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ORIGINAL ARTICLE

Rearrangement in the regulation of *sigD* gene expression promotes 4-hydroxyisoleucine production in *Corynebacterium glutamicum*

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Abstract

Corynebacterium glutamicum is widely used in the production of amino acids. *C. glutamicum* possesses seven sigma factors, among which SigD is responsible for the transcription of genes involved in the synthesis of mycolic acid (MA) and its derivatives, the unique cell envelope of *C. glutamicum*. To understand the influence of MA synthesis on amino acid production and membrane phenotype of *C. glutamicum*, the expression of *sigD* gene and some mycolyltransferase genes, i.e., *cmt1*, *cop1* and *cmt2*, were regulated by several growth-regulated promoters in this study. Except for 2 mutant strains of P_{cg3096}-*sigD* and P_{cg1633}-*cop1*, the growth and 4-hydroxyisoleucine (4-HIL) titer of most modified strains did not change significantly. But the 4-HIL titer of P_{odhI}-*sigD* strain increased by 20.73% (142.45 ± 3.69 mM) compared to that of control strain (117.99 ± 0.34 mM). After it was cultivated in bioreactor, 4-HIL titer reached 372.56 mM. This may be caused by the increase of MA content, and 17% decrease of cell hydrophobicity and 12% increase of membrane permeability were observed at the exponential phase. In conclusion, we proved that rearrangements in regulation of *sigD* expression contributed to the improved fermentation performance of *C. glutamicum* and promoted 4-HIL production.

Keywords *Corynebacterium glutamicum* · SigD · 4-Hydroxyisoleucine · Promoter engineering · Mycolic acid

Introduction

Diabetes is one of the four major non-communicable diseases worldwide. It is usually treated with oral medicines and insulin injection. In recent decades, fenugreek has been shown to have preventive and palliative effects on type I and type II diabetes, because the 4-hydroxyisoleucine (4-HIL) in fenugreek showed unique activity in stimulating insulin secretion as well as ameliorating insulin resistance [1]. 4-HIL was originally extracted from fenugreek seeds. The anti-diabetic activity of 4-HIL depends on glucose concentration, so the side effects caused by other chemicals for treating diabetes, such as hypoglycemia and gastrointestinal discomfort can be avoided [2]. 4-HIL can also enhance insulin sensitivity, thereby can be used for the treatment of type II diabetes [3]. Considering the limited amount of 4-HIL that can be extracted from plants, fermentation methods have been adopted to produce 4-HIL [4]. L-isoleucine dioxygenase (IDO) was found in *Bacillus thuringiensis* that catalyzes the C-4 hydroxylation of L-isoleucine (Ile) to form 4-HIL [5].

Corynebacterium glutamicum is a Gram-positive bacterium widely used for the production of various amino acids, and its product is generally recognized as safe [6]. In a previous study, *ido* gene was introduced into *C. glutamicum* ssp. *lactofermentum* SN01, resulting in a *de novo* synthesis of 65.44 ± 2.27 mM 4-HIL from its own produced Ile [7]. Besides Ile, α-ketoglutaric acid (α-KG) and O_2 are also required for the synthesis of 4-HIL in IDO reaction (Fig. 1). Recently, to increase the supply of co-substrates α-KG and O_2 in an Ile responsive manner, the modified Ile biosensor Lrp-P_{brnFE}N was used to co-express the *ido* gene with *odhI* and *vgb* genes, increasing the 4-HIL titer to 135.3 mM [8]. *odhI* gene encodes the inhibitor of α-KG dehydrogenase complex and *vgb* gene encodes the *Vitreoscilla* hemoglobin VHb. But L-lysine (Lys) was still remained as the main byproduct. To reduce the production of Lys, the Lys-OFF riboswitch was integrated before *dapA* gene, the key gene of Lys

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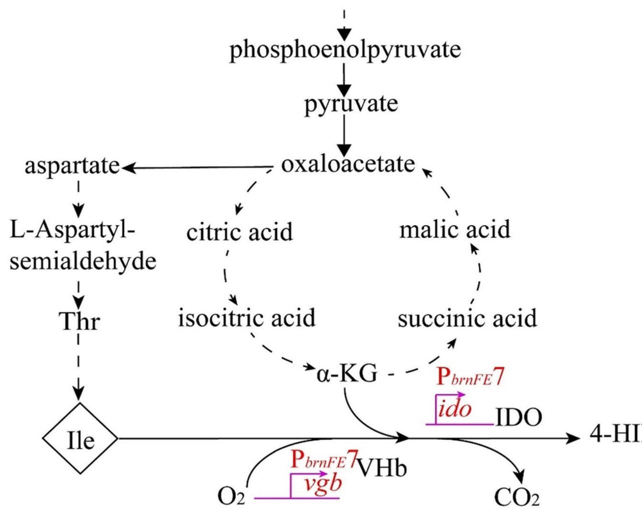

Fig. 1 Metabolic pathways of 4-HIL production in *C. glutamicum*, including TCA cycle and Ile production pathway. Those marked in red are foreign genes carried by plasmids. VHb: *Vitreoscilla* hemoglobin, IDO: Ile dioxygenase

biosynthetic pathway, and Lys content decreased greatly in the resulting D-RS strain [9].

The *C. glutamicum* genome codes for 7 sigma subunits (factors) of RNA polymerase: primary sigma factor SigA (σ^A), primary-like SigB and 5 other alternative sigma factors (SigC, SigD, SigE, SigH and SigM). Each sigma factor is responsible for recognizing promoters of genes belonging to its regulon (sigmulon) involved in specific functions of the cell [10]. Among them, SigD (σ^D) is mainly responsible for recognizing promoters involved in membrane-associated genes such as mycolic acid (MA) synthesis genes (*fadD2*, *pks*), mycolyltransferase genes (*cop1*, *cmt1*, *cmt2*, *cmt3*) [11], L, D-transpeptidase gene (*lppS*), and others [12]. Recently, it has been shown that overexpression of *sigD* influences the cell envelopes of *C. glutamicum* strain, reduces the formation of foams during fermentation, and affects the expression of genes related to MA synthesis [13]. *C. glutamicum* and other *Corynebacteria* have special cell envelopes with a peptidoglycan layer, arabinogalactan [14], an MA layer, and a S layer sequentially arranged on the outside of its phospholipid bilayer (Fig. 2). The MA layer is composed of trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM) formed by mycolic acid and trehalose. Comparison of the transcriptome data of the Ile-producing strain *C. glutamicum* SN01 [15] with that of *C. glutamicum* ATCC13032 showed that the *sigD* and many genes of SigD regulon, such as some mycolyltransferase genes were weakly expressed in SN01 during the exponential phase (Table S1). It was found that regulating the expression of some genes of SigD regulon altered the cell envelope properties of *C. glutamicum* ATCC13032 and affected cell growth [13]. Changes in MA content also have an effect on amino acid synthesis and efflux in *C. glutamicum* [13, 16]. The MA content of the cell wall permeability barrier was implicated in the amino acid excretion process [17].

Considering that the expression of *sigD* and SigD regulon are usually downregulated at the stationary phase, and the *sigD* expression in SN01 was quite weak, several growth-regulated promoters (GRP) with different strengths such as P_{CP_2836} [18] were used here in D-RS strain to increase the expression strength of *sigD* gene during the exponential phase. The changes of membrane properties, cell growth, and 4-HIL production in these modified strains were then analyzed. Next, considering the importance of mycolyltransferase on covalent linkage of MA with other layers and formation of the entire cell envelope, we evaluated the effect of regulation of some crucial mycolyltransferase genes on 4-HIL production and cell envelope properties of

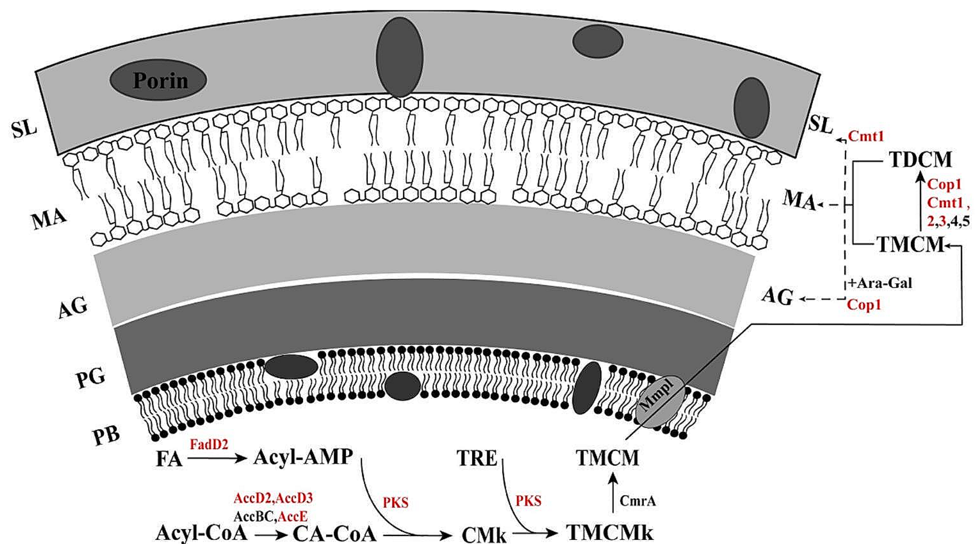

Fig. 2 *C. glutamicum* cell envelope and its mycolic acid synthesis pathway. The enzymes shown in red are regulated by SigD factors during gene transcription. AG: arabinogalactan, CA-CoA: carboxylated acyl-CoA, CMk: keto corynomycolic acid, FA: fatty acid, MA: mycomembrane, PB: phospholipid bilayer, PG: peptidoglycan, SL: S layer, TDCM: trehalose dicorynomycolate, TMCM: trehalose monocorynomycolate, TRE: trehalose

C. glutamicum D-RS. Finally, the best strain P_{odhI}-*sigD* was cultivated for 4-HIL production in the bioreactor.

Materials and methods

Strains, media and culture conditions

The strains used in this study are listed in Table 1. *Escherichia coli* JM109 was used as a host to construct the integrative plasmids and expression plasmid. *E. coli* was cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37 °C, 200 rpm. *C. glutamicum* D-RS was engineered for synthesizing 4-HIL, while *C. glutamicum* ATCC13032 was used for amplifying target genes and promoters. *C. glutamicum* was cultured in LBB medium (18.5 g/L brain-heart infusion, 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl) using a rotary shaker at 30 °C and 200 rpm [19], and 30 mg/L kanamycin, 10 mg/L chloramphenicol, or 100 g/L sucrose were added to medium as needed.

Modification of promoters in the chromosome of SN01

The primers are showed in Table 2. According to our unreported transcriptome data, the five GRP promoters with the higher strength at the exponential phase and lower strength at the stationary phase, i.e. P_{odhI}, P_{cg1206}, P_{cg1633}, P_{cg2705}, and P_{cg3096} (Table S2), were selected to replace the promoter of *sigD* gene (P_{sigD}, the upstream 157 bp of *sigD* gene). The pK18*mobsacB* and CRISPR-Cpf1-assisted genome editing system were used for promoter replacement [20]. The editing plasmid pk18-P_{sigD}::P_{odhI} was constructed by amplifying the upstream homologous arm of *sigD*, the P_{odhI} promoter, and the downstream homologous arm of *sigD* using the primer pairs of *sigD*-U-F/*sigD*-U-R, P_{odhI}-F/P_{odhI}-R, and *sigD*-D-F/*sigD*-D-R, respectively. These fragments were overlapped and ligated into the pK18*mobsacB* vector. The other editing plasmids were constructed in a similar way. Each of the above constructed editing plasmids was then electrotransformed into *C. glutamicum* D-RS for replacing the P_{sigD} promoter using the CRISPR-Cpf1-assisted SacB editing system. After single exchange, pCS-assisted double exchange and pCS curing, the corresponding *sigD* promoter replacement strains, i.e. D-RS P_{sigD}::P_{odhI}, D-RS P_{sigD}::P_{cg1206}, D-RS P_{sigD}::P_{cg1633}, D-RS P_{sigD}::P_{cg2705}, and D-RS P_{sigD}::P_{cg3096} were constructed. P_{cop1}, P_{cmt2}, and P_{cmt1} promoter replacement strains D-RS P_{cop1}::P_{cg1633}, D-RS P_{cmt1}::P_{cg1633}, D-RS P_{cmt2}::P_{cg1633}, D-RS P_{cmt2}::P_{tuf} and the D-RS P_{cmt1}-*cmt1*::P_{cmt1}-*cmt1* (ATCC13032) were also constructed similarly (Table 1).

Construction of *ido* and *vgb* co-expression plasmid and strains

The plasmids are showed in Table 1. The P_{brnFE}7-*ido* fragment was amplified from pIL-$_7$I^U [9] by primer pairs P_{brnFE}7-U/*ido*-R. The P_{brnFE}7 and *vgb* fragments were amplified from pIL-$_7$I^U and pJYW-4-*imi*3-*vgb* [21] by primer pairs P_{brnFE}7-F/P_{brnFE}7-R and *vgb*-F/*vgb*-R, respectively. Then three fragments were overlapped, digested with *Bam*HI, and ligated into *Bam*HI-digested pJYW-4, generating the plasmid P_7P_7. The plasmid P_7P_7 was then transformed into strains D-RS, D-RS P_{sigD}::P_{cg1633}, D-RS P_{sigD}::P_{cg1206}, D-RS P_{sigD}::P_{cg2705}, D-RS P_{sigD}::P_{odhI}, D-RS P_{sigD}::P_{cg3096}, D-RS P_{cop1}::P_{cg1633}, D-RS P_{cmt2}::P_{cg1633}, D-RS P_{cmt2}::P_{tuf}, D-RS P_{cmt1}::P_{cg1633} and D-RS P_{cmt1}-*cmt1*::P_{cmt1}-*cmt1* (ATCC13032) to generate strain RS1, RSS1, RSS2, RSS3, RSS4, RSS5, RSC1, RSC2, RSC3, RSC4 and RSA1, respectively.

Analysis of cell hydrophobicity and permeability

Cell surface hydrophobicity was determined according to the following method [22]. Briefly, cell suspension was collected from the cultivated LBB medium at 8 h and 15 h, washed twice with PBS buffer (0.05 mM, pH 7.4), and resuspended in PBS buffer (0.05 mM, pH 7.4) to a final optical density at 562 nm (OD_{562}) of 0.5 (recorded as OD_{562A}). Then 2 mL xylene was added to 2 mL cell suspension, oscillated for 2 min and stood for 1 h. The OD_{562} of the water phase (recorded as OD_{562B}) was determined after the xylene was removed. The cell hydrophobicity was calculated by formula (OD_{562A}-OD_{562B})/OD_{562A}.

The membrane permeability was determined by N-phenyl-1-naphthylamine (NPN) fluorescent probe method [23]. Briefly, cell suspension was collected from the cultivated LBB medium at 8 h and 15 h, washed twice with PBS buffer (0.05 mM, pH 7.4), and resuspended in PBS buffer (0.05 mM, pH 7.4) to a final OD_{562} of 0.5. Then 80 µL NPN (10 µmol/L) was added to 1.92 mL cell suspension in a 24-well plate. The fluorescence intensity was measured by microplate reader (Excitation wavelength 350 nm, emission wavelength 428 nm, slit width 5 nm), and the cell permeability was expressed by formula fluorescence intensity/OD_{562}.

Chromatography of lipids

Total lipids were extracted according to methods in the literature [24]. Briefly, wet cells were harvested from the fermentation medium, then sequentially resuspended in a mixture of 10 mL $CHCl_3$/CH_3OH (1:2, v/v), 10 mL $CHCl_3$/CH_3OH (1:1, v/v), and 10 mL $CHCl_3$/CH_3OH (2:1, v/v) for 16 h and the supernatants of each suspension were collected and mixed. The $CHCl_3$ and CH_3OH was removed

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Source
Strains		
JM109	*E. coli* gene cloning strain	Novagen
ATCC13869Δ*pks*	*pks* deletion mutant of *C. glutamicum* ATCC13869	[23]
SN01	Ile-producing strain of *C. glutamicum* ssp. *lactofermentum*	CCTCC
D-RS	Integration of Lys-OFF riboswitch before *dapA* gene in SN01	[9]
RS1	D-RS harboring P_7P_7	This work
RSS1	D-RS P_{sigD}::P_{cg1633} harboring P_7P_7	This work
RSS2	D-RS P_{sigD}::P_{cg1206} harboring P_7P_7	This work
RSS3	D-RS P_{sigD}::P_{cg2705} harboring P_7P_7	This work
RSS4	D-RS P_{sigD}::P_{odhI} harboring P_7P_7	This work
RSS5	D-RS P_{sigD}::P_{cg3096} harboring P_7P_7	This work
RSC1	D-RS P_{copI}::P_{cg1633} harboring P_7P_7	This work
RSC2	D-RS P_{cmt2}::P_{cg1633} harboring P_7P_7	This work
RSC3	D-RS P_{cmt2}::P_{tuf} harboring P_7P_7	This work
RSC4	D-RS P_{cmt1}::P_{cg1633} harboring P_7P_7	This work
RSA1	D-RS P_{cmt1}-*cmt1*::P_{cmt1}-*cmt1*(ATCC13032) harboring P_7P_7	This work
Plasmids		
pK18*mobsacB*	Cloning vector of *E. coli*, Km^R	[20]
pCS	SacB gene editing auxiliary plasmid harboring *cpf1* and sgRNA(*kan*), Cm^R	[20]
pJYW-4-*imi*3-*vgb*	pJYW-4 harboring *ido*, *mqo*, *ido*3, and *vgb* genes, Km^R	[21]
pIL-$_7I^U$	pJYW-4 harboring *lrp* and P_{brnFE}7-controlled codon-optimized *ido* gene, Km^R	[9]
P_7P_7	pJYW-4 harboring P_{brnFE}7-controlled codon-optimized *ido* and P_{brnFE}7-controlled *vgb* genes, Km^R	This work
pk18-P_{sigD}::P_{odhI}	pK18*mobsacB* carrying *sigD*-up, P_{odhI} and *sigD*-down	This work
pk18-P_{sigD}::P_{cg1633}	pK18*mobsacB* carrying *sigD*-up, P_{cg1633} and *sigD*-down	This work
pk18-P_{sigD}::P_{cg1206}	pK18*mobsacB* carrying *sigD*-up, P_{cg1206} and *sigD*-down	This work
pk18-P_{sigD}::P_{cg2705}	pK18*mobsacB* carrying *sigD*-up, P_{cg2705} and *sigD*-down	This work
pk18-P_{sigD}::P_{cg3096}	pK18*mobsacB* carrying *sigD*-up, P_{cg3096} and *sigD*-down	This work
pk18-P_{copI}::P_{cg1633}	pK18*mobsacB* carrying *cop1*-up, P_{cg1633} and *cop1*-down	This work
pk18-P_{cmt1}::P_{cg1633}	pK18*mobsacB* carrying *cmt1*-up, P_{cg1633} and *cmt1*-down	This work
pk18-P_{cmt2}::P_{cg1633}	pK18*mobsacB* carrying *cmt2*-up, P_{cg1633} and *cmt2*-down	This work
pk18-P_{cmt2}::P_{tuf}	pK18*mobsacB* carrying *cmt2*-up, P_{tuf} and *cmt2*-down	This work
pk18-*cmt1*::*cmt1*(ATCC13032)	pK18*mobsacB* carrying *cmt1*-up, P_{cmt1}-*cmt1*(ATCC13032) and *cmt1*-down	This work

from the mixed supernatants by rotary evaporation to obtain the crude lipid extract. Then 10 mL of $CHCl_3/H_2O$ mixture (1:1, v/v) was added to the crude lipid extract, fully mixed and stratified. After collecting the organic phase, the solvent was removed by rotary evaporation to obtain the total lipid extract. Finally, 50 μL $CHCl_3/CH_3OH$ (2:1, v/v) was added to dissolve the lipid extract and 2 μL solution was spotted onto thin-layer chromatography (TLC) silica gel plates and chromatographed for 20 min with the spreading agent $CHCl_3/CH_3OH/H_2O$/ammonia (65:25:1:3, v/v/v/v).

4-HIL fermentation in shake flasks and bioreactor

For producing 4-HIL via fermentation, the recombinant *C. glutamicum* were streaked onto plates containing the LBB medium and incubated at 30 °C for 48 h. And then the grown bacteria were inoculated in a 500 mL baffled flask

Table 2 Primers used in this study

Primers	Sequences (5'-3')	Restriction sites	Description
sigD-U-F	AATTCGAGCTCGGTACCCGGACTGGCTACTGCTTTGGG	*Bam*HI	For *sigD*-up amplification
sigD-U-R	CAGAAACTGACAGATAATAC		
sigD-D-F	**AGAAGGAGGG***TTATTAG*TTGGCTGACACTGAGCGC		For *sigD*-down amplification
sigD-D-R	CCTGCAGGTCGACTCTAGACCCGCTTGATCCGCTCTT	*Bam*HI	
sigD-P_{odhI}-F	TTATCTGTCAGTTTCTGCGATCACGAGGGGGCACATT		For P_{odhI} amplification
sigD-P_{odhI}-R	CCAA*CTAATAA***CCCTCCTTCT**TTAAACTTCCTCCGTGTCGA		
sigD-P_{cg1633}-F	TTATCTGTCAGTTTCTGTGGGGGTGTTTTTGGCGTGT		For P_{cg1633} amplification
sigD-P_{cg1633}-R	CCAA*CTAATAA***CCCTCCTTCT**ATTATTCAGCACCCATGTTTTATTC		
sigD-P_{cg1206}-F	TTATCTGTCAGTTTCTGCCGCAGGCGAAATCGCATCG		For P_{cg1206} amplification
sigD-P_{cg1206}-R	CCAA*CTAATAA***CCCTCCTTCT**AAATAAAGTGTGAAGTTGTGCTA		
sigD-P_{cg2705}-F	TTATCTGTCAGTTTCTGTTGTATGTTTTAGGCCCG		For P_{cg2705} amplification
sigD-P_{cg2705}-R	CCAA*CTAATAA***CCCTCCTTCT**CATCTTTCTTGATAAAAAGCGG		
sigD-P_{cg3096}-F	TTATCTGTCAGTTTCTGAAACATGCTTGTCGACGCCG		For P_{cg3096} amplification
sigD-P_{cg3096}-R	CCAA*CTAATAA***CCCTCCTTCT**TGGGTCTCCTTTGGGCCACC		
cmt1-U-F	*TTCGAGCTCGGTACCCGG*AGCCACCAACACCCAACCG	*Bam*HI	For *cmt1*-up amplification
cmt1-U-R	GCAGCCCATAACCCACAAT		
cmt1-D-F	**AGAAGGAGGG***TTATTAG*ATGAAGCTTCTTCGCCGC		For *cmt1*-down amplification
cmt1-D-R	*CTGCAGGTCGACTCTAGA*AAGGGCGAGCCTAGTATGG	*Bam*HI	
cmt2-U-F	*ATTCGAGCTCGGTACCCGG*ACTGACAAGCACTTAGCGA	*Bam*HI	For *cmt2*-up amplification
cmt2-U-R	CTCTTGGGTAGTTATCACAGC		
cmt2-D-F	**AGAAGGAGGG***TTATTAG*ATGTCCGTATTTACACGAG		For *cmt2*-down amplification
cmt2-D-R	GCCTGCAGGTCGACTCTAGATGGGCTTTTGATTTTGGTG	*Bam*HI	
cop1-U-F	AATTCGAGCTCGGTACCCGGGAGGACGGTCACGAAGTCA	*Bam*HI	For *cop1*-up amplification
cop1-U-R	CAAGACTCGCAGCATTTCC		
cop1-D-F	**AGAAGGAGGG***TTATTAG*ATGCGCGACACCGCATTT		For *cop1*-down amplification
cop1-D-R	*GCCTGCAGGTCGACTCTAGA*GGGAAGTCATACGGGAGATA	*Bam*HI	
cmt2-P_{tuf}-F	GTGATAACTACCCAAGAGTAAGTGGGGTAGCGGCTTGT		For P_{tuf} amplification
cmt2-P_{tuf}-R	*CTAATAA***CCCTCCTTCT**AATTGGTTTTGCTTTCACTG		
cmt1-F(13032)	ATTGTGGGTTATGGGCTGCGTTCAGAGATTTTTGGCTCG		For ATCC13032 P_{cmt1}-*cmt1* amplification
cmt1-R(13032)	AATGTTAAATCTGTTTCTAGGCCTC		
cmt1-D-F(13032)	CCTAGAAACAGATTTAACATTAAAACCGGC		For D-RS *cmt1*-down amplification
cmt1-D-R(13032)	CCTGCAGGTCGACTCTAGAGCTCTTTATCTTCCGTTT	*Bam*HI	

The ribosomal binding sites are indicated in boldface. The spacer sequences are italicized. The restriction sites are underlined

containing 40 mL of seed medium and cultivated at 30 °C and 200 rpm for 17 h. The seed cultures were inoculated into a 500 mL baffled flask containing 30 mL of fermentation medium until the final OD_{562} was 1.8 and then shook at 200 rpm and 30 °C for 144 h by a cyclotron shaker [25]. During the fermentation, 1 mL culture samples were taken regularly to determine the OD_{562}, residual glucose and the Ile and 4-HIL concentration.

For producing 4-HIL via fermentation in a bioreactor, the cultivated culture of *C. glutamicum* strain RSS4 in shake flask were inoculated at 10% volumetric inoculum into a 2 L fermenter (T&J-Minbox 2 L*4; T&J Bioengineering Co., Ltd., Shanghai, China) containing 800 mL medium. The medium contains 100 g/L glucose, 20 g/L $(NH_4)_2SO_4$, 15 g/L corn syrup, 1 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 1.1 g/L $FeSO_4 \cdot 7H_2O$, $0.3*10^{-4}$ g/L protocatechuic acid, 3 g/L yeast extract, $1*10^{-3}$ g/L vitamin B1, and $1.5*10^{-3}$ g/L betaine. Fermentation was initially carried out at pH 7.0–7.2, 800 rpm, 30 °C and 2 vvm of aeration rate. After 72 h, the pH, rotational speed and aeration rate were controlled at pH 6.5–7.0, 800 rpm and 1.5 vvm, respectively. Relative dissolved oxygen was controlled at 80–100% for the first 24 h, after which it was coupled to the stirring speed and controlled at 30%. Samples were taken every 12 h to determine the OD_{562} value and residual glucose. Glucose was fed to the final concentration of 40 g/L when the residual glucose concentration in the medium dropped below 20 g/L.

Real-time PCR analysis

Total RNA was extracted from *C. glutamicum* cells growing in LBB medium at 8 h. After removing residual DNA and reversely transcribing RNA into cDNA, real-time (RT)-PCR was performed as described previously [15]. The relative abundance of 16 S rRNA was used as a standard control. The relative gene expression analysis was quantified by the cycle threshold (Ct) and $2^{-\Delta\Delta CT}$ value.

Result and discussion

Regulation of *sigD* expression by growth-regulated promoters

A previously constructed strain, *C. glutamicum* D-RS, was selected as the chassis strain for modifying *sigD* expression. In strain D-RS, a Lys-OFF riboswitch was chromosomally inserted at the upstream of *dapA* gene of strain SN01 to reduce the accumulation of by-product Lys [9]. According to transcriptomic data, the *sigD* gene expression of D-RS was about 80% weaker than that of *C. glutamicum* ATCC13032 (Table S1). Because the growth of ATCC13032 is better than that of SN01, and the expression of *sigD* and SigD regulon in *C. glutamicum* ATCC13032 is usually downregulated at the stationary phase, so in order to enhance *sigD* gene expression at the exponential growth phase of strain *C. glutamicum* D-RS, the GRP with the higher strength at the exponential phase was exploited. Previously, P_{CP_2836} was applied as an exponential up-regulated promoter to promote L-valine production [18], P_{odhI} was used to regulate glutamate decarboxylase expression for γ-aminobutyric acid production and showed high transcription level at exponential phase and significantly low transcription level at stationary phase [26]. Besides P_{odhI} and the identical promoter of P_{CP_2836} in *C. glutamicum* ATCC13032, i.e. P_{cg3096}, several GRP that were up-regulated at the exponential phase and down-regulated at the stationary phase, i.e. P_{cg1206}, P_{cg1633}, P_{cg2705}, were identified from the transcriptome data (Table S2). These 5 GRPs were used to replace the original promoter of the *sigD*, generating 5 GRP-modified strains such as D-RS P_{sigD}::P_{odhI}. Overexpression of *vgb* genes has been demonstrated to be effective to increase O_2 supply and 4-HIL production [8, 25]. Then the P_7P_7 plasmid carrying *ido* and *vgb* genes regulated by an Ile-responsive promoter $P_{brnFE}7$ was electrotransformed into chassis strain D-RS and these GRP-modified strains to obtain the control strain of RS1 and the *sigD*-regulated strains of RSS1–RSS5. These strains were cultured in shake flasks, and the fermentation results were shown in Fig. 3a, b.

The growth and glucose consumption of *sigD*-regulated strains were not affected, except for RSS5 (Fig. 3a), so RSS5 was discarded for subsequent studies. RSS1–RSS4 grew quickly to OD_{562} of 60–70 before 48 h and slowly thereafter. Glucose was consumed quickly and exhausted at 72 h. The RSS4 strain grew better than the RS1 in the first 48 h and achieved a 4-HIL titer of 142.45 ± 3.69 mM, which was 20.73% higher than that of RS1 (117.99 ± 0.34 mM) (Fig. 3b). The growth of other three modified strains were not significantly affected and their 4-HIL titers (102.51–120.72 mM) were similar to RS1. However, the 4-HIL titer of RSS4 was only 7.15 mM higher than that of a previous strain ST17 (135.30 mM) [8], in which the *ido*, *odhI* and *vgb* genes were dynamically overexpressed by the Ile biosensor Lrp-$P_{brnFE}7$. Therefore, altering the cell envelope properties by modifying *sigD* expression may not contribute much to cell growth and 4-HIL production in shake flask fermentation. Subsequently, in order to further understand the metabolic performance of these strains in the bioreactor, some cell properties such as the cell hydrophobicity and permeability of the *sigD*-regulated strains growing in LBB medium were investigated during the exponential (8 h) and stationary phases (15 h), and the results are shown in Fig. 3c, d. Firstly, the hydrophobicity of *C. glutamicum* D-RS was much stronger than that of ATCC13032. This indicates that with the

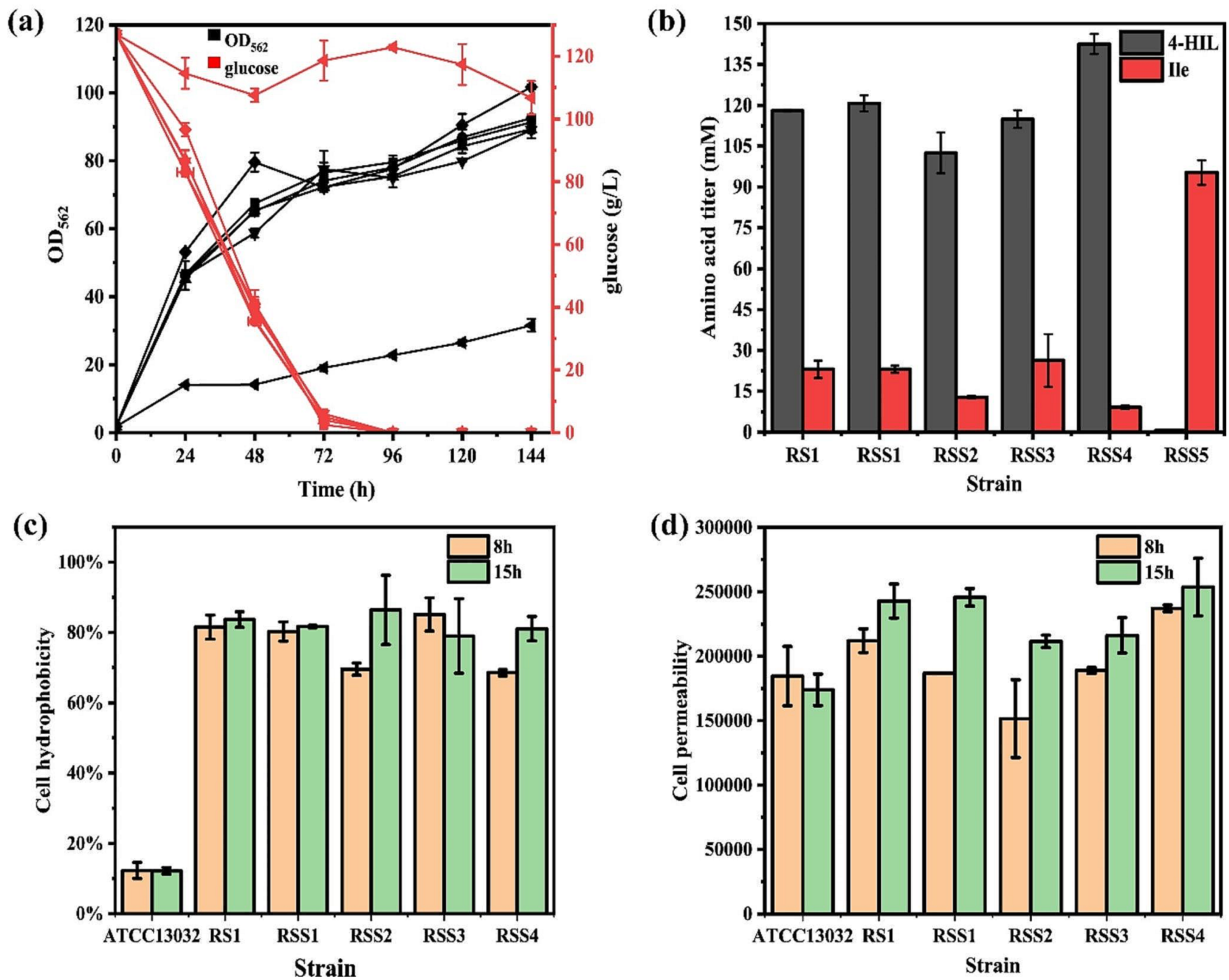

Fig. 3 Fermentation data and cell envelope properties of *sigD*-regulated strains. (**a**) cell growth and glucose consumption, Square: RS1, circle: RSS2, upward triangle: RSS1, downward triangle: RSS3, diamond: RSS4, left triangle: RSS5, (**b**) 4-HIL and Ile concentration, (**c**) cell hydrophobicity at 8 h and 15 h, (**d**) cell permeability at 8 h and 15 h

increase of cell density of D-RS strain, cells will tend to aggregate and adhere to the wall of flasks or bioreactors. Upon comparison between D-RS and *sigD*-modified strains, the hydrophobicity of both RSS2 and RSS4 decreased by about 17% at the exponential phase, while that of the rest of the strains was similar with the control strain RS1. The permeability of RSS4 increased by 12% at the exponential phase, while that of the remaining strains especially RSS2 was lower than that of RS1. In addition, all the permeability of RS1 and RSS1–RSS4 at 15 h were higher than their permeability at 8 h, also with the permeability of RSS4 the highest. As at exponential phase, P_{cg1206}, P_{cg1633} and P_{cg2705} were all moderate promoters with higher strength than P_{sigD}, it can be assumed that the induction of *sigD* expression with moderate promoter (RSS1–RSS3) will lead to an increase in cell envelope synthesis and a decrease in cell permeability. Meanwhile, their hydrophobicity did not reduce and finally their 4-HIL production did not increase. The much higher cell hydrophobicity would cause cell adhesion and attachment and was not conducive to fermentation [27], while the increased cell permeability would facilitate the excretion of amino acids [7]. Therefore, RSS4 with decreased hydrophobicity and increased permeability was considered as a suitable strain for further detecting the 4-HIL titer in a fermenter.

Total lipids of RSS4 strain were extracted and compared to that of the control strain RS1 and a strain unable to synthesize MA, i.e., ATCC13869 Δ*pks* [23]. Two kinds of lipid component in *C. glutamicum* will be shown by chromatography: one is the phospholipids (below the dashed line in Fig. 4a), and the other is the substances with MA (above the dashed line in Fig. 4a). As shown in Fig. 4a, ATCC13869

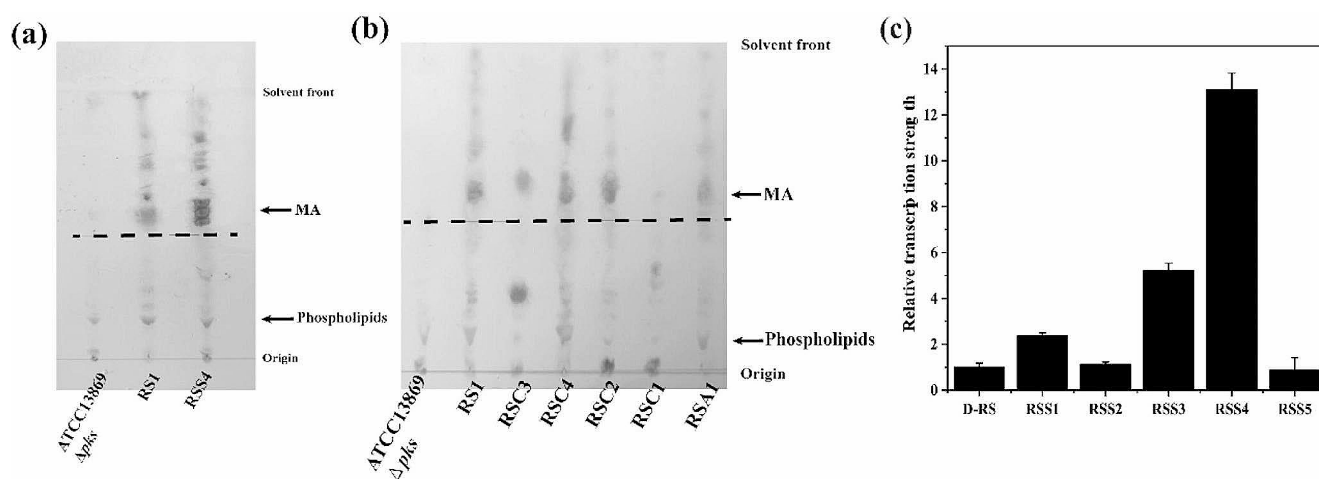

Fig. 4 TLC and RT-PCR analysis of strains. (**a**) TLC of *sigD*-regulated strains. (**b**) TLC of *sigD*-regulated strains. (**c**) TLC of *cop1*-regulated, *cmt2*-regulated and *cmt1*-regulated strains

Δ*pks* did not possess MA, while RS1 and RSS4 contained MA. The MA content of RSS4 increased and its phospholipid content was similar as compared to RS1. Moreover, the *sigD* transcription level of RSS1–RSS5 was basically in accordance with the strength of their modified promoters, as verified by RT-PCR (Fig. 4b). The *sigD* transcription of RSS4 with strong P_{odhI}-regulated *sigD* was the highest, about 12 times that of D-RS, while that of RSS5 with weak P_{cg3096}-regulated *sigD* was the lowest, a little lower than that of D-RS. RSS3 and RSS1 with moderate P_{cg2705} and P_{cg1633}-regulated *sigD* showed 5.2 and 2.4-fold level of D-RS. Therefore, the up-regulation of *sigD* expression during the exponential phase by P_{odhI} can effectively improve the content of MA in the cell envelopes, which might be helpful for increasing the titer of 4-HIL. With *sigD* gene overexpression at exponential phase, the hydrophobicity of RSS4 cell envelope decreased. The increase of MA content promoted the growth performance of strain and the production of amino acids (Fig. 3). In this study, enhancing the expression of genes related to MA synthesis was beneficial to cell growth, as indicated in previous report [28]. The increased permeability also contributed to the production of 4-HIL.

Regulation of *cmt2* and *cop1* expression

Cmt2 and Cop1, the two mycolyltransferases that are functionally close to each other, induces the conversion between TDCM and TMCM. Cop1 has been reported to transfer corynomycolate from TMCM to arabinogalactan layer of *C. glutamicum* [29]. The genes expressing these two enzymes are regulated by SigD factor during transcription (Fig. 2). Considering that the regulation of *sigD* expression may influent the transcription of all genes in SigD regulon, to exploit the effect of only a single crucial gene, the MA transferase genes *cop1* and *cmt2* were selected to be regulated. The transcriptome data showed that the expression intensity of *cmt2* and *cop1* in SN01 was about 90% lower than that in ATCC13032 (Table S1). Therefore, the growth-dependent promoter P_{cg1633} and the strong constitutive promoter P_{tuf} were used in this study to regulate the *cmt2* and *cop1* expression. However, as the promoter replacement process carried out, it was found that the *cop1* gene could not be regulated by the strong promoter P_{tuf}, and thus only three strains, RSC1, RSC2, and RSC3, were finally constructed (Table 1).

The results of 144 h fermentation showed that RSC1 with P_{cg1633}-regulated *cop1* was unable to grow properly and it was unable to utilize glucose for growth in exponential phase (Fig. 5a). The TLC image in Fig. 4c showed that the MA content of RSC1 was very low. This may be due to the non-normal expression of *cop1*, which affects the linkage between MA and AG layers. According to a previous report, a deficiency in AG layer biosynthesis may affect the MA layer stability and is unfavorable to the growth of *C. glutamicum* strain [14]. In addition, as shown by Fig. 5b, there was nearly no 4-HIL titer in RSC1. The growth of other two P_{cg1633}- or P_{tuf}-modified *cmt2* strains (RSC2 and RSC3) were not significantly affected and their 4-HIL titers (88.25–96.44 mM) were a little lower than that of RS1 (Fig. 5a, b). The cell hydrophobicity and cell permeability of RSC2 changed a little compared with RS1 in exponential phase (Fig. 5c, d). However, the cell permeability of RSC3 reduced by 14% in exponential phase, which might lead to the 33.70% decrease in its 4-HIL titer compared to RS1. In addition, the MA content of RSC2 and RSC3 was similar with that of RS1 (Fig. 4c), indicating that the regulation of *cmt2* has little effect on MA content. Mycoloyltransferases has many important functions for the physiological

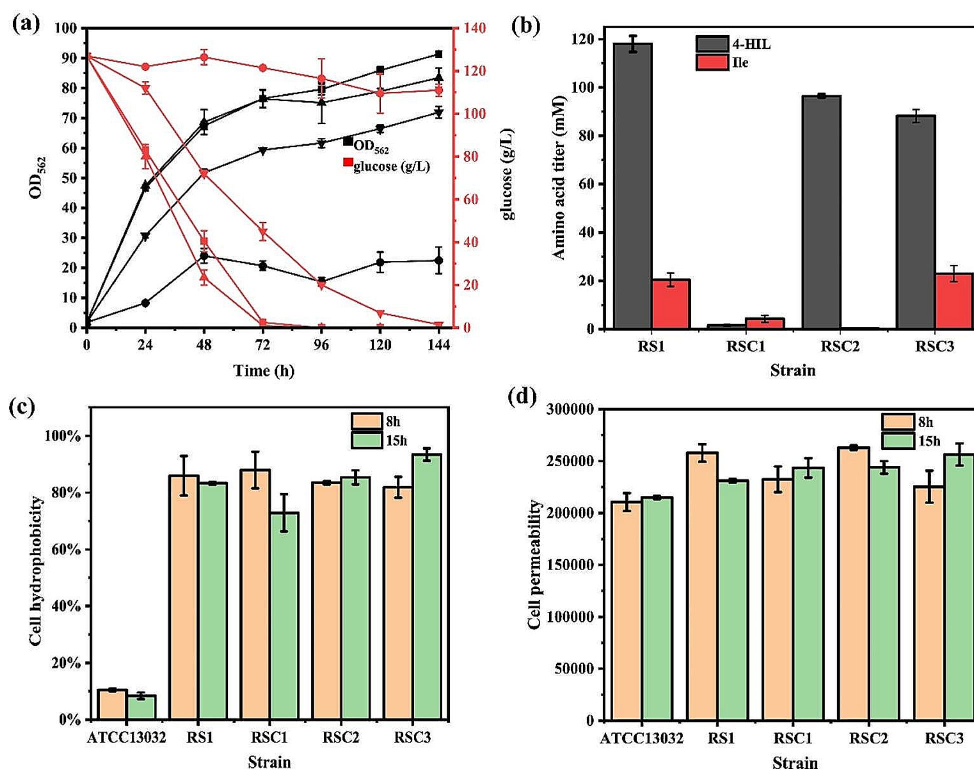

Fig. 5 Fermentation data and cell envelope properties of *cop1*- and *cmt2*-regulated strains. (**a**) cell growth and glucose consumption, Square: RS1, circle: RSC1, upward triangle: RSC2, downward triangle: RSC3, (**b**) 4-HIL and Ile concentration, (**c**) cell hydrophobicity at 8 h and 15 h, (**d**) cell permeability at 8 h and 15 h

process of *Corynebacteriales* [30], the integrity of the cell envelope of *C. glutamicum* is essential for its survival. In summary, the regulation of *cmt2* gene expression did not significantly affect the 4-HIL production, but the regulation of *cop1* expression seriously retarded cell growth and 4-HIL production.

Regulation of *cmt1* expression

Cmt1 also belongs to a type of MA transferase, but its function is distinct from that of Cmt2 and Cop1. Cmt1 is responsible for ligating the corynomycolate of TDCM and TMCM to porin [29, 31] (Fig. 2), and the promoter of the *cmt1* gene is likewise subjected to regulation by the SigD factor. Transcriptome data showed that the expression intensity of *cmt1* gene in SN01 was 84% lower than that in ATCC13032 (Table S1). Comparison of genomic sequences revealed that the sequence of the *cmt1* gene and its promoter in SN01 differed significantly from the sequence of the corresponding gene in ATCC13032, with little continuous identity (Table S3). A difference in sequence may make the function of the enzyme after expression very different. In addition, it can also be seen from Fig. 3c that the hydrophobicity of ATCC13032 is particularly low compared with D-RS strain. Therefore, during the experiment, the *cmt1* gene of D-RS was first regulated by growth-dependent promoter P_{cg1633}, generating the strain RSC4. On the other hand, the P_{cmt1}-*cmt1* gene sequence in D-RS strain was replaced by the corresponding sequence of ATCC13032, generating the strain RSA1.

As shown in Fig. 6a, b, by the results of 144 h shake flask fermentation it can be seen that the growth of the modified strains was not affected, the final OD_{562} value is between 80 and 90. The 4-HIL titer of modified strains (103.88–106.80 mM) did not increase and the titer was even not as high as that of RS1 (117.99 ± 0.34 mM). In terms of cell properties, the cell hydrophobicity of RSA1 was much higher than that of ATCC13032 and was 19% lower than that of RS1 in exponential phase, as shown in Fig. 6c, suggesting that direct substitution of *cmt1* sequences did not change cell hydrophobicity in exponential phase. The cell permeability of RSC4 and RSA1 increased slightly compared with RS1 (Fig. 6d), and did not promote 4-HIL production (Fig. 6b). TLC image showed a slight reduction in MA content in RSA1 and RSC4 (Fig. 4c). Thereby, regulation of *cmt1* expression does not significantly influence cell hydrophobicity and permeability of strain, and may not promote the production of 4-HIL.

4-HIL production in fed-batch fermentation

Considering that the 4-HIL production was relatively low in flasks, fed-batch fermentation of RSS4 shall be carried out to evaluate its potential in scale production. Therefore fed-batch fermentation of RSS4 was carried out in a 2 L fermenter.

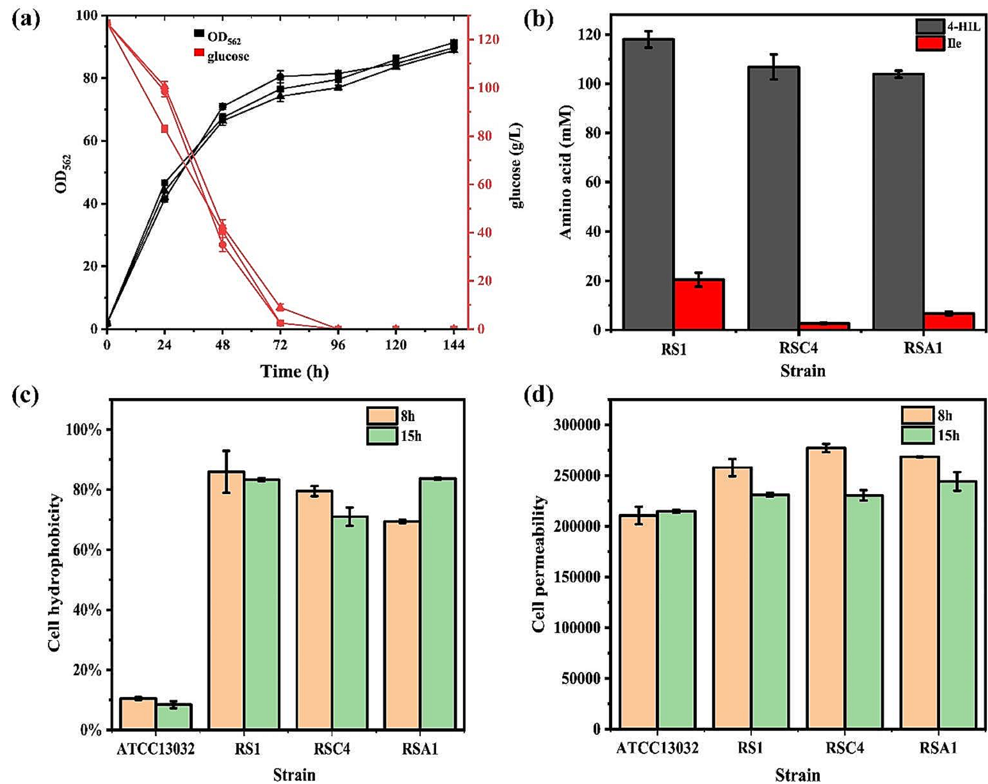

Fig. 6 Fermentation data and cell envelope properties of *cmt1*-regulated strains. (**a**) cell growth and glucose consumption, Square: RS1, circle: RSC4, upward triangle: RSA1, (**b**) 4-HIL and Ile concentration, (**c**) cell hydrophobicity at 8 h and 15 h, (**d**) cell permeability at 8 h and 15 h

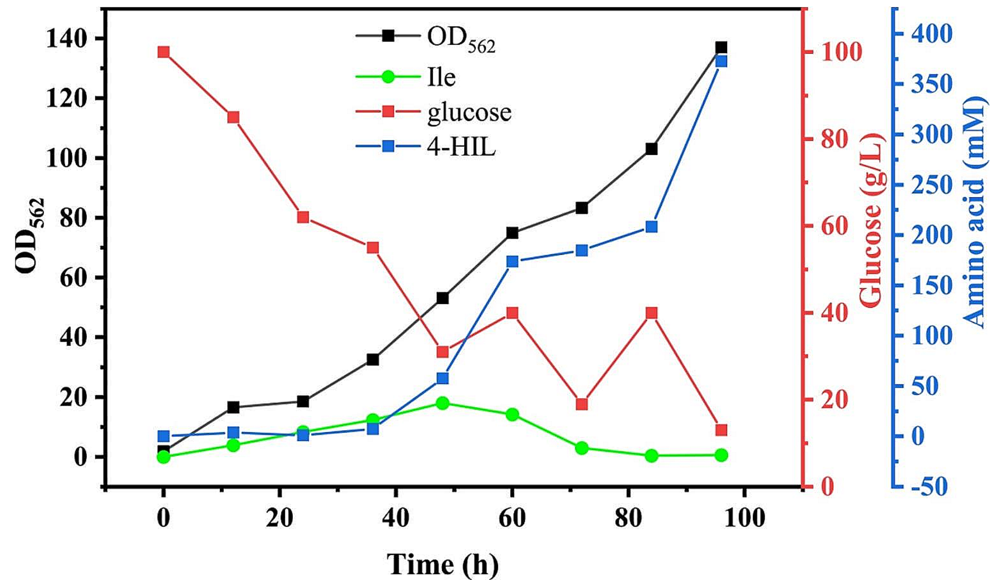

Fig. 7 Data on cell growth, glucose consumption and 4-HIL titer of RSS4 strain in batch fermentation

As shown in Fig. 7, at 72 h, the OD_{562} value was close to the value at the same period of shaking flask fermentation and the residual glucose concentration was close to 20 g/L. Then glucose was supplemented to the fermenter till about 40 g/L to continue the fermentation. During the 84–96 h, the strain consumed glucose fast, and the OD_{562} reached a maximum of 137, which was 1.41 times of the highest OD_{562} of the shake flask. On the one hand, the strain was not easy to stick to the wall of fermenter, likely due to the reduction of cell hydrophobicity [32], and on the other hand, the enhanced cell permeability also helps the strain to use nutrients and excrete products [33]. Therefore, the RSS4 strain developed in this study has certain practical significance for the production of 4-HIL. The modified strain RSS4 achieved a 4-HIL titer of 372.56 mM at the bioreactor level, which is the highest 4-HIL titer reported so far, at the bioreactor fermentation level. Due to the quite slow cell growth before 36 h, 4-HIL was not produced at this stage,

while during 36–60 h and 84–96 h, 4-HIL accumulated quickly, in accordance with the quick cell growth during these periods. Finally, 372.56 mM 4-HILwas accumulated. In addition, only little by-products (15.31 mM Lys and 8.97 mM L-alanine) were accumulated at the end of fermentation. Compared with the 4-HIL titer of *C. glutamicum* strain HIL18 (34.2 g/L, i.e. 232.65 mM) modified by eight-step metabolic engineering in bioreactor previously reported [34], the 4-HIL titer of *C. glutamicum* RSS4 with only P_{odhI}-*sigD* modification in bioreactor here increased by 60.13%. Through the mere regulation of *sigD* gene expression, the cell envelope properties of the strain were changed, which played a good role in supporting and promoting the production of 4-HIL. The modification of other genes involving in cell envelope synthesis such as *accD2* or *fadD2*, *cmrA* and *aftB* and their potentials for enhancing 4-HIL production and stability of *C. glutamicum* strains shall be investigated in the future. To further improve 4-HIL production in RSS4, dynamic engineering of the Ile synthesis and 4-HIL conversion pathways shall be researched. Moreover, integration of multiple-copy *ido* genes in *C. glutamicum* chromosome will be exploited in the future to produce 4-HIL economically.

Conclusion

This study focused on the rearrangements in regulating *sigD* expression by GRP and thereby promoted the 4-HIL production from the Ile-producing strain D-RS. Finally, an improved strain RSS4 with P_{odhI}-regulated *sigD* was obtained. Not only its cell permeability increased by 12%, but also its cell hydrophobicity reduced by 17%. This change of cell envelope properties was favorable to its fermentation in the shake flask and bioreactor, and finally the 4-HIL titer reached the highest titer of 372.56 mM so far under the bioreactor condition, and there were only little by-products. Thereby, it is feasible to improve metabolite production by changing the properties of cell envelope.

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Author contributions FS conceived and designed research. CH, RC and YX conducted experiments. CH and FS analyzed data. FS, CH, RC and YX wrote the manuscript.

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Data availability All data associated with this work is embedded within the manuscript.

Code Availability Not applicable.

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

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

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