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GENETICS AND MOLECULAR BIOLOGY

Contribution of YthA, a PspC Family Transcriptional Regulator of *Lactococcus lactis* F44 Acid Tolerance and Nisin Yield: a Transcriptomic Approach

Hao Wu,[a,b] Jingui Liu,[a,b] Sen Miao,[a,b] Yue Zhao,[a,b] Hongji Zhu,[a] Mingqiang Qiao,[d] Per Erik Joakim Saris,[e] Jianjun Qiao[a,b,c]

[a]Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin, China

[b]Key Laboratory of Systems Bioengineering, Ministry of Education, Tianjin, China

[c]SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering, Tianjin, China

[d]College of Life Sciences, Nankai University, Tianjin, China

[e]Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland

ABSTRACT To overcome the adverse impacts of environmental stresses during growth, different adaptive regulation mechanisms can be activated in *Lactococcus lactis*. In this study, the transcription levels of eight transcriptional regulators of *L. lactis* subsp. *lactis* F44 under acid stress were analyzed using quantitative reverse transcription-PCR. Eight gene-overexpressing strains were then constructed to examine their influences on acid-resistant capability. Overexpressing *ythA*, a PspC family transcriptional regulator, increased the survival rate by 3.2-fold compared to the control at the lethal pH 3.0 acid shock. Moreover, the nisin yield was increased by 45.50%. The *ythA*-overexpressing strain FythA appeared to have higher intracellular pH stability and nisin-resistant ability. Subsequently, transcriptome analysis revealed that the vast majority of genes associated with amino acid biosynthesis, including arginine, serine, phenylalanine, and tyrosine, were predominantly upregulated in FythA. Arginine biosynthesis (*argG* and *argH*), arginine deiminase pathway, and polar amino acid transport (*ysfE* and *ysfF*) were proposed to be the main regulation mechanisms of YthA. Furthermore, the transcription of genes associated with pyrimidine and exopolysaccharide biosynthesis were upregulated. The transcriptional levels of *nisIPRKFEG* genes were substantially higher in FythA, which directly contributed to the yield and resistance of nisin. Three potential DNA-binding sequences were predicted by computer analysis using the upstream regions of genes with prominent changes. This study showed that YthA could increase acid resistance and nisin yield and revealed a putative regulation mechanism of YthA.

IMPORTANCE Nisin, produced by *Lactococcus lactis* subsp. *lactis*, is widely used as a safe food preservative. Acid stress becomes the primary restrictive factor of cell growth and nisin yield. In this research, we found that the transcriptional regulator YthA was conducive to enhancing the acid resistance of *L. lactis* F44. Overexpressing *ythA* could significantly improve the survival rate and nisin yield. The stability of intracellular pH and nisin resistance were also increased. Transcriptome analysis showed that nisin immunity and the biosynthesis of some amino acids, pyrimidine, and exopolysaccharides were enhanced in the engineered strain. This study elucidates the regulation mechanism of YthA and provides a novel strategy for constructing robust industrial *L. lactis* strains.

KEYWORDS *Lactococcus lactis*, transcriptional regulator, YthA, acid stress, nisin yield

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Address correspondence to Jianjun Qiao, jianjunq@tju.edu.cn.

H.W. and J.L. contributed equally to this article.

Lactococcus lactis, a member of lactic acid bacteria (LAB), is extensively employed in the food industry and usually used as a primary building block for applications in biomedicine and other fields (1–3). Nisin production has been optimized, distributed, and used widely in food and biomedical industries for the last 50 years (4, 5). During fermentation, *L. lactis* is confronted with many types of stress, especially acid stress because of the conversion of pyruvate to lactate (6), which probably leads to the suboptimal growth and the decrease in nisin yield. Otherwise, pH values decrease, and the corresponding increase in hydrogen ion concentrations in the cytoplasm might contribute to the stability of nisin (7). Thus, studying acid resistance mechanism and constructing acid-tolerant industrial strains are essential. *L. lactis* has developed a multifactorial response to cope with acid stress, including the ATP-dependent expulsion of protons, the production of basic compounds, the repair of impaired macromolecules, and protection of the cell envelope from various types of damage (8). Moreover, bacteria have established a complicated regulatory network via integrating and coordinating the expression levels of different genes.

A series of transcriptional regulators has been found to contribute to acid resistance. Transcriptional activator GadE controls the most important acid resistance system, the glutamate-dependent (Gad) system, by directly activating the expression of *gadA* and *gadBC* in *Escherichia coli* (9). As a major partner of GadE, regulator RcsB can also directly activate *gadA* transcription via the glutamate decarboxylase (GAD) box (10). In *Streptococcus mutans*, two Spx global regulators, *spxA* and *spxB*, are associated with stress tolerance, and an Δ*spxA* strain shows decreased survival rate under acid stress (11). The transcriptional regulator Ldb0677 has been identified as an acid stress-related regulator by proteomics approach, and its targets have been revealed via bacterial one-hybrid technology in *Lactobacillus delbrueckii* subsp. *bulgaricus* CAUH1 (12). In *Lactobacillus acidophilus*, inactivation of the transcriptional regulator La867 has a remarkable influence on acid tolerance (13).

In *L. lactis*, a range of transcriptional regulation mechanisms have been preliminarily studied. CodY and CcpA are two widely known global regulators in *L. lactis*. CodY controls various cellular functions, including nutrient transport and nitrogen metabolism, especially branched-chain amino acid metabolism (14, 15). CodY also fulfills a prominent role in carbon starvation and near-zero growth (16, 17). Moreover, the catabolite control protein CcpA can repress putrescine synthesis and the utilization of galactose and fructose (18, 19). CcpA also activates the central metabolism and the expression of prolidase PepQ, constituting a link between carbon and nitrogen metabolism regulations. In addition, a significant number of operon-specific transcriptional regulators have been observed. For instance, ClaR can regulate cellobiose and lactose metabolism, and MalR is an activator of the maltose transport system (20, 21). The transcriptional regulator FabT affects *fab* gene expression in fatty acid biosynthesis (22). The transcriptional regulator GadR can control the GAD pathway by activating the transcription of *gadB* and *gadC* (23). CtsR shows a negative regulation of the *clp* genes *clpC*, *clpP*, *clpE*, and *clpB* (24).

The survival of bacteria depends on their ability to sense and respond to cell envelope disturbances. The phage shock protein (Psp) system plays a pivotal role in envelope stress response (25). In *E. coli*, PspC family members participate in the stress response system to maintain the integrity and function of bacterial cell envelope (26). When the cell envelope is damaged, the Psp response cannot be established, and the proton motive force is dissipated (27). A physical and/or biochemical change gives rise to the cell stress response and results in increased expression levels of *psp* genes. PspA, PspB, PspC, and PspF form a signal transduction system in the regulation of *psp* gene expression. PspB and PspC, two cytoplasmic membrane proteins, can form an integral complex to act as sensory activators of the Psp response in both *Yersinia enterocolitica* and *E. coli* (28). The three-gene *ythC-ythB-ythA* operon exists in *L. lactis*, and YthA and YthB contain the conserved region of PspC family transcriptional regulators. YthC is a hypothetical protein and has not been explored. Nevertheless, no other gene related to

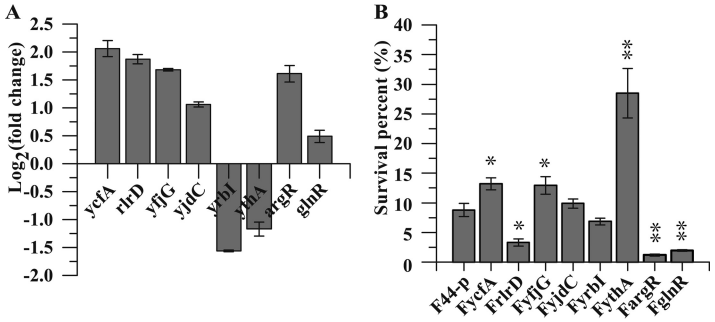

FIG 1 (A) Relative transcription levels of eight regulators under acid shock analyzed by qRT-PCR. The fold changes of qRT-PCR were normalized using 16S rRNA as an internal control gene. The error bars, indicating standard deviations (SD), are from three replicate flasks. (B) Acid tolerance of the strains overexpressing transcriptional regulators. The survival percentage was the ratio of the CFU per milliliter counted after the acid challenge to the CFU/ml at T_0 (start of the challenge). Cells were cultured in fermentation medium (pH 7.2) and challenged in fermentation medium (pH 3.0). Error bars indicate the SD of three independent experiments. *, $P < 0.01$; **, $P < 0.001$ (*t* test).

Psp response system in *L. lactis* has been observed, and the regulation mechanism of YthA remains unclear.

In this study, which is based on a transcription level analysis of *L. lactis* F44 under acid stress using quantitative reverse transcription-PCR (qRT-PCR), eight transcriptional regulators with pronounced expression changes were overexpressed to determine which can increase the acid resistance. Overexpressing *ythA* could increase the survival rate and nisin yield. The stability of intracellular pH and nisin resistance were also confirmed to be improved in FythA. To further investigate the regulation mechanism of YthA, transcriptome sequencing (RNA-seq) was performed. The vast majority of genes in the metabolisms of some amino acids, pyrimidine, exopolysaccharides (EPS), nisin biosynthesis, and nisin immunity were remarkably upregulated. By combining transcriptome data with *in silico* motif predictions, three potential DNA binding motifs were identified in the promoter regions of genes with prominent changes. Thus, we show that YthA contributes to acid resistance and nisin yield and provides a putative acid tolerance mechanism for YthA.

RESULTS

Determination of transcriptional regulators contributing to acid resistance in *L. lactis* F44. Based on transcriptional level analysis of eight transcriptional regulators in *L. lactis* F44 after a pH 4.0 acid shock for 1 h using qRT-PCR (Fig. 1A), these genes were overexpressed in *L. lactis* F44 (Tables 1 and 2). Subsequently, to clarify the effects on the acid resistance of these engineered strains, an acid resistance assay was performed, and strain F44-p harboring an empty vector pLEB124 was used as a control. These analyses showed different survival rates after lethal pH 3.0 acid shock for 2.5 h (Fig. 1B). Notably, *ythA* overexpression enhanced the acid-resistant ability, and the engineered strain FythA showed a 3.2-fold increase of survival rate compared to the control under acid shock. Moreover, the survival rates of FyjdC and FyrbI were slightly higher than that of the control, and FyjdC and FyrbI showed no significant changes. However, the survival rates of FrlrD, FargR, and FglnR were all remarkably lower than that of F44-p. Due to the pronounced improvement in acid resistance, we focused on further investigating the physiological characteristics of FythA during fermentation.

Effects of YthA on physiological characters and nisin yield of *L. lactis* F44. Cell density, pH value, and nisin yield were monitored every 2 h to evaluate the effects of YthA during fermentation. In the fermentation with an initial pH of 7.2, FythA presented a lower growth rate compared to the control after 6 h of incubation (Fig. 2A). Subsequently, the biomass accumulation sharply increased from 6 to 8 h, and the specific growth rate exceeded that of F44-p after 6 h (Fig. 2B). The biomass of FythA peaked (optical density [OD] = 1.76 ± 0.01) at 10 h, and it was 4.65% higher than that

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
L. lactis F44	Parental strain, derived from *L. lactis* YF11 (China General Microbiological Culture Collection Center accession no. CGMCC7.52)	Laboratory stock (64)
L. lactis F44-p	*L. lactis* F44 carrying empty pLEB124; Em^r	This study
L. lactis FycfA	*ycfA*-overexpressing strain; Em^r	This study
L. lactis FrlrD	*rlrD*-overexpressing strain; Em^r	This study
L. lactis FyfjG	*yfjG*-overexpressing strain; Em^r	This study
L. lactis FyjdC	*yjdC*-overexpressing strain; Em^r	This study
L. lactis FyrbI	*yrbI*-overexpressing strain; Em^r	This study
L. lactis FythA	*ythA*-overexpressing strain; Em^r	This study
L. lactis FargR	*argR*-overexpressing strain; Em^r	This study
L. lactis FglnR	*glnR*-overexpressing strain; Em^r	This study
E. coli TG1	Plasmid preparation	Laboratory stock
M. flavus ATCC 10240	Indicator (the Gram-positive pathogen sensitive to nisin)	Laboratory stock
Plasmids		
pLEB124	Expression vector; Em^r	Laboratory stock
pLEBycfA	Derivative of vector pLEB124 carrying *ycfA* from *L. lactis* F44; Em^r	This study
pLEBrlrD	Derivative of vector pLEB124 carrying *rlrD* from *L. lactis* F44; Em^r	This study
pLEByfjG	Derivative of vector pLEB124 carrying *yfjG* from *L. lactis* F44; Em^r	This study
pLEByjdC	Derivative of vector pLEB124 carrying *yjdC* from *L. lactis* F44; Em^r	This study
pLEByrbI	Derivative of vector pLEB124 carrying *yrbI* from *L. lactis* F44; Em^r	This study
pLEBythA	Derivative of vector pLEB124 carrying *ythA* from *L. lactis* F44; Em^r	This study
pLEBargR	Derivative of vector pLEB124 carrying *argR* from *L. lactis* F44; Em^r	This study
pLEBglnR	Derivative of vector pLEB124 carrying *glnR* from *L. lactis* F44; Em^r	This study

[a]Em^r, erythromycin resistance.

of the control (OD = 1.68 ± 0.01) at 8 h. The pH value of culture declined due to the production and accumulation of lactic acid. Intriguingly, the pH values of FythA fermentation broth were higher than that of the control during the entire fermentation. The final pH reached 4.70 ± 0.23 at 14 h, whereas the control was pH 4.49 ± 0.04 (Fig. 2A).

To further elucidate the effects of YthA on acid resistance, the cell density and the pH value were measured in an acidic medium with an initial pH 6.0. As expected, the engineered strain exhibited a 12.6% higher total biomass than the control, and pH values decreased slower than that in the control throughout the fermentation (Fig. 2C). In contrast to the fermentation with an initial pH of 7.2, the specific growth rate of FythA increased from 0.29 to 0.51 h^{-1} (from 2 to 4 h) and was higher than that of the control (0.37 h^{-1}) after 4 h (Fig. 2D). The doubling time of FythA (9.13 h) was lower than that of the control (17.28 h) after 8 h. The difference in physiological characteristics between FythA and F44-p was more significant than that for the initial pH 7.2 ($P < 0.01$, after 10 h). These results further confirmed that YthA was conducive to resisting acid stress. We presumed that YthA might promote the production of certain alkaline substances, such as alkaline amino acids and NH_3.

TABLE 2 Candidates of transcriptional regulators

Gene	Identification no.	Gene length (bp)	Accession no.	Product
ycfA	F44_g0218	648	ATY86861.1	TetR family transcriptional regulator
rlrD	F44_g0345	822	ATY86977.1	LysR family transcriptional regulator
yfjG	F44_g0506	627	ATY87125.1	TetR family transcriptional regulator
yjdC	F44_g0905	465	ATY87505.1	MarR family transcriptional regulator
yrbI	F44_g1581	834	ATY88165.1	XRE family transcriptional regulator
ythA	F44_g1877	465	ATY88441.1	Stress-responsive transcriptional regulator, PspC family
argR	F44_g2007	459	ATY88561.1	Arginine operon repressor
glnR	F44_g2177	369	ATY88875.1	MerR family transcriptional regulator

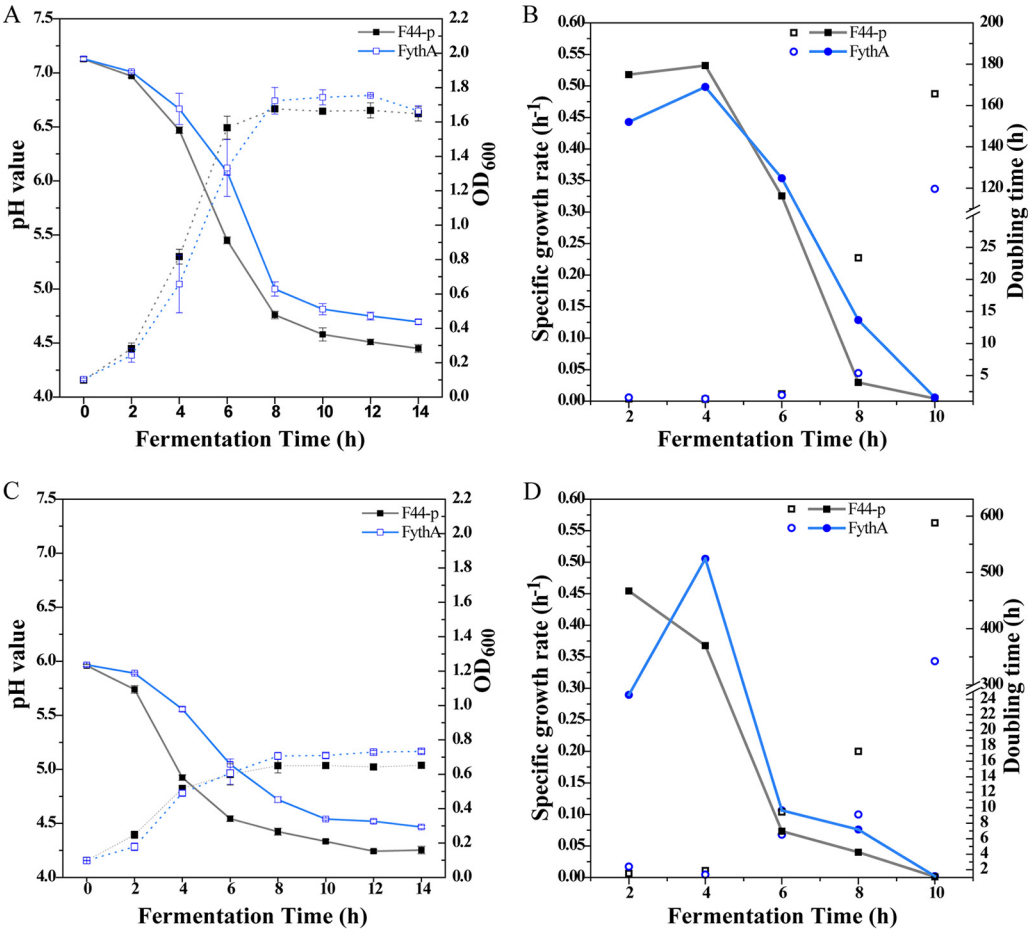

FIG 2 Physiological characteristics of a *ythA*-overexpressing strain. (A) Cell density (dashed lines) and pH value (solid lines) during fermentation at the initial pH 7.2. OD_{600} indicates the optical density measured at 600 nm. (B) Specific growth rate and doubling time during fermentation at the initial pH 7.2. (C) Cell density (dashed lines) and pH value (solid lines) during fermentation at the initial pH 6.0. OD_{600} indicates the optical density measured at 600 nm. (D) Specific growth rate and doubling time during fermentation at the initial pH 6.0.

The nisin yield was determined every 2 h during fermentation with an initial pH of 7.2. Notably, the nisin titer of FythA peaked at 3,380.20 ± 135.40 IU/ml, which was 45.50% higher than that of the control at 8 h, and decreased thereafter (Fig. 3A). This demonstrated that the nisin yield could be improved by increasing the acid resistance, a finding consistent with our previous study (7).

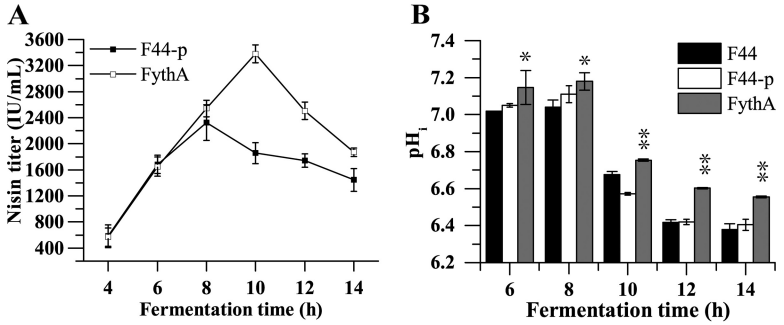

FIG 3 Nisin yield and pH_i of *ythA*-overexpressing strain. (A) Effects of transcriptional regulator YthA on the nisin titer during fermentation at an initial pH of 7.2. Samples were taken every 2 h, ranging from 4 to 14 h. (B) pH_i of FythA, F44, and F44-p during fermentation. Samples were taken every 2 h for 6 to 14 h. Error bars indicate the SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (*t* test).

YthA enhances the intracellular pH stability. Intracellular pH (pH_i) homeostasis plays a major role in maintaining physiological stability under acid stress in LAB. To further investigate the influence of regulator YthA on the acid resistance, the pH_i values of the FythA strain and wild-type strain F44 were measured during fermentation. As shown in Fig. 3B, the pH_i values of both strains decreased, along with the fermentation, and obvious differences of pH_i were detected between FythA and F44. The engineered strain FythA exhibited a higher pH_i than that in F44 during fermentation, especially at the stationary phase. In other words, FythA maintained a relatively stable pH_i during the process. These results indicated that the transcriptional regulator YthA could contribute to maintaining the stability of pH_i in *L. lactis* and guaranteed the growth of strains, which might be beneficial for acid tolerance and nisin yield.

Transcriptome analysis of the YthA-overexpressing strain. To investigate the possible regulation mechanism mediated by YthA, a transcriptomic approach was used with an Illumina HiSeq 4000. FythA and F44 grown in fermentation medium were collected at the exponential phase ($OD_{600} \approx 0.8$). For transcriptomic analysis, at a cutoff of 1.5-fold change (log_2-fold change) and a *P* value of <0.05, 304 and 45 genes, respectively, were upregulated and downregulated compared to the *L. lactis* F44 with empty plasmid. A total of 16 genes were selected to be validated by qRT-PCR. Among these genes, 10 were significantly upregulated, 5 were downregulated, and 1 showed no obvious change. Apparent positive correlations were verified between the transcriptomic results and qRT-PCR for these genes (Fig. 4A). The functional distribution of significantly changed proteins, according to the Cluster of Orthologous Groups (COG) classification for proteins, is shown in Fig. 4B. A number of genes in class E (amino acid transport and metabolism) were upregulated, which suggested that YthA mainly regulated transport and metabolism of amino acids to improve acid resistance.

YthA improves the metabolism and transport of amino acids. We found here that the metabolism of some amino acids, especially arginine, serine, phenylalanine, and tyrosine, was notably enhanced in FythA (Fig. 5 and Table 3). Most genes in the glutamate and arginine biosynthesis pathway, including *gltB*, *argB*, *argC*, *argD*, *argE*, *argF*, *argG*, and *argH*, were consistently upregulated. Among them, the argininosuccinate synthase gene *argG* and the argininosuccinate lyase gene *argH*, respectively, showed dramatic 8.63- and 7.67-fold upregulations. The arginine deiminase (ADI) pathway is a known pivotal mechanism that can withstand acid stress in LAB. Genes associated with the ADI pathway—*arcA* (ADI), *arcB* (ornithine carbamoyltransferase), *arcC2* (carbamate kinase), *arcC3* (carbamate kinase), and *arcD1* (arginine/ornithine antiporter)—were all abundant. Three genes, namely, *proA*, *proB*, and *proC*, that are involved in proline biosynthesis were expressed at elevated levels. The expression of *aspB* gene, which encodes aspartate aminotransferase to generate aspartate from oxaloacetate in the aspartate biosynthesis pathway, was significantly increased by ~1.87-fold.

In addition to the biosynthesis of arginine, the pathways of the other two alkaline amino acids, histidine and lysine, were also improved. Four genes that refer to histidine biosynthesis (*hisZ*, *hisG*, *hisC*, and *hisD*) were also highly upregulated. The genes *hisZ* and *hisG*, which encode ATP-phosphoribosyltransferase, increased 1.86- and 1.97-fold, respectively. These two enzymes catalyze the condensation of ATP with phosphoribosyl pyrophosphate, the first step of histidine biosynthesis (29). Moreover, several lysine biosynthesis genes, including *ychH*, *yciA*, and *lysA*, were upregulated. The expression of the diaminopimelate decarboxylase gene *lysA*, which catalyzes the final step in the lysine biosynthesis pathway of bacteria, was increased by 2.31-fold.

The genes encoding the enzymes responsible for serine biosynthesis (*serA*, *serC*, and *serB*) were upregulated, suggesting that YthA positively regulated the serine biosynthesis. The homoserine kinase gene *thrB* that converts homoserine to *O*-phosphohomoserine in threonine biosynthesis yielded a 2.09-fold increase in abundance. The serine acetyltransferase gene *cysE*, which catalyzes the conversion of serine to *O*-acetyl-

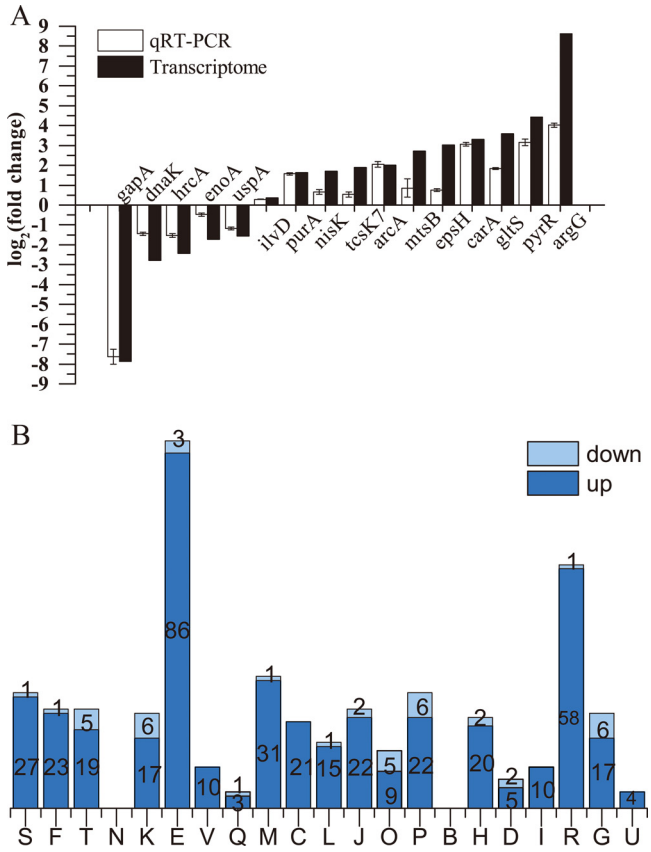

FIG 4 (A) The relative mRNA level changes of 16 genes in FythA were compared between transcriptome data and qRT-PCR results. The fold changes of qRT-PCR were normalized using 16S rRNA as an internal control gene. Error bars indicating the SD were from three replicate flasks. (B) Distribution of significantly changed genes according to the COG classification. The *y* axis indicates the number of genes in various COG categories. Columns: S, function unknown; F, nucleotide transport and metabolism; T, signal transduction mechanisms; N, cell motility; K, transcription; E, amino acid transport and metabolism; V, defense mechanisms; Q, secondary metabolites biosynthesis, transport, and catabolism; M, cell wall/membrane/envelope biogenesis; C, energy production and conversion; L, replication, recombination, and repair; J, translation, ribosomal structure, and biogenesis; O, posttranslational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; B, chromatin structure and dynamics; H, coenzyme transport and metabolism; D, cell cycle control, mitosis, and meiosis; I, lipid transport and metabolism; R, general function prediction only; G, carbohydrate transport and metabolism; U, intracellular trafficking and secretion.

L-serine, was upregulated 1.99-fold. Notably, genes associated with methionine biosynthesis (*metA* and *metE*) were also upregulated (1.87- and 2.19-fold, respectively).

Moreover, the shikimate pathway (including *aroA*, *aroB*, *aroC*, *aroD*, *aroE*, *aroF*, *aroK*, and *aroH*), which is the early step in the biosynthesis of aromatic amino acids, including phenylalanine, tyrosine, and tryptophan, was integrally upregulated. Among these genes, *aroA*, *aroK*, and *aroC*, which encode enzymes that catalyze shikimate to chorismite, were upregulated. The production of pivotal enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) is not only the first step in the synthesis of aromatic compounds, but DAHPS is also the rate-limiting enzyme. The introduction of *E. coli aroH* encoding DAHPS resulted in a significant increase in phenylalanine in *Corynebacterium glutamicum* (30). In the present study, the transcription of *aroH* was dramatically upregulated by 3.16-fold.

Several genes implicated in the polar amino acid transport system (*ysfEF*, *gltS*, *yvdF*, and *yjgC*), the glutamate transport system (*glnPQ*), and the oligopeptide transport system (*optS* and *optA*) were also upregulated. *ysfE*, *ysfF*, and *gltS* expression levels were increased. Overall, the results demonstrate that transcriptional regulator YthA positively mediates the biosynthesis and transport systems of multiple amino acids.

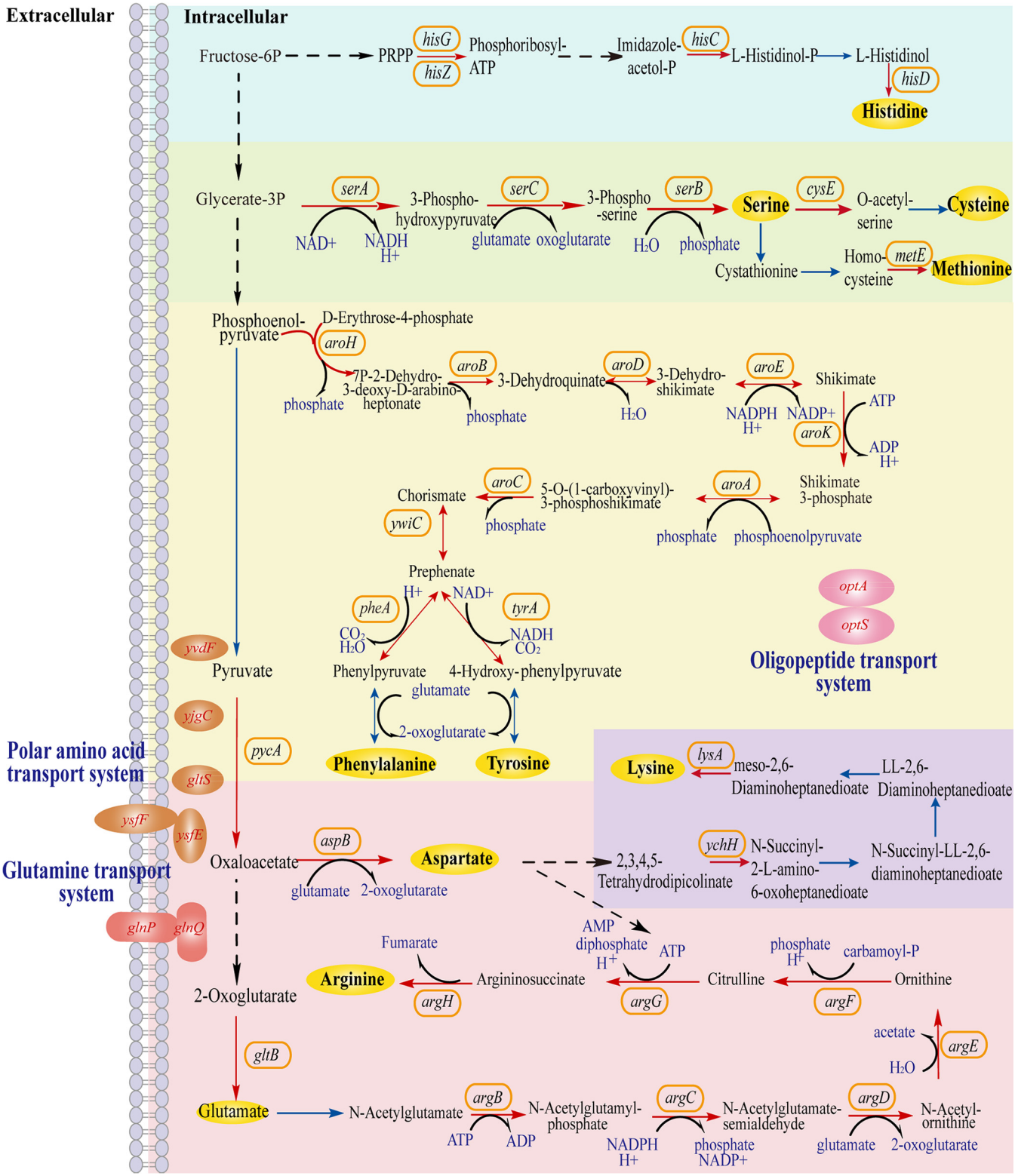

FIG 5 Effects of transcriptional regulator YthA on the metabolism of some amino acids. The upregulated genes are represented by red fonts or solid arrows. Genes with no significant change are represented by blue solid arrows.

Effects of YthA on EPS synthesis and cell division. Exopolysaccharides (EPS) can protect bacterial cells against extreme stresses, including pH, temperature, and osmotic stress. In this study, transcriptional analysis revealed that most EPS synthesis genes (*epsABCDEFGHIJK*) were induced in FythA (Table 4). The *epsA*, *epsB*,

TABLE 3 Significantly changed genes involved in the transport and metabolism of amino acids

Pathway	Gene	Log_2 ratio[a]	Description
Arginine biosynthesis	*argG*	8.62	Argininosuccinate synthase
	argH	7.67	Argininosuccinate lyase
	argF	2.56	Ornithine carbamoyltransferase
	argE	3.45	Acetylornithine deacetylase
	argD	1.65	Acetylornithine aminotransferase
	argC	1.69	*N*-Acetyl-gammaglutamyl-phosphate reductase
	argB	2.65	Acetylglutamate kinase
Arginine deiminase (ADI) pathway	*arcD2*	1.86	Arginine/ornithine antiporter
	arcD1	2.29	Arginine/ornithine antiporter
	arcC3	2.70	Carbamate kinase
	arcC2	2.27	Carbamate kinase
	arcB	2.24	Ornithine carbamoyltransferase
	arcA	2.00	Arginine deiminase
Aromatic amino acids biosynthesis	*aroK*	2.52	Shikimate kinase
	aroH	3.16	Phospho-2-dehydro-3-deoxyheptonate aldolase
	aroF	2.21	3-Deoxy-7-phosphoheptulonate synthase
	aroE	2.07	Shikimate 5-dehydrogenase
	aroD	2.37	3-Dehydroquinate dehydratase
	aroC	1.97	Chorismate synthase
	aroB	2.39	3-Dehydroquinate synthase
	aroA	1.99	3-Phosphoshikimate 1-carboxyvinyltransferase
Proline biosynthesis	*proA*	2.24	Gamma-glutamyl phosphate reductase
	proB	1.51	Glutamate 5-kinase
	proC	1.91	Pyrroline-5-carboxylate reductase
Serine biosynthesis	*serC*	2.74	MFS transporter
	serB	2.21	Phosphoserine phosphatase
	serA	2.14	3-Phosphoglycerate dehydrogenase
Lysine biosynthesis	*ychH*	1.86	2,3,4,5-Tetrahydropyridine-2,6-carboxylate *N*-succinyltransferase
	yciA	2.01	*N*-Acetyldiaminopimelate deacetylase
	lysA	2.31	Diaminopimelate decarboxylase
Histidine biosynthesis	*hisC*	1.67	Histidinol-phosphate aminotransferase
	hisD	1.58	Histidinol dehydrogenase
	hisG	1.86	ATP phosphoribosyltransferase
	hisZ	1.97	ATP phosphoribosyltransferase, regulatory subunit
Glutamate transport system	*glnQ*	1.61	Glutamine ABC transporter ATP-binding protein
	glnP	1.70	Amino acid ABC transporter permease
Methionine biosynthesis	*metE*	2.19	Methyltetrahydropteroyltriglutamate/homocysteine *S*-methyltransferase
	metA	1.87	Homoserine *O*-succinyltransferase
Polar amino acid transport system	*ysfE*	6.32	Glutamate ABC transporter ATP-binding protein
	ysfF	4.45	Glutamate ABC transporter permeas
	gltS	3.58	Glutamate or arginine ABC transporter substrate binding protein
	yjgC	1.53	Amino acid ABC transporter substrate binding protein
	yvdF	1.60	ABC transporter substrate-binding protein
Oligopeptide transport system	*optS*	1.97	ABC transporter substrate-binding protein
	optA	1.55	ABC transporter substrate-binding protein
Others	*tyrA*	1.94	Prephenate dehydrogenase
	asnB	2.15	Asparagine synthase
	pheA	2.34	Prephenate dehydratase
	thrB	2.09	Homoserine kinase
	aspB	1.87	Aspartate aminotransferase
	yddD	1.57	Glyoxalase
	araT	1.51	Aminotransferase A
	murA	1.51	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase
	pepN	1.50	Aminopeptidase N
	axe	−1.80	Acetyl esterase
	lysP1	1.99	Lysine specific permease
	cysE	1.99	Serine acetyltransferase
	gltB	1.79	Glutamate synthase

[a]Values are expressed as the fold change in a log_2 scale.

TABLE 4 Significantly changed genes involved in exopolysaccharide synthesis, cell division, nisin biosynthesis and resistance, and nucleotide metabolism

Pathway	Gene	Log_2 ratio[a]	Description
Exopolysaccharide synthesis	*epsH*	3.01	Group 1 glycosyltransferase
	epsI	2.81	Hypothetical protein
	epsJ	2.30	Polysaccharide biosynthesis protein
	epsG	2.86	Family 2 glycosyltransferase
	epsF	2.51	Hypothetical protein
	epsE	2.29	Group 1 glycosyltransferase
	epsK	1.57	Polysaccharide biosynthesis protein
	epsD	1.87	Sugar transferase
	epsC	1.93	Tyrosine protein phosphatase
	epsB	1.74	Tyrosine protein kinase
	epsA	1.87	Polysaccharide biosynthesis protein
Cell division	*ftsX*	1.77	Cell division permease FtsX
	ftsQ	1.55	Cell division initiation protein DivIB
	ftsE	1.56	Cell division ATP-binding protein
	smc	2.16	Chromosome segregation protein SMC
Nisin synthesis and immunity	*nisI*	1.46	Nisin immunity protein
	nisP	1.46	Peptidase
	nisK	1.69	Nisin biosynthesis sensor protein NisK
	nisR	1.50	Nisin biosynthesis two-component system, response regulator NisR
	nisF	1.29	Lantibiotic ABC transporter ATP-binding protein
	nisE	1.76	Lantibiotic ABC transporter permease
	nisG	2.10	Transporter
Pyrimidine metabolism	*carA*	3.29	Carbamoyl-phosphate synthase small subunit
	pyrB	1.50	Uridine phosphorylase
	pyrC	2.99	Uracil permease
	pyrZ	2.89	Dihydroorotate dehydrogenase electron transfer subunit
	pyrD	3.29	Aspartate carbamoyltransferase catalytic subunit
	pyre	3.22	Dihydroorotate dehydrogenase
	pyrF	2.69	ADP-ribose pyrophosphatase
	pyrR	4.41	Phosphoribosyl transferase
	pyrP	3.19	5′-Methylthioadenosine/*S*-adenosylhomocysteine nucleosidase
	upp	2.51	Orotidine 5′-phosphate decarboxylase
	udp	1.62	Adenylosuccinate synthetase

[a]Values are expressed as the fold change in a log_2 scale.

epsC, *epsD*, and *epsK* genes were all upregulated (>1.5-fold and <2.0-fold). The transcriptional levels of *epsEFGHIJ* increased by more than 2.0-fold. The polysaccharide chain-length-determining genes *epsF* and *epsG* showed 2.51-fold and 2.86-fold abundance. Intriguingly, *epsH* encoding glycosyltransferase showed a 3.01-fold increase in abundance.

A series of genes related to cell division were considerably induced. Gene *smc*, which encodes a chromosome segregation protein, increased by 2.16-fold. The cell division protein gene *ftsQ* displayed a 1.56-fold upregulation. The gene transcription levels of *ftsE* and *ftsX*, which encode ATP-binding protein and permease protein of the cell division transport system, increased 1.56-fold and 1.78-fold, respectively, in abundance.

YthA increases the biosynthesis and immunity of nisin. The nisin gene cluster *nisABTCIPRKFEG* was observed in our strain. The majority of these genes were upregulated in the engineered strain (Table 4). The nisin immunity gene *nisI* and the nisin leader peptide-processing gene *nisP* both increased in expression by 1.46-fold. The *nisR-nisK* two-component system (TCS) genes showed 1.50- and 1.69-fold abundance. Nisin transport system genes *nisFEG*, which are also important for nisin immunity, were induced 1.29-, 1.76-, and 2.10-fold, respectively. To further investigate immunity to nisin, we performed nisin resistance assays for FythA, F44-p, and F44. FythA was more resistant to nisin than either F44-p or F44 (Fig. 6). The maximum valid dilution of FythA on the plate with 18,000 IU/ml nisin was $1:10^6$, whereas those of F44 and F44-p were only $1:10^3$ (Fig. 6A). Moreover, FythA showed better viability than the control on the

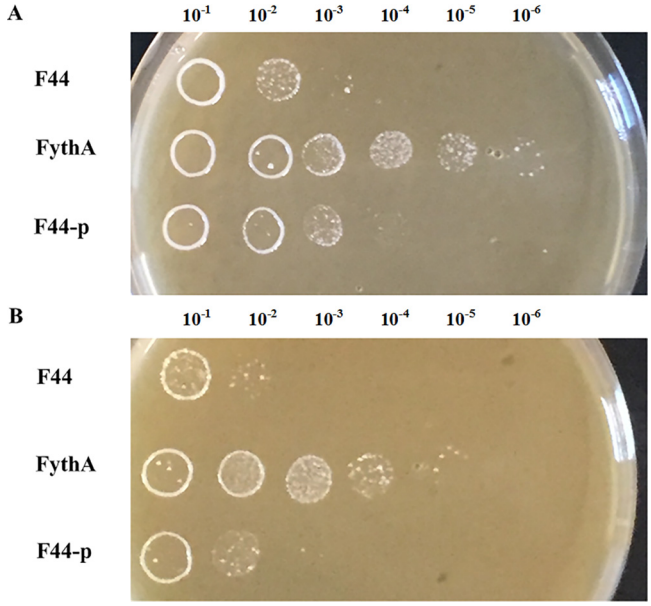

FIG 6 Nisin resistance of FythA, F44-p, and F44. The nisin-resistant capacity was determined by the serial dilutions plated on the fermentation medium agar plates containing 18,000 IU/ml nisin (A) and 20,000 IU/ml nisin (B).

plate containing 20,000 IU/ml nisin (Fig. 6B). These results showed that YthA had a detectable effect on the ability of biosynthesis and immunity of nisin.

Pyrimidine metabolism and other effects of YthA. A total of 11 genes implicated in pyrimidine metabolism (*carA*, *pyrBCDEFPRZ*, *upp*, and *udp*) were prominently upregulated. The *pyrB*, *pyrD*, *pyrE*, *pyrF*, *pyrP*, and *pyrR* genes increased by >3-fold (Table 4). The aminoacyl-tRNA synthetase genes (*cysS*, *asnC*, *argS*, *trpS*, and *lysS1*) were enhanced (see Table S1 in the supplemental material). Moreover, *ylgC* (RNA methyltransferase) was upregulated 3.49-fold. Two ribosomal protein genes, *rpmG3* and *rpsN2*, showed 2.85- and 2.55-fold increases in abundance, respectively, indicating high potential for protein translation.

In addition to NisR/NisK, the KdpD/KdpE and TcsR7/TcsK7 TCSs also yielded increases. Several transcriptional regulators, including *yebF*, *yfdD*, *scrR*, *ybjK*, *rcfB*, *yfdC*, *rex*, *rlrG*, *argR*, and *zitR*, were also upregulated (see Table S1 in the supplemental material). Among them, the first five genes increased by >2-fold. YfdD and YfdC were both annotated as regulatory protein Spx. On the contrary, four regulators—*yohC*, *hrcA*, *tcsR8*, and *ysgA*—were decreased by >2-fold. In particular, *yohC* was downregulated by 3.02-fold. Interestingly, three genes (*uspA2*, *yahB*, and *yjaB*) that encode universal stress proteins were repressed by ~1.5-fold. Moreover, the carbon starvation *cstA* protein decreased 2.05-fold.

Carbohydrate utilization was also significantly affected by YthA (see Table S1 in the supplemental material). Two sucrose utilization genes, transcriptional regulator *scrR* and β-fructofuranosidase *scrB*, were abundant. Two genes related to the glycolysis pathway *pgi* (glucose-6-phosphate isomerase) and *yrjI* (probable phosphoglycerate mutase) were upregulated. Nevertheless, the *gapA* gene that encodes the $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase decreased dramatically by 7.86-fold. In addition, *glk* and *enoA* were both downregulated.

Metal irons implicated in many vital biological processes are essential for the survival of bacteria. As shown in Table S1 in the supplemental material, the expression of the manganese/zinc transport system genes *mtsA*, *mstB*, and *mtsC* dramatically increased (3.71- and 3.29-fold). The expression of *era* (GTPase), which is essential for the cell viability of bacteria, increased by 1.8-fold. Four genes—*secY*, *secA1*, *secA2*, and *secE*—that encode preprotein translocase were all enhanced. In particular, *secE* dem-

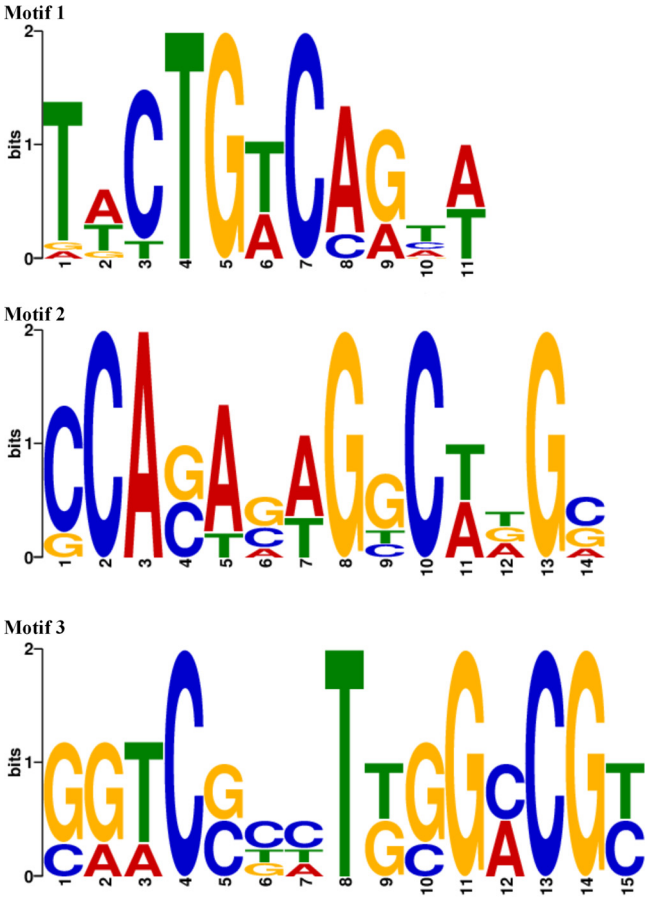

FIG 7 Visualization of putative DNA binding motifs of YthA predicted by MEME. Motif 1 (TWCTGWCAG TW), motif 2 (CCASASWGGCWDGS), and motif 3 (GTCSBHTKGGMCGY) are depicted by sequence logos generated by the MEME suite tools. The relative heights of the letters represent the frequencies of nucleotides at each position.

onstrated a 3.0-fold increase in abundance. Astonishingly, four chaperone genes (*grpE*, *dnaK*, *groES*, and *groEL*) were downregulated.

Proposed binding sites of YthA. To gain further insight into the possible DNA-binding motifs of YthA regulation, 15 promoter regions of the most highly changed genes (most of them up- or downregulated by >3-fold) were analyzed to find putative binding sites of YthA using the motif finder MEME (see Table S2 in the supplemental material). Three putative motifs containing 11 to 15 bp were detected, as shown in Fig. 7. Motif 1, TWCTGWCAGTW, was identified at 18 sites upstream of five candidates: *pyrR*, *pyrE*, *pyrB-carA*, *pyrZD*, and *ylgC*. Motif 2, CCASASWGGCWDGS, was found upstream of *argGH*, *pyrR*, *pyrE*, *pyrB-carA* and *pyrZD*. Motif 3 (GTCSBHTKGGMCGY) was only found upstream of genes, such as *argG*, *argH*, *gltS*, *argE*, *aroH*, and *gapA*, that are related to amino acid biosynthesis. These motifs may be associated with YthA regulation in *L. lactis* F44, and further verification is needed.

DISCUSSION

The protein YthA is a stress-responsive transcriptional regulator of the PspC family, with 154 amino acids and a calculated molecular mass of 18.3 kDa. The overall approach used in the present study research is depicted in Fig. 8. FythA displayed a slow pH decrease, whereas it exhibited a higher total biomass and growth rate compared to the control under acidic fermentation. Moreover, FythA showed an increased acid resistance and nisin yield. A transcriptomic approach was used to identify the regulation mechanism of YthA in *L. lactis* (Fig. 9). We observed that YthA

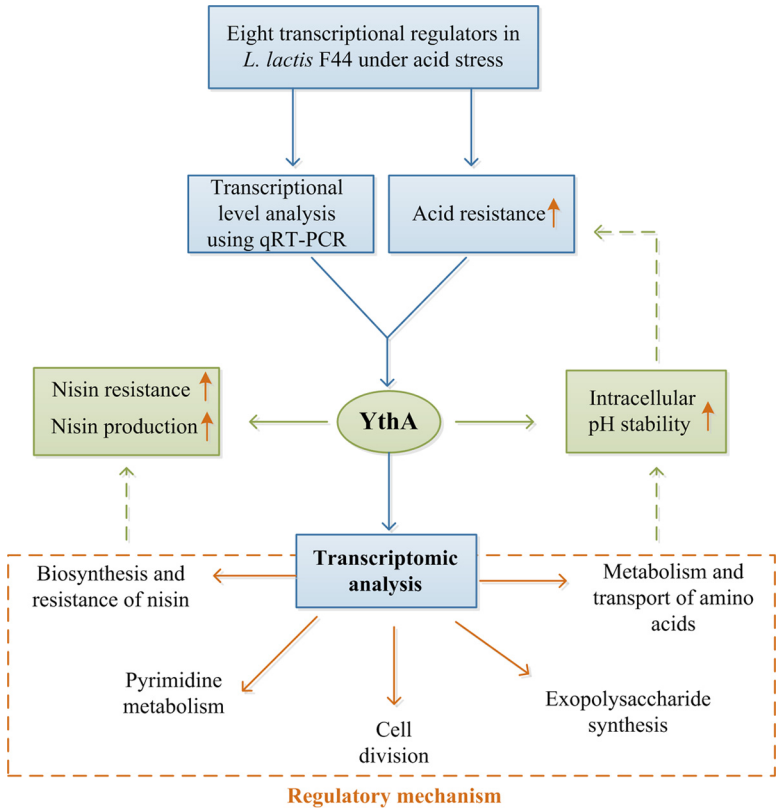

FIG 8 Overall approach used in this research. A summary of the various steps involved in our research workflow is depicted.

was strongly targeted in amino acid biosynthesis, pyrimidine metabolism, exopolysaccharide biosynthesis, and nisin immunity. The stress-responsive transcriptional regulator YthA was found to contribute to acid resistance, and its regulation mechanism is preliminarily revealed in *L. lactis*.

L. lactis encounters acid stress because of the accumulation of lactic acid, which may inhibit or even cease growth. A previous study found that a range of transcriptional regulators could assist bacteria in increasing acid resistance (12). In our study, overexpressing *ythA* improved the acid resistance of *L. lactis*. As shown in Fig. 2C, the decreased pH values in FythA were lower throughout the fermentation, whereas the total biomass was higher than that of the control. Moreover, the engineered strain FythA contributed to the stability of the pH_i value (Fig. 3B). Maintaining the relative pH_i stability is vital to bacteria and guarantees normal physiology and cell metabolism (31, 32). To investigate the regulation mechanism, a transcriptomic approach was used. The expression levels of a large number of genes involved in amino acid biosynthesis increased in FythA. Amino acids participate in a series of LAB physiological process, including protein synthesis, intracellular pH regulation, metabolic energy generation, and stress resistance (33). Six genes in arginine synthesis were significantly upregulated. The levels of *argG* and *argH*, in particular, dramatically increased 8.62- and 7.67-fold, respectively. Previous studies found that *argG* and *argH* were acid inducible, and their upregulation might enhance the metabolic flux from aspartate to arginine, which could increase the production of ATP and ammonia and the consumption of acidic amino acids (aspartate and glutamate) (34, 35). Moreover, the biosynthesis of the basic amino acids histidine (*hisZ*, *hisG*, *hisC*, and *hisD*) and lysine (*ychH*, *yciA*, and *lysA*) was strongly induced. Cytoplasmic buffering, which can sequester or release protons, is important in pH homeostasis (36). In our study, YthA could activate the biosynthesis of basic amino acids (arginine, histidine, and lysine) to sequester protons. In addition,

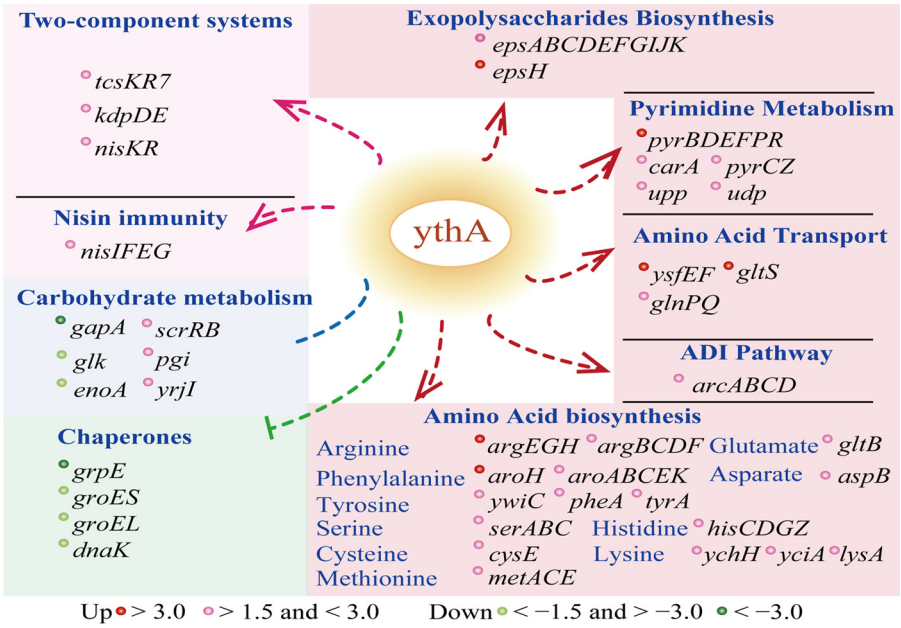

FIG 9 Proposed transcription mechanism of transcriptional regulator YthA on *L. lactis* F44. According to transcriptome analysis, YthA was presumed to regulate the amino acid transport and biosynthesis, ADI pathway, pyrimidine metabolism, exopolysaccharide synthesis, two-component systems, nisin immunity, and chaperones. Red spots represent more abundant genes >3.0-fold. Pink spots represent upregulated genes (between 1.5- and 3.0-fold). Green spots represent downregulated genes (>3.0-fold). Light green spots represent downregulated genes (between 1.5- and 3.0-fold).

YthA enhanced the entire arginine deiminase (ADI) pathway consisting of arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB), and carbamate kinase (ArcC2 and ArcC3). The ADI pathway can convert arginine into ornithine via citrulline. This pathway is one of the most representative mechanisms for cells to cope with acid stress and energy deficiency by producing ammonia and ATP (37). Overall, these results might provide clues to explain the physiological characteristics of the *ythA*-overexpressing strain (Fig. 2). Furthermore, YthA could induce the biosynthesis of arginine, histidine, lysine, and ADI pathway to maintain intracellular pH homeostasis in response to acid stress.

Also, EPS, which are important components influencing cell surface characteristics, are vital in pathogenesis and symbiosis and protect the cell from various environmental stresses (38). A *epsXABCDEFGHIJKL* cluster in *L. lactis* F44 has been observed. EpsA is essential for the EPS biosynthesis and is a positive regulator for the EPS production (39). EspH is a key enzyme that catalyzes the biosynthesis of oligosaccharide-repeating units (40). The protein EpsF, a membrane protein, participates in exporting saccharide, and the protein EpsG exhibits autophosphorylation (41). In *Methylovorus* sp. strain MP688, it was demonstrated that EPS are indispensable to bacterial survival in adverse environments (42). Thus, we presumed that YthA can help bacteria survive acid stress by promoting the EPS biosynthesis.

In addition, YthA might activate several other mechanisms and possibly contribute to withstand acid stress. The two-component system (TCS) for signal transduction acts as an essential mechanism for environmental sensing and signal transduction in most bacteria. In the present study, the transcription levels of TCS KdpD/KdpE were notably upregulated. The system is highly conserved across more than 1,000 bacterial species and is connected to K^+ homeostasis, the enhancement of virulence, and survival against acid, salt, and oxidative stresses (43, 44). In addition, the levels of manganese/zinc transport system genes, namely, *mtsA*, *mstB*, and *mtsC*, were dramatically increased. These genes are responsible for transporting zinc, manganese, and iron in *Streptococcus pyogenes* (45). However, MtsA fulfills a role in the homeostasis of iron and manganese, without controlling zinc homeostasis *in vivo* (46). Manganese is indispens-

able for enzymatic catalysis, and a connection between manganese homeostasis and oxidative stress has been found (46).

In the present study, fermentation results suggest that overexpressing *ythA* was beneficial to the cell growth of *L. lactis* under acid stress (Fig. 2). According to the transcriptome results, YthA could promote the synthesis and transport of some amino acids (Fig. 5). Except for arginine, almost all of the genes involved in the synthesis of serine, phenylalanine, and tyrosine were markedly upregulated in our data sets. Besides its role in protein synthesis, L-serine also works as an important precursor of several essential compounds, such as phosphatidylserine and D-serine. A previous study reported that a deficiency in serine biosynthesis led to a low growth rate of *Mycobacterium tuberculosis* (47). Moreover, several genes in the biosynthesis of threonine, cysteine, histidine, methionine, and lysine were abundant. Notably, genes involved in the transport system of polar amino acids (*ysfEF*, *gltS*, *yvdF*, *yjgC*, and *glnPQ*) and oligopeptide transport system (*opts* and *optA*) were upregulated. Among these examples, GltS is a transporter for glutamate uptake in *Helicobacter pylori*, *E. coli*, and *C. glutamicum* (48, 49). YthA may facilitate glutamate uptake, which could also influence arginine synthesis indirectly.

In addition, the six genes (*carA* and *pyrBCDEF*) responsible for transferring L-glutamine to UMP, the *de novo* synthesis of pyrimidines, were upregulated. UMP, which can be further converted into UTP, CTP, dCTP, and dTTP, plays a vital role in pyrimidine synthesis (50). The pyrimidine biosynthetic pathway is also associated with arginine metabolism through carbamoyl phosphate (51). Furthermore, GTPase Era functions as an RNA chaperone by controlling the processing and maturation of 16S rRNA and the 30S (small) ribosomal subunit (52). Aside from participating in ribosome assembly, GTPases are involved in other cellular processes, including DNA replication, cell metabolism, cell division, and stress response (53). Moreover, the abundant transcripts of *ftsQ*, *ftsE*, and *ftsX* suggested faster cell division compared to the control. The gene *ftsQ*, which encodes a cell division protein, is essential in the assembly of all of the components of the septal region (54). The FtsEX complex is one of the PG hydrolase regulatory systems that recruit and control PG hydrolases during growth (55).

In *L. lactis*, the *nisABTCIPRKFEG* cluster is responsible for the synthesis, modification, regulation, immunity, and secretion of nisin. NisR/NisK is supposed to be a transcription activator of nisin biosynthesis and immunity in *L. lactis* (56, 57). NisR can directly activate *nisA*, *nisR*, and *nisF* and implies a vital role in the virulence of *Streptococcus suis* serotype 2 (58). In our study, FythA exhibited higher nisin resistance and yield than the control (Fig. 6 and 3A). Furthermore, the transcription enhancements of nisin cluster genes were verified. Previous studies have demonstrated that the Psp system is pivotal in bacteriocin resistance, especially in nisin resistance (59–61). Enhancing nisin resistance is a strategy for improving nisin yield (62). The nisin producer *L. lactis* employs two immunity systems, NisI and NisFEG, to protect against nisin (63, 64). In our study, the increase in the level of NisIFEG activated by YthA might be another reason for the high nisin yield. Moreover, the TcsK7/TcsR7 system is conserved with BceSR and BraSR and serves as the key regulator for the resistance of bacitracin and nisin in *Bacillus subtilis* and *Staphylococcus aureus*, respectively (65, 66). The upregulation of the TCS TcsK7/TcsR7 system induced by YthA would be important in nisin resistance. Thus, similar to our previous study, improving the nisin-resistant ability of *L. lactis* F44 could increase the nisin yield significantly (67).

To improve acid tolerance, recent studies have focused on manipulating regulatory genes other than metabolic genes in various industrial microbes (68). However, the regulatory systems related to the acid tolerance of *L. lactis* are rarely known. To further elucidate the issue, eight transcriptional regulators were overexpressed, and the transcriptional regulator YthA was verified to improve acid tolerance, maintain intracellular pH homeostasis, and increase the nisin resistance and nisin yield of *L. lactis* F44. Using transcriptome analysis coupled with qRT-PCR verification, we further investigated the possible regulation mechanism of YthA and assumed three potential DNA-binding motifs. The direct target genes and the DNA-binding motifs need to be further determined.

TABLE 5 Primers used in PCR amplifications

Primer	Oligonucleotide sequence (5′ → 3′)	Length (bp)[a]
argR-F	CGCGGATCCGTCAATATATAGTCCATTATATGGT	495
argR-R	TCCCCCGGGTTATCCTAATAAAATATACTTTATTTCC	
yjdC-F	CGCGGATCCTACCGTTAGGGAGGAGTG	486
yjdC-R	TCCCCCGGGTTAGGCATTTTTTTCCTCCT	
ythA-F	CGCGGATCCCACAAGGTTAATTCAACGAT	489
ythA-R	TCCCCCGGGTTAGAAATCTGACCAGTCATCTT	
glnR-F	CGCGGATCCCTATAATGAAAGGCATGGAGG	438
glnR-R	TCCCCCGGGTTACATCCGCGGTTGGGTA	
ycfA-F	CGGGATCCCTGGAAATGGGGTGAAAA	678
ycfA-R	TCCCCCGGGGTTAGTATTTTTTACGTTTACGTGCT	
yfjG-F	CGGGATCCTTTGATTTATTGGTATTAGCTAGAG	657
yfjG-R	TCCCCCGGGTTAGGTGTTTTTTATATAAATTGGC	
rlrD-F	CGCGGATCCATTTTTGTATTTTAGGGAGAAA	843
rlrD-R	TCCCCCGGGTTAATGAATTTCGGTTTCTTTTT	
rcfA-F	CGCGGATCCTACTCAAATAAGGAGAATAAAAT	702
rcfA-R	TCCCCCGGGTTAAGCAAAATCATCTTCTAATTTC	

[a]That is, the length of the product of PCR.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All of the strains and plasmids used in this study are listed in Table 1. *L. lactis* subsp. *lactis* F44 was used for gene engineering. *E. coli* TG1 was used as the host for genetic manipulation. *E. coli* was incubated in Luria-Bertani (LB) medium (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter) at 37°C, with shaking at 180 rpm. *L. lactis* was cultivated in a fermentation medium containing peptone at 1.5 g/liter, yeast extract at 1.5 g/liter, sucrose at 1.5 g/liter, KH_2PO_4 at 2.0 g/liter, NaCl at 0.15 g/liter, corn steep liquor at 0.3 g/liter, cysteine at 0.26 g/liter, and $MgSO_4{\cdot}7H_2O$ at 0.015 g/liter at an initial pH 7.2, followed by incubation at 30°C without shaking. *Micrococcus flavus* ATCC 10240, used as an indicator strain for nisin assay, was grown in LB medium at 37°C with 180-rpm agitation. Media were supplemented with the antibiotic erythromycin for the selection at concentrations of 100 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*.

Cloning and overexpression of transcriptional regulators. All of the transcriptional regulator genes were amplified by PCR using *L. lactis* subsp. *lactis* F44 genomic DNA as a template. Primers of the relevant genes used in this study were designed by Primer Premier 5 (Premier Biosoft, Canada) and are listed in Table 5.

All of the products were restricted with BamHI and SmaI and subsequently ligated into plasmid pLEB124, which was treated with the same restriction enzymes to yield the expression vector. Vectors were introduced into *E. coli* TG1 by heat shock transformation. Finally, after antibiotic selection and enrichment, the vectors carrying target gene fragments were transformed into *L. lactis* F44 to obtain recombinant strains.

Acid resistance assay. *L. lactis* cells were harvested by centrifugation after culture for 8 h and subsequently resuspended in equal volumes of fermentation medium that had been acidified to pH 3.0 with hydrochloric acid. After incubation at 30°C for 2.5 h, the number of viable strains was determined by plating on agar plates and was used as the final cell density. The number of cells before acid challenge was set as the initial cell density. Plates were incubated at 37°C for 24 h before colony counting. Survival rates were calculated by dividing the number of final cell density after being challenged at pH 3.0 by the number of the initial cell density immediately after resuspension.

Nisin yield assay. Plate diffusion method was performed to determine the nisin yield (69). Standard nisin diluted to 200, 100, 50, and 25 IU/ml with 0.02 M HCl was used to draw the standard curve. Accordingly, the fermentation broth was diluted 2-fold by 0.02 mol/liter HCl, and the mixture was heated at 100°C for 5 min. After cooling at room temperature, the samples were centrifuged at 5,000 × *g* for 5 min to remove the cell pellets. The assay medium (26 ml with 1.5% agar) was pretreated by autoclaving, and 390 μl of Tween 20 (JiangTian, Tianjin, China) was added at approximately 70°C. When the assay medium was cooled to approximately 50°C, the indicator *M. flavus* ATCC 10240 was added at a ratio of 1% (vol/vol), and the total broth was poured into a sterile plate. Eight wells were drilled using a 7-mm-diameter hole punch on each assay agar plate. Portions (100 μl) of each fermentation sample and standard nisin solution were injected into the corresponding wells. After incubation at 37°C for 24 h, the inhibition zones were measured by using a Vernier caliper. A regression equation was calculated based on the measured data. Each sample was performed in triplicate.

Determination of pH_i. The intracellular pH (pH_i) was measured by using a fluorescence method as described previously, and the 5-(and-6)-carboxyfluorescein succimidyl ester (cFDASE) was used as the fluorescent probe (7). Harvested cells were washed three times and resuspended in 50 mM HEPES buffer (pH 8.0), followed by incubation for 10 min at 30°C in the presence of 1.0 μM cFDASE. Subsequently, the cells were washed three times and resuspended in 50 mM potassium phosphate buffer (pH 7.0), and nonconjugated fluorochrome was eliminated by incubation with 10 mM lactose for 30 min at 37°C. The cells were then washed twice and resuspended in 50 mM HEPES buffer (pH 8.0), followed by an additional 10 min at 30°C with 1 μM valinomycin and nigericin. Samples were preserved on ice until required. Calibration curves and pH_i values were determined from the ratio of the fluorescence signal

TABLE 6 Primers used in qRT-PCR

Primer	Oligonucleotide sequence (5′ → 3′)	Length[a] (bp)
argG-F	AGTGGCATTGGGCTCG	127
argG-R	ACACCAGCTTCAATCGCA	
carA-F	AGAGCATCAAATGAGCCAAC	112
carA-R	AACGACAACTTTTCTGCCTG	
dnaK-F	GACCGTAACACAACTATCCCA	113
dnaK-R	TCAGCTGCCATTGGACG	
uspA-F	GGCACAGAAGTTGATTTTGAG	113
uspA-R	AGACCAGTTGACCCAATTACA	
pyrR-F	GACCGTGGACATCGTGAAT	119
pyrR-R	AAAATACTGTCATTGCCATCG	
purA-F	AGAAATCGCTTATCTCGCTG	133
purA-R	AATCTTCTTATCGCCTTTGG	
mtsB-F	AGGAGAACTATCAGGTGGTCAG	112
mtsB-R	GCTTATCGAATCAATCCCAA	
gltS-F	AGGAGCCGTGATTGAAAAC	122
gltS-R	TAGGAAGTGCAACCGCTG	
tcsK7-F	GAGGAGCTGAAGAATCAGGTT	125
tcsK7-R	TTCACAAGTGAAGCCATACTCA	
nisK-F	TCAATATCTTTGTTAATGCCTGT	117
nisK-R	AAGGATGACCATTATTCCAGAT	
arcA-F	CAGGAATTATGGATGGTGCT	113
arcA-R	GACTTTCTTGAGTGCTGCTTTA	
hrcA-F	TTCTTAATGCTCCTCAACGTG	105
hrcA-R	TTACTTCACCAGTCCCTAATGTT	
epsH-F	TTTTTGGATCATACCCCATAA	102
epsH-R	TACTTCTTCCGAAAATTTCATG	
gapA-F	AATGACCCTTGATGGTCCA	120
gapA-R	AGTTCAGGCAAAACAAGACC	
enoA-F	AATTCCACTCTATCGCTATCTTG	134
enoA-R	CCCGTTTGACTGGTGTAATC	

[a]That is, the length of the qRT-PCR fragment.

measurements using a Fluorescence Spectrophotometer F-2700 (Hitachi High-Tech, Japan) at excitation wavelengths of 490 nm (pH-sensitive wavelength) and 440 nm (pH-insensitive wavelength) with a 5-nm slit width. The emission wavelength was 525 nm with a 5-nm slit width. All values were taken from three independent experiments.

RNA extraction and RNA-seq. Total RNA of the strains was isolated by using a Quick-RNA MicroPrepkit (ZYMO Research, Irvine, CA) and treated with DNase I (NEB) according to the manufacturer's instructions. The quality of the total RNA samples, such as concentration, RNA integrity number (RIN), 28S/18S, and size, were analyzed by using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano kit). The purity of the samples was detected by using a NanoDrop apparatus. rRNA depletion was performed using Ribo-Zero magnetic kit (bacteria; Epicentre, Madison, WI), and the sample was purified by using RNAClean XP beads (Agencourt). Afterward, RNA was fragmented into 130 to 170 nucleotides by adding fragment buffer (Ambion) to the samples.

First-strand cDNAs were generated using First-Strand MasterMix and SuperScript II reverse transcription (Invitrogen), and then the second-strand cDNAs were synthesized by adding Second-Strand MasterMix. Subsequently, the purified fragmented cDNAs were combined with End Repair Mix, and an adenine base was added. cDNA fragments were amplified with several rounds of PCR and subsequently purified. Library quantification was performed using an Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 reagents). Subsequently, the qualified cDNA libraries were prepared to generate the cluster on the flow cell (TruSeq PE V3-cBot-HS cluster kit; Illumina) and sequenced on a HiSeq 4000 system (TruSeq SBS KIT-HS V3; Illumina).

Read preprocessing and transcriptome analysis. Raw reads primarily produced by Illumina HiSeq 4000 were subjected to perform quality control by analyzing the base composition of raw reads and quality distribution of the bases along the reads (Q20 $>$ 95% and Q30 $>$ 90%) to ensure data accuracy. After quality control, raw reads were filtered into clean reads, which were then aligned to the reference sequences by using SOAPaligner/SOAP2 (70). The number of perfect clean reads corresponding to each gene was calculated and normalized to the number of reads per kilobase of exon model per million mapped reads. The false discovery rate (FDR) was used to determine the threshold of the *P* value. We identified the genes significantly differential expressed at an FDR of $<$0.001, a *P* value of $<$0.05, and a $\log_2$ ratio (pYthA/control) $\geq$ 1.5-fold normalized change. Furthermore, the COG annotation was determined by the BLAST software against the COG protein database (www.ncbi.nlm.nih.gov/COG/) (71).

Transcriptional verification by quantitative real-time PCR. To determine mRNA levels, reverse transcription was performed using total RNA (0.5 μg) as a template by RevertAid First-Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA). Quantitative real-time PCRs were performed with cDNA (~100 ng), mix (LightCycler 480 SYBR green I Master), and gene-specific primers on a LightCycler 480

real-time PCR system (Roche, Switzerland). The PCR program was run as follows: preincubation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. Specific primers were designed using Primer Premier 5, as listed in Table 6. The 16S rRNA gene in *L. lactis* F44 was used as an internal control. The value of the relative gene quantification was calculated based on the $2^{-\Delta\Delta CT}$ analysis method (72).

Determination of nisin resistance. Nisin resistance was determined by the serial dilution assay, as described by Xie et al. (73). *L. lactis* strains F44, F44-p, and FythA were cultivated approximately to 5×10^8 CFU/ml in the fermentation medium at 30°C without rotation. Afterward, the cultures were serially diluted in 10-fold increments in physiological saline solution. Serial dilutions were plated on fermentation medium agar plates containing 18,000 or 20,000 IU/ml nisin. Plates were incubated at 30°C for 24 h. All experiments were performed in triplicate.

DNA motif mining. MEME suite tools (http://meme-suite.org/) were used to search YthA binding sites in the promoter region of the proposed target genes in the genome of *L. lactis* F44 (74). The most significantly changed genes in transcriptome analysis were used as the data source (see Table S2 in the supplemental material). Binding site searches were performed using the putative promoter regions (regions that contained the intergenic region and 100 bp upstream of the genes) of the selected genes. Any number of sites per sequence was allowed, and sites should be on the given strand.

Statistical analysis. To evaluate the statistical significance of the survival rate under acid stress, a Student *t* test was performed. The statistical significance values for the OD, pH, and pHi were assessed by using a *t* test. SPSS software version 19.0 (IBM) was used for statistically analyzing the data.

Accession number(s). The mRNA-seq data of *L. lactis* F44-p and FythA obtained in this study and used in the sequencing procedures were deposited in the NCBI Gene Expression Omnibus under accession number GSE101424.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02483-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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