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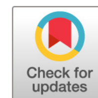

Transcriptional Regulator AcrR Increases Ethanol Tolerance through Regulation of Fatty Acid Synthesis in *Lactobacillus plantarum*

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ABSTRACT *Lactobacillus plantarum* is a versatile bacterium with significant adaptability to harsh habitats containing excessive ethanol concentrations. It was found that the *L. plantarum* NF92-TetR/AcrR family regulator, AcrR, significantly enhanced the growth rate of this lactic acid bacterium in the presence of ethanol. Through screening 172 ethanol-resistant related genes by electrophoretic mobility shift and quantitative reverse transcription-PCR (RT-qPCR) assays, six genes were identified to be regulated by AcrR under ethanol stress. Among these was a gene coding for a 3-hydroxyacyl-ACP dehydratase (*fabZ1*) regulated by AcrR under ethanol stress. AcrR regulated *fabZ1* under ethanol stress by binding to its promoter, P_{fabZ1}. DNase I footprinting analysis indicated that there were two specific AcrR binding sites on P_{fabZ1}. RT-PCR results showed *fabZ1* could cotranscribe with its downstream 12 genes and conform a fatty acid *de novo* biosynthesis (*fab*) gene cluster under the control of P_{fabZ1}. Both RT-qPCR of the *fab* gene cluster in *acrR* knockout and overexpression strains and fatty acid methyl ester analysis of the *acrR* knockout strain showed that AcrR could promote fatty acid synthesis in *L. plantarum* NF92. Membrane fluorescence anisotropy analysis of *acrR* knockout and overexpression strains showed that AcrR could increase membrane fluidity under ethanol stress. Thus, AcrR could regulate fatty acid synthesis and membrane fluidity to promote the adaption of *L. plantarum* NF92 to a high ethanol concentration.

IMPORTANCE Ethanol tolerance is essential for *L. plantarum* strains living in substances with more than 9% ethanol, such as wine and beer. The details regarding how *L. plantarum* adapts to ethanol are still lacking. This study demonstrates that AcrR regulates the *de novo* synthesis of fatty acids in *L. plantarum* adapting to toxic levels of ethanol. We also identified the ability of the TetR/AcrR family regulator to bind to the fatty acid biosynthesis gene promoter, P_{fabZ1}, in *L. plantarum* and defined the binding sites. This finding facilitates the induction of the adaptation of *L. plantarum* strains to ethanol for food fermentation applications.

KEYWORDS *Lactobacillus plantarum*, AcrR, fatty acid *de novo* synthesis, ethanol tolerance

Lactic acid bacteria (LAB) are widely distributed in a range of niches, including vegetables, meat, dairy products, and gastrointestinal tracts, as well as numerous fermented foods and beverages (1–3). Sometimes, these environments are not very suitable for the survival of LAB, and many stress factors in these habitats, such as toxic alcoholic content, suboptimal growth temperature, acidic pH, starvation, and growth-inhibitory compounds originating from bacterial metabolism, are detrimental to LAB. Numerous studies aimed at elucidating the mechanisms of LAB to sense stress signals and protect themselves against acid, oxidative chemical agents, bile, and heat stresses

Citation Yang X, Teng K, Li L, Su R, Zhang J, Ai G, Zhong J. 2019. Transcriptional regulator AcrR increases ethanol tolerance through regulation of fatty acid synthesis in *Lactobacillus plantarum*. Appl Environ Microbiol 85:e01690-19. https://doi.org/10.1128/AEM.01690-19.

Editor Danilo Ercolini, University of Naples Federico II

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Received 24 July 2019
Accepted 11 September 2019
Accepted manuscript posted online 13 September 2019
Published 30 October 2019

have been reported (4–9). These studies have shown that LAB respond rapidly to the habitat by modulating different cellular processes, including stress response pathways, amino acid metabolism, carbohydrate metabolism, cell envelope composition, and others (10).

Lactobacillus plantarum, some representatives of which have been characterized as probiotics, has been used to improve food fermentations, as well as human health (11, 12). *L. plantarum* can survive in many different ecological niches and is able to tolerate varied stressors. Ethanol is a common stress factor in food fermentations, especially in the wine industry, and represents a hurdle for the survival and viability of *L. plantarum*. The toxicity of ethanol is generally attributed to the disturbance of the plasma, resulting in a loss of membrane integrity and an increase in membrane permeability (13, 14). *L. plantarum* is widely distributed in the early, middle, and late stages of fermented grains and has a good adaptability to this environment (15). In the fermentation process, *L. plantarum* plays an important role in the acidification of red grapes, improving flavor and inhibiting pathogens or spoilage bacteria (16–19). It has been reported that *L. plantarum* tolerates up to 13% (vol/vol) ethanol at 18°C (20), and several studies have been conducted to explore the response of *L. plantarum* to this solvent (21–27). For example, overproduction of Hsp 18.55 and Hsp 19.3 can enhance the survival of *L. plantarum* in the presence of butanol (1% [vol/vol]) or ethanol (12% [vol/vol]) (26). Overexpression of the *L. plantarum* peptidoglycan biosynthesis associated gene, *murA2*, can increase the tolerance of *Escherichia coli* to alcohols and enhance ethanol production (24). However, these studies did not reveal a detailed molecular basis for how *L. plantarum* adapts to ethanol stress.

The TetR/AcrR family is a large and important family of regulators and is widespread among bacteria and archaea (28). Regulators in the TetR/AcrR family are always one-component signal transduction systems, which contain a conserved helix-turn-helix DNA-binding domain and a C-terminal ligand regulatory domain. These regulators play important roles in a wide range of cellular activities, including osmotic stress response, biosynthesis of antibiotics, multidrug efflux pumping, catabolic pathways, secondary metabolite biosynthesis, and the pathogenicity of bacteria (29–33). However, the role of AcrR in ethanol tolerance is not documented. It has been reported that AcrR has a very slight negative impact on the ethanol tolerance of *E. coli* (34); however, the exact regulation mechanism is unknown.

TetR/AcrR family regulators not only have various functions in different kinds of microorganisms but also can regulate many cellular activities in the same bacteria (34–36). Our previous report showed that the TetR/AcrR family regulator AcrR could participate in sorbitol or mannitol utilization by regulating the aldehyde-alcohol dehydrogenase encoding gene *adhE* in *L. plantarum* NF92 isolated from the Chinese liquor fermented grains (37). In this study, we found that AcrR could positively regulate fatty acid biosynthesis to enhance the ethanol resistance of *L. plantarum* NF92. The mechanism by which AcrR regulates *L. plantarum* NF92 tolerance to ethanol and adaptation was explored.

RESULTS

AcrR is involved in ethanol tolerance in *L. plantarum* NF92. *L. plantarum* NF92 is a strain that was isolated from Chinese liquor fermented grains. We measured the growth of *L. plantarum* NF92 in MRS supplemented with ethanol. The results showed that the growth of *L. plantarum* NF92 was not affected obviously when grown in 3% (vol/vol) and 6% (vol/vol) ethanol but was completely repressed by 12% (vol/vol) ethanol. Meanwhile, 9% (vol/vol) ethanol obviously repressed the growth of *L. plantarum* NF92; however, the cell density of NF92 could still reach half that of the control. As a result, we chose 9% (vol/vol) as the ethanol stress concentration in this study (Fig. 1A). In our previous report, we found that AcrR could promote the expression of AdhE, which could catalyze acetyl coenzyme A (acetyl-CoA) to ethanol (37). At the same time, we found that it might be also involved in ethanol tolerance of *L. plantarum* NF92. As shown in Fig. 1B, the *acrR* overexpression strain of *L. plantarum* NF92 ($acrR^{+}$) had a

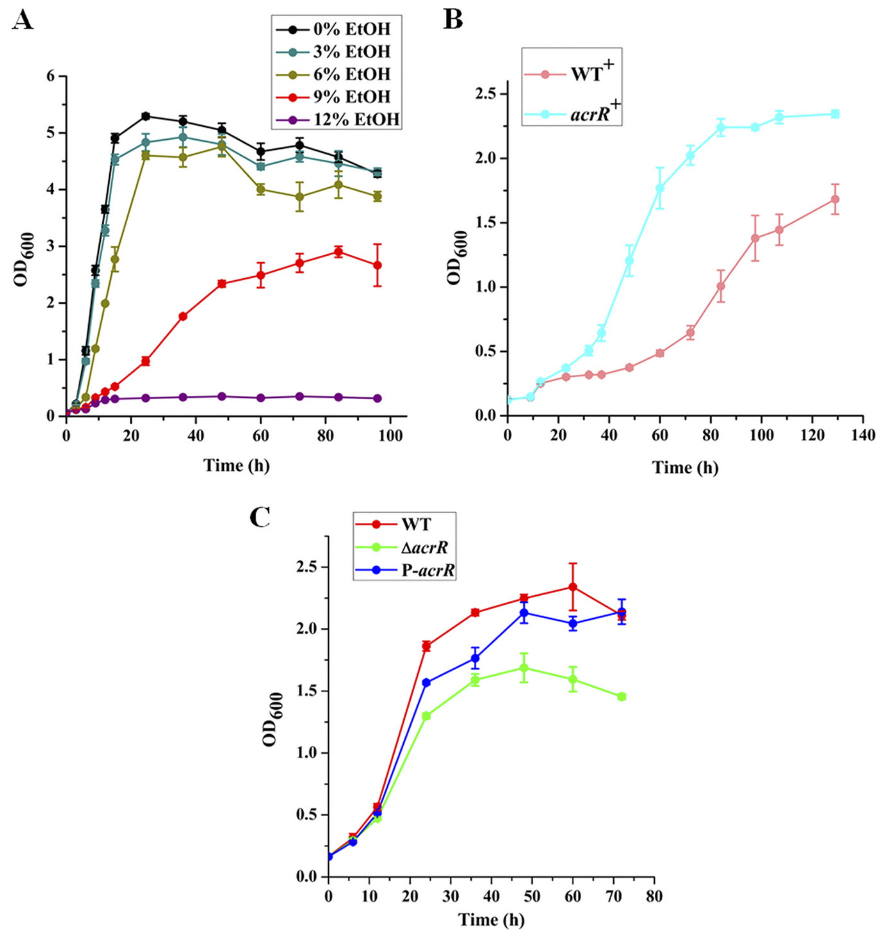

FIG 1 Growth curves of *L. plantarum* NF92. (A) Growth curve of *L. plantarum* NF92 in the presence of various concentrations (vol/vol) of ethanol (EtOH). (B) Effects of *acrR* overexpression on the growth of *L. plantarum* NF92 in MRS supplemented with 9% (vol/vol) ethanol. (C) Effects of *acrR* disruption on the growth of *L. plantarum* NF92 in MRS supplemented with 9% (vol/vol) ethanol. Values and standard deviations were calculated from three repeated samples.

significantly shorter growth time and higher cell densities in 9% (vol/vol) ethanol than that of the empty vector containing wild-type strain (WT^+), suggesting that AcrR might be involved in ethanol resistance in *L. plantarum* NF92. Further, the growth of the *L. plantarum* NF92 wild-type strain, the *acrR* knockout (Δ*acrR*) strain, and the *acrR* complemented strain P-*acrR* was tested in MRS supplemented with 9% (vol/vol) ethanol. Compared to the wild-type strain, the growth of the Δ*acrR* strain was significantly repressed. The growth of the *acrR* complemented strain P-*acrR* was much stronger than that of Δ*acrR* strain in MRS supplemented with 9% (vol/vol) ethanol and partly recovered to the level of wild-type strain (Fig. 1C). These results demonstrate that AcrR plays an important role in ethanol tolerance in *L. plantarum* NF92.

Genes that may be regulated by AcrR during ethanol tolerance in *L. plantarum* NF92. In order to identify ethanol tolerance related genes regulated by AcrR in *L. plantarum* NF92, we checked the binding capabilities of AcrR to 88 promoters. These promoters were referred to 172 genes that are differently expressed after 8% ethanol treatment in *L. plantarum* WCFS1 (21) and the main genes of peptidoglycan and wall teichoic acid biosynthesis related to stress response in LAB (10, 38). These genes are mainly involved in stress response, cell division, cell envelope synthesis, citrate metabolism, pyrimidine synthesis, and so on. The related genes and promoters were found in *L. plantarum* NF92 and are listed in Table S1 in the supplemental material. Electrophoretic mobility shift assays (EMSAs) were used to detect the binding of AcrR to 88

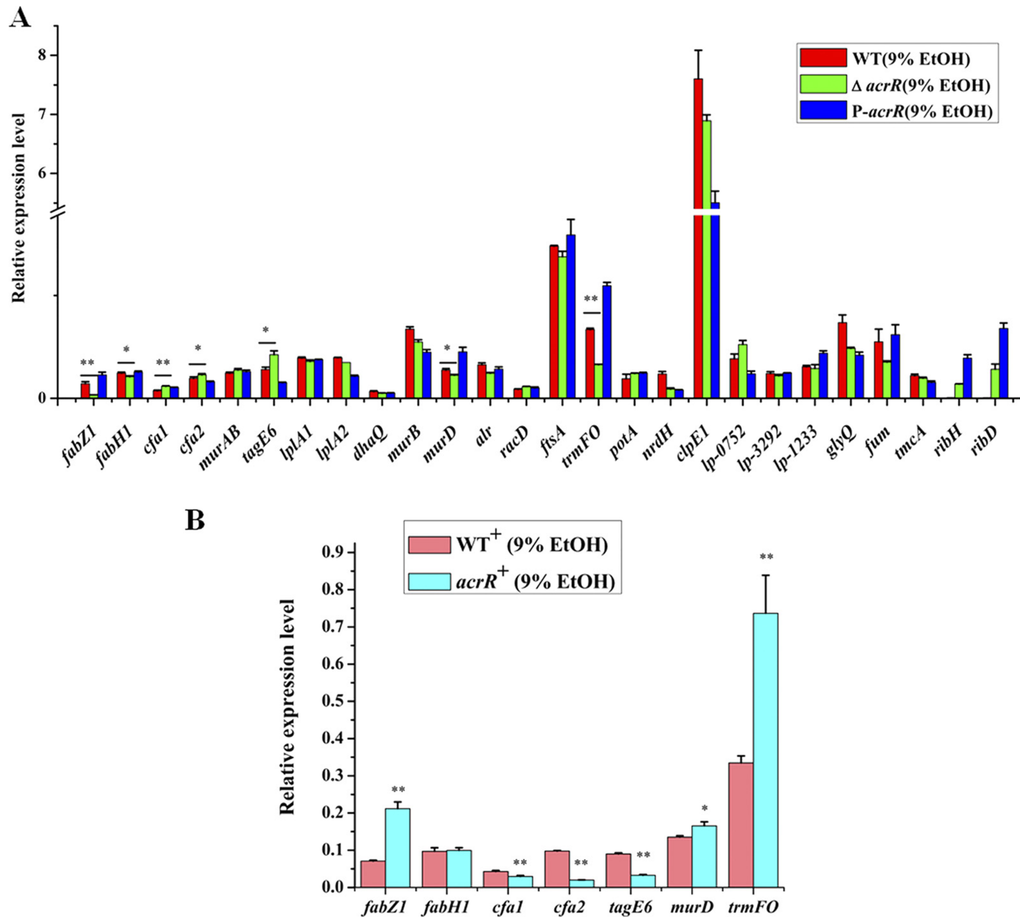

FIG 2 RT-qPCR transcriptional analysis of genes that may be regulated by AcrR under ethanol stress. (A) Transcriptional levels of genes that may be regulated by AcrR in WT, Δ*acrR*, and P-*acrR* strains under 9% (vol/vol) ethanol. (B) Transcriptional levels of genes that may be regulated by AcrR in the WT^+ and *acrR* overexpression ($acrR^+$) strains under 9% (vol/vol) ethanol. Relative values were obtained using the transcription of *gyrB* as reference. cDNA used as template in this experiment was diluted 1,000-fold. The *x* axis represents different genes. Values and standard deviations were calculated from three repeated samples. **, $P < 0.01$; *, $P < 0.05$.

promoters. As shown in Table S1, 51 of these promoters were detected to be bound by AcrR, and among them, 25 promoters showed stronger binding abilities. The expressions of the first genes controlled by these 25 promoters in WT, Δ*acrR*, and P-*acrR* strains grown in 9% (vol/vol) ethanol were further analyzed by quantitative reverse transcription-PCR (RT-qPCR). The results showed that the expression levels of seven genes, namely, *fabZ1* (3-hydroxyacyl-ACP dehydratase, controlled by P_{fabZ1}), *fabH1* (ketoacyl-ACP synthase III, controlled by P_{fabH1}), *cfa1* (cyclopropane-fatty-acyl-phospholipid synthase, controlled by P_{cfa1}), *cfa2* (cyclopropane-fatty-acyl-phospholipid synthase, controlled by P_{cfa2}), *murD* (UDP-*N*-acetylmuramoyl-L-alanine-D-glutamate ligase, controlled by P_{murD}), *trmFO* {[FADH(2)-oxidizing methylenetetrahydrofolate-tRNA-(uracil(54)-C(5)]-methyltransferase, controlled by P_{trmFO}}, and *tagE6* (glycosyltransferase, controlled by P_{tagE6}), were obviously changed in the Δ*acrR* strain compared to those in the WT and recovered in P-*acrR*. In the Δ*acrR* strain grown in 9% ethanol, the expression levels of *fabZ1*, *fabH1*, *murD*, and *trmFO* were significantly downregulated, whereas *cfa1*, *cfa2*, and *tagE6* were significantly upregulated, compared to those in the WT strain grown in 9% ethanol. In the $acrR^+$ strain grown in 9% (vol/vol) ethanol, the transcriptional levels of *fabZ1*, *murD*, and *trmFO* were significantly upregulated and *cfa1*, *cfa2*, and *tagE6* were significantly downregulated compared to those in the WT strain grown in 9% ethanol, whereas the expression of *fabH1* did not show obvious differences before or after *acrR* was overexpressed (Fig. 2B). These results demonstrate that *fabZ1*,

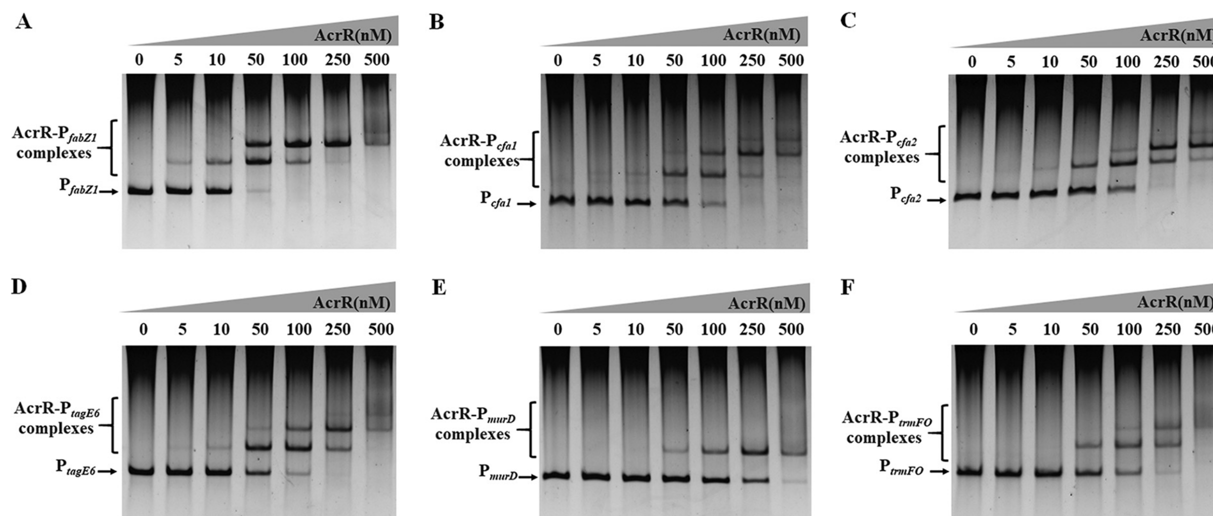

FIG 3 Binding ability analysis of AcrR to promoters by EMSA. (A) EMSA of AcrR binding to the upstream region of *fabZ1* (P$_{fabZ1}$). (B) EMSA of AcrR binding to the upstream region of *cfa1* (P$_{cfa1}$). (C) EMSA of AcrR binding to the upstream region of *cfa2* (P$_{cfa2}$). (D) EMSA of AcrR binding to the upstream region of *tagE6* (P$_{tagE6}$). (E) EMSA of AcrR binding to the upstream region of *murD* (P$_{murD}$). (F) EMSA of AcrR binding to the upstream region of *trmFO* (P$_{trmFO}$). Each lane contained 20 ng of DNA probe. Concentrations of AcrR ranged from 0 to 500 nM.

murD, and *trmFO* may be positively regulated by AcrR, while *cfa1*, *cfa2*, and *tagE6* may be negatively regulated by AcrR, when *L. plantarum* NF92 is grown in 9% (vol/vol) ethanol. The EMSA results also showed that AcrR had strong binding abilities to the promoters of these six genes (Fig. 3). AcrR could bind to P$_{fabZ1}$, P$_{cfa1}$, P$_{cfa2}$, and P$_{tagE6}$ at a concentration of 10 nM and to P$_{murD}$ and P$_{trmFO}$ at a concentration of 50 nM. Among these genes, *fabZ1*, which is related to fatty acid *de novo* biosynthesis, was most obviously affected by AcrR under ethanol stress. The expression of *fabZ1* decreased 3.28-fold when *acrR* was knocked out and increased 2-fold when *acrR* was overexpressed. Moreover, the *fabZ1* promoter P$_{fabZ1}$ had the strongest binding ability to AcrR, and a clear binding band could be seen when the concentration of AcrR was 5 nM (Fig. 3A). The possible binding sites of AcrR to P$_{fabZ1}$ were identified by a DNase I footprinting assay. Two regions located on P$_{fabZ1}$ at bp −221 to bp −178 (site I) and bp −92 to bp −62 (site II) upstream of the *fabZ1* start codon ATG were identified (Fig. 4A). The site I (TGCTAAACTGTGCCAGTTCGTTGACGGATTATGACCGCAAAAAA, 44 bp) and site II (CTGTAAATTAATCTGCTGAAATCAATTGACA, 31 bp) fragments were inserted into a nonspecific DNA fragment (fragment 0 [F0]), respectively creating fragment 1 (F1) and fragment 2 (F2). EMSA showed that AcrR could bind to F1 and F2 but not to F0 (Fig. 4B), indicating that AcrR can bind to the site I and site II regions of P$_{fabZ1}$. The second structure of P$_{fabZ1}$ was predicted by DNAMAN, and both site I and site II were included in the hairpin structures in the structure of P$_{fabZ1}$ (Fig. 4C). Site I contains a 5-bp (AACTG) repeat sequence and site II contains an 8-bp (TGTAAATT) imperfect repeat sequence. We proposed that *fabZ1* and the possible genes controlled by *fabZ1* promoter might be regulated by AcrR to participate in ethanol tolerance in *L. plantarum* NF92.

Fatty acid *de novo* biosynthesis gene cluster is regulated by AcrR under ethanol. FabZ1 is a 3-hydroxyacyl-ACP dehydratase that is involved in the dehydration of 3-hydroxyacyl-ACPs of all chain lengths in the basic type II fatty acid biosynthetic pathway (39). The location of *fabZ1* in the genome of *L. plantarum* NF92 was analyzed, and another 12 genes were located downstream of it. Twelve of these thirteen genes are involved in type II fatty acid synthesis, and another is necessary for the activity of ACP during fatty acid biosynthesis; details are listed in Table S1 in the supplemental material. These 13 genes composed the fatty acid biosynthesis (*fab*) gene cluster, with a total length of 9,890 bp. The transcriptional units of the *fab* gene cluster were determined by RT-PCR, which detected two parts of the *fab* gene cluster, namely, the

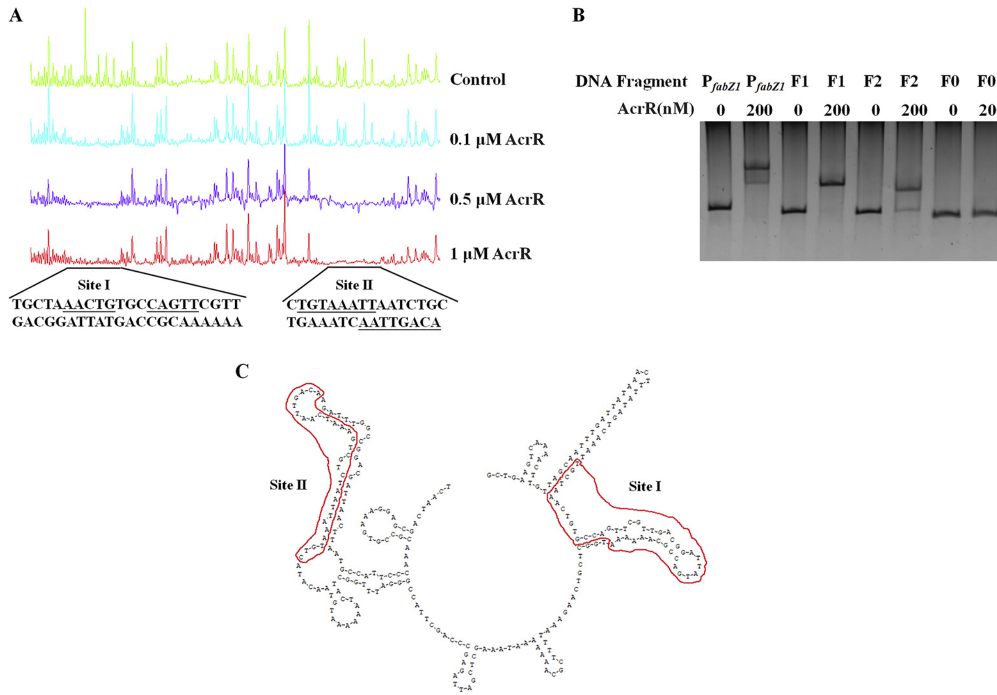

FIG 4 Identification of AcrR binding site on P_{fabZ1}. (A) Determination of AcrR binding sites on P_{fabZ1} by DNase I footprinting. Each lane contained 1.5 μg of DNA probe, which was incubated with increasing concentrations of AcrR protein (0.1, 0.5, and 1 μM). DNA probe incubated with 7 μM bovine serum albumin was used as a negative control. (B) Binding of AcrR to the 44-bp sequence (site I) and 31-bp sequence (site II). F1 contained the 44-bp sequence; F2 contained the 31-bp sequence. F0, nonspecific DNA as a negative control; P_{fabZ1}, the positive control. Each lane was loaded with a 20-ng DNA fragment and either 0 or 200 nM AcrR, as indicated in the figure. (C) Second structure of a part of P_{fabZ1}, which was predicted by DNAMAN. Red lines indicate the positions of site I and site II.

fragments from *fabZ1* to *accC1* and from *accC1* to *sfp*. As shown in Fig. 5, single bands of the expected size were obtained for genes from *fabZ1* to *accC1* and from *accC1* to *sfp*. This indicated that these 13 genes could form a cotranscribed unit that was controlled by the same promoter P_{fabZ1}. It is proposed that the *fab* gene cluster might

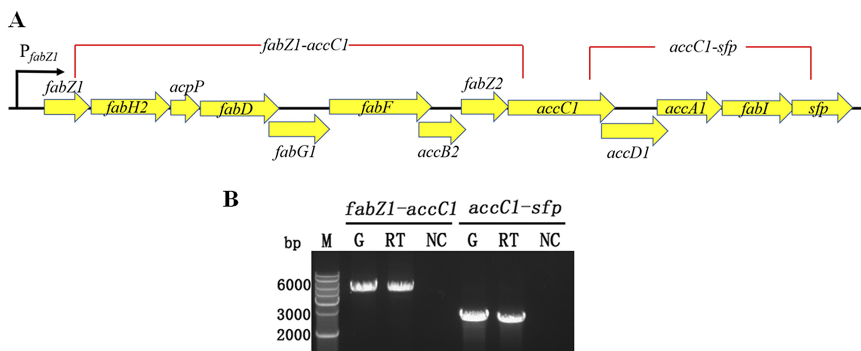

FIG 5 Organization and transcription unit of *fab* gene cluster in *L. plantarum* NF92. (A) Organization of the *fab* gene cluster in *L. plantarum* NF92. The gene cluster was divided into two parts (red lines) for RT-PCR to determine potential cotranscription. *fabZ1*, 3-hydroxyacyl-ACP dehydratase FabZ; *fabH2*, ketoacyl-ACP synthase III; *acpP*, acyl carrier protein; *fabD*, ACP *S*-malonyltransferase; *fabG1*, SDR family oxidoreductase; *fabF*, β-ketoacyl-[acyl-carrier-protein] synthase II; *accB2*, acetyl-CoA carboxylase biotin carboxyl carrier protein subunit; *fabZ2*, β-hydroxyacyl-ACP dehydratase; *accC1*, acetyl-CoA carboxylase biotin carboxylase subunit; *accD1*, acetyl-CoA carboxylase carboxyl transferase subunit beta; *accA1*, acetyl-CoA carboxylase carboxyl transferase subunit alpha; *fabI*, enoyl-[acyl-carrier-protein] reductase FabI; *sfp*, 4′-phosphopantetheinyl transferase superfamily protein. (B) RT-PCR for cotranscription analysis of genes in *fab* gene cluster. *fabZ1-accC1*, 5,354 bp; *accC1* to *sfp*, 2,610 bp. RNA was extracted from the WT strain. Lanes: G, positive controls with genomic DNA; RT, cDNA from the RNA sample; NC, negative controls consisting of DNase I-treated RNA sample; M, DNA marker.

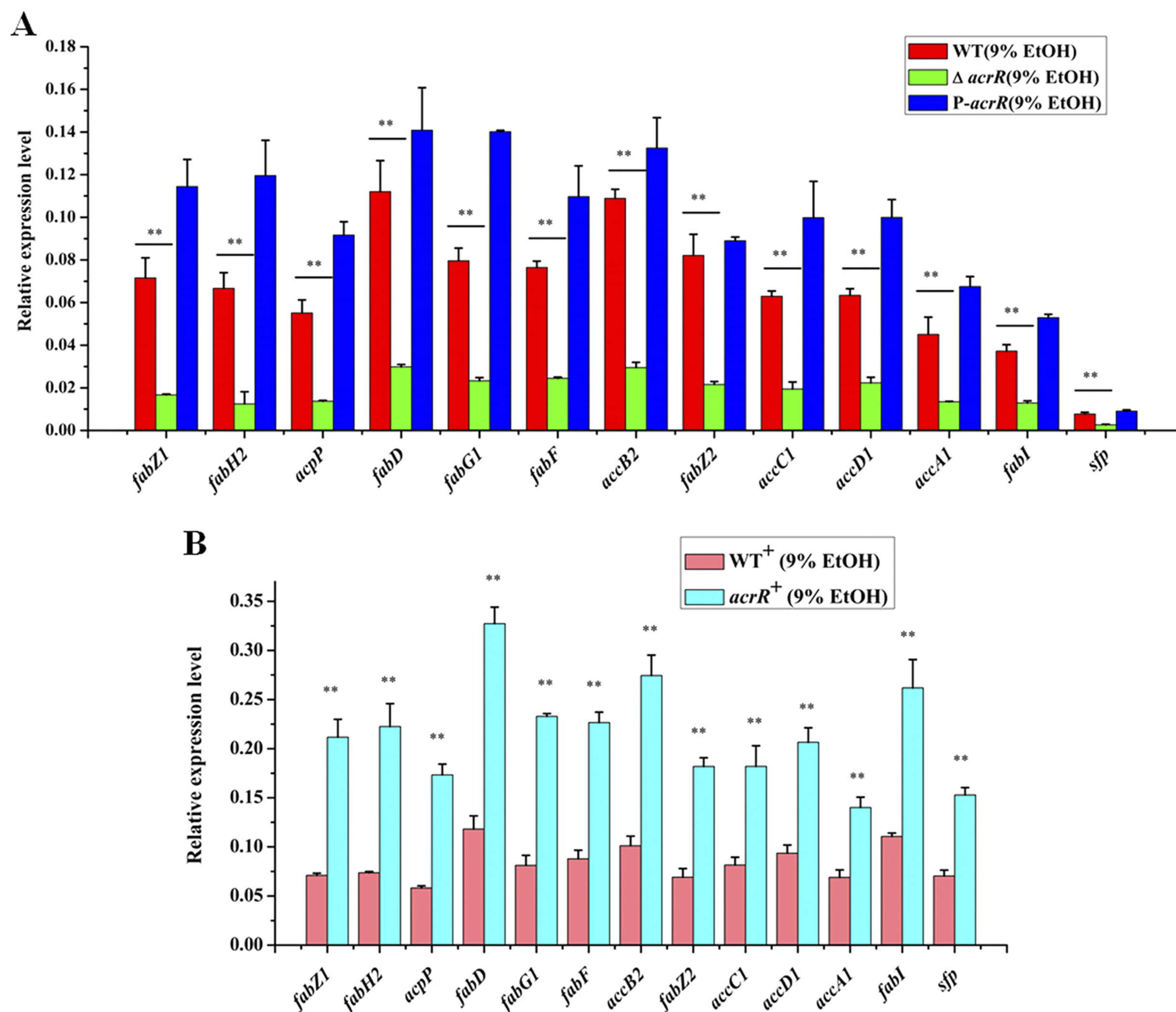

FIG 6 RT-qPCR transcriptional analysis of *fab* gene cluster in WT, Δ*acrR*, P-*acrR*, WT$^+$, and *acrR*$^+$ strains under ethanol stress. (A) Effects of *acrR* disruption on *fab* transcription in WT, Δ*acrR*, and P-*acrR* strains under 9% (vol/vol) ethanol stress. (B) Effects of *acrR* overexpression on *fab* transcription in WT$^+$ and *acrR*$^+$ strains under 9% (vol/vol) ethanol stress. Relative values were obtained using the transcription of *gyrB* as a reference. cDNA used as template in this experiment was diluted 1,000-fold. The *x* axis represents different genes. Values and standard deviations were calculated from three repeated samples. **, $P < 0.01$.

be regulated by AcrR during ethanol stress. RT-qPCR analysis was performed using total RNAs from WT, Δ*acrR*, P-*acrR*, WT$^+$, and *acrR*$^+$ strains. The results showed that the expression levels of these 13 genes were all significantly reduced in the *acrR* knockout strain (Δ*acrR*) and increased in the overexpression strain (*acrR*$^+$) compared to the wild type (Fig. 6). These results suggest AcrR is a positive regulator of *fab* gene cluster and may promote fatty acid *de novo* biosynthesis in *L. plantarum* NF92 under ethanol stress.

AcrR promotes fatty acid synthesis under ethanol stress. In order to test whether AcrR affect the fatty acid contents of *L. plantarum* NF92 under ethanol stress, fatty acid methyl ester (FAME) analysis was used to detect the fatty acid contents in WT, Δ*acrR*, and P-*acrR* strains grown in 9% (vol/vol) ethanol. Analysis of the fatty acid composition of *L. plantarum* NF92 during ethanol stress revealed that mainly ten kinds of fatty acids were detected, including saturated fatty acid (SFA; $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, and $C_{18:0}$), unsaturated fatty acid (UFA; $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$), and cyclopropane fatty acid (CFA; cyc$C_{17:0}$ and cyc$C_{19:0}$). Compared to the WT and complemented P-*acrR* strains, most of the fatty acids, especially $C_{18:1}$, were decreased in the *acrR* disruption strain. The relative content of total fatty acid was also significantly decreased in the Δ*acrR* strain (Fig. 7).

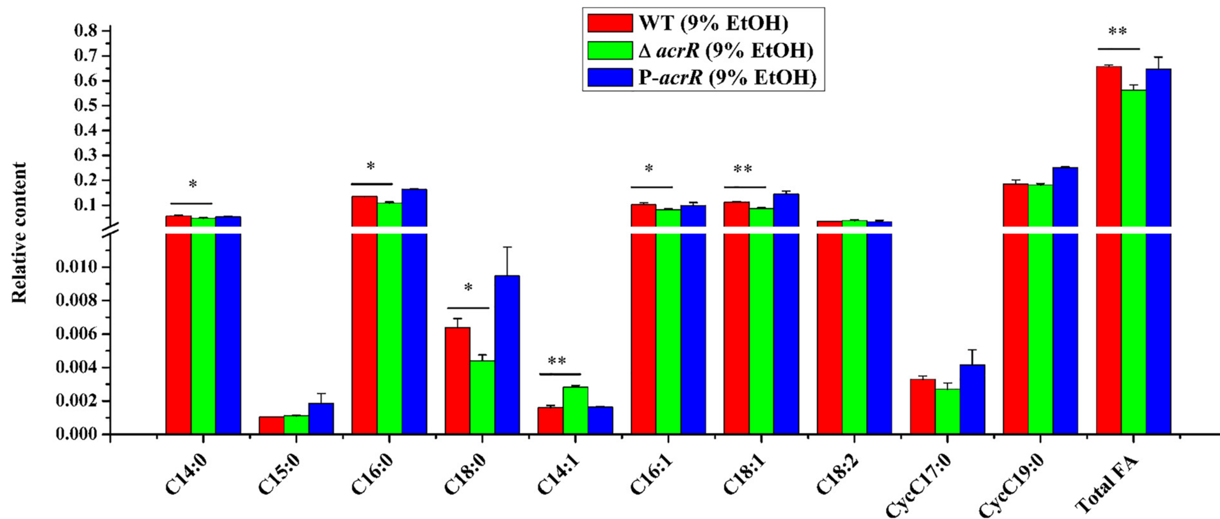

FIG 7 Effects of AcrR on composition and contents of fatty acids of *L. plantarum* NF92 under 9% (vol/vol) ethanol stress. Fatty acids of WT, Δ*acrR*, and P-*acrR* strains were determined in logarithmic cultures grown in MRS containing 9% (vol/vol) ethanol. The *y* axis represents the relative content of fatty acid per mg of dried cells. Values and standard deviations were calculated from three repeated samples. *, $P < 0.05$; **, $P < 0.01$.

This demonstrates that AcrR can promote fatty acid synthesis under ethanol stress, which is consistent with its influence on *fab* gene cluster.

AcrR increases membrane fluidity of *L. plantarum* NF92 under ethanol stress. Changes in fatty acids always influence cell membrane fluidity. We hypothesized that AcrR might be involved in regulating membrane fluidity of *L. plantarum* NF92 under ethanol stress. In order to elucidate the possible effects of AcrR on membrane-related alterations, membrane fluidity was evaluated by fluorescence anisotropy using TMA-DPH {1-[4 (trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene} as a probe. Samples were obtained from the exponential phase of WT, Δ*acrR*, P-*acrR*, WT$^+$, and *acrR*$^+$ strains under 9% (vol/vol) ethanol. Higher fluorescence anisotropy values were observed in the Δ*acrR* strain than in the WT and P-*acrR* strains (Fig. 8A), which meant that disruption of *acrR* reduced the membrane fluidity of *L. plantarum* NF92. Furthermore, the fluores-

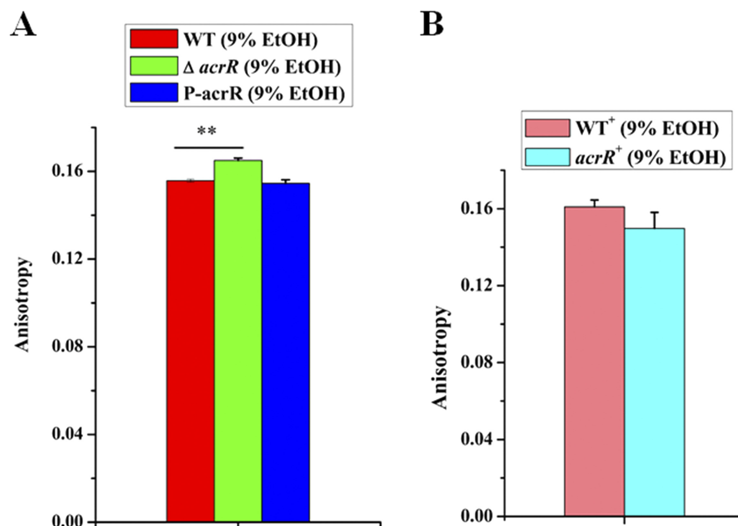

FIG 8 Effects of AcrR on membrane fluidity of *L. plantarum* NF92 under 9% (vol/vol) ethanol stress. (A) Effects of *acrR* disruption on membrane fluidity of *L. plantarum* NF92 under 9% (vol/vol) ethanol stress. (B) Effects of *acrR* overexpression on membrane fluidity of *L. plantarum* NF92 under 9% (vol/vol) ethanol stress. The *y* axis represents the fluorescence anisotropy. Values and standard deviations were calculated from three repeated samples. **, $P < 0.01$.

cence anisotropy values in the *acrR*$^+$ strain were lower than in the WT$^+$ strain (Fig. 8B), indicating that overexpression of *acrR* increased the membrane fluidity of *L. plantarum* NF92. These results demonstrate that AcrR may enhance membrane fluidity through upregulation of fatty acid biosynthesis in *L. plantarum* NF92 under ethanol stress.

DISCUSSION

AcrR, as a member of the TetR/AcrR family, was proved to participate in sorbitol or mannitol utilization in our previous report (37). In the present study, we found that AcrR could enhance the tolerance of *L. plantarum* NF92 to ethanol through regulating fatty acid *de novo* biosynthesis. We show a novel regulation mechanism of AcrR in *L. plantarum* that has not been reported.

Cell envelope acts as a physical barrier between the cell and its surroundings, and it is the first barrier of bacteria to defend environmental stress. The ability to adjust lipid composition to adapt to different environments is crucially important for bacterial survival. Fatty acids are central to microbial environmental adaptation. For example, *Bacillus* species are able to adapt to a wide range of habitat changes, including changes in the growth medium, temperature, and pH, by modifying their fatty acid patterns (40). *L. plantarum* also changes fatty acid contents to survive in harsh environmental conditions, including heat, acid, and ethanol (27, 41). It is essential for the cell to keep a tight regulation of fatty acid biosynthesis to efficiently respond to stresses. Regulation of fatty acid *de novo* synthesis has been studied in several species, such as *Escherichia coli*, *Bacillus subtilis*, mycobacteria, *Lactococcus lactis*, and *Streptococcus pneumoniae* (42–48). However, there is little report about the regulation of fatty acid synthesis in *Lactobacillus*. Here, we proved that AcrR could regulate fatty acid synthesis in *L. plantarum* NF92 under ethanol stress. In the previous reports, several regulators have been identified to regulate fatty acid synthesis, such as FabR in *E. coli*, FapR in *B. subtilis*, FabT in *Streptococcus pneumoniae*, and FasR in *Streptococcus coelicolor*, *Mycobacterium tuberculosis*, or *Corynebacterium glutamicum*. Among these regulators, FasR from *C. glutamicum*, FasR from *Mycobacterium tuberculosis*, and FabR from *E. coli* are all TetR family regulators. These findings indicate that several TetR family regulators are involved in regulating fatty acid synthesis, which supports our conclusion in *L. plantarum*. However, AcrR showed only 10.8, 12.61, and 13.19% identities with FasR from *C. glutamicum*, FasR from *Mycobacterium tuberculosis*, and FabR from *E. coli*, respectively. We analyzed the binding sites on the promoters that were recognized by AcrR, FasR, and FabR, and no conserved sequences were found among them (data not shown). Meanwhile, AcrR in *L. plantarum* NF92 could regulate all of the genes related to the type II fatty acid *de novo* biosynthesis pathway, whereas FabR from *E. coli* and FasR from *C. glutamicum* could only regulate parts of related genes. For example, FabR functions as a repressor controlling the expression of *fabA* (β-hydroxydecanoyl-ACP dehydratase) and *fabB* (β-ketoacyl-ACP synthase I) from *E. coli*. The function and regulation of AcrR in *L. plantarum* NF92 are different from the reported TetR family regulators involving in fatty acid synthesis.

Ethanol tolerance has been associated with high plasma membrane fluidity (49–51). Changes of fatty acids always lead to the fluidization response based on the hypothesis of "homeoviscous adaptation" (52). In our study, we found when *acrR* was knocked out, the CFA/SFA ratio and the CFA/UFA ratio were both significantly increased, and the UFA/SFA ratio was decreased (Table S2), which meant the membrane fluidity would be decreased. This was consistent with the membrane fluidity evaluation results that *acrR* knockout strain showed a lower membrane fluidity. Thus, AcrR could enhance membrane fluidity under ethanol stress. It has been reported that the increase of membrane fluidity may help to protect *Lactobacillus hilgardii* avoid rigidity disruption induced by ethanol (51). We proposed that in *L. plantarum* NF92, the membrane fluidity was increased through regulating fatty acid biosynthesis by AcrR to adapt to the ethanol stress. In addition, in the Δ*acrR* strain, the percentage of cyc$C_{19:0}$ was significantly increased, and $C_{18:1}$ was significantly decreased (Table S2). Both $C_{18:1}$ and cyc$C_{19:0}$ are important in overcoming the toxic effects of ethanol (53, 54). Methylenoctadecenoic

acid cyc$C_{19:0}$ could be synthesized *in situ* by the transfer of a methylene group from *S*-adenosyl-L-methionine to the double bond of unsaturated fatty acid by the Cfa synthase enzymes (55), whose transcriptional levels were increased in the Δ*acrR* strain under ethanol stress. AcrR may also have a central role in maintaining the balance between $C_{18:1}$ and cyc$C_{19:0}$ during ethanol stress.

TetR family regulators are known to regulate numerous aspects of bacterial physiology and some of them are global regulators (28). In our study, we found that AcrR could bind to promoters of various genes. In addition to the *fab* gene cluster, AcrR could regulate *murD*, *trmFO*, and *tagE6* in *L. plantarum* NF92 under ethanol stress. Peptidoglycan (PG) is the main component of the Gram-positive cell wall. MurD, which could catalyze the addition of D-Glu and help to form UDP-MurNAc-pentapeptide, has a central role in PG synthesis (38). RNA modification plays a key role in regulating the cellular response to various stressors (56). Methylation is one of the most common chemical modifications in RNA; for example, tRNA contains abundant methylated nucleotides. These methylated nucleotides can stabilize the L-shaped tRNA structure and improve molecular recognition. One such modification involves a conserved uridine at position 54 (U54) in the T-loop of tRNA, which is often modified to 5-methyluridine (m^5U; ribothymidine) by the folate-dependent tRNA methyltransferase TrmFO (57, 58). TagE6 is a glycosyltransferase, which is required for protein O-glycosylation. Protein glycosylation is a highly ubiquitous protein modification in nature and is considered to be one of the posttranslational modifications involved in protein stability, signaling and response to stress, adaptation to changing environments, regulation of toxic and damaged proteins, protein localization, and host-pathogen interactions (59, 60). In *L. plantarum* strains, protein glycosylation is a common feature, and the cell wall teichoic acid biosynthesis could also require the activity of specific TagE (teichoic acid glycosylation) proteins (61). In *L. plantarum* NF92 there is another glycosyltransferase-encoding gene, *tagE5*, located downstream of *tagE6*. Both TagE6 and TagE5 are required for the glycosylation of proteins, especially those O-glycosylated with *N*-acetylhexosamine, likely *N*-acetylglucosamine (GlcNAc) in *L. plantarum* WCFS1 (61). In *L. plantarum* NF92, the amino acid sequences of TagE6 and TagE5 showed identities of 99.81 and 99%, respectively, with those in *L. plantarum* WCFS1. They might also have a central role in protein glycosylation. Above all, in addition to regulating fatty acid synthesis, AcrR might participate in ethanol tolerance by promoting cell wall synthesis, enhancing tRNA stabilization and molecular recognition ability and influencing protein glycosylation. Much more additional research on the relationship of these genes with ethanol tolerance is needed.

Conclusion. AcrR is a TetR/AcrR family regulator that controls the expression of fatty acid synthesis in *L. plantarum* NF92. AcrR can bind to multiple gene promoters, among which the promoter of the *fab* gene cluster (P_{fabZ1}) shows the strongest ability to bind AcrR. All the genes on *fab* gene cluster are strongly regulated by AcrR in *L. plantarum* NF92 under ethanol stress. AcrR enhances fatty acid synthesis by directly binding to site I (TGCTAAACTGTGCCAGTTCGTTGACGGATTATGACCGCAAAAAA) and site II (CTGTAAAT TAATCTGCTGAAATCAATTGACA) of P_{fabZ1} in *L. plantarum* NF92 under ethanol stress. The adaption of *L. plantarum* NF92 to ethanol is mainly regulated by AcrR through promoting fatty acid synthesis and improving membrane fluidity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. plantarum* NF92 and its derivatives—the Δ*acrR* (the *acrR* knockout strain), P-*acrR* (the *acrR* complemented strain), WT^+ (the empty vector-containing strain), and *acrR*$^+$ (the *acrR* overexpressed strain) strains—were grown in De Man-Rogosa-Sharp (MRS) medium at 30°C. Chloramphenicol (20 μg/ml) was used for the WT^+ and *acrR*$^+$ strains. The growth of *L. plantarum* NF92 in MRS supplemented with ethanol was determined by measuring the cell optical density at 600 nm with a spectrophotometer (model 680; Bio-Rad). The AcrR *E. coli* expression strain (*acrR*) was grown in Luria broth (LB) with aeration and 50 μg/ml kanamycin at 37°C. All the bacterial strains used in this study were constructed as described in our previous study (37).

Protein expression and purification. The expression and purification of AcrR were conducted according to a previous report (37). Briefly, AcrR was induced and expressed using IPTG (isopropyl-β-D-thiogalactopyranoside), purified on an immobilized metal affinity chromatography Ni^{2+} column (GE

TABLE 1 Primers used in this study

Primer[a]	Function	Nucleotide sequence (5′–3′)
P(cfa1)-F	Cloning of P_{cfa1} for EMSA	GCTGAATAGTCTCGATGTCTGGCAACAGAC
P(cfa1)-R		CCTGAATTTGATTTTCTGGAAGATTGATTGTC
P(cfa2)-F	Cloning of P_{cfa2} for EMSA	ACAGCGATAATTACTGCAAACACGATC
P(cfa2)-R		GGTGTGGTAAAAGGTTTTTTCTAGCATTG
P(tagE6)-F	Cloning of P_{tagE6} for EMSA	GTTGTGTTGTTATTTCGATTGAATGGTGAT
P(tagE6)-R		TATTACTCGCTCCCTTACACGACGTTCTG
P(fabZ1)-F	Cloning of P_{fabZ1} for EMSA	GCGTGCATCTAAGCTAGTGACAGTTGAAC
P(fabZ1)-R		AGTTAGTCGCTCCTTTCACGGCGTTTG
P(trmFO)-F	Cloning of P_{trmFO} for EMSA	ATGAAAAAAAGGTCATCTACGGATGATCT
P(trmFO)-R		CCAGAATTCCTCTTTTCTTGTTGTCATACC
P(murD)-F	Cloning of P_{murD} for EMSA	CATTCTTGTCCGAGTTAGCTGTACATAATTG
P(murD)-R		AAACCAAGATCTCCCTATAATGTTTGTAGATG
QgyrB-F2	qRT-PCR analysis of *gyrB*	AGGTGGGACGCATGAAGAAG
QgyrB-R2		CCGTCATCCCTTCACGAACA
Q-0752-F	qRT-PCR analysis of *lp_0752*	ACCCATGGCTTTGGTGTTTTG
Q-0752-R		GCTTGCGGTTTAGCTTGAGT
Q-murA1-F	qRT-PCR analysis of *murA1*	CGCTAAAATTCGGGGTGCTG
Q-murA1-R		TCGAAGCAGCCAGGGATAGA
Q-3292-F	qRT-PCR analysis of *lp_3292*	CATCATCAATCGCGTTATCGGT
Q-3292-R		GCAACAGCCACATCGTTACC
Q-1233-F	qRT-PCR analysis of *lp_1233*	AGATTGGGCGATTCATCCGT
Q-1233-R		TCGACTTCTCGTGGCAATGG
Q-nrdH-F	qRT-PCR analysis of *nrdH*	GTTCCTCACGGCGCACAATA
Q-nrdH-R		TTTCAACGACCGGAACAGCA
Q-glyQ-F	qRT-PCR analysis of *glyQ*	GGAACGATGAGCCCCTACAC
Q-glyQ-R		GGGTTCTCACCATACCGACC
Q-potA-F	qRT-PCR analysis of *potA*	CCCACGATCAGGAAGAAGCC
Q-potA-R		GCCACGAAATGGTTGATCGG
Q-alr-F	qRT-PCR analysis of *alr*	GCGATTCTAGCGAAGGGACT
Q-alr-R		CGCCCTGCCATAAACTCGAT
Q-cfa1-F2	qRT-PCR analysis of *cfa1*	TTTCCCAGGTGGCTACGTTC
Q-cfa1-R2		CCCAGATTTCAGTCGTCCGT
Q-cfa2-F	qRT-PCR analysis of *cfa2*	AGGCATCCGTCGTTTGATGA
Q-cfa2-R		CAGCAAATTCCAACGCCACA
Q-ftsA-F	qRT-PCR analysis of *ftsA*	GACGGAAGCCGAATTTGACG
Q-ftsA-R		CCACGTTGTGCCATCACTTC
Q-trmFO-F	qRT-PCR analysis of *trmFO*	CAGCGGGTCAGCTTAAAACC
Q-trmFO-R		CGCGCTTGAATCTTGGTCTG
Q-murB-F	qRT-PCR analysis of *murB*	GCCGGGCGTGTTGATTATTG
Q-murB-R		TTTTGCGGAACTTGGCATCC
Q-fabZ1-F	qRT-PCR analysis of *fabZ1*	GGTCATTACGCGACGTTTGG
Q-fabZ1-R		AAGGCCCAGCGGTTAGAATC
Q-fabH2-F	qRT-PCR analysis of *fabH2*	CTGCGACGGCTTGTTTAGTG
Q-fabH2-R		TTTGGTATTGCCCACTGCGA
Q-fabH1-F	qRT-PCR analysis of *fabH1*	CGCGACAGGGTTATCAGCAA
Q-fabH1-R		TCAGTCTCCGCAACTATGCT
Q-accB1-F	qRT-PCR analysis of *accB1*	TCCTGCTAAGATGGTCACGC
Q-accB1-R		TAGTCGTTGGCCGTTCACTG
Q-tagE6-F	qRT-PCR analysis of *tagE6*	ACCCATGGCTTTGGTGTTTTG
Q-tagE6-R		GCTTGCGGTTTAGCTTGAGT
Q-fum-F	qRT-PCR analysis of *fum*	CCATCAAGACCCTAGCAGCC
Q-fum-R		GGGTTCGTTAGCCGGAATGT
Q-tmcA-F	qRT-PCR analysis of *tmcA*	TTCCAAAAGCCCACTCCGTT
Q-tmcA-R		GCGGTTATAGCGGTCTGGTT
Q-ribD-F	qRT-PCR analysis of *ribD*	GTGGCGACGTACCAAAATCC
Q-ribD-R		ACATCCACCTCAGCATGGTC
Q-lplA1-F	qRT-PCR analysis of *lplA1*	CTCGGTTGATGACGGCTTGA
Q-lplA1-R		TTTAGCGATGGCCTCATGGT
Q-lplA2-F	qRT-PCR analysis of *lplA2*	TTAACGCCCCCTGCATCATT
Q-lplA2-R		ATAAACCGCTCCACCACCAG
Q-racD-F	qRT-PCR analysis of *racD*	GACCGTTACCCATCGTGACC
Q-racD-R		TGCACATCCGCTTCCAATGA
Q-clpE1-F	qRT-PCR analysis of *clpE1*	GGTGAAGCTGGGGTTGGTAA
Q-clpE1-R		TGAACAAGGGAAGCCACGTC
Q-murD-F	qRT-PCR analysis of *murD*	CGCAAATCCTAGCGGGAGAG
Q-murD-R		AGCTTTACCGGCATTTGTGC

(Continued on next page)

TABLE 1 (Continued)

Primer[a]	Function	Nucleotide sequence (5′–3′)
Q-dak1A-F	qRT-PCR analysis of *dak1A*	GGAGTTGCTGGCACGATTTT
Q-dak1A-R		AGCAACCCCAATCGTGTGAA
Q-acpP-F	qRT-PCR analysis of *acpP*	ACGATGACAACGAACTTTACCG
Q-acpP-R		ATCACCAACCGTGGCTAACG
Q-fabD-F	qRT-PCR analysis of *fabD*	ACCCCCTTTAGCCAGGAAAC
Q-fabD-R		AAACTATGCCCCGGTCCAAC
Q-fabG1-F	qRT-PCR analysis of *fabG1*	GGCAAGCATTAACGGCTGAA
Q-fabG1-R		GAGTCCATCCAAGCGACCAA
Q-fabF-F	qRT-PCR analysis of *fabF*	GTATTGCAGGGTTTGCGTCC
Q-fabF-R		TAAGACTAACGTCGCACCCC
Q-accB2-F	qRT-PCR analysis of *accB2*	ACGGATACGGAAGCGATGAC
Q-accB2-R		TGTGAACGCGCTGAATTTGT
Q-FabZ2-F	qRT-PCR analysis of *fabZ2*	CCGTATCGTAGCGGTCCAAC
Q-FabZ2-R		GCCGAATCATCGTTCTTCCG
Q-accC1-F	qRT-PCR analysis of *accC1*	CCGGCGAAGCATATCGAAGT
Q-accC1-R		GACCGCACATGGACTTTCCT
Q-accD1-F	qRT-PCR analysis of *accD1*	TATGTTGAGCGAGCCACGAG
Q-accD1-R		CCAATAAGGTTTCCGCCCGT
Q-accA1-F	qRT-PCR analysis of *accA1*	TCATGGTGATCGTCAACGGG
Q-accA1-R		GCCAAAATGCCGAGCTTGAT
Q-FabI-F	qRT-PCR analysis of *fabI*	CAGTATTGCCTGGGGGTGTA
Q-FabI-R		CACAGGCAATTAACGGCACA
Q-sfp-F	qRT-PCR analysis of *sfp*	CGGTGCAAGATCGTTATCGG
Q-sfp-R		TCAAGAACCTCAACACGGCT
murD-Q-F	Transcriptional unit analysis of *murD*, *murG*, and *ftsQ* gene cluster	GCTCTAGAGATGAAGTCGGTTGAGCAGTATCGTA
murD-Q-R		AAAACTGCAGTCAATTATTCTGATTCTTGAACGG
tagE6-5-F	Transcriptional unit analysis of *tagE6* and *tagE5* gene cluster	GCTCTAGAGATGTATTATTTTGTGAATACCAGCATT
tagE6-5-R		AAAACTGCAGTCACTGAACAACCGGCTGCCACT
fabZ1-accC1-F	Transcriptional unit analysis of *fab* gene cluster	TCCAACATGGGAACGGTGAGTTGTCAG
fabZ1-accC1-R		CACTGAATAGACAGCGACCGCTTTAATCC
accC1-sfp-F		GTTCAAACGAACCGGATCTTCTTAGCAGG
accC1-sfp-R		TTGCTGAATTAATTGGCGACTCACGGT

[a]F, forward; R, reverse.

Healthcare Life Science), confirmed by SDS-PAGE, and quantified using a Pierce BCA protein assay kit (Thermo Scientific).

Screening genes that may be regulated by AcrR during ethanol tolerance. According to the microarray data of *L. plantarum* WCFS1 (21) and the peptidoglycan and wall teichoic acid biosynthesis in LAB (10, 38), 172 genes whose transcription was differently influenced by ethanol or may be related to stress response were chosen to screen the genes that might be regulated by AcrR under ethanol stress. The main screening methods and processes were as follows. For step 1, the promoters of these genes were deduced according to the functions and locations of these genes in the genome of *L. plantarum* NF92 and predicted by BDGP (http://www.fruitfly.org/seq_tools/promoter.html). An EMSA was used to detect the binding abilities of promoters of these genes to AcrR. A high binding ability was defined as promoters that could bind when the concentration of AcrR was ≤50 nM. For step 2, the transcriptional levels of genes whose promoters had a high affinity for AcrR were analyzed in WT, Δ*acrR*, and P-*acrR* strains under ethanol stress by RT-qPCR. For step 3, the transcriptional levels of the selected genes in step 2 were confirmed in WT$^+$ [containing an empty vector pMG36c(M)] and *acrR*$^+$ strains under ethanol stress by RT-qPCR. Genes that were differentially expressed in both steps 2 and 3 were considered to be regulated by AcrR under ethanol stress. EMSA and RT-qPCR were performed according to a previous report, with some modifications (37). As DNA probes, promoters of these genes were amplified by PCR from the genomic DNA of *L. plantarum* NF92. Total RNAs were extracted from the cells of *L. plantarum* NF92 and its derivative strains (Δ*acrR*, P-*acrR*, WT$^+$, and *acrR*$^+$), which were cultured to exponential phase in 9% (vol/vol) ethanol containing MRS broth in triplicate. cDNAs were synthesized by using a RevertAid first-strand cDNA synthesis kit (Thermo Scientific) and used as templates for the target genes and the internal control (i.e., the DNA gyrase B-encoding gene *gyrB*) (62). H_2O was used as a negative control. The primers used in this study are described in Table 1.

DNase I footprinting assays. DNase I footprinting assays were conducted according to a previous report (37), with some modifications. As a DNA probe, the spacer sequence between *fabZ1* and its upstream gene (P_{fabZ1}) was amplified by PCR using the fluorescently labeled primers FAM-P_{fabZ1}-F [5′-(6-FAM)-AGCGTTGCCGTTTAGGTTAAAAGTTATGGG-3′] and HEX–P_{fabZ1}-R [5′-(HEX)-AGTTAGTCGCTCCT TTCACGGCGTTTG-3′]. To confirm the binding sites, two binding sequences from P_{fabZ1} were inserted into the fragment of the 16S rRNA gene (F0), generating fragment 1 (F1) and fragment 2 (F2). Their binding abilities with AcrR were determined by EMSA.

RT-PCR. RT-PCR was performed to analyze transcription units using primers (see Table 1) designed to amplify across the intergenic regions of neighboring genes. RNA samples were extracted from cells of *L. plantarum* NF92. cDNA was used as the template for PCR amplification. Genomic DNA was used as the positive control to verify the amplicon size, and DNase I-treated total RNA was used as a negative control to rule out possible contamination of the RNA sample with genomic DNA.

FAME analysis. Preparation and analysis of FAMEs were performed according to a previous report (63), with some modifications. *L. plantarum* NF92 and its derivatives (the Δ*acrR* and P-*acrR* strains) were cultured in MRS with 9% (vol/vol) ethanol, independently. Exponential-phase cells were harvested and washed with phosphate-buffered saline twice. After freezing for 24 h at −80°C, the samples were immediately freeze-dried in a freeze dryer with a condenser temperature at −96°C and a chamber pressure of <0.20 hPa for 48 h. Portions (150 mg) of dried cells were saponified with 3 ml of 1 mol/liter NaOH-methanol (MeOH) at 70°C for 10 min and methylated with 6 ml of 10% H_2SO_4-MeOH at 70°C for 15 min, and then FAMEs were extracted by using *n*-hexane. A gas chromatograph (Agilent, 7890A) and a 5975C mass spectrometer with an HP-5 MS capillary column (30 m by 0.25 mm by 0.25-μm film) were used to analyze the FAMEs. Fatty acids were identified according to the NIST database. Relative quantification of each peak was performed by using benzoic acid as an internal standard (IS). For quantification, the area ratios of a protonated molecule of a given FAME versus the IS were calculated.

Measurement of membrane fluidity. The membrane fluidity of *L. plantarum* NF92 was investigated by fluorescence anisotropy as previously described (64), with some modifications. Briefly, cells from WT, Δ*acrR*, P-*acrR*, WT^+, and $acrR^+$ strains grown in MRS containing 9% (vol/vol) ethanol were harvested at exponential phase. The samples were incubated for 1 h at 37°C with TMA-DPH at a final concentration of 5 μM. The fluorescence anisotropy was measured at 37°C using an F-7000 spectrofluorometer (Hitachi, Tokyo, Japan) with excitation at 360 nm and emission at 430 nm using 5- and 5-nm slits, respectively, and a 3-s integration time. Anisotropy values (*r*) were calculated according to the following equation:

$$r = \frac{I_{VV} - I_{VH}(I_{HV}/I_{HH})}{I_{VV} + 2I_{VH}(I_{HV}/I_{HH})}$$

where *I* is the corrected fluorescence intensity and subscripts *V* and *H* indicate the values obtained with vertical or horizontal orientation, respectively, of the excitation polarizer and emission analyzer (in that order). In these experiments, decreases in the degree of fluorescence anisotropy reflected increases in the fluidity of the lipid bilayer, which controls or alters the mobility of TMA-DPH in the membrane.

Statistical analysis. Determinations were performed from three independent cultures of each bacterial strain. A one-way analysis of variance was performed by OriginPro 8.0 to test whether there were any significant differences in RT-qPCR results, fatty acid compositions, and membrane fluidities. If the *P* value was <0.05, the differences were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01690-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (grant 31570114), the Special Fund for Agro-scientific Research in the Public Interest (grant 201503134), and the Guangxi Major Science and Technology Project (grant AA18118041).

J. Zhong and K. Teng were involved in the conception and design of the study and manuscript revision. X. Yang drafted the manuscript. X. Yang, L. Li, R. Su, and J. Zhang were involved in the acquisition, analysis, and interpretation of the data. G. Ai analyzed the fatty acid methyl esters. All authors discussed the results and reviewed the manuscript.

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