Chapter 6
The Improved Stress Tolerance of *Escherichia coli* by Directed Evolution of IrrE

Jianmei Luo, Jiajia Liu, Yuanyuan Zheng and Min Wang

Abstract IrrE, a global regulator of *Deinococcus radiodurans*, has proven to be effective in enhancing microbial stress tolerance. In this paper, IrrE from *D. radiodurans* R1, was introduced into *Escherichia coli* and directed evolved by error-prone PCR. The influence of mutation of IrrE on the cell growth and tolerance to various stresses was further investigated. First, one of the mutations, designated M4 with higher ethanol tolerance was obtained by error-prone PCR using the pET-28a(+)-*irrE*(W) as a template. The OD_{600} value of M4 reached 1.6 after 28 h cultivation under 8 % ethanol, while no obvious cell growth was observed in the recombinant strain harboring plasmid pET-28a(+)-*irrE*(W) and the control strain harboring plasmid pET-28a(+). The cell viability of M4 under different stress shock conditions (such as pH = 5.0, pH = 10.0, 3 mmol/L sorbitol and 15 % methanol), the cell growth rate, and the final biomass were improved obviously. The sequence comparison of *irrE* revealed that there were two sense substitutions (C24T and G530A). The substitution of G530A caused one amino acid change Gly177Glu. The homology modeling of IrrE was built according to the known structure of IrrE protein from *D. deserti*, which showed that the amino acid mutation located in HTH motif of IrrE. The work laid a foundation for further research on the relationship of IrrE structure and host cell tolerance to stress.

Keywords IrrE · *Escherichia coli* · *Deinoeoccus radiodurans* · Stress tolerance · Error-prone PCR

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6.1 Introduction

Recently, global transcriptional machinery engineering (gTME) has been widely explored to improve strain phenotypes by engineering transcription factors or RNA polymerase subunits, thereby altering transcriptional profiles of target strains; various degrees of success have been achieved [1–3]. The genus *Deinococcus* is known for its ability to survive extreme stress conditions, conferred by a unique pool of genes.

Deinococcus radiodurans is one of the most radio-resistant organisms known. This bacterium can survive extreme cold and dehydration as well as exposure to vacuum and acidic conditions [4]. Genetic analysis of a DNA damage-sensitive strain of *D. radiodurans* R1 led to the discovery of a novel regulatory protein, IrrE (GeneID: 7738927) [5, 6]. IrrE, a newly identified gene switch responsible for extreme radio-resistance of *D. radiodurans*, plays a central regulatory role in multiple DNA damage repair and protection pathways in response to radiation stress [6]. Interestingly, the heterologous expression of *irrE* could enhance the tolerance to osmotic pressure in *Escherichia coli* [7], salt tolerance in *E. coli* and *Brassica napus* [8], the anti-oxidation and anti-ultraviolet irradiation in *Bacillus subtilis* [9].

Escherichia coli is one representative strain of prokaryotes and model micro-organism, which has been widely used for the production of bio-fuels and other chemicals. But the strain always faces various abiotic stresses in the industrial application, such as organic solvents, extreme pH, osmotic pressure, and so on, which seriously restricts its application efficiency.

In our previous work, the wild *irrE* gene from *D. radiodurans* R1 has been heterologously expressed in *E. coli* BL21(DE3). The results showed that the growth rate and the final biomass of recombinant strain containing pET-28a(+)-*irrE*(W) under stress were significantly higher than the control strain (harboring the empty vector pET-28a(+)). In order to obtain the mutants with higher tolerance to stress, IrrE was further directed evolved by error-prone PCR using pET-28a(+)-*irrE*(W) as a template in this paper. The cell growth performance and tolerance to stress of the evolved strain were investigated. The sequence comparison between the wild and evolved IrrE, and homologous modeling analysis by SWISS MODEL were further studied. The results could better understand the relationship of IrrE structure and host cell tolerance to stress.

6.2 Materials and Methods

6.2.1 Materials

Restriction enzymes, DNA-modifying enzymes, DNA markers, and T4 Ligases were purchased from TaKaRa (Dalian, China). The kits for DNA purification, gel recovery, and plasmid mini-prep were purchased from Omega Bio-Tek (USA).

Protein Marker II was purchased from Biomed (Beijing, China). Taq DNA polymerase, dNTPs was purchased from Sangon (Shanghai, China). The kit for protein purification was purchased from ComWin (Beijing, China). Sequence analysis was performed by BGI (Beijing, China). All cloning steps were performed in *E. coli* DH5α or BL21(DE3). The plasmid pET-28a(+) was stored in Key Laboratory of Industrial Fermentation Microbiology (Tianjin University of Science & Technology).

6.2.2 Strains

Escherichia coli BL21(DE3)/pET-28a(+) and *E. coli* BL21(DE3)/pET-28a(+)-*irrE* (W) have been successfully constructed in our previous work, which were grown in Luria-Bertani (LB) medium containing 50 μg/mL kanamycin at 37 °C. When required, 2 mmol/L IPTG was added to the medium to induce the expression of IrrE.

6.2.3 Library Construction and Selection

Using the plasmid pET-28a(+)-*irrE*(W) as a template, error-prone PCR method was employed to construct the library. The mutation ratio was controlled by adjusting the concentration of manganese ions and magnesium ions.

A total volume of 100 μL of reaction mixture contained 10 μL 10 × Taq DNA polymerase buffer, 0.2 mmol/L dATP and dGTP, 1 mmol/L dCTP and dTTP, 10 ng of template, 0.05–0.25 mmol/L $MnCl_2$, 1–7 mmol/L $MgCl_2$ and 5 units (U) of Taq DNA polymerase. A pair of primers used was F1 (5′-CCCATGGCTGCAGAAAT-GCCCAGTGC-3′) and R1 (5′-CATGCCATGGTCTAGATGTGCAGCG-3′). PCR was carried out at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 1 min for a total of 30 cycles. The products of error-prone PCR were purified, digested, and inserted into *Nco*I and *Eco*RI sites of plasmid pET-28a(+)-*irrE*(W) replacing the wild-type *irrE* gene. The ligation products were transformed into *E. coli* BL21(DE3) competent cells. Cells were plated on LB-agar plates containing 50 mg/mL kanamycin, incubated at 37 °C overnight, and scraped off to create a liquid library. The library size was approximately 10^6.

The liquid library was inoculated into the challenging medium. After cultivation at 37 °C on a rotary shaker (180 r/min) to the late logarithmic phase, the cultures were transferred into the fresh challenging medium to cultivate for 2–3 times under the same conditions. The unique difference was that the ethanol concentration in the challenging medium was gradually increased until the final concentration reached 8 %. Finally, the cultures were spread onto LB agar plates containing 50 mg/mL kanamycin. After incubation at 37 °C overnight, the grown colonies were

preliminary considered as mutants with ethanol tolerance, which should be further screened.

The single colony on the LB-agar plates was, respectively, inoculated into LB liquid medium containing 50 μg/mL kanamycin. After incubation overnight at 37 °C on a rotary shaker (180 r/min), the OD_{600} value of culture was adjusted to the same level and transferred into the fresh challenging medium (LB medium containing 50 μg/mL kanamycin, 2 mmol/L IPTG and different concentration of ethanol (4, 8, 12 % (v/v)) using 0.5 % (v/v) of the seed culture. The cell was grown at 37 °C on a rotary shaker (180 r/min). The culture was withdrawn during growth and OD_{600} value was detected. The higher ethanol tolerance mutants were selected by comparison of cell growth rate and the final biomass.

During the above selection process, the plasmid of the mutant with higher ethanol tolerance was verified by PCR and enzyme digest methods, and then retransformed into fresh *E. coli* DH5a cells. The mutant growth in challenging media was retested to separate the effects of the mutated irrE gene from any possible spontaneous chromosomal mutations acquired during the selection process.

6.2.4 Cell Growth Assay

To test the influence of *irrE* mumants on the cell growth, strains were inoculated into LB liquid medium containing 50 μg/mL kanamycin. After cultivation overnight at 37 °C on a rotary shaker at 180 r/min, the OD_{600} value of cultures were adjusted to the same level and transferred into the fresh challenging medium (LB medium containing 50 μg/mL kanamycin and 2 mmol/L IPTG using 0.5 % (v/v) of the seed culture. The cells were grown at 37 °C on a rotary shaker at 180 r/min. The culture was withdrawn during growth and OD_{600} value was detected to obtain the growth curve.

6.2.5 Shock Experiments and Cell Growth Under Different Stress Conditions

To test the tolerance of strain toward extremely stresses, strains were grown overnight at 37 °C on a rotary shaker at 180 r/min in LB medium containing 50 mg/mL kanamycin. The cultures were transferred into the fresh LB medium containing 50 mg/mL kanamycin using 1 % (v/v) of the seed culture and grown at 37 °C on a rotary shaker at 180 r/min. When reaching an OD_{600} value of 0.6, the cultures were added 2 mmol/L IPTG and inoculated for 6 h to induce the expression of IrrE. Then 1 mL of culture was withdrawn and the cells were harvested by centrifugation at 6,000 r/min for 10 min. The same mass cells were transferred to 50 mL challenging

Table 6.1 Shock experiment conditions of the control strain (*E. coli* BL21(DE3) harboring plasmid pET-28a(+)), the recombinant strain harboring pET-28a(+)-*irrE*(W) and the mutant harboring pET-28a(+)-*irrE*(M)

Stresses	Sorbitol (mol/L)	pH	Methanol (%, v/v)	Ethanol (%, v/v)	H_2O_2 (%, v/v)
Condition	3.0	pH = 2; pH = 12	10	12	1
Time (min)	30	30	30	30	30

medium containing 50 μg/mL kanamycin and different kinds of stresses (Table 6.1). During incubation, optical density at 600 nm was used to measure cell growth.

To test the cell growth performance in challenging medium, strains were grown overnight at 37 °C on a rotary shaker at 180 r/min in LB medium containing 50 mg/mL kanamycin, respectively. The OD_{600} values of culture were adjusted to the same level and transferred to the fresh challenging medium (LB medium containing 50 μg/mL kanamycin, 2 mmol/L IPTG and different challenging substances (4 % ethanol, 5 % methanol and 1 M sorbitol)) using 0.5 % (v/v) of the seed culture. During incubation, optical density at 600 nm was used to measure cell growth.

6.2.6 Mutational Sites Analysis of IrrE

The mutated *irrE* gene was sequenced and then translated into the amino acids by software DNAMAN. To analyze the influence of the mutant sites on the function and structure of IrrE, the homology modeling was built based on the solved structure of IrrE protein from *Deinoeoccus deserti* by SWISS-MODEL.

6.3 Results and Discussion

6.3.1 Library Construction and Selection of Ethanol-Tolerant Mutants

6.3.1.1 The Development of Error-Prone PCR Conditions

Low concentration of Mg^{2+} is required for Taq DNA polymerase while high level can promote the stability and the complementarity of base pairs. Mn^{2+} can reduce the specificity of the polymerase to the template, which is advantageous for mutation. Therefore, error-prone PCR conditions were optimized by adjusting the concentration of Mg^{2+} (1–7 mmol/L) and Mn^{2+}(0.05–0.25 mmol/L). When the concentration of Mg^{2+} was set at 3 mmol/L, all stripes were clear and bright, and no

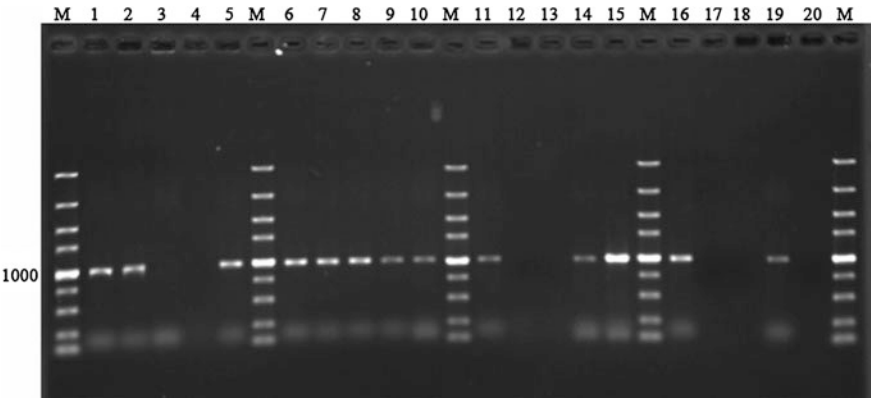

Fig. 6.1 Error-prone PCR conducted with different concentrations of Mn^{2+} and Mg^{2+}. *M* DL5000 DNA Marker. *1–5* The concentration of Mn^{2+} is 0.05, 0.1, 0.15, 0.2, 0.25 mmol/L, respectively, at Mg^{2+} is 1 mmol/L. *6–10* The concentration of Mn^{2+} is 0.05, 0.1, 0.15, 0.2, 0.25 mmol/L, respectively, at Mg^{2+} is 3 mmol/L. *11–15* The concentration of Mn^{2+} is 0.05, 0.1, 0.15, 0.2, 0.25 mmol/L, respectively, at Mg^{2+} is 5 mmol/L. *15–20* The concentration of Mn^{2+} is 0.05, 0.1, 0.15, 0.2, 0.25 mmol/L, respectively, at Mg^{2+} is 7 mmol/L

obvious dispersion was observed under different concentrations of Mn^{2+} (Fig. 6.1). So 3 mmol/L of Mg^{2+} was used for the error-prone PCR.

6.3.1.2 Library Construction

Error-prone PCR was employed to construct the library using the plasmid pET-28a (+)-*irrE*(W) as a template. PCR was carried out under different Mn^{2+} concentrations (0.05–0.25 mmol/L) and 3 mmol/L Mg^{2+}. As presented in Fig. 6.2, the target band was clear and bright. The size of *irrE* was about 1,000 bp [8]. Therefore, IrrE library (about 10^6) was constructed by using the above PCR conditions and the detailed procedures are described in Sect. 6.2.3.

6.3.1.3 Selection of Ethanol-Tolerant Mutants

The liquid library was screened according to the detailed procedures described in Sect. 6.2.3. After incubation in the challenging medium (LB medium containing 50 μg/mL kanamycin, 2 mmol/L IPTG and 8 % ethanol), the cultures were spread onto LB agar plates containing 50 mg/mL kanamycin. After incubation at 37 °C overnight, 24 single colonies (named M1-M24) were grown and preliminary considered as mutants with higher ethanol tolerance.

24 single colonies were respectively inoculated into LB medium and the cell growth curves under different ethanol stresses were determined according to the detailed procedure shown in Sect. 6.2.3. By comparison of cell growth rate and the

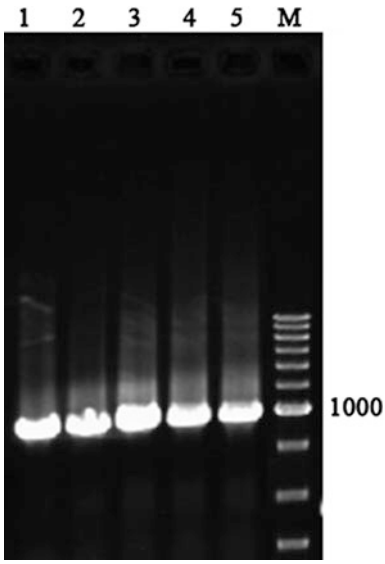

Fig. 6.2 Error-prone PCR conducted with different Mn^{2+} concentrations. *M* DL500 DNA marker. *1–5* The concentration of Mn^{2+} is 0.05, 0.1, 0.15, 0.2, 0.25 mmol/L, respectively, at Mg^{2+} is 3 mmol/L

final biomass, four mutants named M1-M4 were screened and grown well in the presence of 4 % ethanol (Fig. 6.3a). When the ethanol concentration was increased to 8 %, one mutant (M4) showed good growth performance and a maximum OD_{600} of 1.6 was reached after 28 h (Fig. 6.3b). Further increase in ethanol concentration (12 %) and no obvious cell growth was observed in all mutants. So mutant M4 was considered as the better ethanol-tolerant strain.

The cell growth performance of the control strain (*E. coli* BL21(DE3) harboring plasmid pET-28a(+)), the recombinant strain harboring pET-28a(+)-*irrE*(W), and the mutant M4 harboring pET-28a(+)-*irrE*(M4) were compared in LB medium containing 8 % ethanol. As shown in Fig. 6.4, the cell growth of the control strain harboring plasmid pET-28a(+) and recombinant strain harboring pET-28a(+)-*irrE* (W) were completely inhibited, while the *irrE* mutant M4 strain showed rapid growth after a 20 h lag phase. The maximum OD_{600} reached 1.6 after 28 h cultivation.

6.3.2 *The Influence of Expression of IrrE on Cell Growth Under No Stress*

As shown in Fig. 6.5, the lag phase of the control strain (*E. coli* BL21(DE3) harboring plasmid pET-28a(+)) and the recombinant strain harboring pET-28a(+)-*irrE*(W) under no stress condition were 0–8 h, while the lag phase of the *irrE* mutant M4 was 2 h shorter than the other two strains. After 14 h cultivation, the

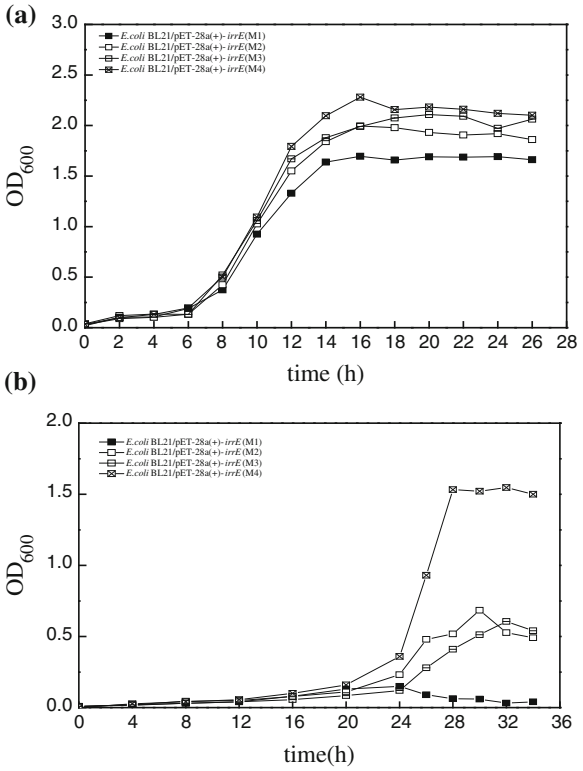

Fig. 6.3 The growth curves of *E. coli* BL21/pET-28a(+)-*irrE*(M1-M4) under various concentrations of ethanol. **a** 4 % ethanol. **b** 8 % ethanol

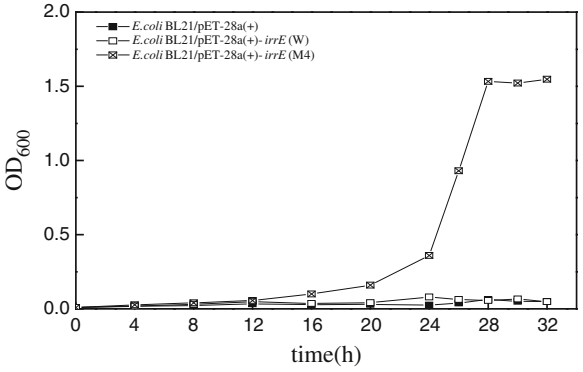

Fig. 6.4 The growth curves of *E. coli* BL21/pET-28a(+)-*irrE*(M4), *E. coli* BL21/pET-28a(+)-*irrE*(W) and the control strain under 8 % ethanol

final biomass of the recombinant strain harboring pET-28a(+)-*irrE*(W) and the *irrE* mutant M4 was silimilar and 10 % more than the control strain harboring plasmid pET-28a(+). This indicated that the expression of *irrE* shortened the cell lag phase and increased the final biomass.

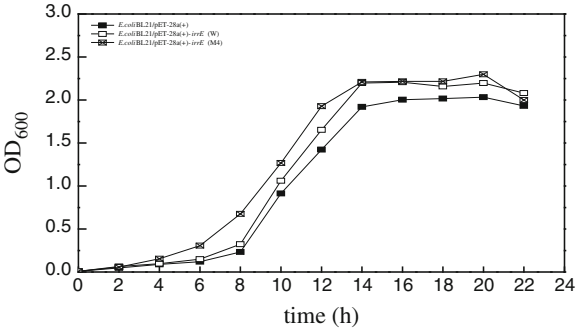

Fig. 6.5 The growth curves of *E. coli* BL21/pET-28a(+)-*irrE*(M4), *E. coli* BL21/pET-28a(+)-*irrE* (W) and the control strain under no stress condition

6.3.3 The Influence of Expression of IrrE on Cell Viability and Growth Performance Under Different Stress Conditions

6.3.3.1 The Influence of Expression of IrrE on Cell Viability Under Stress Shock

After shocked with 1 % H_2O_2 and 10 % ethanol for 30 min, the cell viability of the mutant M4 was less than 15 %, which was higher than those of the control strain and wild type strain. For other shock conditions (pH = 5, pH = 10, 15 % methanol and 3 M sorbitol), the cell viabilities of mutant M4 were 56.9, 70, 55, 51.6 %, respectively, which increased by 25.0, 30.4, 17.5, and 6.7 %, respectively, compared with the wild type strain and increased by 36.4, 45.4, 35.0, and 16.0 % compared with the control strain (shown in Fig. 6.6). These results indicate that the expression of mutant *irrE* could remarkably enhance the tolerance of *E. coli* to stresses of 3 M sorbitol, pH = 5, pH = 10, and 15 % methanol.

6.3.3.2 The Influence of Expression of IrrE on Cell Growth Performance Under Different Stress Conditions

In the presence of 4 % ethanol, the lag phase of the control strain and the wild-type stain was 10 h, while the lag phase of the *irrE* mutant M4 was shortened to 6 h and its cell growth rate and final biomass were obviously increased, indicating that the IrrE mutation enhanced the strain tolerance to 4 % ethanol (Fig. 6.7a). Under the other stress conditions (5 % methanol and 1 M sorbitol), the control strain, the wild-type stain and the *irrE* mutant M4 showed the similar growth tendency. However, the *irrE* mutant M4 showed higher growth rate and final biomass. In the presence of 5 % methanol, the *irrE* mutant M4 reached a maximum OD_{600} of 2.2 after 18 h

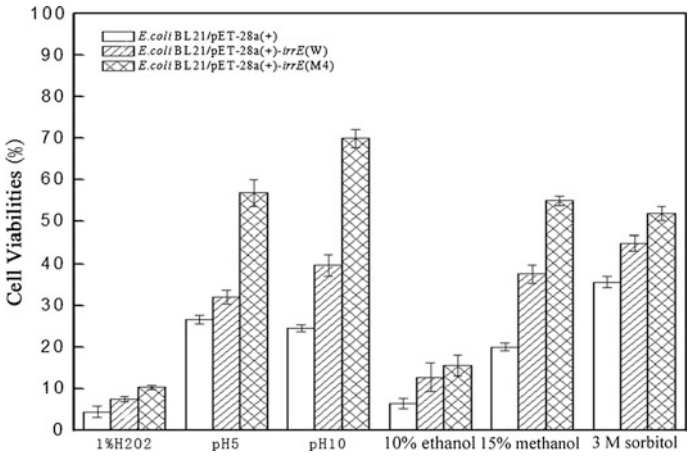

Fig. 6.6 The cell viability of *E. coli* BL21/pET-28a(+)-*irrE*(M4), *E. coli* BL21/pET-28a(+)-*irrE* (W) and the control strain under various kinds of shock conditions

cultivation, which was 18 and 25 % higher than the control strain and wild-type strain, respectively (Fig. 6.7b). In the presence of 1 M sorbitol, the *irrE* mutant M4 reached a maximum OD_{600} of 2.0 after 16 h cultivation, which was 17 and 42 % higher than the control strain and wild-type strain, respectively (Fig. 6.7c). These results indicate that the expression of the mutated IrrE could promote host cell tolerance to organic solvents and osmotic pressure.

6.3.4 The Mutation Site Analysis of IrrE

The *irrE* sequence of mutant M4 was analyzed. The whole length of *irrE* was 987 bp, encoding 328 amino acids. The comparison result between the wild-type and mutated *irrE* showed that there were two sense substitutions (C24T and G530A). The substitution of G530A caused one amino acid change Gly177Glu.

The IrrE protein structure of *D. deserti* has been solved, which shows 64 % homology with the IrrE protein from *D. radiodurans*. Therefore the homology structural model of IrrE from mutant M4 was built using SWISS-MODEL according to the known structure of IrrE protein from *D. deserti.* The domain boundary of IrrE from *D. radiodurans* was as follows: the N-terminal domain (residues 1–161); the middle domain (residues 162–203); and the C-terminal domain (residues 204–328) (Fig. 6.8a). The results show that the mutational amino acid was located in helix-turn-helix (HTH) motif of IrrE, which was considered to be involved in DNA binding and recognition [10] (Fig. 6.8b).

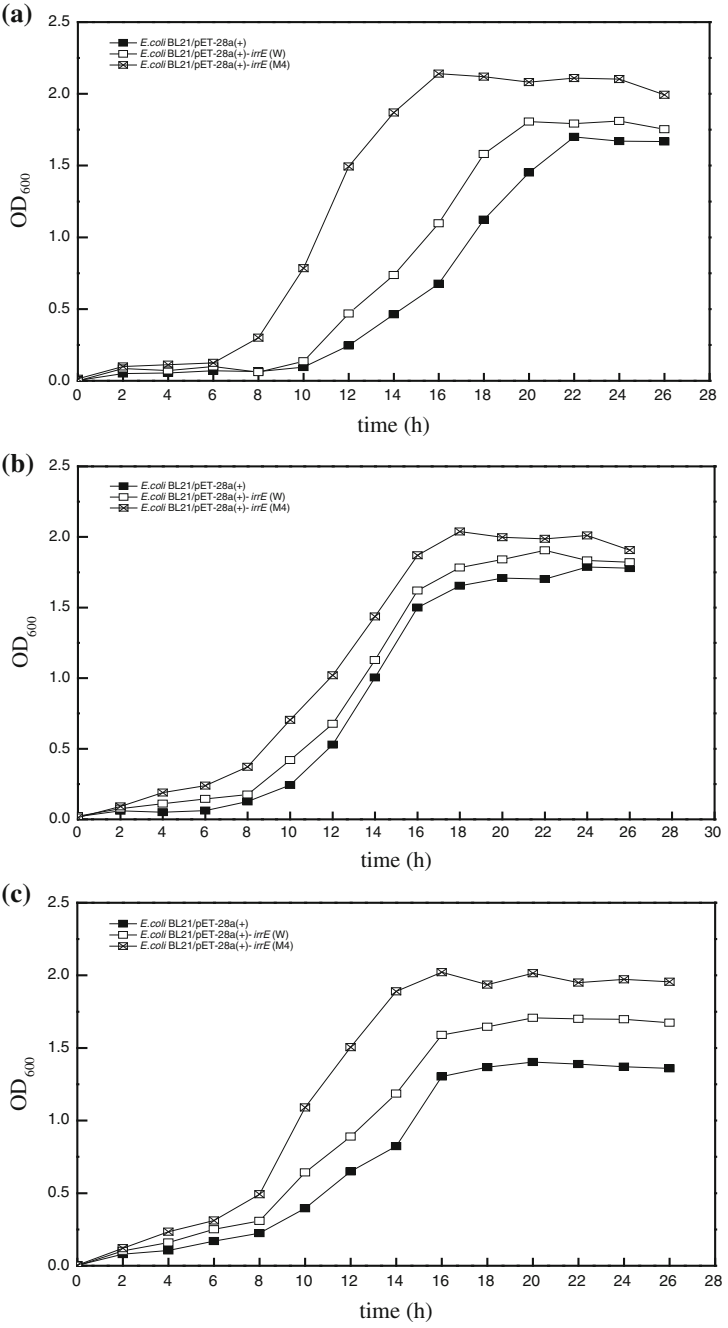

Fig. 6.7 The growth curves of *E. coli* BL21/pET-28a(+)-*irrE*(M4), *E. coli* BL21/pET-28a(+)-*irrE* (W) and the control strain under certain concentration of stress conditions. **a** 4 % ethanol. **b** 5 % methanol. **c** 1 M sorbitol

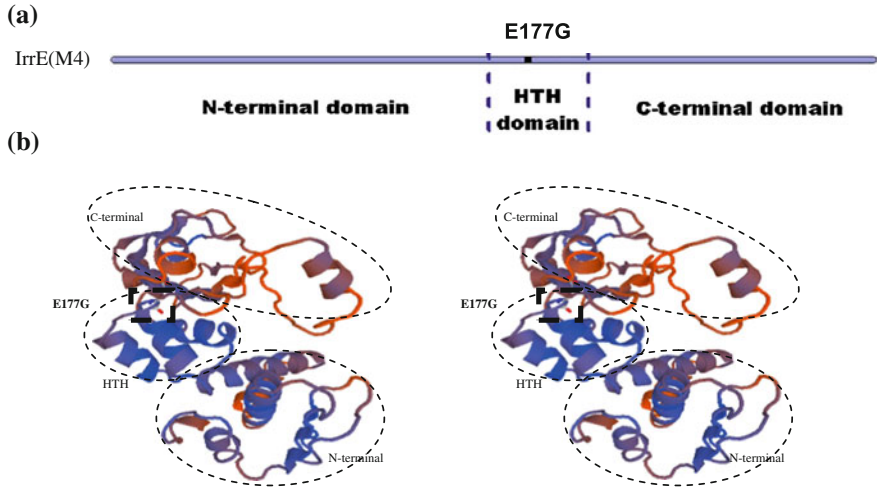

Fig. 6.8 Summary and location of mutation sites of IrrE. **a** Summary of mutation site in the amino acid sequence of the mutant M4, the full sequence is divided into three parts by two *dashed lines*. Each part represents a separate domain of *irrE* (from *left* to *right* N-terminal domain, HTH domain, C-terminal domain). **b** Location of mutation sites of the mutant M4 in a modeled structure of *irrE* from *D. radiodurans*

6.4 Conclusions

The *irrE* gene from *D. radiodurans* R1 was directed evolved by error-prone PCR method and was introduced into *E. coli*. One mutant M4 with better stress tolerance was obtained, which showed good cell growth performance in the presence of 8 % ethanol. Also, the cell viabilities of mutant M4 were promoted under extremely stress shock conditions (pH = 5, pH = 10, 15 % methanol and 3 M sorbitol). On the other hand, mutant M4 exhibited higher growth rate and final biomass in the presence of challenging medium (4 % ethanol, 5 % methanol and 1 M sorbitol) compared with the control and wild type strains. These results indicate that the expression of mutational IrrE could further improve the tolerance of *E. coli* to various stresses.

The comparison result between the wild-type and mutated *irrE* showed that there were two sense substitutions (C24T and G530A). The substitution of G530A caused one amino acid change, Gly177Glu. The homology modeling of IrrE was constructed using the known IrrE from *D. desertias* as a template, indicating the mutational amino acid was located in HTH motif of IrrE, which was thought to be involved in DNA binding and recognition [10].

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