250 rpm for 72 h; a fermentation broth is collected and centrifuged at 5000 rpm for 15 min, and after the centrifugation, supernatant and bacteria precipitate are separated. The bacteria are freeze-dried and 20 mg of dry powder of the bacteria is taken for methylation for 1 h. After cooling to room temperature, 1 mL of double distilled water is added to extract cell debris in the aqueous phase. 10 mg·ml⁻¹ of pentadecanoic acid is dissolved in ethanol and added as an internal standard. The mixed solution is vortexed and centrifuged at 12,000 rpm for 10 min. After standing for layer separation, 500 µL of organic (chloroform) phase is transferred into a new GC detection bottle. Afterwards, sample analysis is performed by gas chromatography and the content and monomer composition of mcl-PHA in the bacteria are analyzed by GC detection.

GC analysis procedure settings are shown in Table 1:

Table 1: GC analysis procedure settings

column temperature	retention time
80°C	1 min
raising the temperature to 230°C at a rate of 10°C/min	10 min

Table 2: monomer composition and content of mcl-PHA in bacteria

plasmid	pQQ05, pZQ12
dry cell weight (g/L)	5.16 ± 0.53
mcl-PHA content (wt%)	3.92 ± 0.17
3HHx (mol%)	0.37
3HHp (mol%)	5.01
3HO (mol%)	31.75
3HN (mol%)	1.96
3HD (mol%)	32.56
3HUD (mol%)	1.23
3HDD (mol%)	19.77
3HTRD (mol%)	1.06
3HTD (mol%)	6.29

Note: 3HHx, C6 monomer; 3HHp, C7 monomer; 3HO, C8 monomer; 3HN, C9 monomer; 3HD, C10 monomer; 3HUD, C11 monomer; 3HDD, C12 monomer; 3HTRD, C13 monomer; 3HTD, C14 monomer.

As can be seen from Table 2, the synthesis amount of the odd and even carbon chain

monomer copolymerized mcl-PHA is about 3.9% of the dry cell weight, i.e., 317 mg/L (shake flask culture), and the obtained copolymer is rich in odd carbon chain monomers and has a complex structure. The experimental results show that the recombinant bacterium prepared by the present invention can utilize only glucose as a carbon source for fermentation to synthesize mcl-PHA in which odd and even carbon chain monomers are copolymerized.

The foregoing descriptions are only preferred implementation manners of the present invention. It should be noted that for a person of ordinary skill in the art, several improvements and modifications may further be made without departing from the principle of the present invention. These improvements and modifications should also be deemed as falling within the protection scope of the present invention.

Sequence Table

<110> Qilu University of Technology

<120> RECOMBINANT PLASMID pZQ12, RECOMBINANT BACTERIUM FOR
SYNTHESIZING ODD AND EVEN CARBON CHAIN MONOMER COPOLYMERIZED

5 MCL-PHA AND APPLICATION THEREOF

<141> 2018-12-29

<160> 30

<170> SIPOSequenceListing 1.0

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10 <211> 1500

<212> DNA

<213> 2 *Ambystoma laterale* x *Ambystoma jeffersonianum*

<400> 1

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What is claimed is:

1. A recombinant plasmid pZQ12, wherein a *cimA* gene is inserted into NcoI and BamHI multiple cloning sites of a plasmid pACYCDuet-1; a *leuBCD* gene is inserted into BamHI and
5 SacI multiple cloning sites of the plasmid pACYCDuet-1; an *ilvA* gene is inserted into SacI and AscI multiple cloning sites of the plasmid pACYCDuet-1; and a *thrA*BC* gene is inserted into the AscI multiple cloning site of the plasmid pACYCDuet-1;

the *cimA* gene has a nucleotide sequence represented by SEQ ID No. 1 in the sequence table;

10 the *leuBCD* gene has a nucleotide sequence represented by SEQ ID No. 2 in the sequence table;

the *ilvA* gene has a nucleotide sequence represented by SEQ ID No. 3 in the sequence table.

the *thrA*BC* gene has a nucleotide sequence represented by SEQ ID No. 4 in the sequence
15 table.

2. A preparation method for the recombinant plasmid pZQ12 according to claim 1, comprising the following steps:

1) performing double enzyme digestion on the plasmid pACYCDuet-1 and the *cimA* gene by using NcoI and BamHI respectively, to obtain enzyme-digested pACYCDuet-1 and *cimA*
20 gene;

2) ligating the enzyme-digested pACYCDuet-1 and *cimA* gene of step 1) to obtain recombinant plasmid pACYCDuet-1-*cimA*;

3) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-*cimA* of step 2) and the *leuBCD* gene by using BamHI and SacI respectively, to obtain
25 enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene;

4) ligating the enzyme-digested pACYCDuet-1-*cimA* and *leuBCD* gene of step 3) to obtain recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD*;

5) performing double enzyme digestion on the recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD* of step 4) and the *ilvA* gene by using SacI and AscI respectively,
30 to obtain enzyme-digested pACYCDuet-1-*cimA*-*leuBCD* and *ilvA* gene;

6) ligating the enzyme-digested pACYCDuet-1-*cimA*-*leuBCD* and *ilvA* gene of step 5) to obtain recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD*-*ilvA*;

7) performing single enzyme digestion on the recombinant plasmid pACYCDuet-1-*cimA*-*leuBCD*-*ilvA* of step 6) and the *thrA*BC* gene by using AscI respectively,

to obtain enzyme-digested pACYCDuet-1-cimA-leuBCD-ilvA and *thrA*BC* gene; and

8) ligating the enzyme-digested pACYCDuet-1-cimA-leuBCD-ilvA and *thrA*BC* gene of step 7) to obtain the recombinant plasmid pZQ12.

3. The preparation method according to claim 2, wherein the preparation method for the *cimA* gene in step 1) comprises the following steps:

a. extracting total DNA of *Methanobacterium* sp. MB1; and

b. by using the total DNA of step a as a template, amplifying the *cimA* gene with primers *cimA*-F and *cimA*-R to obtain the *cimA* gene; wherein

the primer *cimA*-F has a nucleotide sequence represented by SEQ ID No. 5 in the sequence table; and

the primer *cimA*-R has a nucleotide sequence represented by SEQ ID No. 6 in the sequence table.

4. The preparation method according to claim 2, wherein the preparation method for the *leuBCD* gene in step 3) comprises the following steps:

(1) extracting total DNA of *Escherichia coli* MG1655; and

(2) by using the total DNA of step (1) as a template, amplifying the *leuBCD* gene with primers *leuBCD*-F and *leuBCD*-R to obtain the *leuBCD* gene; wherein

the primer *leuBCD*-F has a nucleotide sequence represented by SEQ ID No. 7 in the sequence table; and

the primer *leuBCD*-R has a nucleotide sequence represented by SEQ ID No. 8 in the sequence table.

5. The preparation method according to claim 2, wherein the preparation method for the *ilvA* gene in step 5) comprises the following steps:

[1] extracting total DNA of *Pseudomonas fluorescens* PICF7; and

[2] by using the total DNA of step [1] as a template, amplifying the *ilvA* gene with primers *ilvA*-F and *ilvA*-R to obtain the *ilvA* gene; wherein

the primer *ilvA*-F has a nucleotide sequence represented by SEQ ID No. 9 in the sequence table; and

the primer *ilvA*-R has a nucleotide sequence represented by SEQ ID No. 10 in the sequence table.

6. The preparation method according to any one of claims 3-5, wherein amplification procedures for the amplification in steps b, (2), and [2] are independently: pre-denaturation: 94°C, 5 min, 1 cycle; denaturation: 94°C, 45 s; annealing: 55-60°C, 45 s; extension: 72°C, 2 min, wherein the denaturation, annealing, and extension all experience 30 cycles; and final

extension: 72°C, 10 min.

7. The preparation method according to claim 2, wherein the preparation method for the *thrA*BC* gene in step 7) comprises the following steps:

(S1) extracting total DNA of *Escherichia coli* MDS42; and

5 (S2) by using the total DNA of step S1 as a template, performing amplification with primers *thrA*-F1/*thrA*-R1 and *thrA*-F2/*thrA*-R2 respectively to obtain two fragments;

(S3) after mixing the two fragments obtained in step S2, by using the obtained mixed fragment as a template, performing amplification with primers *thrA*-F1 and *thrA*-R2 to obtain a *thrA** gene subjected to site-directed mutagenesis;

10 (S4) by using the total DNA of step S1 as a template, performing amplification with a primer *thrBC*-F/*thrBC*-R to obtain a *thrBC* gene; and

(S5) by using the *thrA** gene of step S3 and the *thrBC* gene of step S4 as templates, performing amplification with a primer *thrA*BC*-F/*thrA*BC*-R to obtain the *thrA*BC* gene; wherein

15 the primer *thrA*-F1 has a nucleotide sequence represented by SEQ ID No. 11 in the sequence table;

the primer *thrA*-R1 has a nucleotide sequence represented by SEQ ID No. 12 in the sequence table;

20 the primer *thrA*-F2 has a nucleotide sequence represented by SEQ ID No. 13 in the sequence table;

the primer *thrA*-R2 has a nucleotide sequence represented by SEQ ID No. 14 in the sequence table;

the primer *thrBC*-F has a nucleotide sequence represented by SEQ ID No. 15 in the sequence table;

25 the primer *thrBC*-R has a nucleotide sequence represented by SEQ ID No. 16 in the sequence table;

the primer *thrA*BC*-F has a nucleotide sequence represented by SEQ ID No. 17 in the sequence table; and

30 the primer *thrA*BC*-R has a nucleotide sequence represented by SEQ ID No. 18 in the sequence table.

8. A recombinant bacterium for synthesizing odd and even carbon chain monomer copolymerized mcl-PHA, wherein the recombinant bacterium is prepared using the following method: transferring the recombinant plasmid pZQ12 according to any one of claims 1-7 and a

recombinant plasmid pQQ05 into *Escherichia coli* LZ09;

the *Escherichia coli* LZ09 is obtained by knocking out acetic acid synthesis genes *ackA* and *poxB*, and a lactate synthesis gene *ldhA* from *Escherichia coli* LZ05.

9. An application of the recombinant bacterium according to claim 8 in the synthesis of
5 odd and even carbon chain monomer copolymerized mcl-PHA.