High-specificity synthesis of novel monomers by remodeled alcohol hydroxylase

Yanning Zheng¹, Lingling Li¹,², Qiang Liu¹,², Haibo Zhang¹, Yujin Cao¹, Mo Xian* and Huizhou Liu*¹

Abstract

Background: Diols are important monomers for the production of plastics and polyurethanes, which are widely used in our daily life. The medium-chain diols with one hydroxyl group at its subterminal end are able to confer more flexibility upon the synthesized materials. But unfortunately, this type of diols has not been synthesized so far. The strong need for advanced materials impelled us to develop a new strategy for the production of these novel diols. In this study, we use the remodeled P450BM3 for high-specificity production of 1,7-decanediol.

Results: The native P450BM3 was capable of converting medium-chain alcohols into corresponding α,ω1-, α,ω2- and α,ω3-diols, with each of them accounting for about one third of the total diols, but it exhibited a little or no activity on the short-chain alcohols. Greatly improved regiospecificity of alcohol hydroxylation was obtained by laboratory evolution of P450BM3. After substitution of 12 amino acid residues (J2-F87A), the ratio of 1,7-decanediol (∑ω3 hydroxylation) to total decanediols increased to 86.8% from 34.0%. Structure modeling and site-directed mutagenesis demonstrated that the heme end residues such as Ala78, Phe87 and Arg255 play a key role in controlling the regioselectivity of the alcohol hydroxylation, while the residues at the mouth of substrate binding site is not responsible for the regioselectivity.

Conclusions: Herein we employ an engineered P450BM3 for the first time to enable the high-specificity biosynthesis of 1,7-decanediol, which is a promising monomer for the development of advanced materials. Several key amino acid residues that control the regioselectivity of alcohol hydroxylation were identified, providing some new insights into how to improve the regiospecificity of alcohol hydroxylation. This report not only provides a good strategy for the biosynthesis of 1,7-decanediol, but also gives a promising approach for the production of other useful diols.

Keywords: P450BM3, Alcohol hydroxylation, Escherichia coli, Diols, 1,7-decanediol, Regiospecificity

Abbreviations: E. coli, Escherichia coli; GC-MS, Gas chromatography-mass spectrometry; IPTG, L-3-0-propyl β-D-thiogalactoside; Kan, Kanamycin; PCR, Polymerase chain reaction.

Background

Diols are of great importance in the manufacture of plastics and polyurethanes, which have molded our society in many ways that make our life much easier [1]. The biosynthesis of short-chain diols such as 1,3-propanediol and 1,4-butanediol has been well developed [2–5], while the long-chain α,ω-diols such as 1,14-tetradecanediol and 1,16-hexadecanediol can be made by the catalytic hydrogenation of long-chain dicarboxylate esters. The medium-chain diols with one subterminal hydroxyl group, for example, 1,7-decanediol, which confer more flexibility upon the synthesized materials, are promising monomers for the synthesis of polymers with better properties. But unfortunately, this type of diols has not yet been synthesized with either chemical or biological method. The strong market demand for new polymers drives us to develop a strategy for the synthesis of these diols.

To obtain the carbon backbones, we paid attention to the straight-chain fatty alcohols, whose biosynthesis has...
been well developed in recent years [6–8]. To finally get the desired diols, an additional hydroxyl group needs to be added to the subterminal carbon of the monohydric alcohols. But it is too difficult for the inorganic catalysts to catalyze the oxidation of a specific subterminal carbon of the monohydric alcohols, as each of the subterminal carbons almost has the same chemical contexts, and the inorganic catalysts usually exhibit poor selectivity. So the biocatalyst was considered as a priority. In seeking such a biocatalyst for the conversion of monohydric alcohols to corresponding diols, P450BM3 seems to be a promising enzyme, which naturally catalyzes the conversion of long-chain fatty acids to corresponding hydroxyfatty acids [9, 10]. P450BM3 is highly soluble in cytosolic environment, and has high catalytic rates and expression level in engineered E. coli, which is the most widely used host for the bioproduction of chemicals [11, 12]. These advantages make it an ideal biocatalyst for biotechnological application. And more importantly, its variants have shown to be able to utilize a wide range of other substrates. The P450BM3 variant 35-E11, whose 17 amino acid residues were substituted, was found to be capable of converting ethane to ethanol [11], and a series of P450BM3 variants were reported the improved activities on non-natural substrates naphthalene, pentane, p-cymene and propylbenzene [13]. These findings suggest that P450BM3 has stunning flexibility in substrate preference. In addition, protein engineering has been widely used as a strategy in the biological production of chemicals [14, 15]. Therefore, an improved regioselectivity for alcohol hydroxylation at ω-3 position can be expected by remodeled P450BM3 (Fig. 1).

In this study, we are reporting a strategy for high-specificity synthesis of α, ω3-diols from renewable medium-chain fatty alcohols. To improve the regioselectivity for alcohol hydroxylation at ω-3 position, we generated a series of P450BM3 variants by laboratory evolution. We also identified several key amino acid residues that control the regioselectivity of alcohol hydroxylation, and discussed the reason why these residues play a key role in determining the regioselectivity by structural analysis.

**Results and discussion**

**Substrate specificity and regioselectivity of P450BM3**

The P450BM3 was chosen as the starting enzyme for the conversion of alcohols to diols. So the engineered E. coli that overexpresses the native P450BM3 was constructed as the biocatalyst to test P450BM3’s ability of alcohol hydroxylation. The heptanol and decanol were firstly used as the substrates for testing the activity of P450BM3, as the medium chain diols are of more interests for the development of new polymers. P450BM3 exhibited high activities towards these substrates and produced almost an equivalent amount of α, ω1-, α, ω2- and α, ω3-diols, exhibiting the same regioselectivity as its native fatty acid substrates (Figs. 2 and 3). But when using pentanol as the substrate, only a tiny amount of 1,4-pentanediol

---

**Fig. 1** Schematic representation of the substrate specificities and regioselectivities of the native P450BM3 and the remodeled P450BM3. The native P450BM3 has a substrate preference for C12-C18 fatty acids and evenly oxidizes the ω-1, ω-2 and ω-3 carbons of the fatty acid substrates (a), while the remodeled P450BM3 exhibits a greatly improved regiospecificity of decanol hydroxylation, with 1,7-decanediol as the dominant product (b).
was obtained (Fig. 2), suggesting P450\textsubscript{BM3} has a different regioselectivity for short-chain alcohols. No activity was observed when using propanol and butanol as the substrates.

As the typical substrates of P450\textsubscript{BM3} are long-chain fatty acids [16], it is not surprising that the short-chain fatty alcohols are poor substrates for P450\textsubscript{BM3}. Arg\textsuperscript{47} and Tyr\textsuperscript{51} were thought to interact with the carboxylate group of the fatty acid substrates [17, 18]. Our finding that P450\textsubscript{BM3} is also capable of utilizing fatty alcohols demonstrates that the interaction between Arg\textsuperscript{47}/Tyr\textsuperscript{51} and carboxylate group is not so strong, and Arg\textsuperscript{47}/Tyr\textsuperscript{51} is not involved in determining the substrate specificity of P450\textsubscript{BM3}. If Arg\textsuperscript{47}/Tyr\textsuperscript{51} is responsible for stabilizing the carboxylate group of the fatty acid substrates, the carboxylate group needs to be recognized by the two amino acid residues, and P450\textsubscript{BM3} will not be able to oxidize those hydrocarbons without carboxylate group. Therefore, the size of the substrate-binding pocket plays an important role in determining the substrate specificity of P450\textsubscript{BM3}, given that the P450\textsubscript{BM3} has no activity towards short-chain alcohols and alkanes [19].

**Substrate specificity and regioselectivity of P450\textsubscript{BM3}\textsuperscript{J}**

For the high-specificity production of the α, ω3-diols, the native P450\textsubscript{BM3} needs to be modified to change its
regioselectivity for alcohol hydroxylation. The regioselectivity is connected with the substrate orientation, which could be changed when the substrate channel of P450\textsubscript{BM3} is constrained. It was found that a P450\textsubscript{BM3} variant (P450\textsubscript{BM3J}), which contains 10 amino acid substitutions (V78A, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V) with respect to the native P450\textsubscript{BM3}, changed its substrate preferences for shorter alkanes (C4-C8) when compared with the native P450\textsubscript{BM3}, which exhibited a substrate preference for C\textsubscript{n} > 8 alkanes [19]. It could be expected that P450\textsubscript{BM3J} will have a higher priority for \(\omega\)-3 hydroxylation when using a longer-chain alcohol substrate. So we generated another engineered E. coli BM3J that overexpressed P450\textsubscript{BM3J}. The same as BM3, BM3J had no activity on propanol and butanol, and exhibited a low activity on pentanol, with 1,4-pentanediol as the only product (Fig. 2). But when using heptanol and decanol as substrates, BM3J produced more 1,4-heptanediol and 1,7-decanediol than BM3, with 1,4-heptanediol and 1,7-decanediol accounting for 50.1 and 64.5 \% of total heptanediols and decanediols, respectively (Figs. 2 and 3).

To examine if the increased \(\omega\)-3 hydroxylation is mainly attributed to a specific amino acid substitution, we made 10 single mutants that correspond to the 10 amino acid substitutions of P450\textsubscript{BM3J}, namely, V78A, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V and L353V. The ratio of 1,7-decanediol to total decanediols increased from 34.0 \% in BM3 to \(~50\ %\) in R255S, suggesting the increased ratio of 1,7-decanediol to total decanediols in BM3J is mainly attributed to the substitution of Arg\textsuperscript{255} to Ser\textsuperscript{255}. The other 9 amino acid substitutions may have a combined effect on the constriction of the substrate-binding channel and the change of the substrate orientation (Fig. 4b).

The effect of heme end residues on the regioselectivity of P450\textsubscript{BM3J}
To further improve the regioselectivity of P450\textsubscript{BM3J}, we need to further constrict the substrate-binding channel. The Val\textsuperscript{78} in P450\textsubscript{BM3} is located near the \(\omega\) terminus of fatty acid substrates, so it may be vital in controlling the substrate orientation. Fatty alcohol substrates should have a quite similar substrate orientation in the active site to the fatty acid substrates, which can be reflected by the regioselectivity for heptanol and decanol hydroxylation. The substitution of Val\textsuperscript{78} in P450\textsubscript{BM3} to Ala\textsuperscript{78} in P450\textsubscript{V78A} nearly does not change the regioselectivity for decanol, as alanine has a similar property to valine. But when the Ala\textsuperscript{78} in P450\textsubscript{BM3J} was substituted to Phe\textsuperscript{78}, yielding a variant J2, the ratio of 1,7-decanediol (\(\omega\)-3 hydroxylation) to total decanediols further increased to 76.3 \% from 64.5 \% in BM3J (Fig. 5). The phenyl group of Phe\textsuperscript{78} creates a narrower space between Phe\textsuperscript{78} and the heme. This conformational change forced the carbon chain bend, made the \(\omega\), \(\omega\)-1 and \(\omega\)-2 terminal carbons move farther away from the heme, and finally resulted in the decrease of undesired \(\omega\)-1 and \(\omega\)-2 hydroxylation (Fig. 4c). Therefore, the residues located around the heme end of the substrate-binding channel indeed play more important roles in determining the regioselectivity.
of the alcohol hydroxylases for medium-chain alcohol substrates.

To test this hypothesis, two residues, Arg\textsuperscript{47} and Phe\textsuperscript{87}, were chosen for further modification. Arg\textsuperscript{47} is located at the mouth of substrate binding site and its guanidinium group is thought to provide an important ion-pair interaction with the carboxylate group of the fatty acid substrates [20], while Phe\textsuperscript{87} is located above the heme and is known to be an important factor in determining the regioselectivity of substrate hydroxylation [21]. The substitution of Arg\textsuperscript{47} to Leu\textsuperscript{47} was found to increase the hydroxylase activity towards pentane and propylbenzene [13]. The R47L mutation was then incorporated into the variant J2, generating a new variant J2-R47L. As expected, the regiospecificity profile of J2-R47L was nearly the same as that of J2 (Fig. 5), demonstrating the substitution of Arg\textsuperscript{47} to Leu\textsuperscript{47} did not change the substrate orientation and had little connection with the regioselectivity for alcohol substrates. But when the Phe\textsuperscript{87} was substituted to Ala\textsuperscript{87} (J2-F87A), the ratio of 1,7-decanedioi to total decanediols further increased to 86.8 % (Fig. 5). The substitution of Phe\textsuperscript{87} to Ala\textsuperscript{87}
released the space for the α-terminus of decanol to move towards the heme, and in the meanwhile made the benzene ring of Phe \(^{78}\) rotate away from the ω terminus of decanol (Fig. 4d). This incident allowed the ω, ω-1 and ω-2 terminal carbons of decanol to further move away from the heme, leading to the increased distribution of 1,7-decanediol. The heme end residues are responsible for contraction or expansion of the hydrophobic pocket, so they can affect the alcohol orientation in the substrate-binding channel and finally control the regio-selectivity of the alcohol hydroxylation.

Conclusions
The engineering strategy described above inaugurates a new realm for the high-specificity production of 1,7-decanediol, which is a promising monomer for the development of advanced materials. The desired 1,7-decanediol was finally produced, being the first successful report on the biosynthesis of diols with one hydroxyl group at the subterminus. The regiospecificity of alcohol hydroxylation was greatly improved by laboratory evolution. Conservative structural models of the P450 \(_{BM3}\) variants demonstrate that the heme end residues in the substrate-binding channel play a key role in determining the regioselectivity for medium-chain alcohols. This study not only provides a good strategy for the biosynthesis of 1,7-decanediol, but also gives a promising approach for the production of other useful diols. More and more advanced materials can be expected once these new diol monomers are available.

Methods
Plasmid construction
The P450 \(_{BM3}\) gene was amplified from genomic DNA of Bacillus megaterium ATCC 14581 (NZ_CP009920) with the primer set BM3-NcoF and BM3-BamHR. The PCR product digested with NcoI and BamHI was cloned into pCOLADuet-1 (Novagen, Darmstadt, Germany) cut with the same restriction enzymes, creating pLQ12. P450 \(_{BM3J}\) was generated by introducing 10 amino acid mutations (V78A, T175I, A184V, F205C, S226R, H236Q, E252G, F205C, A290V, L353V) on P450 \(_{BM3}\) [19]. The codon-optimized 1129 bp nucleic acids coding for the N-terminal P450 \(_{BM3J}\) were chemically synthesized, amplified with the primer set BM3-J-NcoF and BM3-J-EcoR, and integrated into the pCOLADuet-1, creating the plasmid pZZ1-up.

The other part of the P450 \(_{BM3J}\) gene, which is the same as that of P450 \(_{BM3}\) gene, was amplified from genomic DNA of B. megaterium (ATCC 14581) with the primer set BM3-J-EcoF and BM3-J-NotR. The PCR product digested with EcoRI and NotI was cloned into pJ2-F87A [22] with EcoRI and NotI, creating pJ2. The whole P450 \(_{BM3J}\) gene sequence is shown in [Additional file 1: Figure S1].

Structure modeling and site-directed mutagenesis
The models of the P450 variant structures were built on a public server Swiss-Model, using 1FAG as the template [22–24]. A method based on the amplification of the entire plasmid using primers that include the desired changes was employed for the site-directed mutagenesis [25]. All the plasmids and strains used in this work are listed in Table 1, and the oligonucleotide primers are given in Table 2.

| Plasmid or strain | Relevant genotype or description | Reference |
|------------------|----------------------------------|-----------|
| pCOLADuet-1      | ColA origin; Kan\(^{r}\); P\(_{TT}\) | Novagen   |
| pLQ12            | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450 \(_{BM3}\) | This study|
| pZJ1             | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450 \(_{BM3J}\) | This study|
| pV78A            | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3V78A | This study|
| pT175I           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3T175I | This study|
| pA184V           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3A184V | This study|
| pf205G           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3F205G | This study|
| pS226R           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3S226R | This study|
| pH236Q           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3H236Q | This study|
| pE252G           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3E252G | This study|
| pR255S           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3R255S | This study|
| pA290V           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3A290V | This study|
| pL353V           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3L353V | This study|
| pJ2              | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2 | This study|
| pJ2-R47L         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2R47L | This study|
| pJ2-F87A         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2F87A | This study|

| Bacterial strains and plasmids used in this study |
|----------------------------------|------------------------|
| Plasmids                        | Relevant genotype or description |
| pCOLADuet-1                    | ColA origin; Kan\(^{r}\); P\(_{TT}\) |
| pLQ12                          | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450 \(_{BM3}\) |
| pZJ1                           | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450 \(_{BM3J}\) |
| pV78A                          | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3V78A |
| pT175I                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3T175I |
| pA184V                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3A184V |
| pf205G                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3F205G |
| pS226R                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3S226R |
| pH236Q                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3H236Q |
| pE252G                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3E252G |
| pR255S                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3R255S |
| pA290V                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3A290V |
| pL353V                         | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3L353V |
| pJ2                            | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2 |
| pJ2-R47L                       | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2R47L |
| pJ2-F87A                       | ColA origin; Kan\(^{r}\); P\(_{TT}\): P450BM3J2F87A |

| Strains                        | Relevant genotype or description |
|--------------------------------|------------------------|
| BL21(DE3)                      | E. coli B dcm ampT hisS5(\(_{m}\)g) gal |
| LQ12                           | BL21(DE3) harboring pLQ12 |
| ZZ1                            | BL21(DE3) harboring pZZ1 |
| V78A                           | BL21(DE3) harboring pV78A |
| T175I                          | BL21(DE3) harboring pT175I |
| A184V                          | BL21(DE3) harboring pA184V |
| F205C                          | BL21(DE3) harboring pF205C |
| S226R                          | BL21(DE3) harboring pS226R |
| H236Q                          | BL21(DE3) harboring pH236Q |
| E252G                          | BL21(DE3) harboring pE252G |
| R255S                          | BL21(DE3) harboring pR255S |
| A290V                          | BL21(DE3) harboring pA290V |
| L353V                          | BL21(DE3) harboring pL353V |
| J2                             | BL21(DE3) harboring pJ2 |
| J2-R47L                        | BL21(DE3) harboring pJ2-R47L |
| J2-F87A                        | BL21(DE3) harboring pJ2-F87A |
Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table S1. *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA) was used as the host to overproduce proteins. During strain construction, cultures were grown aerobically at 37 °C in LB medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract). Kanamycin (50 mg/L) was added if necessary. For initial protein over-production in shake flasks, cultures were firstly grown in LB medium containing 50 mg/L kanamycin, then induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), next incubated at 30 °C for 10 h, and finally harvested by centrifugation. The cell catalysts obtained from 50 ml cultures were transferred into 25 ml M9 medium (37.8 g/L Na₂HPO₄, 12H₂O, 7.5 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 4 mM MgSO₄) supplemented with 50 mg/L kanamycin, 0.25 mM IPTG and 5 mM alcohols, and incubated at 30 °C for 48 h.

Analysis of diols by GC-MS

Different diols produced by the engineered strains were identified by gas chromatography–mass spectrometry (GC–MS). These diols were isolated by ethyl acetate extraction. After concentrated by a rotary evaporator and redissolved with ethanol, 1 μL sample was injected for GC-MS analysis. The system consisted of model 7890A network GC system (Agilent Technologies) and a model 5975C network mass selective detector (Agilent Technologies, Santa Clara, CA). A HP-INNOWAX capillary column (30 m × 0.25 mm; 0.25 μm film thickness; Agilent Technologies) was used, with helium as the carrier gas. The following oven temperature program was carried out: 50 °C for 2 min, increase of 10 °C/min to 240 °C, 240 °C for 5 min. The injector was maintained at 250 °C.

Additional file

Additional file 1: Figure S1. Nucleotide sequence of P450BM3J.

DQ0 30 kb

Acknowledgements

The authors thank National Natural Science Foundation of China (21376255 and 21572242) and Taishan Scholars Climbing Program of Shandong (tspd20150210) for the financial support of this project.

Funding

National Natural Science Foundation of China (21376255 and 21572242) and Taishan Scholars Climbing Program of Shandong (tspd20150210).

Availability of supporting data and materials

Supporting data could be found in Additional file 1.

Authors’ contributions

YZ carried out the product analysis, participated in strain cultivation, site-directed mutagenesis and drafted the manuscript. LL carried out the plasmid construction, participated in strain cultivation, site-directed mutagenesis and drafted the manuscript. QL participated in strain cultivation, site-directed mutagenesis and helped to draft the manuscript. HZ participated in the site-directed mutagenesis and helped to draft the manuscript. YC participated in strain cultivation and helped to draft the manuscript. HL and MX conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All the authors consented on the publication of this work.

Ethical approval and consent to participate

Not applicable.

Table 2 Primers used in this study

| Name       | Sequence (5′ → 3′) |
|------------|--------------------|
| BM3-NcoF   | CTTGACATGGGCATGCAATAGAAATGCCTCAG |
| BM3-BamR   | CGGGACCTTCCCCGAGCCACGGCTTGTCCATG |
| BM3-NotR   | GACGGGAATTCTTCCACATCG |
| BM3-EcoF   | GAAGAATTCGTCCAGAGGCTTGTCCATG |
| BM3-F205C-R| GATGACGGGAACATGTCGCTCTACAAATTAGTTAC |
| BM3-A290-F | GTTTCATTATTTTGAAAGTACGCAAGAAGGACG |
| BM3-A290-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |
| BM3-175-I-F | CTTAATGTCGAGTTTGGTCAAGGAGGAG |
| BM3-175-I-R | CGGTAACATGGATGAGGAACGCAAGGACG |
| BM3-F205C-F | CTTTCATACCTTCCGACTTCTACAAATTAGTTAC |
| BM3-A290-V-F | GTATTACAAAAAGTAGCAGCAAGAAGGACG |
| BM3-A290-V-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |
| BM3-175-I-F | CTTAATGTCGAGTTTGGTCAAGGAGGAG |
| BM3-175-I-R | CGGTAACATGGATGAGGAACGCAAGGACG |
| BM3-F205C-F | CTTTCATACCTTCCGACTTCTACAAATTAGTTAC |
| BM3-A290-V-F | GTATTACAAAAAGTAGCAGCAAGAAGGACG |
| BM3-A290-V-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |
| BM3-175-I-F | CTTAATGTCGAGTTTGGTCAAGGAGGAG |
| BM3-175-I-R | CGGTAACATGGATGAGGAACGCAAGGACG |
| BM3-F205C-F | CTTTCATACCTTCCGACTTCTACAAATTAGTTAC |
| BM3-A290-V-F | GTATTACAAAAAGTAGCAGCAAGAAGGACG |
| BM3-A290-V-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |
| BM3-175-I-F | CTTAATGTCGAGTTTGGTCAAGGAGGAG |
| BM3-175-I-R | CGGTAACATGGATGAGGAACGCAAGGACG |
| BM3-F205C-F | CTTTCATACCTTCCGACTTCTACAAATTAGTTAC |
| BM3-A290-V-F | GTATTACAAAAAGTAGCAGCAAGAAGGACG |
| BM3-A290-V-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |
| BM3-175-I-F | CTTAATGTCGAGTTTGGTCAAGGAGGAG |
| BM3-175-I-R | CGGTAACATGGATGAGGAACGCAAGGACG |
| BM3-F205C-F | CTTTCATACCTTCCGACTTCTACAAATTAGTTAC |
| BM3-A290-V-F | GTATTACAAAAAGTAGCAGCAAGAAGGACG |
| BM3-A290-V-R | CATTATGGTCGCAATGGATGAGGAACGCAAGGACG |
| BM3-L353-V | GCGGACGAAATTATGTTCTGCTTCCTCAGC |
| BM3-L353-R | GAACACATGGTCGCTCTACAAATTAGTTAC |

Underlines indicate restriction enzyme sites
Bacillus megaterium – Escherichia coli

Bacillus subtilis – for direct production of 1,4-butanediol.

Escherichia coli

Escherichia coli

BM3

BM-3

for the production of hydroxy fatty acids from glucose.

for the efficient production of cytochrome P-450 BM3: 2.

Microb Cell Fact. 2012;11:65.

11. Meinhold P, Peters MW, Chen MM, Takahashi R, Haselbeck R, Niu W, Pujolet-Baxley C, Burgard A, Boldt J, Khanduriina J, Travick JD, Osterhout RE, Stephen R, et al. Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. Nat Chem Bio. 2011;7:445–52.

8. Mattam AJ, Yazdani SS. Engineering chain fatty acids to bioalcohols. Biotechnol Biofuels. 2013;6:128.

7. Zheng YN, Li LL, Liu Q, Yang JM, Wang XW, Liu W, Xu X, Liu H, Zhao G, Xian M. Optimization of fatty alcohol biosynthesis pathway for selectively converting of short chain diols. Microb Cell Fact. 2014;13:165.

10. Cao Y, Cheng T, Zhao G, Niu W, Guo J, Xian M, Liu H. Metabolic engineering of Escherichia coli for the production of hydroxy fatty acids from glucose. BMC Biotechnol. 2016;16:26.

11. Meinhold P, Peters MW, Chen MM, Takahashi K, Arnold FH. Direct conversion of ethane to ethanol by engineered cytochrome P450 BM3. Biochem J. 1999;339:371

5. Moscoviz R, Trably E, Bernet N. Consistent 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH. Biotechnol Biofuels. 2016;9:32.

6. Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature. 2010;463:599–62.

3. Nakamura CE, Whited GM. Metabolic engineering for the microbial production of 1,3-propanediol. Curr Opin Biotech. 2003;14:454–9.

4. Yim H, Haselbeck R, Niu W, Pujol-Baxley C, Burgard A, Boldt J, Khanduriuna J, Travick JD, Osterhout RE, Stephen R, et al. Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. Nat Chem Bio. 2011;7:445–52.