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主编点评文章

The effect of the structure of lipopolysaccharide on the permeability of *Escherichia coli* cell membranes

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Abstract: Lipopolysaccharide is the main component of outer membranes, which serve as a permeability barrier. In this study, we investigated the role of the structure of lipopolysaccharide on the permeability of bacterial cell membranes. Nine *Escherichia coli* strains which make different structures of LPS were selected or constructed. Lipopolysaccharide and lipid A were extracted from these strains, and their structures were analyzed by using thin-layer chromatography and electrospray ionization mass spectrometry. The membrane permeability of these strains was analyzed by using N-phenyl-1-naphthylamine fluorescent probe. Wild type *E. coli* showed the least permeability, while mutants in which LPS structures were changed by the deletion or expression of related genes showed higher permeability. The number of phosphate groups and the acyl chains, and the length of the polysaccharide of lipopolysaccharide all affect the permeability of *E. coli*. The chain length of the polysaccharide has the largest effect on the permeability, followed by the number of the acyl chains. The results indicate that the cellular membrane permeability is related to the structure of lipopolysaccharide.
Keywords: Lipopolysaccharide, Lipid A, Membrane permeability, *Escherichia coli*

大肠杆菌细胞外膜渗透性与脂多糖结构的关系

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摘 要: 细胞外膜是大肠杆菌的半透膜屏障，其主要成分是脂多糖。选取并构造共 9 种具有不同脂多糖结构的大肠杆菌，用于考察脂多糖结构对细胞外膜渗透性的影响。从 9 种菌株中提取出脂多糖和类脂 A，并且用薄层层析色谱和离子源质谱来鉴定其结构。用 N-苯基-1-萘胺作为荧光探针来测定细胞外膜渗透性大小。野生型大肠杆菌表现出最小的渗透性，因敲除或表达某些基因而导致脂多糖结构改变的突变株均表现出较高的渗透性。脂多糖上的磷酸基团、脂肪酸链和多糖链的改变都影响了大肠杆菌的渗透性，其中多糖链长度的改变对渗透性影响最大，其次是脂肪酸链的数目变化。实验结果表明渗透性和脂多糖的结构具有较强的相关性。

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关键词: 脂多糖, 类脂 A, 膜渗透性, 大肠杆菌

1 Introduction

Outer membranes of gram-negative bacteria serve as a permeability barrier[1−2], it retards the entry of substrate into the cell and prevents the product from being released. Reducing this barrier would accelerate the efficiency of whole-cell catalyzed reactions and the production yield of industrial microorganism[3]. Several methods to improve the membrane permeability have been reported, including the physical and chemical treatments, and structure modification on membrane proteins by genetic engineering[3−6]. Lipopolysaccharide (LPS), as the major component of outer membrane in most gram-negative bacteria, should be a key contributor to the permeability of outer membranes. LPS consists of three moieties: the hydrophobic lipid A, the core region and the O-antigen repeats[1−2]. The core oligosaccharide and the phosphate group of LPS are negatively charged, resulting in a strong affinity for divalent cations[7]. The hydrophilic polysaccharide component is responsible for the exclusion of hydrophobic molecules and the hydrophobic lipid A of LPS limits the entry of hydrophilic compounds[3]. Mutations on genes *lpxA*, *lpxD*, *lpxC*, *lpxM* and *lpxL* involved on the lipid A of LPS biosynthesis changed the antibiotic resistance of bacterial cells[8], suggesting that the permeability of cell membranes is related to the structure of lipid A of LPS. However, the detailed relationship between the structures of lipid A and the permeability of membranes is not clear.

In this study, the effect of LPS on the permeability of outer membranes of *Escherichia coli* was investigated by using nine *E. coli* strains that make different structures of LPS. The outer membranes of wild type *E. coli* showed the least permeability, while mutants showed higher permeability. The number of phosphate groups and the fatty acid chains in the lipid A of LPS, and the length of the polysaccharide of LPS all affected the permeability of the outer membranes. Considering *E. coli* as a model strain for gene engineering and industrial fermentation, our results would be useful for redesigning the membrane structure of *E. coli* and other Gram-negative bacteria to improve their permeability and increase the yield of products.

2 Materials and methods

2.1 Materials

Glass-backed silica gel 60 thin-layer chromatography (TLC) plates were purchased from Jiangyou Silica Development Company (Yantai, China). Chloroform, ammonium acetate, ethanol sodium acetate, methanol, ammonia solution and sodium chloride were obtained from Sinopharm Chemical Reagent Company. Luria broth, yeast extract and tryptone were from Oxoid. Kanamycin, ampicillin, tetracycline, chloramphenicol, N-phenyl-1-naphthylamine (NPN), restrictions enzymes, calf intestine alkaline phosphatase (CIAP), T4 DNA ligase, isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sangon (Shanghai, China). Plasmid DNA was prepared by using the Plasmid Minipreps Purification System B (BioDev-Tech, Beijing, China). DNA was separated on agarose gels and purified with the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, Canada). DNA synthesis and sequencing were performed by Sangon (Shanghai, China).

2.2 Strains, plasmids and growth conditions

All bacterial strains and plasmids used are listed in Table 1. *E. coli* grew at 37 °C in Luria-Bertani medium supplemented with the appropriate antibiotics (100 mg/L ampicillin, 12.5 mg/L tetracycline or 25 mg/L chloramphenicol).

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Table 1 Bacterial strains and plasmids used in this study
表 1 文中使用的菌株和质粒

Strains and plasmids 菌株和质粒	Description 描述	Source/Reference 来源/参考文献
Strains 菌株		
E. coli W3110	Wild type *E. coli*, F^-, λ^-	*E. coli* Genetic Stock Center
WBB06	W3110 mutant with a deletion of *waaC* and *waaF* genes	[9]
MLK1067	W3110 mutant with a deletion of *lpxM*	[10]
MKV15b	W3110 mutant with a deletion of *lpxL*, *lpxM* and *lpxP* genes	[11]
W3110/pWSK29	W3110 transformed by pWSK29	[12]
W3110/pWSK29-LpxE	W3110 transformed by pWSK29-LpxE	[13]
W3110/pWSK29-LpxF	W3110 transformed by pWSK29-LpxF	[14]
W3110/pWSK29-PagL	W3110 transformed by pWSK29-PagL	This work
W3110/pWSK29-PagP	W3110 transformed by pWSK29-PagP	This work
Plasmids 质粒		
pWSK29	Low copy vector, Amp^r	[15]
pWSK29-LpxE	pWSK29 harboring *lpxE*	[13]
pWSK29-LpxF	pWSK29 harboring *lpxF*	[14]
pWSK29-PagL	pWSK29 harboring *pagL*	This work
pWSK29-PagP	pWSK29 harboring *pagP*	This work

LB medium supplemented with appropriate antibiotics (as described below) was used in all cultivations. The medium for plasmid-carrying strains was supplemented with appropriate antibiotics. For cell growth study, 50 mL of medium in a 250 mL flask was inoculated with an overnight culture (2% inoculum). The flasks were shaken in a rotary shaker at 200 r/min at 37 °C. Samples were taken from each culture every two hours to monitor the growth pattern by measuring optical density at 600 nm with a UV/vis spectrophotometer (UV-1800, Shimadzu). For cell growth study with induction, the same medium as described above was used and initial cultivation was carried out at 37 °C until OD_{600} reached 0.5−0.7, when IPTG was added to a final concentration of 1 mmol/L.

2.3 Construction of plasmids pWSK29-PagL and pWSK29-PagP

The gene *pagL* was amplified by PCR, using the genomic DNA of *Salmonella typhimurium* as the template. The forward primer was 5′-GCTCTAGAGGTGGAGTGTATATGAAGAG-3′ in which an *Xba* I restriction site (underlined) was designed. The reverse primer was 5′-CGGGATCCCTCGCTAATTGTTATTCAAC-3′ in which a *Bam*H I restriction site (underlined) was designed. The PCR product was digested with *Xba* I and *Bam*H I and ligated into the vector pWSK29 that had been similarly digested and treated with CIAP. The ligation mixture was transformed into *E. coli* W3110, and the positive transformants, designated W3110/pWSK29-PagL, were selected on LB plates containing 100 mg/L ampicillin.

The strain W3110/pWSK29-PagP was constructed in a similar way. The gene *pagP* was PCR amplified from the genomic DNA of *E. coli* W3110. The forward primer was 5′-CGGAATTCAAGGAGATATAAATGAACGTGAGTAAATATGT-3′ in which an *Eco*R I restriction site (underlined) was designed, and the reverse primer was 5′-CCGCTCGAGTCAAAACTGAAAGCGCATCC-3′ in which an *Xho* I restriction site (underlined) was designed. The PCR product was then digested using *Eco*R I

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and *Xho* I and ligated into the vector pWSK29 that had been similarly digested and treated with CIAP. The ligation mixture was transformed into *E. coli* W3110 and ampicillin-resistant transformants, designated W3110/pWSK29-PagP, were selected on LB plates.

2.4 Extraction of lipid A and Kdo_2-lipid A

Lipid A and Kdo_2-lipid A were extracted according to the published method[16]. Briefly, the overnight culture was inoculated in 200 mL fresh LB broth with an initial OD_{600} of 0.02, and grew until OD_{600} of 1.2. The cells were harvested and washed twice with phosphate-buffered saline. The cell pellet was re-suspended in 76 mL of a single-phase Bligh-Dyer mixture[17], incubated at room temperature for 60 min, and centrifuged at 2 000×*g* for 10 min. Kdo_2-lipid A in WBB06 could be collected from the supernatant, while LPS for other *E. coli* strains should be in the pellets. The pellets were re-suspended in 27 mL of 12.5 mmol/L sodium acetate (pH 4.5), heated at 100 °C for 30 min, followed by sonic irradiation in a bath apparatus[18]. After being cooled to room temperature, the suspension was converted into a two-phase Bligh-Dyer system by addition of 30 mL of chloroform and 30 mL of methanol. After mixed thoroughly and centrifuged, the lower phases containing lipid A were pooled and dried under a stream of nitrogen.

2.5 Electrospray ionization mass spectrometry (ESI/MS) analysis of lipid A

All mass spectra were acquired on a Waters SYNAPT Q-TOF mass spectrometer equipped with an ESI source. Lipid A samples were dissolved in chloroform and methanol (4:1, *V/V*) and subjected to ESI/MS in the negative ion mode. Data acquisition and analysis were performed using MassLynx V4.1 software.

2.6 NPN assay for cell membrane permeability

Membrane permeability of *E. coli* was determined by the NPN assay[19]. *E. coli* cultures were harvested by centrifugation at 8 000 ×*g* for 10 min, washed and resuspended in 10 mmol/L phosphate buffer (pH 7.4). The final cell suspension was adjusted to OD_{600} of 0.5. NPN was added to a final concentration of 40 μmol/L into quartz cuvettes containing 2 mL of cell suspension, followed by immediate inversion of the cuvettes to mix up. The fluorescence was recorded by a 650-60 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The slit width was set at 5 mm. The excitation wavelength and emission wavelength were set at 350 nm and 420 nm, respectively. An increase in fluorescence due to partitioning of NPN into the outer membrane was recorded immediately as a function of time until there was no further increase.

3 Results and Discussion

3.1 Structure analysis of LPS extracted from nine different *E. coli* strains

To study the effect of LPS structure on the membrane permeability of *E. coli*, strains of *E. coli* which make different structures of LPS were selected or constructed. The involved genes are listed in Table 2, and their function sites on the structure of LPS are represented in Fig. 1.

Wild type strain W3110 and W3110/pWSK29 makes LPS consisting of lipid A and core polysaccharide (Fig. 1). Mutant strain WBB06 makes Kdo_2-lipid A because the genes *waaC* and *waaF* were inactivated[9]. W3110/pWSK29-LpxE and W3110/pWSK29-LpxF produce lipid A lacking the 1-phosphate[13] or the 4′-phosphate[14], respectively. MLK1067 produces LPS containing a penta-acylated lipid A due to the inactivation of gene *lpxM*[10]. MKV15b produces tetra-acylated lipid A because three genes *lpxL*, *lpxM* and *lpxP* which encode enzymes adding the secondary fatty acid chains to the lipid A were inactivated[11,22−23]. In addition, we

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Table 2 The enzymes and genes related to LPS modifications
表 2 修饰脂多糖结构的酶和基因

Enzymes 酶	Genes 基因	Function 功能
WaaC	*waaC*	WaaC catalyzes the addition of the first l-*glycero*-D-*manno*-heptose (heptose) molecule to one 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue of the Kdo-lipid A molecule[20]
WaaF	*waaF*	WaaF adds Hep II to Hep I to complete inner-core backbone[21]
LpxL	*lpxL*	LpxL adds a secondary lauroyl residue to the fatty acid chain at 2′-position of lipid A[22]
LpxM	*lpxM*	LpxM adds a secondary myristoyl residue to the fatty acid chain at 3′-position of lipid A[22]
LpxP	*lpxP*	LpxP adds palmitoleate residue to the fatty acid chain at 2′-position of lipid A[23]
LpxE	*lpxE*	LpxE removes the phosphate group from the 1-position of lipid A[13]
LpxF	*lpxF*	LpxF removes the phosphate group from the 4′-position of lipid A[14]
PagL	*pagL*	PagL removes the 3-O-linked acyl chain of lipid A[24]
PagP	*pagP*	PagP transfers a palmitate from glycerophospholipids to the 2-position of of lipid A[25]

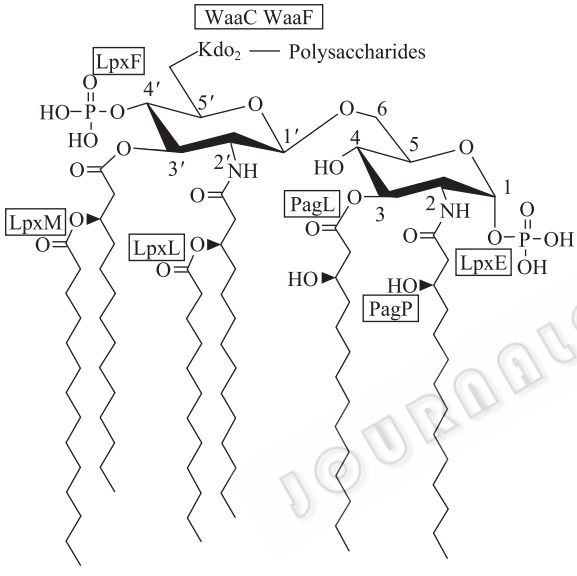

Fig. 1 Structure of LPS of wild type *E. coli* W3110
图 1 野生型大肠杆菌 W3110 脂多糖的结构图

Note: The detailed structure of lipid A is given, and the function sites for enzymes mentioned in the text are labeled.

constructed strains of W3110/pWSK29-PagL and W3110/pWSK29-PagP. The former produces penta-acylated lipid A[26], while the latter produces a hepta-acylated lipid A of LPS.

The growth curves of all *E. coli* strains used in this study are shown in Fig. 2. All strains grew well, although there are differences. The best growth is the wild type; and the worst is W3110/pWSK29-LpxF.

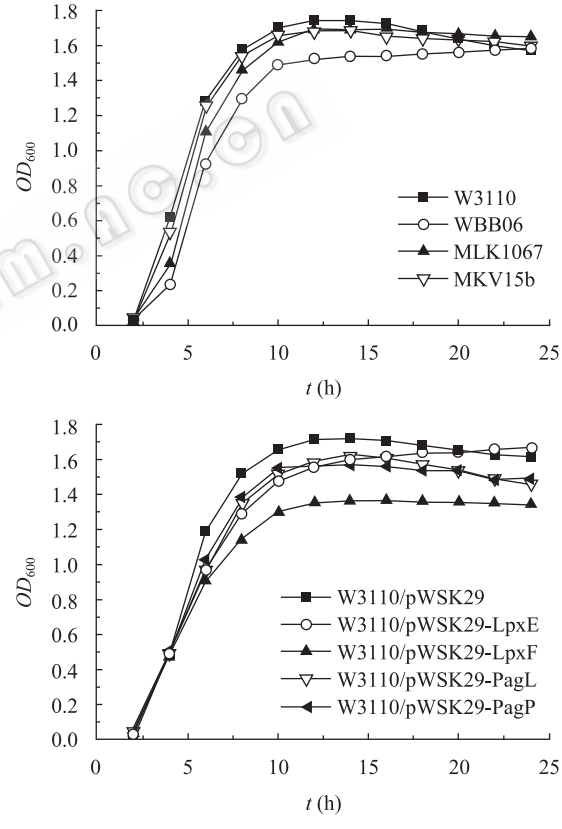

Fig. 2 Growth curves of nine different *E. coli* strains
图 2 9 种大肠杆菌的生长曲线

The major differences of LPS structure of the above nine *E. coli* strains were focused on the Kdo_2-lipid A moiety. The lipid A or Kdo_2-lipid A were extracted from the above strains and their structures were confirmed by ESI/MS (Fig.

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3)[9−11,13−14,22−25]. The peak at *m/z* 1 797.3 was derived from the wild type hexa-acylated lipid A. For W3110, W3110/pWSK29, W3110/pWSK29-LpxE and W3110/pWSK29-PagP, the peak at *m/z* 1 717.3 could be derived from the wild type hexa-acylated lipid A by the loss of the 1-phosphate. Other labeled peaks corresponded to the major lipid A of the strains. Compared with the spectrum of lipid A from W3110/pWSK29 (Fig. 3), a major peak at *m/z* 1 490.1 was shown in the spectrum of lipid A from W3110/pWSK29-PagL, it could be the molecular ion of a penta-acylated lipid A. The minor peak at *m/z* 1 717.3 could be derived from the wild type hexa-acylated lipid A by the loss of the 1-phosphate during hydrolysis[18], suggesting that there were some lipid A not hydrolyzed by PagL in the cell. In the spectrum of lipid A from W3110/pWSK29-PagP, the minor peak at *m/z* 1 955.4 could be the PagP product, the hepta-acylated lipid A, while the major peak at *m/z* 1 717.3 could be from the wild type hexa-acylated lipid A by the loss of the 1-phosphate. This suggests that the function of PagP is somehow inhibited in *E. coli*, consistent with the previous publication[27].

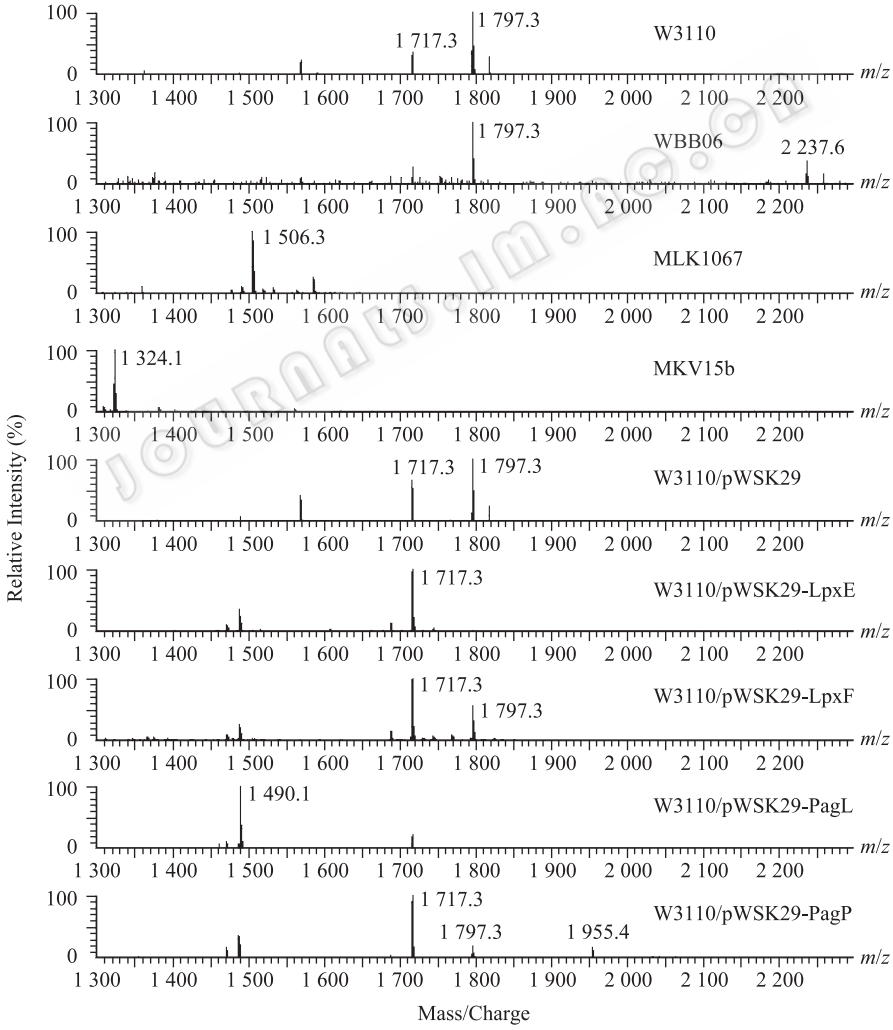

Fig. 3 ESI/MS analysis of lipid A extracted from nine *E. coli* strains

图 3 9 种大肠杆菌的类脂 A 质谱分析图

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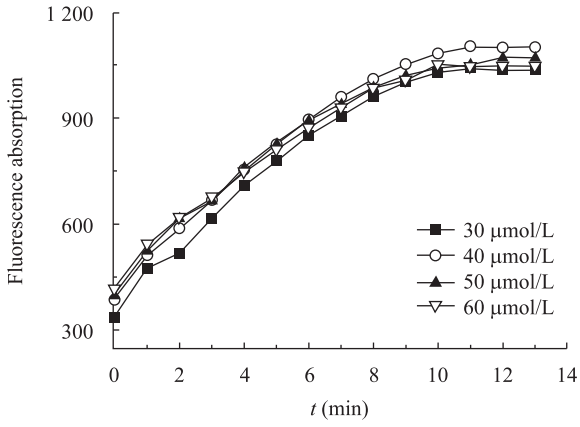

Fig. 4 The effect of NPN concentration on the fluorescence absorption of *E. coli*

图 4 不同浓度 NPN 对大肠杆菌荧光吸收值的影响

Note: Using the strain MLK1067 as an example.

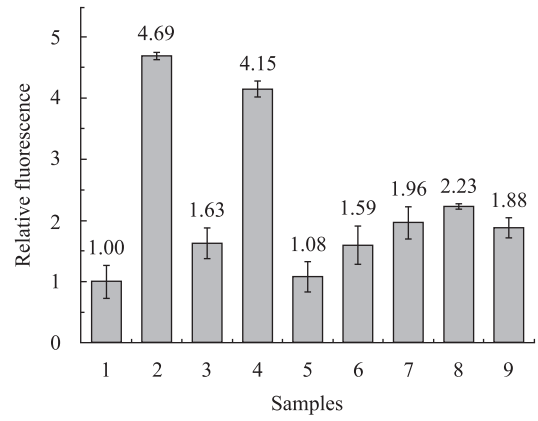

Fig. 5 Relative fluorescence of nine different *E. coli* strains after uptaking NPN

图 5 9 种大肠杆菌经 NPN 处理之后的荧光吸收值

Note: 1: W3110; 2: WBB06; 3: MLK1067; 4: MKV15b; 5: W3110/pWSK29; 6: W3110/pWSK29-LpxE; 7: W3110/pWSK29-LpxF; 8: W3110/pWSK29-PagL; 9: W3110/pWSK29-PagP.

3.2 The effect of the structure of lipid A on the cell membrane permeability

To study the effect of the structure of LPS on the membrane permeability of *E. coli*, nine strains which could make different structures of LPS were investigated by using the hydrophobic NPN as a probe[19]. First, the concentration of NPN added into the assay was optimized. Different concentrations of 30, 40, 50, 60 μmol/L NPN were tested, and the optimum concentration was 40 μmol/L. Fig. 4 showed the dependence of permeability on the concentration of NPN, using *E. coli* mutant MLK1067 as an example.

The *E. coli* mutant WBB06 makes Kdo_2-lipid A, the shortest version of LPS. The membrane permeability of WBB06 was about 5 times larger than that of the wild-type strain W3110 (Fig. 5), suggesting that length of polysaccharide in LPS plays an important role in the permeability barrier of *E. coli*. Decreasing the length of polysaccharide in LPS not only increases the accessibility of the outer membrane, but also changes the phase behavior[28] and fluidity of lipid A[29].

Based on the NPN assay, *E. coli* mutants MLK1067 and W3110/pWSK29-PagL had larger permeability than wild type W3110 and W3110/pWSK29, indicating that the number of fatty acid chains in LPS plays a role on the permeability of cell membranes. This is further supported by the NPN assay on MKV15b. MKV15b produced tetra-acylated lipid A, and its permeability is larger that that of MLK1067 and W3110/pWSK29-PagL. The latter two mutant strains produced penta-acylated lipid A. The permeability of W3110/pWSK29-PagP is also larger than that of W3110/pWSK29, even though most of the lipid A in W3110/pWSK29-PagP were not modified (Fig. 3). Put together, any changes on the number and positions of fatty acid chains on lipid A of LPS could increase the permeability of *E. coli* cells, possibly by enhancing the fluidity of membranes[29].

There are two phosphate groups in the structure of lipid A, 1-phosphate and 4′-phosphate (Fig. 1). Their effect on the permeability of *E. coli* were examined by using mutant strains of W3110/pWSK29-LpxE and W3110/pWSK29-LpxF. LpxE and LpxF are enzymes found in *Francisella no-*

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vicida which selectively remove the 1-phosphate and 4'-phosphate, respectively[13−14]. The permeability of both mutant cells was slightly higher than that of W3110/pWSK29 as shown in Fig. 4. The phosphate groups of LPS might bind divalent cations to protect the integrality of cell membrane. The permeability of W3110/pWSK29-LpxF is larger than that of W3110/pWSK29-LpxE, suggesting the 4'-phosphate group of lipid A is more critical than the 1-phosphate for the integrality of outer membrane.

4 Conclusions

In this study, the effect of LPS structure on the permeability of outer membranes of *E. coli* has been systematically studied. Nine *E. coli* strains which make different structures of LPS were selected or constructed, and their outer membrane permeability analyzed. The outer membranes of wild type *E. coli* showed the least permeability, while mutants of *E. coli* in which LPS structures were changed by the mutation of genes involved in LPS biosynthesis pathway showed higher permeability, suggesting that *E. coli* has evolved the perfect LPS structure as the permeability barrier. The number of phosphate groups and the fatty acid chains in the lipid A of LPS, and the length of the polysaccharide of LPS all affect the permeability of the outer membranes of *E. coli*. The chain length of the polysaccharide of LPS has the largest effect on the permeability, followed by the number of the fatty acid chains of LPS. These results would be useful for designing the membrane structure of industrial microorganism to improve their permeability and increase the yield of products.

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