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Transcriptional Control of the Citrate-Inducible *citMCDEFGRP* Operon, Encoding Genes Involved in Citrate Fermentation in *Leuconostoc paramesenteroides*

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In this study we describe the expression pattern of the *Leuconostoc paramesenteroides citMCDEFGRP* operon in response to the addition of citrate to the growth medium. An 8.8-kb polycistronic transcript, which includes the *citMCDEFGRP* genes, was identified; its synthesis was dramatically induced upon addition of citrate to the growth medium. We also found that expression of the *cit* operon is subjected to posttranscriptional regulation, since processing sites included in four complex secondary structures (I, II, III, and IV) were identified by Northern blot analysis and mapped by primer extension. Upstream of the *citMCDEFGRP* operon a divergent open reading frame, whose expression was also increased by citrate, was identified by DNA sequencing and designated *citI*. The start and end sites of transcription of the *cit* operon and *citI* gene were mapped. The start sites are separated by a stretch of 188 bp with a very high A+T content of 77% and are preceded by transcriptional promoters. The end sites of the transcripts are located next to the 3′ end of two secondary structures characteristic of ρ-independent transcriptional terminators. The effect of the *citI* gene on expression of the *cit* operon was studied in *Escherichia coli*. The presence of the *citI* gene in *cis* and in *trans* resulted in increased activity of the *cit* promoter. These data provide the first evidence that citrate fermentation in *Leuconostoc* is regulated at the transcriptional level by a transcriptional activator rather than by a repressor.

Citrate metabolism is carried out by only a few strains of lactic acid bacteria. This metabolic ability is invariable linked to endogenous plasmids that contain the gene encoding the transporter responsible for citrate uptake from the medium. Citrate transporters (CitPs) have been found in strains belonging to the genera *Lactococcus* and *Leuconostoc*, bacteria in which the mechanism of citrate fermentation has been studied in detail (1, 14–16). The first step in the breakdown of citrate inside the cell involves its conversion to acetate and oxalacetate by citrate lyase, a three-subunit enzyme (2). In the next step, oxalacetate is decarboxylated by oxalacetate decarboxylase, yielding pyruvate and carbon dioxide (for a review, see reference 9). The pathway generates a proton motive force (PMF) by a secondary mechanism (10). Electrogenic exchange of divalent citrate and monovalent lactate, catalyzed by CitP, efficiently generates a membrane potential, inside negative (17). Moreover a pH gradient (inside alkaline) is formed by the consumption of scalar protons in the decarboxylation of oxalacetate (10). Together, the membrane potential and pH gradient constitute the PMF, which seems to contribute significantly to the growth advantage observed during cometabolism of citrate and glucose in both *Lactococcus* and *Leuconostoc* (14, 17).

Like all the known citrate lyases, the *Leuconostoc* enzyme forms a functional complex of three proteins: a γ subunit (acyl carrier protein [ACP]), a β subunit (citryl-S-ACP lyase), and an α subunit (citrate:acetyl-ACP transferase) (2). This enzymatic complex is active only if the thioester residue of the prostethic group linked to the γ subunit is acetylated. This activation is catalyzed by an acetate:SH-citrate lyase ligase which converts HS-ACP in the presence of ATP and acetate to acetyl-S-ACP (2).

The genes encoding CitP (22) and the subunits of citrate lyase (2) have been independently cloned and sequenced from genomic DNA of two different strains of *Leuconostoc mesenteroides*. Moreover, it has been shown that the *citCDEFG* genes coding for the *L. mesenteroides* citrate lyase together with a putative malic enzyme gene constitute an operon, which is induced by citrate at the transcriptional level (3). However, little is known about the molecular mechanism(s) involved in regulation of the synthesis of the CitP permease. Marty-Teysset et al. (16) reported that in *L. mesenteroides* the activity of the transporter was increased when citrate was added to the growth medium. In agreement with these experiments, we recently found that the utilization of citrate by *L. paramesenteroides* was stimulated when cells were grown in a medium containing citrate (15). These observations suggest that the mechanism of regulation of *Leuconostoc* CitP is different from the one demonstrated for the 99% identical CitP from *Lactococcus*. In the latter organism, the presence of citrate in the growth medium does not influence the expression of *citP* (13); instead, expression is transcriptionally induced at acidic pHs (8).

To investigate the regulation of *Leuconostoc* CitP synthesis, we recently cloned the *citP* gene from *L. paramesenteroides* (15). This gene is carried by a 22-kb plasmid. It is included in an operon together with five genes coding for the citrate lyase multienzymatic complex (*citCDEFG*) (15) and two open reading frames (ORFs), named *citM* and *citR*, coding for a putative malic enzyme and a polypeptide with homology to a *Lactococcus lactis cit* regulator (12).

In the work presented here, we analyzed the expression

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TABLE 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Reference or source
Bacterial strains		
L. paramesenteroides J1	Lac^+ Cit^+	15
E. coli DH5-α	*supE44* Δ*lacU169* (ϕ80 *lacZM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
Plasmids		
pCITJ1	21-kb Cit^+ plasmid from *L. paramesenteroides* J1	15
pMM13	5.8-kb fragment from pCITJ1 including *citI*, *citCDE*, and the 3′ end of *citF* cloned into pBluescript SK(+)	15
pMM8	2.4-kb fragment from pCITJ1 including the 3′ end of *citR* and *citP* cloned in pBluescript SK(+)	15
pJM116	Promoter probe plasmid derivative of pBR322 containing a promoterless *lacZ* gene	5
pJMM1	pJM116 derivative containing *lacZ* under control of the *citMCDEFGRP* promoter and *citI*	This work
pJMM12	Deletion of a 0.75-kb fragment from *citI* contained in pJMM1	This work
pSU39	Low-copy-number cloning vector derivative of pACYC184	4
pSUI	pSU39 derivative containing *citI* under control of the *lacUV5* promoter	This work

pattern of the *L. paramesenteroides citMCDEFGRP* operon and showed unequivocally that its transcription is induced by citrate independently of the pH of the growth medium. We also present evidence that a regulatory protein, named CitI, encoded by an ORF found in the upstream region of *citMC DEFGRP* is a transcriptional activator of the *cit* operon. The proposed mechanism of *citMCDEFGRP* transcriptional activation provides an explanation for the induction of citrate fermentation in *Leuconostoc* when citrate is added to the growth medium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *L. paramesenteroides* J1 was grown at 30°C without shaking in modified MRS medium supplemented with 2% glucose (MRSG) as described previously (15). *Escherichia coli* was routinely grown in Luria-Bertani medium (19) and transformed as previously described. Ampicillin and kanamycin were added at a final concentrations of 100 and 30 μg/ml, respectively.

RNA analysis. After growth overnight in MRSG medium, *L. paramesenteroides* J1 was sedimented by centrifugation and resuspended in saline solution. Appropriate aliquots of the cultures were used to inoculate MRSG fresh medium to give an initial A_{660} of approximately 0.05. For Northern analysis, the cultures were grown to an A_{660} of 0.2, then supplemented with 1% sodium citrate, and further incubated at 30°C. At the times indicated in the figure legends, aliquots were withdrawn and used for analysis of mRNA. For primer extension experiments, endonuclease S1 mapping, and dot blot analysis, MRSG or MRSG supplemented with 1% sodium citrate was inoculated with the overnight cultures as indicated above. Cultures were grown until they reached an A_{660} of 0.2 and then used for analysis of RNA. To adapt *Leuconostoc* cultures to acidic pHs, stock cultures previously grown at pH 7.0 and kept frozen at −70°C were used to inoculate MRSG medium adjusted at pH 5.0 and grown overnight. The overnight cultures were sedimented and resuspended in saline solution, and appropriate aliquots were used to inoculate fresh medium at the pH required to give an A_{660} of approximately 0.05 as indicated. These conditions of growth and dilution allowed the latent period of the cultures to be reduced and the contributions of the mRNA present in the overnight cultures to be minimized.

RNA manipulations. For primer extension and endonuclease S1 mapping, RNA from *L. paramesenteroides* was isolated as previously described for *L. lactis* (12) except that cell lysis was performed by the addition of lysozyme at 30 μg/ml. For Northern blot hybridization, RNA was isolated with a Ribolyser and Recovery kit from Hybaid as specified by the supplier. The RNAs were checked for the integrity and yield of the rRNAs in all samples. The patterns of rRNAs were similar in all preparations. The total RNA concentrations were determined and quantified by UV spectrophotometry and by Gel Doc 1000 (Bio-Rad). Primer extension analysis was performed as previously described (12). The primers used for detection of the start sites of *citMCDEFGRP* and *citI* mRNAs were 5′-TG GGATTTGTGCACCTT-3′ and 5′-TCTTCGGCAATTTTAGC-3′, respectively, complementary to nucleotides (nt) +32 to +16 of *citM* and +35 to +19 of *citI*. The primers used for detection of the 5′-end of mRNA processed species were 5′-GTGTGCCGGCGACTGCA-3′, 5′-CCGGCCTTGACCATCGC-3′, 5′-CGT CATGCCATCGCGGA-3′, and 5′-GGCATGTGACCAACCTG-3′, complementary to nt +34 to +18 of *citD*, +272 to +256 of *citE*, +54 to +38 of *citF*, and +728 to +712 of *citR*, respectively. One picomole of either primer was annealed to 15 μg of total RNA. Primer extension reactions were performed by incubation of the annealing mixture with 20 U of avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 30 min. Endonuclease S1 mapping was performed as previously described (12). The probe used for determination of the 3′ end of *citMCDEFGRP* or *citI* was a 720-nt *Sty*I-*Eco*RI or a 535-nt *Ban*I-*Pst*I DNA fragment from pMM8 or pMM13, respectively. Probes were ^{32}P labeled at their unique 3′ recessive ends by fill-in with *E. coli* Klenow fragment and used to detect the *citMCDEFGRP* or *citI* transcripts. Size determination of the reaction products of primer extension and endonuclease S1 mapping were carried out in 8% polyacrylamide gels containing 7 M urea. Bands labeled with ^{32}P were detected by autoradiography on Kodak X-Omat S films and were directly quantified with a PhosphorImager system (Molecular Dynamics). For Northern blot analysis, samples containing 6 μg of total RNA were fractionated in a 1% agarose gel. Transfer of nucleic acids to nitrocellulose membranes and Northern blot hybridization with 0.2 pmol of the appropriate probes were performed as previously described (12). The single-stranded probes used were synthesized as follows. The *Bgl*II-*Pst*I fragment from pMM10 and the *Pst*I insert of pMM8 were purified and ^{32}P labeled in one strand with T7 DNA polymerase. Primers 5′-C TTTACTTGCTTGCTCG-3′ and 5′-AGCAAGCAATGCGTGCG-3′, complementary to nt +517 to 500 of *cit*C and +1014 to 998 of *citP*, were used to give probes I and II (Fig. 1A). Bands labeled with ^{32}P were detected and quantified as indicated above.

DNA analysis and manipulation. Plasmid DNA preparations for cloning and sequencing experiments as well as transformations of *E. coli* were performed as described elsewhere (21). Treatment of DNA with restriction enzymes and T4 DNA ligase was performed as recommended by the suppliers. DNA sequence of both strands of the *citI* gene was determined from plasmid pMM13 with automated DNA sequencing instrumentation (ABI PRISM; Perkin-Elmer) at the Centro de Investigaciones Biológicas.

Construction of plasmids pJMM1, pJMM12, and pSUI. To construct a transcriptional fusion of the promoter of the *citMCDEFGRP* operon to the *E. coli lacZ* gene, a 2.2-kb *Eco*RI-*Eco*RI fragment from pMM13 including the promoter region and the *citI* gene under the control of its own promoter was purified and cloned into the unique *Eco*RI site of plasmid pJM116 to give pJMM1. To construct plasmid pJMM12, pJMM1 was digested with *Hin*dIII and the 0.8-kb fragment including the *citMCDEFGRP* and *citI* promoters was purified and ligated to *Hin*dIII-linearized pJM116. To construct plasmid pSUI, the *citI* gene was amplified by PCR by using pMM13 as the template and primers RegU (5′-GTGCAGAATTCGTCATCGACGGTGGATAC-3′) and RegM (5′-AAAA AAACTGCAGAATTTCCAGTTTAAATCTCG-3′). The underlined sequences indicate sites *Eco*RI and *Pst*I, respectively. The PCR product was purified, digested with *Eco*RI and *Pst*I, and ligated to *Eco*RI- and *Pst*I-digested pSU39. The constructs were established in *E. coli* DH5-α by transformation.

β-Galactosidase assay. *E. coli* cells were grown overnight in Luria-Bertani medium with aeration at 37°C, then diluted 1:100 in 10 ml of fresh medium, and grown to an A_{600} of 0.3. The final absorbance was measured after 10 min in ice bath to stop the cell growth, and aliquots of 1 ml were harvested. Pellets were frozen at −20°C until they were used. To induce *citI* expression from pSUI, *E. coli* cells were grown to an A_{600} of 0.2 and then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). At the times indicated in Table 2, samples were withdrawn and processed as described above. β-Galactosidase activity was measured by the method of Miller (19). Specific activities were expressed in units per absorbance of the cultures at 600 nm.

Nucleotide sequence accession number. The nucleotide sequence of *L. paramesenteroides* J1 that contains the *citI* gene has been deposited at the EMBL database under accession no. AJ132782.

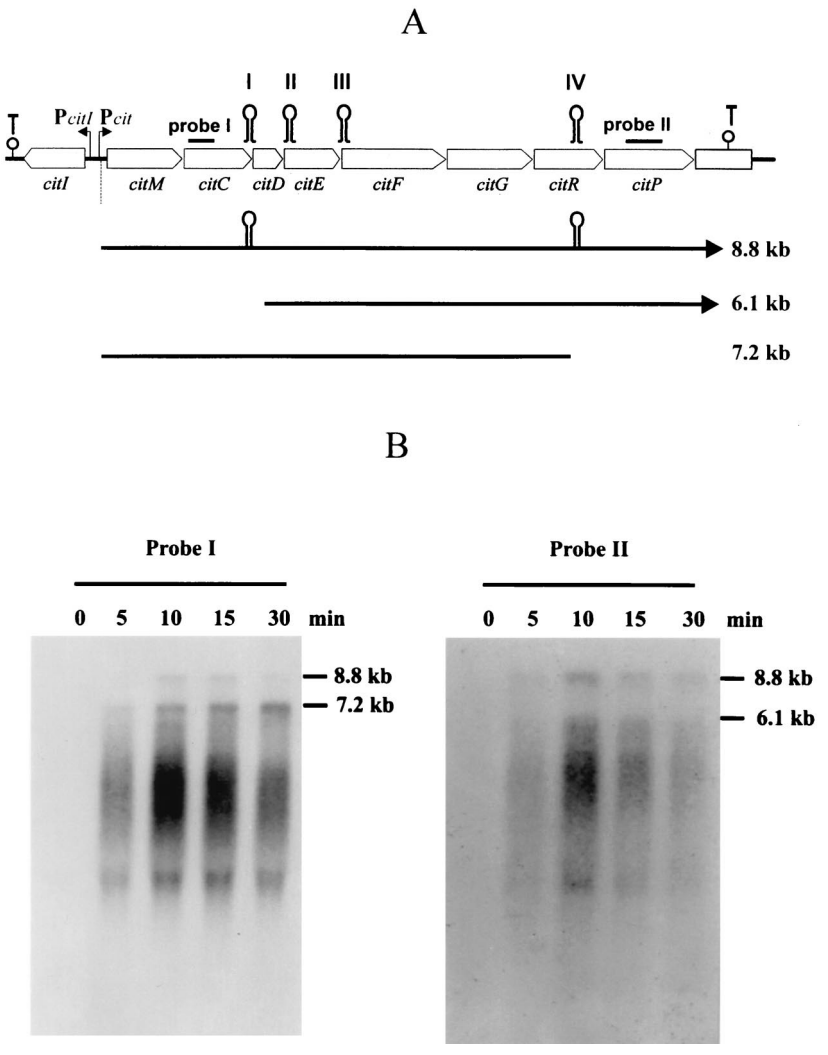


FIG. 1. Organization of the citrate fermentation genes in *L. paramesenteroides* J1 (A) and Northern blot analysis of the cit*MCDEEFGRP* operon (B). (A) The 11-kb DNA cluster encompassing nine genes involved in citrate utilization is shown in at the top. P_{cit}, promoter of the *citMCDEEFGRP* operon, P_{citI}, promoter of the *citI* gene. The secondary structures downstream of *citP* and *citI* represent ρ-independent transcriptional terminators. The stem-loop structures named I, II, III, and IV include the processing sites of *citMCDEEFGRP* mRNA mapped in Fig. 2. Probe I includes a 0.5-kb fragment of *citC*. Probe II includes a 0.5-kb fragment of *citP*. The major RNA species observed in the Northern blot shown in panel B are indicated. (B) Northern blot analysis was carried out as described in Materials and Methods. Strain J1 was grown to an A_{660} of 0.2 in MRSG medium. At this time cells, were supplemented with 1% citrate; total RNA was isolated at different times and probed with probe I or probe II.

RESULTS

Transcription of the *citMCDEFGRP* operon is induced by citrate. The clustering of the *citMCDEFGRP* genes (Fig. 1A) and their functional relationship suggest that these eight genes form a single transcriptional unit (15). To verify the operon structure and to test whether its transcription is regulated by citrate, Northern blot analysis was performed. Total cellular RNA was isolated from cultures of the *L. mesenteroides* J1 grown in medium lacking citrate and then supplemented with citrate for various times. The RNA was hybridized with two ^{32}P-labeled probes (Fig. 1B). Probe I includes a 0.5-kb fragment covering the 5′ end of *citC*, while probe II covers a 0.57-kb internal fragment of *citP* (Fig. 1A). The larger RNA species detected with probe I also hybridized with probe II (Fig. 1B). This mRNA species could correspond to a 8.8-kb transcript starting upstream of *citM* and ending downstream of *citP*. Quantification of total *citMCDEFGRP* RNA by dot blotting allowed the detection of basal levels of transcription before the addition of citrate (time zero) and revealed a 13-fold increase of transcription after 10 min of induction (data not shown). These experiments demonstrated that the *citMCDEFGRP* operon is induced by citrate at the transcriptional level. Probe I also revealed another RNA specie of 7.2 kb (Fig. 1B), which was predominant 10 min after addition of citrate, while probe II detected a second band of 6.1 kb (Fig. 1B). The differential pattern obtained with both probes suggested that the 8.8-kb transcript is subjected to specific processing at several locations. Furthermore, analysis of this mRNA with the Fold program (23) using the University of Wisconsin Genetics Computer Group software package (7) predicted the existence of four complex secondary structures named I, II, III, and IV (Fig. 1A and 2) with predicted free energies of −31, −29, −31, and −42 kcal/mol, respectively. A cleavage at structures I and IV (Fig. 1A) could account for the existence of RNA species of 6.1 and 7.2 kb, respectively (Fig. 1B). To test that these structures as well as structures II and III were specific cleavage sites

for endoribonucleases, a primer extension analysis was performed with four different primers located proximal to the 3′ end of these putative cleavage sites (for details, see Materials and Methods). The results obtained (Fig. 2) revealed that indeed processing at these secondary structures occurred. The two cleavages at structure I should disrupt *citC* and as a consequence impair its translation. Moreover, they could enhance expression of *citD*, since its predicted ribosomal binding site (RBS) is located at the base of the structure and it will be exposed to the ribosomes in the processed species. The three cleavages detected at structure II are located within *citE*, and they should abolish synthesis of CitE. Cleavages at structure III could determine expression of *citF*, since its RBS is buried in the structure. Finally, the three cleavages at structure IV should result in disruption of *citR* translation. Therefore, expression of the *citMCDEFGRP* operon seems to be subjected to posttranscriptional regulation, and the specific cleavages may determine that the cell synthesizes the different proteins, required for citrate utilization, in suitable proportions.

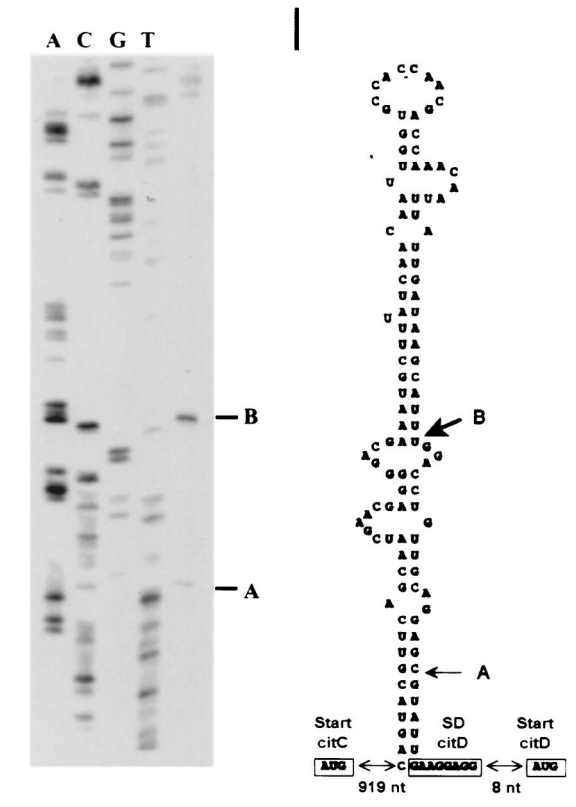
Identification of the *citI* gene and determination of the transcriptional signals of the *citMCDEFGRP* operon. Northern analysis revealed that addition of citrate to *Leuconostoc* cultures induced the transcription of the *citMCDEFGRP* operon (Fig. 1B). In an attempt to identify a possible regulator responsible for this transcriptional induction, the DNA sequence of the region located upstream of the *citMCDEFGRP* operon was determined. An ORF of 1,095 nt located upstream and oriented inversely to the *cit* operon was detected and designated *citI* (Fig. 1A and 3C). A potential RBS (5′-AAGGA-3′) is located 9 nt upstream of the first ATG of *citI* (Fig. 3C). Thus, the gene should encode a protein of 322 amino acids with a predicted M_r of 36,488. Data bank searches revealed significant homology of this peptide with 11 characterized and putative transcriptional regulators belonging to the SorC family and included in the ProDom domain PD006970 (5). Among them, ClyR (accession no. O86289), a putative regulator of the *maecitCDEFG* from *L. mesenteroides*, showed the highest identity (54% in a 309-amino-acid overlap), while the other members of the family showed homology ranging from 26 to 20% (data not shown). These homologies strongly suggested that CitI could be a regulator involved in transcriptional induction of the *cit* operon by citrate.

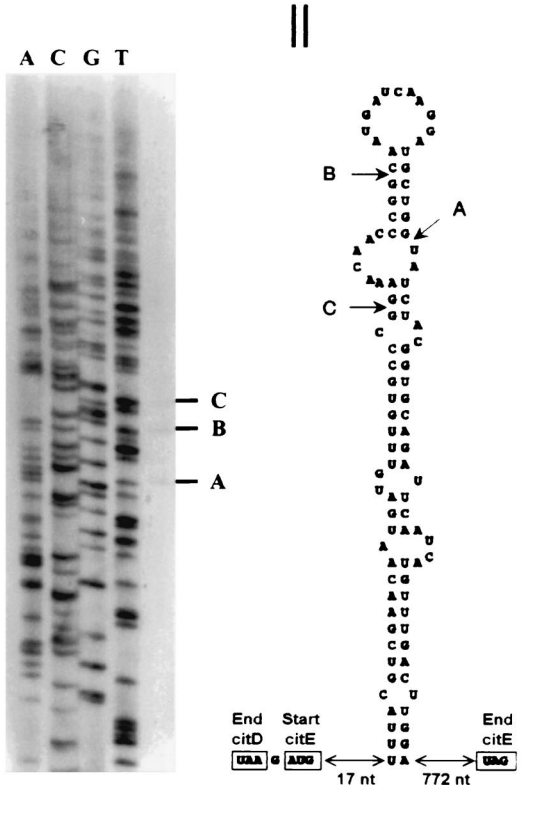
The region of DNA between the start sites of *citI* and *citM* is 188 nt in length and has an unusually high A+T content of 77% (Fig. 3C). Moreover, analysis of this stretch of DNA with the Bend program (Dnastar) predicts that this region has an intrinsic bending (data not shown), suggesting that transcription of these genes could be modulated by alteration of the curvature mediated by a regulatory protein. The overall results indicated that CitI could be a regulator involved in transcriptional induction of the *cit* operon by citrate. Therefore, to determine the steady-state levels of transcription of the *citI* gene and the *cit* operon, and to establish whether synthesis of the transcripts was driven from promoters localized in the intergenic region, we determined the start sites of transcription of *citI* (Fig. 3A) and *citM* (Fig. 3B) by primer extension. Taking into account that *citP* expression as well as citrate fermentation in *L. lactis* is induced at acidic external pHs (8), we extracted RNA from cultures of strain J1 grown in medium buffered at pH 7.0, 5.0, or 4.5 and containing or lacking citrate (Fig. 3A and B). Both transcripts start with an adenosine residue located 31 and 58 nt upstream of the start codons of *citI* and *citM*, respectively (Fig. 3C). In both cases and at all pHs tested, the detected extended products were more abundant in RNA preparations from cultures grown in the presence of citrate (Fig. 3A and B). Quantification of the extended products and of total RNA blotted to specific probes indicated that the levels of *citI* and of *citM* mRNAs were about 2.5 ± 0.75- and 30.0 ± 9-fold higher in cultures grown in the presence of citrate (data not shown). These experiments also showed that external pH did not affect expression of the *citI* gene or the *cit* operon. Therefore, we conclude that citrate is a transcriptional inductor of both the *L. paramesenteroides cit* operon and the *citI* gene and that these genes are not subjected to the pH regulation observed for the *citQRP* operon from *L. lactis* (8).

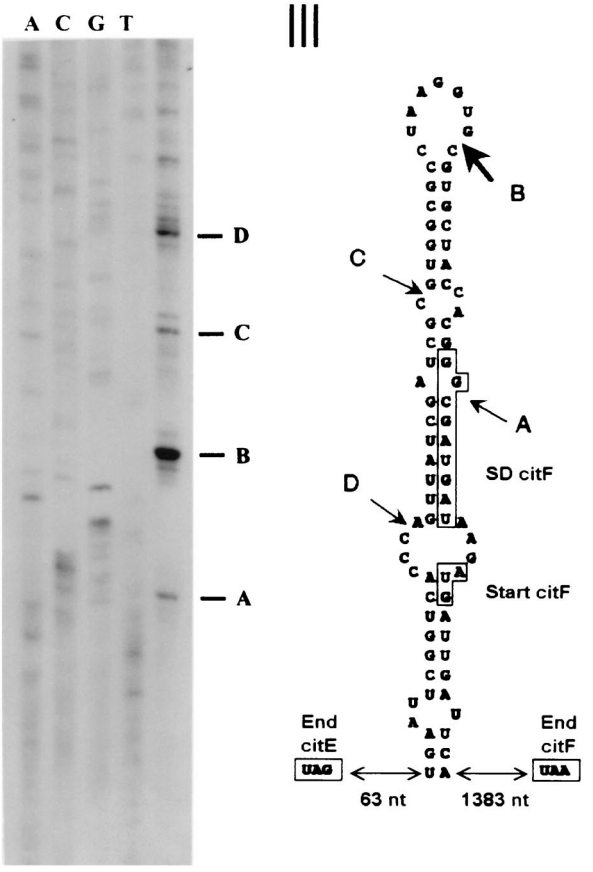
Preceding the start site of *citM* mRNA, we detected a canonical −10 hexamer TATAAT and a −35 hexamer TTtACA which shares five residues with the consensus sequence of σ^{70} promoters (Fig. 3C). Preceding the start site of *citI* mRNA, a −10 hexamer TATgAT, containing five residues identical to the consensus, was observed. However, no obvious −35 sequence was found at the appropriate distance (Fig. 3C). This lack of −35 hexamer is not unusual for promoters requiring an activator for binding of the RNA polymerase.

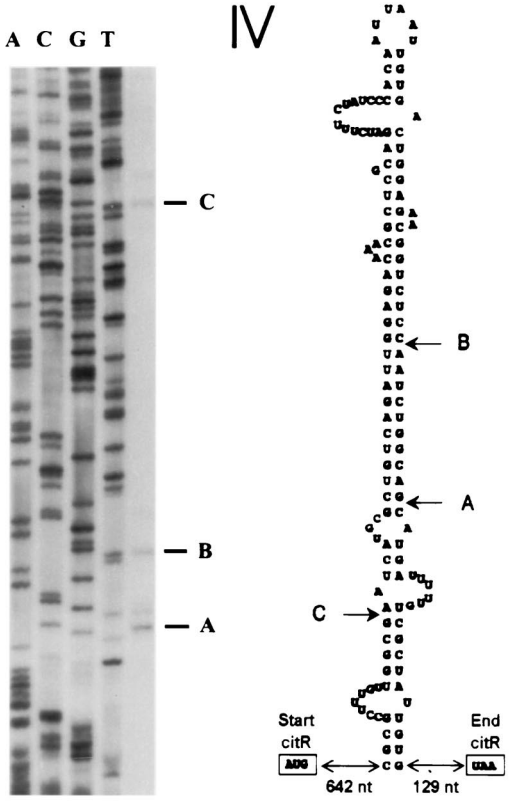
Analysis of the DNA sequence localized downstream of *citI* and *citP* showed the presence of putative ρ-independent transcriptional terminators (Fig. 1A). To assess whether these terminators were functional, the 3′ end of the *cit* transcripts was determined by endonuclease S1 mapping of total RNA extracted from citrate-induced cells (Fig. 4A). Both transcripts ended at two nucleotides (C and U for *citMCDEFGRP* mRNA or G and A for *citI* mRNA) located next to the 3′ end of the terminators (Fig. 4B and C). Thus, the location of the 5′ and 3′ ends of the *cit* transcripts confirmed the nature of the *citMC DEFGRP* operon and revealed that *citI* is included in a monocistronic mRNA.

CitI is a transcriptional activator of the *cit* operon. Since no useful techniques are available to analyze regulation of gene expression in *Leuconostoc*, we attempted to examine the role of the *citI* gene product in determining *citMCDEFGRP* promoter activity in an *E. coli* host. To this end, we constructed plasmids pJMM1 and pJMM12, based on the ColE1 replicon (Fig. 5). Plasmid pJMM1 contains the *citI* gene under the control of its own promoter and the promoter of the *cit* operon fused to the *E. coli lacZ* gene, while plasmid pJMM12 is a pJMM1 derivative in which most of the *citI* gene has been deleted. These constructs were used to transform *E. coli* DH5-α, and β-galactosidase activities of the resultant strains were measured. Cells of strain DH5-α harboring pJMM1 showed about 3.5-fold-higher β-galactosidase activity than pJMM12-containing cells (Table 2), suggesting that the presence of the *citI* gene increases the activity of the *cit* promoter. To determine whether the enhanced activity of the *cit* promoter in plasmid pJMM1 is due to *cis*-acting sequences contained in *citI* or if the observed effect is caused by the *citI* gene product, we constructed plasmid pSUI. This plasmid contains the *Leuconostoc citI* gene under the control of the *E. coli lacZ* promoter and bears the P15a replicon, which is compatible with ColE1 derivatives (Fig. 5). To test the induction of the *cit-lacZ* transcriptional fusion upon expression of CitI in *trans*, we assayed the β-galactosidase activities of strain DH5-α/pJMM12 transformed either with the parental vector pSU39 or with plasmid pSUI (Table 2). The levels of activity in DH5-α carrying plasmids pJMM12 and pSUI were more than threefold higher, upon treatment with IPTG, than those detected in DH5-α harboring plasmids pJMM12 and pSU39 (Table 2). Thus, induction of *cit* promoter (P_{cit})-*lacZ* expression was increased upon overexpression of *citI* supplemented in *trans*. Since *E. coli* is unable to transport citrate due to lack of a functional transport system, these experiments were performed with cells growing in a medium devoid of citrate. Therefore, our results strongly suggest that CitI functions as a









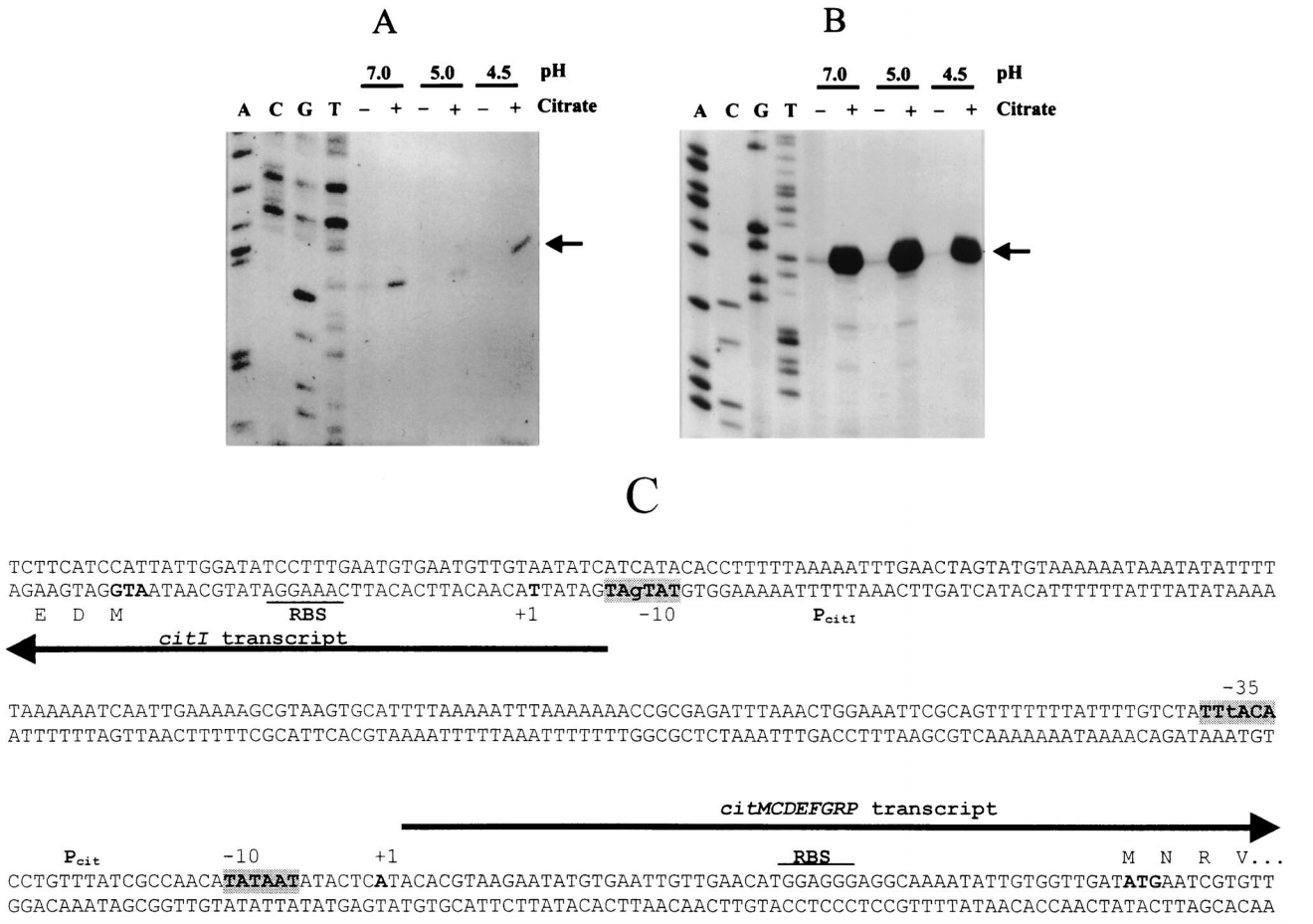


FIG. 3. Primer extension analysis of the transcription start sites of *citI* (A) and *citMCDEFGRP* (B). The autoradiographs show primer extension experiments performed with RNA extracted from strain J1 grown with or without citrate in media buffered at pH 4.5, 5.0, and 7.0. Lanes A, C, G, and T show sequencing reactions performed with the same primers used in the extension reactions. Arrows indicate the 5′-extended fragments of *citI* (A) and *citMCDEFGRP* (B), respectively. (C) Nucleotide sequence of the *citM-citI* intergenic region containing the bidirectional promoter region of the *citMCDEFGRP* and *citI* genes. The −10 and −35 regions are indicated in grey boxes. The transcription initiation sites (+1) and the ATGs of *citM* and *citI* are in boldface. The putative RBSs are underlined. Arrows indicate the direction of transcription.

transcriptional activator of the *citMCDEFGRP* operon in the absence of citrate transport and utilization.

DISCUSSION

The proteins specifically required for the first two steps of citrate fermentation (the citrate transporter and the citrate lyase) and the CitI regulator are encoded by a plasmid-borne 11-kb cluster harboring nine genes which are organized into two divergently transcribed units (Fig. 1A). The physical arrangement of the eight *citMCDEFGRP* genes suggested that they constitute an operon (15). This assumption was confirmed in this work by detection of the 8.8-kb *cit* transcript and by determination of its start and termination sites. In addition to the full-length transcript, we detected distinct smaller mRNA species. We demonstrated that they are formed by specific processing at complex structures, which seems to be target for endonucleolytic cleavage (Fig. 2). Northern blot analysis indicated that the *cit* transcript is predominantly processed at structures I and IV and that the more abundant RNA species are those including either the *citMCDEFG* or *citDEFGRP* cluster. These two RNAs should be suitable for synthesis of citrate lyase, and they could support the translation of either the citrate lyase ligase (CitC) or the citrate permease (CitP). Thus, the cell through processing might be able to regulate the synthesis of the different proteins in suitable proportions. The early degradation of the *citC* mRNA could make sense physiologically. It has been reported that *Klebsiella pneumoniae* cells require more copies of citrate lyase than the ligase necessary for activation (18). If *citC* is subjected to rapid degradation, as suggested by the Northern experiment, processing of the primary mRNA transcript would provide an appropriate mechanism to reduce the level of *citC*. Thus, RNA processing could be a mechanism ensuring that the catabolic enzyme citrate

FIG. 2. Analysis of processing of *citMCDEFGRP* mRNA in *L. paramesenteroides* by primer extension. Detection of the 5′ ends of processed species as well as the proposed putative structures (I, II, III, and IV) of the regions involved in the processing of *citMCDEFGRP* are depicted. The primer extension reactions were carried out as described in Materials and Methods. The specific cleavage sites (marked by arrows) at each structure were determined by comparing the extended fragments with a sequence reaction carried out with the same primer used in the extension reaction.

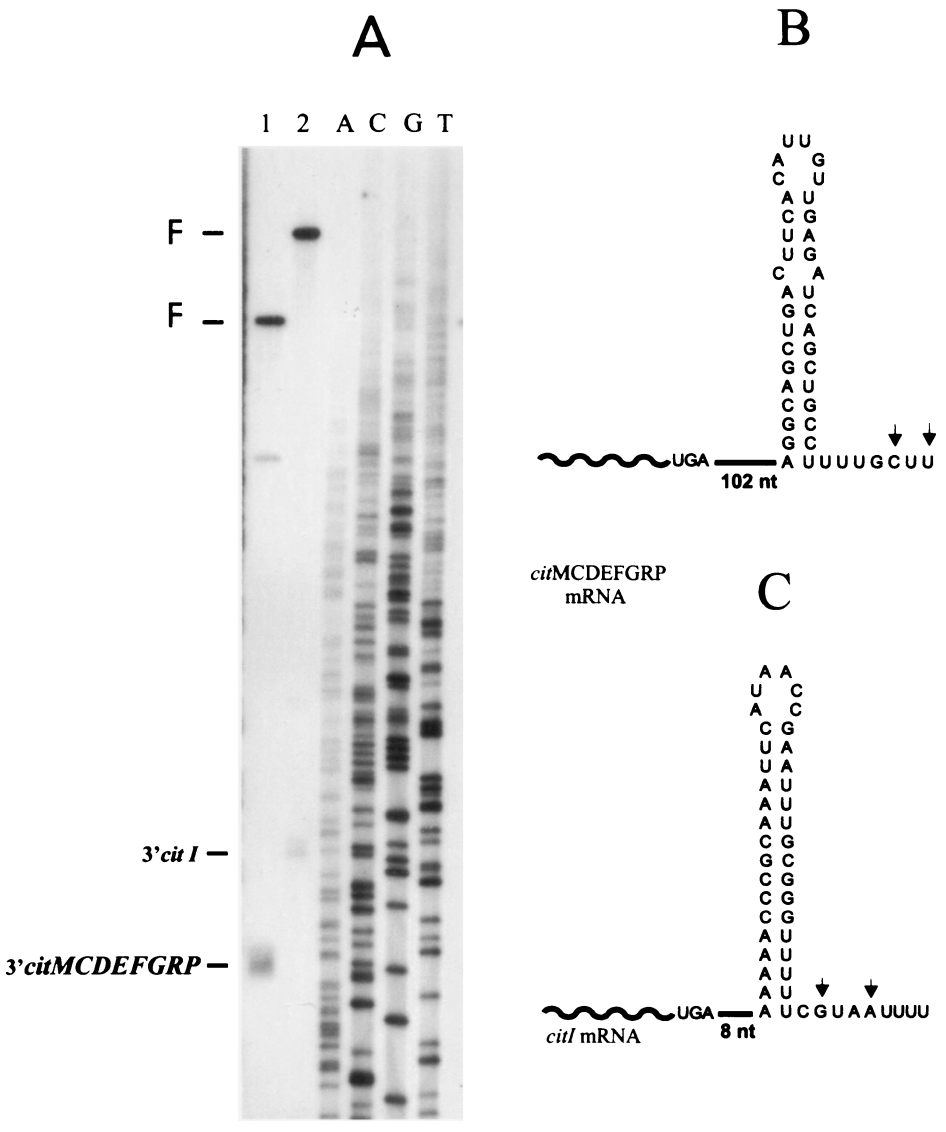


FIG. 4. Endonuclease S1 analysis of the transcription termination sites of *citMCDEFGRP* and *citI*. (A) Autoradiograph showing reactions performed with RNA extracted from strain J1 grown in medium supplemented with citrate. Lane 1, 3′ *citI*-protected fragment; lane 2, 3′ *citMCDEFGRP*-protected fragment. F, full-length restriction fragments. Lanes A, C, G, and T show unrelated sequencing reactions. (B and C) Predicted ρ-independent terminators of *citMCDEFGRP* and *citI*, respectively. Arrows indicate termination sites of the transcripts.

lyase is synthesized in excess to the citrate lyase ligase. Similarly, this mode of posttranscriptional regulation could be necessary to decrease and to uncouple the expression of *citP* from the *citDEFG* genes. If this is the case, this fate of *citP* could be correlated with the lack of linkage, and as a consequence presumably different levels of expression, of citrate permease and citrate lyase genes in *L. mesenteroides* (2) and *L. lactis* (12).

How could the transcription of the *Leuconostoc citMCDEF GRP* be regulated by CitI and by citrate? In this report, we have shown that transcription of the *cit* operon is induced about 30-fold when *Leuconostoc* cells are grown in citrate. Moreover, we have provided evidence that expression of *cit* operon is effected by transcriptional activation mediated by the *citI* gene product. The involvement of *citI* in the induction of the *citMCDEFGRP* promoter is evident from the increase in β-galactosidase activity of *E. coli* DH5-α expressing a P_{cit}-*lacZ* transcriptional fusion in the presence of the *citI* gene either in *cis* or in *trans*. This result indicates that the promoter region of the *cit* operon is a target for the CitI transcriptional activator.

E. coli is unable to transport citrate under aerobic conditions (20), and we were able to detect CitI-mediated activation of the *cit* promoter in *E. coli* cultures grown in medium lacking citrate and in the absence of CitP. Moreover, the induction of transcription of *citI* from P_{lac} resulted in an enhanced expression of the P_{cit}-*lacZ* transcriptional fusion (Table 2). These facts suggested that citrate is not directly required for the activation of P_{cit}. In *Leuconostoc*, *citI* transcription is increased about threefold when cells are grown in a citrate-containing media. Thus, citrate could be necessary to stimulate the transcription of *citI* by binding either to its gene product, CitI, or to a not yet identified citrate sensor. If this is the case, a small increase in *citI* transcription could account for the large increase of the *citMCDEFGRP* mRNA detected in *Leuconostoc* (Fig. 3A and B). Why is the regulation of the *cit* operons

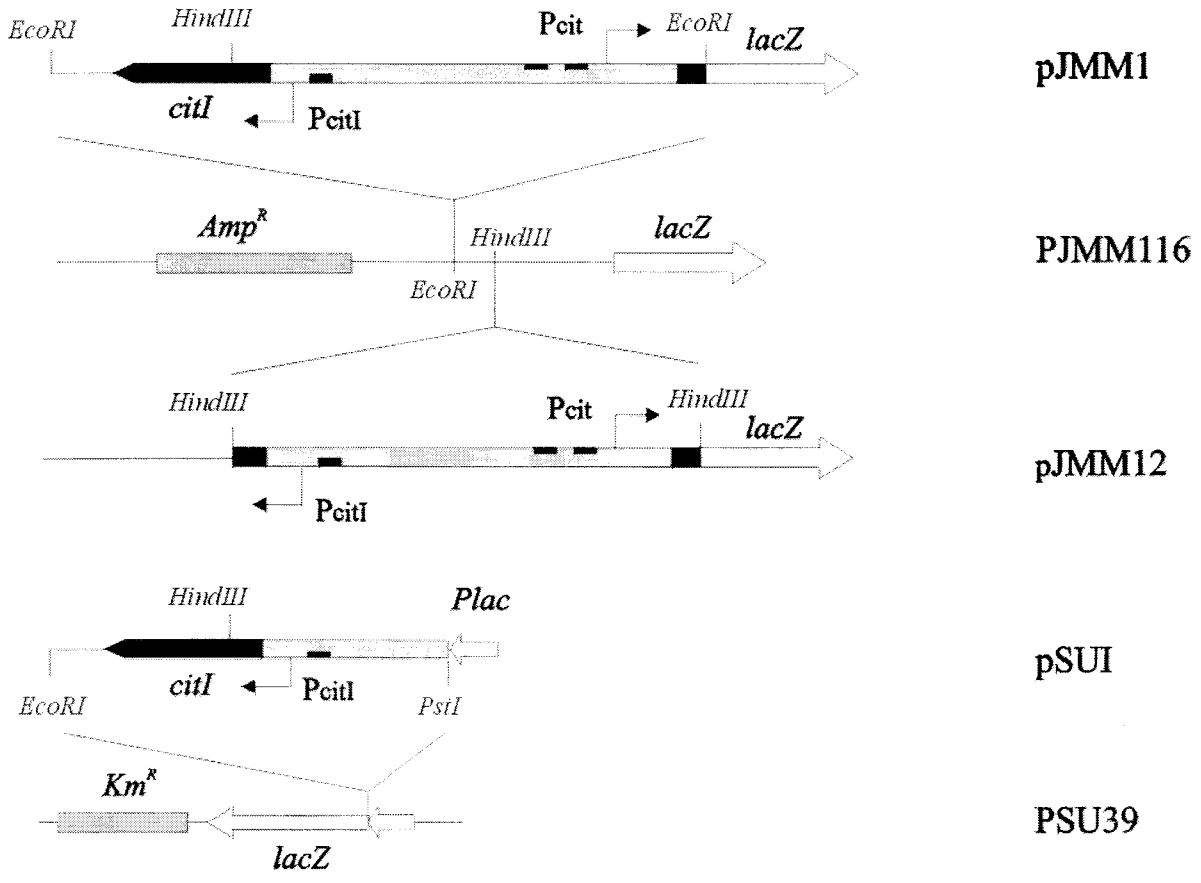


FIG. 5. Schematic representation of plasmids used to study the role of the *citI* gene product in expression of the *citMCDEFGRP* operon. The construction of plasmids pJMM1, pJMM12, and pSU1 is detailed in Materials and Methods. P_{cit}, promoter of the *citQMCDEEFGRP* operon, P_{citI}, promoter of the *citI* gene; *Plac*, *lacUV5* promoter.

different in *Leuconostoc* and *Lactococcus*? We have determined that transcription of the *Leuconostoc cit* operon is not increased by growing cells at acidic pHs. These experiments directly demonstrate that in addition to the difference in gene organization of the *cit* operons in *Lactococcus* and *Leuconostoc*, the mechanisms controlling their expression are different. While expression of the *Lactococcus citQRP* operon is transcriptionally regulated by external pH (8), transcription of the *Leuconostoc citMCDEFGRP* operon is regulated by citrate. The difference in regulation of expression is likely to reflect different physiological functions of citrate metabolism in the two bacteria. In the heterofermentative bacterium *Leuconostoc*, citrate degradation is induced by citrate in cultures growing exponentially. This results in a cometabolism of citrate and glucose leading to a growth advantage relative to growth of glucose alone (9, 17). This growth stimulation is attributed to a metabolic shift in the heterofermentative pathway for glucose breakdown yielding additional ATP (9, 17). On the other hand, induction of the citrate metabolic pathway under acidic conditions by the homofermentative bacterium *L. lactis* is used in the late exponential growth phase for alkalinization of the growth medium (8). In addition, the increased metabolism of citrate seems to make *L. lactis* cells more resistant to the inhibitory effect of the glucose fermentation product, lactate, that accumulates under these conditions (14).

TABLE 2. β-Galactosidase activities of *E. coli* DH5-α cells containing the indicated plasmids[a]

Plasmid(s)	Time of induction[b] (min)	β-Gal activity (Miller units/ A_{600})
pJMM1	NI	27,097 ± 1,046
pJMM12	NI	8,613 ± 279
pJM116	NI	1,188 ± 40
pJM116 + pSUI	NI	1,152 ± 56
pJM116 + pSU39	NI	1,900 ± 212
pJMM12 + pSUI	NI	26,633 ± 473
	20	30,443 ± 3,480
	40	37,110 ± 3,860
	60	50,806 ± 3,947
pJMM12 + pSU39	NI	7,343 ± 217
	20	9,291 ± 440
	40	11,177 ± 826
	60	11,931 ± 1,181

[a] Each value is the average of the values from at least three experiments.
[b] Induction with IPTG was performed as described in Materials and Methods. NI, noninduced.

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