Construction of leaky strains and extracellular production of exogenous proteins in recombinant Escherichia coli

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Summary
In this study, a strategy of the construction of leaky strains for the extracellular production of target proteins was exploited, in which the genes mrcA, mrcB, pal and ipp (as a control) from Escherichia coli were knocked out by using single- and/or double-gene deletion methods. Then the recombinant strains for the expression of exogenous target proteins including Trx-hPTH (human parathyroid hormone 1–84 coupled with thioredoxin as a fusion partner) and reteplase were reconstructed to test the secretory efficiency of the leaky strains. Finally, the fermentation experiments of the target proteins from these recombinant leaky strains were carried out in basic media (Modified R media) and complex media (Terrific Broth media) in flasks or fermenters. The results demonstrated that the resultant leaky strains were genetically stable and had a similar growth profile in the complex media as compared with the original strain, and the secretory levels of target proteins into Modified R media from the strains with double-gene deletion (up to 88.9%/mrcA ipp-pth) are higher than the excretory levels from the strains with single-gene deletion (up to 71.1%/ipp-pth) and the host E. coli JM109 (DE3) (near zero). The highest level of extracellular production of Trx-hPTH in fermenters is up to 680 mg l⁻¹.

Introduction
In the past decades, the number of recombinant proteins used for therapeutic applications has increased dramatically. Many of these applications involve cell factors with low molecular weight [e.g. human parathyroid hormone (hPTH)] and diverse proteins [e.g. reteplase (rPA), a thrombolytic agent derived from human tissue plasminogen activator]. As a drug used for treating patients with osteoporosis, hPTH secreted by parathyroid gland is composed of 84 amino acid residues (Vad et al., 2005; Möricke et al., 2011). rPA with approximately 39 kDa, as an effective drug used in thrombolytic and antiplatelet therapy in peripheral vascular disease, consists of the kringle-2, and protease domains of tissue type plasminogen activator after the kringle-1, finger and growth factor domains are removed (Bode et al., 1995; Benenati et al., 2001).

A variety of alternative expression systems are being developed and evaluated to produce recombinant protein drugs effectively. Escherichia coli is the most commonly used host for the production of recombinant proteins. In order to obtain the target exogenous proteins expressed intracellularly in recombinant E. coli, cell disruption is necessary, which often results in the increase of pyrogen level (mainly from the cell membrane composition), the increase of sample impurities and the decrease of protein activities. Particularly, the formation of inclusion body often occurs when the target protein is intracellularly overexpressed. To overcome these problems in the production of exogenous proteins, extracellular secretion of exogenous proteins in recombinant E. coli is increasingly becoming an important choice. In large-scale industrial production of exogenous proteins, the extracellular excretion of target proteins can remove the cell disruption step, offer a better environment for protein folding and reduce the risk of intracellular enzyme degradation (Mergulhao et al., 2005). Besides, the extracellular secretion of target proteins can improve the recombinant protein yield because the target protein accumulation is not limited in periplasmic or intracellular space (Makrides, 1996; Fu et al., 2005).

Normally, extracellular secretion of recombinant proteins is blocked mainly due to the difficulties in protein translocation across the two membranes – the cell membrane and the outer membrane of E. coli cells (Koebnik et al., 2000; Choi and Lee, 2004). Although periplasmic expression of recombinant proteins can often be achieved with the help of a signal peptide, the available methods to overcome the outer membrane barrier for extracellular
production of recombinant proteins are limited. In order to solve this problem, various genetic attempts have been made to facilitate the extracellular secretion of recombinant proteins in *E. coli*, including manipulation of transport pathways (Sugamata and Shiba, 2005), optimization of codon and signal sequence (Takemori et al., 2012), fusion expression of carrier protein which can be normally secreted extracellularly (Fernandez et al., 2000; Choi and Lee, 2004) and fusion expression of outer membrane protein F (Jeong and Lee, 2002), YebF (Zhang et al., 2006) or osmotically inducible protein Y (Qian et al., 2008). In addition, the coexpression of lysis-promoting proteins such as bacteriocin release protein (BRP) (van der Wal et al., 1995) or colicin E1 lysin protein (Kil) (Robbens et al., 1995), as well as the use of wall-less strains (the so-called L-forms) (Gumpert and Hoischen, 1998) have also been reported.

Meanwhile, many fermentation techniques, including changes of culture medium compositions (Fu, 2010), temperature (Rinas and Hoffmann, 2004), aeration and calcium ion (Shokri et al., 2003), osmotic pressure and induction conditions (Orr et al., 2012), as well as the addition of supplements such as glycine (Yang et al., 1998) and Triton X-100 (Fu et al., 2005; Fu, 2010), have been explored to achieve the extracellular production of recombinant proteins in *E. coli*. The main disadvantage of the fermentation control for the extracellular production of target proteins is that the fermentation conditions vary greatly with different target proteins.

To overcome the uncertainty of the fermentation conditions, the construction of leaky strains (including the *E. coli* Sec pathway) will become a main alternative to transport periplasmic-directed recombinant proteins into media. Leaky strains can be constructed by knocking out of genes related to the biosynthesis of cell wall and membrane, especially of the outer membrane genes such as *lpp* encoding Braun’s lipoprotein (Shin and Chen, 2008) of *E. coli*. A lot of studies on the secretion of recombinant proteins in *E. coli* have been reported (Rinas and Hoffmann, 2004; Nandakumar et al., 2006; Xia et al., 2008; Ostendorp et al., 2009); however, the secretory efficiency into media for most of recombinant proteins was not high. Further studies to improve the extracellular production of the target proteins have become inevitable.

In this study, *E. coli* JM109 (DE3), as a popular host for the expression of recombinant proteins, was selected. The genes *pal* (encoding peptidoglycan-associated outer membrane lipoprotein) (Bernadac et al., 1998; Cascales et al., 2002), *mrcA* (Ishino et al., 1980; Broome-Smith et al., 1985) and *mrcB* (encoding peptidoglycan synthetase) (Bertsche et al., 2005) from this strain were selected as target genes for genetic manipulation; gene *lpp* (encoding Braun’s lipoprotein) (Shin and Chen, 2008) was selected as a control.

Several leaky strains including *mrcA*, *mrcB*, *pal* and *lpp* (single-gene knock-out) as well as *lpp* *mrcB*, *mrcA* *lpp*, *lpp* *pal*, *mrcA* *pal* and *mrcB* *pal* (double-gene knock-out) were constructed by inframe deletion method to improve the extracellularly secretory levels of their target proteins. All the strains were tested for the secretory levels of their target proteins. Then, the recombinant strains of *E. coli* for the extracellular production of the two proteins Trx-hPTH and rPA were built up. Further, the fermentation experiments of target proteins in both flasks and fermenters were conducted and the yields of the target proteins inside and outside cells were determined.

**Results**

**Construction of single or double-gene deleted mutants**

In theory, the disruption of genes *mrcA* (2553 bp) or *mrcB* (2535 bp) (Banzhaf et al., 2012), both encoding the peptidoglycan synthetase, or the disruption of gene *pal* (522 bp) (Bernadac et al., 1998; Cascales et al., 2002) encoding the peptidoglycan-associated outer membrane lipoprotein may cause the deficiencies in the structures of their cell walls and the outer membranes, therefore causing the leakage of the intracellular proteins in *E. coli*. To construct leaky strains for extracellular production of target protein(s), *mrcA*, *mrcB* and *pal* as well as *lpp* (237 bp, as a control) (Shin and Chen, 2008) from *E. coli* JM109 (DE3) were selected as target genes for the genetic modification.

Nine mutants named *E. coli* *mrcA*, *mrcB*, *pal*, *lpp* *mrcB*, *mrcA* *lpp*, *lpp* *pal*, *mrcA* *pal* and *mrcB* *pal* were obtained by gene manipulation as described in *Experimental procedures*. To identify each mutant, polymerase chain reaction (PCR) amplification of the target genes from leaky mutants of single- or double-gene deletion was carried out by using the corresponding specific ‘verification’ primers (Supporting Information Table S1), and the results of the PCR experiments were illustrated in Supporting Information Fig. S1. The strains *mrcA* N, *mrcB* N and *lpp* N for the construction of double-gene-deleted mutants were also confirmed by sequencing (Supporting Information Table S4). In addition, the morphology of the mutants was observed and compared with their original strain of *E. coli* JM109 (DE3) (Fig. 1). The result demonstrated that the morphological change of strains *pal* and *lpp* was significant [shorter and thicker compared with JM109 (DE3)], suggesting the genes *pal* and *lpp* associated with outer membranes are very important in cell morphology.

As shown in Supporting Information Fig. S1, by using corresponding ‘verification’ primers, the colony PCR products of all the mutants, with single or double deletion of genes *mrcA*, *mrcB*, *pal* and *lpp* had the expected band size. Subsequently, the gene sequencing of the PCR
products of strains mrcA N, IBL16-02mrcB N and IBL16-04lpp N was carried out (Supporting Information Table S4). The experimental results demonstrated that all the mutants including mrcA, mrcB, pal, lpp, lpp mrcB, mrcA lpp, lpp pal, mrcA pal and mrcB pal have been correctly constructed. However, the mutant of double deletion of gene mrcB and mrcA was not obtained, suggesting that such a mutation may be lethal to E. coli (Denome et al., 1999).

Expression of recombinant protein Trx-hPTH in leaky strains with single-gene deletion

To test the secretory efficiency of these leaky strains, the plasmid pET32a-pth was first transformed into the leaky strains with single-gene deletion (mrcA, mrcB, pal and lpp). Four recombinant leaky strains mrcA-pth, mrcB-pth, pal-pth and lpp-pth were obtained. After fermentation in flasks with Modified R (MR) media, expressed protein samples of each strain were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A) and bicinchoninic acid (BCA) protein assay (Table 1A).

As shown in Table 1A and Fig. 2A, approximately 220 mg l\(^{-1}\) Trx-hPTH (51.8% of total Trx-hPTH protein) and 420 mg l\(^{-1}\) Trx-hPTH (71.1% of total soluble Trx-hPTH protein) were obtained from the culture media of pal-pth and lpp-pth respectively. No Trx-hPTH was obtained from the culture media of mrcA-pth and mrcB-pth. This observation suggested that the single deletion of gene pal encoding peptidoglycan-associated outer mem-

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brane lipoprotein or lpp encoding Braun’s lipoprotein can significantly result in the leakage of the target protein into media, while the single deletion of the gene mrcA or mrcB encoding peptidoglycan synthetase is not efficient for this leakage under these conditions. The present study also demonstrated that the growth rate of all these strains [especially pal-pth incubated for 49 h before isopropyl-β-D-thiogalactopyranoside (IPTG) induction] in MR media was very slow, suggesting nutritional components of MR media are not complete to these strains as compared with Luria–Bertani (LB) or Terrific Broth (TB) media. The growth of strain lpp-pth is the best among all the four mutants under the same condition, suggesting that gene lpp is not important for the growth of E. coli.

To accelerate cell growth and improve the extracellular yield of Trx-hPTH, TB media were selected (Fig. 2B). The final optical density of all these strains was significantly improved after 11 h cultivation. As shown in Supporting Information Table S5, the final optical densities of mrcA-pth and mrcB-pth were higher than those of pal-pth and lpp-pth, indicating that the growth limitation of strains pal and lpp can be greatly relieved in rich media. Also, from Fig. 2A and B, we found that the secretory profiles (i.e. the compositions of proteins on SDS-PAGE) of expressed proteins of these leaky strains in both MR media and TB media were similar. Furthermore, the yields of the Trx-hPTH produced by these mutants in TB media are significantly higher than those in MR media, suggesting that the expression efficiency of exogenous proteins are closely related to the cell growth.

It was reported that the supplemented glycine could be incorporated into the nucleotide-activated peptidoglycan precursors (Hammes et al., 1973) and thus affected the biosynthesis of peptidoglycan, resulting in the structural defects of cell wall and finally enhancing the extracellular secretion of recombinant proteins in E. coli (Li et al., 2010). Based on the similar secretory mechanism that partly disrupts the synthesis of cell wall and further indirectly affects outer membrane permeability or both, it is possible for constructing leaky strains with double-gene deletion (genes mrcA and mrcB for cell wall biosynthesis as well as pal and lpp for outer membrane biosynthesis) to improve the extracellular secretion of the recombinant proteins (Bertsche et al., 2005; Born et al., 2006).

**Expression of recombinant protein Trx-hPTH in leaky strains with double-gene deletion**

Generally speaking, the mutants with double deletion of the genes mrcA, mrcB, pal and lpp may result in higher

| Strains       | OD<sub>600(A)</sub> | OD<sub>600(B)</sub> | Trx-hPTH<sup>d</sup> | Total proteins<sup>d</sup> |
|---------------|---------------------|---------------------|------------------------|--------------------------|
|               |                     |                     | EPS (mg l<sup>-1</sup>) | IPS (mg l<sup>-1</sup>)  | EPS (mg l<sup>-1</sup>) | IPS (mg l<sup>-1</sup>) |
| mrcA-pth      | 0.92 ± 0.06         | 2.24 ± 0.06         | –                      | 80 ± 1                   | 960 ± 40                 | 320 ± 5                 |
| mrcB-pth      | 0.49 ± 0.03         | 1.46 ± 0.06         | –                      | 50 ± 3                   | 600 ± 30                 | 140 ± 5                 |
| pal-pth       | 1.82 ± 0.44 (49 h)  | 6.24 ± 1.49 (55 h)  | 220 ± 40               | 200 ± 50                 | 1190 ± 200               | 670 ± 180               |
| lpp-pth       | 2.39 ± 0.07         | 3.73 ± 0.37         | 420 ± 20               | 170 ± 20                 | 2040 ± 2                | 700 ± 60                |
| JM109 (DE3)-pth | 0.70 ± 0.02       | 1.79 ± 0.06         | –                      | 90 ± 3                   | 750 ± 40                 | 240 ± 10                |
| JM109 (DE3)   | 2.21 ± 0.01         | 10.38 ± 0.05        | –                      | –                       | 380 ± 50                 | 2110 ± 70               |

**Table 1A.** Growth analysis and protein detection from the recombinant leaky strains with single-gene deletion in MR medium.

| Strains       | OD<sub>600(A)</sub> | OD<sub>600(B)</sub> | Trx-hPTH<sup>d</sup> | Total proteins<sup>d</sup> |
|---------------|---------------------|---------------------|------------------------|--------------------------|
|               |                     |                     | EPS (mg l<sup>-1</sup>) | IPS (mg l<sup>-1</sup>)  | EPS (mg l<sup>-1</sup>) | IPS (mg l<sup>-1</sup>) |
| lpp-pth       | 1.98 ± 0.35         | 1.98 ± 0.47         | 150 ± 40               | 40 ± 10                  | 1370 ± 160              | 260 ± 80                |
| mrcB-lpp-pth  | 0.80 ± 0.002        | 4.65 ± 0.02         | 410 ± 1                | 50 ± 1                   | 1140 ± 20               | 800 ± 10                |
| lpp-pal-pth   | 0.24 ± 0.001        | 0.25 ± 0.02         | 90 ± 0                 | –                       | 290 ± 1                 | 120 ± 10                |
| mrcB-pal-pth  | 2.42 ± 0.14 (14 h)  | 4.04 ± 0.28 (24 h)  | 170 ± 10               | 140 ± 10                 | 1440 ± 100              | 700 ± 50                |
| lpp-mrcB-pal-pth | 1.68 ± 0.05 (13 h) | 3.85 ± 0.13 (21 h)  | 130 ± 10               | 90 ± 10                  | 1050 ± 100              | 400 ± 40                |
| JM109 (DE3)-pth | 1.20 ± 0.01       | 3.44 ± 0.02         | –                      | 140 ± 4                  | 750 ± 10                | 500 ± 20                |
| JM109 (DE3)   | 2.99 ± 0.02         | 15.24 ± 1.62        | –                      | –                       | 310 ± 20                | 2510 ± 160              |

**Table 1B.** Growth analysis and protein detection from the recombinant leaky strains with double-gene deletion in MR medium.
double-gene-deleted leaky strains lpp mrcB-pth, mrcA lpp-pth, mrcA pal-pth and mrcB pal-pth in MR media respectively. The extracellular yields of the target protein from mutants lpp mrcB-pth, mrcA lpp-pth, mrcA pal-pth and mrcB pal-pth with double-gene deletion are higher than that from mutant lpp pal-pth, suggesting that the deletion mutants of double genes associated with the biosynthesis of outer membrane and cell wall may be more suitable for the extracellular production of target proteins than the deletion mutants of double genes associated with the biosynthesis of outer membrane only.

Judging from the band migration shown in Fig. 3A and B and Supporting Information Fig. S3, it was revealed that the extracellular levels of recombinant protein Trx-hPTH produced by these leaky strains (lpp mrcB-pth, mrcA lpp-pth, lpp pal-pth, mrcA pal-pth and mrcB pal-pth) in TB media are higher than those in MR media, especially those produced by the strains lpp mrcB-pth and mrcA lpp-pth.

Batch cultivation in a fermenter

To further test the secretory performances of these mutants in a 5 l fermenter (SY3005B), we chose lpp mrcB-pth as a model strain for the extracellular production of Trx-hPTH. The strain was induced with IPTG (0.5 mM) at 4.5 h after inoculation, and the fermentation was continued for 6 h in TB media. The extracellular Trx-hPTH levels increased with the time during the fermentation. Particularly, almost all the Trx-hPTH was transferred into the media after 7 h fermentation and the yield was up to 680 mg l⁻¹ after 10 h cultivation, displaying a better secretory efficiency of Trx-hPTH in fermentor than that in flasks. The SDS-PAGE profiles of Trx-hPTH production in fermenters were shown in Fig. 4. Judging from the bands migration shown in Fig. 4, it appeared that the extracellular levels of proteins than the mutants with single-gene deletion. To obtain more efficient leaky strains for extracellular production of the target protein hPTH (9.4 kDa) (Prarahlad et al., 2006), the plasmid pET32a-pth expressing Trx-hPTH (~30 kDa) (Fu et al., 2005) was transformed into the leaky strains with double-gene deletion: lpp mrcB, mrcA lpp, lpp pal, mrcA pal and mrcB pal. Five recombinant leaky strains lpp mrcB-pth, mrcA lpp-pth, lpp pal-pth, mrcA pal-pth and mrcB pal-pth were obtained. After fermentation in flasks with MR media, expressed protein samples of each strain were analysed by SDS-PAGE (Fig. 3) and BCA protein assay (Table 1B).

As shown in Table 1B, approximately 150 mg l⁻¹ Trx-hPTH (77.7% of total Trx-hPTH protein), 410 mg l⁻¹ Trx-hPTH (88.9% of total Trx-hPTH protein), 90 mg l⁻¹ Trx-hPTH (concentration of intracellular target protein is undetectable), 170 mg l⁻¹ Trx-hPTH (54.7% of total Trx-hPTH protein) and 130 mg l⁻¹ Trx-hPTH (58.9% of total Trx-hPTH protein) were obtained extracellularly from the
accumulation of intracellular Trx-hPTH protein (lanes 8 to 14) stopped at 6 h.

**Expression of recombinant protein rPA in leaky strains**

In order to test the leakage efficiency of exogenous proteins expressed in cytoplasm from the mutants with single/double deletion of genes mrcA and mrcB as well as pal and lpp, the recombinant strains of *E. coli* harbouring pET22b-rpa for the expression of the exogenous protein rPA (in cytoplasm owing to no signal peptide) were constructed and then fermented in TB medium. The results demonstrated, as expected, that mutants with single/double deletion of genes *mrcB* and *lpp* caused a little leakage of intracellular proteins (including rPA) into media due to the deficiencies in mutants’ cell wall and outer membrane structures, as compared with strain JM109 (DE3)-rpa (Supporting Information Fig. S3, Band 1). However, the leakage efficiency for the exogenous protein (rPA) expressed in cytoplasm is much lower than that (Trx-hPTH) expressed in periplasm.

**Discussion**

Cell lysis is a main factor affecting the construction and application of leaky strains. To exclude the effect of cell lysis on the extracellular product of exogenous proteins, the growth curves of some leaky mutants were measured (Supporting Information Fig. S4). The mutants of *lpp mrcB-pth* and *mrcA lpp-pth* had normal growth courses like typical *E. coli* (but having relatively low OD₆₀₀ valves at the same time), and no cell lysis occurred within 12 h. Also from Figs 2 and 3, and Supporting Information Figs S2 and S3, we found that the intracellular protein levels for the majority of the samples are significantly higher than the extracellular protein levels, suggesting that the cell lysis does not play a key role in the extracellular production of the recombinant proteins in leaky strains.

The normal cell growth is another important factor in the application of leaky strains. The growth of all leaky stains, as expected, was worse than that of their host *E. coli* JM109 (DE3) (Table 1A). However, the optical density at 600 nm (OD₆₀₀) and intracellular protein sample (IPS) of the recombinant leaky strains, except for mrcB-pth, were higher than those of JM109 (DE3)-pth. In addition, we found that after 25 h cultivation in MR medium, the OD₆₀₀ of almost all these mutants (*lpp mrcB-pth*, *mrcA lpp-pth*, *mrcA pal-pth* and *mrcB pal-pth*) with double-gene deletion in MR medium was higher than their corresponding mutants (*mrcA-pth*, *mrcB-pth*, *pal-pth* and *lpp-pth*) with single-gene deletion, suggesting that the double deletion of genes *mrcA* or *mrcB* (associated with the biosynthesis of the outer membranes of *E. coli*) are more suitable for the growth of *E. coli* than the single deletion of genes *mrcA* or *mrcB* in MR medium. The growth of strain *lpp pal-pth* was retarded in MR medium. The reason for this remained unclear at the present, but it may be due to the removal of two lipoprotein genes related to the outer membrane integrity and cell division cycle (Gerding *et al.*, 2007).

When TB medium was used for extracellular protein production, the final optical density of all these strains was improved after 11 h cultivation (Supporting Information Table S6). The final optical density of *lpp pal-pth* was higher than that of *mrcA pal-pth* and *mrcB pal-pth*, suggesting that the growth limitation of Δ*lpp* and Δ*pal* strains can be relieved in TB medium (a complete medium). The further study on their mechanisms is warranted. The present study demonstrated that the extracellular yields of recombinant protein Trx-hPTH from the mutants with double deletion were significantly higher than those from the mutants with single deletion under the same condition (Supporting Information Fig. S2). In addition, our results showed, as expected, that mutants with single/ double deletion of genes, *mrcB* and *lpp*, could not cause the efficient leakage of the target protein rPA due to the protein expression in cytoplasm, not in periplasmic space (Supporting Information Fig. S3).

The comparison between the intracellular and extracellular production of Trx-hPTH using mutant *lpp mrcB-pth* as a model strain revealed that the extracellular production of Trx-hPTH continued to rise after the intracellular yield achieved a steady level (Fig. 4). In comparison, the intracellular accumulation of Trx-hPTH began to decrease after 6 h of fermentation. The probable reason is that the increasing accumulation of Trx-hPTH in periplasmic space results in the leaky capability increase of outer membrane of mutant cells. In addition, the extracellular excretion of target proteins can remove the cell disruption step, offer a better environment for protein folding, reduce the risk of intracellular enzyme degradation (Mergulhao *et al.*, 2005) and has unlimited periplasmic or intracellular space (Makrides, 1996; Fu *et al.*, 2005). Thus, the extracellular yield of target proteins could continuously increase even after the accumulation of intracellular production stops.

In conclusion, there was no apparent cell autolysis during the fermentation process of exogenous proteins. High leaky levels of Trx-hPTH were achieved from *lpp mrcB-pth* (77.7%) and *mrcA lpp-pth* (88.9%) with the double-gene deletion of *lpp* and *mrcB* as well as *mrcA* and *lpp* in MR medium. Among all the mutants with single/double-gene deletion, the extracellular production levels of target proteins in TB medium were much higher than those in MR medium. Particularly, almost all the Trx-hPTH has been transferred into the medium after 7 h fermentation in fermenters. All the results above
suggested that double deletion of peptidoglycan synthetase genes and outer membrane genes may increase the outer membrane permeability enough for the leakage of periplasmic protein without affecting the growth of these strains significantly in complex media.

Herein, the main advantage of leaky strains is that no any additive is needed to induce extracellular protein production and the main disadvantage is that the secretory selectivity is not high, suggesting that these genes affect the structure of the outer membrane but do not participate in the active transport of target protein(s) (Mergelhao et al., 2005).

**Experimental procedures**

**Strains and plasmids and primers**

The strain *E. coli* JM109 (DE3) was used as a host for genetic modification. The plasmid pKD46 (Amp<sup>+</sup>) was used as a helper plasmid for increasing the efficiency of homologous recombination, and pCP20 (Cm<sup>+</sup>/Amp<sup>+</sup>) was used for eliminating the resistance marker of the mutants in the construction of leaky strains (Datsenko and Wanner, 2000). The plasmids pET32a-pth and pET22b-rpa, both constructed without signal sequences, were used for the expression of exogenous proteins Trx-hPTH (a thioredoxin fusion protein used for its residence at the periplasmic space of *E. coli*) (Bayer et al., 1987; Fu et al., 2005) and rPA (Wang et al., 2009) in periplasmic space or in cytoplasm. The primers were designed by Primer Premier 5.0 and verified by Oligo 7.0. All the strains, plasmids, target fragments (TFs) and ‘verification’ primers used in this study are shown in Supporting Information Table S1.

**Media**

To activate and select strains, LB medium containing 10 g l<sup>−1</sup> tryptone (Oxoid, England), 5 g l<sup>−1</sup> yeast extract (Oxoid) and 5 g l<sup>−1</sup> NaCl was prepared (1.5% agar added in solid medium). The media were supplemented with ampicillin (Amp, a final concentration of 50 μg ml<sup>−1</sup>), kanamycin (Kan, 30 μg ml<sup>−1</sup>) or chloramphenicol (Cm, 25 μg ml<sup>−1</sup>) as required. To quantitatively detect target proteins, MR medium (a basic medium without proteins) was utilized in this study (Fu et al., 2005). After primary screening, TB medium (a complex medium) (Fu et al., 2005) was selected for improving the yield of extracellular production of recombinant proteins.

**Gene manipulation**

The leaky strains were constructed by using one-step inactivation method (Datsenko and Wanner, 2000; Baba et al., 2006). Briefly, the specific TFs with the flanking flippase recognition target sites used for the gene knockout, which spans the chloramphenicol acetyltransferase (*cat*) or kanamycin (*kan*) resistance cassettes, were amplified by a PCR method using corresponding primers shown in Supporting Information Table S1. Plasmid pKD3 (Cm<sup>+</sup>) or pKD4 (Kan<sup>+</sup>) were used as templates. The TFs have 50-nucleotide extensions that are homologous to the adjacent regions of the genomic *mrcB*, *mrcA*, *lpp* and *pal* genes (Supporting Information Table S2).

To construct leaky strains (*mrcA*, *mrcB*, *pal* and *lpp*) with inframe single deletion of genes *mrcA*, *mrcB*, *pal* and *lpp*, each corresponding TF was electro-transformed (MicroPulser Electroporator, Bio-Rad, Philadelphia, USA) into *E. coli* JM109 (DE3) harbouring pKD46 (Amp<sup>+</sup>) which can express the λ Red recombinase after L-arabinose induction. The resulting mutants were selected on LB agar plates containing 30 μg ml<sup>−1</sup> Kan or 25 μg ml<sup>−1</sup> Cm, and subsequently verified by a colony PCR method using the ‘verification’ primers listed in Supporting Information Table S1. In the processing of single deletion of genes, plasmid pKD4 or pKD3 (only for the single deletion of the gene *pal*), primer couples *mrcA-F/mrcA-R*, *mrcB-F/mrcB-R*, *pal-F/pal-R* and *lpp-F/lpp-R* as well as ‘verification’ primers *mrcA-vF/mrcA-vR*, *mrcB-vF/mrcB-vR*, *pal-vF/pal-vR* and *lpp-vF/lpp-vR* were correspondingly used.

To construct leaky strains (*lpp mrcB*, *mrcA lpp*, *mrcA pal* and *mrcB pal*) with inframe double-gene deletion, the antibiotic marker of single-gene-deleted strains was first removed by cloning a helper plasmid pCP20 (Amp<sup>+</sup>, Cm<sup>+</sup>) using competent cell preparation kit GK6031 (Shanghai Generay Biotech, Shanghai, China). The pCP20 contains the gene encoding the flippase recognition target (FLP) recombinase and a temperature-sensitive replication origin that creates thermal induction of FLP synthesis (Datsenko and Wanner, 2000); therefore, the positive colonies losing the resistance to ampicillin, kanamycin or chloramphenicol can be selected under suitable temperatures. More specifically, the positive mutants must be able to grow on the agar plates containing 50 μg ml<sup>−1</sup> Amp at 30°C and can grow on the same plates without Amp at 39°C due to the thermal induction of FLP synthesis and the thermal blockage of the replication of pCP20. The mutants without antibiotic resistance markers were further identified using the colony PCR and sequencing methods. The procedures for the construction of leaky strains with double deletion of genes were similar to those described above except for the TFs and the original strains. (For more details about the plasmids, primers, resistance markers, TFs as well as the kinship of strains, please see Supporting Information Table S3.)

To test the performance of the leaky strains (Supporting Information Table S1) with single- or double-gene deletion, the previously constructed plasmids (Table 1) expressing Trx-hPTH and rPA were transformed into these strains using the competent cell preparation kit GK6031 (Shanghai Generay Biotech) and then identified by DNA analysis and protein assay described as below. To test the genetic stability of them, the expression analysis of exogenous proteins were conducted in LB medium with the addition of ampicillin after the seventh generation culture of the recombinant leaky strains, and their growth curves were obtained. Finally, the leaky efficiency of target proteins from these strains was tested by fermentation experiments and protein assay.

**Flask fermentation**

To prepare inoculums, a single colony of each recombinant leaky strain (Supporting Information Table S3) with expres-
**Batch fermentation in a 5 l fermenter**

One single colony of *E. coli* pET32a-pth from a LB plate was inoculated into a 250 ml flask containing 30 ml of LB medium supplemented with 50 μg ml⁻¹ ampicillin and then incubated at 37°C in a rotary shaker at 200 r.p.m. The overnight culture was inoculated into a 250 ml flask containing 30 ml of TB medium supplemented with ampicillin (50 μg ml⁻¹) and then incubated overnight at 37°C in a rotary shaker at 200 r.p.m. After cultivation for 7 h, IPTG (Sangon Biotech) was added into the culture with a final concentration of 0.5 mM to induce recombinant protein expression and the culture was incubated for an additional 3.5–4 h. During the fermentation process, the cell density was determined by measuring the optical density at 600 nm (OD₆₀₀) with a BioPhotometer plus spectrophotometer (Eppendorf, Hamburg, Germany).

To improve the secretory efficiency of target proteins in the leaky strains, complex LB media and TB media were used. To prepare inoculums, a single colony of each recombinant leaky strain (Supporting Information Table S3) with expression plasmid from LB plates was inoculated into each 250 ml flask containing 30 ml of LB medium supplemented with ampicillin (50 μg ml⁻¹) and incubated overnight at 37°C in a rotary shaker at 200 r.p.m. The overnight culture was inoculated into a 250 ml flask containing 30 ml TB medium supplemented with ampicillin (50 μg ml⁻¹) to reach an OD₆₀₀ of 0.1, followed by incubation at 37°C in a rotary shaker at 200 r.p.m. After cultivation for 25 h (single-gene mutants) or 16 h (double-gene mutants), IPTG (Sangon Biotech) was added into the culture with a final concentration of 0.5 mM to induce recombinant protein expression and the culture was cultivated for an additional 8–9 h. During the fermentation process, the cell density was determined by measuring the optical density at 600 nm (OD₆₀₀) with a BioPhotometer plus spectrophotometer (Eppendorf).

**Sample preparation**

To prepare samples for the analysis of recombinant proteins, 1 ml of each culture was pelleted by centrifugation at 10 000 r.p.m. for 5 min at room temperature. The resulting supernatant fraction was defined as extracellular protein sample (EPS) and the pellet fraction as IPS. To differentiate IPS further, the pellet was washed twice by phosphate-buffered saline (PBS) (Na₂HPO₄·12H₂O 7.16 g l⁻¹, KH₂PO₄ 2.72 g l⁻¹; 0.02 M, pH 8.0), centrifuged at 10 000 r.p.m. for 5 min, resuspended in 1 ml PBS, subjected to ultrasonication and centrifuged at 10 000 r.p.m. for 5 min again. The supernatant fraction collected was defined as intracellular soluble protein sample and the precipitate fraction as intracellular insoluble protein sample. After that, the EPSs and IPSs were analysed by SDS-PAGE (Fu et al., 2005). The total protein levels of protein samples were measured by BCA protein assay as described below.

**DNA gel and SDS-PAGE**

DNA electrophoresis for PCR product analysis was carried out at 110 V for 30 min (Sambrook and Russel, 2011). Briefly, the samples of PCR products were mixed with 5× loading buffer (Shanghai Generay Biotech, China), and then loaded onto agarose gel (1%) with ethidium bromide. After electrophoresis, the gel was scanned by TANON gel image system 1600 (TANON Science and Technology, Shanghai, China).

SDS-PAGE was run at 110 V for 2 h according to Schägger’s publication (Schägger and von Jagow, 1987). Briefly, 40 μl protein sample was mixed with 10 μl Sample Loading Buffer (5×) SD8320 (Sangon Biotech) and boiled at 100°C for 5 min, and then the mixture was loaded onto SDS-PAGE gels (15%) with a volume of 15 μl. After electrophoresis, the gels were stained with Coomassie R250 (Shanghai Solarbio Bioscience & Technology, Shanghai, China) and then scanned by the TANON gel image system 1600. To determine the proportion of target protein in extracellular or IPS, the protein bands were normalized and quantified by a GIS 1D analysis software 4.1.2 (TANON Science & Technology).

**BCA protein assay**

The protein levels (including Trx-hPTH and total proteins) of all protein samples were measured by BCA method (protein assay kit-SK3051, Sangon Biotech) using bovine serum albumin (BSA) as standard. Serial dilutions of commercial BSA (Sangon Biotech) were used for constructing a standard curve. Samples with appropriate dilutions were prepared in duplicates, each with 50 μl. After addition of 1 ml working stock AB solution (Sangon Biotech), each sample in a tube was incubated at 37°C for 30 min. After cooling down to the room temperature, the absorbance OD₅₆₂ of each sample was measured by a BioPhotometer plus spectrophotometer (Eppendorf). The mean concentration of each protein sample was determined by using absorbance A₅₆₂ value according to the BSA protein standard curve.

**Authors’ contributions**

Zhao-Yuan Chen carried out the experiments and interpretation of results and drafted the manuscript. Li Xie, Jie Cao, Xiao-Fei Li, Zhen-Hai Yu and Jie Yu participated in the
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Conflict of interest

None declared.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Verification of the mutants by colony PCR (Lane M: representing standard marker). (A) Single knockout of gene mrcA (kan/1700 bp): Lane 1, JM109 (DE3) as a template; Lane 2, 3 and 4, mrcA as a template; (B) Single knockout of gene mrcB (kan/1700 bp): Lane 1 and 2, JM109 (DE3) as a template; Lane 3 and 4, mrcB as a template; (C) Single knockout of gene pal (cat’1600 bp): Lane 1 and 2, JM109 (DE3) as a template; Lane 3, 4 and 5, pal as a template; (D) Single knockout of gene lpp (kan/1800 bp): Lane 1, 2, 3 and 4, lpp N as a template; Lane 5 JM109 (DE3) as a template; Lane 6, 7 and 8, lpp as a template; (E) Double knockout of genes lpp and mrcB (cat’1200 bp) as well as mrcA and lpp (kan/1800 bp); Lane 1 and 6, others, Lane 3, JM109 (DE3) as a template; Lane 4, lpp mrcB (Cm’3) as a template (mrcB-vF and mrcB-vR as primers); Lane 5, lpp mrcB (Kan’3) as a template (mrcB-vF and mrcB-vR as primers); Lane 6, lpp N as a template (lpp-vF and lpp-vR as primers); Lane 7, mrcA lpp (Kan’3) as a template (lpp-vF and lpp-vR as primers); (F) Double knockout of genes lpp and pal (kan/1200 bp); Lane 1 and 2, lpp pal (Kan’5) as a template (pal-vF and pal-vR as primers); Lane 3 and 4, (Cm’3) as a template (pal-vF and pal-vR as primers); Lane 5, nothing; lane 6, JM109 (DE3) as a template; (G) Double knockout of genes pal and mrcB (kan/1700 bp) as well as pal and mrcA (kan/1700 bp); Lane 1, 3 and 5, mrcB pal as a template (pal-vF and pal-vR as primers); Lane 2 and 4, false positive colonies as a template (pal-vF and pal-vR as primers); Lane 6, 7, 8 and 9, JM109 (DE3) as a template; Lane 10, mrcA pal as a template (pal-vF and pal-vR as primers).
Fig. S2. SDS-PAGE analysis of protein samples in TB medium. Lane 1 and 2, the extracellular proteins of the recombinant leaky strains lpp mrcB with pET32a-pth; Lane 3, 4, 5 and 6, the extracellular proteins of the recombinant leaky strains mrcA lpp, lpp, mrcB and mrcA with pET32a-pth; Lane 7, the extracellular proteins of the strains JM109 (DE3) harbouring pET32a-pth; M, protein ladder; Lane 8, 9, 10, 11, 12, 13 and 14, intracellular soluble proteins corresponding to lane 1, 2, 3, 4, 5, 6 and 7.

Fig. S3. SDS-PAGE analysis of protein samples of lpp mrcB with pET22b-rpa in TB medium (M, protein ladder). Lane 1, 2, 3, and 4, the extracellular proteins of the recombinant strains lpp mrcB, mrcB, lpp and JM109 (DE3) harbouring pET22b-rpa; Lane 5, 6, 7 and 8, intracellular soluble proteins corresponding to lane 1, 2, 3 and 4; Lane 9, 10, 11 and 12, intracellular insoluble proteins corresponding to lane 1, 2, 3 and 4.

Fig. S4. Growth curve of the strains in flasks in LB medium. (A) ■, strain lpp mrcB with pET32a-pth; ●, Strain lpp mrcB harbouring pET32a-pth with IPTG induction after four hour cultivation; ▲, Strain mrcA lpp with pET32a-pth. (B) ■, strain mrcB; ●, strain JM109 (DE3).

Table S1. Strains, plasmid and primers used in this study.
Table S2. The sequences of TFs (targeting fragments).
Table S3. The plasmids, primers, resistance markers, targeting fragments and hosts used for mutant construct.
Table S4. Sequences of the PCR products of the mutants of mrcAN, mrcBN and lppN.
Table S5. Growth analysis and protein detection from the recombinant leaky strains with single-gene deletion in TB medium.
Table S6. Growth analysis and protein detection from the recombinant leaky strains with double deletion in TB medium.