The Arginine Repressor ArgR2 Controls Conjugated Linoleic Acid Biosynthesis by Activating the clα Operon in Lactiplantibacillus plantarum

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ABSTRACT CLA (conjugated linoleic acid) has attracted substantial attention due to its physiological functions, including regulating immunity, reducing obesity, and contributing to cancer suppression. In Lactiplantibacillus plantarum, CLA oleate hydratase (CLα-HY), CLA short-chain dehydrogenase (CLα-DH), and CLA acetoacetate decarboxylase (CLα-DC) catalyze the biotransformation of linoleic acid (LA) to CLA. However, the underlying transcriptional regulation mechanism of this pathway remains largely unknown. In this study, the potential transcriptional regulators that might bind to the clα promoter of L. plantarum AR195 were investigated by DNA pulldown. Interestingly, ArgR2, the transcriptional regulator of arginine metabolism, was identified as a potential regulator involved in the regulation of CLA biotransformation. Electrophoretic mobility shift assay (EMSA) and molecular interaction results demonstrated the specific binding of ArgR2 to the regulatory region of the clα operon. The knockout of argR2 led to the downregulation of clα-dh and clα-dc by 91% and 34%, respectively, resulting in a decline in the CLA yield by 14%. A segmental EMSA revealed that ArgR2 bound to three distinct sites in the clα regulatory region, and these binding sites were highly conserved and rich in AT. The regulatory mechanism of ArgR2 on CLA biosynthesis further expanded our knowledge of the regulatory mechanism of CLA biosynthesis in L. plantarum and laid the theoretical foundation for the production and application of CLA.

IMPORTANCE CLA (conjugated linoleic acid) has received extensive attention owing to its important physiological functions. CLA from natural sources is far from meeting people’s demands. Lactic acid bacteria can efficiently synthesize cis-9, trans-11-CLA and trans-10,cis-12-CLA, which possess physiological activities. However, little is known about the regulatory mechanism. In this study, we identified that the biosynthesis of CLA in L. plantarum AR195 was transcriptionally regulated by the arginine biosynthesis regulatory protein ArgR2. The regulation mechanism of ArgR2 on CLA biosynthesis lays a theoretical foundation for the regulation of CLA synthesis and industrial production.

KEYWORDS CLA, conjugated linoleic acid, Lactiplantibacillus plantarum, biosynthesis, ArgR2, arginine repressor, transcriptional regulation

CLA (conjugated linoleic acid) is the general term for a variety of octadecadienoic acid isomers containing conjugated double bonds. CLA has gained much attention for its various health-related physiological activities, including its anticancer properties, antiatherosclerosis properties, inhibition of inflammation, inhibition of obesity, inhibition of diabetes, and prevention of cardiovascular diseases (1–3). There has been sufficient evidence to prove that the physiological functions of CLA are attributed mainly to two isomers: cis-9,trans-11-CLA (c9,t11-CLA) and trans-10,cis-12-CLA (1, 4).
Rumen dairy products and meat products are the main sources of CLA in people's daily life. However, the content of CLA in these products is too low to have a physiological function. Moreover, there is a lack of research on its biosynthesis and regulation mechanisms. The typical microorganisms with CLA biosynthesis capabilities include rumen bacteria, *Propionibacterium*, and *Lactobacillus* (1). Recently, an increasing number of studies have focused on CLA biosynthesis by *Lactobacillus*. The main reasons include the following. First, compared with rumen bacteria and *Propionibacterium*, *Lactobacillus* has the advantages of being safe and amenable to easy large-scale culture (5). Functional foods including CLA produced by *Lactobacillus* are more acceptable to people. Second, *Lactobacillus* can synthesize e9,t11-CLA and r10,c12-CLA with higher isomer selectivity. The CLA synthesized by *Lactobacillus* has broad application prospects in the food industry due to its specific functions.

The lactobacilli with CLA biotransformation abilities cover almost all species and genera of lactic acid bacteria, including *Lactiplantibacillus plantarum* (6–8), *Lactobacillus acidophilus* (9), *Lactobacillus pentosus* (10), *Lactobacillus reuteri* (11), *Lactococcus lactis* (12), and *Bifidobacterium* (13), etc. CLA isomerase is considered the key enzyme in CLA biosynthesis. Rosson et al. heterologously expressed CLA isomerase of *Lactobacillus reuteri* ATCC 55739. However, no CLA was detected (14). Furthermore, hydroxyl fatty acid was identified as the intermediate product during CLA bioconversion in *L. plantarum* AKU 1137, demonstrating that the biotransformation of CLA from linoleic acid (LA) in *Lactobacillus* was not a one-step isomerization (15). After that, Kishino et al. identified the genes encoding the enzymes involved in CLA biotransformation in *L. plantarum* AKU 1009a and revealed the synthetic pathway in detail (16).

In *L. plantarum*, linoleic acid was first hydrated to 10-hydroxy-cis12-octadecenoic acid (10-HOE) under the catalysis of CLA oleate hydratase (CLA-HY) (hydration/dehydration). After dehydration, double-bond shift, hydration, and dehydration reactions, 10-HOE was finally transformed into CLA. The enzymes involved in this process included CLA-HY, CLA short-chain dehydrogenase (CLA-DH), and CLA acetooacetate decarboxylase (CLA-DC) (16). Yang et al. also verified this pathway in *L. plantarum* ZS2058 based on cre-lox gene-editing technology (17, 18). Moreover, our previous work had proven that strains with different CLA biosynthesis abilities possessed different transcriptional levels of *cla-hy*, *cla-dh*, and *cla-dc*, suggesting that the upregulation of the CLA yield may be achieved by regulating the transcription of these genes (19). Except for *LpLtrR*, the LysR-type transcriptional regulator of *L. plantarum* WCFS1 (20), we speculated that there may be other regulators involved in CLA synthesis.

In this study, we found that ArgR2 bound to the regulatory region of the *cla* operon in *L. plantarum* AR195. The knockout of *argR2*, showed no obvious effect on bacterial growth, but it showed an inhibitory effect on CLA biosynthesis. The regulation of CLA biotransformation by ArgR2 was achieved by binding to three different sites on the regulatory region of the *cla* operon. This study broadened the understanding of ArgR2 in *L. plantarum* and provided a theoretical basis for the regulation and production of CLA.

**RESULTS**

ArgR2 was the potential regulator of CLA biosynthesis. In our previous study, *L. plantarum* AR195 showed the highest CLA biotransformation capability among the *L. plantarum* strains in our laboratory stock (19). To investigate the biosynthesis and regulation mechanisms of CLA in *L. plantarum* AR195, the detailed genome was sequenced. The genome size was 5.28 Mb, and it also contained multiple plasmids. The genome length is 3,219,240 bp, and 3,235 coding genes were predicted. The genes encoding CLA-HY, CLA-DH, and CLA-DC in *L. plantarum* AR195 are AR0148, AR0080, and AR0081, respectively. Amplification of the overlapping regions was performed to investigate the gene structure and verify the promoter. RNA and cDNA were used as the templates for PCR amplification, respectively, and the genomic DNA was used as a control. As shown in Fig. 1A, *cla-dh* and *cla-dc* were located in one transcriptional unit, which was similar to that in *L. plantarum* WCFS1. Moreover, *cla-er* (AR0082) was also located in this operon.
To explore the potential transcriptional regulators that might regulate CLA biotransformation in *L. plantarum* AR195, a DNA pulldown experiment was carried out. Interestingly, an arginine metabolism repressor, ArgR2, was captured, indicating that ArgR2 was most likely the potential regulator of CLA biosynthesis. cis-element analysis of the *cla* operon was also performed using the Softberry website (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). A potential ArgR binding box was found in the *cla* promoter at a distance of 12 bp from the -35 region (Fig. 1B). According to previous reports, ArgR2 mainly regulated the expression of genes related to arginine metabolism, glutamic acid metabolism, lysine metabolism, purine and pyrimidine synthesis, and cell morphology. It has not been reported that it participated in fatty acid metabolism, especially the biosynthesis of CLA. To investigate the function of ArgR2, argR2 heterologous expression and knockout mutant strains were constructed.

**Heterologous expression of ArgR2.** ArgR2 of *L. plantarum* AR195 contains 459 bases, encoding 152 amino acids. The molecular weight was 17.5 kDa, predicted by the amino acid sequence using ExPASY (https://web.expasy.org/compute_pi/). The positive clones were screened by colony PCR and sequence verification (Fig. 2A). The positive colonies (Fig. 2A, lane 4) were used for protein expression. As shown in Fig. 2B, ArgR2 was successfully expressed as a soluble product. The molecular weight of ArgR2 matched well with the theoretical molecular weight, and protein purification was successful, without further contamination.

**ArgR2 bound to the promoter of the cla operon.** To investigate whether ArgR2 bound to the regulatory region of the *cla* operon in vitro, electrophoretic mobility shift assays (EMSAs) and Octet experiments were performed. As shown in Fig. 3A, a DNA-protein complex band was detected when ArgR2 was incubated with the biotin-labeled probes. Moreover, the binding band showed a concentration-dependent increase. When the protein reached 0.085 mg/mL, binding was saturated. Binding was further verified by an Octet experiment. The ArgR2-*cla* promoter interaction showed that the affinity coefficient (Kd) was less than 1.0 pM (Fig. 3B). These results indicated that ArgR2 stably
bound to the cla promoter, suggesting that ArgR2 might be involved in the regulation of CLA biotransformation.

**Construction of the argR2 knockout strain.** The argR2 knockout strain was necessary to investigate the regulatory effect of ArgR2 on CLA biotransformation. Therefore, we constructed an argR2 knockout strain of L. plantarum AR195, ΔargR2, using the CRISPR-Cas9 system (Fig. 4A). According to previous studies, ArgR2 is involved in nitrogen metabolism, purine synthesis, pyrimidine synthesis, cell morphology, and so on. The effect of the argR2 mutation on the growth of L. plantarum AR195 was investigated in this study. As shown in Fig. 4B, the deletion of argR2 slowed bacterial growth in the first 24 h, but the final biomass was close to that when bacteria were cultured in MRS broth without LA. However, when the medium was supplemented with LA, the growth ability of the knockout strain (ΔargR2) was suppressed to a modest degree, indicating that ArgR2 played a role in the biotransformation of CLA from LA.

**ArgR2 controls CLA biotransformation by regulating the transcription of the cla operon.** To investigate the effect of ArgR2 on CLA biosynthesis, the levels of CLA production in the wild-type (WT) and ΔargR2 strains were determined. As shown in Fig. 5A, the knockout of argR2 decreased CLA production from 107.3 to 92.4 mg/mL, reducing the CLA yield by 14%. We speculated that the inhibitory effect of argR2 on the CLA yield might be achieved by regulating the expression of CLA synthesis genes. To test this hypothesis, the transcriptional levels of CLA synthesis genes in the WT and ΔargR2 strains were examined. RNA was isolated when the cells were grown to the
mid-exponential phase. Compared with those in the WT, the knockout of argR2 decreased the transcription levels of cla-dh and cla-dc by 91% and 34%, respectively (Fig. 5B), suggesting that ArgR2 regulated the biosynthesis of CLA by positively regulating the transcription of cla-dh and cla-dc.

Identification of the regulatory site. ArgR was involved in the metabolism of l-arginine, which usually bound to a 16- to 20-bp conserved palindrome sequence (named the ARG box) in the promoter of the arginine biosynthetic genes. ArgR inhibited the expression of these genes, thereby regulating the arginine concentration. As shown in Fig. 6A, the ARG box is composed of 18 bases, and the AT content is relatively high (https://regprecise.lbl.gov/sites.jsp?regulog_id=4344). According to the conserved motif, two typical ARG boxes, ArgR2 box 2 (ArgR2-2) and ArgR2-3, were identified within the regulatory region of the cla operon in addition to ArgR2-1 predicted by Softberry (Fig. 6B).

To identify the precise binding site of ArgR2, EMSAs were performed using purified His-ArgR2 with three biotin-labeled probes (P1, P2, and P3) containing the predicted ArgR2 binding boxes separately (Fig. 6B). As shown in Fig. 6C, all three biotin-labeled probes contributed to binding to His-ArgR2, and P1 possessed the strongest binding sites. To further verify the potential binding sequence, the key sites were mutated. The conserved T was mutated to C, and A was mutated to G. The results showed that the mutated probe still bound to ArgR2 (data not shown). However, under the same conditions, the mutational probes P1* and P3* showed a reduction in binding, while muta-
tion of the second site showed no significant difference, suggesting that the ArgR-1 and ArgR-3 boxes might be the main binding sites of ArgR2.

**DISCUSSION**

CLA has gained much attention due to its important physiological functions. But little is known about its biosynthesis and regulation mechanisms. In this study, we found that the arginine repressor ArgR2 bound to the promoter of the *cla* operon and regulated the biosynthesis of CLA in *L. plantarum* AR195. The deletion of *argR2* inhibited the transcription of *cla-dh* and *cla-dc*, thereby reducing the CLA yield. Through cis-element analysis and segmental EMSAs, three AT-rich ArgR2 binding boxes were identified. Among them, the ArgR-1 and ArgR-3 boxes played a major role in this regulation. Our previous work showed that LttR positively regulates CLA production in *L. plantarum* WCFS1. Taking this one step further, the current study focused on *L. plantarum* AR195 (the high-CLA-producing strain screened in our laboratory bank) and found that in addition to LttR, ArgR2 also positively regulated the transcription of *cla-dh* and *cla-dc*. This work demonstrated that ArgR2 mediated the interplay between fatty acid metabolism and amino acid metabolism and suggested that there were fine regulatory mechanisms during CLA biosynthesis (Fig. 7).

ArgR is a hexamer protein that can inhibit the transcription of arginine biosynthesis-related genes. ArgR in *Escherichia coli* has been studied thoroughly: an "SR" sequence in the N-terminal domain participates in DNA binding, and a conserved sequence, “GTIAGDDTL/I,” at the C terminus is considered to be the arginine binding domain (21–23). The binding of ArgR and ArgR boxes in the DNA regulatory region caused topological and structural changes in genes, thus regulating the transcription of target genes.

**FIG 6** Identification of the binding site of ArgR2. (A) ArgR binding motif predicted by regprecise. (B) Putative binding sites of ArgR2. P1, indicated by the blue underlining, contains the predicted ArgR2 binding box 1 (ArgR2-1). P2, indicated by the red underlining, contains the predicted ArgR2 binding box 2 (ArgR2-2). P3, indicated by the green underlining, contains the predicted ArgR2 binding box 3 (ArgR3-3). (C) EMSAs of His-ArgR2 with biotin-labeled P1, P2, and P3. S represents the negative control using the unlabeled probe.
addition to regulating arginine synthesis, ArgR is involved in the regulation of nitrogen metabolism, purine and pyrimidine biosynthesis, cell morphology, and antibiotic biosynthesis in *Streptomyces coelicolor* (24). In this study, ArgR₂ of *L. plantarum* acted as a transcriptional activator promoting CLA biosynthesis, suggesting possible cross talk between arginine and fatty acid metabolisms mediated by ArgR₂ (Fig. 7).

This study furthers our understanding of CLA biotransformation in *L. plantarum* and broadens our knowledge of ArgR. It also lays a theoretical foundation for the biosynthesis and regulation of CLA.

**MATERIALS AND METHODS**

**Strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *E. coli* and its derived strains were cultured in Luria-Bertani (LB) medium at 37°C at 200 rpm. The seed activation of *L. plantarum* AR195 and the derived strains were cultured at 37°C on MRS medium agar plates under anaerobic conditions. The activated colonies were inoculated into MRS liquid medium containing LA for CLA biotransformation as described previously (20).

**Genome sequencing.** The genome of *L. plantarum* AR195 was sequenced using high-throughput sequencing technology. The genome sequence was assembled from scratch by means of bioinformatics. The complete genomic sequence was sequenced by the second- and third-generation Illumina HiSeq and PacBio platforms. Genome sequencing and analysis were performed by Shanghai Majorbio Biopharm Technology Co. Ltd. (Shanghai, China).

**TABLE 1 Strains and plasmids used in this study**

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **Strains**       |             |                     |
| *E. coli* BL21(DE3) | Protein expression host | Novagen |
| *L. plantarum* AR195 | Wild-type strain | 19 |
| *L. plantarum* AR195 ΔargR₂ | argR₂ knockout strain | This study |
| *L. plantarum* AR195/pIB184-argR₂ | argR₂ overexpression strain | This study |
| **Plasmids**      |             |                     |
| pET30a            | Protein expression vector | Novagen |
| pET30a-argR₂      | ArgR₂ expression recombinant vector | This study |
| pLCNICK-0537      | Knockout plasmid | 26 |
| pLCNICK-argR₂      | Used for argR₂ knockout | This study |
| pIB184            | Overexpression plasmid | 20 |
| pIB184-argR₂      | Used for argR₂ overexpression | This study |

**FIG 7** The transcriptional regulatory mechanisms of CLA biosynthesis in *L. plantarum*. 
TABLE 2 Primers used in this study

| Primer | Sequence |
|--------|----------|
| pET30a-argR2-S | 5'-CGGCTACGGAGGCTTAAAGTG-3' |
| pET30a-argR2-A | 5'-CGGCTACTGGCTGTTAATAGTC-3' |
| PET-YZ-S | 5'-CATCATTCTCTGAGCTGGTTG-3' |
| PET-YZ-A | 5'-ACCCCTCAAGACCGCTTTAG-3' |
| PIB184-argR2-S | 5'-ATGCAATAAGATGGATTGGACGAAAGCAAGGGC-3' |
| PIB184-argR2-A | 5'-GCTTATCGAGGCTGACCTGCTGTTTATTGAGCAGCTGTAAGACGATT-3' |
| PIB-YZ-S | 5'-GAAAGGCGGAAAGGCGGCT-3' |
| PIB-YZ-A | 5'-GCGAGTAGGCGAAAGGCGGCT-3' |
| argR2-up-S | 5'-CTTTTTCTAAACTGAGGCCTAAGCAGTTGTTATAGGCTGTAAGACGATT-3' |
| argR2-up-A | 5'-GCGAGTAGGCGAAAGGCGGCT-3' |
| argR2-down-S | 5'-TAGCTACCGGGGGGAAGCTGTTAAGCTTACCTGACCTG-3' |
| argR2-down-A | 5'-ACCGAGTAGGCGTCTCTCTCAAGGATTTTCTGCTGAACTGCT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| PIB-YZ-S | 5'-AAGAGTAGAATTCTTATCG-3' |
| PIB-YZ-A | 5'-AAGAGTAGAATTCTTATCG-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| PIB-YZ-S | 5'-AAGAGTAGAATTCTTATCG-3' |
| PIB-YZ-A | 5'-AAGAGTAGAATTCTTATCG-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| PIB-YZ-S | 5'-AAGAGTAGAATTCTTATCG-3' |
| PIB-YZ-A | 5'-AAGAGTAGAATTCTTATCG-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |
| argR2-sgRNA-S | 5'-TTTGGCAAAATGTAGCAGAAGGAAAAAGACCGGACTCGA3' |
| argR2-sgRNA-A | 5'-ATAGCTATGATAATATCGTACTATGCTCGAAGACTGCTTATTAGAGCAGCTGTAAGACGATT-3' |

DNA pulldown. The promoter regions of the cla operon of L. plantarum AR195 were amplified by PCR using the primer pair qd-0080-A/S listed in Table 2. The PCR products were labeled with biotin using a universal biotin-labeled primer (5'-biotin-AGCCAGTGGCGATAAG-3'). The biotin-labeled DNAs were bound to streptavidin beads for affinity chromatography. The L. plantarum AR195 genomic DNA was amplified by PCR using primers (pET30a-argR2-S/A) listed in Table 2. The purified argR2 genes were inserted into pET30a digested by EcoRI and KpnI, generating the recombinant vector. For ArgR2 expression, the recombinant plasmids were extracted from the transformant and verified by PCR using the primer pair PET-YZ-S/A. The positive plasmids verified by PCR and sequencing were introduced into the expression host, E. coli BL21(DE3). Colony PCR was performed using a 2x colony PCR mixture, and the products were detected by agarose gel electrophoresis. Protein expression and purification were performed as described previously (25).

EMSA. The biotin-labeled cla regulatory regions were amplified by two-step PCRs, the same as that described above for the DNA pulldown assay. The quality and concentration of the probes were determined by agarose gel electrophoresis and by using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). EMSAs were carried out using a chemiluminescent EMSA kit (Beyotime Biotechnology, China) as described previously (25).

Octet. The preparation of biotin-labeled probes used for Octet analysis was the same as that used for EMSAs. The biotin-labeled probes were dissolved in BSA containing 10 mmol/L HEPES, 2 mmol/L MgCl2, 0.1 mmol/L EDTA, and 200 mmol/L KCl (pH 8.0). Different concentrations of ArgR2 were dissolved...
in buffer B containing 1% (v/v) bovine serum albumin (BSA) and 2% (v/v) Tween 20; the other components were the same as those in buffer A. The samples were added to the detection plate and determined by Octet analysis based on the theory of biolayer interferometry (BLI). The determination procedure was as follows: balancing in buffer A for 10 min, loading of DNA probes for 10 min, balancing in buffer B until the baseline was flat, association with protein for 10 min, and dissociation in buffer B for 10 min (25).

**Mutant strain construction.** The argR<sup>2</sup> deletion strain of *L. plantarum* was constructed using CRISPR-Cas9 gene-editing technology. The upstream and downstream homologous arms of argR<sup>2</sup> and the single guide RNA (sgRNA) were inserted into pLCNICK-0537, which was digested by XbaI and Apal (Thermo Fisher Scientific, USA) (26). The positive recombinant knockout plasmid pLCNICK-argR<sup>2</sup> was verified by colony PCR and sequencing. The upstream verification primers were designed at the upstream 249 bp of the upper homologous arm, and the downstream primer designs at the lower homologous arm. The expected PCR product length of wild bacteria is 2.907 bp. If argR<sup>2</sup> is successfully knocked out, the length should be 2,448 bp. The positive recombinant plasmids were introduced into *L. plantarum* competent cells (20).

**CLA determination.** CLA has a special absorption peak at 233 nm, while linoleic acid does not. According to the optical properties of CLA, a UV spectrophotometer was used to determine the CLA concentration. Different concentrations of a CLA standard were dissolved in n-hexane, and a standard curve was drawn according to the absorbance at 233 nm. Two milliliters of isopropanol and 1.5 mL of anhydrous sodium sulfate. Next, it was dissolved in 5 mL n-hexane for UV absorption detection. The concentration of CLA was calculated according to the standard curve.

**qPCR.** The primers used for quantitative PCR (qPCR) are listed in Table 2. The strains were harvested for RNA purification at the exponential phase. The WT strains were sampled at the 6th hour, and the mutant bacteria were sampled at the 12th hour. Cell culture and RNA extraction were performed using a total RNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions. DNase digestion was introduced to remove the genomic DNA. The cDNA was prepared and analyzed by PCR, and the protein was quantified by BLI. The concentration of CLA was calculated according to the standard curve.

**Data availability.** The FASTQ format raw data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) database (accession no. PRJNA765134).

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**REFERENCES**

1. Yang B, Gao H, Stanton C, Ross RP, Zhang H, Chen YQ, Chen H, Chen W. 2017. Bacterial conjugated linoleic acid production and their applications. Prog Lipid Res 68:26–36. https://doi.org/10.1016/j.plipres.2017.09.002

2. Liu Y, Xiao W, Yu L, Tian F, Wang G, Lu W, Narbad A, Chen W, Zhai Q. 2021. Evidence from comparative genomic analyses indicating that *Lactobacillus*-mediated irritable bowel syndrome alleviation is mediated by conjugated linoleic acid synthesis. Food Funct 12:1121–1134. https://doi.org/10.1039/d0fo02616f.

3. Rojas MM, Villalpando DM, Alexander-Aguilera A, Ferrer M, Garcia HS. 2021. Effect of CLA supplementation on factors related to vascular dysfunction in arteries of orchidectomized rats. Prostaglandins Other Lipid Mediat 157:106586. https://doi.org/10.1016/j.prostaglandins.2021.106586.

4. Salsinha AS, Pimentel LL, Fontes AL, Gomes AM, Rodriguez-Alcala LM. 2018. Microbial production of conjugated linoleic acid and conjugated linoleic acid relies on a multienzymatic system. Microbiol Mol Biol Rev 82:e00019-18. https://doi.org/10.1099/mmb.0.00019-18.

5. Wang G, Huang W, Xia Y, Xiong Z, Ai L. 2019. Cholesterol-lowering potential of *Lactobacillus* strain overexpression of bile salt hydrolase on high cholesterol diet-induced hypercholesteremic mice. Food Funct 10:1684–1695. https://doi.org/10.1039/c8fo02181c.

6. Liu P, Shen S-R, Ruan H, Zhou Q, Ma L-L, He G-Q. 2011. Production of conjugated linoleic acids by *Lactobacillus plantarum* strains isolated from naturally fermented Chinese pickles. J Zhejiang Univ Sci B 12:923–930. https://doi.org/10.1016/j.jzus.2011.09.007.

7. Kishino S, Ogawa J, Omura Y, Matsumura K, Shimizu S. 2002. Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. J Am Oil Chem Soc 79:159–163. https://doi.org/10.1007/s11746-002-0451-4.

8. Zeng Z, Lin J, Gong D. 2009. Identification of lactic acid bacterial strains with high conjugated linoleic acid-producing ability from natural sauerkraut fermentations. J Food Sci 74:M154–M158. https://doi.org/10.1111/j.1750-3841.2009.01123.x.

9. Lin TY, Lin C-W, Lee C-H. 1999. Conjugated linoleic acid concentration as affected by lactic cultures and additives. Food Chem 67:1–5. https://doi.org/10.1016/S0308-8146(99)00077-1.

10. Gurovic MSV, Gentili AR, Olivera NL, Rodriguez MS. 2014. Lactic acid bacteria isolated from fish gut produce conjugated linoleic acid without the addition of exogenous substrate. Process Biochem 49:1071–1077. https://doi.org/10.1016/j.procbio.2014.04.004.

11. Yang B, Chen H, Gu Z, Tian F, Ross RP, Stanton C, Chen YQ, Chen W, Zhang H. 2014. Synthesis of conjugated linoleic acid by the linoleate isomerase complex in food-derived lactic acid bacteria. J Appl Microbiol 117:430–439. https://doi.org/10.1111/j.1365-2621.2009.03392.x.

12. Kim YJ, Liu RH. 2002. Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. J Food Sci 67:1731–1737. https://doi.org/10.1111/j.1365-2621.2002.tb08714.x.
13. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. 2003. Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. J Appl Microbiol 94:138–145. https://doi.org/10.1046/j.1365-2672.2003.01814.x.

14. Rosson RA, Deng M-D, Grund AD. March 2004. Polynucleotide encoding a propionibacterium linolate isomerase and uses thereof. US patent 6,743,609.

15. Ogawa J, Matsumura K, Kishino S, Omura Y, Shimizu S. 2001. Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by Lactobacillus acidophilus. Appl Environ Microbiol 67:1246–1252. https://doi.org/10.1128/AEM.67.3.1246-1252.2001.

16. Kishino S, Takeuchi M, Park S-B, Hirata A, Kitamura N, Kunisawa J, Kiyono H, Ishimoto R, Isobe Y, Arita M, Ueda K, Shima J, Takahashi S, Yokozeiki K, Shimizu S, Ogawa J. 2013. Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. Proc Natl Acad Sci U S A 110:17808–17813. https://doi.org/10.1073/pnas.1312937110.

17. Yang B, Chen H, Gao H, Ren Q, Zhang H, Chen W. 2020. Genetic determines for conjugated linolenic acid production in Lactobacillus plantarum ZS2058. J Appl Microbiol 128:191–201. https://doi.org/10.1111/jam.14466.

18. Yang B, Qi H, Gu Z, Zhang H, Chen H, Chen YQ. 2017. Characterization of the triple-component linoleic acid isomerase in Lactobacillus plantarum ZS2058 by genetic manipulation. J Appl Microbiol 123:1263–1273. https://doi.org/10.1111/jam.13570.

19. Liu X-X, Zhang H-Y, Song X, Yang Y, Xiong Z-Q, Xia Y-J, Ai L-Z. 2021. Reasons for the differences in biotransformation of conjugated linoleic acid by Lactobacillus plantarum. J Dairy Sci 104:11466–11473. https://doi.org/10.3168/jds.2021-20532.

20. Liu X-X, Xiong Z-Q, Wang G-Q, Wang L-F, Xia Y-J, Song X, Ai L-Z. 2021. LysR family regulator LttR controls production of conjugated linoleic acid in Lactobacillus plantarum by directly activating the cla operon. Appl Environ Microbiol 87:e02798-20. https://doi.org/10.1128/AEM.02798-20.

21. Cho S, Cho YB, Kang TJ, Kim SC, Palsson B, Cho BK. 2015. The architecture of ArgR-DNA complexes at the genome-scale in Escherichia coli. Nucleic Acids Res 43:3079–3088. https://doi.org/10.1093/nar/gkv150.

22. Tian G, Maas WK. 1994. Mutational analysis of the arginine repressor of Escherichia coli. Mol Microbiol 13:599–608. https://doi.org/10.1111/j.1365-2958.1994.tb00454.x.

23. Burke M, Merican AF, Sherratt DJ. 1994. Mutant Escherichia coli arginine repressor proteins that fail to bind L-arginine, yet retain the ability to bind their normal DNA-binding sites. Mol Microbiol 13:609–618. https://doi.org/10.1111/j.1365-2958.1994.tb00455.x.

24. Pérez-Redondo R, Rodriguez-Garcia A, Botas A, Santamarta I, Martin JF, Liras P. 2012. ArgR of Streptomyces coelicolor is a versatile regulator. PLoS One 7:e32697. https://doi.org/10.1371/journal.pone.0032697.

25. Liu XX, Shen MJ, Liu WB, Ye BC. 2018. GlnR-mediated regulation of short-chain fatty acid assimilation in Mycobacterium smegmatis. Front Microbiol 9:1311. https://doi.org/10.3389/fmicb.2018.01311.

26. Huang H, Song X, Yang S. 2019. Development of a RecET/T-assisted CRISPR-Cas9 toolbox for Lactobacillus. Biotechnol J 14:e1800690. https://doi.org/10.1002/biot.201800690.