ORIGINAL RESEARCH PAPER

Construction of an *Escherichia coli* mutant producing monophosphoryl lipid A

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Abstract Lipid A is a major component in the outer membrane of most Gram-negative bacteria. Monophosphoryl lipid A contains no phosphate group at 1-position and can be used as an adjuvant. We constructed an *Escherichia coli* mutant CW001 by integrating a gene *lpxE* into the chromosome of *E. coli* W3110. The gene *lpxE* encodes an enzyme LpxE which removes the 1-phosphate group of lipid A. CW001 predominantly produces 1-dephosphorylated lipid A in vivo, as adjudged by thin layer chromatography and electro-spray ionization mass spectrometry. This study not only is important for the development of lipid A adjuvants but also provides a novel method for integration of heterologous genes into the chromosome of *E. coli*.

Keywords Adjuvant · Chromosomal integration · Endotoxin · Lipid A · Lipopolysaccharide · Monophosphoryl lipid A · *Salmonella minnesota*

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Introduction

Lipopolysaccharide (LPS), known as endotoxin, forms the outer monolayer of the outer membrane in most Gram-negative bacteria. Lipid A is the hydrophobic anchor of LPS and is responsible for the activity of the endotoxin (Wang and Quinn 2010; Raetz et al. 2007; Raetz and Whitfield 2002). Lipid A can be recognized by Toll-Like Receptor 4 (TLR4) existing on the surface of immune cells and trigger various pathophysiological responses, resulting in serious endotoxin shock (Bosshart and Heinzelmann 2007; Munford 2006). The toxicity of lipid A is closely related to its precise chemical structure (Wang et al. 2004, 2006, 2007, 2009).

Escherichia coli lipid A contains two phosphate groups and six fatty acid chains (Fig. 1a). The two phosphate groups in lipid A are crucial for its bioactivity; they interact with a cluster of positively charged residues from dimeric TLR4 and MD-2 (Park et al. 2009). Monophosphoryl lipid A (MPLA), a lipid A derivative, which contains only one phosphate group, is much less toxic than the wild type lipid A but still retain many of the immunomodulatory properties (Takayama et al. 1981; Persing et al. 2002). Monophosphoryl lipid A cannot activate caspase-1, one of the key enzymes involved in the synthesis of interleukin-1β (Okemoto et al. 2006). Vaccine development has focused on MPLA analogues which could induce altered cytokine profiles that retain adjuvant activity without toxicity (Baldridge and Crane 1999).
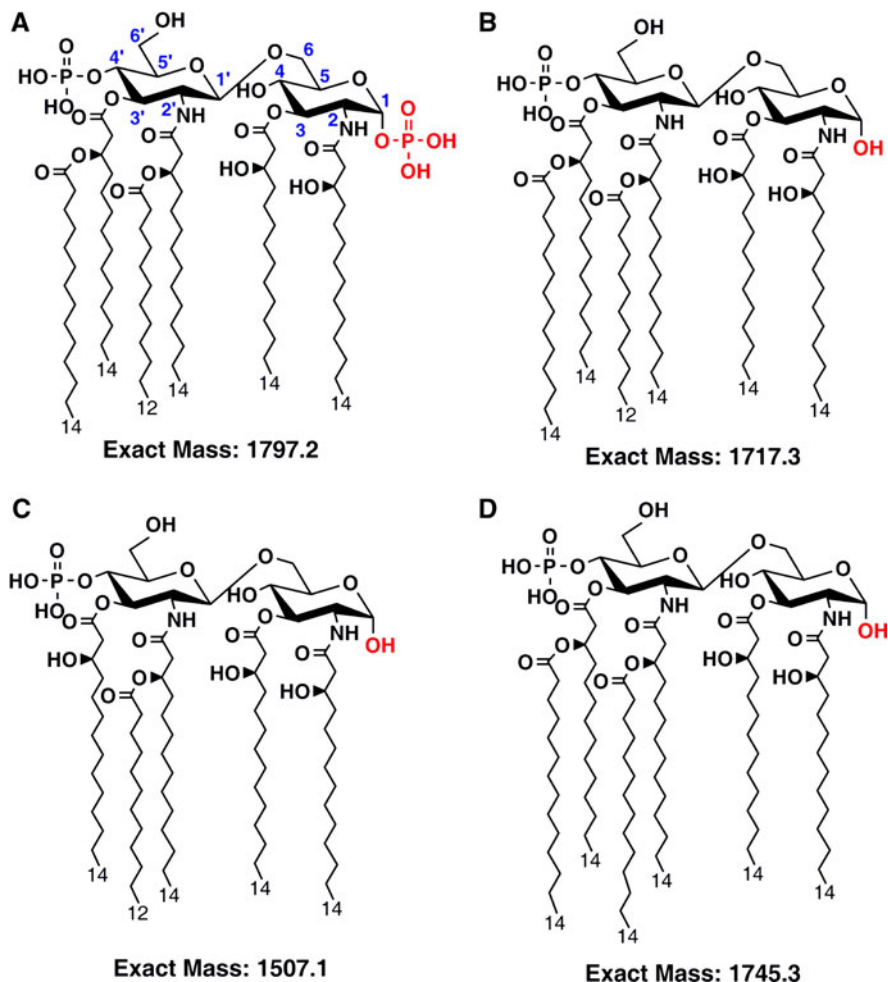
Recently, MPLA analogues have been used as vaccine adjuvants and have recently been approved for use in the USA (Mata-Haro et al. 2007; Reed et al. 2009).

Currently, MPLA analogues are prepared either by extracting the lipid A from *Salmonella minnesota* R595 (Fig. 1c and then removing the 1-phosphate group chemically (Persing et al. 2002), or by chemical synthesis. *Salmonella minnesota* R595 makes multiple lipid A species which are difficult to separate from each other. Chemically-synthetic lipid A has a slightly different structure from *E. coli* lipid A (Fig. 1d). Unlike *S. minnesota* R595, *E. coli* can only produce one type of lipid A. Here we have constructed an *E. coli* mutant CW001 which produces MPLA in vivo, by expressing the gene *lpxE* from *Francisella novicida* (Wang et al. 2004). The gene *lpxE* was inserted in the chromosome of CW001. The production of MPLA in CW001 was confirmed by TLC and ESI–MS. This

provides a solid foundation for the development of lipid A vaccine adjuvants and the attenuated living vaccine strains. In addition, a rapid and efficient method was also developed for integrating heterologous genes into the *E. coli* chromosome.

Materials and methods

DNA preparation and PCR techniques

All bacterial strains and plasmids used are listed in Supplementary Table 1. Restrictions enzymes, calf intestine alkaline phosphatase, T4 DNA ligase, and DNA Ladder were from Sangon (Shanghai, China). Plasmid DNA was prepared by using the EZ-10 spin column plasmid mini-preps kit form Bio Basic Inc (Markham, Canada). All the PCR reaction mixtures

used in this study are 50 μl containing 10 μl 5×
PrimerSTAR buffer, 4 μl dNTP mixture (2.5 mM
each), 1 μl plasmid template (100 mg l^{-1}), 1 μl
forward primer (20 μM), 1 μl reverse primer
(20 μM), 0.5 μl DNA polymerase. The PCR reaction
was usually started at 94°C for 5 min, followed by 35
cycles of denaturation (30 s at 94°C), annealing, and
extension. At the end, an additional 10 min incuba-
tion at 72°C was used. PCR products were purified
by using the TIANgel midi purification kit from
Tiangen (Beijing, China). Primers synthesis and
DNA sequencing were performed by Sangon. The
sequences of primers used in this study are listed in
Supplementary Table 2.

Chromosomal integration of the lpxE gene
in *Escherichia coli*

The detailed procedure to construct the *lpxE* mutant
strain is shown in Fig. 2. DNA fragment, *Fkan*, a
kanamycin resistance gene flanked by FRT sites, was
amplified from the plasmid pKD13 by using primers
Fkan-F and Fkan-R. The PCR product was digested
with *Xho*I, and ligated into pWSK29-*FnlpxE*, which
was similarly digested and treated with calf intestine
alkaline phosphatase. The resulting plasmid is desig-
nated as pWSK29-*FnlpxE-Fkan*. The *lpxE-Fkan* cas-
sette was PCR amplified from the plasmid pWSK29-
FnlpxE-Fkan by using primers LacZ-F and LacZ-R.
During PCR, the annealing was at 55°C for 15 s, and
the extension was at 72°C for 150 s. Both ends of the
PCR product are the two split fragments from *lacZ-α*.
The PCR products were purified from the gel and
transformed into W3110 carrying a plasmid pKD46
(Datsenko and Wanner 2000). The cell suspension was
then spread onto agar plates containing 30 μg kana-
mycin/ml, 24 μg IPTG/ml and 40 μg X-Gal/ml, and
the white colonies were selected. The strain containing
the correct recombination was designated W3110-
lpxE-kan. Next, the antibiotic resistance cassette *kan*
was eliminated from the chromosome using the
plasmid pCP20 (Cherepanov and Wackernagel 1995)
and the final strain was designated CW001.

Isolation of lipid A from *Escherichia coli*

An overnight culture of *E. coli* was inoculated in
200 ml LB broth, grown to an OD$_{600}$ of 1, harvested
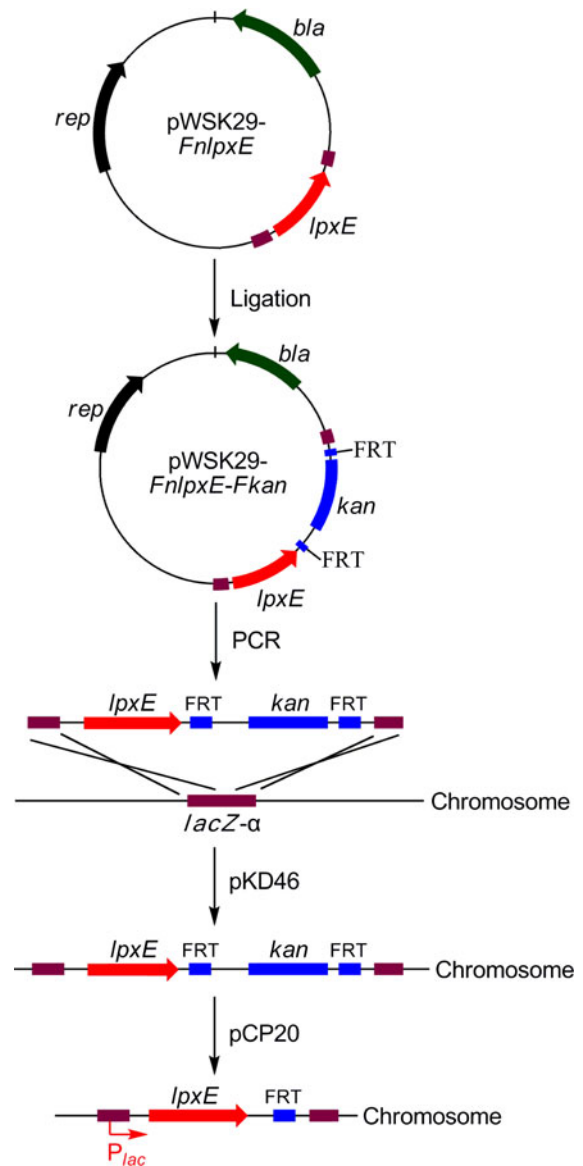and washed twice with phosphate-buffered saline.

Fig. 2 The procedure of the chromosomal integration of *lpxE*
gene in *E. coli*. DNA fragment *Fkan*, a kanamycin resistance
gene flanked by FRT sites, was ligated into pWSK29-*FnlpxE*
to form the plasmid pWSK29-*FnlpxE-Fkan*. The *lpxE-Fkan*
cassette was PCR amplified and transformed into W3110
carrying a plasmid pKD46. The recombination occurred and
the *lpxE-Fkan* cassette was inserted in the chromosome. At the
end, the cassette *kan* was eliminated from the chromosome
using the plasmid pCP20. *Rep*, replication initiation protein;
lacZ-α, a fragment encodes the α subunit of LacZ protein; *bla*,
ampicillin resistance gene

Cell pellets were re-suspended in 76 ml chloroform/
methanol/water (1:2:0.8, by vol), and held at room
temperature for 1 h. Insoluble material was collected

by centrifugation at $1000 \times g$ for 20 min and washed once with the same solvent. To cleave the glycosidic linkage between polysaccharide and lipid A in LPS, the pellets were re-suspended in 27 ml 12.5 mM sodium acetate (pH 4.5), heated at 100°C for 30 min, followed by sonication. After cooling to room temperature, 30 ml chloroform and 30 ml methanol were added, mixed thoroughly and centrifuged. The lower phase containing lipid A was pooled and dried using a rotary evaporator.

Chromosomal integration of the cat gene in Escherichia coli

Briefly, *Fkan* was ligated into pBluescript II SK (+), resulting the plasmid pBSK-*Fkan*. The *cat* gene was PCR amplified from the plasmid pACYC184, using forward primer Cat-F and reverse primer Cat-R. The primer Cat-F carries a SD sequence and a *Not*I restriction site at the 5′-end, while the primer Cat-R carries a *Sal*I restriction site at the 5′-end. During PCR, the annealing was at 58°C for 15 s, and the extension was at 72°C for 60 s. The PCR product was digested with *Not*I and *Sal*I, and ligated into pBSK-*Fkan* which was similarly digested. The resulting plasmid is designated as pBSK-*cat-Fkan*, which contains the *cat-Fkan* cassette under the *lac* promoter. Next, the *cat-Fkan* cassette was PCR amplified by using primers lacZ-F and lacZ-R. The PCR products were purified from the gel, and transformed into W3110/pKD46. The cell suspension was spread onto agar plates containing kanamycin, IPTG and X-Gal, and the white colonies were selected. The correct recombination was confirmed by PCR and the strain containing correct recombination is designated W3110-*cat-kan*. The *kan* cassette was then eliminated using the plasmid pCP20 and the final strain was designated W3110-*cat*.

Tachypleus amebocyte lysate assay

Chromogenic end-point *Tachypleus amebocyte* lysate kit was from Xiamen Houshiji (Xiamen, China). *E. coli* W3110 and CW001 were grown in LB broth containing IPTG, harvested by centrifugation, and washed three times with phosphate-buffered saline. LPS were prepared using the hot phenol/water method (Westphal and Jann 1965), and quantified using the method described by Lee and Tsai (1999).

LPS samples and standard were mixed separately with *Tachypleus amebocyte* lysate reagent, and incubated at 37°C for 8 min. The activities were determined using *p*-nitroanilide as chromogenic substrate. The experiment was repeated thrice.

Results

Construction of a MPLA production strain CW001 by chromosomal integration of lpxE gene in Escherichia coli

Monophosphoryl lipid A adjuvants are currently prepared either by chemical synthesis, or by extracting the lipid A from bacteria and then removing the 1-phosphate group chemically (Persing et al. 2002). To construct bacteria that could produce MPLA in vivo, the *lpxE* gene was integrated in the chromosome of *E. coli* as shown in Fig. 2. The *Fkan* DNA fragment was first cloned into the plasmid pWSK29-*FnlpxE*, to form a plasmid pWSK29-*FnlpxE-Fkan* which contains a *lac* promoter and Shine-Dalgarno sequence (SD). The *lpxE-Fkan* cassette was then amplified by PCR, and transformed into W3110/pKD46. The Red enzymes expressed by pKD46 facilitated the recombination of the *lpxE-Fkan* cassette at the *lacZ* locus in the chromosome. The insertion of *lpxE-Fkan* cassette would break the *lacZ* gene; therefore *E. coli* containing correct insertion of the *lpxE-Fkan* in the chromosome would show white colonies on plates containing IPTG and X-gal. At the end, the plasmid pCP20 which can transiently express FLP recombinase was introduced into the host cell, and the *kan* was removed. The correct insertion of the *lpxE* and the later removal of *kan* were confirmed by the loss of kanamycin resistance and PCR analysis. This *lpxE* mutant *E. coli* is designated CW001.

The growth rate of CW001 was found similar to that of wild type W3110. The stability studies showed that the integrated *lpxE* could stay at the *lacZ* locus in the chromosome of CW001 even after 50 generations.

Analysis of MPLA produced by CW001

Lipid A were isolated from strains of W3110, CW001 and W3110/pWSK29-*FnlpxE*, and separated on TLC.
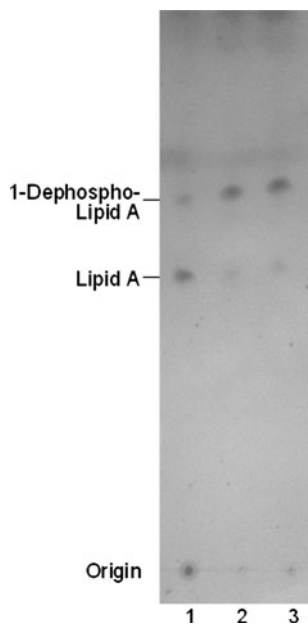
Fig. 3 Analysis of lipid A and 1-dephospho-lipid A on TLC. The lipid A samples were dissolved in chloroform/methanol (4:1, by vol), spotted onto a silica gel 60 TLC plate, which was developed in chloroform/methanol/acetic acid/water (40:25: 4:2, by vol). After drying, the lipid A bands on the TLC plate were visualized by spraying a 10% H_2SO_4 (by vol) in ethanol, followed by charring at 180°C. *Lane 1*, sample from W3110; *lane 2*, sample from CW001; *lane 3*, sample from W3110/ pWSK29-*FnlpxE*

Fig. 4 ESI/MS analysis of lipid A or 1-dephospho-lipid A isolated from *E. coli* W3110 (**a**), CW001 (**b**) and W3110/ pWSK29-*FnlpxE* (**c**). Lipid A samples were dissolved in chloroform/methanol (4:1, by vol) and subjected to ESI/MS in the negative ion mode. The mass spectra were acquired on a Waters SYNAPT Q-TOF mass spectrometer equipped with an ESI source. Data acquisition and analysis were performed by using MassLynx V4.1 software

A major band, the typical lipid A band, was shown on the TLC in samples isolated from W3110 (Fig. 3, lane 1), but it is significantly weaker in samples isolated from W3110/pWSK29-*FnlpxE* and CW001 (Fig. 3, lanes 2, 3). However, a more rapidly migrating substance, presumed to be 1-dephospho-lipid A was present in both W3110/pWSK29-*FnlpxE* and CW001 (Fig. 3, lanes 2, 3). Due to the hydrophilic property of phosphate, lipid A containing two phosphate groups should migrate slower on TLC than 1-dephospho-lipid A. This experiment suggests that LpxE expressed from the chromosome of CW001 functions equally as well as that expressed from the plasmid pWSK29 in the strain W3110/pWSK29-*FnlpxE* (Fig. 3, lanes 2, 3).

Lipid A and its derivatives isolated from W3110, CW001 and W3110/pWSK29-*FnlpxE* were further analyzed by ESI/MS. The lipid A isolated from W3110 created a major peak at m/z 1797.5 (Fig. 4a), suggesting the peak is created by the molecular ion
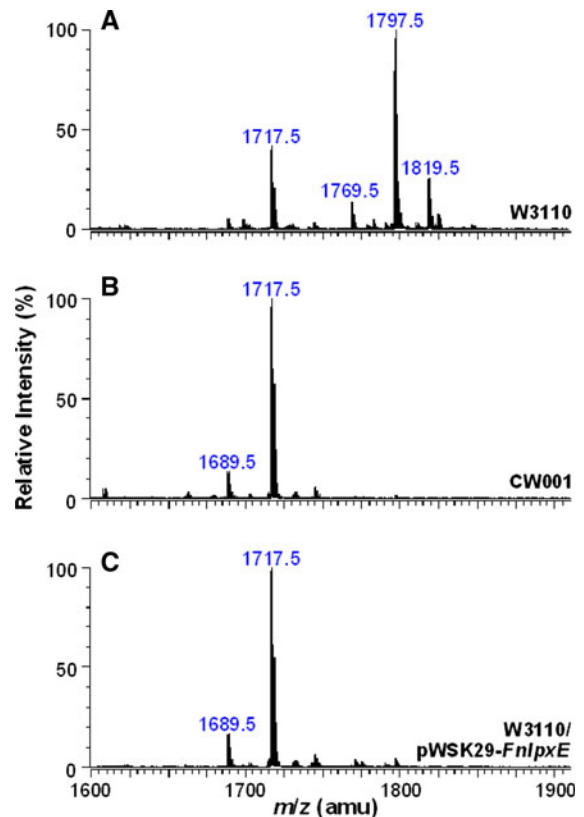
[M-H]⁻ of lipid A (Fig. 1a). The peak at m/z 1717.5 might arises by loss of a phosphate during the extraction procedure. The peaks at m/z 1819.5 likely arises by the sodium adduct of the molecular ion of lipid A; the peak at m/z 1769.5 could be created by minor lipid A species which differ by 28 Da, that is, two methylene units. The 1-dephospho-lipid A isolated from CW001 and W3110/pWSK29-*FnlpxE* showed the similar spectra which contain a major peak at m/z 1717.5 as expected (Fig. 4b, c). These results indicate that the *lpxE* gene in the chromosome of CW001 was expressed and its product LpxE can modify the structure of lipid A in vivo.

Comparison of the stimulation activities of LPS isolated from W3110 and CW001

LPS was purified from both W3110 and CW001, and their stimulation activities were measured by *tachypleus amebocyte* lysate. The stimulation activity of W3110 LPS (0.005 μM) was 0.3 EU ml^{-1}, while the activity of the same amount of CW001 LPS was 0.64 EU ml^{-1}. The dephosphorylation of lipid A in LPS produced by CW001 might have higher binding affinity to the Factor C, the LPS-sensitive protein, and facilitate the agglutination reaction. Earlier studies have shown that the molecular interaction between LPS and Sushi 1 domain of Factor C is basically the hydrophobic interaction between the lipid tail of LPS and the Sushi 1 peptide (Tan et al. 2000; Li et al. 2006). Therefore, the enhanced hydrophobicity of MPLA produced in CW001 due to the absence of 1-phosphate might cause the stronger interaction with Sushi 1 peptide of Factor C.

Chromosome integration of the report gene cat in *Escherichia coli*

To test the generality of the method we used for the integration of *lpxE* in the chromosome of *E. coli*, we also integrated the reporter gene *cat* into the chromosome of *E. coli*. The integration of the *cat* gene into the chromosome of *E. coli* was evaluated by antibiotics. As shown in Fig. 5, wide type W3110 could not grow on plates containing chloramphenicol or kanamycin, but W3110-cat-kan and W3110/pBSK-cat-Fkan grew well on all plates. W3110-cat, however, could grow on the plate containing chloramphenicol and IPTG, but not on the plate containing kanamycin. This indicates that the reporter gene *cat* has been inserted and the *kan* gene has been removed in the chromosome of W3110-cat. This experiment proves that the method we used in this study has a generality for the insertion of a heterologous gene in the chromosome of *E. coli*.

Discussion

MPLA has become an important target molecule for the development of novel vaccine adjuvants. To obtain MPLA, the 1-phosphate group in lipid A is usually removed by chemical method. In this study, we
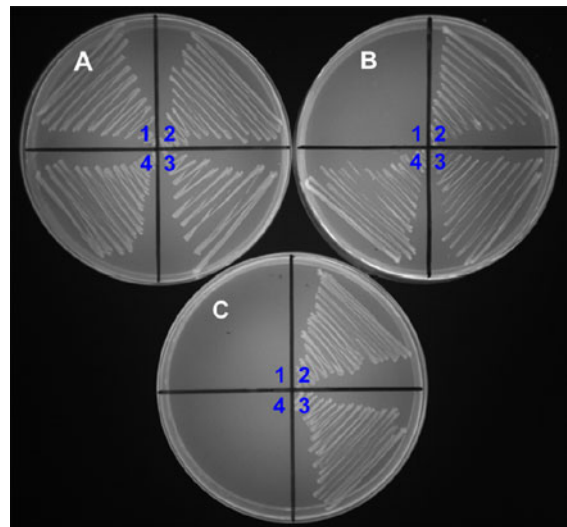

Fig. 5 Analysis of different *E. coli* strains containing the reporter gene *cat*. (*A*) The plain LB plate. (*B*) The LB plate containing 30 μg ml^{-1} chlorophenicol. (*C*) The LB plate containing 30 μg ml^{-1} kanamycin. The plates were divided into four section on which the following four *E. coli* strains were spread. *1*, W3110; *2*, W3110/pBSK-cat -Fkan; *3*, W3110-cat-kan; *4*, W3110-cat

constructed a recombinant, plasmid-free *E. coli* strain CW001 that predominantly produce 1-dephosphorylated lipid A, by expressing *lpxE* in the chromosome of W3110. The *lpxE* gene in the chromosome of CW001 is stable and its expression does not affect the growth of the cell. The stimulation activity of CW001 LPS is higher than W3110. Strains expressing *lpxE* possess 1-dephospho-lipid A in their LPS and could have many potential applications. Lipid A detoxification by LpxE may be a useful tool for improving live vaccines to prevent infectious disease.

In addition, we also developed a rapid and efficient method to integrate heterologous genes in the chromosome of *E. coli* by combining the Red recombination system (Sharan et al. 2009; Albermann et al. 2010; De Mey et al. 2010) and the blue-white screen. Heterologous genes are usually expressed in *E. coli* using plasmids as the vector. But plasmids in bacteria are not stable and usually need antibiotics as selection markers. Therefore, chromosomal integration is the ideal approach to express heterologous genes in *E. coli*.

Because many *E. coli* plasmids, such as pMD18-T, pMD19-T, pGEM-T, pBluescript II SK(±), pUC18, pUC19, and pWSK29, contain the *lacZ* gene or the *lacZ*-α segment, the *lacZ* locus in the chromosome of

E. coli could be selected at the special location for the integration of heterologous genes. The *lacZ* segment in plasmids usually contains the *lac* promoter and an internal multiple cloning site which facilitates the expression and insertion of heterologous genes. After insertion of the heterologous gene, the *lacZ* segment is separated into two parts which simply become the homology extensions for the integration of the gene at the *lacZ* locus of chromosome in *E. coli*. The homology extensions produce by this way are long, which significantly reduces the rate of false-positive clones by the Red recombination system. By combining the Red recombination and the blue-white screen, almost all white colonies showed accurate integration of *lpxE* or *cat* at the *lacZ* locus in the chromosome of *E. coli*, suggesting that the method has the generality for the insertion of genes into the chromosome of *E. coli*.

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