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Transcriptional Regulation of the Citrate Gene Cluster of *Enterococcus faecalis* Involves the GntR Family Transcriptional Activator CitO▽†

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The genome of the gram-positive bacterium *Enterococcus faecalis* contains the genes that encode the citrate lyase complex. This complex splits citrate into oxaloacetate and acetate and is involved in all the known anaerobic bacterial citrate fermentation pathways. Although citrate fermentation in *E. faecalis* has been investigated before, the regulation and transcriptional pattern of the *cit* locus has still not been fully explored. To fill this gap, in this paper we demonstrate that the GntR transcriptional regulator CitO is a novel positive regulator involved in the expression of the *cit* operons. The transcriptional analysis of the *cit* clusters revealed two divergent operons: *citHO*, which codes for the transporter (*citH*) and the regulatory protein (*citO*), and upstream from it and in the opposite direction the *oadHDB-citCDEFX-oadA-citMG* operon, which includes the citrate lyase subunits (*citD*, *citE*, and *citF*), the soluble oxaloacetate decarboxylase (*citM*), and also the genes encoding a putative oxaloacetate decarboxylase complex (*oadB*, *oadA*, *oadD* and *oadH*). This analysis also showed that both operons are specifically activated by the addition of citrate to the medium. In order to study the functional role of CitO, a mutant strain with an interrupted *citO* gene was constructed, causing a total loss of the ability to degrade citrate. Reintroduction of a functional copy of *citO* to the *citO*-deficient strain restored the response to citrate and the Cit$^+$ phenotype. Furthermore, we present evidence that CitO binds to the *cis*-acting sequences O_1 and O_2, located in the *cit* intergenic region, increasing its affinity for these binding sites when citrate is present and allowing the induction of both *cit* promoters.

Enterococcus faecalis, a gram-positive catalase-negative coccus, is a natural member of the human and animal microflora. This ubiquitous microorganism has numerous fields of interest due to its importance as a cause of nosocomial infections and to its utilization in the food industry. With respect to the latter, the bacteria are involved in the ripening process and in aroma development of diverse cheeses. These positive effects have been attributed to specific biochemical traits such as lipolytic activity and citrate utilization. Citrate metabolism has been extensively studied in bacteria, both from a basic and applied point of view, since citrate fermentation plays an important role during the production of diverse kinds of drinks and foods (1–3, 7, 8, 12, 15, 16, 21, 28, 29, 31–33, 48–51).

Citrate fermentation was recently investigated in *E. faecalis*, and the contribution to aroma development was characterized (18, 46, 47). In this microorganism, the first steps of the citrate degradative pathway are shared with other citrate-fermenting bacteria. Initially, citrate is converted to oxaloacetate and acetate by the enzyme citrate lyase. Then, oxaloacetate is decarboxylated to pyruvate. This compound is subsequently degraded to acetate, CO_2, and formate with the generation of ATP by the acetate kinase enzyme. In addition, small quantities of lactate, acetoin, and ethanol are produced, suggesting a low activity of lactate dehydrogenase, acetolactate synthase, and alcohol dehydrogenase enzymes (46, 47). Although the genes involved in the citrate fermentation pathway have been identified, the regulation of citrate metabolism remains unclear in this microorganism.

To date, two different mechanisms for sensing citrate and controlling its utilization have been described in bacteria. These mechanisms ensure that selected genes are expressed under the appropriate conditions and at the right time. The expression of the specific transporters and the citrate lyase complex, required for citrate fermentation, are regulated through the control of transcription initiation by the two-component sensor-regulator system CitA/B (6, 37) and by the transcriptional activator protein CitI (34).

The CitA/CitB two-component system was initially described in enterobacteria (6–8, 37). CitA functions as a sensor of extracellular citrate, whereas CitB acts as a transcriptional activator of the citrate-specific fermentation genes (*cit* operons). In *Klebsiella pneumoniae*, the *citS-oadGAB-citAB* operon encodes the citrate carrier CitS, the three subunits of the oxaloacetate decarboxylase (OadA, OadB, and OadG), and the CitA/B two-component system (6, 7, 37, 41). The divergent operon *citCDEFG* encodes the citrate lyase complex (CitD, CitE, and CitF) and the auxiliary proteins CitC and CitG (7, 8, 48, 49). The expression of the citrate fermentation genes requires not only citrate but also anoxic conditions and Na^+ ions (8, 36).

Recently, the transcriptional factor CitI from *Weissella*

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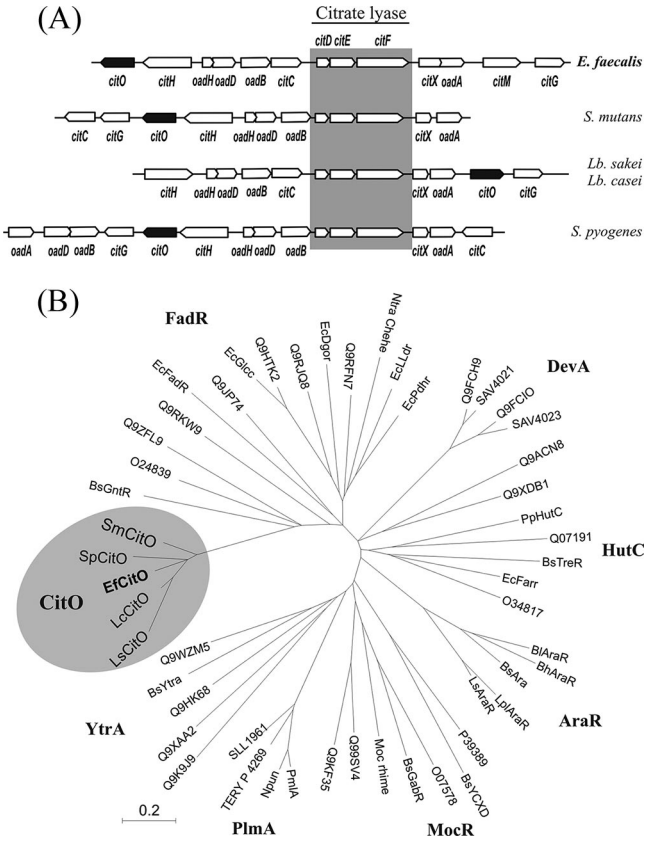


FIG. 1. (A) Genetic organization of *cit* genes involved in citrate utilization in *E. faecalis*, *S. mutans*, *L. sakei*, *L. casei*, and *S. pyogenes*. The shaded box indicates the organization of the highly conserved *citDEF* genes in the different *cit* clusters. (B) Phylogenetic tree corresponding to proteins of the GntR family. A multiple sequence alignment was computed using MEGA3. GntR-like regulators were classified in seven subfamilies according to the clusters of branches that emerged from the constructed tree. The shaded ellipse shows the new subfamily constituted by *citO* homologs.

paramesenteroides was reported to function as a switch of the *cit* genes, which are directly activated by citrate (34). CitI, a member of DeoR family, is highly conserved in different lactic acid bacteria such as *W. paramesenteroides*, *Leuconosctoc mesenteroides*, *Lactococcus lactis*, and *Lactobacillus plantarum* (31–34).

Interestingly, the presence of an alternative transcriptional factor associated with citrate breakdown emerged from gene context analysis of citrate fermentation gene clusters. In fact, we found that the genetic organization of *cit* operons was different in *E. faecalis*, *Streptococcus mutants*, *Lactobacillus casei*, *Lactobacillus sakei*, and *Streptococcus pyogenes* (Fig. 1A). In this group of microorganisms, *citO* is a common element. It is predicted to encode a GntR family transcriptional regulator. The members of this family consist of a conserved N-terminal helix-turn-helix DNA binding domain, and at the C terminus, there is usually a variable effector-binding/oligomerization domain (19, 43).

Another important feature of the *cit* operons from this group is the presence of the alternative citrate transporter named CitH (Fig. 1A). In a previous work, we reported the functional characterization of the citrate transporter, CitH, from *E. faecalis.* This protein was expressed in *Escherichia coli* and functionally characterized in membrane vesicles prepared from these cells. Citrate uptake was dependent on the presence of divalent metal ions (Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+}, and Pb^{2+}) (5).

In this report, we demonstrate that the *citO* gene encodes a novel transcriptional factor necessary for the activation of the citrate fermentation pathway in *E. faecalis.* When citrate is present, CitO induces the transcription of the *citHO* and *oadHDB-citCDEFX-oadA-citMG* operons by binding to the intergenic region (IR) between them. In this way, CitO allows *E. faecalis* cells to coordinate the expression of the genes involved in synthesizing the enzymes necessary for citrate transport and degradation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. faecalis* ATCC 29212 strain was selected based on its ability to metabolize citrate detected by using Kempler and McKay medium (25). *E. faecalis* JH2-2 was utilized for genetic manipulation since the *E. faecalis* ATCC 29212 strain has been refractory to the usual techniques of transformation. *E. faecalis* ATCC 29212, JH2-2, and V583 conserve the *cit* locus, as estimated by PCR and sequence reactions. Cultures of *E. faecalis* were grown at 37°C without shaking in 100-ml sealed bottles containing 20 to 50 ml of Luria-Bertani medium (LB) (45) supplemented with 33 mM trisodium citrate (LBC medium); initial pH was 7.0.

E. coli strain DH5α was used as an intermediate host for cloning, *E. coli* BL21(DE3) was used for overproduction of CitO with a six-His tag (His_6-CitO), and *E. coli* EC101 was used as host for pGh9 constructs. *E. coli* strains were routinely grown aerobically at 37°C in LB medium and transformed as previously described (45). Growth was monitored by measuring absorbance at 600 nm in a Beckman DU640 spectrophotometer. Aerobic growth was achieved by gyratory shaking at 250 rpm. Ampicillin (100 μg/ml), erythromycin (150 μg/ml), or kanamycin (50 μg/ml) was included in the medium to select cells harboring ampicillin-, erythromycin-, or kanamycin-resistant plasmids. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg/ml) was used to identify recombinant plasmids with DNA insertions that impaired β-galactosidase activity in the DH5α strain induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Construction of the *E. faecalis* JH2-2 CitO mutant strain. The *E. faecalis* JH2-2 strain was constructed by interrupting the *citO* gene by a single recombination event using the thermosensitive vector pGh9 (30). An internal fragment of *citO* was amplified by PCR using chromosomal DNA of *E. faecalis* ATCC 29212 as a template. The forward primer fcitOU introduced an EcoRI site, and the reverse primer fcitOL introduced an HindIII site (see Table S1 in the supplemental material). The PCR product was digested with these two enzymes and ligated into the corresponding sites of the pGh9 vector. The resulting plasmid, named pmCitO (Table 1), was introduced into *E. coli* EC101, isolated, and then electroporated into the *E. faecalis* JH2-2 strain as described elsewhere (14). The transformant strain was grown overnight at a permissive temperature of 30°C in LB medium plus glucose with 5 μg/ml erythromycin for plasmid maintenance. The saturated culture was diluted 500-fold into fresh medium and incubated at the restrictive temperature of 37°C at which plasmid replication is disabled. When the culture reached an optical density at 600 nm (OD_{660}) of 0.5, serial dilutions were plated on LB medium plus glucose and antibiotic. Next, the colonies were transferred to LB plates with or without 33 mM citrate. Colonies with no differential growth in these media were further analyzed for a citrate utilization phenotype. The resultant *citO*-deficient strain was named JHB1 (Table 2). The interruption of *citO* was confirmed by PCR and Southern blotting.

Construction of plasmids with P*cit-lacZ* transcriptional fusions. The plasmids bearing the promoter-*lacZ* transcriptional fusions, listed in Table 1 and Fig. 4, are all derivatives of the pTCV-*lac* vector (40), and the oligonucleotides used in their construction are given in Table S1 in the supplemental material. To construct the pTCV-*lac*-derivative plasmids, DNA fragments from the promoter regions of the *citHO* and *oadHDB-citCDEFX-oadA-citMG* operons were obtained by PCR using chromosomal DNA of *E. faecalis* ATCC 29212 as the template and cloned into the pCR-Blunt II-TOPO vector (Invitrogen). pTOPO-derivative plasmids were digested with EcoRI, and each released fragment was ligated into the corresponding site of pTCV-*lac* vector. The desired orientation of the fragments was determined by PCR.

TABLE 1. Plasmids used in this study

Plasmid	Comments	Oligonucleotides	Reference or source
pGh9	Thermosensitive plasmid carrying erythromycin resistance		30
pGEMT-easy			Promega
pCR-Blunt II-TOPO			Invitrogen
pET28a			Novagen
pBM02	pUC18 derivative carrying CRL264 replicon, P*cit* (promoter) and chloramphenicol resistance		B. Marelli et al.
pTCV-lac	Promoterless vector which allows *lacZ* fusion construction		40
pmCitO	pGh9 derivative carrying a 500 bp *citO* internal fragment	fcitOU, fcitOL	This work
pCitO	pBM02 derivative for expressing CitO in *E. faecalis*		This work
pET-CitO	pET28a derivative expressing His_6-CitO	CitOU, CitOL	This work
pTCV-P*citHO*		EfHpromU, EfDpromL	This work
pTCV-P*citHO*1		Efint2U, EfDpromL	This work
pTCV-P*citHO*2		EfHpromU, Efint3L	This work
pTCV-P*citHO*3		EfHpromU, Efint4L	This work
pTCV-P*citCL*		EfHpromU, EfDpromL	This work
pTCV-P*citCL*1		EfHpromU, EfDpromL	This work
pTCV-P*citCL*2		EfbsintU, EfDpromL	This work
pTCV-P*citCL*3		EfbsPoadHU, EfDpromL	This work
pTO_1O_2	pTOPO derivative carrying a DNA fragment including the O_1 and O_2 binding sites	Efint2U, EfbsoadHL	This work
pCO_1O_2	pBM02 derivative carrying a DNA fragment including the O_1 and O_2 binding sites		This work

To mutate the −10 box of the P*citCL* promoter, the oligonucleotides EfHpromU-EfPCLmutL and EfPCLmutU-EfDpromL (see Table S1 in the supplemental material) were used for the amplification of two overlap extension PCRs. A mixture of these PCR products was used as a DNA template for another PCR using the oligonucleotides EfHpromU and EfDpromL (see Table S1 in the supplemental material). The amplification product was cloned into pCR-Blunt II-TOPO vector and then subcloned into pTCV-*lac* vector as described above, yielding plasmid pTCV-P*citCL*1 (Table 1).

Plasmid pCO_1O_2 used for the competence assay was obtained by subcloning from the pTO_1O_2 plasmid the DNA fragment (obtained with the primers Efint2U and EfbsoadHL) (see Table S1 in the supplemental material) which contains the binding sites (designated O_1 and O_2) of CitO. pTO_1O_2 was digested with EcoRI, and the released fragment was ligated into the same restriction site of pBM02 (B. Marelli et al., unpublished results) (Table 1).

TABLE 2. *E. faecalis* strains used in this study

Strain	Genotype or description	Source or reference
ATCC 29212	*cit*$^+$	ATCC
JH2-2	*cit*$^+$	11, 24
JHB1	JH2-2 *citO*::*pmCitO*	This work
JHB2	JH2-2 (pTCV-P*citHO*)	This work
JHB3	JH2-2 (pTCV-P*citHO*1)	This work
JHB4	JH2-2 (pTCV-P*citHO*2)	This work
JHB5	JH2-2 (pTCV-P*citHO*3)	This work
JHB6	JH2-2 (pTCV-P*citCL*)	This work
JHB7	JH2-2 (pTCV-P*citCL*1)	This work
JHB8	JH2-2 (pTCV-P*citCL*2)	This work
JHB9	JH2-2 (pTCV-P*citCL*3)	This work
JHB10	JH2-2 (pTCV-lac)	This work
JHB11	JHB1 (pCitO)	This work
JHB12	JHB1 (pBM02)	This work
JHB13	JHB1 (pTCV-P*citHO*)	This work
JHB14	JHB1 (pTCV-P*citCL*)	This work
JHB15	JHB1 (pTCV-P*citHO*) (pCitO)	This work
JHB16	JHB1 (pTCV-P*citCL*) (pCitO)	This work
JHB17	JHB2 (pBM02)	This work
JHB18	JHB2 (pCO_1O_2)	This work
JHB19	JHB6 (pBM02)	This work
JHB20	JHB6 (pCO_1O_2)	This work

The cloned fragments were checked by sequencing at the University of Maine DNA sequencing facility.

β-Galactosidase assays. Cells were grown overnight in LB broth containing 0.5% glucose and kanamycin (1,000 μg/ml). Overnight cultures were diluted to an OD_{660} of 0.08 and grown in LB medium supplemented with kanamycin until the cells were harvested in early stationary phase. When indicated in the figure legends, different carbon sources were added to the growth medium at specified concentrations. β-Galactosidase was assayed as described by Israelsen et al. (23), except that the cells were permeabilized by treatment with 20 U/ml of mutanolysin (Sigma) for 10 min at 37°C.

Citrate lyase activity. Citrate lyase activity was determined at 25°C in a coupled spectrophotometric assay with malate dehydrogenase as previously described (33). Briefly, the assay mixture contained, in a final volume of 1 ml, 100 mM potassium phosphate buffer (pH 7.2), 5 mM trisodium citrate, 3 mM $MgCl_2$, 0.23 mM NADH, 25 U of malate dehydrogenase, and 20 μg of total protein from different cell extracts. The oxidation of NADH was measured in a spectrophotometer at 340 nm. One unit of enzyme activity is defined as 1 pmol of citrate converted to acetate and oxaloacetate per min under the conditions used. For chemical acetylation of citrate lyase, 20 μg of protein was incubated for 5 min at 25°C with 5 mM acetic anhydride and then used immediately for determination of citrate lyase activity.

RNA isolation and analysis. For Northern blotting and primer extension analysis, total RNA was isolated from *E. faecalis* cells grown for 9 h in LB or LBC medium, as previously described (33). The RNAs were checked for their integrity and yield of the rRNA in all samples. The patterns of the rRNAs were similar in all preparations. Total RNA concentration was determined by UV spectrophotometry and by gel quantification with Gel Doc 1000 (Bio-Rad). Primer extension analysis was performed as previously described (33). The primers used for detection of the start sites of *citH*, *oadH*, *citC*, and *citM* were pecitH, pecitH2, peoadH, peoadH2, pecitC, and pecitM (see Table S1 in the supplemental material), respectively. One picomole of primer was annealed to 15 μg of RNA. Primer extension reactions were performed by incubation of the annealing mixture with 200 U of Moloney murine leukemia virus reverse transcriptase (Promega) at 42°C for 60 min. Determination of the size of the reaction products was carried out in 6% polyacrylamide gels containing 8 M urea. Extension products were detected in a GE Healthcare Life Sciences Phosphorimager.

For Northern blot analysis, samples containing 20 μg of total RNA were separated in a 1.2% agarose gel. Transfer of nucleic acids to nylon membranes and hybridization with radioactive probes were performed as previously described (33). The double-stranded probes hybridizing with *citH*, *citEF*, and *citM* were labeled by incorporation of [α-^{32}P]dATP in the PCR amplification using oligonucleotides citHU-citHL, citEFU-citEFL, and citMU-citML, respectively

(see Table S1 in the supplemental material). mRNA molecular sizes were estimated by using a 0.5- to 9-kb RNA ladder (NEB).

Cloning of *citO*. The gene encoding the putative regulator CitO was amplified by PCR using genomic DNA of *E. faecalis* ATCC 29212 as the template, following a standard protocol. The forward primer citOU introduced an NdeI site around the initiation codon of the *citO* gene, and the reverse primer citOL introduced a BamHI site downstream of the stop codon (see Table S1 in the supplemental material). The PCR product was double digested and ligated into the corresponding restriction sites of vector pET-28a(+) (Novagen). The resulting plasmid, named pET-CitO, codes for His_6-CitO at the N terminus (Table 1). The sequence of the insert was confirmed, and the plasmid was subsequently introduced into *E. coli* BL21(DE3) for *citO* overexpression.

pET-CitO was then digested with NdeI and EcoRI. The released *citO* fragment was ligated into the corresponding sites of the pBM02 vector (B. Marelli et al., unpublished). The resulting plasmid, named pCitO, has the *citO* gene downstream of a promoter that allows the expression of CitO in *trans* in *E. faecalis* (Table 1).

Purification of His_6-CitO and gel mobility shift assays. *E. coli* BL21(DE3) harboring pET-CitO was grown in 500 ml of LB medium at 37°C until an OD_{600} of 0.6. Then, CitO expression was subsequently induced by addition of 0.5 mM IPTG. After 5 h, the culture was harvested by centrifugation and resuspended in ice-cold Tris-HCl buffer (100 mM, pH 8.0), containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 300 mM NaCl, and 5% glycerol. The cells were disrupted by passing them three times through a French pressure cell at 10,000 $lb/inch^2$. The suspension was centrifuged at 30,000 × *g* for 25 min to remove cell debris, and the supernatant was mixed with nickel-nitrilotriacetic acid agarose (Novagen) and incubated on ice for 1 h. The matrix was washed with buffer (100 mM Tris-HCl buffer, pH 8.0,1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 300 mM NaCl, and 5% glycerol) containing 25 mM imidazole. The proteins were eluted with the same buffer, containing 100 mM imidazole. The purified His_6-CitO was then dialyzed against binding buffer (25 mM Tris-HCl, pH 6.6) containing 150 mM NaCl and 10% glycerol and stored at −80°C for further studies. Protein purity was estimated at >90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its concentration was determined by the Bradford method (9).

Gel mobility shift assays were performed as follows. A DNA fragment including the *citH* and *oadH* promoter regions was radiolabeled by incorporation of [α-^{32}P]dATP in a PCR mixture, for which the EfbscitHU and EfbsPoadH (see Table S1 in the supplemental material) primers were used. The amplicon was purified from a 5% polyacrylamide gel prior to its use for binding reactions, which were performed in a 10-μl reaction mixture containing 25 mM Tris-HCl, pH 6.6, 150 mM NaCl, 1 mM $CaCl_2$, 0.05% Tween 20, and 10% glycerol. DNA fragments (0.5 nM) and proteins (75 to 800 nM) were incubated for 15 min at 30°C. The samples were applied to a 5% polyacrylamide gel, which had been prerun for 1 h at 4°C in 45 mM Tris-borate (pH 8.0)–1 mM EDTA. Gels were dried onto Whatman 3MM paper and exposed to a storage phosphor screen, and band patterns were detected in a GE Healthcare Life Sciences Phosphorimager.

DNase I footprinting. For DNase I footprinting, labeled target DNA fragments (0.7 nM) were incubated in the presence of different amounts of CitO in 70 μl of binding buffer consisting of 25 mM Tris-HCl (pH 6.6), 150 mM NaCl, 1 mM $CaCl_2$, 0.05% Tween 20, and 10% glycerol with a 100-fold excess of competitor DNA [poly(dI-dC)]. After 15 min of binding at 30°C, 9 μl of 27.5 mM $CaCl_2$ was added to each tube. RQ1 RNase-free DNase (Promega) was added at a final concentration of 0.003 U ml^{-1}. Digestion was allowed for 2 min at room temperature and quenched by the addition of 125 μl of reaction stop solution (200 mM NaCl, 20 mM EDTA, pH 8.0, 0.25 mg ml^{-1} tRNA). After phenol-chloroform extraction, DNA fragments were precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 95% (vol/vol) formamide. Fragments were then denatured at 95°C for 2 min and fractionated in a polyacrylamide–6% urea (wt/vol) gel. Gels were dried onto Whatman 3MM paper and exposed to a storage phosphor screen, and band patterns were detected in a GE Healthcare Life Sciences Phosphorimager.

Cloning of Cit cluster genes. In order to sequence the *cit* cluster from *E. faecalis* ATCC 29212, we used the annotated sequence of *E. faecalis* V583 (www.tigr.org) to design oligonucleotide primers. A series of pGEM-T easy (Promega)-derived plasmids (pBV01 to pBV07) was constructed by cloning the product of the PCR amplification using the oligonucleotide pairs Efs1 to Efs7 indicated in Table S1 in the supplemental material. The complete DNA sequence of the *cit* cluster was determined by sequencing these plasmids through the automated DNA sequencing instrumentation at the University of Maine DNA sequencing facility.

RESULTS

Transcription of the *cit* divergon from *E. faecalis* is induced by citrate. The ability of different *E. faecalis* strains to metabolize citrate was evaluated using Kempler and McKay differential medium. *E. faecalis* ATCC 29212 strain was selected to begin with the genetic characterization due to a strong positive reaction observed in this medium. After that, we determined the complete nucleotide sequence of a 12.5-kb region comprising the *cit* locus present in *E. faecalis* ATCC 29212. A sequence analysis showed that this locus possesses 99.3% identity with the corresponding *E. faecalis* V583 genes (www.tigr.org) (see Materials and Methods). On the basis of their homology we identified 13 genes belonging to the *cit* locus (Fig. 1A). In a central position, we found the *citD*, *citE*, and *citF* genes, which encode the three citrate lyase subunits, γ (acyl carrier protein [ACP]), β (citryl-ACP oxaloacetate lyase), and α (acetyl-ACP: citrate ACP-transferase), respectively. In addition, in this region we identified the *citC*, *citX*, and *citG* accessories genes, which encode the acetate:SH-citrate lyase ligase, apo-citrate lyase phosphoribosyl-dephospho-coenzyme A transferase, and triphosphoribosyl-dephospho-coenzyme A synthase, respectively (7, 8, 48–50).

In this cluster, we also identified a putative β-subunit of a membrane oxaloacetate decarboxylase (*oadB*). Moreover, we found two related open reading frames (ORFs), OadA and OadD, with a high degree of similarity to the N-terminal domain (carboxyltransferase activity) and the C-terminal domain (biotin carrier protein) of the OAD α-subunit from *K. pneumoniae*, respectively (12, 51). Upstream from *oadD*, an ORF named *oadH* was found that encodes a protein that did not show homology with any protein of known function. However, it is conserved in the group of bacteria in which the *cit* cluster is present (Fig. 1A). Since the gene coding for a homolog of the *K. pneumoniae* OAD complex γ-subunit is missing in these *cit* clusters and since neither was found elsewhere on the genome of this group of lactic acid bacteria (Fig. 1A), we propose that the *oadH* gene encodes a protein with functional properties analogous to the product of the *oadG* gene from *K. pneumoniae*.

Moreover, an additional ORF is present in the *cit* cluster, CitM, which is homologous to the characterized soluble oxaloacetate decarboxylase from *L. lactis* (52). Upstream from *oadH* and on the complementary strand, we found two ORFs, CitH, encoding the citrate transporter (5), and CitO, which shows a high degree of similarity to transcriptional regulatory proteins (Fig. 1A).

In order to determine the transcriptional pattern of the *cit* cluster in *E. faecalis*, Northern blot analysis was performed. Total RNA was isolated from cultures of the *E. faecalis* ATCC 29212 strain grown in LB or LBC medium. Total RNA was hybridized against α-^{32}P-labeled DNA probes: probe I consists of a 1,400-bp fragment covering the *citH* gene, probe II covers the *citEF* region (1,200 bp), while probe III corresponds to *citM* gene (1,200 bp) (Fig. 2A). Northern blot analysis revealed that the citrate fermentation gene cluster in *E. faecalis* is transcribed in two different units (Fig. 2B). The *citHO* operon (2,400 nucleotides [nt]) and the *oadHDB-citCDEFX-oadA-citMG* operon (10,200 nt). Both operons were clearly induced when cells were grown in the presence of citrate (Fig. 2B, lanes 2, 4, and 6). In fact, there were no detect-

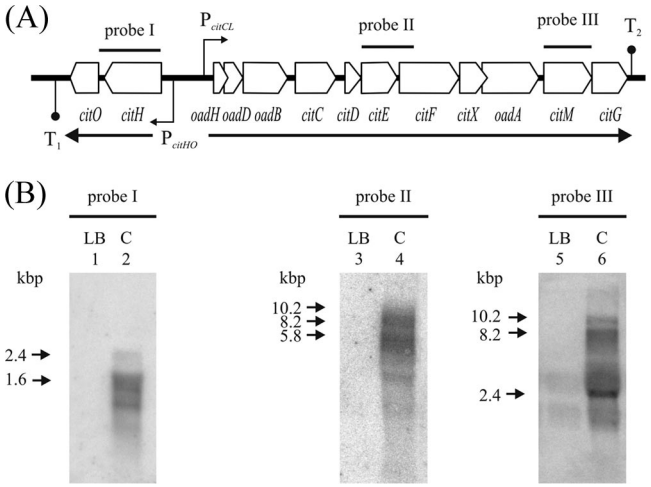


FIG. 2. Transcriptional analysis of the *cit* operons in *E. faecalis*. (A) Schematic representation of the *citHO* and *oadHDB-citCDEFX-oadA-citMG* operons. P*citHO* and P*citCL* indicate the promoters of the *cit* operons. The secondary structures T1 and T2 represent putative Rho-independent transcriptional terminators. (B) Northern blot analysis of *E. faecalis* ATCC 29212 strain. Cells were cultivated in LB or LBC (C) medium. Total RNA was hybridized against specific probes: *citH* (probe I), *citEF* (probe II), and *citM* (probe III). The major RNA species observed in the Northern blot are indicated by arrows.

able transcripts of *cit* operons when cells were grown in LB medium (Fig. 2B, lanes 1, 3, and 5). In addition to the 2,400-nt *citHO* mRNA, smaller mRNA species were detected with probe I, which could be the result of the larger transcript processing. A putative secondary structure was found in the IR between both genes (cauuuuGUCUUuuCUugcGUUAUaUUUUcAUuuuUAAUA uuauauuUAUUAucuaUAAAAAUAACcuauAGGGAGGuGC cucaua, with a ΔG of −8.3 kcal/mol; here and below, uppercase letters correspond to nucleotides involved in the complementary interaction, and lowercase letters represent internal loops) using the Mfold program (35, 58). The transcriptional start site of the *citHO* operon was determined by primer extension experiments using two independent oligonucleotides (pecitH and pecitH2) (see Table S1 in the supplemental material). The transcript starts with a cytosine residue located 134 nt upstream from ATG of *citH*. The promoter is situated upstream of this sequence, with the −10 (TATATT) and −35 (TATTTA) putative boxes separated by 17 nt (Fig. 3A, lane 1, and C). A putative Rho-independent terminator sequence was identified in the *citO* 3′ region (gcAA AAAAGACCUGCAGUcGCucguGCaACUGCAGGUCUUU UUUcauuuuuuuu, with a ΔG of −27.1 kcal/mol) (35, 58) (Fig. 2A).

DNA probes II and III revealed the presence of a larger mRNA transcript that could correspond to a 10,200-nt mRNA starting upstream of *oadH* and ending downstream of *citG*. We identified a putative Rho-independent terminator sequence in the *citG* 3′ region (uAAAAGGCGGCAuuCGUUcaaaAACGgcUG UCGCCUUUUuuuaugaaauuuuuuu, with a ΔG of −19.8 kcal/mol) (35, 58) (Fig. 2A). The differential pattern observed with both probes suggested a specific processing of the larger transcript. In the blots hybridized with probes II and III, we identified smaller products that probably correspond to transcripts processed in three putative secondary structures. The first putative secondary structure was identified in the 3′ region of the *oadH* gene (GUUGCcauuauuGCAGC, with a ΔG of −4.5 kcal/mol), the second one was found in the *oadB-citC* IR (UGUUAGCUu

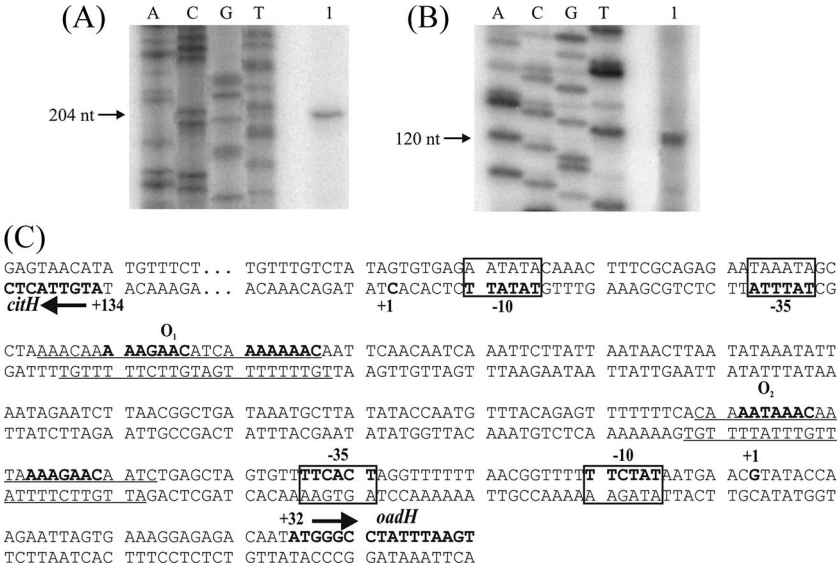
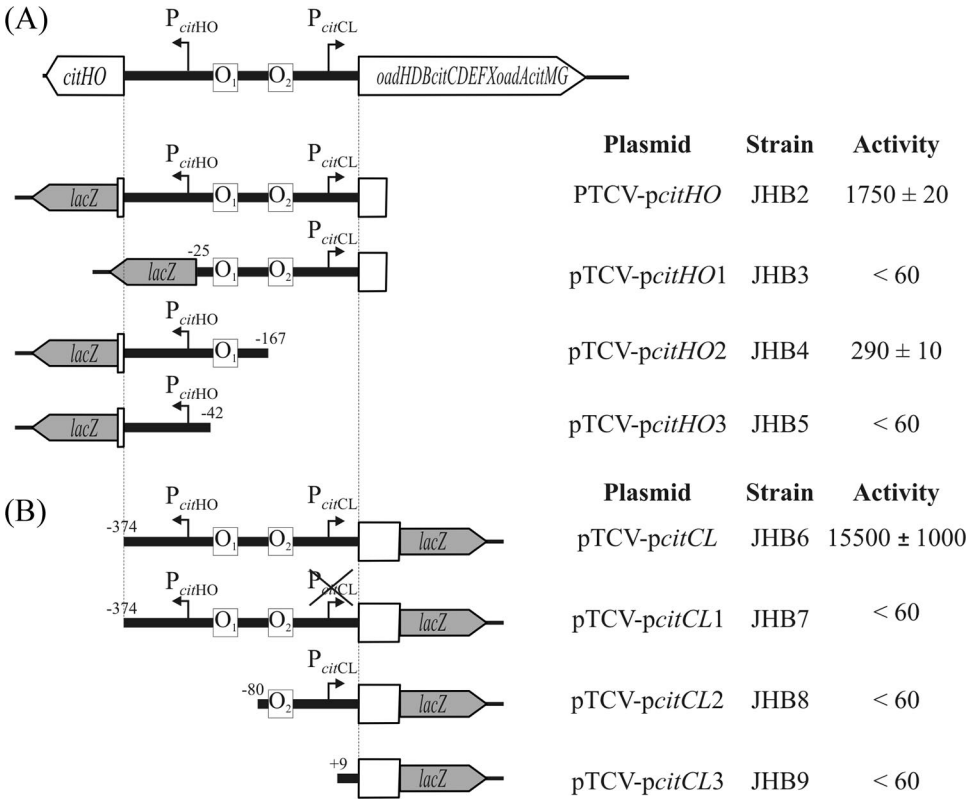


FIG. 3. Primer extension analysis of the transcriptional start sites of *citHO* (A) and *oadHDB-citCDEFX-oadA-citMG* operons (B). The images show primer extension experiments performed with RNA extracted from strain ATCC 29212 grown in LBC medium. Lanes A, C, G, and T show unrelated sequencing ladders. The length of the fragments is indicated on the left side of the sequence ladder. (A) Lane 1 corresponds to detection of the 5′ end of *citHO* mRNA using the pecitH oligonucleotide. (B) Lane 1 corresponds to detection of the 5′ end of *oadHDB-citCDEFX-oadA-citMG* mRNA using the peoadH2 oligonucleotide. (C) Nucleotide sequence of the *citH-oadH* promoter regions. Transcriptional start sites are indicated (+1); −10 and −35 regions are shown by boxes. The directions of transcription and translation are indicated by horizontal arrows. The coding sequences for CitH and OadH are shown in bold. The sequences protected by CitO in DNase I footprinting experiments on the top and bottom DNA strands are underlined. The direct repeat sequence, which is possibly involved in CitO binding, is in boldface.



Plasmid	Strain	Activity
PTCV-p*citHO*	JHB2	1750 ± 20
pTCV-p*citHO*1	JHB3	< 60
pTCV-p*citHO*2	JHB4	290 ± 10
pTCV-p*citHO*3	JHB5	< 60

Plasmid	Strain	Activity
pTCV-p*citCL*	JHB6	15500 ± 1000
pTCV-p*citCL*1	JHB7	< 60
pTCV-p*citCL*2	JHB8	< 60
pTCV-p*citCL*3	JHB9	< 60

FIG. 4. Schematic representation of the pTCV-*lac*-derived plasmids. The promoter regions of the *citHO* (A) and *oadHDB-citCDEFX-oadA-citMG* (B) operons are depicted. The CitO binding sites identified in vitro (O_1 and O_2) are represented by boxes. The end of each DNA fragment is labeled relative to the transcriptional start site. The levels of accumulated β-galactosidase activity were measured in cell extracts from cultures grown in LBC medium for 8 h.

uuUGGUUGGGGCGGCGuuuuCGUUGUUUUAuuuAUCA uuuAUC UAGC, with a ΔG of −15.6 kcal/mol), and the third was found between *oadA* and *citM* (GAAGCAAGGGUUUGA GCGACgauaacgGUUGUUCAAACCCUUGCUU, with a ΔG of −34.6 kcal/mol). Therefore, in addition to the *citHO* (2.4 kbp) and the *oadHDB-citCDEFX-oadA-citMG* (10.2 kbp) transcripts, smaller mRNA species were observed, suggesting posttranscriptional regulation, as previously reported for other microorganisms (3, 6, 32, 33). Thus, RNA processing could be used as a mechanism to ensure that the catabolic citrate lyase complex (*citDEF*) and the decarboxylases (*oadHDB*, *oadA*, and *citM*) are synthesized in a differential manner. Similarly, this mode of processing could be necessary to maintain the balance between the levels of expression of the citrate transporter (*citH*) and the regulatory protein (*citO*).

Next, primer extension experiments using two independent oligonucleotides (peoadH and peoadH2) (see Table S1 in the supplemental material), showed that the transcriptional start site of the *oadHDB-citCDEFX-oadA-citMG* operon corresponds to a guanosine residue located 32 nt upstream of the assumed *oadH* start codon. A putative promoter was detected with −10 (TTCTAT) and a −35 (TTCACT) hexamers (Fig. 3B and C). The location of the promoter was confirmed by reverse transcription-PCR (not shown) and by transcriptional fusion analysis (see below).

To validate the data observed by Northern blotting, the intergenic promoter region was studied by using the promoterless vector pTCV-*lac* (40), which allows *lacZ* fusion construction. Since attempts to transform *E. faecalis* ATCC 29212 failed, the laboratory strain JH2-2 was used for genetic manipulation. β-Galactosidase activity was determined in cell extracts of *E. faecalis* JH2-2 harboring plasmid pTCV-P*citHO* or pTCV-P*citCL* (Fig. 4) (strains JHB2 and JHB6, respectively) grown under the same conditions described for Northern blotting. The β-galactosidase activities measured in *E. faecalis* JHB2 and JHB6 grown in LBC were 1,750 ± 20 Miller units and 15,500 ± 1,000 Miller units, respectively. No significant β-galactosidase activity (less than 60 MU) was found in *E. faecalis* JHB10 (harboring pTCV-*lac* empty vector) cell extracts grown in LBC medium or in JHB2, JHB6, and JHB10 cell extracts grown in LB medium.

Deletion of P*citHO* promoter (strain JHB3) (Fig. 4A) resulted in no significant β-galactosidase activity, implying that this is the only promoter that directs the transcription of *citHO* operon. In addition, the P*citCL* promoter −10 box TTCTAT was mutated to TGGCGC (strain JHB7) (Fig. 4B), producing no significant β-galactosidase activity in *E. faecalis* JHB7 cell extracts. This indicates that P*citCL* is the only promoter driving the transcription of the *oadHDB-citCDEFX-oadA-citMG* operon.

Hence, from these experiments we conclude that the expression of the *cit* divergon is controlled by P*citHO* and P*citCL* promoters and is induced at the transcriptional level by the presence of citrate.

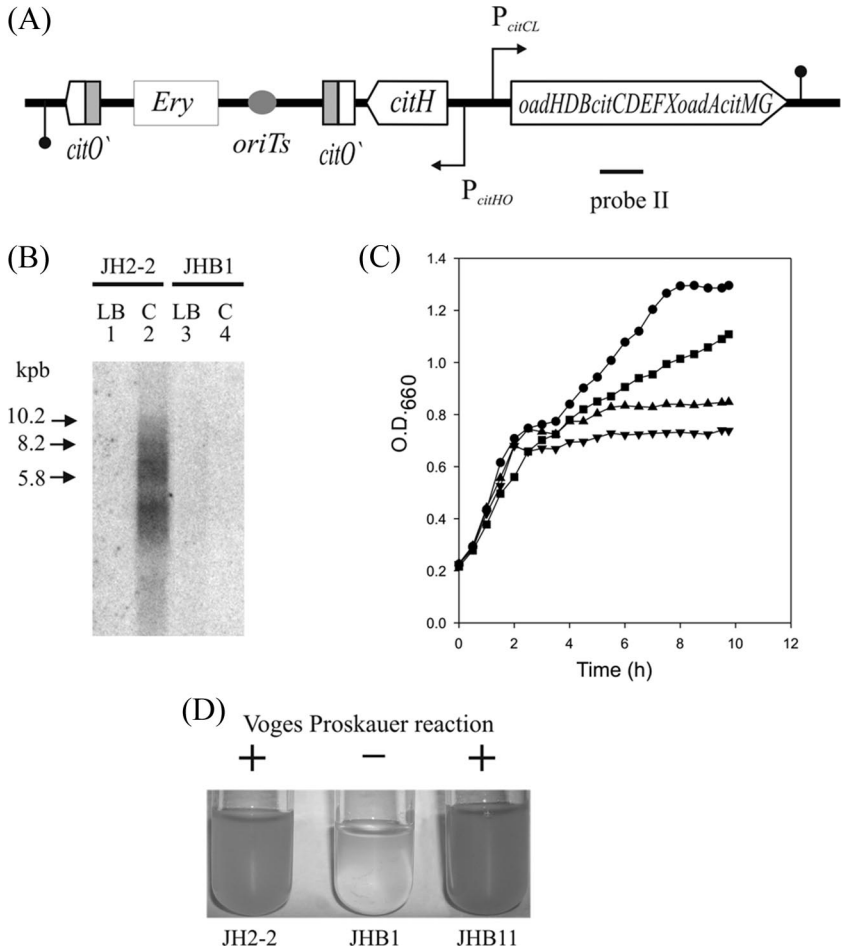


FIG. 5. Construction and characterization of an *E. faecalis citO*-defective strain (JHB1). (A) Schematic representation of *citO* inactivation by insertion of the pmCitO plasmid. The fragments labeled *citO′* are parts of the *citO* gene; *ery* and *ori*(Ts) are the erythromycin cassette and the thermosensitive replication origin of pGh9 plasmid, respectively. (B) Northern blot analysis in *citO*$^+$ (JH2-2) and *citO* mutant (JHB1) strains. Cells were grown in LB (lanes 1 and 3) or LBC (lanes 2 and 4) medium. The assay was performed as described in the legend of Fig. 2 but using only probe II. Transcripts were detected only for strain JH2-2 grown in the presence of citrate (lane 2) and not in the mutant strain (lane 4) or in the absence of citrate (lanes 1 and 3). (C) Growth curves in LBC medium of *E. faecalis* JH2-2 and derivative strains, *citO*$^+$ (JH2-2; ●), *citO* mutant(JHB1; ▼), *citO* mutant harboring the plasmid pCitO (JHB11; ■) and *citO* mutant harboring the plasmid pBM02 (JHB12; ▲). (D) Voges-Proskauer assays. Strains JH2-2, JHB1, and JHB11 were grown in LBC medium. A positive reaction was obtained in the wild type (JH2-2) and in the complemented strain (JHB11).

A new cluster of GntR-like transcriptional regulators. CitO belongs to the GntR-like transcriptional regulator family, which includes more than 5,000 proteins annotated in the Swiss-Prot database. Members of this family of regulators are distributed among very diverse bacterial groups and regulate a variety of biological processes. GntR-like proteins were classified into four major subfamilies (FadR, MocR, HutC, and YtrA) and into three minor subfamilies (DevA, PlmA, and AraR) (20, 27, 43) (Fig. 1B). A multiple alignment of 51 full-length GntR sequences (NCBI) was constructed using the native implementation of CLUSTAL W in the alignment explorer tool of MEGA3 (26). Phylogenetic trees were constructed with MEGA3 by using the neighbor-joining method (44). The resulting trees were visualized using the tree explorer tool of MEGA3. The phylogenetic tree of the family reveals that the five members associated with the citrate fermentation pathways of *S. mutants*, *S. pyogenes*, *L. casei*, *L. sakei*, and *E. faecalis* are on a separate branch of the FadR subfamily (Fig. 1B). We infer from this result that the CitO cluster arose from an ancestral sequence shared with the FadR subfamily that diverged through a replacement process in the effector binding domain. Even though several FadR subfamily members were characterized and reported to be involved in the regulation of aspartate (AnsR) (53), pyruvate (PdhR) (38), glycolate (GlcC) (39), lactate (LldR) (17), and gluconate (GntR) (42) metabolism, no evidence concerning to the role of the CitO transcriptional factor cluster was found.

CitO is the positive activator of the *cit* divergon. In order to investigate the role of CitO in the regulation of the *cit* operons, we constructed a *citO* mutant by interrupting this gene using single-crossover chromosomal integration of the pmCitO plasmid (Fig. 5A). The appropriate disruption of *citO* was confirmed by Southern blot hybridization (data not shown). The resulting strain, JHB1, was tested in its ability to use citrate. While the parental *E. faecalis* JH2-2 strain showed a citrate lyase activity of 1.5 ± 0.2 U μg^{-1} in a protein cell extract

obtained from cells grown in LBC medium, the *citO* mutant strain JHB1 was unable to express this activity under the same conditions.

We subsequently performed a Northern blot assay using probe II (Fig. 5A) with total RNA obtained from cells grown as described above. As expected, the presence of transcript was detected in the lane corresponding to RNA from JH2-2 grown in LBC medium, and no signal was observed in the samples corresponding to JHB1 (Fig. 5B). In agreement with this transcriptional evidence, growth curves showed that citrate metabolism allowed the parental strain to reach a final OD_{660} of 1.3 in LBC medium while strain JHB1 reached an OD_{660} of only 0.75 (Fig. 5C).

Finally, the ability to degrade citrate was evaluated by the production of acetoin through the Voges-Proskauer test. Liquid cultures (LBC medium) of wild-type JH2-2 produced large quantities of acetoin, whereas the level of acetoin was undetectable in the supernatant of the strain JHB1 analyzed in the same conditions (Fig. 5D).

To confirm the role of CitO as the activator of the pathway, we proceeded to complement the deficient strain JHB1 by expressing CitO in *trans* using the pCitO plasmid (see Materials and Methods) (Table 1). JHB1 cells harboring pCitO (JHB11) recovered the ability to metabolize citrate, which was evidenced by the increase in the final OD_{660} for cells grown in LBC medium (Fig. 5C) (OD_{660} of 1.0), the detection of citrate lyase activity in extracts of cells grown in LBC medium (1.1 ± 0.1 U μg^{-1} of protein), and the production of C_4 compounds measured by the Voges-Proskauer reaction (Fig. 5D).

Moreover, we analyzed the P*citHO* and P*citCL lacZ* fusions in the *citO* mutant strains (JHB13 and JHB14, respectively). The β-galactosidase levels in these strains were less than 60 MU, even when cells were grown in the presence of citrate, but normal activity was restored in the JHB15 and JHB16 complemented strains (harboring pTCV-P*citHO* and pTCV-P*citCL*, respectively) when cells were grown in LBC medium (see also Fig. 8B and C).

Binding of CitO to the promoter regions. To address whether the regulatory effect of the *citO* gene product involved a direct interaction with the *citH* and *oadH* promoter regions, a His_6-CitO fusion protein was constructed and expressed in *E. coli*. This fusion protein was subsequently used in gel mobility shift assays using a DNA fragment corresponding to the *citH-oadH* IR (Fig. 6A). The assay was performed by incubating the IR amplicon (427 bp) with increasing concentrations of purified CitO. As a result, a CitO-IR complex was identified (Fig. 6B, C1). Binding specificity was confirmed by determining that CitO did not bind to an unrelated 174-bp fragment belonging to the *B. subtilis acp* promoter (data not shown).

The results presented to this point indicate that induction of *cit* genes occurs upon growth of *E. faecalis* in LB medium in the presence of citrate. This led us to think that citrate could interact with CitO, modifying its binding capability to the DNA. To confirm this hypothesis, we evaluated the effect of citrate in a gel mobility shift assay, using this compound at a final concentration of 1.1 mM and increasing concentrations of CitO. With this approach a subtle effect of citrate presence was detected. Figure 6C shows that with 185 nM CitO, a small amount of labeled probe is retarded, while in the absence of citrate all the DNA is in the free form (Fig. 6B). These experiments suggest that the presence of citrate might increase the affinity of the regulator for its binding sites. This assumption was confirmed by DNase I footprinting experiments (see below).

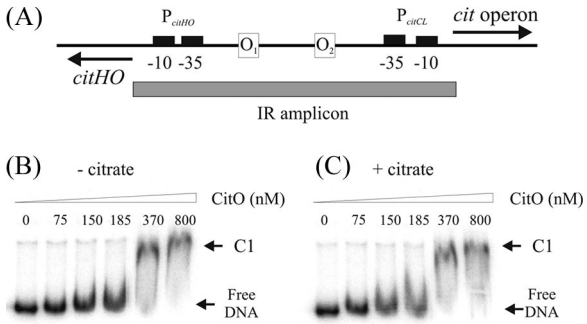


FIG. 6. Binding of CitO to the *citHO-oadHDB-citCDEFX-oadA-citMG* IR. (A) Schematic representation of the *cit* operon IR. P*citHO* and P*citCL* promoters are indicated. The thick line on the top indicates the location of the 427-bp DNA probe (IR amplicon) used in the gel shift experiments. (B and C) Image of gel shift assay performed with 0.5 nM IR amplicon and increasing concentrations of CitO without (B) and with (C) 1.1 mM citrate. Arrows indicate the positions of the retarded complex C1 and free DNA.

The localization of the CitO binding sites within the *cit* promoters was determined by DNase I footprinting on both strands of the DNA using a 100-fold excess of competitor DNA (Fig. 7). A 427-bp fragment containing the promoter regions of the *citHO* and *oadHDB-citCDEFX-oadA-citMG* operons was used as target DNA in the experiments. This fragment carries all the sequences necessary for CitO to exert its regulation on the *cit* divergon, as was demonstrated by transcriptional fusions (Fig. 4). Two binding sites were detected in the promoter regions of the *cit* operons when citrate was present. In the *citHO* operon coding strand (Fig. 7), CitO protects the region between positions −41 and −65 (binding site O_1) while in the *oadHDB-citCDEFX-oadA-citMG* operon coding strand (Fig. 7), CitO protects the region between positions −51 and −74 (binding site O_2). The same sequences are protected in the noncoding strand. The exact position of O_2 was also determined using a different 247-bp fragment that allowed a better resolution of the protected nucleotides (not shown). The position of the binding sites relative to the *cit* operon promoters is depicted in Fig. 3C.

To further demonstrate that CitO specifically recognizes the IR in vivo, we cloned a 247-bp DNA fragment containing the O_1 and O_2 sites into plasmid pBM02 to generate the pCO_1O_2 plasmid (Table 1). Both pBM02 and pCO_1O_2 were used to transform the JHB2 and JHB6 strains (harboring pTCV-P*citHO* and pTCV-P*citCL*, respectively). Transformation with the empty vector resulted in normal transcriptional induction of both strains (JHB17 and JHB19), while transformation with plasmid pCO_1O_2 (strains JHB18 and JHB20) reduced the level of β-galactosidase activity 4.3- and 5-fold, respectively. We concluded that the presence in *trans* of the O_1 and O_2 regions titrates the intracellular CitO molecules, leading to a failure in the induction of the *cit* operons in the presence of equal amounts of inducer.

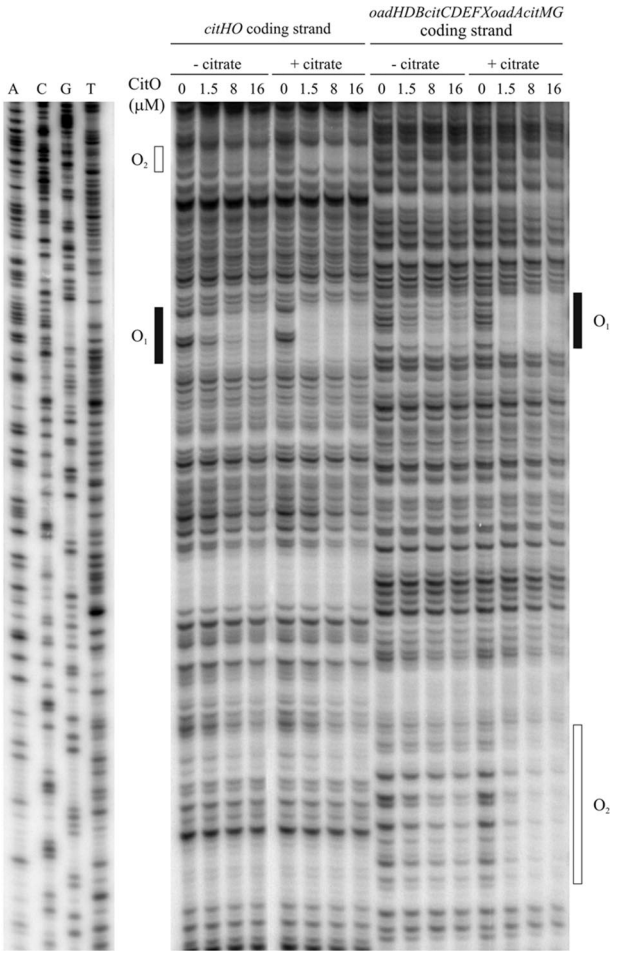


FIG. 7. DNase I protection experiments of the operons promoters by CitO. A 427-bp DNA fragment including the *citHO-oadHDB-citCDEFX-oadA-citMG* operon promoter regions was end labeled with [γ-^{32}P]ATP and used as target DNA in the assays. Each strand was labeled in separate experiments. Unrelated sequencing reactions were run at the side with the DNase I footprinting reactions. Protected regions (O_1 and O_2) are indicated at the sides of the gel lanes.

Citrate specifically induces binding of CitO to the O_1 and O_2 DNA sequences. The results presented in Fig. 6 suggest that the presence of citrate could modify the interaction of CitO with its binding sites. To confirm this hypothesis, we tested the effect of citrate addition in a DNase I footprinting assay (using this compound at a final concentration of 1.1 mM) and increasing concentrations of CitO (Fig. 7). The experiment showed that at higher concentrations (8 to 16 μM), CitO is able to bind to DNA even in the absence of citrate (as observed in the electrophoretic mobility shift assay), while in the presence of 1.1 mM citrate the protection pattern of the binding sites (O_1 and O_2) is already observed with the lowest concentration of CitO (1.5 μM), indicating that citrate increases the affinity of CitO for its binding sequences. Further analysis of the protected regions showed that CitO has a higher affinity for the O_1 site since full protection is observed with 1.5 μM, whereas for O_2 only partial protection is observed when 16 μM CitO is employed.

Moreover, the effect of citrate analog compounds (malate, succinate, and isocitrate, among others) was tested (Fig. 8A). No effect on the affinity of CitO for its binding regions was observed with these compounds, indicating that citrate is the specific inducer that modulates CitO binding to DNA.

Furthermore, we determined the specific substrate/effector for CitO in vivo by using strains JHB15 and JHB16 (derivatives of the JH2-2 *citO* mutant strain and carrying the pCitO plasmid and pTCV-P*citHO* or pTCV-P*citCL* plasmid, respectively). Cell extracts from these two strains grown in LBC medium were able to express the *lacZ* gene present in the pTCV-*lac*-derived vector, while cells growing with citrate-analogous compounds, such as malate, succinate, α-ketoglutarate, fumarate, pyruvate, or isocitrate, were not (Fig. 8B and C).

It is worth noting that although CitO is expressed from the pCitO plasmid in strains JHB15 and JHB16, under these conditions induction of the P*citHO* and P*citCL* promoters is detected only in the presence of citrate. These results suggest that while CitO is able to bind to DNA in the absence of citrate (Fig. 6 and 7), it might be incapable of activating the transcription of the *cit* genes without interaction with the specific inducer.

Deletion analysis of the promoter region of the *cit* divergon. To define the role of CitO binding regions in the mechanism of transcription regulation mediated by CitO, a set of DNA fragments covering different regions of the *cit* promoters were PCR amplified and fused to the promoterless *lacZ* reporter gene of pTCV-*lac* vector (Fig. 4). The plasmids harboring the *cit-lacZ* transcriptional fusions were electroporated into the *E. faecalis* JH2-2 strain. Then, the level of accumulated β-galactosidase activity of the resulting strains was subsequently examined in vivo in the presence of the inducer citrate, and the results are shown in Fig. 4.

Deletion of the CitO binding site O_2 on the P*citHO* promoter (strain JHB4) resulted in a sixfold decrease in *lacZ* activity relative to the full promoter fusion. When both sites (O_1 and O_2) were absent (strain JHB5), no significant activity was detected. On the other hand, deletion of O_1 or both sites on the P*citCL* promoter fusion (strains JHB8 or JHB9, respectively) results in no significant activity of this promoter.

In conclusion, the two CitO binding sites identified in vitro are very likely to be the fundamental *cis*-acting elements for the regulation of the citrate metabolic operon promoters by CitO in vivo. Furthermore, the lower level of induction or the total loss of activity observed in a promoter with only one of the binding sites present suggests a regulatory mechanism dependent on cooperative binding of the regulatory protein to its DNA targets.

DISCUSSION

Citrate metabolism is widespread among microorganisms, and it constitutes an alternative carbon and energy source in prokaryotes. In addition, citrate metabolism is an important industrial feature due to its contribution to the synthesis of aroma compounds such as acetoin, diacetyl, and butanediol (21). Moreover, citrate metabolism by *Enterococcus* spp. has recently received increasing attention (13, 47) since many Mediterranean cheeses naturally contain high numbers of enterococci, which are also being used as starter cultures (13). Although citrate metabolism has been extensively explored in

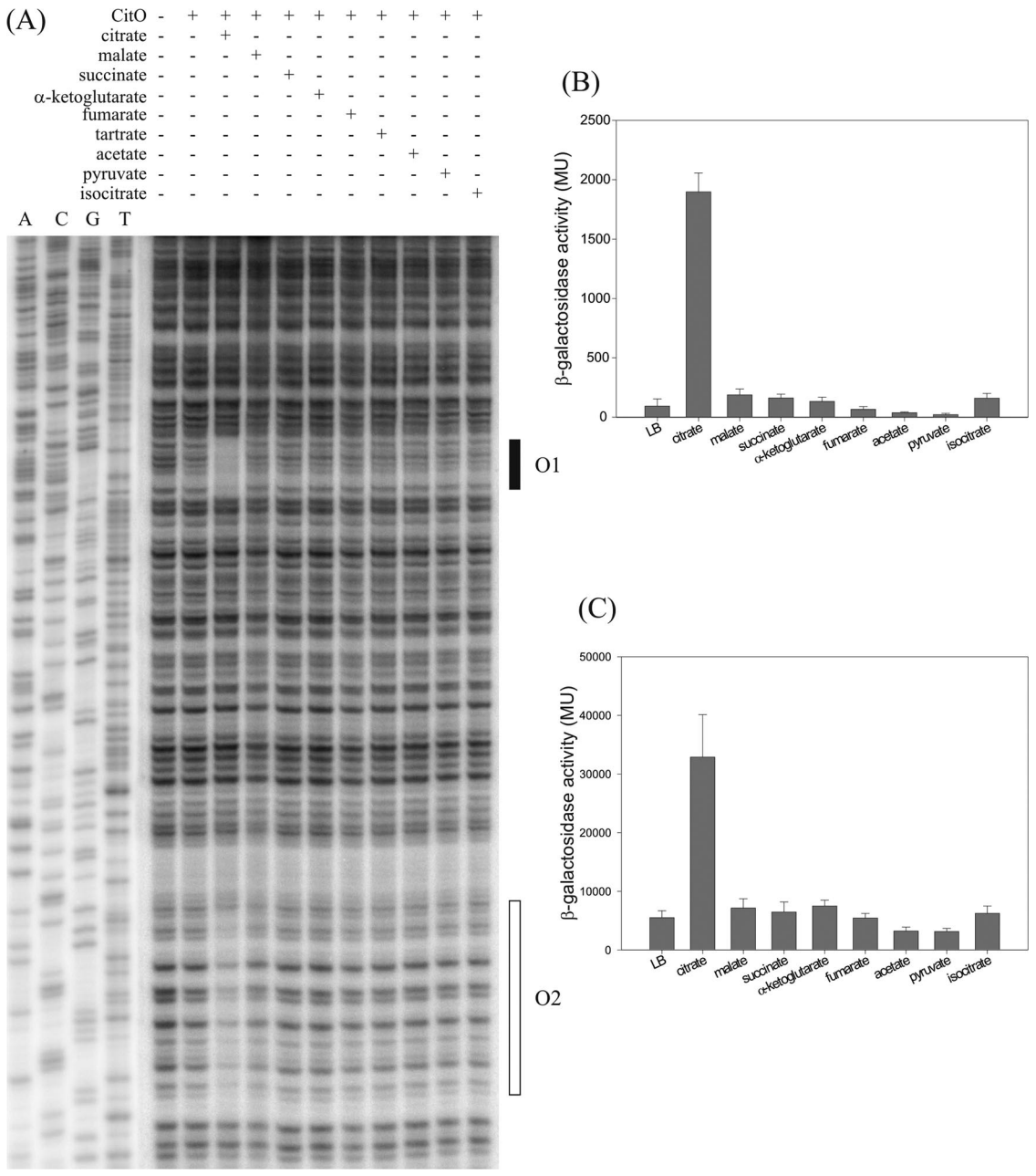


FIG. 8. (A) Effect of diverse citrate analogs on DNase I protection of the *cit* promoters by CitO. The *oadHDB-citCDEFX-oadA-citMG* coding strand was employed as target DNA. The CitO protein was used at a concentration of 1.5 μM. The different citrate analogs were used at a final concentration of 1.1 mM each. The presence (+) or absence (−) of the compounds and of CitO is shown in the top panel. The boxes indicate the extent of the protected regions. Influence of diverse citrate analogs on the expression of P*citHO-lacZ* (B) and P*citCL-lacZ* (C) fusions. Strain JHB15 (JH2-2 *citO* mutant harboring plasmids pCitO/pTCV-P*citHO*) and strain JHB16 (JH2-2 *citO* mutant harboring plasmids pCitO/pTCV-P*citCL*) were grown in LB medium and LB medium supplemented with initial concentrations of 30 mM citrate, malate, succinate, α-ketoglutarate, fumarate, acetate, or pyruvate or 10 mM isocitrate. The levels of accumulated β-galactosidase activity were measured 8 h after inoculation. Error bars represents the standard deviation of triplicate measurements.

enterobacteria and other microorganisms, this is the first time the transcriptional and regulatory mechanisms have been described in *E. faecalis*.

First, citrate fermentation genes were characterized in *E. faecalis* by means of Northern blot assays, which showed the presence of two polycistronic units constituted by the *citHO* and *oadHDB-citCDEFX-oadA-citMG* genes. Both mRNA transcripts were specifically processed in smaller species. This posttranscriptional regulation could allow cells to coordinate the level of different activities such as regulatory (CitO), transport (CitH), and degradative (citrate lyase complex or membrane or soluble oxaloacetate decarboxylase) activities. This kind of processing was also described in *K. pneumoniae* (36), *L. mesenteroides* (3), *W. paramesenteroides* (32), and *L. lactis* (33).

We then identified two promoter regions which drive the expression of the *cit* operons, P*citHO* and P*citCL*.

Our work showed that expression of the *citHO* and *oadHDB-citCDEFX-oadA-citMG* operons in bacteria grown in the presence of citrate was mediated by the transcriptional activator CitO. A *citO* mutant strain was constructed that was unable to metabolize citrate. Furthermore, this mutant strain was also unable to produce C_4 compounds, suggesting a relationship between the production of these compounds and citrate fermentation. The Cit^+ phenotype was restored when *citO* was complemented in *trans*. Moreover, the expression of a wild-type copy of *citO* in this strain enabled the production of C_4 compounds.

E. faecalis CitO belongs to the FadR subfamily of the GntR family of transcriptional regulators that includes activators, repressors, and molecules which both activate and repress a wide range of bacterial operons (43). Members of the GntR family, which was first described by Haydon and Guest (19), share similar N-terminal DNA-binding domains but show heterogeneous C-terminal effector-binding and oligomerization domains. FadR from *E. coli* is the only member of the FadR group whose crystal structure has been determined (22, 54, 55, 57). FadR plays a dual role in transcriptional regulation of the enzymes involved in fatty acid metabolism. Its function as a repressor or an activator is readily explained by the location of its binding sites within the promoter regions. When the binding site either overlaps the −10 or −35 regions or lies just downstream of the −10 sequence, FadR represses transcription. On the other hand, when the binding sites are located immediately upstream of the −35 region of the promoter, FadR binding activates transcription, presumably by promoting the binding or action of RNA polymerase. Consensus sequence for the *cis*-acting element recognized by the FadR family members was used as a tool to predict a putative operator sequence. No palindromic sequences related to the consensus sequence (Ny GTNxACNy, where x 4p = 3 nucleotides and y = 5 nucleotides) were found in the *citH-oadH* intergenic region. Although most members of the GntR family recognize palindromic or pseudopalindromic sequences, transcriptional activators of the MocR subfamily of the GntR family, GabR (ATACCA) (4) and TauR (CTGGACYTAA) (Y is T or C) (56), are able to recognize direct repeat sequences in the promoter regions. Even though none of these GntR consensus sequences was found in the *cit* intergenic region, our genetic and biochemical data clearly indicated that CitO activates the gene expression by binding to DNA at the P*citHO* and P*citCL* promoter regions. In fact, DNase I protection studies determined two CitO binding sites which were positioned at −41 through −65 relative to the transcription start site of *citHO* (site O_1) and −51 through −74 relative to the transcription start site of *citCL* operon (site O_2). The O_1 sequence on the coding strand was 5′-TGT TTT TTT GAT GTT CTT TTT GTT T-′3, while the O_2 protected sequence on the coding strand was 5′-ACA AAA TAA ACA ATA AAA GAA CAA TC-′3. The O_1 binding site is located only 5 bp from the −35 box of the P*citHO*, while the O_2 site is at 14 bp from the −35 box of the P*citCL*. Thus, CitO bound at these positions promotes the transcriptional activity of the bound RNA polymerase by protein-protein interactions. These interactions most commonly include contacts to the α-subunit C-terminal domain of RNA polymerase but can also involve contacts to the N-terminal domain of the α-, σ-, β-, or β′-subunit (10). Nucleotide sequence comparison between the O_1 and O_2 sites shows that these regions are direct repeat sequences, where it is possible to identify two similar short putative binding sites, AAAARAAC (R is A or G) in each protected region (Fig. 3). Short runs of repeated adenine or thymine residues, such as AAAA or TTTT, which are known to lead to intrinsic DNA bending are also present in the intergenic region. However, in the DNase I protection assays, we could not observe hypersensitive sites. Site-directed mutagenesis of the O_1 and O_2 sites will be necessary to determine the role of the relevant sequences and analyze if bending is involved in regulation.

The results presented in this work led us to hypothesize that CitO could be able to interact with intracellular citrate, increasing in this way CitO affinity for its binding sites; this fact is supported by electrophoretic mobility shift assay (Fig. 6) and DNase I protection assays (Fig. 7). Deletion experiments indicated that the O_1 DNA region is required for the expression of P*citCL*, suggesting cooperativity between the O_1 and O_2 sites. Moreover, the O_1 binding site seems to be occupied at lower CitO concentrations than the O_2 site (Fig. 7). This could imply that the *citHO* operon is transcriptionally activated at lower concentrations of CitO and citrate.

All together, these results suggest a model for the activation of the *cit* divergon in *E. faecalis*. When the enterococcal cells are grown in the absence of citrate, both *cit* operons are switched off, and there is no basal transcription of these genes. The presence of citrate in the medium induces the activation of the *citHO* operon. Then, it is possible that in the presence of citrate the intracellular concentrations of CitO (activator) and the inducer (citrate) increase, reaching the level at which CitO can bind to O_2, which results in the full expression of the degradative pathway. Clearly, more experiments will be necessary to understand the fine-tuning of this activation mechanism.

In conclusion, this is the first time a transcriptional activator belonging to GntR family has been studied in *E. faecalis*, offering an attractive system to explore the molecular basis of the specific interactions of the CitO activator to DNA and providing different regulatory models for other industrial and pathogenic microorganisms.

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