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Effects of lipopolysaccharide structure on lycopene production in *Escherichia coli*

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**Abstract**

Lipopolysaccharides, the major molecules in the outer membrane of *Escherichia coli*, affect the behavior of bacteria including outer membrane permeability, but its influence on lycopene production in *E. coli* has never been reported. In this study, the effects of lipopolysaccharides with different structures on lycopene biosynthesis were investigated. Firstly, the heterogenous *crtEBI* operon were overexpressed in 10 LPS mutant strains of *E. coli* W3110 (Δ*waaC*, Δ*waaP*, Δ*waaY*, Δ*waaG*, Δ*waaU*, Δ*waaO*, Δ*waaR*) and their ability to produce lycopene were compared. Δ*waaC*:pWSK29-*crtEBI*, Δ*waaF*:pWSK29-*crtEBI* and Δ*waaY*:pWSK29-*crtEBI* produced 4.19, 4.20, and 3.81 mg/g lycopene, respectively, while the control W3110/pWSK29-*crtEBI* produced 3.71 mg/g lycopene; the other strains produced less lycopene than the control. In order to enhance lycopene production, genes *dxr*, *dxr*, *ispA*, and *idi* were overexpressed in Δ*waaC*:pWSK29-*crtEBI*, Δ*waaF*:pWSK29-*crtEBI* individually or in combination, and the lycopene production in each strain was analyzed. The maximum yield of 5.39 mg/g was achieved in Δ*waaC*:pWSK29-*crtEBI*-SRA, which is 142% higher than that in W3110/pWSK29-*crtEBI*. The results indicate that the length of lipopolysaccharide affects lycopene biosynthesis in *E. coli*, and the shorter lipopolysaccharide and higher outer membrane permeability might be beneficial to lycopene bio-synthesis.

1. Introduction

*Escherichia coli* is an important platform for protein expressing and various product biosynthesis [1], however, the limited permeability of its outer membrane hinders the performance of *E. coli* as whole-cell biocatalyst [2]. The expression of SARS coronavirus small envelope protein [2] and deletion of *lpp* encoding lipoprotein in *E. coli* [3] were effective for increasing the outer membrane permeability. We have constructed *E. coli* mutants that could synthesize different length of lipopolysaccharides (LPS), the major molecules in the outer membrane, and found that the structure of LPS is closely relevant to the outer membrane permeability [4].

Lycopene is a bright red carotenoid pigment with 11 conjugated double bonds, and is an efficient singlet oxygen quencher. Recent advance in metabolic engineering has provided a very promising route for the heterologous production of lycopene within various microorganisms. *E. coli* has been widely utilized as microbial cell factory for the synthesis of various carotenoids [5]. *E. coli* can naturally synthesize (2E, 6E)-farnesyl diphosphate (FPP) from 3-phospho-α-glycerate and pyruvate through a series of catalytic reactions; expressing heterologous *crtE*, *crtB* and *crtI* in *E. coli* can convert FPP into lycopene (Fig. 1).

Different types of plasmids have been used to carry the genes *crtE*, *crtB* and *crtI* in *E. coli* [6]. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are synthesized via MEP pathway in *E. coli* [7]. In the MEP pathway, isopentenyl diphosphate isomerase encoded by *idi*, 1-deoxyxylulose-5-phosphate synthase encoded by *dxr*, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase encoded by *ispD*, and 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase encoded by *ispF* play important roles; therefore, over-expressing these genes has been used to elevate isoprenoid accumulation [5,8,9].

For example, overexpressing *idi* could significantly stimulate carotenoid synthesis [10–13]. Enhancing the gene expression by chromosomal promoter replacement [14] or introduction of a heterologous mevalonate pathway to increase IPP and DMAPP supply [15] have also been used for carotenoid synthesis.

In this study, a series of *E. coli* LPS mutants strains [4] were used to...
phosphate synthase; CrtE, geranylgeranyl pyrophosphate synthase subunit; CrtB, phytoene synthase; CrtI, phytoene dehydrogenase; G3P, 3-phospho-1-glycerate; DXP, 1-deoxy-d-xylulose 5-phosphate; MEP, 2-C-methyl-d-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; DMAAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, (2E,6E)-farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

Table 1

| Strains or Plasmids | Description | Source |
|---------------------|-------------|--------|
| W3110               | Wild type E. coli, F−, λ− | Novagen |
| ATCC14067           | Wild type Corynebacterium glutamicum | ATCC |
| ΔwaoC               | W3110 ΔwaoC | [4] |
| ΔwaoF               | W3110 ΔwaoF | [4] |
| ΔwaoG               | W3110 ΔwaoG | [4] |
| ΔwaoO               | W3110 ΔwaoO | [4] |
| ΔwaoR               | W3110 ΔwaoR | [4] |
| ΔwaoU               | W3110 ΔwaoU | [4] |
| ΔwaoP               | W3110 ΔwaoP | [4] |
| ΔwaoQ               | W3110 ΔwaoQ | [4] |
| ΔwaoY               | W3110 ΔwaoY | [4] |
| ΔwaoB               | W3110 ΔwaoB | [4] |
| ΔwaoC/pWSK29-crtEBI | ΔwaoC harboring pWSK29-crtEBI | This work |
| ΔwaoF/pWSK29-crtEBI | ΔwaoF harboring pWSK29-crtEBI | This work |
| ΔwaoG/pWSK29-crtEBI | ΔwaoG harboring pWSK29-crtEBI | This work |
| ΔwaoO/pWSK29-crtEBI | ΔwaoO harboring pWSK29-crtEBI | This work |
| ΔwaoR/pWSK29-crtEBI | ΔwaoR harboring pWSK29-crtEBI | This work |
| ΔwaoU/pWSK29-crtEBI | ΔwaoU harboring pWSK29-crtEBI | This work |
| ΔwaoP/pWSK29-crtEBI | ΔwaoP harboring pWSK29-crtEBI | This work |
| ΔwaoQ/pWSK29-crtEBI | ΔwaoQ harboring pWSK29-crtEBI | This work |
| ΔwaoY/pWSK29-crtEBI | ΔwaoY harboring pWSK29-crtEBI | This work |
| ΔwaoB/pWSK29-crtEBI | ΔwaoB harboring pWSK29-crtEBI | This work |
| ΔwaoC/pWSK29-crtEBI-ispA | ΔwaoC harboring pWSK29-crtEBI-ispA | This work |
| ΔwaoF/pWSK29-crtEBI-ispA | ΔwaoF harboring pWSK29-crtEBI-ispA | This work |
| ΔwaoG/pWSK29-crtEBI-dxs | ΔwaoG harboring pWSK29-crtEBI-dxs | This work |
| ΔwaoF/pWSK29-crtEBI-dxs | ΔwaoF harboring pWSK29-crtEBI-dxs | This work |
| ΔwaoC/pWSK29-crtEBI-dxr | ΔwaoC harboring pWSK29-crtEBI-dxr | This work |
| ΔwaoF/pWSK29-crtEBI-dxr | ΔwaoF harboring pWSK29-crtEBI-dxr | This work |
| ΔwaoC/pWSK29-crtEBI-SR | ΔwaoC harboring pWSK29-crtEBI-SR | This work |
| ΔwaoF/pWSK29-crtEBI-SR | ΔwaoF harboring pWSK29-crtEBI-SR | This work |
| ΔwaoC/pWSK29-crtEBI-SRA | ΔwaoC harboring pWSK29-crtEBI-SRA | This work |
| ΔwaoF/pWSK29-crtEBI-SRA | ΔwaoF harboring pWSK29-crtEBI-SRA | This work |
| ΔwaoC/pWSK29-crtEBI-ispA/pACYC184-SRA | ΔwaoC harboring pWSK29-crtEBI and pACYC184-SRA | This work |
| ΔwaoF/pWSK29-crtEBI-pACYC184-SRA | ΔwaoF harboring the pWSK29-crtEBI and pACYC184-SRA | This work |
| ΔwaoC/pWSK29-crtEBI/pACYC184-SRAI | ΔwaoC harboring the pWSK29-crtEBI and pACYC184-SRAI | This work |
| ΔwaoF/pWSK29-crtEBI/pACYC184-SRAI | ΔwaoF harboring the pWSK29-crtEBI and pACYC184-SRAI | This work |

Plasmids

| plp1282 | Expression vector | [26] |
| pACYC184 | Expression vector | [27] |
| pBlueScript II SK+ | Expression vector | Stratagene |
| pWSK29 | pWSK29 harboring the crtEBI operon from C. glutamicum ATCC14067 | This work |
| pACYC184-pACYC184 | pACYC184 harboring the genes ispA, dxa and dxr from W3110 | This work |
| pACYC184-DA-1| pACYC184 harboring the genes dxa, ddr and ddi from W3110 | This work |
Fig. 2. Maps of different plasmids used in this study. The genes *crtE*, *crtI*, and *crtB* were amplified from the genome of *Corynebacterium glutamicum* ATCC14067.
synthesize lycopene by overexpressing the genes \textit{crtE}, \textit{crtB}, \textit{dxr}, \textit{dxs} and \textit{ispA}, and the effects of different structures of LPS on lycopene biosynthesis were investigated.

2. Material and methods

2.1. Strains, media and growth conditions

All \textit{E. coli} strains used in this study were listed in Table 1. \textit{E. coli} strains were grown in LB medium (10 g/L trypton, 5 g/L yeast extract, 10 g/L NaCl) at 37°C or 30°C at 200 rpm. LB medium or 2×YT+G (10 g/L NaCl, 1 g/L glycerol), was used for shaking flask. When necessary, the medium was supplemented with ampicillin (100 μg/mL), kanamycin (30 μg/mL) or chloramphenicol (30 μg/mL) for plasmid maintenance or strain selection. Isopropyl-β-D-thiogalactoside (IPTG) or arabinose were used as the inducer.

2.2. Construction of expression plasmids and recombinant strains

The maps of different plasmids carrying the genes \textit{crtE}, \textit{crtB}, \textit{dxr}, \textit{dxs} or \textit{ispA} individually or in combination are shown in Fig. 2. Restriction enzymes, shrimp alkaline phosphatase, T4 DNA ligase and plasmid DNA ladder were purchased from Sangon (Shanghai, China). Plasmid DNA was prepared by using the EZ-10 spin column plasmid mini-preps (Beijing, China). Primersynthesis and DNA sequencing were performed with the corresponding restriction enzymes and ligated with the vector pWSK29 or pACYC184 similarly digested, resulting in the plasmids pWSK29-\textit{crtEBI}, pWSK29-\textit{crtEBI-ispA}, pWSK29-\textit{crtEBI-dxr}, pWSK29-\textit{crtEBI-SRA}, pACYC184-SRA, and pACYC184-SRAI, respectively.

The plasmid pWSK29-\textit{crtEBI} was transformed into \textit{E. coli} W3110 and its mutant strains \textit{ΔwaaC}, \textit{ΔwaaF}, \textit{ΔwaaG}, \textit{ΔwaaO}, \textit{ΔwaaR}, \textit{ΔwaaU}, \textit{ΔwaaP}, \textit{ΔwaaQ}, \textit{ΔwaaY}, and \textit{ΔwaaB}, resulting in the strains \textit{ΔwaaC}/pWSK29-\textit{crtEBI}, \textit{ΔwaaF}/pWSK29-\textit{crtEBI}, \textit{ΔwaaG}/pWSK29-\textit{crtEBI}, \textit{ΔwaaO}/pWSK29-\textit{crtEBI}, \textit{ΔwaaR}/pWSK29-\textit{crtEBI}, \textit{ΔwaaU}/pWSK29-\textit{crtEBI}, \textit{ΔwaaP}/pWSK29-\textit{crtEBI}, \textit{ΔwaaQ}/pWSK29-\textit{crtEBI}, \textit{ΔwaaA}/pWSK29-\textit{crtEBI}, \textit{ΔwaaB}/pWSK29-\textit{crtEBI}. The plasmids pWSK29-\textit{crtEBI-ispA}, pWSK29-\textit{crtEBI-dxr}, pWSK29-\textit{crtEBI-SRA} and pWSK29-\textit{crtEBI-SRAI} was transformed into \textit{ΔwaaC} and \textit{ΔwaaF}, respectively, resulting in the strains \textit{ΔwaaC}/pWSK29-\textit{crtEBI}, \textit{ΔwaaF}/pWSK29-\textit{crtEBI}, \textit{ΔwaaC}/pWSK29-\textit{crtEBI-ispA}, \textit{ΔwaaF}/pWSK29-\textit{crtEBI-ispA}, \textit{ΔwaaC}/pACYC184-SRAI, \textit{ΔwaaF}/pACYC184-SRAI was transformed into \textit{ΔwaaC}/pWSK29-\textit{crtEBI}, \textit{ΔwaaF}/pACYC184-SRAI, \textit{ΔwaaC}/pACYC184-SRAI, \textit{ΔwaaF}/pACYC184-SRAI, and \textit{ΔwaaB}/pACYC184-SRAI, Transformation of \textit{E. coli} was performed according to the published protocol [16].

2.3. Analytical methods

Cell growth during the cultivations was monitored by measuring the optical density at 600 nm (OD\textsubscript{600}). For dry cell weight (DCW) determination, a known volume of fermentation broth was centrifuged for 10 min in pre-weighted test tubes at 4°C and 4000 rpm, washed once with water, and dried for 24 h at 90°C to a constant weight.

To extract lycopene, the \textit{E. coli} cells were grown in a 500-mL shaking flask at 37°C and 200 rpm, harvested by centrifugation at 4000 rpm and 4°C for 10 min, and rinsed twice with deionized water. Then the cells were suspended in 1 mL of acetone and incubated at 55°C for 15 min in the dark. The samples were centrifuged at 4000 rpm for 10 min, and the acetone supernatant containing lycopene was transferred to a clean tube. The lycopene content of the extracts was measured according to the previous reported method [8]. Lycopene (purchased from Sigma) dissolved in acetone was used as the standard. The results were the mean from three independent determinations, and the standard deviations were in the range of ± 10% of the means.

2.4. Outer membrane permeability assay

To determined outer membrane permeability of \textit{E. coli}, the fluorescent probe 1-N-phenyl-1-naphthylamine (NPN) assay was used [17]. \textit{E. coli} strains were cultivated in LB broth at 37°C, harvested by centrifugation at 4000 rpm for 10 min, washed and resuspended in phosphate buffer (10 mM, pH = 7.4). The value of OD\textsubscript{600} was adjusted to 0.5 in the final cell suspension. Then 1.92 mL of cell suspension was mixed with 80 μL NPN (1 mM) into quartz cuvette, immediately. Fluorescence
Fig. 3. A. Color comparison of different *E. coli* cells. B. Comparison of cell growth and lycopene yield in different *E. coli* cells. Data represent the average of three experiments and the error bars represent the standard deviation.
Fig. 4. Comparison of cell outer membrane permeability of different E. coli cells. Data represent the average of three experiments and the error bars represent the standard deviation.

was measured using a Fluorescence Spectrophotometer (650-60, Hitachi, Japan), using a slit width of 5 nm, an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

3. Results

3.1. Lycopene biosynthesis in E. coli W3110 overexpressing the genes crtE, crtB, and crtI from C. glutamicum is affected by LPS structure

LPS mutants ΔwaaC, ΔwaaF, ΔwaaG, ΔwaaO, ΔwaaR, ΔwaaU, ΔwaaP, ΔwaaQ, ΔwaaY and ΔwaaB from E. coli W3110 synthesized different length of LPS and showed different outer membrane permeability [4]. In order to effect LPS structure on lycopene biosynthesis, pWSK29-crtEBI and pWSK29 were transformed into these strains and their lycopene productions were investigated [19].

As shown in Fig. 3A, the color of the bacterial cells can be used to determine the amount of lycopene. W3110/pWSK29-crtEBI cells were red, but not W3110 and W3110/pWSK29 cells, suggesting that lycopene was produced only when the genes crtE, crtB, and crtI from C. glutamicum were overexpressed. ΔwaaC/pWSK29-crtEBI, ΔwaaF/pWSK29-crtEBI and ΔwaaY/pWSK29-crtEBI cells were also red, ΔwaaG/pWSK29-crtEBI cells were light red, suggesting lycopene was synthesized in these cells. These results indicate that LPS structure affects lycopene synthesis in E. coli. These cells were broken and lycopene was extracted to quantify. As shown in Fig. 3B, all the strains harboring pWSK29-crtEBI can synthesize lycopene, but more lycopene was produced in ΔwaaC/pWSK29-crtEBI (4.19 mg/g), ΔwaaF/pWSK29-crtEBI (4.20 mg/g), ΔwaaY/pWSK29-crtEBI (3.81 mg/g). This quantification is consistent with the red shades of the cells (Fig. 3A vs B). Based on their OD600 value, the cell growth was retarded when pWSK29-crtEBI was introduced in E. coli. By comparison, ΔwaaC/pWSK29-crtEBI and ΔwaaF/pWSK29-crtEBI grew better and produced more lycopene than other strains, therefore, they were used in further study.

The outer membrane permeability of the 10 E. coli LPS mutants ΔwaaC, ΔwaaF, ΔwaaG, ΔwaaO, ΔwaaR, ΔwaaU, ΔwaaP, ΔwaaQ, ΔwaaY and ΔwaaB with or without pWSK29-crtEBI were analyzed, using the wild type W3110 as the control (Fig. 4). Similar outer membrane permeabilities were observe for the same strain with or without pWSK29-crtEBI, suggesting that the lycopene produced in the strains ΔwaaC/pWSK29-crtEBI, ΔwaaF/pWSK29-crtEBI, ΔwaaG/pWSK29-crtEBI, ΔwaaO/pWSK29-crtEBI, ΔwaaR/pWSK29-crtEBI, ΔwaaU/pWSK29-crtEBI, ΔwaaP/pWSK29-crtEBI, ΔwaaQ/pWSK29-crtEBI, ΔwaaY/pWSK29-crtEBI and ΔwaaB/pWSK29-crtEBI does not affect the outer membrane permeability and might locate in the inner membranes. ΔwaaC/pWSK29-crtEBI showed high outer membrane permeability and produced a large amount of lycopene, but the outer membrane permeability and the lycopene production were not proportionally increased for other strains such as ΔwaaY/pWSK29-crtEBI and ΔwaaF/pWSK29-crtEBI. The results suggested that outer membrane permeability is not the only factor affecting lycopene production in E. coli.

3.2. More lycopene was produced in ΔwaaC/pWSK29-crtEBI and ΔwaaF/pWSK29-crtEBI after enhancing the MEP pathway

To further improve lycopene yield, the key genes dxr, dxr, ispA and idi in the MEP pathway were overexpressed, individually or in combination, in ΔwaaC/pWSK29-crtEBI and ΔwaaF/pWSK29-crtEBI; the cell growth and lycopene production in these recombinant strains were investigated (Fig. 5).

ΔwaaC/pWSK29-crtEBI-ispA, ΔwaaC/pWSK29-crtEBI-dxS, and ΔwaaC/pWSK29-crtEBI-dxr produced 4.53, 4.81 and 4.61 mg/g lycopene, respectively; ΔwaaC/pWSK29-crtEBI-SRA, ΔwaaC/pWSK29-crtEBI-SRA, ΔwaaC/pWSK29-crtEBI/SRA, and ΔwaaC/pWSK29-crtEBI/SRA produced 5.17, 5.39, 5.08 and 5.14 mg/g lycopene, respectively (Fig. 5). Compared to ΔwaaC/pWSK29-crtEBI, these strains grew better and produced more lycopene, suggesting that enhancing the MEP pathway facilitates lycopene synthesis in E. coli.

ΔwaaF/pWSK29-crtEBI-ispA, ΔwaaF/pWSK29-crtEBI-dxS, and ΔwaaF/pWSK29-crtEBI-dxr produced 4.35, 4.29 and 4.22 mg/g lycopene, respectively; ΔwaaF/pWSK29-crtEBI-SRA, ΔwaaF/pWSK29-crtEBI/SRA, ΔwaaF/pWSK29-crtEBI/SRA, and ΔwaaF/pWSK29-crtEBI/SRA produced 4.95, 5.28, 4.87 and 5.08 mg/g lycopene, respectively. Compared to ΔwaaF/pWSK29-crtEBI, these strains produced more lycopene, suggesting again that enhancing the MEP pathway facilitates lycopene synthesis in E. coli.

4. Discussion

Biocatalysis employing the whole cell had been increasingly developed as a green technology in the synthesis of various valuable products, while low permeability of cell membrane always lead to the low productivities [3]. The outer membrane of gram-negative bacteria provided the cell with an effective permeability barrier against external noxious agents [18], and at the same time to allow the influx of nutrient molecules [19]. In the previous publications, lpp deletion was developed as a general permeabilization method. The lpp mutant had higher permeability of outer membrane and higher ability to synthesize L-carnitine [3]. Besides, expression of SCVE viroporins in E. coli also improved the diffusivity of small molecules across outer membrane through introducing additional pores within the outer membrane. As expected, the biocatalysts efficiency of E. coli was enhanced [2].

LPS, as the major molecule in outer membrane, played important roles on membrane behavior. In E. coli, LPS typically consists of a hydrophobic domain known as lipid A, a nonrepeating core oligosaccharide, and a distal polysaccharide known as O-antigen repeats. The core oligosaccharide is assembled on lipid A via sequential glycosyl transfer from nucleotide sugar precursors. In E. coli, the chromosomal waa locus encodes enzymes required for biosynthesis of the core oligosaccharide [20]. Mutations in LPS could alter outer membrane stability, giving rise to pleiotropic phenotype [21,22]. Among 10 W3110 Δwaa mutant strains, W3110 ΔwaaC has the simplest LPS structure. In compared to W3110, W3110 ΔwaaC had four-fold higher membrane permeability, and this likely benefited for lycopene accumulation. Comparative transcriptome showed that mRNA levels of dxr and ispA in ΔwaaC and ΔwaaF were up-regulated, compared to W3110 (data not shown). The over-expression of the dxr gene provided more IPP precursors in the MEP pathway in tobacco, which consequently stimulated synthesis efficiency of isoprenoid downstream [23]. IPP and DMAPP
supply likely limited lycopene biosynthesis [9]. Besides, the accumulation of metabolites in the MEP pathway stimulated the transcription of \( dxs \) and \( dxr \) in \textit{Arabidopsis} cell culture [24]. In the current study, therefore, the genes \( dxs \), \( dxr \) and \( idi \) were overexpressed individually or in combination, to increase these precursors supply. As expected, the overexpression of genes in MEP pathway stimulated lycopene synthesis [25].

A recombinant \textit{E. coli} strain overexpressing the genes \( crtE \), \( crtB \), and \( crtI \) from \textit{Deinococcus radiodurans} R12 and optimizing the Shine-Dalgarno regions and aligned spacing sequence could produce 88 mg/g lycopene after 40 h fermentation [28]. \textit{E. coli} K12f could efficiently uptake fructose; overexpressing the \( crtEBI \) operon from \textit{Pantoea anaerobia} in K12f could produce 192 mg/g lycopene when grown on LB medium containing 10 g/L fructose [29]. Therefore, overexpressing the \( crtEBI \) operon from other bacteria in \( \Delta \text{waaC} \) or \( \Delta \text{waaF} \) might increase lycopene production.

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