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# A Novel Efficient L-Lysine Exporter Identified by Functional Metagenomics

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Lack of active export system often limits the industrial bio-based production processes accumulating the intracellular product and hence complexing the purification steps. L-lysine, an essential amino acid, is produced biologically in quantities exceeding two million tons per year; yet, L-lysine production is challenged by efficient export system at high titers during fermentation. To address this issue, new exporter candidates for efficient efflux of L-lysine are needed. Using metagenomic functional selection, we identified 58 genes encoded on 28 unique metagenomic fragments from cow gut microbiome library that improved L-lysine tolerance. These genes include a novel L-lysine transporter, belonging to a previously uncharacterized EamA superfamily, which is further *in vivo* characterized as L-lysine exporter using *Xenopus oocyte* expression system as well as *Escherichia coli* host. This novel exporter improved L-lysine tolerance in *E. coli* by 40% and enhanced yield, titer, and the specific production of L-lysine in an industrial *Corynebacterium glutamicum* strain by 7.8%, 9.5%, and 12%, respectively. Our approach allows the sequence-independent discovery of novel exporters and can be deployed to increase titers and productivity of toxicity-limited bioprocesses.

**Keywords:** amino acid, transporter, *E. coli*, *C. glutamicum*, *Xenopus oocytes*

## INTRODUCTION

The global chemical industry is transitioning from reliance majorly on petrochemical processes to more sustainable bio-based production. This development holds promise to improve the sustainability of the chemical industry while also reducing the overall production costs of chemical products. In order to establish a cost-competitive bioprocess, titers of fermentations frequently exceed 100 g l<sup>-1</sup>, which leads to a significant stress on the host organism. Indeed, a majority of industrial bioprocesses are limited in production due to several stresses, for example, osmotic stress, leading to product toxicity.

One of the most significant bio-based chemical products is L-lysine. The global bio-based L-lysine production is estimated to reach 3.0 million tons in 2022 corresponding to 5.6 billion USD of market value according to the current L-lysine market report (Elder, 2018). Industrial L-lysine bioprocesses entirely rely on *Corynebacterium glutamicum* and *Escherichia coli* production strains that achieve titers over 1.2M (Becker and Wittmann, 2012; Lee and Kim, 2015).

It was observed that the L-lysine export rate is inhibited by 50% upon exceeding the extracellular concentration of 400 mM compared to that at 80 mM (Kelle et al., 1996), indicating the substantial inhibition of the L-lysine-specific export in industrial fermentation. Indeed, several studies have demonstrated the benefit of incorporating active efflux systems to address intracellular product accumulation in bio-based production (Malla et al., 2010; Hemberger et al., 2011; Borodina, 2019). Hence, L-lysine export system is an obvious target to maintain the producer organism at high lysine concentration as well as easing the downstream process. Despite its importance, there are only two identified lysine-specific exporters: (i) LysE as a member of the lysine efflux permease (LysE; 2.A.75) family (Vrljic et al., 1999; Bellmann et al., 2001) and (ii) lysine outward permease (LysO or YbjE in *E. coli*; Vrljic et al., 1996; Pathania and Sardesai, 2015) achieved five-fold higher lysine export rate upon overexpression of LysE in *C. glutamicum* (Vrljic et al., 1996). Similarly, Yasueda and coworker have deployed this strategy for 10-fold improvement in L-lysine production by expressing a spontaneously mutated LysE from *C. glutamicum* in *Methylophilis methylotrophus* (Gunji and Yasueda, 2006). In addition to the rational engineering of the existing exporters, there is an urgent demand for new genetic building blocks to further improve L-lysine tolerance and production.

Functional metagenomic selection is an effective approach to discover novel genes and enzymes due to its ability to access the wide range of genetic elements present in a particular environmental niche (Sommer et al., 2009, 2010; Munck et al., 2015; Forsberg et al., 2016). Hence, we set out to use functional metagenomic selection to identify novel L-lysine transporter candidates from a cow fecal library, with the goal of improving industrial L-lysine production (Figure 1A). Using C13-labeled L-lysine and mRNA expression of the screened transporter in *Xenopus oocyte*, the transporter candidate was confirmed as L-lysine exporter. Expression of the metagenomic derived L-lysine transporter improved titer and productivity in both Gram-positive and Gram-negative production strains.

## MATERIALS AND METHODS

### Bacterial Strains, Growth Conditions, and Chemicals

All bacterial strains, vectors, and plasmids used in this study are listed in Table 1. All oligonucleotide primers (synthesized by Integrated DNA Technologies, Inc.) used are presented in Table 2. *E. coli* strains were routinely cultured at 37°C in Luria-Bertani (LB) broth or on agar supplemented with kanamycin (35 µg mL<sup>-1</sup>) when necessary (hereafter referred as LB-km). For the L-lysine production in *E. coli* LB as well as M9 minimal media supplemented with 2 g l<sup>-1</sup> of yeast extract were used and the strains were cultivated in 96-deep well plate in 600 µl of media. *Corynebacterium glutamicum* strains were cultured in modified CGXII medium (Keilhauer et al., 1993) supplemented with 5% sucrose, 1% BHI, 0.5 mM IPTG, and kanamycin (when necessary), at 30°C and 250 RPM. Growth media were supplemented with 1.5% agar for plate assays.

DNA manipulations were carried out following standard protocols (Sambrook and Russell, 2001). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States). Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Denmark) and New England Biolabs (Hertfordshire, United Kingdom). DNA sequencing was performed using an automated DNA sequence analyzer.

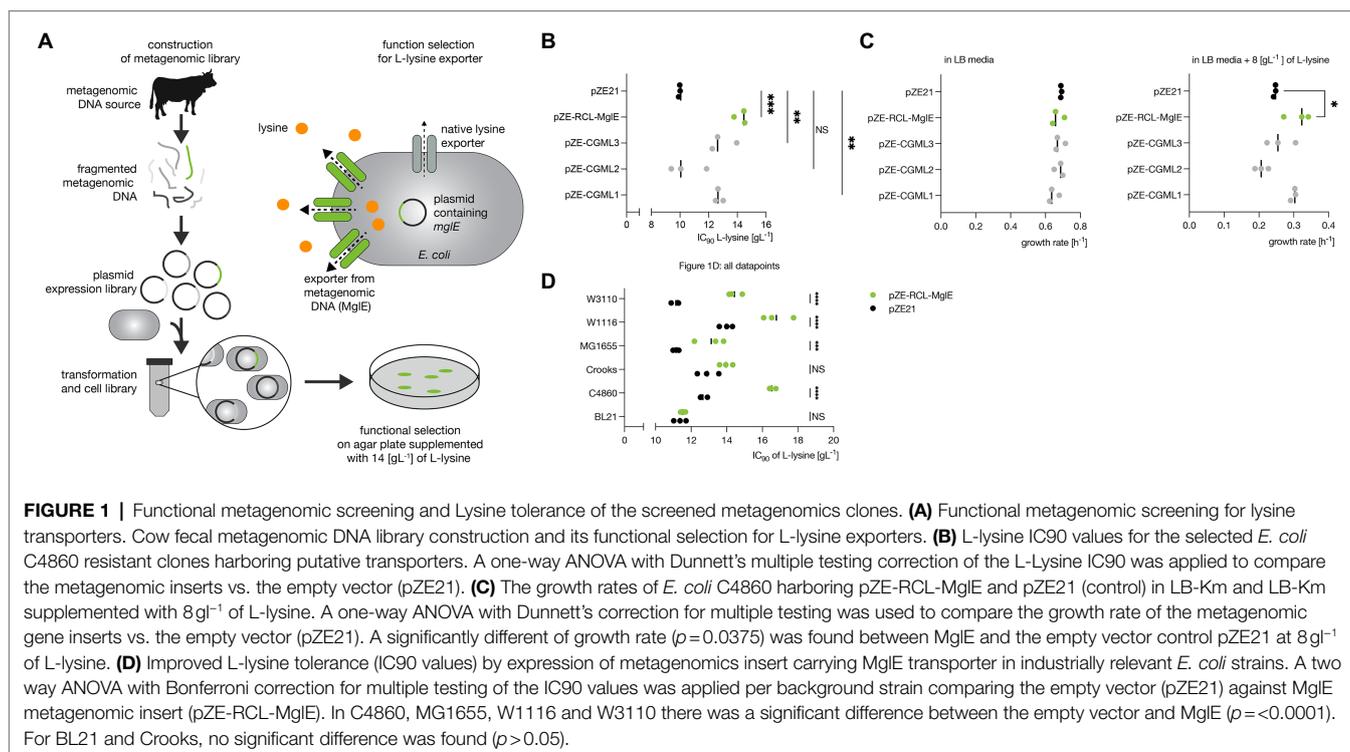
### Metagenomic Library Construction and Functional Screening for L-Lysine

A metagenomic expression library of total DNA extracted from a cow fecal sample was constructed as described previously (Sommer et al., 2009). Briefly, library construction involves (i) the isolation of total DNA from 5g of fecal material using the PowerMax Soil DNA Isolation Kit (MO BIO Laboratories Inc.), (ii) fragmentation of extracted DNA into pieces of an average size of 2 kb by sonication using a Covaris E210 (Massachusetts, United States) followed by end-repair using the End-It end-repair kit (Epicentre), and (iii) blunt-end ligation into the pZE21 MCS1 expression vector with constitutive promoter pLteo-1 (Lutz and Bujard, 1997) at the *HincII* site using the Fast Link ligation kit (Epicentre), and (iv) transformation of the ligated sample into electrocompetent *E. coli* top10 cells by the standard method (One Shot TOP10 Electrocomp Cells, Invitrogen).

After electroporation, cells were recovered in 1 ml of SOC medium for 1 h at 37°C, and the library was titered by plating out 1, 0.1, and 0.01 µl of recovered cells onto LB-km plates. The insert size distribution was estimated by gel electrophoresis of the PCR products obtained by amplifying inserts using primers annealing to the vector backbone flanking the *HincII* site. The average insert size for the library was 1.7 kb. The total size of the metagenomics library was determined by multiplying the average insert size by the number of colony-forming units (CFU) per ml (5 × 10<sup>8</sup> bp). The remainder of the recovered cells were inoculated into 10 ml of LB-km liquid media and grown overnight; the library was frozen down in 15% glycerol and stored at -80°C.

The *E. coli* C4860 strain was used for the functional screening of L-lysine exporters. First, a plasmid prep of the metagenomics library was carried out from the *E. coli* top10 cells harboring the library. Then, 400 ng of metagenomics plasmid DNA was transformed into electrocompetent *E. coli* C4860 cells, and the library titer was determined as described above. On the basis of the determined library sizes and the titer of the library, 10<sup>6</sup> cells (i.e., 100 µl of the library cells) were plated on LB-km agar supplemented with L-lysine at the selective concentration (14 g l<sup>-1</sup>). Plates were incubated at 37°C, and the growth of colonies (likely lysine-tolerant clones) was assayed after 48–65 h of incubation.

The metagenomic inserts present in L-lysine-tolerant clones were Sanger sequenced using the pZE21\_F and pZE21\_R primer pair, which annealed to the vector backbone. The resulting raw sequencing chromatogram files were analyzed and functionally annotated using the deFUME web server (van der Helm et al., 2015): <http://www.cbs.dtu.dk/services/deFUME/>.



## Minimum Inhibitory Concentration and IC<sub>90</sub> Determination

For minimum inhibitory concentration (MIC) determination, the *E. coli* strains were cultured in LB liquid media at 37°C overnight, and then approximately  $1 \times 10^4$  cells were inoculated from the overnight cultures into LB liquid media and grown at 30°C and 300 RPM in 96-well microtiter plates containing 150 µl of medium per well. MICs were determined using a logarithmic L-lysine (or chemical) concentration gradient with two-fold serial dilutions. Endpoint absorbance measurements ( $A_{600nm}$ ) were taken with a plate reader (Synergy H1, BioTek) after 24 or 48 h of incubation and were background-subtracted. Growth inhibition of the *E. coli* strains was plotted against L-lysine (chemical) concentration with a polynomial interpolation between neighboring data points using R software.<sup>1</sup> The percentage of inhibition was calculated using the formula:  $1 - [A_{600nm \text{ lysine (or chemical)}}/A_{600nm \text{ control}}]$ . The inhibitory concentration was defined as the lowest concentration of the chemical that inhibited 90% of the growth of the strain tested (IC<sub>90</sub>).

## Growth Experiments

Single colonies of the tolerant clone(s) harboring transporter homologues were grown overnight at 37°C with shaking in liquid LB-km medium. The OD values at 600 nm [(OD)<sub>600nm</sub>] were determined by 10-fold dilution. Then, the cultures were diluted to adjust the (OD)<sub>600nm</sub> to 0.1, and 5 µl of each culture was inoculated in 150 µl fresh media containing L-lysine (0 and 8 g l<sup>-1</sup>) and kanamycin in a 96-well microtiter plate.

The plate was incubated at 37°C for 24 h in an automated spectrophotometer (ELx808, BioTek) that recorded the (OD)<sub>600nm</sub> at an interval of 60 min. The data were subsequently retrieved and analyzed to determine growth rates. The growth rate data are the average of triplicate experiments, with error bars representing the standard error of the mean (SEM).

## PCR and *Escherichia coli* Transformation

PCR was performed in a total volume of 50 µl under the following DNA amplification conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C–65°C for 30 s, and 72°C for 1 min, and finally 72°C for 5 min. Electrocompetent or chemically competent *E. coli* cells were transformed with the ligation mixture using a standard protocol and plated onto LB-km agar plates for selection.

## In silico Analysis

The amino acid sequence of MglE (Metagenomics gene for lysine Export) was analyzed using Interpro,<sup>2</sup> its 2D membrane topology was predicted using Phobius (Käll et al., 2007) and visualized using Protter (Omasits et al., 2014), and the phylogenetic tree was plotted using iTOL. The UniProtKB database (UniProt Consortium, 2014) was accessed on June 1 2016 to query the closest homologs of MglE. Pfam version 29.0 (December 2015; Finn et al., 2014) was used to extract the LysE family (Pfam id: PF01810) members. The Maximum Likelihood Phylogenetic tree was constructed using CLC Main Workbench 7 with the neighbor-joining construction method.

<sup>1</sup><http://www.r-project.org>

<sup>2</sup><http://www.ebi.ac.uk/interpro/>

**TABLE 1** | Bacterial strains and plasmids used in this study.

Strains/Plasmids	Description	Source/reference
<b>Strains</b>		
<i>Escherichia coli</i>		
DH5 $\alpha$	General cloning host	Invitrogen
Top10	Highly efficient host for cloning and plasmid propagation	Invitrogen
BL21(DE3)	<i>ompT hsdT hsdS</i> ( $r_b^- m_b^-$ ) <i>gal</i> (DE3)	Novagen
C4860	Host for phage PhiX174, sup-, lambda-, F-, (now known as DSM 13127)	DSMZ
MG1655	F <sup>-</sup> <i>ilvG</i> <sup>-</sup> <i>rfb</i> -50 <i>rph</i> -1	Blattner et al., 1997
W3110	F <sup>-</sup> lambda <sup>-</sup> IN(rrnD-rrnE)1 <i>rph</i> -1	Hayashi et al., 2006
W1116	strain W and ATCC 9637	Archer et al., 2011
Crooks	<i>E. coli</i> ATCC 8739	DSMZ
BL21/pZE21	<i>E. coli</i> BL21 carrying pZE21	This study
C4860/pZE21	<i>E. coli</i> C4860 carrying pZE21	This study
MG1655/pZE21	<i>E. coli</i> MG1655 carrying pZE21	This study
W3110/pZE21	<i>E. coli</i> W3110 carrying pZE21	This study
W1116/pZE21	<i>E. coli</i> W1116 carrying pZE21	This study
Crooks/pZE21	<i>E. coli</i> Crooks carrying pZE21	This study
BL21(DE3)/pZE-RCL-MgIE	<i>E. coli</i> BL21 carrying pZE-RCL-MgIE	This study
C4860/pZE-RCL-MgIE	<i>E. coli</i> C4860 carrying pZE-RCL-MgIE	This study
MG1655/pZE-RCL-MgIE	<i>E. coli</i> MG1655 carrying pZE-RCL-MgIE	This study
W3110/pZE-RCL-MgIE	<i>E. coli</i> W3110 carrying pZE-RCL-MgIE	This study
W1116/pZE-RCL-MgIE	<i>E. coli</i> W1116 carrying pZE-RCL-MgIE	This study
Crooks/pZE-RCL-MgIE	<i>E. coli</i> Crooks carrying pZE-RCL-MgIE	This study
C4860/pZE-MgIE	<i>E. coli</i> C4860 carrying pZE-MgIE	This study
C4860/pZE0-P-MgIE-T	<i>E. coli</i> C4860 carrying pZE0-P-MgIE-T	This study
C4860/pZE-YbjE	<i>E. coli</i> C4860 carrying pZE-YbjE	This study
C4860/pZE-Gene1	<i>E. coli</i> C4860 carrying pZE-Gene1	This study
C4860/pZE-Gene2	<i>E. coli</i> C4860 carrying pZE-Gene2	This study
C4860/pZE-Gene3	<i>E. coli</i> C4860 carrying pZE-Gene3	This study
C4860/pZE-Gene4	<i>E. coli</i> C4860 carrying pZE-Gene4	This study
W3110/pSIJ8	<i>E. coli</i> W3110 carrying pSIJ8	This study
W3110:: $\Delta$ ldcC/pSIJ8	<i>ldcC</i> deletion mutant of <i>E. coli</i> W3110 carrying pSIJ8	This study
W3110:: $\Delta$ ldcC. $\Delta$ cadA/pSIJ8	<i>ldcC</i> and <i>cadA</i> deletion mutant of <i>E. coli</i> W3110 (DMLC) carrying pSIJ8	This study
DMLC	<i>ldcC</i> and <i>cadA</i> deletion mutant of <i>E. coli</i> W3110	This study
DMLC/pZE21	<i>E. coli</i> DMLC carrying pZE21	This study
DMLC/pZE-RCL-MgIE	<i>E. coli</i> DMLC carrying pZE-RCL-MgIE	This study
Keio strain		
b0186	<i>ldcC</i> replaced by kanamycin cassette <i>E. coli</i> K-12 BW25113	Baba et al., 2006
b4131	<i>cadA</i> replaced by kanamycin cassette in <i>E. coli</i> K-12 BW25113	Baba et al., 2006
<i>Corynebacterium glutamicum</i>		
VL5	Industrial L-lysine production strain of <i>C. glutamicum</i>	Vitalys
VL5/pEKEx2	<i>C. glutamicum</i> VL5 carrying pEKEx2	This study
VL5/pEK-RCL-MgIE	<i>C. glutamicum</i> VL5 carrying pEK-RCL-MgIE	This study
<b>Plasmids and vectors</b>		
pZE21	<i>E. coli</i> expression vector with pLtet promoter, pBR322 origin of replication. and kanamycin cassette	Lutz and Bujard, 1997
pSIJ8	pKD46 based vector with rhaRS-prha-FLP and ampicillin cassette	Jensen et al., 2015
pUSER016	pNB1u based vector with T7 promoter for <i>in vitro</i> transcription	Darbani et al., 2016
pEKEx2	IPTG inducible <i>C. glutamicum</i> expression vector with kanamycin cassette from pUC4 K; Ptrc, lacl, pUC18 mcs, and pBL1 origin of replications.	Eikmanns et al., 1991
pZE-RCL-MgIE	pZE21 carrying L-lysine resistant metagenomic insert with MgIE operon from cow fecal sample	This study
pZE-MgIE	pZE21 carrying <i>mgIE</i> gene	This study
pZE0-P-MgIE-T	Promoter less pZE21 carrying native promoter and <i>mgIE</i> gene and native terminator region	This study
pUSER016-pT7-MgIE	Codon-optimized MgIE gene cloned into pUSER016 for functional studies in <i>Xenopus</i> oocytes	This study
pEK-RCL-MgIE	pEKEx2 carrying L-lysine resistant metagenomic insert with MgIE operon from cow fecal sample	This study
pZE-YbjE	pZE21 carrying <i>ybjE</i> gene from <i>E. coli</i> BL21(DE3)	This study
pZE-Gene1	pZE21 carrying gene from <i>Bacteroides coprophilus</i> CAG:333 having 82% identity with <i>mgIE</i>	This study
pZE-Gene2	pZE21 carrying codon-optimized gene from <i>Bacteroides barnesiae</i> having 78% identity with <i>mgIE</i>	This study
pZE-Gene3	pZE21 carrying gene from <i>Bacteroides</i> sp. CAG:770 having 54% identity with <i>mgIE</i>	This study
pZE-Gene4	pZE21 carrying codon-optimized gene from <i>Alistipes fingoldii</i> CAG:68 having 43% identity with <i>mgIE</i>	This study

The gene sequences of *mgIE*, *ybjE*, *gene1*, *gene2*, *gene3* and *gene4*, are provided in the **Supplementary Information**.

**TABLE 2** | Oligonucleotides used in this study.

Primers	Oligonucleotide sequences (5'-3')	Restriction site
pZE21_F	ATCAGTGATAGAGACTGAGCAC	
pZE21_R	TTTCGTTTTATTGATGCCTCTAG	
MgIE_F	AAAGGT <u>ACC</u> ATGAGAAATTTAAGTAAAAAGC	<i>KpnI</i>
MgIE_R	TTAAAGC <u>TTT</u> CTATCTCTTTGTTACTGAAATCAT	<i>HindIII</i>
RCL-MgIE-F	CGGG <u>GTA</u> CCCAAGAGGAGAAAGGTACCGGGC	<i>KpnI</i>
RCL-MgIE-R	CGAGCTCCCGGGCTGCAGGAATTCG	<i>SacI</i>
YbjE_F	AAA <u>GGTACC</u> ATGTTTTCTGGGCTGTTAATCA	<i>KpnI</i>
YbjE_R	TAT <u>GGATCC</u> TTACGCAGAGAAAAAGGCGAT	<i>BamHI</i>
linear_cassette F	TGCAAGGCATTAAGTTGGGTAACGC	
linear_cassette R	CCATGATTACGCCAAGCTATTTAGGTGACAC	
<b>For user cloning</b>		
pZE21_User_F	ATAAGACGGU ATCGATAAGCTTGATATCGAATTCC	
pZE21_User_R	AGGGTACCCU CGAGGTGAAGACGAAAGGGCCTCG	
P-MgIE-T_User_F	AGGGTACCCU GAAGTCAAGCATCTCAAAAACTAC	
P-MgIE-T_User_R	ACCGTCTTAU TTACGATTTACTACGGAGTATTA	
pZE21_User_F4	ATAAGCTU GATATCGAATTCCTGCAGCCC	
pZE21_User_R4	AATGAATU CGGTCAGTGCGTCTGCTGAT	
<b>For gene deletion and confirmation</b>		
ldcC_F1	TCAGCGCTGATGAGCTACG	
ldcC_F2	AGTTCTGAAAAAGGGTCACTTC	
ldcC_R	TCGCAATATGGTGAACCTGTT	
CadA_F1	TGAAGTACTCCCAGATTTGGATC	
CadA_F2	CGGCTGTGAGGGTGTTTTCA	
CadA_R	TTAATTTAAAAGTATTTCCGAGGCTCC	

Restriction sites are indicated by underlined and *italics*.

## Transport Assays in *Xenopus* Oocytes

The *Xenopus laevis* oocytes were obtained from EcoCyte Bioscience (Germany). Oocytes were kept in Kulori buffer (pH 7.4) and at 18°C. A linear cassette (including T7 promoter, *mgIE* as the gene of interest, and 3'UTR) was amplified from the plasmid pUSER016-T7-MgIE using Phusion Hot Start polymerase (Thermo Fisher Scientific) and the primers linear\_cassette F and linear\_cassette R. The linear cassette was used as template for *in vitro* transcription. Capped cRNAs were synthesized using the mMESSAGE mMACHINE T7 Transcription Kit (AM1344; Thermo Fisher). The quality and quantity of the RNA were determined by Agilent 2,100 Bioanalyzer. For microinjection of cRNA and 13C-labeled L-lysine, we used the RoboInject (Multi Channel Systems, Reutlingen, Germany) automatic injection system (Darbani et al., 2019). Injection needles with opening of 25 µm were used (Multi Channel Systems, MCS GmbH). For expression in oocytes, 25 ng of the *in vitro* transcribed cRNA for the MgIE transporter was microinjected into the oocytes 3 days prior to transport assays. To perform the export assay, 50 nl of the 13C-labeled L-lysine stock solution was injected into the oocytes to obtain estimated internal concentrations of 6 mM, assuming an after-injection dilution factor of 20 (Darbani et al., 2016). Following four washing steps, each batch of 20 oocytes was incubated for 180 min in 90 µl Kulori buffer at pH 5 or pH 7.4. After incubation, 70 µl of the medium was collected from each batch with intact oocytes and added onto 70 µl of 60% MeOH before

LC-MS analysis. The oocytes were washed four times with Kulori buffer pH 7.4 and intracellular metabolites were extracted in 30% MeOH to analyzed on LC-MS.

## *Escherichia coli* Double Deletion Mutant Construction

The chromosomal lysine decarboxylases *ldcC* and *cadA* in *E. coli* W3110 were successively knocked out by PCR targeting to create *E. coli* DMLC strain. The gene disruption process was carried out as described previously using an ampicillin-resistant pSIJ8 helper plasmid containing both the λ Red and FLP systems (Jensen et al., 2015). The primer pair *ldcC\_F2/ldcC\_R* (situated 64bp away from the start/stop codon *ldcC* gene) was used to amplify the kanamycin cassette from the genomic DNA of the *ldcC* inframe knocked out Keio strain b0186 whereas the primer pair *CadA\_F2/CadA\_R* (situated 56bp away from the start/stop codon *cadA* gene) was used to amplify the kanamycin cassette from the genomic DNA of the *cadA* inframe knock-out Keio strain b4131. These PCR products were used to delete *ldcC* and *cadA* in *E. coli* W3110 strain. The detailed process of construction of *E. coli* DMLC is given in **Supplementary Material**.

## Plasmid Construction

### Subcloning the Metagenomic Insert Implicated in Tolerance Phenotypes

The recombinant plasmids pZE-MgIE and pZE0-P-MgIE-T were constructed as described below. The construction of recombinant plasmids was verified by both restriction mapping and direct nucleotide sequencing of the respective genes in the recombinant plasmids.

Using the oligonucleotide pair MgIE\_F/MgIE\_R and the pZE-RCL-MgIE plasmid as template DNA, the exact *orf* of the putative carboxylate/amino acid transporter homologue (referred to as MgIE) was amplified. The amplicon was purified with a Qiagen PCR purification kit and digested with the restriction enzymes *KpnI* and *HindIII* (NEB, United Kingdom), followed by ligation using T4 DNA ligase (Fermentas, Denmark) into the corresponding restriction sites of the multiple cloning site (MCS) of the pZE21 vector to construct the recombinant plasmids pZE-MgIE.

To construct the recombinant plasmid pZE0-P-MgIE-T, USER cloning was applied. The pZE21-vector backbone without its promoter (pZE0) was amplified using the oligonucleotide pair pZE21\_User\_F/pZE21\_User\_R. The putative carboxylate/amino acid transporter homologue and the native promoter and terminator sequences (P-MgIE-T fragment) were amplified from the pZE-RCL-MgIE plasmid using the primer pair P-MgIE-T\_User\_F/P-MgIE-T\_User\_F. The amplified PCR products were ligated into the recombinant plasmid pZE0-P-MgIE-T using the recommended standard USER cloning protocol (NEB, United Kingdom).

### Codon-Optimized *mgIE* Plasmids Construction for Oocytes Expression Study

For functional analysis of MgIE in *Xenopus* oocytes, we constructed recombinant plasmid pUSER016-pT7-MgIE by USER cloning. The pUSER016 vector backbone was digested with *PacI* for 18 h and the ends were further nicked with *Nt.BbvCI*

for 2h. The codon-optimized *mglE* (sequence provided in **Supplementary Data**) for *Xenopus laevis* was synthesized along with N-terminal linker sequence—GGCTTAAU and C-terminal linker sequences—ATTAAACC (in the complementary sequence, T is replaced by U for pairing with A) including uracil with compatible USER sites easing direct cloning into linearized and nicked pUSER016 vector by USER cloning (USER, NEB), resulting in plasmid pUSER016-pT7-MglE.

### Recombinant Plasmids With *Escherichia coli* Lysine Exporter and Homologous Genes of *mglE*

The lysine exporter, *ybjE* (900bp, GenBank accession no. CAQ31402), from *E. coli* BL21 (DE3) was amplified using oligonucleotides YbjE-F and YbjE-R. The PCR product of *ybjE* was cloned into pZE21 vector excised with *KpnI* and *BamHI* restriction enzymes to construct pZE-YbjE expression plasmid.

Similarly, four *mglE* homologous genes: (i) Gene1 (891 bp, 82% identity, GenBank accession no. CDC57518) from *Bacteroides coprophilus* CAG:333, (ii) Gene2 (891 bp, 78% identity, GenBank accession no. WP\_018711839) from *Bacteroides barnesiae*, (iii) Gene3 (870 bp, 54% identity, GenBank accession no. CDC66277) from *Bacteroides* sp. CAG:770, and (iv) Gene4 (912 bp, 43% identity, GenBank accession no. CCZ76555) from *Alistipes fingoldii* CAG, were synthesized along with N-terminal linker sequence—AATTCATU AAAGAGGAGAAAGGTACC and C-terminal linker sequence—GTCGACGGTATCG ATAAGCTT (in the complementary sequence, T is replaced by U for pairing with A) including uracil easing direct user cloning. These four synthesized gene fragments were cloned into the linear PCR fragment of pZE21 vector amplified by using oligonucleotides pZE21\_User-F4 and pZE21\_User-R4 (**Table 2**) to construct pZE-Gene1, pZE-Gene2, pZE-Gene3 and pZE-Gene4 recombinant plasmids, respectively.

### Construction of Recombinant Industrial *Corynebacterium glutamicum* Strains

The full metagenomics insert derived from pZE-RCL-MglE plasmid was amplified using the primer pair RCL-MglE-F/RCL-MglE-R. The resulting PCR product was cloned into pEKEx2 vector, using the *KpnI* and *SacI* restriction sites, yielding expression plasmid pEK-RCL-MglE. The newly constructed expression plasmid was introduced into the industrial L-lysine producing strain *C. glutamicum* VL5 by electroporation and concomitant selection on kanamycin containing 2xTY-Agar. Successful construction of the new strain, named *C. glutamicum* VL5/pEK-RCL-MglE, was verified by re-isolation of the plasmid, followed by control digest and PCR on the length of the insert. The same procedure was performed to introduce the empty pEKEx2 expression vector into *C. glutamicum* VL5, yielding control strain *C. glutamicum* VL5/pEKEx2.

### Analysis of L-Lysine in Supernatant of Bacterial Culture

A calibration curve of an authentic standard was generated using concentrations ranging from 0.4 to 77 mg L<sup>-1</sup>. The accurate

mass of L-lysine from 20-fold diluted samples (supernatants containing L-lysine for quantification) was analyzed using an LC-MS Fusion (Thermo Fisher Scientific, United States) with positive electrospray ionization (ESI+). The final concentration was adjusted for the dilution factor. Bracketing calibration was used for the quantification of the external concentration. For the quantification of L-lysine, an LC-MS/MS, EVOQ (Bruker, Fremont United States) was used with multiple reaction monitoring (MRM) transition in positive ionization mode (ESI+), with the quantified transition 147 → 84 (CE 10) and qualifier transition 147 → 130 (CE 7). The significance of the specific lysine production was calculated using a Student *t*-test.

## RESULTS AND DISCUSSION

### Identification of Metagenomic L-Lysine Tolerance Genes

The gut microbiota from industrial livestock animals are conceivably enriched with the microbes capable to survive at higher L-lysine concentration which is supplied as food additives. A metagenomic library derived from cow feces was constructed and used to find candidate genes that could lead to a higher L-lysine tolerance in *E. coli* C4860 strain. The resulting library was screened for L-lysine tolerance by plating on LB agar plates supplemented with selective concentrations of L-lysine (14 g l<sup>-1</sup>; **Figure 1A**; section “Materials and Methods”). Colonies appeared only on the plates with the metagenomic library but not on the control plates with *E. coli* C4860 harboring empty vector. Eighty L-lysine-tolerant clones were selected for further analysis.

Metagenomic inserts present in those L-lysine-tolerant clones were PCR amplified, sequenced, and annotated using deFUME (van der Helm et al., 2015). In total, 28 unique clones containing 58 complete or partial open reading frames were identified in this analysis. The sequence analysis revealed that resistant clones contained *orfs* homologous to genes encoding L-lysine modification/degradation enzymes, membrane proteins, signaling proteins, and hypothetical proteins (**Supplementary Table S1**).

### Selection of Transporter Candidates

For the purpose of improving industrial production strains, novel efflux systems constitute more relevant genetic building blocks than those involved in degradation and modification, which would counteract the objective of L-lysine production. Accordingly, we focused our subsequent analysis on *orfs* encoding potential efflux systems. We identified six unique metagenomic inserts encoding putative membrane proteins, some of which are annotated in GenBank as hypothetical proteins (**Supplementary Table S1**). Four inserts were selected for further testing based on the absence of putative degradation enzymes flanking the putative transporters on the metagenomic insert.

We determined the L-lysine IC<sub>90</sub> values for *E. coli* transformants where each of the four selected metagenomic inserts have been introduced. The L-lysine IC<sub>90</sub> values for these transformants ranged from 10.44 ± 1.277 to 14.25 ± 0.415

$\text{gl}^{-1}$ , corresponding to a more than 40% (value of  $p=0.0001$ ) increase in the L-lysine IC90 for the clone harboring pZE-RCL-MglE compared to the empty vector control strain (Figure 1B). All of these strains have similar growth profiles in LB medium, whereas the growth rates varied in the liquid LB supplemented with  $8\text{gl}^{-1}$  of L-lysine. At  $8\text{gl}^{-1}$  of L-lysine supplementation, the growth rate of the highest tolerant metagenomic clone harboring pZE-RCL-MglE was 30% higher (value of  $p=0.0375$ ) than that of the empty vector control (Figure 1C).

## Lysine Tolerance by MglE in *Escherichia coli* Strains

To test whether the pZE-RCL-MglE plasmid carrying the *mglE* gene could confer L-lysine tolerance to industrially relevant *E. coli* strains, we introduced the plasmid into various *E. coli* strains; BL21 (DE3), MG1655, Crooks, W1116, and W3110 and IC90 values of L-lysine were determined for all of the constructed *E. coli* strains. Interestingly, the IC90 values of L-lysine for most of these *E. coli* strains (except Crooks and BL21) were increased ( $p<0.0001$ ) upon introduction of the pZE-RCL-MglE plasmid (Figure 1D). Of particular interest, the L-lysine IC90 was increased by 30% ( $p<0.0001$ ) in W3110, which is a widely used background strain for L-lysine production (Imaizumi et al., 2005). These data demonstrate that the mechanism of L-lysine tolerance mediated by pZE-RCL-MglE is effective across the industrially relevant *E. coli* strains and accordingly should be generally applicable to industrial *E. coli*-based L-lysine fermentations.

## Protein Sequence Analysis of the Metagenomic Insert Carrying MglE

Sequence analysis of the 1.6kb PCR amplicon from pZE-RCL-MglE metagenomics insert contained an open reading frame *mglE* flanked by native promoter and terminator sequences (Figure 2A). The MglE protein consists of 297 amino acids, and its closest homolog is a hypothetical protein from *Bacteroides coprophilus* (GenBank accession no. WP\_008140691) with 82% identity at the amino acid level. The MglE protein is a member of RhaT/EamA-like transporter family of the drug/metabolite transporter (DMT; 2.A.7) superfamily. The MglE contains two copies of the EamA domain, which is found in transporters belonging to the EamA family, at 9–143 aa (pfam (Finn et al., 2014) e-value:  $3e^{-11}$ ) and 152–292 aa (pfam e-value  $9.6e^{-12}$ ). The members of the EamA family are diverse, and most of their functions are unknown (Franke et al., 2003). Nevertheless, a few proteins belonging to EamA family are well characterized as exporters, such as PecM exports a pigmented compound indigoidine in *Erwinia chrysanthemi* (Rouanet and Nasser, 2001) and YdeD export metabolites of the cysteine pathway in *E. coli* (Daßler et al., 2000). The predicted two-dimensional topology of the MglE transporter possesses six cytoplasmic regions, five periplasmic regions, and 10 transmembrane domains, with both the N- and C-terminals in the cytoplasmic region (Figure 2B).

Phylogenetic analysis shows that the closest MglE homologs (>50% sequence identity) are present in the phyla bacteroidetes

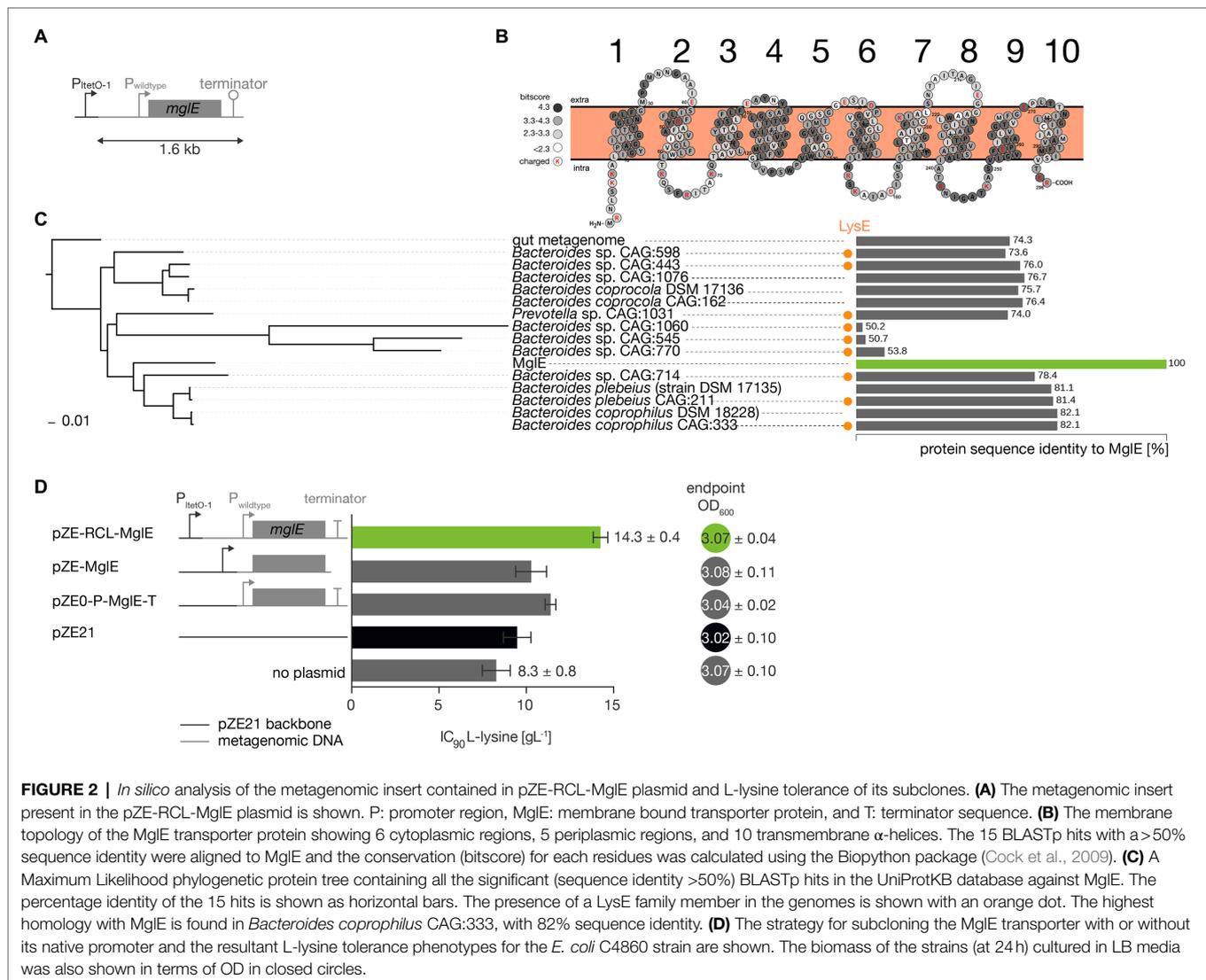
and are annotated as uncharacterized proteins (Figure 2C). Members of the LysE family (pfam id: PF01810) are mainly (>5 species per phyla) found in the phyla: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria. The full list of phyla that contain species with a LysE domain are Proteobacteria: 102, Bacteroidetes: 73, Firmicutes: 45, Actinobacteria: 28, Chloroflexi: 1, Cyanobacteria: 3, Spirochaetes: 1, Verrucomicrobia: 1, and Gemmatimonadetes: 1. Interestingly, nine genomes containing the MglE homolog also contain additional LysE family (pfam id: PF01810, TC code: 2.A.75) genes (Figure 2C orange dot).

## Effect of Regions in the Metagenomics Insert on L-Lysine Tolerance

To investigate the effects of promoter region of the metagenomic clone on lysine tolerance, the recombinant plasmids pZE-MglE (*mglE* cloned into pZE21), and pZE0-P-MglE-T (MglE along with its native promoter and terminator cloned into pZE21 without the pLteo-1 vector promoter) were subcloned from pZE-RCL-MglE plasmid. The constructed plasmids were transformed into *E. coli* C4860, and IC90 values of L-lysine were determined (Figure 2D). Although these recombinant C4860 strains grew in the same extent in LB media, they showed different L-lysine tolerance. We observed that the IC90 for the strain harboring the pZE0-P-MglE-T plasmid was higher than that of the strain harboring the pZE-MglE plasmid, indicating that the native promoter resulted in higher lysine tolerance than the vector promoter. However, the synergistic effect of these two promoters was better still (i.e., the IC90 values of the pZE-RCL-MglE plasmid were higher than those of the strains harboring pZE-MglE or the pZE0-P-MglE-T plasmids). Hence, the lysine tolerance of the *E. coli* C4860 strain was the highest upon harboring the full metagenomic insert (i.e., pZE-RCL-MglE).

## MglE Exports L-Lysine When Expressed in *Xenopus* Oocytes

For functional analysis, MglE was expressed in *Xenopus* oocytes and the export of C13-labeled L-lysine was measured. The water-injected oocytes were used as negative control. L-lysine export assay was performed by injecting C13-labeled L-lysine to obtain a final cytosolic concentration of 6mM in the oocytes. The oocytes were incubated in Kulori buffers with two different pH (pH 5 and 7.4) for 3h, and L-lysine concentrations were measured in the buffer and within the oocytes. MglE-expressing oocytes resulted in 6.6-fold and 8.5-fold higher extracellular concentrations of C13-labeled L-lysine and natural C12 L-lysine, respectively, than control oocytes, when buffer with pH 5 was used (Figure 3Ai). The intracellular concentrations of C13-labeled and natural C12 L-lysine were correspondingly 2.1-fold and 1.4-fold lower than in control oocytes (Figure 3Ai). On the other hand, there was no significant change in L-lysine quantities when oocytes were incubated at pH 7.4 (Figure 3Aii). This indicates a proton-gradient dependent export mechanism for MglE transporter.



## MglE Assists L-Lysine Export in *Escherichia coli*

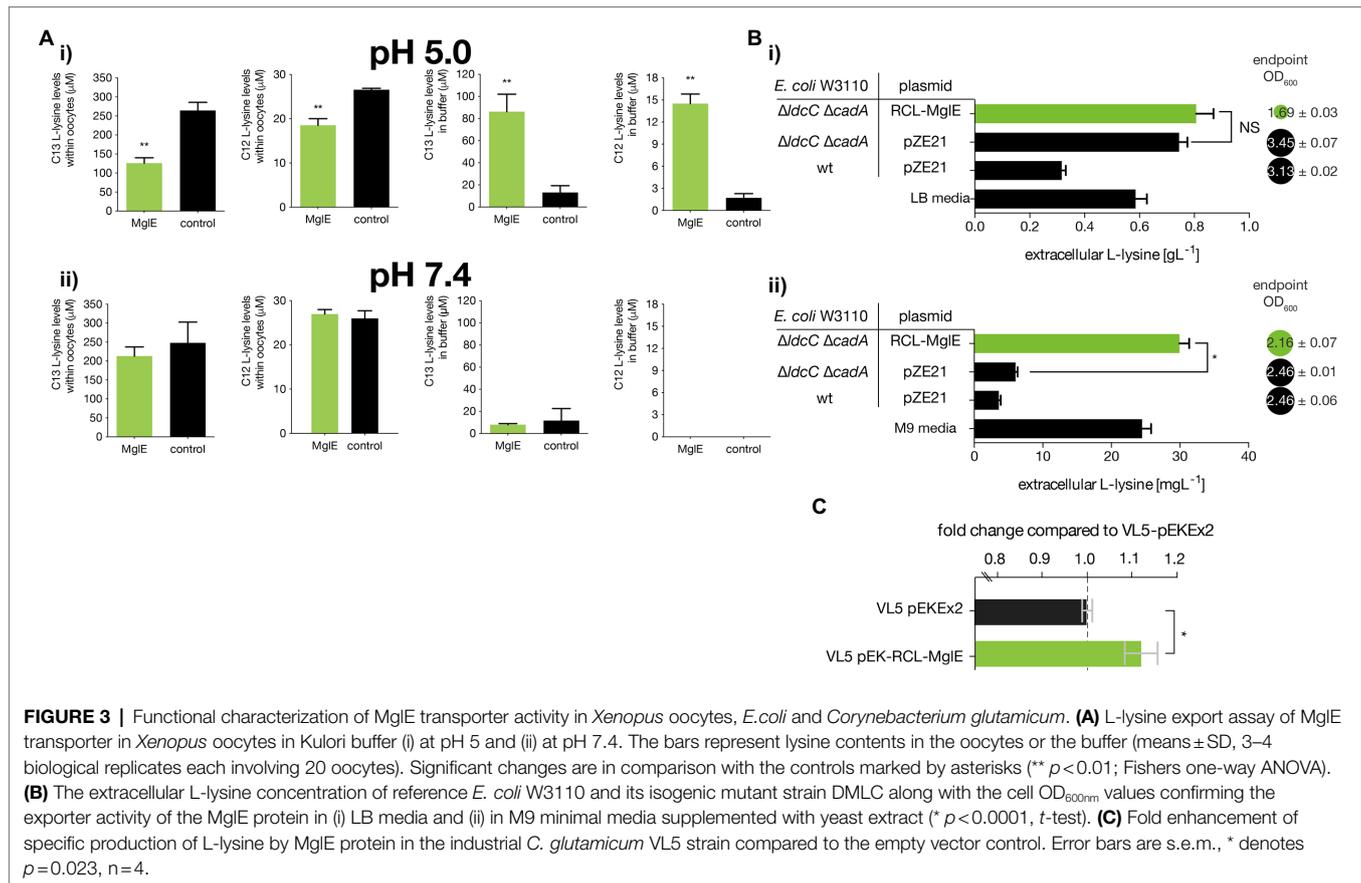
To analyze the function of MglE for L-lysine efflux, *E. coli* DMLC strain was constructed by knocking out two lysine decarboxylase genes, constitutive (*ldcC*) and acid-inducible (*cadA*). These deletions should prevent L-lysine degradation, thereby leading to increased L-lysine production (Kikuchi et al., 1997). Then, the pZE-RCL-MglE plasmid and pZE21 empty vector were transformed into *E. coli* DMLC, yielding *E. coli* DMLC/pZE-RCL-MglE and *E. coli* DMLC/pZE21, respectively. Subsequently, these strains along with *E. coli* W3110/pZE21, were cultured in LB media as well as M9 minimal media supplemented with yeast extract in 96 deep well plates with culture volume of 600  $\mu$ l.

The bacterial growth and extracellular L-lysine concentrations were measured after 24h.

In both media, upon knocking out *ldcC* and *cadA*, there were higher L-lysine concentration in supernatant as compared to the wild-type strain and further the extracellular L-lysine concentration was increased upon expression of MglE operon

in the deletion mutant. However, the difference of extracellular L-lysine concentration between *E. coli* DMLC and *E. coli* DMLC/pZE-RCL-MglE is not significant in LB media as compared to M9 minimal media because of the presence of higher amount of L-lysine in LB media.

We found that in LB media the absolute extracellular L-lysine titer was increased from 744 to 806  $\text{mg l}^{-1}$  in the presence of the metagenomic insert consisting MglE operon that is, 8.3% higher L-lysine production in the *E. coli* DMLC/pZE-RCL-MglE strain as compared to *E. coli* DMLC/pZE21 in LB. However, the biomass of the strain expressing MglE was decreased by about 50% as compared to the control strain (Figure 3Bi). Furthermore, the L-lysine production was analyzed in minimal media supplementing yeast extract. In minimal media, the extracellular L-lysine accumulation was increased from 6 to 30  $\text{mg l}^{-1}$  ( $p < 0.0001$ , t-test) whereas there was 12% reduced in biomass upon expressing the MglE metagenomic fragment (Figure 3Bii). The difference in extracellular L-lysine concentration among the strain expressing MglE and the control



strain is prominent in minimal media (\* $p < 0.0001$ , *t*-test) than in LB media ( $p = 0.1997$ , *t*-test). Hence, the above results demonstrate that MglE assists exporting L-lysine from *E. coli*.

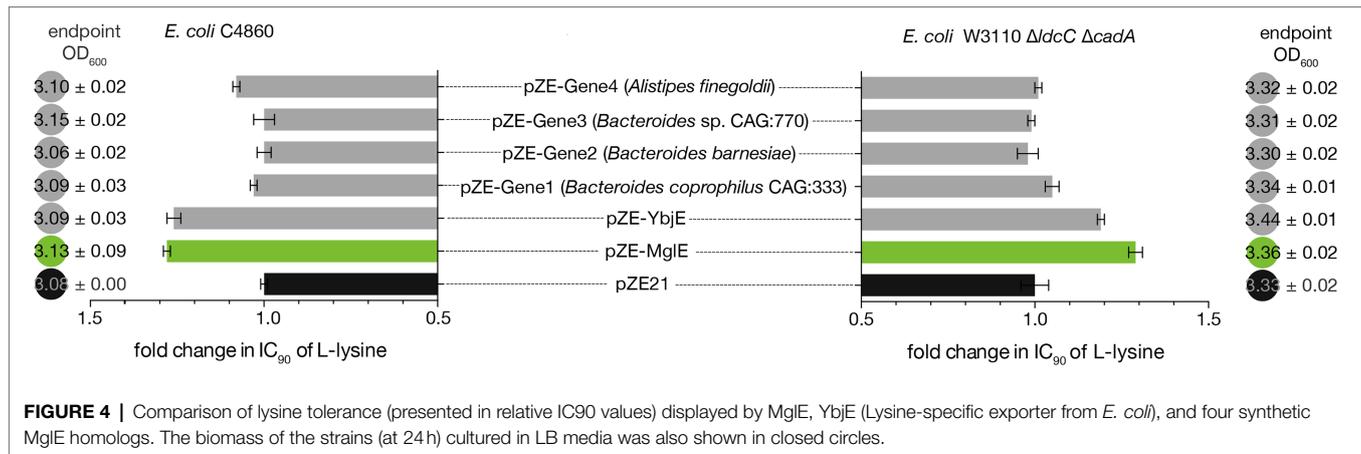
The reduced biomass might be due to the combined effect of expression of membrane protein in high copy number and active export of L-lysine by MglE which could have resulted in less available energy for the cell to build biomass. It is worth noting that a big proportion of total cellular energy-demand is for the transport machinery which has accordingly been under evolutionary selection toward a higher energetic efficiency (Darbani et al., 2018).

## Improvement of L-Lysine Productivity in Industrial *Corynebacterium glutamicum* Strain

Expression of a functionally active gene(s) from one strain to another often requires special optimizations. For the practical application in industrial L-lysine bioprocesses, retaining the activity of the discovered exporter into the production host, *C. glutamicum*, is very essential. To test whether the MglE can boost the industrial L-lysine bioprocesses, we cloned and expressed it in *C. glutamicum* production strain.

The full metagenomic insert carrying the MglE along with its native promoter and terminator was amplified from the pZE-RCL-MglE plasmid and cloned into pEKEx2 expression vector to construct pEK-RCL-MglE expression plasmid.

The constructed plasmid was introduced into *C. glutamicum* VL5 strain (Materials and methods), a producer strain currently used for industrial L-lysine production. Using sucrose as a carbon source, L-lysine production from the constructed *C. glutamicum* recombinant strain was analyzed at 30h. The *C. glutamicum* VL5 parent strain and VL5 transformed with empty plasmid (i.e., VL5/pEKEx2) produced 26.59 and 27.13  $\text{g l}^{-1}$  of L-lysine respectively, whereas 29.72  $\text{g l}^{-1}$  of L-lysine titer was produced by the strain expressing MglE operon (i.e., VL5/pEX-RCL-MglE). Furthermore, L-lysine yield with respect to amount of sucrose consumed were calculated for these three strains which resulted 0.76, 0.77, and 0.83  $\text{g g}^{-1}$  for the strains VL5, VL5/pEKEx2, and VL5/pEK-RCL-MglE, respectively. Hence, expression of MglE operon into *C. glutamicum* VL5 improved the L-lysine yield and titer by 7.8% and 9.5%, respectively in comparison to the empty vector control. Unlike in *E. coli* strains, expression of the full metagenomics insert harboring MglE did not have any growth inhibition effect as the vector control and the recombinant strains both have nearly equal biomass. Notably, upon incorporation of the MglE operon into *C. glutamicum*, the specific L-lysine production was improved by 12%  $\pm$  0.07% relative to the empty vector control (Figure 3C). By expressing the operon in this highly efficient producer strain, we showed not only the functionality of the MglE in a Gram-positive species but also the benefit of the efflux system on an already highly optimized producer strain.



## Comparison of L-Lysine Tolerance Conferred by MglE and Its Homologous Genes

The YbjE transporter from *E. coli* is one of the functionally characterized lysine-specific exporters. Based upon the homology search, four genes in the NCBI database having different homologies (ranging from 43% to 82% in amino acid level) with MglE were also selected and gene synthesized. To find out the lysine tolerance of MglE as compared to that of YbjE and the four selected MglE homologous proteins (with 82%, 78%, 54%, and 43% amino acid sequence identity), recombinant expression plasmids pZE-YbjE, pZE-Gene1, pZE-Gene2, pZE-Gene3, and pZE-Gene4 were constructed. The nucleotide sequences of these genes are provided in the **Supplementary Information**. The constructed plasmids, pZE-MglE and pZE21 vector control were introduced into *E. coli* C4860 and *E. coli* DMLC strains and determined the IC<sub>90</sub> of L-lysine for the constructed recombinant strains as described in early experiments.

Interestingly, we found that the expression of MglE provided similar L-lysine tolerance in *E. coli* C4860 strain as that of YbjE whereas in *E. coli* DMLC strain, MglE displayed even higher tolerance than YbjE. On the other hand, all of the four MglE homologs showed lower lysine tolerance than MglE in both strains (**Figure 4**).

## CONCLUSION

We deployed functional metagenomic selections to identify novel genetic building blocks encoding product efflux systems. Through applying this approach to the problem of L-lysine toxicity at high concentration, we discovered MglE, which is confirmed as a novel type of L-lysine exporter that belongs to the EamA superfamily and this gene could not have been identified by only sequence analysis or homology to known L-lysine transporters. We demonstrated the benefits of MglE in both Gram-negative (*E. coli*) and Gram-positive (*C. glutamicum*) strains and showed that MglE expression improves the productivity of an industrial L-lysine production strain. If incorporated into industrial-scale bioprocesses, this discovery has the potential to enhance L-lysine production by about 10%–12%, representing an increased profit

on the order of 200–500 million USD per year. Our approach of using functional metagenomics to identify novel transporters is independent of prior knowledge of transporter gene sequences and can be generally applied to most of the toxic compounds/chemicals production by fermentation.

## PROTEIN ACCESSION NUMBERS

The sequence information of full metagenomics insert harboring *mglE* is deposited in the NCBI GenBank database with the accession number KU708839.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

MS and SM conceived the study and drafted the manuscript. SM designed and performed the *E. coli* experiments. BD and IB conceived the experiments on *Xenopus* oocytes. BD performed the experiments. SW performed *C. glutamicum* experiments. EH performed *in silico* analysis. MS, IB, and JF led the research teams. EH, BD, SW, JF, and IB contributed to the writing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.855736/full#supplementary-material>

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