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ORIGINAL RESEARCH PAPER

Expression of lycopene biosynthesis genes fused in line with Shine-Dalgarno sequences improves the stress-tolerance of *Lactococcus lactis*

Xiangrong Dong · Yanping Wang · Fengyuan Yang · Shanshan Zhao · Bing Tian · Tao Li

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Abstract
Objectives Lycopene biosynthetic genes from *Deinococcus radiodurans* were co-expressed in *Lactococcus lactis* to produce lycopene and improve its tolerance to stress.
Results Lycopene-related genes from *D. radiodurans*, DR1395 (*crtE*), DR0862 (*crtB*), and DR0861 (*crtI*), were fused in line with S hine-Dalgarno (SD) sequences and co-expressed in *L. lactis*. The recombinant strain produced 0.36 mg lycopene g^{-1} dry cell wt after 48 h fermentation. The survival rate to UV irradiation of the recombinant strain was higher than that of the non-transformed strain.
Conclusion The *L. lactis* with co-expressed genes responsible for lycopene biosynthesis from *D. radiodurans* produced lycopene and exhibited increased resistance to UV stress, suggesting that the recombinant strain has important application potential in food industry.

Keywords *Deinococcus radiodurans* · *Lactococcus lactis* · Lycopene biosynthesis genes · Shine-Dalgarno sequence · Stress-tolerance

Introduction

Lycopene is a C40 carotenoid with scavenging activity of reactive oxygen species for cellular protection. In non-phototrophic bacteria, lycopene is synthesized by geranylgeranyl diphosphate synthase (CrtE), phytoene synthase (CrtB) and phytoene desaturase (CrtI). Microorganisms without related carotenoid biosynthetic genes, e.g., *Escherichia coli* and *Pichia pastoris*, have been used for large-scale carotenoid production by the heterologous expression of carotenoid-related genes (Jin et al. 2015; Misawa et al. 1990; Araya-Garay et al. 2012). *Lactococcus lactis*, a non-lycopene-producing bacterium, can produce lactic acid which is widely used as a flavorant and preservative in foods and as a raw material for pharmaceuticals (Gaspar et al. 2013). As one of the probiotics in the human intestine, lactic acid bacteria (LABs) provide nutritive benefits for human or animal health. LABs are often exposed to oxidative stress and osmotic pressure (e.g., salt and acid) during industrial fermentation and transfer in gastrointestinal tract

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X. Dong · Y. Wang (✉) · F. Yang · S. Zhao
Henan Key Laboratory of Ion-beam Bioengineering, College of Physics and Engineering, Zhengzhou University, Zhengzhou 450001, China
e-mail: wyp@zzu.edu.cn

B. Tian · T. Li
Key Laboratory for Nuclear-Agricultural Sciences of Chinese Ministry of Agriculture and Zhejiang Province, Institute of Nuclear-Agricultural Sciences, Zhejiang University, Hangzhou 310029, China

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(Corcoran et al. 2008). Lycopene production via the heterologous expression of lycopene genes might improve the stress-tolerance and functional potential of LABs.

Lycopene biosynthetic genes encoding DR1395 (CrtE), DR0862 (CrtB) and DR0861 (CrtI) have been identified in *Deinococcus radiodurans* (Bing et al. 2007), which is a well-known for its extreme resistance to radiation and oxidants, In the present study, the lycopene biosynthetic genes were co-expressed in *L. lactis* MG1363 by fusion in line with Shine-Dalgarno (SD) sequences. Lycopene production and survival of the lycopene-expressing LAB under stresses (UV radiation, salt and extreme pH) were investigated. Lycopene production of the recombinant strain was optimized by changing fermentation conditions.

Materials and methods

Strains and growth conditions

Wild-type *D. radiodurans* strain R1 (lab stock) was grown in TGY broth (0.5 % Bactotryptone, 0.1 % glucose, 0.3 % Bacto yeast extract) at 32 °C with shaking (200 rpm) or on TGY plates solidified with 1.5 % (w/v) agar. pGEM-T Easy vector was purchased from Promega (Madison, WI, USA). *E. coli* DH5 cells was grown at 37 °C in lysogeny broth (LB) or on LB plates solidified with 1.5 % (w/v) agar. *Lactococcus lactis* MG1363 was cultured in M17 medium (Difco) supplemented with 0.5 % (w/v) glucose (GM17) and broth at 30 °C as stand cultures. Bacterial growth was monitored from the OD_{600} value.

Lycopene gene cloning, vector construction and transformation

Genomic DNA from *D. radiodurans* was extracted following the standard protocol of a DNA extraction kit. PCR primers were designed based on *D. radiodurans* lycopene- synthesizing genes deposited in GenBank (DR1395, DR0861 and DR0862) (Supplementary Table 1). Thermal cycling conditions were: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 50 s, 60 °C for 40 s and 72 °C for 90 s; then extension at 72 °C for 5 min. The purified PCR product was cloned into a pMD18-T vector to form a recombinant plasmid, pMD-EBI, which was transformed into competent *E. coli* DH5. Agarose plates containing 100 μg ampicillin ml^{-1} were used for antibiotic selection. *E. coli* was transformed using a modified $CaCl_2$ technique. After screening using ampicillin-containing agarose plates, positive clones were selected. Plasmids were isolated, digested with restriction enzymes for identification, and sequenced. Clones with the correct sequence were isolated and digested with *Pst*I and *Kpn*I to form gene fragments with overhanging ends. After the shuttle vector (pMG36e) was digested with the same enzymes, the recovered vector fragment and target fragment were ligated overnight. The ligated product was transformed into competent *E. coli* MC1061; positive clones were selected for expanded culture. Plasmids were isolated and digested with restriction enzymes for identification by 1 % agarose gel electrophoresis at 120 V for 50 min, followed by DNA sequencing for verification. The verified and correct clone was transformed into *L. lactis* MG1363 by electroporation and transformed with the empty vector (pMG36e) was obtained as a control.

Table 1 Lycopene yield of the MG(EBI) strain under different pH conditions

pH	Lycopene yield (mg g^{-1} DCW) (X ± SD)
4	0.19 ± 0.02
5	0.23 ± 0.01
6	0.34 ± 0.02
7	0.36 ± 0.01
8	0.32 ± 0.01
9	0.27 ± 0.01
10	0.21 ± 0.02

Values are presented as the mean ± standard deviation (SD) of three independent experiments

Lycopene production in Lactococcus lactis MG1363

L. lactis MG(EBI) was grown in 50 ml GM17 medium at 30 °C for 24 h. Ten ml was then inoculated into 1 l LB and grown at 30 °C for 48 h. Cells were collected centrifuging at 12,000×*g* at 4 °C for 5 min, washed three times with sterilize water and the cell pellet was extracted three times with cool acetone in the dark. The extraction was repeated until the cells were

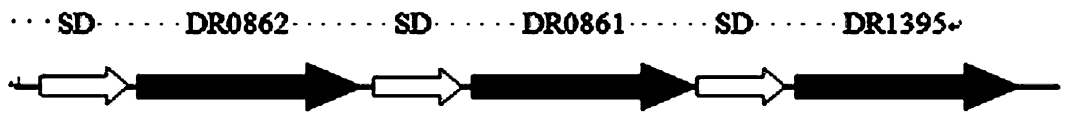

Fig. 1 The expression of lycopene biosynthetic genes in *L. lactis*. A schematic representation of *crtE*, *crtB* and *crtI*; The lycopene synthetic genes was ligated into the shuttle vector pMG36e and obtained the pMG-EBI, which was transferred into *L. lactis* MG1363

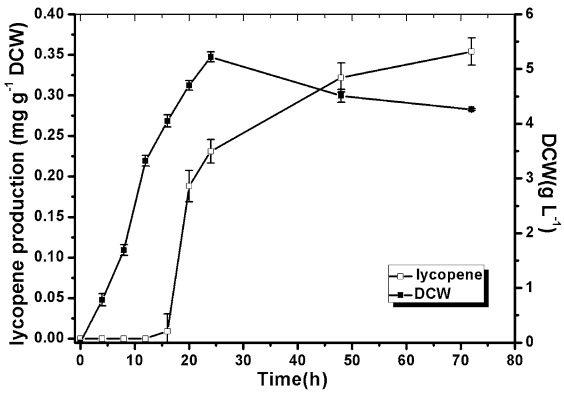

Fig. 2 Cell growth and lycopene production of the strain MG(EBI) in GM17 medium at 30 °C for 72 h. Values are presented as the mean ± standard deviation (SD) of three independent experiments

colorless (Bing et al. 2007). The collected supernatants were analyzed by HPLC using a Hypersil ODS-C18 column (4.6 × 150 mm^2, 5 μm) at 470 nm and eluted with acetonitrile/methanol/2-propanol (40:50:10, by vol) at 1 ml min^{-1}. Data were processed and analyzed using the Empower software.

Lycopene expression in LAB at different stages

The positive clones of LAB expressing the lycopene biosynthesis genes were selected and inoculated into 10 ml GM17 liquid medium (containing 500 mg erythromycin ml^{-1}). After culture overnight at 30 °C, the culture was inoculated into 1 l fresh GM17 medium and then cultured at 30 °C up to 72 h. Cell dry weights were determined gravimetrically. Lycopene was extracted from cells harvested from 10 ml culture as indicated above and measured by HPLC.

Effects of different culture conditions on the cell growth and lycopene production of LAB

To optimize the lycopene yield, 5 g sucrose or glucose l^{-1} and inorganic salts (0.1 g $CaCl_2$, KCl or $MgCl_2$ l^{-1}) were added into the GM17 medium. After 48 h growth, bacteria were collected and growth and the lycopene yields were measured. To investigate the effects of pH on the pigment synthesis and bacterial growth, the initial pH of the culture medium was adjusted to between 4 and 10 using HCl or NaOH.

Effects of NaCl on the cell growth and lycopene production of LAB

LAB expressing the lycopene-related genes was inoculated into 10 ml GM17 medium. After culture overnight at 30 °C, the bacteria were inoculated into fresh GM17 medium with 3 % (w/v) NaCl and then stationary-cultured at 30 °C. The dry cell weights and lycopene contents were then determined.

Survival of lycopene-produced LAB after exposure to UV radiation

The recombinant strains of LAB and the corresponding control strains were cultured overnight and inoculated into fresh broth until the OD_{600} was 0.5, followed by determination of the survival rate under UV radiation. Briefly, 100 μl diluted bacterial culture was spread onto plates. To ensure that there was stable irradiation from the UV light source, we conducted a 30 min preheating followed by UV irradiation for up to 60 s. Three parallel plates were used for each duration of UV irradiation and the plates without exposure to UV irradiation were used as controls. The survival rate was calculated after culture at 30 °C for 24 h.

Results

Expressions of lycopene synthetic genes in LAB

The lycopene synthetic genes were ligated into the shuttle vector pMG36e and obtained the pMG-EBI (Fig. 1). The plasmid was extracted and transferred into *L. lactis* MG1363. The lycopene gene expressing

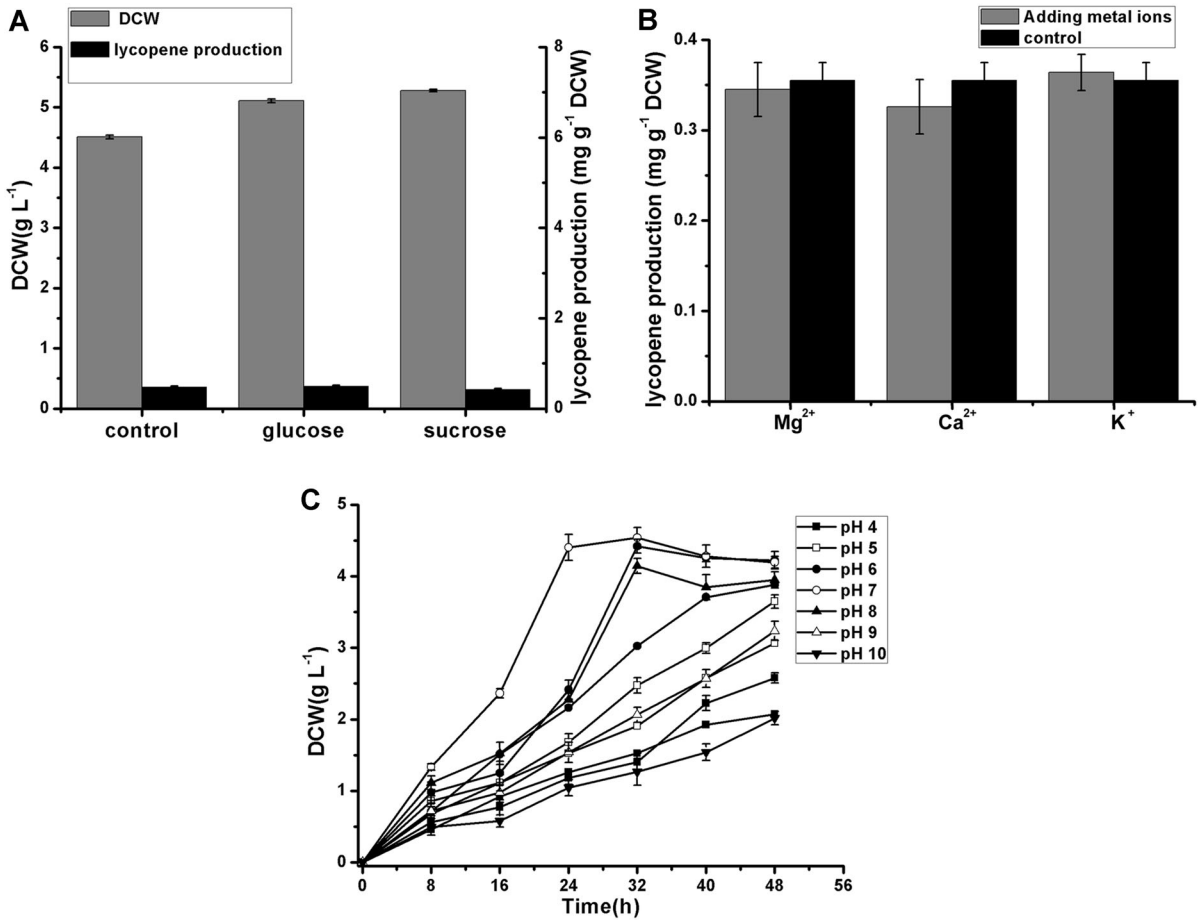

Fig. 3 Effects of culture conditions on cell growth and lycopene yield of MG(EBI). **a** Cell growth and lycopene yield of the strain MG(EBI) in GM17 medium with different carbon sources at 30 °C for 48 h. **b** The lycopene yield of strain MG(EBI) in GM17 medium with different metal ions at 30 °C for 48 h. **c** Cell growth of the strain MG(EBI) in GM17 medium with different initial pH conditions at 30 °C for 48 h. Values are presented as the mean ± standard deviation (SD) of three independent experiments

L. lactis MG1363 was designated MG(EBI), while the strain transferred with the empty plasmid pMG36e was designated MG(Vector), which was served as the control strain.

Lycopene production in LAB at different stages

Lycopene production was detected in the recombinant LAB cultures after 16 h fermentation (Fig. 2). Lycopene reached a maximum of 0.35 mg g^{-1} DCW after 72 h. This result was lower than that obtained with some other reported strains, probably due to that the LAB-*E. coli* shuttle plasmid might have a low copy number when expressed in the LAB.

Effects of different fermentation conditions on lycopene production by the recombinant strain

Compared with the control strain, strain MG(EBI) cultured in the LAB medium added with glucose or sucrose showed no significant changes in either the biomass or lycopene yield (Fig. 3a), indicating that the addition of extra carbon sources has no significant effect on the growth and lycopene production of the recombinant strain. Moreover, the supplement of K^+, Mg^{2+} and Ca^{2+} ions did not significantly affect the cell growth and lycopene yield of LAB (Fig. 3b).

Addition of 5 g glucose l^{-1} or 0.1 g KCl l^{-1} in the culture medium enhanced the lycopene yield

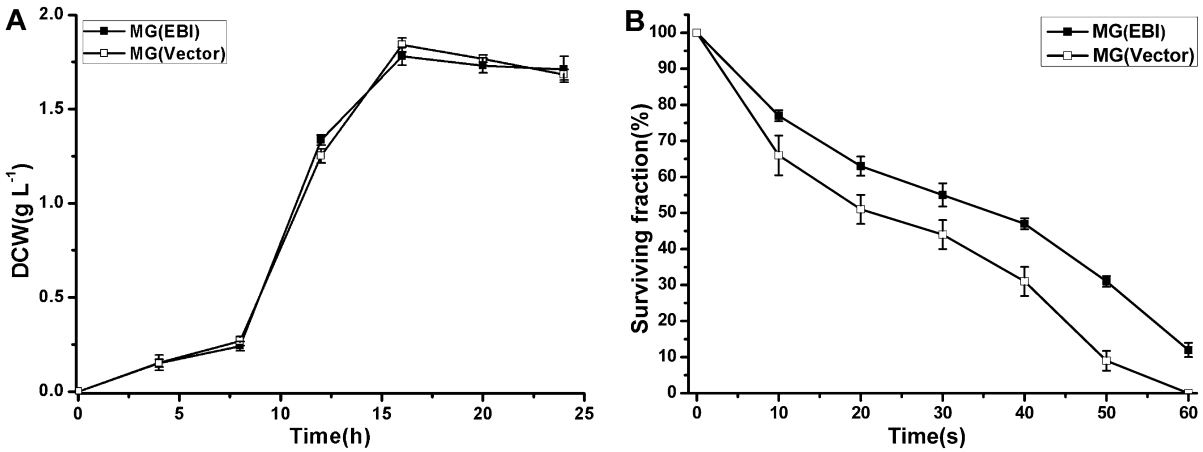

Fig. 4 Effects of different stresses on the growth of the MG(EBI) and MG(Vector) strains. **a** Cell growth of the MG(EBI) and MG(Vector) strains in GM17 medium under 3 % NaCl stress at 30 °C for 24 h. **b** Survival rates of the MG(EBI) and MG(Vector) strains after exposure to UV radiation. Values are presented as the mean ± standard deviation (SD) of three independent experiments

(0.37 mg lycopene g^{-1} DCW and 0.36 mg lycopene g^{-1} DCW, respectively).

The recombinant strain could grow in the medium at an initial pH from 4 to 10 (Fig. 3c). The optimum of cell growth was from pH 6 to 8. At pH 7, the strain reached the stationary growth phase after 24 h and the pigment yield after 48 h reached its maximum (0.36 mg g^{-1} DCW) (Table 1). However, the cell growth was slower at pH 4 and 10 and the pigment yields after 48 h were 0.19 and 0.21 mg g^{-1} DCW, respectively.

Survival of recombinant strains under different stress conditions

As shown by the biomass of bacteria present at different time points (Fig. 4a), there were no significant differences in the tolerance to 3 % (w/v) NaCl between the MG(EBI) and MG(Vector) strains.

Under UV radiation, the survival rates of MG(EBI) were higher compared with those of MG(Vector). (Fig. 4b). The survival rates of MG(EBI) and MG(Vector) were, respectively, approx.y 50 and 32 % after irradiation for 40 s; 31 and 14 % after 50 s and 9 % and almost 0 % (failed to survive) after 60 s irradiation, indicating that the lycopene-synthesizing ability of the MG(EBI) significantly enhanced its tolerance against UV stress.

Discussion

Lycopene has many physiological benefits (e.g., cancer prevention, immune enhancement and anti-aging effects) and has great value for application in food and medicine. Many efforts have been done for lycopene biosynthesis using genetic engineering techniques. Rose et al. constructed the pACCRT-EIB plasmid by inserting the lycopene-synthesizing genes originating from *Erwinia* and transferred it into *E.coli* JMl01, achieving a lycopene yield of 200–500 μg g^{-1} DCW (Rose 1988). Yamano et al. (1994) transferred the lycopene synthetic genes (crtE, crtB, and crtI) and a promoter into yeast and obtained the cells with lycopene biosynthetic ability. In this study, a lycopene-producing recombinant strain of LAB was constructed which produced lycopene and was tolerant to UV radiation. Addition of glucose or sucrose or different metal ions to the growth medium did not significantly change either the growth or lycopene yield.

Lycopene-produced LAB have great potential to improve the nutritional value of LAB as well as to explore their potential for the production of multi-functional foods. The present problems are the relatively low lycopene yield and the presence of antibiotic-resistant label in the vector carrying the lycopene genes. Further research is required to improve the

yield of lycopene in LAB by constructing a gene expression system with stronger promoter elements and use a food-grade label for recombinant screening.

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Supporting information Supplementary Table 1—The primer sequences used.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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