

Multiplex Design of the Metabolic Network for Production of L-Homoserine in *Escherichia coli*

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ABSTRACT L-Homoserine, which is one of the few amino acids that is not produced on a large scale by microbial fermentation, plays a significant role in the synthesis of a series of valuable chemicals. In this study, systematic metabolic engineering was applied to target Escherichia coli W3110 for the production of L-homoserine. Initially, a basic L-homoserine producer was engineered through the strategies of overexpressing thrA (encoding homoserine dehydrogenase), removing the degradative and competitive pathways by knocking out metA (encoding homoserine O-succinyltransferase) and thrB (encoding homoserine kinase), reinforcing the transport system, and redirecting the carbon flux by deleting *iclR* (encoding the isocitrate lyase regulator). The resulting strain constructed by these strategies yielded 3.21 g/liter of L-homoserine in batch cultures. Moreover, based on CRISPR-Cas9/dCas9 (nuclease-dead Cas9)-mediated gene repression for 50 genes, the iterative genetic modifications of biosynthesis pathways improved the L-homoserine yield in a stepwise manner. The rational integration of glucose uptake and recovery of L-glutamate increased L-homoserine production to 7.25 g/liter in shake flask cultivation. Furthermore, the intracellular metabolic analysis further provided targets for strain modification by introducing the anaplerotic route afforded by pyruvate carboxylase to oxaloacetate formation, which resulted in accumulating 8.54 g/liter L-homoserine (0.33 g/g glucose, 62.4% of the maximum theoretical yield) in shake flask cultivation. Finally, a rationally designed strain gave 37.57 g/liter L-homoserine under fed-batch fermentation, with a yield of 0.31 g/g glucose.

IMPORTANCE In this study, the bottlenecks that sequentially limit L-homoserine biosynthesis were identified and resolved, based on rational and efficient metabolicengineering strategies, coupled with CRISPR interference (CRISPRi)-based systematic analysis. The metabolomics data largely expanded our understanding of metabolic effects and revealed relevant targets for further modification to achieve better performance. The systematic analysis strategy, as well as metabolomics analysis, can be used to rationally design cell factories for the production of highly valuable chemicals.

KEYWORDS L-homoserine, metabolic engineering, CRISPR interference system, intracellular metabolite profiling, microbial cell factory

A nonessential amino acid for the biosynthesis of L-threonine and L-methionine (1, 2), L-homoserine (L-hydroxybutyric acid), was first synthesized in 1907 by Fischer and Blumenthal. L-Homoserine is also an important precursor for the production of isobutanol, 1,4-butanediol (3), L-phosphinothricin (4), 2,4-dihydroxybutyrate (5), and 1,3-propanediol (6). Therefore, maximizing the productivity of L-homoserine based on new genetic-engineering tools would further extend its potential chemical and biolog-ical applications (3, 7). The biosynthesis of L-homoserine has been classified as a linear biosynthetic pathway consisting of glycolysis, the tricarboxylic acid (TCA) cycle, and the

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FIG 1 Key metabolic pathway for construction of an inducer-free L-homoserine-producing strain. The red crosses indicate that the genes are disrupted. The green arrows indicate that pathways are overexpressed. Glu, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6P, fructose-1,6-diphosphate; 6PG, 6-phosphategluconolactone; XSP, xylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; G3P, 3-phosphate-glycerate; G2P, 2-phosphate-glycerate; SER, serine; PEP, phosphoenolpyruvate; Ace-CoA, acetyl-CoA; PYR, pyruvate; LAC, lactate; ACE, acetate; CIT, citrate; ICL, isocitrate; α -KG, α -ketoglutarate; SUCC, succinyl-CoA; SUC, succinate; FUM, fumarate; MAL, L-malate; OAA, oxaloacetate; ASP, L-aspartate 4-semialdehyde; ASP-SA, L-aspartate 4-semialdehyde; HS, L-homoserine; LYS, L-lysine; THR, L-threonine; OSH, *O*-succinyl homoserine.

aspartate metabolic pathway (Fig. 1). The accumulation of L-homoserine was derived from an L-threonine- or L-methionine-producing strain. It was found that most efforts were focused on regulating gene expression of the L-aspartate metabolic pathway (8, 9). Generally, three aspartokinase isoenzymes (AKI, AKII, and AKIII) that catalyze the first step in L-homoserine biosynthesis were overexpressed to enhance the carbon flux to L-homoserine production. In addition, the transporter system was enhanced to improve survivability under the stress of L-homoserine (10). Due to the lack of optimization upstream of glycolysis, as well as branched pathways (11), L-homoserine was produced with a low yield by mutant and metabolically engineered strains, including *Corynebacterium glutamicum* (12) and *Escherichia coli* (13, 14). Until now, the optimization of each gene participating in different pathways and the identification of bottlenecks limiting L-homoserine biosynthesis in *E. coli* have rarely been reported.

Currently, pathway design and optimization are essential to obtain microbial cell factories with excellent performance for industrial production of commercial chemicals (15, 16). A series of attempts have been made to construct novel producers for biobased chemicals as the technologies in support of synthesizing new materials and manipulating genes have matured (17–19). Indeed, some strategies have been applied to optimize the biosynthetic pathways, which consist of large numbers of genes, or to reprogram gene expression to manipulate complex phenotypes. Prominent examples of such methods include the multidimensional heuristic process (MHP) (20), multiplex navigation of global regulatory networks (MINR) (21), and multibranched and multilevel regulated biosynthetic pathways (MBMRPs) (22), which rapidly engineer organisms with desired capabilities without the trial and error of iterative experimentation. On the other hand, "omics" profiling technologies, such as metabolomics, have facilitated an overview of cell metabolism, allowing a more in-depth insight into intracellular mechanisms in modified-organism analysis (23).

Here, the initial L-homoserine-producing strain was obtained by blocking the degradative and competitive pathways and overexpressing *thrA* (encoding homoserine dehydrogenase) based on an *O*-succinyl homoserine-producing strain (24). Then, ratio-



FIG 2 Comparison of fermentation performances of the engineered strains. Cells were cultured in MS medium at 28°C for 48 h. Con, control. The error bars represent SD.

nal design, including reinforcement of the transport system, redirection of the carbon flux, the CRISPR interference (CRISPRi) system, and module integration strategy, were used to construct an L-homoserine-producing strain. The iteratively modified strain HS33 could produce 7.25 g/liter L-homoserine from glucose in a shake flask. Metabolomics analysis further revealed the intracellular behaviors (metabolic differences) in response to genetic modifications to direct carbon to L-homoserine formation. Based on the metabolomics analysis, the anaplerotic route afforded by pyruvate carboxylase was introduced to direct carbon toward L-homoserine, which resulted in accumulating 8.54 g/liter L-homoserine (0.33 g/g glucose, 62.4% of the maximum theoretical yield) in shake flask cultivation. Finally, 37.57 g/liter L-homoserine was produced under fedbatch fermentation, with a yield of 0.31 g/g glucose.

RESULTS

Construction of an initial L-homoserine-producing strain. The "pull-push-block" strategy is an efficient method to engineer microorganisms involved in biosynthesizing target products by modifying metabolic networks (25, 26). In our previous study, the E. coli strain ΔJIB* TrcmetL with overexpression of metL and deletion of the metJ, metl, and metB genes was constructed to produce O-succinyl homoserine from L-homoserine and succinyl-coenzyme A (CoA) (24). To construct the basic L-homoserine-producing strain HS1, L-homoserine-converting pathway-related genes (thrB, encoding homoserine kinase, and metA, encoding homoserine O-succinyltransferase) were successively deleted to "block" L-homoserine degradation. Then, the L-homoserine-converting pathway was further strengthened by overexpression of thrA to "push" the carbon flux to L-homoserine production. Subsequently, the lysine-auxotrophic strain HS2 was generated by deleting lysA to investigate the effect of eliminating a precursor competing metabolic pathway on L-homoserine production. As shown in Fig. 2, HS1 could accumulate 1.85 g/liter of L-homoserine in MS (minimal salt) medium. The disruption of L-lysine biosynthesis increased the production of HS2 to 2.01 g/liter with the same specific production of 0.33 g/g cell dry weight (CDW) when L-lysine (0.025 g/liter, optimized amount) was added (see Fig. S1 in the supplemental material).

Modification of the transport system for improvement of the L-homoserine titer. Based on observations that L-homoserine inhibits the activity of aspartokinase (encoded by *metL*) and L-glutamate dehydrogenase (encoded by *gdhA*) (27, 28), toxicity stress arising from gradual accumulation of product results in repressing cell growth and product yield (29, 30). Therefore, strengthening the capability of the L-homoserine transport system and the transformation of other toxic intermediate metabolites are top priorities. *rhtA*, encoding the inner membrane transporter that is involved in the



FIG 3 Effect of transport system (RhtA and EamA) modification on ∟-homoserine production. Cells were cultured in MS medium at 28°C for 48 h. The error bars represent SD.

export of L-homoserine (31), was overexpressed chromosomally by replacing the native promoter with the trc promoter to obtain strain HS3 (Trc-rhtA). Strain HS3 increased the production of L-homoserine by 30.9% to 2.63 g/liter (Fig. 3). It was reported that overexpression of eamA confers resistance to toxic chemicals (32). In order to further increase the L-homoserine export capacity and relieve the growth burden of homoserine-producing strains to enable survival, the native promoter of the eamA gene was replaced by the trc promoter in strain HS4 (Trc-eamA). Batch cultivation of HS4 resulted in the production of 2.17 g/liter L-homoserine. Moreover, two rhtA gene copies (the native rhtA gene and replacement of the lacl gene) and eamA were overexpressed under the trc promoter in the chromosome to construct strain HS5 (*Clacl*::Trc-*rhtA* Trc-*rhtA* Trc-*eamA*). Under batch culture, strain HS5, with modification of the transport system and construction of a constitutive expression system, could produce 3.14 g/liter L-homoserine, which was 54.2% higher than strain HS2 production. In addition, the specific production of strain HS5 was also increased (Fig. 3), which confirmed that enhancing the transport system was beneficial for the improvement of L-homoserine productivity.

Effect of a glyoxylate shunt on L-homoserine production. The deletion of the *iclR* gene can activate the glyoxylate shunt, which increases the availability of malate as the precursor of oxaloacetate (2, 33). To further direct the carbon flux to L-homoserine, the transcriptional regulator IcIR was removed to construct strain HS6. As shown in Fig. 4, the final L-homoserine concentration obtained in strain HS6 with *icIR* deleted was equal to that in the control strain. Additionally, the AspA-catalyzed reaction is an efficient alternative to the AspC-catalyzed reaction in the conversion of fumarate and oxaloacetate to L-aspartate (34, 35). Therefore, the deletion of fumarase genes (*fumA*, *fumC*, and *fumB*) and overexpression of *aspA* were implemented. Under batch culture, both strains HS7 (with *fumAC* deleted) and HS8 (with *aspA* overexpressed) showed lower L-homoserine production than HS6 (Fig. 4).

Repression of candidate genes by the CRISPRi system to further enhance L-homoserine production. To identify the target genes to be further manipulated, the CRISPRi system for sequence-specific control of gene expression was used on the L-homoserine production strain HS6. Specifically, the homoserine-forming pathway was partitioned into three modules, which were separated at GAP (D-glyceraldehyde 3-phosphate) and OAA (oxaloacetate). These metabolic nodes are involved in the glycolytic pathway, by-product production, and amino acid biosynthesis. The results for strains with target gene downregulation were measured and compared with the control harboring the pTarget-null plasmid without an N20 sequence [HS6 (pdCas9, pTarget-null)]. As shown in Fig. 5a, 39 genes were chosen to study the effects



FIG 4 Effects of TCA cycle perturbation on fermentation performances of different engineered strains. Cells were cultured in MS medium at 28° C for 48 h. The error bars represent SD.

of target gene repression on L-homoserine production. Compared with the control group, HS6(pdCas9, pTarget-null), the strains with single guide RNAs (sgRNAs) targeting genes exhibited differences in the accumulation of L-homoserine. The L-homoserine production of strains with sgRNA-directed downregulation of *ptsH*, *ptsl*, *crr*, *ptsG*, *tktA*, *rpe*, *talB*, *argA*, *argG*, *proB*, and *gadA* in three modules were increased by more than 100%. On the other hand, the sgRNAs targeting *zwf*, *pta*, and *poxB* also increased the accumulation of L-homoserine by 50% to 100% (Fig. 5a).

On the basis of these results, candidate genes with promising positive targets for L-homoserine yields were selected for further individual deletion. As such, *ptsG*, *ptsH*, *ptsl*, *crr*, *tktA*, *rpe*, *talB*, *zwf*, *ackA*, *pta*, *ilvA*, *poxB*, *argG*, *gadA*, and *proB* were removed individually based on strain HS6 to construct a series of novel L-homoserine producers (strains HS9 to HS23). As shown in Fig. 5b, the cell growth of these engineered strains was variable; in particular, growth of the strain with deletion of *ptsl* was seriously inhibited. Additionally, the results showed that the strains with individual deletions of *ptsG*, *ptsH*, *crr*, *tktA*, *rpe*, *pta*, *argG*, and *proB* accumulated higher L-homoserine content than the control strain. In particular, strains HS9 ($\Delta ptsG$) and HS12 (Δcrr) with perturbation of the phosphotransferase system (PTS) showed L-homoserine production at 5.27 g/liter and 5.52 g/liter, respectively, which was higher than that of other mutations. These positive genes, which were screened out by the CRISPRi system, should be further investigated to identify the rate-limiting steps blocking L-homoserine biosynthesis.

Restoration of glucose uptake by non-PTS sugar transporter modification. On the basis of the above-mentioned results, the effects of the iterative genetic modifications of the selected genes not participating in L-homoserine biosynthesis were investigated. In this study, combinational genetic perturbation targeting the PTS and the pentose phosphate pathway was performed. To provide ample upstream pathway strength, a new set of five strains (HS24 to HS28) were engineered on the basis of perturbation of the PTS equilibrium, on which iterative genetic modifications were carried out between the PTS and the pentose phosphate pathway. Unexpectedly, the results showed that the L-homoserine yield was reduced in these further engineered strains (Fig. 6a). Given the differences in cell growth and major by-product accumulation between HS9 and HS12, the $\Delta ptsG$ strain (HS9) was chosen as the candidate for further investigation (Fig. 6b). Due to the decrease in glucose uptake caused by the ptsG gene deletion, the promoter region of the *alk* gene (encoding glucokinase) was replaced with the trc promoter, and inactivation of galR led to constitutive expression of a non-PTS sugar transporter to restore glucose transport capacity for efficient L-homoserine production (see Fig. S2 in the supplemental material). The L-homoserine



FIG 5 CRISPR-dCas9-based strategy for systematic screening and fine tuning of gene expression for L-homoserine overproduction. (a) Schematic of CRISPR-dCas9-based gene regulation in the L-homoserine biosynthesis pathway. The 39 target genes, including glycolysis, by-products, and branch pathways, were investigated in this study. The results were drawn from combined data for the single gene target among the genes. Relative changes in L-homoserine yield compared with the negative control (HS5 harboring a pTarget-null plasmid without N20 sequence) are represented as colored circles. Blue circles represent no significant change (-20% to 5%) in L-homoserine production compared to the negative control. Targets that increased L-homoserine production are shown in red. (b) Cell growth and homoserine production in deletion of positive targets. Control (con) refers to strain HS6. The cells were cultured in MS medium at 28°C for 48 h. The error bars represent SD.

titer observed with the HS29 strain was 6.27 g/liter, which was a 19.0% increase compared to the HS9 strain. The major by-products, including acetic acid and α -ketoglutarate, were produced at 1.86 g/liter and 2.39 g/liter, respectively, in flask fermentations.

Flux reinforcement by enhancing circulation of the amino donor. It should be clear that glutamate functions as the amino donor for the transformation of



FIG 6 Modification targets for enhancement of the glycolysis pathway. (a) Effects of disrupting the PTS and pentose phosphate pathway on L-homoserine production and cell growth. (b) Effects of modification of a non-PTS glucose uptake system on cell growth and homoserine production. KG, ketoglutarate; ACE, acetate. Cells were cultured in MS medium at 28°C for 48 h. The error bars represent SD.

oxaloacetate into L-aspartate. On the other hand, α -ketoglutarate would be accumulated from L-glutamate under conditions of nitrogen limitation. Not only is α -ketoglutarate responsible for catalyzing the first reaction in ammonia assimilation, it coordinates carbon and nitrogen utilization by rapid modulation of the glycolytic flux to alter import of glucose and consumption of phosphoenolpyruvate (PEP) (11). Therefore, in order to convert α -ketoglutarate into L-glutamate, strain HS30 was constructed by replacing the wild-type promoter of the gltBD operon (encoding L-glutamate synthase) with a strong trc promoter in strain HS6, and the L-homoserine yield was increased by 23.1% to 3.95 g/liter. This showed that the amino donor (L-glutamate) supply was considered another bottleneck in the biosynthesis of L-homoserine. Additionally, another set of two strains (HS31 and HS32) were constructed by deleting *argG* and *proB* based on the *gltBD* gene upregulation strain HS30 to increase the availability of intracellular L-aspartate and L-glutamate, respectively. However, not only were cell growth and glucose consumption severely repressed, but less accumulation of L-homoserine was observed, especially for strain HS32 (Fig. 7a).

These observations prompted the construction of strain HS33, which was iteratively modified by upregulating the expression of L-glutamate synthase based on strain HS29 to remove the deeper rate-limiting step. The successful overexpression of selected genes was subsequently verified by reverse transcription-quantitative PCR (RT-qPCR) (see Fig. S3 in the supplemental material). Notably, the observed L-homoserine production by strain HS33 was increased by 15.6% to 7.25 g/liter without affecting cell growth and the consumption of glucose (Fig. 7b). Also, the concentration of the by-product α -ketoglutarate was further decreased to 1.63 g/liter (Fig. 7b), indicating that upregulation of the *gltBD* operon could effectively facilitate the conversion of α -ketoglutarate into L-glutamate. It was concluded that the multidirectional control of



FIG 7 Modification of metabolic flux at the glutamate node. (a) Comparison of L-homoserine production, cell growth, and glucose consumption in different engineered strains. (b) Effects of *gltBD* operon overexpression on L-homoserine and by-product production. The error bars represent SD.

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metabolic pathways associated with metabolic strategies and CRISPR-based transcriptional regulators enabled efficient carbon utilization and recovery of the amino donor for production of L-homoserine.

Metabolic variation response to iterative genetic modifications. An intracellularmetabolite profiling study was performed in E. coli W3110 and HS33 to further investigate the effectiveness of the above-mentioned iterative genetic modifications and to identify important metabolic pathways that are closely associated with overproduction of L-homoserine (Fig. 8). In strain HS33, the intracellular levels of glycolytic intermediates, such as glucose-6-phosphate (G6P), pyruvate, and acetyl-CoA, increased 2.3-, 2.2-, and 18.2-fold, respectively. Additionally, a 3.3-fold increase in AMP was also detected in the strain. These results indicated that in strain HS33, disruption of the PTS (deletion of *ptsG*) effectively reduced consumption of PEP, which was beneficial in increasing the formation of precursor for L-homoserine biosynthesis. Moreover, the activation of a non-PTS by glk gene promoter replacement and galR gene deletion increased the consumption of ATP, resulting in the elevation of AMP. Also, 2.3- and 2.2-fold increases of fumarate and malate, as well as a 35.0% decrease in isocitrate, were observed in strain HS33, which indicated the activation of a glyoxylate shunt in the TCA cycle. The concentrations of α -ketoglutarate and L-glutamate increased 5.9- and 3.2fold, respectively, in strain HS33. For two metabolites, aspartate and α -ketoglutarate, biosynthesis occurs through transamination between oxaloacetate and L-glutamate. Also, L-glutamate, as the donor of an α -amino for the biosynthesis of amino acids, can be synthesized from α -ketoglutarate by a transamination reaction. The increased L-glutamate and α -ketoglutarate pools indicated that the overexpression of the *gltBD* cluster could effectively provide an amino donor for the L-homoserine biosynthetic pathway and adjust the circulation of their pools within the proper range. With the increase of the L-homoserine pool, a decrease of aspartate was also observed. These results demonstrated that enhancement of the L-aspartate metabolism pathway associated with disruption of competitive pathways could effectively activate the biosynthesis of L-homoserine from L-aspartate. The concentration of the by-product 2,6diaminopimelate (DAP) increased 2.2-fold due to knocking out lysA. Based on metabolome analyses, a better understanding is needed of the new limitations, such as carbon redirection and L-homoserine efflux, which would facilitate flux reinforcement by introducing an anaplerotic pathway or other regulatory mechanisms for moreefficient production of L-homoserine.

Replenishment of oxaloacetate from pyruvate toward further improvement based on metabolomics analysis. While obtaining a global overview of metabolic variation, we found that intracellular levels of acetyl-CoA for strain HS33 were higher (more than 18-fold) than those for the wild-type strain, suggesting the carbon flux was not efficiently diverted to oxaloacetate or citrate for production. To address this new bottleneck, two strategies were investigated: (i) introducing the pyruvate carboxylase gene into the engineered strain to direct pyruvate to oxaloacetate rather than acetyl-CoA and (ii) introducing the citrate synthase gene into the engineered strain to direct acetyl-CoA to the TCA cycle. Since E. coli lacks the anaplerotic enzyme pyruvate carboxylase, pyruvate accumulation in the strain could not be directly altered by directing pyruvate to the aspartate branch (Fig. 9a). Although the thrA gene was previously overexpressed by replacing the trc promoter, the transcription level was increased by about 2-fold (see Fig. S3). To further enhance the flux of the L-aspartate branch, we overexpressed an aspartate-insensitive pyruvate carboxylase (encoded by pycP458S) from C. glutamicum and threonine-insensitive bifunctional aspartokinase/ homoserine dehydrogenase (encoded by *thrA*^{G433R}) and aspartokinase (encoded by lysC) from E. coli in plasmid pACYC 184. Additionally, E. coli citrate synthase functions as a trimer of dimeric subunits, and its activity is allosterically inhibited by NADH and 2-oxoglutarate (36). In contrast, citrate synthase from Gram-positive bacteria and all eukaryotes is a simple dimer that is not allosterically regulated (37). Therefore, we overexpressed various citrate synthase genes, including citA_{bs} (Bacillus subtilis), gltA_{ca}



FIG 8 Systems metabolic engineering of E. coli for homoserine production. The data comprise relative concentrations of intracellular intermediates and metabolic flux predictions in the central metabolic pathways, as well as the genomic traits engineered into the strain. The thickness of the reaction arrows represents the predicted flux amount. The relative pool sizes of selected metabolites within two strains are given as REL values in the tables, with concentrations in E. coli W3110 taken as the reference for normalization between samples. The genomic traits include deletion of the transcriptional repressor (Δ metJ), deletion of O-succinylhomoserine lyase (Δ metB), overexpression of aspartate kinase (P_{tre} metL), deletion of homoserine kinase ($\Delta thr B$), deletion of homoserine O-succinyltransferase ($\Delta met A$), overexpression of homoserine dehydrogenase (P_{rrc} thrA), deletion of diaminopimelate decarboxylase (Δ /ysA), insertion of *rhtA* into the *lacl* locus (Δ*lacl::rhtA*), overexpression of the L-homoserine exporter (P_{trc} rhtA), overexpression of the cysteine/O-acetylserine exporter (P_{trc} eamA), deletion of the isocitrate lyase regulator ($\Delta iclR$), deletion of the glucose-specific PTS enzyme IIBC component ($\Delta ptsG$), deletion of the galactose operon repressor ($\Delta galR$), overexpression of glucokinase (P_{trc} glk), and overexpression of the glutamate synthase operon (P_{trc} gltBD). REL, relative values; Glu, L-glutamate; α-KG, α-ketoglutarate; Asp-SA, L-aspartate 4-semialdehyde; DAP, diaminopimelic acid; GOX, glyoxylate cycle. The screened metabolomics data were subjected to PLS-DA. SCM (defined as follows: base VIP, >1.0; fold change, >1.2 or <0.8333; q < 0.05) were selected for subsequent chemical structure identification.



FIG 9 Introducing the anaplerotic route afforded by pyruvate carboxylase further improved the production of homoserine. (a) An overview of the central metabolic pathway in the strategy for diverting carbon to L-homoserine from acetyl-CoA. Blue arrows represent the paths (genes) introduced in the construction of a further improved strain. (b) The effects of overexpression of pyruvate carboxylase or citrate synthase on fermentation performance of L-homoserine. $gltA_{ecr}$ citrate synthase-encoding gene from *E. coli; citA*_{bsr} encoding citrate synthase from *Bacillus subtilis; gltA*_{cgr} citrate synthase-encoding gene from *Corynebacterium glutamicum*. The error bars represent SD.

(*C. glutamicum*), and *gltA*_{ec} (*E. coli*), in plasmid pTrc99A. Four plasmids were transformed into strain HS33, resulting in strain HS33/pACYC-*pyc*^{P458S}-*thrA*^{G433R}-*lysC*, HS33/pTrc-*citA*_{bs}, HS33/pTrc-*gltA*_{cq}, and HS33/pTrc-*gltA*_{ec} (Table 1).

Five strains, including HS33, were cultivated in MS medium for 48 h. Among the tested strains, HS33/pACYC-*pyc*^{P4585}-*thrA*^{G433R}-*lysC* could produce 8.54 g/liter L-homoserine (0.33 g/g glucose), which was increased by 17.8% compared to strain HS33. Pyruvate carboxylase plays an anaplerotic role for carbon redistribution by directing pyruvate to the aspartate branch, which was shown to be efficient in reducing the accumulation of α -ketoglutarate (0.26 g/liter) and acetate (1.35 g/liter). However, the other three strains that overexpressed citrate synthase produced smaller amounts of L-homoserine than the corresponding strain, HS33 (Fig. 9b). The overexpression of citrate synthase by *B. subtilis* resulted in reducing the accumulation of α -ketoglutarate, but the L-homoserine yield was higher than that of the control (0.30 versus 0.23 g/g glucose). In contrast, the overexpression of citrate synthase by *C. glutamicum* and *E. coli* resulted in improved growth and reduced by-product (acetate) accumulation. However, α -ketoglutarate accumulated significantly, which was probably due to the fact that the flux was altered by the enzyme by directing oxaloacetate to the citrate branch.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
E. coli BL21	E. coli B F ⁻ dcm ompT hsdS _p (r _p ⁻ m _p ⁻) gal [malB ⁺] K-12(λ S)	Trans BL21 ^a
E. coli W3110	Wild type: $F^- \lambda^- IN (rrnD-rrnE) 1 rph-1$	CGSC ^b
E. coli AJIB* TrcmetL	W3110: AmetJ AmetB Trc-metL	24
HS1	E. coli ΔJIB* TrcmetL: ΔthrB ΔmetA Trc-thrA	This study
HS2	HS1· //vsA	This study
HS3	HS? Trc-rbtA	This study
HSA	HS2: Trc-pamA	This study
HSS	HS2: Machimer and Tro-rhta Tro-rama	This study
НСС	HS5. AidD	This study
Н50	HS6: AfumAC	This study
	HSG; April	This study
		This study
HS13	HS6; $\Delta I K I A$	This study
HS14	HS6; $\Delta r p e$	This study
HS15	HS6; ΔtalB	This study
HS16	HS6; $\Delta z w t$	This study
HS1/	HS6; $\Delta ackA$	This study
HS18	HS6; Δpta	This study
HS19	HS6; Δ <i>ilvA</i>	This study
HS20	HS6; $\Delta poxB$	This study
HS21	HS6; $\Delta argG$	This study
HS22	HS6; <i>∆gadA</i>	This study
HS23	HS6; Δ <i>proB</i>	This study
HS24	HS6; $\Delta crr \Delta ptsH$	This study
HS25	HS6; $\Delta crr \Delta ptsl$	This study
HS26	HS6; $\Delta crr \Delta ptsG$	This study
HS27	HS6; $\Delta crr \Delta rpe$	This study
HS28	HS6; $\Delta crr \Delta t ktA$	This study
HS29	HS9; $\Delta galR$ Trc-glk	This study
HS30	HS6; Trc- <i>gltB</i>	This study
HS31	HS30; $\Delta argG$	This study
HS32	HS30; $\Delta proB$	This study
HS33	HS9; $\Delta galR$ Trc-glk Trc-gltB	This study
Plasmids		
pTarget-X	A plasmid used to transcribe sgRNA targeting gene X in the genome. X refers to the genes in central metabolism and selected amino acid biosynthetic pathways.	This study
pACYC-pyc ^{P458S} -thrA ^{G433R} -lysC	Cm ^r ; pACYC184 containing pyc (P458S), thrA (G433R), and lysC	This study
pTrc-gltA _{oc}	pTrc99A containing <i>gltA</i> from <i>E. coli</i>	This study
pTrc-citAbe	pTrc99A containing citA from B. subtilis	This study
pTrc-gltA _{ca}	pTrc99A containing <i>gltA</i> from <i>C. glutamicum</i>	This study
TransGen Biotech, Shanghai, China.		

^bCGSC, *E. coli* Genetic Stock Center.

Fed-batch production of L-homoserine. In order to scale up for L-homoserine production by HS33/pACYC-*pyc*^{P4585}-*thrA*^{G433R}-*lysC* from glucose, fed-batch culture was implemented in a 5-liter bioreactor containing 2 liters of medium. Cell growth, L-homoserine production, and residual glucose during the fed-batch culture are shown in Fig. 10. A total of 450 ml feeding medium containing 500 g/liter glucose was added to the bioreactor during the cultivation, and the residual glucose concentration was maintained below 10 g/liter. The cell growth entered stationary phase within 40 h, and the optical density at 600 nm (OD₆₀₀) fluctuated around 30 till the end of cultivation. Within 108 h of the fermentation, L-homoserine production continuously increased and was independent of cell growth, and the maximum titer achieved was 37.57 g/liter with a yield of 0.31 g/g glucose, representing a 4.4-fold increase compared to the titer achieved in flask shake cultivation. By-products of amino acids were almost undetectable. However, further effort is needed to increase the titer and shorten the fermentation time, such as optimizing the expression of the recognized genes and the culture conditions.

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FIG 10 Fed-batch fermentation profile of HS33/pACYC- pyc^{P458S} - $thrA^{G433R}$ -lysC in a 5-liter bioreactor. Time profiles of L-homoserine production, glucose concentrations, and cell growth during the fed-batch cultivation are shown. The error bars represent SD.

DISCUSSION

This work showed the construction of a basic homoserine producer through a combination of the traditional pull-push-block effects of metabolic engineering and rational genetic modifications. In accordance with the results reported previously, the disruption of competitive and degradative pathways (L-threonine, L-lysine, and L-methionine pathways) contributed to increasing the production of L-homoserine (13). When it comes to the accumulation of target products that are toxic to cells or that inhibit the activity of enzymes in the metabolic pathway, strengthening the exportation of these metabolites is of great importance to relieve the cell burden and enhance production (32, 38–40). Here, strengthening the transport system and constructing a constitutive expression system not only allowed constitutive expression of modified genes without the addition of inducer, it also avoided suppressing cell growth caused by the inducer and L-homoserine (32). Additionally, observations after deleting the transcriptional regulator IcIR indicated that the TCA cycle could operate smoothly when activating the glyoxylate shunt flux. However, the diversion of flux away from forming oxaloacetate from fumaric acid by deletion of fumarase genes or overexpression of aspA dramatically reduced the production of L-homoserine, which may be attributed to the toxicity of fumaric acids (41).

In addition to the above-mentioned rather obvious genetic modifications, we also tested the impacts of another 39 different genes on L-homoserine production by using CRISPRi-based gene repression methodology. The results of gene repression indicated that use of the CRISPRi system was a feasible method to regulate gene expression with less labor and time consumption. Specifically, downregulation of the PTS is widely approved for reinforcing the biosynthesis of PEP-derived products (42, 43). The repression of zwf, tktA, rpe, and talB disrupted the pentose phosphate pathway, which can direct more carbon to the synthesis of precursors in the glycolytic pathway. araA-, argG-, proB-, and gadA-repressing regulation may contribute to accumulating L-glutamate and L-aspartate and therefore provide more precursors for L-homoserine biosynthesis (44). Also, the repression of *ackA*, *pta*, and *poxB* contributed to reducing secretion of acetate, which blocked the by-product formation pathway and released acid stress. Moreover, validation of screened candidate genes by individual gene deletion further indicated that sequence-specific control of gene expression on a genome-wide scale is a practical tool for engineering gene-regulatory systems in L-homoserine biosynthesis (45). However, we observed that deletions of some genes

led to a decrease in L-homoserine production, which was not consistent with the results showing that these genes were repressed by CRISPR-dCas9. The possibility of completely inhibiting gene expression with CRISPRi is low, and in some cases, even a low expression level of certain genes located in metabolic pathways could potentially contribute enough enzyme activity to maintain its flux and thus cover the effect of targeting such genes (46).

Based on CRISPRi-based systematic analysis, integration of glucose uptake and recovery of L-glutamate were adopted to develop a stable strain with better performance. In the metabolism of the carbon skeletons of L-homoserine, PTS disruption is beneficial to improve the size of the intracellular pool of PEP, which is a direct precursor of L-aspartate family amino acids (42). However, the observed phenotype of PTS disruption mutations other than *ptsG* mutation was in accord with the reports that low growth rates were observed as the strains would consume ATP for phosphorylation of glucose through a non-PTS system (47). Therefore, restoration of glucose transport capacity could efficiently improve L-homoserine production. In terms of engineering amino acid supply pathways, enhancing circulation of amino donors by converting α -ketoglutarate to L-glutamate could also efficiently enhance L-homoserine production. However, increasing the availability of the intracellular amino acids L-aspartate and L-glutamate by blocking the metabolic pathways failed to increase the accumulation of L-homoserine. The results indicated that the regulation of L-aspartate and L-glutamate metabolism is complicated, as they are precursors involved in more than 10 pathways, which made it difficult to identify the major bottleneck for L-homoserine accumulation.

Metabolomics analysis revealed that the multiplex design of the metabolic network caused less flexibility of flux distribution on the metabolome level. The significant variations of strain HS33 directed more carbon toward L-homoserine biosynthesis after the multiple genetic modifications. Additionally, they facilitated understanding of the underlying metabolic effects and provided relevant targets for strain modification to achieve a preferable performance. The results demonstrated that introducing the anaplerotic pathway to direct pyruvate to oxaloacetate was superior to the strategy of directing acetyl-CoA to the TCA cycle. The metabolic bottleneck for the production of L-homoserine could be efficiently relieved by modification of metabolic flux at both the oxaloacetate and pyruvate nodes. Directing pyruvate to the L-aspartate branch could directly realize carbon redistribution, while regulation of the TCA cycle was quite complex, as it is a crucial component of metabolism in organisms (48).

In conclusion, 5 levels of bottlenecks, i.e., the transport system, transcriptional regulation, carbon utilization, amino donor circulation, and the anaplerotic path, that sequentially limited L-homoserine biosynthesis were identified and resolved after systematic searching and multiplex design of the L-homoserine biosynthesis pathway. The L-homoserine production of the optimal strain HS33/pACYC-*pyc*^{P4585}-*thrA*G433R-*lysC* reached 8.54 g/liter (0.33 g/g glucose, 62.4% of the maximum theoretical yield) and 37.57 g/liter (0.31 g/g glucose, 58.6% of the maximum theoretical yield) in shake flask cultivation and fed-batch fermentation without optimizing the expression of the selected genes or the culture conditions (Table 2). Further, it is expected that L-homoserine production by the optimal strain constructed in this study can be further enhanced by optimizing the expression of the positive genes and the fermentation process, which would unlock the potential of the strain for the production of L-homoserine and its derivatives on a large scale.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All the strains and plasmids used in this study are listed in Table 1. *E. coli* BL21 was used for plasmid construction, and the OSH-producing strain (24) was used as the original strain. The low-copy-number plasmid pACYC184 was used as the backbone for overexpressing the mutated *thrA* gene (G433R) and *lysC* gene from *E. coli* W3110 and the mutated *pyc* gene (P458S) from *C. glutamicum* ATCC 13032. Plasmid pTrc99A was used for overexpressing citrate synthase genes from *E. coli* W3110, *B. subtilis* 168, and *C. glutamicum* ATCC 13032. Luria-Bertani medium (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl) was used for cell cultivation. When needed, kanamycin and chloramphenicol were supplemented at final concentrations of 50 μg/ml.

	L-Homoserine titer	Cell growth			
Strain	(g/liter) (±SD)	(OD ₆₀₀)	Cultivation mode	Analytical method ^a	Source
E. coli NZ10	10.6		Shake flask (glucose)	HPLC (<i>o</i> -phthalaldehyde)	14
C. glutamicum 9366-EMS	14.5		Batch cultivation (sucrose)	Amino analyzer	53
C. glutamicum MH20-22B	3.0		Shake flask (glucose)	HPLC (phenylisothiocyanate)	12
HM5(pBRmetL–pNrhtA)	39.54 ± 1.10	38.99 ± 0.55	Fed-batch (glucose)	HPLC (<i>o</i> -phthalaldehyde)	13
HS1	1.85 ± 0.11	14.01 ± 0.14	Shake flask (glucose)	Amino analyzer	This study
HS2	2.01 ± 0.11	15.21 ± 0.11	Shake flask (glucose)	Amino analyzer	This study
HS5	3.14 ± 0.01	15.17 ± 0.27	Shake flask (glucose)	Amino analyzer	This study
HS6	3.26 ± 0.13	14.67 ± 0.44	Shake flask (glucose)	Amino analyzer	This study
HS9	5.28 ± 0.10	17.65 ± 0.27	Shake flask (glucose)	Amino analyzer	This study
HS12	5.52 ± 0.04	12.06 ± 0.47	Shake flask (glucose)	Amino analyzer	This study
HS29	6.27 ± 0.05	17.32 ± 0.44	Shake flask (glucose)	Amino analyzer	This study
HS30	3.95 ± 0.07	17.07 ± 0.35	Shake flask (glucose)	Amino analyzer	This study
HS33	7.25 ± 0.20	15.58 ± 0.55	Shake flask (glucose)	Amino analyzer	This study
HS33/pACYC-pycP458S-thrAG433R-lysC	8.54 ± 0.10	15.48 ± 0.34	Shake flask (glucose)	Amino analyzer	This study
HS33/pACYC-pycP4585-thrAG433R-lysC	37.57 ± 0.66	29.3 ± 0.14	Fed-batch (glucose)	Amino analyzer	This study

TABLE 2 L-Homoserine production	and relevant fermentation	parameters of the engineered	strains
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^aHPLC, high-performance liquid chromatography.

Plasmid construction. To construct plasmid pACYC-*pyc*^{P4585}-*thrA*^{G433R}-*lysC*, the mutated *pyc* gene (P4585) (NCBI gene ID 3344537) was amplified from *C. glutamicum*, and the *thrA* gene (G433R) and *lysC* gene were amplified from *E. coli* (49). The three fragments were ligated into pACYC184 by the standard protocol of Gibson assembly (50).

For expression of citrate synthase, the corresponding genes, g/tA_{ec} (NCBI gene ID 12930947), $citA_{bs}$ (NCBI gene ID 936270), and g/tA_{cg} (NCBI gene ID 3344398), were amplified from the genomic DNA of *E. coli, B. subtilis*, and *C. glutamicum* using the primers $GltA_{ec}$ –F/Glt A_{ec} –R, Cit A_{bs} –F/Cit A_{bs} –R, and $GltA_{cg}$ –F/Glt A_{cg} –R. Then, the three fragments were assembled with the pTrc99A backbone fragment obtained using the primers pTrc99A-F/pTrc99A-R to yield pTrc- $gltA_{ec'}$ pTrc- $citA_{bs'}$ and pTrc- $gltA_{cg}$. The primers used for plasmid construction are listed in Table 3.

Construction of an sgRNA-expressing plasmid for the CRISPRi system. The pdCas9 plasmid with a catalytically dead Cas9 mutant was stored in our collection (45). The original plasmid, pCas, and pTargetF, containing the Cas9 gene (51) and sgRNA, were donated by Sheng Yang from the Institute of Plant Physiology and Ecology (Chinese Academy of Sciences, Shanghai, China). The sgRNA-expressing plasmid library targeting selected genes was constructed according to previously reported methods (22). Briefly, sgRNA cassettes were generated through PCR-based site-directed mutagenesis using the original pTarget as a template. Primer sequences used for generation of sgRNA cassettes and the corresponding sgRNA expression vectors are listed in Table 4. Every sgRNA-expressing plasmid pdCas9 to obtain a series of strains: H56(pdCas9, pTarget-X), where X refers to the genes in the central metabolism and selected amino acid biosynthetic pathways.

Genetic manipulation of *E. coli* **derivatives using CRISPR-Cas9.** Gene insertion and deletion and chromosomal promoter replacement were achieved by using the CRISPR-Cas9 system, as previously described (24). The detailed protocol of gene editing is described in the supplemental material. The primers used in this study are listed in Table 5. Electrocompetent cells were prepared as previously described (51).

Primer	Sequence (5'–3')	Source
pACYC-F	CCTGATGAATGCTCATCCGG	This study
pACYC-R	GCAAATATTATACGCAAGG	This study
Pyc-F	CCTTGCGTATAATATTTGCATGTCGACTCACACATCTTC	This study
Pyc-R	ACACTCGCATGTATATCTCCTTTTAGGAAACGACGACGATCA	This study
ThrA-F	AAGGAGATATACATGCGAGTGTTGAAGTTCGG	This study
ThrA-R	TTTCAGACATGTATATCTCCTTTTAGACTCCTAACTTCCATG	This study
LysC-F	AGGAGTCTGAAAGGAGATATACATGTCTGAAATTGTTGTCTC	This study
LysC-R	CCTGATGAATGCTCATCCGGTTACTCAAACAAATTACTAT	This study
pTrc99A-F	TCTGTTTCCTGTGTGAAATT	This study
pTrc99A-R	TCTAGAGTCGACCTGCAG	This study
GltA _{ec} _F	AATTTCACACAGGAAACAGAATGGCTGATACAAAAGCAAA	This study
GltA _{ec} _R	GCCTGCAGGTCGACTCTAGATTAACGCTTGATATCGCTTT	This study
CitA _{bs} _F	AATTTCACACAGGAAACAGAATGGTACATTACGGATTAAA	This study
CitA _{bs} _R	GCCTGCAGGTCGACTCTAGATTATGAAAGAACTTCCTCGG	This study
GltA _{ca} _F	AATTTCACACAGGAAACAGAATGTTTGAAAGGGATATCGT	This study
GltA _{cg} _R	GCCTGCAGGTCGACTCTAGATTAGCGCTCCTCGCGAGGAA	This study

TABLE 3 Primers used for plasmid construction

TABLE 4 Primers used for CRISPRi

Primer ^a	Target	Sequence (5'-3') ^c
pTarget-ptsH	ptsH	ATACTAGT GGTGTGCAGACCGTTCGGAG GTTTTAGAGCTAGAAATAGC
pTarget-ptsG	ptsG	ATACTAGT GCGATTTACCGACCTTTTGC GTTTTAGAGCTAGAAATAGC
pTarget-ptsl	ptsl	ATACTAGT CGGGTATCGCTTTCGGTAAA GTTTTAGAGCTAGAAATAGC
pTarget-crr	crr	ATACTAGT TCCGGTATCCTTCTTGTCGT GTTTTAGAGCTAGAAATAGC
pTarget-tktA	tktA	ATACTAGTATGCTATTCGTGCGCTGAGCGTTTTAGAGCTAGAAATAGC
pTarget-tktB	tktB	ATACTAGTATGCGATTCGCGCACTCAGTGTTTTAGAGCTAGAAATAGC
pTarget-rpe	rpe	ATACTAGTCTCAATTCTGTCGGCTGATTGTTTTAGAGCTAGAAATAGC
pTarget-rpiA	rpiA	ATACTAGTCCTACACCAACAATGGTGCCGTTTTAGAGCTAGAAATAGC
pTarget-rpiB	rpiB	ATACTAGTATCAGTACGCTCTGACGACCGTTTTAGAGCTAGAAATAGC
pTarget-talA	talA	ATACTAGTCTGTCGTGGCAGACAGCGGCGTTTTAGAGCTAGAAATAGC
pTarget-talB	talB	ATACTAGT TTCGTCAGTACACCACCGTA GTTTTAGAGCTAGAAATAGC
pTarget-zwf	zwf	ATACTAGT GGTCATTTTCGGCGCGAAAG GTTTTAGAGCTAGAAATAGC
pTarget-ackA	ackA	ATACTAGT TCATCGATGCAGTAAATGGT GTTTTAGAGCTAGAAATAGC
pTarget-pta	pta	ATACTAGT TACCGGAACCAGCGTCGGTC GTTTTAGAGCTAGAAATAGC
pTarget-poxB	рохВ	ATACTAGT CCCTGCCGATTCGAGTGTTT GTTTTAGAGCTAGAAATAGC
pTarget-ldhA	ldhA	ATACTAGT GTACTGTTTTGTGCTATAAA GTTTTAGAGCTAGAAATAGC
pTarget-dld	dld	ATACTAGTACGAGCAAGTTCATTCAAAAAGTTTTAGAGCTAGAAATAGC
pTarget-adhE	adhE	ATACTAGT GAAACTGGCATATTCACGCT GTTTTAGAGCTAGAAATAGC
pTarget-mdh	mdh	ATACTAGT CAATACCGCCAGCAGCGCCG GTTTTAGAGCTAGAAATAGC
pTarget-serA	serA	ATACTAGT GAAGGCTTTCCAGCGCCTTT GTTTTAGAGCTAGAAATAGC
pTarget-pck	pck	ATACTAGTATAAGCCTCGAGTTCTTGCGGTTTTAGAGCTAGAAATAGC
pTarget-pps	pps	ATACTAGT TGGTTATACCAAAGCACCAG GTTTTAGAGCTAGAAATAGC
pTarget-maeB	таеВ	ATACTAGT TCCCTGGAACTGGAAATTCA GTTTTAGAGCTAGAAATAGC
pTarget-ilvA	ilvA	ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC
pTarget-tdcB	tdcB	ATACTAGTATAATGTCATCAATAGCAACGTTTTAGAGCTAGAAATAGC
pTarget-pyrL	pyrL	ATACTAGT CCAGCGTCTTTTTTCAGACG GTTTTAGAGCTAGAAATAGC
pTarget-pyrB	pyrB	ATACTAGT GATTAAGGTCATCGCGACTA GTTTTAGAGCTAGAAATAGC
pTarget-asnA	asnA	ATACTAGTACGTTGTTTGGCAATGTAAGGTTTTAGAGCTAGAAATAGC
pTarget-asnB	asnB	ATACTAGTCATCAGGCGTGACAGCTCGAGTTTTAGAGCTAGAAATAGC
pTarget-purA	purA	ATACTAGT TTTACCTTCGTCACCCCATT GTTTTAGAGCTAGAAATAGC
pTarget-putA	putA	ATACTAGT GTCGTCCAGCTTAACCCCCA GTTTTAGAGCTAGAAATAGC
pTarget-argG	argG	ATACTAGTATACCAATACGTTGACCTACGTTTTAGAGCTAGAAATAGC
pTarget-glnA	gInA	ATACTAGT CATCGTCAGTACGTGTTCAG GTTTTAGAGCTAGAAATAGC
pTarget-ltaE	ltaE	ATACTAGT TCATCGCTTCGAGCATGGCG GTTTTAGAGCTAGAAATAGC
pTarget-tdh	tdh	ATACTAGT GCCCTCTTCCGCTTTCAGTT GTTTTAGAGCTAGAAATAGC
pTarget-argA	argA	ATACTAGT TGATATAGGGAACCGAATGG GTTTTAGAGCTAGAAATAGC
pTarget-proB	proB	ATACTAGT CGAGTTTTACCACCAGCGTC GTTTTAGAGCTAGAAATAGC
pTarget-aspA	aspA	ATACTAGTACACCATAGTAGGCATCAGCGTTTTAGAGCTAGAAATAGC
pTarget-gadA	gadA	ATACTAGT GTGAATCGAGTAGTTCTGAG GTTTTAGAGCTAGAAATAGC
pTarget-null ^b		AT <i>ACTAGT</i> GTTTTAGAGCTAGAAATAGC
pTarget R-common		ACTAGTATTATACCTAGGACTGAGC

^aEach pTarget primer has the same reverse primer, pTarget R-common (listed last).

^bpTarget-null represents the primer used to construct pTarget-null without the N20 sequence, used as a negative control.

^cThe italic letters represent the restriction enzyme cutting site (Spel), and the boldface letters represent the 20-bp region complementary to the targeting region of the selected gene.

Culture conditions. MS medium was used for L-homoserine production by *E. coli* W3110-derived strains, consisting of (per liter) 40 g glucose, 17 g $(NH_4)_2SO_4$, 4 g yeast extract, 1 g KH_2PO_4 , 1 g $MgSO_4$, 25 g $CaCO_3$, 5 mg $FeSO_4$.7H₂O, 5 mg $MnSO_4$.7H₂O, and 5 mg $ZnSO_4$. Shake flask fermentation was performed in 500-ml flasks containing 20 ml MS medium at 28°C for 48 h at 150 rpm. When necessary, the medium was supplemented with 50 μ g/ml chloramphenicol or kanamycin. L-Methionine and L-threonine were supplemented to final concentrations of 0.05 and 0.2 g/liter. Additionally, L-lysine was added to MS medium at final concentrations ranging from 0 to 0.5 g/liter (0, 0.0125, 0.025, 0.05, 0.125, 0.25, and 0.5 g/liter), depending on the experiment, as described in the text. For expression of the CRISPRi system, strains were induced with 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) when the OD₆₀₀ reached 0.4 to 0.6. The cells were cultivated aerobically at 28°C in a shaking incubator at 150 rpm and supplemented with 50 μ g/ml antibiotics when necessary.

In fed-batch fermentation, a 5-liter bioreactor (Baoxing, Shanghai, China) was used for L-homoserine, with an effective working volume of 2.0 liters. The medium for fed-batch fermentations consisted of (per liter) 15 g glucose, 14 g $(NH_4)_2SO_4$, 2 g yeast extract, 2 g KH_2PO_4 , 0.5 g $MgSO_4$, 0.5 g L-threonine, 0.2 g L-methionine, 0.1 g L-lysine, 5 mg FeSO₄:7H₂O, 5 mg $MnSO_4$:7H₂O, and 5 mg ZnSO₄. The feeding medium contained 500 g/liter glucose, 12.5 g/liter KH_2PO_4 , 10.0 g/liter NaHCO₃, 4 g/liter L-threonine, 1 g/liter L-methionine, and 0.5 g/liter L-lysine, and the feed was started when the residual glucose concentration was below 10 g/liter. The fermentation temperature was maintained at 28°C, and the pH was adjusted to 6.8 with 40% ammonia water throughout the process. The dissolved oxygen level was maintained above 20% by adjusting aeration and agitation rates.

TABLE 5 Primers used for genome editing

Purpose	Target	Primer ^a	Sequence (5'-3')
Gene knockout	metA	pTarget- <i>metA</i>	TAATACTAGTTCTGGCAGAGACGGAAGAAGGTTTTAGAGCTAGAAATAGC
		L- <i>metA</i> -F	ATGAGAAAAAATCGCCTACGCCCCC
		L- <i>metA</i> -R	TCACAGAAGAAACCTGATTACCTCACTACA
		R- <i>metA</i> -F	TAATCAGGTTTCTTCTGTGATAGTCGATCG
		R-metA-R	ATAAAGACTTTCACATTGGCGTTGA
		T-metA-F	TGCTTAAGCAGAAATAATCG
	L A	I-metA-R	
	IYSA	plarget-lysA	
		L-IYSA-F	
		L-IYSA-R P_lucA_E	CLAACCOCAGAACAAACTCCAGATAAGTGC
		R-lysA-I R-lysA-R	
		T-/vsA-F	ATAGCTGTCAGTACGGGAAA
		T-/vsA-R	TTACCACGAACGAACAAAAG
	Δ lacl::Trc-rhtA	pTarget-lacl	GCTCTAAAACAATGAGTGAGCTAACTCACAACTAGTATTATACCTAGGAC
		L-lacl-F	AAGTGGTATGGCCGACAGAT
		L- <i>lacl</i> -R	TAATTGTCAAATTCACCACCCTGAATTGAC
		<i>lacl</i> ::Trc- <i>metA</i> -F	GGTGGTGAATTTGACAATTAATCATCCGGC
		<i>lacl</i> ::Trc- <i>metA</i> -R	AAGTGTAAAGTTAATTAATGTCTAATTCTT
		R-lacl-F	CATTAATTAACTTTACACTTTATGCTTCCG
		R-lacl-R	GGTCAAATTCAGACGGCAAA
		T-lacl-F	ATGATAGTTTTCGACTGACC
		T- <i>lacl-</i> R	AAAAGATAATTCATCCCACC
	iclR	pTarget- <i>iclR</i>	TAATACTAGTCTAACCACGATGCAACAGCAGTTTTAGAGCTAGAAATAGC
		L-icIR-F	GATCGTTGACGTCCGGAGAAATAGA
		L- <i>iclR</i> -R	CCAGAAAAAGGACAGTCTCTTTTTCTGTA
		R-icIR-F	AGAGACTGTCCTTTTTCTGGCGGGCAGAGG
		R-icIR-R	AGTGTCGTGTTTTCGTTTTTATTAA
			GGTTTCAATCAGGATCAGAT
	fum D	I-ICIK-K	
	TUTTIB	prarget-iumb	
		L-IUIIID-F	
		L-IUIIID-N R-fumR-E	
		R-fumB-R	
		T-fumB-F	
		T-fumB-R	ACCGGATAAGGAATTCACGC
	fumAC	pTarget-fumAC	TAATACTAGTGATGCCTGTAAAAAACACGGGTTTTAGAGCTAGAAATAGC
		L-fumAC-F	TTGTGGTTAGGATCTTTATAGTTAC
		L-fumAC-R	TTGCAGATTACATTGTTCTCTCACTTACTG
		R-fumAC-F	GAGAACAATGTAATCTGCAACATACAGGTG
		R-fumAC-R	CCAGGTCGATAACCTTTCGCAAGCA
		T-fumAC-F	ATTTTCTTCACCCTGCATA
		T-fumAC-R	CTTGAGCAACACAAGCTATT
	ptsG	pTarget- <i>ptsG</i>	ATACTAGTGCGATTTACCGACCTTTTGCGTTTTAGAGCTAGAAATAGC
		L-ptsG-F	TGGATCTCGGATTTTACATC
		L-ptsG-R	TTACGGATTACATAATTGAGAGTGCTCCTG
		R-ptsG-F	CTCAATTATGTAATCCGTAAGACGTTGGGG
		R-ptsG-R	CTCAATTATGTAATCCGTAAGACGTTGGGG
		I-ptsG-F	
		I-ptsG-R	
	ptsH	plarget-ptsH	
		L-pism-r	
		L-pish-R	
		R-plsn-r R-ptcH-P	
		T-ntsH-F	TGGCAGGTGAAGAGATTAAA
		T-ptsH-R	TIGGTIGGATIGACGTAAAC
	ntsl	pTarget-ntsl	
	pusi	I -ntsl-F	AAAGCCAAAGCTGAATCGAT
		L-pts/-R	TCGTGGATTACATAACCCTACCTTACTTGT
		R-ptsl-F	TAGGGTTATGTAATCCACGAGATGCGGCCC
		R-ptsl-R	TCCATGTTGGAGATAACAAC
		T-ptsl-F	AAAGCACCTTTTTAGGTGCT
		T-ptsl-R	GAGCGATGAATTGATTTTGC

(Continued on next page)

or plageser ATACTACTICGGTATCCTTCTGCGTGTTTTAGAGCTAGAAATAGC Lerr R GCAAGAATGCAAATGCAATCTAC Lerr R GCAAGAATGCAATCTACTCAC Rerr R AAACAAGATGCAAATGCAACGGA Rerr R AAACAGAATGCAATGCAACTGCAACGCGA Rerr R AAACAGAATGCAATGCACGCGAAAGGGTTTAGAGCTAGAAATAGC Lerr R ATACTAGTCGGGAACCAGGGGTTTAGAGCTAGAAATAGC Lerr R AAACAGTGTACTCTCCCGGCGAAACAGGGTTTAGAGCTAGAAATAGC Lerr R ATACTGACTGGGGAACCAGGGGTTTAGAGCTAGAAATAGC Lerr R ATACTGACTGGGAACCAGGGATTAGGAGCTAGAAATAGC Lerr R Rerr R AAACGGGAA Lerr R AAACAGTGGGAACGGGA Lerr R AAACGGGAATGCGGAACGGGGTTTAGAGCTAGAAATAGC Lerr R Rerr R AAACGGGGAATGCGGAACAGGGGTTTAGAGCTAGAAATAGC Rerr R RERR TCCGGGTGGCGCAAACAGGGTTTAGAGCTAGAAATAGC Rerr R RERR TCCGGGTGGTTAGGGGA Rerr R RERR TCCGGTGGGTGCAAACAGGGTTTAGGGGAA Rerr R RERR R AATGCGGGGTGTAGGGAA RERR R RAGGGTAGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGTTAGGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGGTTAGGGGAA RERR R AGAGGGGGGTGAGGGGAA RERR R AGAGGGGGGTGAGGGGGGGAA RERR R AGAGGGGGGTGAGGGGAA RERR R AGAGGGGGGAGGGGAA RERR R AGAGGGGGGAGGGGAA RERR R AGAGGGGGGAGGGGAA RERR R AGAGAGGGGGAGGGAG RERR R AGAGGGGGGAGGGGAG RERR R AGAGGGGGGAGGGGAG RERR R AGAGGGGGGAGGGGAG RERR R AGAGGGGGGAGGGGGGGGAA RERR R AGAGGGGGGAGGGGGGAG RERR R AGAGGGGGGAGGGAGGGGAG RERR R AGAGGGGGGAGGGAGGGGAGGGGAG RERR R AGAGGGGGGAGGGGAGGGGAGGGGAGGGGAGGG	Purpose	Target	Primer ^a	Sequence (5'-3')
L-or-F GCAMAGATIGANATICIAC L-or-F GCAMAGATIGANATICITACCIMAGNA R-or-F GAACATICATATICITCCCAGA R-or-F GAACATICATATICICCGAGA T-or-F ANACATICATATICICCGAACA R-or-F ANACATICATATICICCGAACA T-or-F ANACATICATATICICCGAACAACCGGGTTTTAGAGCTAGAAATAGC L-galle F CTCCGTTACTAGAACCGAACCAGCGGTTTTAGAGCTAGAAATAGC L-galle F CTCCGTTACTAGAACCGAACCAGACCAAACCGGGTTTAGAGCTAGAAATAGC H-galle F CTCCGGTTACTAGATAGACGA R-galle F CATCAGTACGGTAATAGAGG T-galle F GATAGGGGTAATAGAGG T-galle F GATAGGGGTAATAGAGG T-galle F GATAGGGGTAATAGAGG H-galle F CATCAGTAGCGGTAATAGAGG H-galle F GATAGGGGTAATAGAGG T-galle F GATAGGGGTAATAGAGG H-galle F GATAGGGGTAATAGAGG H-galle F GATAGGGGTAATAGAGG H-galle F GATAGGGGTAATAGAGG H-galle F GATAGGGGTAATAGGAT F H-galle F GATAGGGGTAATAGGAT F H-galle F GATAGGGGTAATAGGAT F H-galle F GATAGGGGTGATAGGAACAGGGTTTAGAGCTAGAAATAGC H-HA-H C GGACATATATAGATTIGACCTCGGGTAA H-HA-H C GGACATATATAGATTIGGCTGGGTAAA H-HA-H C GGACATATATAGATTIGGGCTGAATAGGTAGAAATAGC H-HA-H F GACGGGAGTGAGTAAT F H-GAACGCGGATGGAGGTAAT F H-HA-H C GGACGGAGTGAGTAGT F H-F GAACGCGGATGGAGGTGAATATGGGT F H-Pe-F GACCGAGTGGAGTGATATT F H-GACCGGATGGAGGGTGATTATAGGGT H-HA-H R GACGCGGATGGAGGTGAATATGGGG H-HA-H R GACGGGGGTGATGGGGTGATGGTGAGAAATAGGC H-HA-H R GACGCGGATGGAGGTGATTATAGAGCTAGGAAATAGGC H-HA-H R GACGGGGGTGATATTAGAGGCTGGGTGATGGGAAA H-HA-H R GACGAGGGGGTGATTATAGAG H-HA-H R GACGGGGGTGATTATAGAG H-HA-H R GACGAGGGTGGTTGTTAAGAGCTAGGGTGATGGG H-HA-H R AACGGGTGGTGTTTAAGAGCTAGGGTGATTAGGGGTGAT H-HB-R TTCCAGGGTGGTTGTTAAGAGCTAGGGGTGATTAGGGGGTGATTAGGGGGGAAGGGTTTAGGAGCTAGAAATAGGC H-HB-R TTCCAGGGTGGTTGTTAAGAGGTGGGGTTTAGGAGCTAGAAATAGGC H-HB-R TTCCAGGGTGGTTGTTAAGGGGGGAAGGGTTTAGGAGCTAGAAATAGGC H-HB-R TTCCAGGGTGGTGTTTAAGGGGGGAAGGGGTTTAGGAGCTAGAAATAGGC H-HB-R TTGCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG		crr	pTarget-crr	ATACTAGTTCCGGTATCCTTCTTGTCGTGTTTTAGAGCTAGAAATAGC
L-or-R GCAAGAATTACATGATCATCCTAAGCAG R-or-R AAACTACTATACATTCTCCCCGAG T-or-R AAATCAAAATCCTTCCCCGAG T-or-R AAATCAAATCCTTCCCCGAG T-or-R CAATGAACTTCTCACGG golf C-or-R CAATGACTTCTCACGG golf C-or-R CCCCACAACAACTTCTCACGG R-golf F TACCTGCGTTACATGAAATACCTCACTAAGGCTGGAAATAGC L-golf F TACCGCCAACAACAACCCACAACAG R-golf F TACCGCCAACAACAACCCACAACAGCTTTAGAGCTAGAAATAGC H-golf F TACCGCCAACAACAACACCCACACAGC R-golf F TACCGCCAACAACAACCCACACAGCACAGCACAGCACTTCCGCG R-golf F TACCGCCACACAACACCCCACACAGCACTTAGAGCTAGAAATAGC L-dick F TTGCGGCTTCAATGGACTCCGGGCTGCAACAACAGCTTTAGAGCTAGAAATAGC L-dick F TTGCGGCTGCAACAACAGCGTTTAGAGCTAGAAATAGC L-dick F TTGCGGCTGCAACACAGCGTTTAGAGCTAGAAATAGC L-dick F AGCCAAATTGCTCCGGGCGCGCACAACAGCGTTTAGAGCTAGAAATAGC L-dick F AGCCAAATGGCTCCGGGCGCGCACAACAGGGTTTAGAGCTAGAAATAGC L-dick F AGCCAAATGGCTAGATGGCCG R-Rick F AGCCAAATGGCATGGCGCGACACACAGGGTTTAGAGCTAGAAATAGC L-dick F ATGCGGCGCGCGAACACAGGGTTTAGAGCTAGAAATAGC L-dick F ATGCGGCGCGCGACACACAGGGTTTAGAGCTAGAAATAGC L-dick F AGCCGCACGCGGTGCAACACAGGGTTAGGAGGAGGAAATAGC L-dick F AGCCGCGCGCGGAACGCGGTGGAGGCGGAGGGCGGAGGGGGGGG			L- <i>crr</i> -F	GCAAAGAGATCGAAATCTAC
R-cr-F GAAGATCATICTICTICCCCAGTGAAA R-cr-F ATACATGAACTTCCCCGAGTGAA R-cr-F ATACATGACATTCCCCGAAG GG T-cr-F ATACATGACATCCCCGAAGAG GG T-cr-F CATTAGCAACTTCCCCGAAGA gaiR plarget gall TATACTAGCACTTCACGA gaiR TATACCAGTGGCGAACCAGCGAACCAGCGGGTTTTAGAGCTAGAAATAGC L-gaiR+F CTCTCGGTGAAGAACGG L-gaiR+F CTCCGGGCGGCAACCAGCGGGGGGGGGGGGGGGGGGGGG			L-crr-R	GCAAGAATTACATGATCTTCTCCTAAGCAG
R-cr-R AMATCAMATCCTTCCCGAAG T-cr-R CATTATGACACCTTCCGAAG T-cr-R CATTATGACACCTTCCGAAG gdiR Pirget-gdiR T-cr-R CATTATGACACCAGACCGAGCGAACCGAGCGAAACTGACAAAATGAC L-gdiR+F TCACTGATAAAACGCA R-gdiR+F TACCGCGAGAAACTGCAGATAAGGCG R-gdiR+F TACCGCGAGAACGCCCAAAACTGCAGTAAAGGC R-gdiR+F TACCGCGAGAACGCCCAAACACGCATAGGCTTTGGAGACTGGAGAATGGC T-gdiR+F TATTCGGTAGGTCGTGTTGTC L-tidA+R TTGTCAATGCGTTGGACGGCGGCGACAACAGGGTTTAGAGCTAGAAATAGC L-tidA+R TTGTCAAGGGCTGAAACTGCGGAGAACAGGGTTTAGAGCTAGAAATAGC L-tidA+R TTGTCAAGGGCTGAAACTGCGATGAGATGGAAA R-tidA+R CACCTAGTAGTCCTTGGGCAGAA R-tidA+R ATGCCAATTATACATTTGCGGGTAAA R-tidA+R ATGCCAATTATACATTGCGGGGGAAA R-tidA+R ATGCCAGTCAATTAGGACGGGGTAGATATT rpe Pirget-triR rpe+R CACTATTGCCCAGGTATATTCGGGGGGAAA rpe+R CGACTTTCTCCAGTCAGGAA rpe+R CGACTATTCGCCAGTAAATTCGGGGGAAA rpe+R CGACTTTCCCCAGTGAAATTCGGGGGAAA rpe+R CGACTATTCGCCGCAGACAGGATTTAGAGCTAGAAATAGC L-pe+R TCCAACGTCGAGAACGGCTGAAA rpe+R CGACTTTCGCCAGTAGAATTCGGGGAAA rpe+R CGACTATTCGCGTGAACAGGCTGGAAA rpe+			R- <i>crr</i> -F	GAAGATCATGTAATTCTTGCCGCAGTGAAA
I-cr-F ALACITCACCGAMAG G-C-F-G ALACITCACCGAMAGCGGGTTTTAGAGCTAGAAATAGC L-galiR TATACTAGTGGGCGAACCGACCGACCGAAGCGGGTTTTAGAGCTAGAAATAGC L-galiR TATGCGGTGATTAGGAGCGG B-galiR TAGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG			R-crr-R	AAATCAAAATCCTTGCCGAG
I-Cri-K CATTACATCATGGEGGAACCAGAACCGAACCGAACCGGGCTTTAGAGCTAGAAATAGC gdiR PTagerg-gdiR CTCTGATTCAGTAAAACCGA L-gdiR-R CTCTGATTCAGTAAAACCGA L-gdiR-R TCTGGGTTACCTAGAAATAGCTAGGAACTAGACGA R-gdiR-R TCTGGGTTACCTAGAAATAGCTAGGAACTAGCAGGA R-gdiR-R TCTGGGTGACGACGACGACCGACCGACAGGTTTGAGGTCAGAAATAGC T-gdiR-R CATTAGGTGACGACGATGGGGA R-gdiR-R CATTAGGTGACGACGACGACGACGACGGACGGACGGACGG			T-crr-F	ATACATGAACTTCCCGAAAG
gan plage-gan (IATIAL INGEGUSANAACCGA LGAAACUGG ITIAGAACUAAGUGG ITIAGAACUAAGUGG ITIAGAACUAAGUGA ITIAGAAGUGA ITIAGAAGUGA ITIAGAAGUGA ITIAGAAGUGAAGUGA ITIAGAAGUGA ITIAGAAGUGAAGUGAAGUGA ITIAGAAGUGAAGUGAAGUGAAGUGAAGUGAAGUGAAGUG		10	I-crr-R	
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regum Transfer regum Transfer regum GATTAGGCGTGATTCCT regum GATTAGGCGTGATTCCT regum GATTAGGCGTGATTCACT regum GATTAGGCGTGCTCCCAAACAAGGTTTTAGAGCTAGAAATAGC regum Catact regum GATTAGTCATTAGTTAGTTAGCGGT regum GATAGTAGTAGTTAGTTAGTTGAGGTATT regum GATAGTAGTAGTAGTAGTAGTAGGGCA regum Regum regum Tattata regum GACAAAGCCACCATAGGCGATTATATGAGCTAGAAATAGC regum Tattata regum Tattata regum Tattata regum Tattata regum Tattatata regum Tattatatatatatatatatata regum Tattatatatatata <td></td> <td></td> <td>L-YUIK-K</td> <td></td>			L-YUIK-K	
Hydum Hydum Hydum HARTINGGGTARTHAGAKG Hydum HARTINGGTARGGTARTHAGAKG Hydum HartingGTARTAGAKG Hydum CartradigGCTCGGACTCGAAACAAGGTTTAGAGCTAGAAATAGC Litth+ HTGTCAATAGCGTAT Litth+ HTGTCAATAGCGTAT Hitth+ ATGCTAATTAGAGCGTAA Ritth+ ATGCTAATTAGAGCGTAGATAA Ritth+ ATGCTAATTAGAGCGTAGCGAA Titth+ ATGCGTAATTAGAGCGCA Titth+ ATGCGTAATTAGAGCGTAGGTAAA Titth+ ATGCGTAATTAGAGCGTAGGTAAA Titth+ ATGCGTAATTAGAGCGTAGGTAAA Titth+ ATGCGTAGTTAGAGTAATTGGGCGAAAATAGC Litth+ TATATTAGAGCGTAGGTAATTAGAGCGAA PPE Planget-type Planget-type ATACTAGTTCGTGGTAGGTAATTGGAGAA Right+ TGCAATTGCGTGTAGAGAAATAGC LingFF ATGCAGTTGGTGAGTAGAGAATATGCG Right+ TGCAATTGCTGTGTAAGCGTAAGAAATAGC LingFF ATGCAGGTCAATTAGAACCGGTAGGAGATTTGGGGTAGAAATAGC LingFF TGGTAGCAAGAGGTCATTATGAGCTAGAAATAGC LingFF TGGTAGCAAGAGGTCATTATGGTGGTAGGAGAATAGC LingFF TGGTAGCAAGAGGAGCTGTATTCTTA RingFF ATGCGTAGCGCAGGAGAATTGGGGTATTGGGGTATGGAGAAATAGC LingFF TTGGTGCGAAGAGGTGATTATGGTGGGAAATAGGC LingFF <td></td> <td></td> <td>R-guin-F</td> <td></td>			R-guin-F	
Table R TATTGGTRCGTTCGGTC 1404 PTarget-WA CCACTATGTCTCGGGCTGCAAACAAGGTTTAGAGCTAGAAATAGC 1404 TTGTCAGACGTTTGGGTCGCAAACAAGGTTTAGAGCTAGAAATAGC 1404 ATGCTAATTACATTTGCACTCCAGATCGGA R4tbA+ CGCAGANTATACATTTGCACTCCAGATCGGA R4tbA+ CGCAGANTATACATTACGATCGCGACAA R4tbA+ CGCAGANTATACATTCGGTCGATAG T4tbA+ AGCAGATCAATTCGTCGGCGGATTGTTTAGAGCTAGAAATAGC 14tbA+ CGCAGATTGTTGCGGCTGATTGTTTAGAGCTAGAAATAGC 14tbA+ AGCAGTCCGATGATTATGAGCGGTCGGTGATTGTTAGAGCTAGAAATAGC 14tbA+ AGCAGTCGGATCAATTCGGCGGGTTGATGAGAAATAGC 14tbA+ AGCAGTCGGTAGATTATAGA 14tbA+ AGCAGTCGGTAGAATAGCGGGCGGGATGTTTAGAGCTAGAAATAGC 14tbA+ AGCAGTCGGTAGAATAGCGGGCGGGTTTGAGAGCTAGGAAATAGC 14tbB+ TAGTAGTCGTCGTAGAAGATGACGGACGGGAAAGTGGTGGAAAATAGC 14tbB+ ATGCTAGTCAGCAATGACTGAA 14tbB+ AGCAGATGATTATAGGGTCGCTGAAA 14tbB+ AGCAGTCGTTGGCAATGACCC 14tbB+ AGCAGGTCCTTTTGGCGCGCGAAAGGTTTTAGAGCTAGAAATAGC 14tbB+ TCGAACTGCTGCAAGAAC 14tbB+ AGCAGGTCCTTTTGGCGCGCGCAAAGGTTTAGAGCTAGAAATAGC 14tbB+ TCGAACTGCTGTAGCCCCCCGTTAGGCTTAGAGCTGAAAATAGC </td <td></td> <td></td> <td>T-galR-F</td> <td>GATTAGGCGTAATTAGAACG</td>			T-galR-F	GATTAGGCGTAATTAGAACG
tkiA pTarge-itka CACTAGTIGCTTCCGGCCTGCAAACAGGTTTTAGAGCTAGAAATAGC L.kkA+F TTGCCAAACGTTTAGCATTTGACTCCGAA R.tkkA+F ATGCCAAATAGATTAGCATTTGACGGAA R.tkkA+F ATGCCAAATAGATTAGCATTTGACGGAAA T-tkkA+R AGGCAGTCCAGTAGTAGTAGTAGTAGTAGAGAAATAGCCAAGGTTTTGAGGCTAGAAATAGC T-tkkA+R AGGAGTCCCAGTGCAGTAGT rpe PTarget-rpe ATACTAGTCCCAGTGCGTTGGTTTTGAGGCTAGAAATAGC L-rpe-F TTCAAACGCGTCGCGCTGCGTTGGTTGGGAGAAATAGC L-rpe-R TGCAAACGCGTCGCGTTGGTTGGGGAGAAATAGGC L-rpe-R GGCAGAAGGGCGTTAGTGGGAGAGAAATAGC L-rpe-R GGCAGAAGGGCGTTAGTGGGAGAAGAAGAGC T-rpe-R GGCAGAAGGTCCTTTATAGA T-rpe-R GGCAGAAGGTCCTTTATAGA T-rpe-R GGCAGAAGGTCCTTTATAGA T-rpe-R GGCAGAAGGTCCTTTATAGA T-rabF-F ACTATGGTACACATGAGAGGAC T-rabF-F TCCTAGCTCCTTTATAGA T-rabF-F TCCTAGCTCCTTTAGAGACCATGGGAGACC T-rabF-F TCCTAGCTCCTCTTAGAGGGGAC T-rabF-F TCTATGCTGCTGCATAAGGTTTAGAGCTTAGAGAATAGC L-rabF-F ACCTAGTAGATGTCGCCCCAAGGGGATTAGAGCTAGAAATAGC L-rabF-F TCCTAGCTCCCCCCTTTAGAGGGGGAC T-rabF-F<			T-aalR-R	TAATTCGGTACGTTCTGTTC
 Linki, P. And T. TIGTCAGACGTTTAGGGTAT Linki, P. ANGCTATATTACATTIGCATTCCAGATCGAA Rinki, P. ANGCTATATACATTIGCATTCCGAGATCGAA Rinki, P. CGAGAATATAATAAGGGCCA Tichki, P. ATCCGTCIACTCTATCTGCGCCGATAAA Rinki, P. ATCCGTCIACTCGATCGAT Tichki, P. ATCCGTCIACTCGATCGATCGAGAAATAGC Linge P. TICAAACTICTCGCGCTGATCGTCTCCTGTGAGAA Ringe F. GAAGAGCGATGTAAGTTGAGAGATATCGCG Ringe F. GAAGAGCGATGTAAGTTGAGAGAATAGC Tinge B. Pargeet-tail B. ATACTAGTTCGCTGATCGGT Tinge G. GCAGAAGGGTCATTTAATAGA Ringe F. GAAGAGCGATGTAAGTTGAGAGATTGGCGAGAAATAGC Lindib P. GACGAAGGGTCATTTAAGAG Tinge G. GCAGAAGGGTCATTTAAGAG Tinge G. GCAGAAGGGTCATTTAAGAGAGTAGTTAGAGAGTAGAAATAGC Lindib P. ACTAGTAGCACAATGACTGGAGAGTAGTTAAGAGCAGGGTAGTAAATAGGC Lindib P. ACTAGTTGGCCAGAGAGTCATTTAAGA Lindib P. ACTAGTAGCACAATGACTTAGAGAGTTTAGGGGGCC Rindib P. TCTTCACTGTAATACTTTGGGTGAGACAGC Lindib P. TCTTCACTGTAATACTTTGGGGGAGCAGACT Tindib P. TCTTCACTGTAATCACTTAGGGTGACAGAC Lindib P. TCTGCAGAGTGATTTAAGAGCC Rindib P. TCTTGACGAATGACTTAGAGGCTAGAAATAGC Lindib P. TCTGACGCGCAAGAGGTTTAGGGGGCATTAGAGAGC Tindib P. TCTGACGCGCAGAAGGGTTTAGGGGCATGGAAATAGC Lindib P. TCTGGATGCGCAGAAGGCTTAAGAGCC Rindib P. TCTGCAGAGGGAGTTTAGGGGGGATTAGAGGCTAGGAAATAGC Lindib P. TCTGGATGGCGAGAGGGTTTAGGGGGGATTGGCGGGAGAAGAGCCTAGGAAATAGC Lindib P. TACTGCTGTATGGGGGAGAGAGGTTTAGGGGCTAGGAAATAGC Lindib P. TACTGCTGTATGGCGGAGAGGTTTAGGGGCTAGGAAATAGC Lindib P. TACTGCTGTATAGGGGGAGATTACAGGGTGGTGTTTAGGAGCTAGGAAATAGC Lindib P. TACTGCTGTAGGGGAAGATACCGGTGGTGTTTAGAGCTAGGAAATAGC Lindib P. TACTGCTATAGGGGAACATAGCGCTAGAAATAGC Lindib P. TACTGCTATGTGGGAAAGAGGCT Ting P. TACAGAGAGATTACCAGGGTTTAGAGGCTAGGAA		tktA	nTarget-tktA	GCACTAGTGCTTCCGGGCTGCAAACAAGGTTTTAGAGCTAGAAATAGC
L. MARA AGG CAAAATGA CAAAATGA CAAAATGA CAAAATAGA CAAATAGA CAATAGA CAATAGA CAATAGA CAATAGA CAATAGA CAATAGA CAATAGA CAATAGA CAATAGAATAG			I -tktA-F	TIGTCAGACGTTTAGCGTAT
R-rku-FF ACTCANANTGTANTTAGCATTTGGGGTAAA R-rku-F ACGCANTTAATAGGGCCA H-rku-F ACGCGTATTATATAGGCCCA T-rku-F ACGGTCATTATATAGGCCCA T-rku-R AGAGACCCATGATT T-rku-R AGAGACCCATGGTCATTGTTGTGGCGCTGATGTTTTAGAGCTAGAAATAGC L-rpe-F TATATTGACTCGCTTCTCCTGAGAA R-rpe-F GACAACCGATGTAGTTGAGAGTATTCGCG R-rpe-F GGACGACGCGTCTTTCTGTGG T-rpe-F CGCCGAGAGGTCATTTAGGA CollB Plaget-viB ATACTAGTTCGCTCAGAGT ACTGGTCACACATGGACTGTGTGTGG T-rpe-F GGCCGAGAGGTCATTTAGGA CollB Plaget-viB ACTGGTCACACATGGACTTTGGCTGAGACC H-rabP-R ACGCGTCACTTAGAGACCCAGTGTTTAGGAGCC R-rabP-R TCACTACACATGGACTGACC R-rabP-R TCACACCGTCTCTTTAATAGC R-rabP-R TCACACCGTCCTTAATAGC R-rabP-R TCACACCGTCCTTAATGGAGCC R-rabP-R TCACACCGTCGCTAATGGACC R-rabP-R TCACACCGTCGCTTAATGGAGCC R-rabP-R TCACACCGTCGCCCGAAAGCC R-rabP-R TCACACCGTCGCCCGAAACCACCC R-rabP-R TCCCACCGTCCTTAATGGAGCC R-rabP-R TCCCACCGTCCTTAATGGAGCCGCGAAACCCCCCGAAAGGTTTTAGAGCTAGGAAATAGC L-rabP-R TCCACACCGTCCCCGCAAAGGGTTTAGAGCTAGGAAATAGC L-rabP-R			L- <i>tktA</i> -R	ATGCTAATTACATTTTGACTCCAGATCGGA
R+tick-RCGACAATATAATAAGGGCCAT+tick-RAGAGAGTCCAATATCATCAT+tick-RAGAGAGTCCAATATCATCAT-tick-RATACTAGTCACAATTCGTCGGCGTGTGTGTTTTAGAGGTAGAAATAGCL-rpe-FTATATTGTCAACTGTCAGGGTL-rpe-RTTCAAACTTACAATCGGGTGGAGAGAATAGCCR+rpe-RGACAAACGGCATGTAGTTTGTGGAGATATTCGCGR-rpe-RGGACAACAGCATTTATATCCT-rpe-RGGACGAAGGTCCATTTATATCCT-rpe-RGGACGAAGGTCCATTTATATCCL-tollb-FACAAGTACACACCCCGTAGTTTAGAGGATAGGCL-tollb-FACAATGACTGAACACCACCGTAGGAAATAGCL-tollb-FACAATGACTGAACACCACCGTAGTTTAGAGGACAGAAATAGCL-tollb-RACCAGTGCTACAAATGACTGAACCACCCGTAGGAAATAGCL-tollb-RTCCGAGATGGTCATTCGGCGGAAGGTTTTAGAGGCTAGAAATAGCL-tollb-RTCCGAGATGGTCACCAACCACCTT-tollb-RTCCGAGATGGTCACCACCACCTT-tollb-RTCCGAGATGGGAGATTCGGCGGAAAGGTTTAGGAGCTAGAAATAGCL-tollb-RTCCGAGATGGGCATTTCGCGGGAAAGGTTTAGGAGCTAGAAATAGCL-zwl-FGCAGATATACATGTCACTCCCTTAATGGGGCGATZwlfPIarget-zwlACAGGAGCCCCACAACAATR-zwl-FGCAGATTACAGGGATGTCTCCTTAAGGGGGGATT-zwl-FTTTATCCAGTGAATGCGGGATT-zwl-FTTTATCCAGGGAAGGGCATAACAACATR-zwl-FGCAGAATGCGGGGGATAACGGGGGGGGGGGGGGGGGGGG			R- <i>tktA</i> -F	AGTCAAAATGTAATTAGCATTTCGGGTAAA
T-tick4FATCCGTCATCATATICCATCAT+tick4FACACGATCCGATAGTATTT+tick4FATACTAGTCCGATAGTATTTreeFlarget-rpeATACTAGTCCAGTAGTAGTTL-rpeFTTCAAACTTACATCCGCTCCTTCAGAAR-rpeFGACACACGCATGTAAGTTGAAGATATCCGCT-rpeFGCCCAAACGCATCTGTGTGT-rpeFGCCCAAACGCATCTTATATACAAL-t0BBATACTAGTTCCCTCAGTACACCACCGCTGGTTTAGAGGCTAGAAATAGCL-t0BBATACTAGTTTCCTCAGTACCACACGCTGGTTATAGAGCTAGAAATAGCL-t0BBAGAATGATTACAGATGACGTATATAGAL-t0BFAGAATGGTTATATAGAR-t0BFTTCCAGCCGTCCTTATATAGGT-t0BFTTCCAGCCGCAGAAGGTTTTAGAGGCTAGAAAAGGCTT-t0BFTTCCAGCCGCAAAGGGTTTAGAGGCTAGAAAATAGCT-t0BFTTCCAGCCAGAAGGTTTAGAGGCTAGAAATAGCT-t0BFTTCCAGCCGCAAAGGTTTAGAGGCTAGAAATAGCT-t0BFTCCAGCCAGATATTACATGTCATTTCCGGCCGGAAAGGTTTAGAGGCTAGAAATAGCT-t0BFTTCAGCCGCAAAGGTTTAGAGGCTAGAAATAGCT-t0BFCAATGTCATTTCCGCTCAGAAAGGTTTAGAGGCTAGAAATAGCT-t0BFCAATGTCATTTCCGCTTCAGAAGGTTTAGAGGCTAGAAATAGCT-t0BFCAATGACAGTGTAATTCCGCGCAAAGGTTTAGAGGCTAGAAATAGCT-t0BFCAATGACACTGTAATTCCGCGCAAAGGTTTAGAGGCTAGAAATAGCT-t0BFCATACAAFGCACACTCAAACAACATT-t0BFCATACAAFGACCACTTACAACGGGTGTTTAGAGGCTAGAAATAGCCACAAFCATGTAGACCACATGGAAATGGCTTTAGAGGCTAGAAATAGCT-t0BFTAATCAGTTGCAAACACATCACAAFCATGTAGACCACATGGAACACATCACAAFCATGTAGACCACATGGAGGTATAACCACAAFCATGTAGACCACATGGAGGTATAACGAGGTCTTAGGGCTAGAAATAGCL-t0CAFTACTAGTTGGTGTATTAC			R-tktA-R	CGAGAATATAATAAGGGCCA
T-tkk-RKAGAGACTCGATGAGTAATTrpePJarget-rpeATACTGGCTGATGTGGTGGGCGATGTATTTMGAGGCTAGAAATAGCL-rpe-RTTCAAACTTACATCGGCTTGTGGGGAGAGAATAGCGL-rpe-RGACAACGGCATGTAGTTGTGGAGATATTCCGR-rpe-FGACAACGGCATGTAGTTGTGGAGATATTCCGT-rpe-FGGCAGAAGGTCCATTTATATCCL-talBAPJarget-talBACGGTACACACACCCGTAGTTAGTTAGAGATATGGGGGAGAAATAGCL-talB-RTTCCAACTGTACACCACCGGTGGTTTAGAGGCTAGAAATAGCL-talB-RTTCCAACGTGTCTTTATAGAGCTAGAAATAGCL-talB-RTTCCAACGTGGCGGAAAGGTTTAGGGGGAAAGTAGGCTAGAAATAGCL-talB-RTTCCAACGTGTCTTTATAGT-talB-RTTCCAACGTGGGACACCCT-talB-RTTCCAACGTGGCGGAAAGGTTTAGGGGCGAAAGGTTTAGGGGCGAAAGGTTTAGGGGCGGAAAGGTTTAGGGGGGAAGGATGAGAAATAGCL-zwFRGAATGACGGGGGATGTTGGGGGGAAAGGTTTAGGGGCGGAAAGGTTTAGGGGCGGAAAGGTTTAGGGGGGAAGGATGAGAAATAGCL-zwFRGAATGACGGAGGGATACACAGGGACGGATATACAGGGAGGGATT-zwFRACACGAGGACGCATCAAACGGAT-zwFRACAGAGGACCGATAAACGGT-zwFRACAGAGGACGCATAAACGGT-zwFRACAGAGGACGCATAAACGGAGGTGGTTTAGGGGGTGGTTTAGAGGCTAGAAATAGCL-zwFRAACCAGGAGGCGCCAAGGAGGCCGGAGGAGGATGTAGAAATAGCL-zwFRAACCAGGAGGCGCGGACGGGGGGTGTTTAGAGGCTAGAAATAGCPacAAATCGTGTGGAGAAGGAGCCCCGACGGCGGGGGGGTGTTAGAGGCTAGAAATAGCL-zwFRAACCGTGTACCAGGACGGGGGGGGTGTTTAGAGGCTAGAAATAGCL-zwFRAACCGGTGGGTGGTTAGGGGTGGTTTAGAGGCTAGAAATAGCL-zwFRAACCGGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG			T- <i>tktA</i> -F	ATCCGTCATCATATCCATCA
pe plaget-pe ATACTAGTCTCAATTCTGTCGCGTAGTGTTTTAGAGCTAGAAATAGC L-pe-R TTCAAACTTACAGCGT L-pe-R TCCAAACTTACATCCGCGTTGAGAA R-pe-F GACACGGTGTAGTTGAAGATATTCGCG R-pe-F GGCAGAGAGGTCATTTGTGTG T-pe-F GGCAGAGAGGTCATTTATGGA talB plaget-talB ATACTAGTTTCGTCAGTCACCGTGACCGTAGAAATAGC L-talb+R ACAATGATTACATGATACTTCTTTAGAGCTAGAAATAGC L-talb+R ACAATGATTACATGATACTTCTTAGCGTGGACC R-talB+R TTCCAGCGTCCTTTAATAG R-talB+R TTCCAGCGTCCTTTAATAG R-talB+R TTCCAGCGTCCTTTAATAG R-talB+R TTCCAGCGTCCTTTAATAG R-talB+R TTCCAGCGTCCTTTAATAG R-talB+R TTCCAGCGTCATTTCGTCGGCAAAGGTTTTAGAGCTAGAAATAGC L-zwif-R TCCAGCGTCATTCCTTAAGT R-talB+R TTCCAGCGTCATTTCGCCGCAAGAGGTTTAGAGCTAGAAATAGC L-zwif-R GCAGCATATTACATGCACACCTTA R-talB+R TTCCAGCGGCAAGGTTATAC L-zwif-R GGCAGATATACCTGCAAGAGTATATACTGCGCAAAGGTTTAGAGCTAGAAATAGC L-zwif-R GGCAGATATACATGCATGAAATACTGCGCAAGGTTTTAGAGCCTAGAAATAGC L-zwif-R CATCAGAGGACCATCAAATGCGGTATACC L-zwif-R TTTAGCCGCAAGACACCATTAATGCGGTATACC L-zwif-R ATACTAGTTGCGCATAAACGGGTGTATTAGAGCCTAGAAATAGC L-zwif-R TTTAGCGTGGAAGAAATAGCGCTAAAAT			T- <i>tktA</i> -R	AGAGAGTCCGATGAGTAATT
L-mpeFTATATTTGAACCGCTACGGTL-mpeFTTCAAACTACCTCCGCTCTTCGTGAGAAR-mpeFGACAAACAGCGCTTTGTGTGAGAATATTCGCGR-mpeFGGCAGAAGGTCATTTATAGAT-mpeFGGCAGAAGGTCATTTATAGApTarget-talBATACTACTTTCGTCATGATCACCACCGTAGTTTAGAGCTAGAAATAGCL-talB-FACTGGTACACAATGACTGACACCACGTAGTTTAGAGGTAGAAATAGCL-talB-FACTGGTACACAATGACTGACACACGGTGACCR-talB-FTACTACTAGTGTACTACACACGGGGACCR-talB-FTACAACTGGCCACAGAGACCR-talB-FTCTGAATGGTCATTAATAGGT-talB-FTCTGAATGGTGCAAAGGGTTTAGGAGGTAGAAATAGCL-zwf-FGACAATGACGGCGAAAGGTTTAGGAGGTAGAAATAGCL-zwf-FGCACAATGACATGTGACCACGGAAGGTTTAGGAGGTAGAAATAGCL-zwf-FGCACAATTACAGTGGTCATTTCGGCGCGAAAGGTTTAGGAGGTAGAAATAGCL-zwf-FGAATGACATGTAATTACTGGCGCTAAACTTR-zwf-FGAATGACATGTAATTACTGGCGGAATGCAGGAATAGGCR-zwf-FGAAGTATACACTGCAGGAGAR-zwf-FGAAGTATACACTGCAGGAGAR-zwf-FGAAGTATACACTGCGGAAR-zwf-FGAAGTATACCGGGAR-zwf-FGAAGTATACCGGGAR-zwf-FTTAGAGGCGGGATR-zwf-FTGAATGCCGGGAATGCCGGAGAGCTAGAAATAGCL-zwf-FTGAATGCGTGCGGTGCATTACCGGGAATGCACGGCGGAGGAGGTGCATGAACR-zwf-FGCAGAGAGTGCCATGCACACGGGGGGTGGTTTTAGAGGCTAGAAATAGCL-zwf-FTGAATAACGGTGTACACCGGCGCGGGGCGGGGTGGTGTTAGGAGCTAGAAATAGCL-zwf-FTGACGGGAGCCGCGGGGCGGGGGTGGGGGGGTGGGGGGGG		rpe	pTarget- <i>rpe</i>	ATACTAGTCTCAATTCTGTCGGCTGATTGTTTTAGAGCTAGAAATAGC
L-pe-RTTCAAACTTACATCCGTTCTCCTTAGAACR-ppe-RGACGGAATGTACACTTGAACATTAGAACATAGGGAR-ppe-RGGCGAAAGGTCATTTATAGAtalBPTarget-talBAtCTAGTTTCGTCAGTACACCACCGTAGTTTAGAGCTAGAAATAGCL-talB-RACAATGATTACATGATGACAAL-talB-RACAATGATTACATGATGACAAR-talB-RTTCAACCTTGTACATGACT-talB-RTTCGAGCGTCTCTTAATAGCT-talB-RTTCGAGCGGTCCTTTAATAGAT-talB-RTTCGAGCGGTCCTTTAATAGAT-talB-RTTCGAATGGTGACACCACTT-talB-RTTCGAATGGTGCACCACTT-talB-RTTCGAATGGTGCACCACTT-talB-RTTCGAATGGTCACCACCACTZwfPTarget-zwfATACTAGTGGTCATTTAGCGCGCGAAAGGTTTAGAGCTAGAAATAGCL-zwFRGCAGATATTACATGGTCATTTAGCGCGCGAAAGGTTTAGAGCTAGAAATAGCL-zwFRGAATGACATGTAATATCTGCGCGCTAATCTR-zwFRGAATGACATGTAATATCTGCGCGCTAATCTR-zwFRGAATGACATGTAATATCTGCGCGCTAATCTR-zwFRGAATGACATGTAATATCTGCGCGTAATCCTR-zwFRGAATGACATGTAATACTGGCGTAATCCTR-zwFRGAATGACATGTAATACTGGCGTAATCCTR-zwFRGAATGACTCATGAACACATR-zwFRGAATGACTGGAGATGCCTAAAATGGGTTTTAGAGCTAGAAATAGCL-zwFRCATGAATGCGGGAATCCCCATAAACGTR-zwFRGAATGACTGGGAGATACCTATAATGR-zwFRGAATGACTGGGGAATACCCAGGGAAR-zwFRGATGGTGGCAATGCGCGGAACACCCTATAATGR-zwFRGATGGTGGCAATGCCGGAACACCTATAATGGR-zwFRCATGAGTGGGAAGGGGTGATR-zwFRGATGAATGCGGGAATCCCAGAGAGTTTAGAGCTAGAAATAGCL-zckA+RTACTGGTGGCAATACGGGAATCCCTATAATG<			L- <i>rpe</i> -F	TATATTTGAACCGCTACGGT
Pre-FGAAGCGGATGTAGATTTCGCGR-pe-FGGCAAACAGCACTCTGTGTGT-pe-FCGACTTTCTCGTTATTCCT-pe-FGGCAACAGCACTCGTACTCCLabParaget-talBATACTAGTTCGTCAGTACACCACCGTAGATTTAGAGCTAGAAATAGCL-talB-FACGTGGTACACAATGATGATGATGATGATGATGATGATGATGACACACCGCGTAGAHalBParaget-talBATACTAGTAGTAGACAATGACGACCGCGTAGACACATGACGACACCGTAGAGAATAGCL-talB-FTACTATCAATGTAATCATTCTTAGAGGTGACCR-talB-FTACTAGGTGGCAACCACTTR-talB-FTCGGAATGCGCGCGAACAGCTT-talB-FTCGGAATGCGCGCGAAAGGGTTTTAGAGCTAGAAATAGCL-zwrFTCGGAATGCGCGCGAACAGCTL-zwrFTCGGAATGCGCGCGAAAGGTTTTAGAGCTAGAAATAGCL-zwrFTCGGTGCCGAGAAGGTGTATTCGCGCGCGAAAGGGTTTTAGAGCTAGAAATAGCL-zwrFTCTGTGCCGAGATGTAATATCGCGCGCGAAAGGGTTTTAGAGCTAGAAATAGCL-zwrFTCTGTGCCGAGATGTAATATCGCGCGCTAATCCTTR-zwFFGAAGCAGCACCATCAAAATGGCATCCTTAGATGR-zwFFGAAGCAGCACCACTGAAATACCGGATT-zwrFTTACCAGTGAATGGGGGTATT-zwrFTTACCAGTGGAATGCACTATAATGL-zckA+FCATAAAACGGGATGCCATTAATGR-zckA+FTACTGCGTGGGGATACCTATAATGR-zckA+FTACTGCGTGGGGATACCCTATAATGGT-zckA+RGGTGTGGGGATACCCGCGGAACCAGCGTCGAGAAATAGCL-pto-FTAAATGCGTGGAACCACCGCGGCGGGTGTTTAGAGCTAGAAATAGCL-pto-FTAAATGCGTGGAACACCCTGAACCAGCGTCGGACAGGATGGAAATAGCL-pto-FTAAATGCGTGGGAATATCGGGAACAGGGTTTAGAGCTAGAAATAGCL-pto-FTAAATGCGTGGGAATATCGGGAACAGGGTTTAGAGCTAGAAATAGCL-pto-FTAAATGCGTGGGAATATCGGGAACAGGGGACGGACGGAGGGAG			L- <i>rpe</i> -R	TTCAAACTTACATCCGCTTCTCCTTGAGAA
Prpe-R GACAAACAGCATCTGTTGTG T-ppe-R GGCAGAAGGTCATTTATACC T-ppe-R GGCAGAAGGTCATTTATAGA talB pTarget-talB ATACTAGTTTCGTCAGTACCACCCCGTGAGTTTAGAGCTAGAAATAGC L-talB-R AGAATGATTACATGACTGACA R-talB-R TCCAGCGTCGTTTTATAG T-talB-R TTCCAGCGTCGCTTTTATAG T-talB-R TTCCAGCGTCGCTTTAATAG T-talB-R TCTGAATGCATGCAGAC T-talB-R TCTGACAGATGAGAC T-talB-R TCTGGCCAGATGCGCGCGAAAGGTTTTAGAGCTAGAAATAGC L-zwi-R GGCAGATATACATGTCATTCTGCGCCGAAAGGTTTAGAGCTAGAAATAGC L-zwi-R GCAGATATACATGTCATGCGCGCGAAAGGTTTTAGAGCTAGAAATAGC L-zwi-R GGCAGATATACATGTGAATTCGCGCCGCAAAGGTTTTAGAGCTAGAAATAGC L-zwi-R GGCAGATATACATGTGAAGTTA T-zwi-R ATGCCGTTATAGCAGGTGAT T-zwi-R ATGCCGTTATAGCAGGAACACAT R-zwi-R ATGCTAGTGAATACACGGAAATAGCGTAAATGGTGTTTTAGAGCTAGAAATAGC L-zwi-R CATCAAGGACACCCACTCAAAACG R-zwi-R ATGCTGGTTATAGGAGGTAACTGAAATGGGTTTTAGAGCTAGAAATAGC L-zwi-R CATCAGTGAAACCACTCAAAACG R-zwi-R TGGTGAAATCACGGAACCCGCACACGCGCGCGTCGTTTAGAGCTAGAAATAGC L-zwi-F TACTAG			R- <i>rpe</i> -F	GAAGCGGATGTAAGTTTGAAGATATTCGCG
T-pe-F = GGACTITICTCGTTATATCC T-pe-R = GGCAGAAGGTCATTTATAGA talB = pTarget-talB ATACTAGTTTCGTCAGTACACCACCGTAGTTTTAGAGCTAGAAATAGC L-talB-F ACTGGTACACATGACTGGA R-talB-F TACTATCATGTACATGTATTCTCTTTA R-talB-F TACTATCATGTAATCATTCTTAGCGGGACC R-talB-R TTCCAGCGTCTCTTTAGAGCTAGAAATAGC T-talB-R TCTGAATCGTCCAGAGACC T-talB-R TCTGAATCGTCCCAGAGAAGGTTTTAGAGCTAGAAATAGC L-zwf-F GCAGATGATCTCCCGGGAAAGGTTTTAGAGCTAGAAATAGC L-zwf-F GCAGATGATCATGTCGCCGGAAAGGTTTAGAGCTAGAAATAGC L-zwf-F GAATGACTGTAATCGTGCATTTCGCGCGGAAAGGTTTAGAGCTAGAAATAGC L-zwf-F GAATGACTGTAATGGTGCATTTC R-zwf-F GAATGACATGTAATTCCGCGGAAAGGTTTAGAGCTAGAAATAGC L-zwf-F TTATCCAGTGTATTACGGGAGCCTATAG R-zwf-F GAATGACATGTAATTCGCGCTATCCTT R-zwf-F TTATCCAGTGGTATT T-zwf-F TTATCCAGTGCATGAATGACGA T-zwf-F TTATCCAGTGCAGTAATGGTGAT T-zwf-F TTATCCAGTGCGCATAACAT ACACAA CACAAAF TCATAGTGCGTGCCATAACC L-ackA-F CATAAAACGGGGACGCCATAACC L-ackA-F CATAAAACGGTGCATAATGGTGCTTTAGAGCTAGAAATAGC L-ackA-F CATAAAACGGAGTCCCATAACC L-ackA-F CATAAAACGGAGTCCCATAACG T-ackA-F CATGAAATGACCAGTGAATGGCTGCTATAGG T-ackA-F CATGAAATGCACGGTGCCCATAAC pta PTarget-pta ATACTAGTGAGTGCCTGAGGAGTAGCTGCTTTAGAGCTAGAAATAGC L-pta-F TAAAAGCGTGGAATACCGGGTGCGCTGGTCGTTTAGAGCTAGAAATAGC L-pta-F TAAAAGCGTGGATTACCGGACCGCGGCGCGATAACG pta PTarget-pta ATACTAGTTACCGGAACCAGGTCGGCTGGTCGTTTAGAGCTAGAAATAGC L-pta-F TAAAAGCGTGGAATACCGGTTACCGCAACGGTCGGTGGTTTAGAGCTAGAAATAGC L-pta-F TAAAAGGTTGGAAAGAGT ACACAACAGTTACCAGGTTAACCGGATCAGGTCGCAGGAAATAGC L-pta-F TAAAAGCGTGGAAAAAGG T-pta-F TAAAACGGTGGAAAAAGG T-pta-F TACAAGCGTGGAACAATAGGGT ACACAACCGTGGAACATTACGGAACCAGTGACGAGGTTTAGAGCTAGAAATAGC L-iWA-F TACCGTGTACAATGGGTCCT T-pta-F CCTTACAACCGTGTAACTGCGCAAGGTTTAGAGCTAGAAATAGC L-iWA-F TACCGTGGCAACAATAGGGT ACACACCTGGGAAAATGCCGCAGGTTTAGAGCTAGAAATAGGC ACACACCTGGGAAAATAGCGCACGAAAAAGGCTAGAAAATAGC ACACACTGGGAAAATAGCGTGAACATTACGGAAAAAGCCTGGAACAATAGGA ACACGGGAAAATAGCCTGGAACAATACGGG ACACACAGCGGACAATACGGGACAATACGGGACAATACGGACAATACGGACAATACGGACAATACGGACAATACGGGACAATACGGGACAATACGGACAATACGGGACAATACGGACAATACGGGACAATACGGGACAATACGGACAATACGG			R- <i>rpe</i> -R	GACAAACAGCATCTGTTGTG
Trpe-RGGCAGAAGGTCATITATAGAtalBPTarget-talBACTAGTITCGTCAGTACACCACGTAGTITAGAGCTAGAAATAGCL-talB-FACTGGTACACAATGACTGAAL-talB-RAGAATGATTACATGGATAGTATTCTCTTTAR-talB-RTACTATCATGTAATCATTCTTAGCGTGACCR-talB-RTTCCAGCGTCCTTTAATAGT-talB-RTTCCAGCGTCCCCAAAGACT-talB-RTCTGAATGGTGCAACCACTTZwfPTarget-zwfATACTAGTGGTCATTTCGGCGCGAAAGGTTTTAGAGCTAGAAATAGCL-zwf-RGCAGATATACATGTCGTATTCTCCTTAAGTTR-zwf-RGCAGATATACATGTCGTATATCTGCGCTAAGAATTGCR-zwf-RATGTCGTTATAGGAGGTGATR-zwf-RATGTCGTTATAGGAGGTGATR-zwf-RATGCGTTATAGGAGGGGAT-zwf-RAACAGGCCCCCATAACCCackATGTGAAACCATCGAGAAGTCCCATAAATGGTGTTTAGAGCTAGAAATAGCL-ackA+RTGTGAAATCACATGGAAGTCCCATAAATGGTGTTTAGAGCTAGAAATAGCL-ackA+RTGTGAAATCACATGGAAGTCCCATAAATGGTGTTTAGAGCTAGAAATAGCL-ackA+RTGTGAAATCACATGGAAGTACCCATAAATGGPTarget-ackAATACTAGTTCATGGAGCAGTCACTATAATTGR-ackA+RTGTGAAATCACATGGAAGACCPtarget-ptaATACTAGTTACCGGAACCAGCGCGCGGTCGTTTAGAGCTAGAAATAGCL-pta-FCATCAACCGTGTAACCCGTTACCCCGCAPtarget-ptaATACTAGTTACCGGGAACAATGGTPtarget-ptaATACTAGTTCCCGGAACCAGGGTTTAGAGCTAGAAATAGCL-pta-FCCTTACAACCGTGTAACTCTCTCTCGGAAAAAGGCL-pta-FCCTTACAACCGTGTAACATGCCCTAACCGGACAGGATTTAGAGCTAGAAATAGCL-pta-FATACTAGCTGCAACATAGGTHahaPTarget-ptaATACTAGCTCTCCGGAACAATGGCTGAAAAAL-i/wA-FTACCAGTGTACATTGCCGGAAAAAGGC </td <td></td> <td></td> <td>T-<i>rpe</i>-F</td> <td>CGACTTTTCTCGTTATATCC</td>			T- <i>rpe</i> -F	CGACTTTTCTCGTTATATCC
talB pTarget-talB ATACTAGTITICGTCACACCACCACCACCACTAGATHTTAGAGCTAGAAAATAGC L-talB-F ACTGGTTACCAATGACAGTGAA L-talB-R AGAATGATTACATGATAGATAGTAGTTICTTACGAC R-talB-R TTCCAGCCGTCCTTTAATAG T-talB-R TTCCAGCCGTCCTTTAATAG T-talB-R TTCCAGCCGTCCTTTAATAG T-talB-R TCTGAATGCGCAACCACTT T-talB-R TCTGATGCGCAAGAGATTA Zwif pTarget-zwif ATACTAGTGTGCCAAGAGAGTTA L-zwi-F TCGTGTCCCAGATGAAGTTAGCT L-zwi-F CGCAGATATTACATGCACTCTCTTAGGT R-zwi-F GAATGACAGGAGTGCA R-zwi-F GAATGACAGGAGAGTGCA R-zwi-F GAATGACAGAGAGTGCAATAGAGGAG R-zwi-F ATACTAGTTCACGTGAATGAGCGA R-zwi-F ATACTAGTTCACCGGAAAACAT R-zwi-F TACCAGTACAGGAAGTGCCATAAC CackA Target-zakA TGGTAAATGGAGTAGCACATCAAACAT R-zwi-F CATAAAACGGATCGCATAAC R-zwi-F TCTCATCAGTGTATGTGATACCAGAAAACGC L-ackA-F TACTAGTTCACCAGGAAGATACCAGCACTCAAACAT R-ackA-F TGAAATCACAGGTAGAAGTACCAGCTGAAAATGGC L-ackA-F CTTAAAACCGGATTACACCAGGTAAACAGCCGCGGTCGTTTAGAGCTAGAAATAGC <			T- <i>rpe</i> -R	GGCAGAAGGTCATTTATAGA
L-tall&F ACTGGTACACAATGACTGAA L-tall&R ACGATGATACATGATGATATTCTCTTTA R-tall&F TACTATCATGATAGTATTCTCTTTA R-tall&F TACAATGCTGCCAGAGAAC T-tall&F ATAAAGTTGGCCAGATGACCACTT 2w/f pTarget-zw/ ATACTAGTGGCAGCACACTT L-zw/FR CCGAGATGAAGTTTA L-zw/FR GCAGATATTACATGATATCTCTCTTAGAGCTAGAAATAGC L-zw/FR GCAGATATTACATGATCATTCTCCTTAGCT R-zw/FR ACGAGCACCATCATTCCCTTATCCT R-zw/FR ACGAGCACCATCATTCCCTTAGCT R-zw/FR TTTATCCAGTGAATGATG T-zw/FR ACGAGCACCATCAAACAT ACGAGCACCATCAAAACGGA T-zw/FR ACGAGCACCATCAAACAT ACCAAGCACCCATCAAACAT ACCAAGCACCATCAAAACGGA T-zw/FR ACGAGCACCATCAAACAT ACCAGACCATCAAAACGGAT C-zw/FR ACGAGCACCATCAAACAT ACCAGACCATCAAAACGGATGCTATAGGCTAGAAATAGC L-ackA-R TGTGAATACCAGTGGAAGTTTAGGCATGGAAATAGC L-ackA-R TGGTAATACCAGTGGAAGTACCTATAATTG R-ackA-F TACTTCCATGGAAGTACCTATAATTG R-ackA-F TACTTCCATGGAAGTACCTATAATTG R-ackA-F TACTTGCATGGAAGTACCTATAAGG T-ackA-R ACGAGCAGCATCAACGGCCAGCCAGCCAGCAGCAGCAGAAATAGC L-pta-F TAAATCCGGTGACCATTAAGG T-ackA-R CTTTAGCTTTGGAAGGTTACCCATGAGAAATAGC L-pta-F TAAATCCGGTGACCCTCTA R-pta-F TACCGTGACCCTCTA R-pta-F TACCGTTAACCGGGAACCAGCGCCGCGCAGCCAGCAGCAGCAGCAGAAATAGC L-pta-R ACCGTGACACCTGTAACCAGCGCGCGCGCAGCAGCAGCAGCAGCAGCAGCAGCA		talB	pTarget- <i>talB</i>	ATACTAGTTTCGTCAGTACACCACCGTAGTTTTAGAGCTAGAAATAGC
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T-zwf-R AACAGAGCACCATCAAACAT ackA pTarget-ackA ATACTAGTTCATCGATGCAGTAAATGGTGTTTTAGAGCTAGAAATAGC L-ackA-F CATAAAACGGATCGCATAAC L-ackA-R TGTGAAATCACATGGAAGTACCTATAATTG R-ackA-R TGATGTTGGTGTTTTCACACGCGCAGCTC R-ackA-R TGATGTTGGTGTTTTGGCA T-ackA-R CTTCATAAAACCAGTTAAGG T-ackA-R ACTTTAGCTTGGAAGGTC pta pTarget-pta ATACTAGTTACCGGAACCAGCGTCGGTCGTTTAGAGCTAGAAATAGC L-pta-F TAAATGCGTTGACACCGCTCGTTTAGAGCTAGAAATAGC L-pta-F AAATGCGTTGACACCTCTTA R-pta-F ATAAACCGGTTATCCTCGTCATCATCCGCA R-pta-R GACGAGATTACAGGGTTATCCTCGTCATCATCCGCA R-pta-R TACCGGTGAATCTCGTCATCATCCGCA R-pta-R TACCGTGACAAGAG T-pta-R TACCGGTGACAATGGTTCCT R-pta-R TACCGTGACAAGGA T-pta-R ATTGCGTTGACAAAGA T-pta-R ATTGCGTGGCAATATGGTCCT R-pta-R TACCGTGACAAAGA T-pta-R ATTGCGTGGCAATATAGGT i/vA pTarget-i/vA ATACTAGTCCTCCGCGACCGGACAGGTTTTAGAGCTAGAAATAGC L-i/vA-F TACGGTAACTTGCGGAAAAA R-i/vA-F TACGGTAACTTGCGGAAAAA R-i/vA-F AATGCGTTAGCAGCCCCGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC R-i/vA-F AATACTAGTCTTCCGGAAAAAAGCCCCCGAACAGGTTTTAGAGCTAGAAATAGC L-i/vA-F AATGTAGTCTCTCCGGAAAAAAGCCCCCGGACAGGTTTTAGAGCTAGAAATAGC R-i/vA-F AATACTAGTCTTCCGGAAAAAAGCCCCCCGAGTAGGAAATAGG R-i/vA-F AATACTAGTAGTAGTCGTGCGGAAAA R-i/vA-F AAACGCGTAAATGCTGACGGACACCGGACAGGTTTAGAGCTAGAAATAGG R-i/vA-F AAACGCTGAAAATGCCTGATAGG R-i/vA-F AAACGCTGAAAATGCCGGAACAATGCCGGAAAAACGC R-i/vA-F AAACGGTAAAACTTACTACTGG R-i/vA-F AAACGCTGGAAAAATGCCTGATAGGA R-i/vA-F AAACGGTGAAAAATGCCACCGGAACAATGCGAAGA R-i/vA-F AAACGGTGAAAAATGCACGAC R-i/vA-F AAACGGTGAAAAATGCAC R-i/vA-F AAACGGTGAACAATACGAC			T- <i>zwf</i> -F	TTTATCCAGTGAATGACGGA
ackApTarget-ackAATACTAGTTCATCGATGCAGTAAATGGTGTTTTAGAGCTAGAAATAGCL-ackA-FCATAAAACGGATCGCATAACL-ackA-RTGGAAATCACATGGAAGTACCTATAATTGR-ackA-RTGATGTTGGTGTTTTCACACCGCCAGCTCR-ackA-RTGATGTTGGTGTTTTGGCAT-ackA-RTGATGTTGGTGTTTTGGACACCGGTCGTTTTAGAGCTAGAAATAGCptapTarget-ptaATACTAGTTACCGGAACCAGCGTCGTTTAGAGCTAGAAATAGCL-pta-RAACAGGTTGACCCTCTAL-pta-RGACGAGATTACACGGTTATCTCGTCATCAGCAR-pta-RTACCGTTATCAATGGTTCCTT-pta-RTACCGTGGCAATATAGGTT-pta-RATACCGTGGCAATATAGGTI/vATarget-i/vAATACTAGTTCCTGTACAAGGTI/vA-FTACGGTAACTCTCGGAACAAGGAGTI/vA-FTACGGTAACTTGCGGAAAAAL-i/vA-FTACGGTAACTTTGCGGAACAAGGTI/vA-FTACGGTAACTTTGCGGAAAAAI/vA-FGAGAACAATAGCCCTGAAAAAAGCCCGGACAGGTTTAGAGCTAGAAATAGCI/vA-FTACGGTAACTTTGCGGAAAAAATGCCCGGACAGGTTTAGAGCTAGAAATAGCI/vA-FGAGATAAACTTACACGGGAAAAAAGCCCGGACAGGTTTI/vA-FGAGATAAACTAGGGAAAAAAGCCCGGACAGGTTTI/vA-FAAACCGTGGAAAAAACGGCTGAAAAAGCCCCGGACAGGTTTI/vA-FAAACCGTGGAACAATAGCGI/vA-FAAACCGTGGAACAATACGAC			T- <i>zwf</i> -R	AACAGAGCACCATCAAACAT
L-ackA-F CATAAAACGGATCGCATAAC L-ackA-R TGTGAAATCACATGGAAGTACCTATAATTG R-ackA-F TACTTCCATGTGATTTCACACCGCCAGCTC R-ackA-R TGATGTTGGTGTTTTGGCA T-ackA-R CTTCATAAAACCAGTTAAGG T-ackA-R ACTTTAGCTTTGGAAGAGTC pta pTarget-pta ATACTAGTTACCGGAACCAGCGTCGGTCGTTTTAGAGCTAGAAATAGC L-pta-F TAAATGCGTTGACACCTCTA L-pta-F GACGAGATTACACGGTTTACCGCAC R-pta-F ATAAACGCGTGAACCAGCGTCGCGTCGTCGTCGTAGAAATAGC R-pta-F ATAAACGCGTGAACACCTCTA R-pta-F CCTTACAACGGTTTACCCGCA R-pta-R TACCGTTATCAATGGTTCCT T-pta-R ACCGTTATCAATGGTTCCT T-pta-R ATTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTGCGGAACA L-ilvA-F GTTAATGATCGGGAAAA L-ilvA-F GTTAATGAGGGAAAAATGCCCCGGACAGGTTTAGAGCTAGAAATAGC R-ilvA-F GTTAATGTAGTGCGGAAAAATGCCCGGTAGGAACAGGTT R-ilvA-F GTTAATGTAGTGCGGAAAAATGCCCGGAACAGGTTTAGAGCTAGAAATAGC R-ilvA-F GTTAATAGTGAGGAAAAATGCCCGGATAGG R-ilvA-F GTTAATGTAGTGGGAAAAATGCCTGATAGA R-ilvA-F GTTAATGTAGTGCGGAAAAATGCCTGATAGG R-ilvA-F AACGCTGGAACAATACGGG		ackA	pTarget-ackA	ATACTAGTTCATCGATGCAGTAAATGGTGTTTTAGAGCTAGAAATAGC
L-ackA-R TGTGAAATCACATGGAAGTACCTATAATTG R-ackA-F TACTTCCATGTGATTTCACACCGCCAGCTC R-ackA-R TGATGTTGGTGTTTTTGGCA T-ackA-R TGATGTTGGAGATCAGG T-ackA-R ACTTTAGCTTTGGAAGAGTC pta pTarget-pta ATACTAGTTACCGGAACCAGCGTCGTTTTAGAGCTAGAAATAGC L-pta-F TAAATGCGTTGACACCTCTA R-pta-F GACGAGATTACACGGTTATCCTCTTTCGT R-pta-F ATAAACCGTGTAATCTCGTCATCATCCGCA R-pta-F TACCGTTATCATGGTTCCT T-pta-R ACCGTGACCAGCGTCGATCATCGCA R-pta-R TACCGTTATCAATGGTTCCT T-pta-R ATTTGCGTGGCAATATAGGT <i>ilvA</i> pTarget- <i>ilvA</i> ATACTAGTCTCCGGACAGGACTAGAAATAGC L- <i>ilvA</i> -F TACGGTAACTCTCGGCAATATAGGT <i>ilvA</i> PTarget- <i>ilvA</i> ATACTAGTCTTCCGGACAGGTTTTAGAGCTAGAAATAGC L- <i>ilvA</i> -F TACGGTAGCACCGGAAAA <i>ilvA</i> PTarget- <i>ilvA</i> ATACTAGTCTTCCGGACACGGACAGGTTTTAGAGCTAGAAATAGC <i>ilvA ilvA ilta</i> CGGTAACTTGCGGAAAA <i>ilvA ilta</i> CGGTAACTTGCGGAAAA <i>ilvA ilta</i> CGGTAACTTGCGGAAAA			L-ackA-F	CATAAAACGGATCGCATAAC
R-ackA-FTACTTCCATGTGATTTCACACCGCCAGCTCR-ackA-RTGATGTTGGTGTTTTGGCAT-ackA-FCTTCATAAAACCAGTTAAGGT-ackA-RACTTTAGCTTTGGAAGAGTCptapTarget-ptaATACTAGTTACCGGAACCAGCGTCGGTCGTTTAGAGCTAGAAATAGCL-pta-FTAAATGCGTTGACACCTCTAR-pta-RGACGAGATTACCGGGTAATCTCGTCATCATCCGCAR-pta-FATAACCGTTATCAATGGTTCCTT-pta-RTACCGTTATCAATGGTTCCTT-pta-RCCTTACAACCTGTACAAAGAT-pta-RATACTGGTGGCAATATAGGTilvApTarget-ilvAATACTAGTTCCTTCGGAGCACGGACAGGTTTTAGAGCTAGAAATAGCL-ilvA-FTACCGTAACTTGCGGAAAAL-ilvA-FTACCGTAACTTGCGGAAAAR-ilvA-FGACGAAATATGCCCCCCAGTTR-ilvA-FGAGATTAAATGTAGGGAAAAATGCCTGATAGR-ilvA-FGAGATTAAATGTAGGGAAAAATGCCTGATAGR-ilvA-FGAGATTAAATGTAGGAAAAATGCCTGATAGR-ilvA-FGAGATTAAACTTACTACTGGR-ilvA-FAAACGCTGGAACAATACGACR-ilvA-FAAACGCTGGAACAATACGAC			L-ackA-R	TGTGAAATCACATGGAAGTACCTATAATTG
R-ackA-RTGATGTTGGTGTTTTTGGCAT-ackA-FCTTCATAAAACCAGTTAAGGT-ackA-RACTTTAGCTTTGGAAGAGTCptapTarget-ptaATACTAGTTACCGGAACCAGCGTCGGTCGTTTAGAGCTAGAAATAGCL-pta-FTAAATGCGTTGACACCTCTAL-pta-RGACGAGATTACACGGTTTATCCTCTTTCGTR-pta-FATAACCGTGTAATCTCGTCATCATCCGCAR-pta-FTACCGTTATCAATGGTTCCTT-pta-RCCTTACAACCGTGTACATCGTCATCATCGCAAT-pta-RTACCGTTATCAATGGTTCCTT-pta-RATTTGCGTGGCAATATAGGTI/vAPTarget-i/vAATACTAGTCCTTCCGGAACACGGGTTTAGAGCTAGAAATAGCL-i/vA-FTACCGTAACTTGCGGAAAAL-i/vA-FTACCGTATATAAGGAAAAL-i/vA-RTTTTCCCTACATTATGAGGAAAAATGCCTGATAGGR-i/vA-RGTTAATAATGAGGAAAAATGCCTGATAGGR-i/vA-FGAGATTAAACTTACTACTGGR-i/vA-FAAACGCTGGAACAATACGAC			R-ackA-F	TACTTCCATGTGATTTCACACCGCCAGCTC
T-ackA-FCTTCATAAAACCAGTTAAGGT-ackA-RACTTTAGCTTTGGAAGAGTCpTarget-ptaPTarget-ptaATACTAGTTACCGGAACCAGCGTCGGTCGTTTAGAGCTAGAAATAGCL-pta-FTAAATGCGTTGACACCTCTAL-pta-RGACGAGATTACACGGTTTATCCTCTTTCGTR-pta-FATAAACCGTGTAATCTCGTCATCATCCGCAR-pta-FATAACCGTGTAATCTCGTCATCATCCGCAR-pta-RTACCGTTATCAATGGTTCCTT-pta-RCCTTACAACCGTGACAAGAT-pta-RATTTGCGTGGCAATATAGGTilvAPTarget-ilvAATACTAGTCCTTCCGGAACAL-ilvA-FACGGTAACTTATCACTGGAAAAL-ilvA-FR-ilvA-FGTTAATAATGAGGAAAAATGCCTGATAGGR-ilvA-FGTTAATAATGAGGAAAAATGCCTGATAGGR-ilvA-FGAGATTAAACTTACTACTGGR-ilvA-FAAACGCTGGAACAATACGAC			R-ackA-R	TGATGTTGGTGTTTTTGGCA
T-ackA-RACTTTAGCTTTGGAAGAGTCptapTarget-ptaATACTAGTTACCGGAACCAGCGTCGGTCGTTTAGAGCTAGAAATAGCL-pta-FTAAATGCGTTGACACCTCTAL-pta-RGACGAGATTACACGGTTTATCCTCTTTCGTR-pta-FATAAACCGTGTAATCTCGTCATCATCCGCAR-pta-RTACCGTTATCAATGGTTCCTT-pta-FCCTTACAACCGTGACAAGAT-pta-RATTTGCGTGGCAATATAGGTilvApTarget-ilvAATACTAGTCCTTCCGGAACAL-ilvA-FIIVAFarget-ilvAR-ilvA-RTTTTCCCTACATTATCACTGGAAAAIIVACTTACAACTTATGAGGAAAAATGCCCCGAACAGGTTTTAGAGCTAGAAATAGCIIVA-FTACGGTAACTTATGAGGAAAAATGCCTGATAGIIVA-FGAGATTAAACTTACGGAAAAATGCCTGATAGGR-ilvA-FGTTAATAATGATGGGAAAAATGCCTGATAGR-ilvA-FGAGATTAAACTTACTACTGGT-ilvA-FAAACGCTGGAACAATACGAC			T-ackA-F	CTTCATAAAACCAGTTAAGG
ptapTarget-ptaATACTAGTTACCGGAACCAGCGTCGGTCGTTTTAGAGCTAGAAATAGCL-pta-FTAAATGCGTTGACACCTCTAL-pta-RGACGAGATTACACGGTTTATCCTCTTTCGTR-pta-FATAAACCGTGTAATCTCGTCATCATCGCCAR-pta-RTACCGTTATCAATGGTTCCTT-pta-FCCTTACAACGTGACACCGGACAGGACAGGCTTTAGAGCTAGAAATAGCI/vApTarget-i/vAATACTAGTCCTTCCGGAAAAL-i/vA-FI/vA-RTTTTCCCTACATTGGGAAAAR-i/vA-RGTTAATAATGAGGAAAAATGCCTGATAGR-i/vA-FGTTAATAATGTAGGGAAAAATGCCTGATAGR-i/vA-FGTTAATAATGTAGGGAAAAATGCCTGATAGR-i/vA-FGAGATTAAACTTACTACTGGR-i/vA-FAAACGCTGGAACAATACGAC			T-ackA-R	ACTTTAGCTTTGGAAGAGTC
L-pta-F TAAATGCGTTGACACCTCTA L-pta-R GACGAGATTACACGGTTTATCCTCTTTCGT R-pta-F ATAAACCGTGTAATCTCGTCATCATCGCCA R-pta-R TACCGTTATCAATGGTTCCT T-pta-F CCTTACAACGTGACAAGA T-pta-R ATTTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTTGCGGAAAA L-ilvA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACTGG T-ilvA-F AAACGCTGGAACATACGAC		pta	pTarget- <i>pta</i>	ATACTAGTTACCGGAACCAGCGTCGGTCGTTTTAGAGCTAGAAATAGC
L-pta-R GACGAGATTACACGGTTTATCCTCTTTCGT R-pta-F ATAAACCGTGTAATCTCGTCATCATCGCA R-pta-R TACCGTTATCAATGGTTCCT T-pta-F CCTTACAACCTGTACAAAGA T-pta-R ATTTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTTGCGGAAAA L-ilvA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACTGG R-ilvA-F AAACGCTGGAACAATACGAC			L-pta-F	TAAATGCGTTGACACCTCTA
R-pta-F ATAAACCGTGTAATCTCGTCATCATCGCCAC R-pta-R TACCGTTATCAATGGTTCCT T-pta-F CCTTACAACCTGTACAAAGA T-pta-R ATTTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACCGGTAACTTGCGGAAAA L-ilvA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACTGG R-ilvA-F AAACGCTGGAACAATACGAC			L-pta-R	GACGAGATTACACGGTTTATCCTCTTTCGT
R-pta-R TACCGTTATCAATGGTTCTT T-pta-F CCTTACAACCTGTACAAAGA T-pta-R ATTTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTTGCGGAAAA L-ilvA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTTAGGGAAAAATGCCTGATAG R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACTGG T-ilvA-F AAACGCTGGAACAATACGAC			R-pta-F	
I-pta-F CCTTACAACGTGTACAAAGA T-pta-R ATTTGCGTGGCAATATAGGT ilvA pTarget-ilvA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTTGCGGAAAA L-ilvA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACGG T-ilvA-F AAACGCTGGAACAATACGAC			R-pta-R	
i/vA pTarget-i/vA ATTAGCGTGGCAATATAGGT i/vA pTarget-i/vA ATACTAGTCCTTCCGGAGCACCGGACAGGTTTTAGAGCTAGAAATAGC L-i/vA-F TACGGTAACTTTGCGGAAAA L-i/vA-R TTTTCCCTACATTATTAACCCCCCCAGTTT R-i/vA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-i/vA-F GAGATTAAACTTACTAGGG T-i/vA-F AAACGCTGGAACAATACGAC			I-pta-F	
IIVA prarget-IIVA ATACTAGTECTTECEGGACACCEGGACAGCTTTAGAGCTAGAAATAGC L-ilvA-F TACGGTAACTTTGCGGAAAA L-ilvA-R TTTTTCCCTACATTATTAACCCCCCCAGTTT R-ilvA-F GTTAATAATGTAGGGAAAAATGCCTGATAG R-ilvA-F GAGATTAAACTTACTACTGG T-ilvA-F AAACGCTGGAACAATACGAC		:1 A	I-pta-K	
L- <i>ilvA</i> -F TTTTTCCCTACATTATTAACCCCCCAGTTT L- <i>ilvA</i> -R GAGATTAATGTAGGGAAAAATGCCTGATAG R- <i>ilvA</i> -R GAGATTAAACTTACTACTGG T- <i>ilvA</i> -F AAACGCTGGAACAATACGAC		IIVA	prarget-IIVA	
R- <i>ilvA</i> -F GTTAATAATGTAGGGAAAAATGCCTGATAG R- <i>ilvA</i> -R GAGATTAAACTTACTACTGG T- <i>ilvA</i> -F AAACGCTGGAACAATACGAC				
R- <i>ilvA</i> -R GAGATTAAIGTAGGGAAAAAIGCCTGATAG T- <i>ilvA</i> -F AAACGCTGGAACAATACGAC			R-ih/A-F	
T- <i>ilvA</i> -F AAACGCTGGAACAATACGAC			R-ilvA-R	GAGATTAAACTTACTACTAG
			T- <i>ilvA</i> -F	AAACGCTGGAACAATACGAC
T- <i>ilvA</i> -R AAACGCTGTTGCAGTATCAG			T- <i>ilvA</i> -R	AAACGCTGTTGCAGTATCAG

(Continued on next page)

TABLE 5 (Continued)

Purpose	Target	Primer ^a	Sequence (5'-3')
	рохВ	pTarget- <i>poxB</i>	ATACTAGTCCCTGCCGATTCGAGTGTTTGTTTTAGAGCTAGAAATAGC
		L-poxB-F	ATAACGTTACCGAAGGCTTT
		L- <i>poxB</i> -R	ACCCTTTTTACATGGTTCTCCATCTCCTGA
		R- <i>poxB</i> -F	GAGAACCATGTAAAAAGGGTGGCATTTCCC
		R- <i>poxB</i> -R	AATTCCCATGCTTCTTTCAG
		T- <i>poxB</i> -F	AGATAAATTCCTGTTGCTGG
		T- <i>poxB</i> -R	CAAATGGTGAACGAATCACA
	argG	pTarget- <i>argG</i>	ATACTAGTATACCAATACGTTGACCTACGTTTTAGAGCTAGAAATAGC
		L-argG-F	TTTCAAATCCCACTACGAAG
		L-argG-R	TGTCGAATTACATAAAATAACACCCTGCTT
		R- <i>argG</i> -F	TTATTTTATGTAATTCGACATCAACCCTGC
		R- <i>argG</i> -R	TCTTCATTTAAATGGAAGCC
		T-argG-F	AAAGCCCAGTTATTCTGTAG
		T- <i>argG</i> -R	GCCATGGAAACAGTTTATGT
	gadA	pTarget-gadA	ATACTAGTGTGAATCGAGTAGTTCTGAGGTTTTAGAGCTAGAAATAGC
		L-gadA-F	AATGTGCAACTTGTCATGGT
		L-gadA-R	CGTTATGTTACATTTCGAACTCCTTAAATT
		R-gadA-F	GTTCGAAATGTAACATAACGTTGTAAAAAC
		R-gadA-R	TCCCTTGACACGAATACAAA
		I-gadA-F	
	0	I-gadA-R	
	proB	plarget-prob	
		L-proB-F	
		L-proB-R	
		R-proB-F	GAGAAICAIGIAAGGAGCAGGCIGAIGCIG
		К- <i>ргов</i> -К	
		I-proB-F	AGGITACGACCATAATCGAA
In situ promotor replacement	rh+A	I-prob-R	
in situ promoter replacement	mia	prarget-ma	
		L-MA-F	
		L-IIIIA-N D_rbtA_E	
	eamA	nTarget-eamA	ΤΑΔΤΑΓΤΑΓΛΟΛΟΕΟΟΊΛΑ ΤΑΔΤΑΓΤΑΓΓΑΔΑΔΑΓΑΤΓΑΔΑΔΤΤΓΑΓΑΓΑΓΓΤΤΤΑΓΑΓΓΤΑΓΑΔΑΤΑΓΓ
	eunia	L-camA-E	
		L-eamA-R	
		R-pamA-F	
		R-eamA-R	
		T-eamA-F	TCACCATCACATATTGTGAA
	asnA	nTarget-asnA	TAATACTAGTTCGATGCAGGGGATAATCGTGTTTTAGAGCTAGAAATAGC
	aspri	I -asnA-F	TCATCATCCTTTCGCTGGTACTCAC
		L-aspA-R	ACCACACATTATACGAGCCGGATGATTAATTGTCAATCTGGATCACTTTAAGTGTC
		R-aspA-F	CGGCTCGTATAATGTGTGGTCACAAAGGAGAGATATACATGTCAAACAACATTCGTAT
		R-aspA-R	CTTCTTCTTCAGCAGGATGCTGAA
		T-aspA-F	TGTTATGCGTTCTGTTACTG
	alk	pTargetF-alk	TAATACTAGTAAGAATTATTTTGACTTTAGGTTTTAGAGCTAGAAATAGC
	5	L-alk-F	CAGCAAGACCGAGAATTAAT
		L-alk-R	CATTATACGAGCCGGATGATTAATTGTCAAATACCTGGGGGGCATAACAAC
		R- <i>qlk</i> -F	GTATAATGTGTGGTCACAAAGGAGATATACATGACAAAGTATGCATTAGT
		R-alk-R	ATAATGGCCTCTTCTTCACT
		T-glk-F	TTTGCGAAAATATCAACGCC
	altB	pTarget- <i>gltB</i>	TAATACTAGTCCGATAAGTTGGAAATCCGCGTTTTAGAGCTAGAAATAGC
	5	L-gltB-F	TTTTGCCCATAACGACGGGT
		L-gltB-R	CATTATACGAGCCGGATGATTAATTGTCAAATGGCAAGCTTATTGGTACA
		R- <i>qltB</i> -F	GTATAATGTGTGGTCACAAAGGAGATATAC ATGACACGCAAACCCCGTCG
		R- <i>qltB</i> -R	TTTGCTCAATGCGTGGCAGA
		T-gltB-F	AAACGAGGCAACATTACAGA
		TrcV ^b	GTGACCACACATTATACGAGCCGGATGA

^aEach pTarget primer shares the same reverse primer, pTarget R-common, listed in Table 4.

^bTrcV represents a primer complementary to the *trc* promoter.

Analytical method. The OD₆₀₀ was measured using an Eppendorf BioPhotometer D30 (Amersham Biosciences, Uppsala, Sweden). Concentrations of glucose were determined by using a glucose analyzer (YSI model 2300; Xylem Inc., Rye Brook, NY, USA) with an IC Sep Ion-300 column (Transgenomic, San Jose, CA, USA). A Sykam S-433D amino acid analyzer (Sykam, Munich, Germany) was used to determine the

concentrations of amino acids. By-product measurement was performed with an Aminex HPX-87H column (300 by 7.8 mm), using 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.6 ml/min and detection via refractive index or UV absorption at 210 nm (6).

RT-qPCR for mRNA quantification. *E. coli* strains W3110 and HS33 were cultured for 18 h in 20 ml MS medium at 150 rpm and 28°C. Samples were collected during the exponential growth phase, prepared by centrifugation, and flash frozen in liquid nitrogen and were sent to Tsingke Biological Technology (Wuhan, China) for RT-qPCR analysis with 16S rRNA as a control for normalization between samples. The fold changes of target genes were calculated as $2^{-\Delta\Delta CT}$ according to the method of Schmittgen and Livak (52).

Metabolome analysis. Metabolomics was applied to characterize the biological variation of the intercellular metabolites between *E. coli* W3110 and HS33. The samples were collected from cultures grown in 6 technical replicates per strain during the exponential growth phase. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and flash frozen in liquid nitrogen for further metabolite extraction. Statistical analysis of metabolite profiles was performed by BGI Tech Solutions Co., Ltd. (Shenzhen, China). The intracellular metabolite concentrations of *E. coli* W3110 were used as a control for normalization between samples. The detailed protocol of the liquid chromatography-mass spectrometry (LC-MS) system is described in the supplemental material. The screened metabolomics data were subjected to partial least squares discrimination analysis (PLS-DA). Significantly changed metabolites (SCM) (defined as follows: base VIP [variable important for the projection], >1.0; fold change, >1.20 or <0.83; *q*-value, <0.05) were selected for subsequent chemical structure identification.

Statistical analysis. All experiments were conducted in triplicate. The data were averaged and are presented as means \pm standard deviations (SD). *P* values of <0.01 were considered statistically significant. All the figures were prepared using Origin Software version 8.0 (OriginLab Corp., Northampton, MA, USA).

Data availability. The raw data supporting the conclusions of this article will be made available, without undue reservation, to any qualified researcher.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. **SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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We declare that we have no conflict of interest.

Z.-Q.L., Y.-G.Z., and B.Z. initiated and supervised the project. P.L. and Z.-H.Y. carried out all the experiments and data analyses. P.L. and Z.-Q.L. were responsible for the preparation and revision of the manuscript. We all read and approved the final manuscript.

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