Nucleotide Sequence and Expression in Escherichia coli of the Lactococcus lactis Citrate Permease Gene

David, Silke; van der Rest, M; Driessen, Arnold J.M.; Simons, Guus; Vos, Willem M. de

Published in:
Journal of Bacteriology

Publication date:
1990

JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5789–5794
0021-9193/90/105789-06$02.00/0

Vol. 172, No. 10

Nucleotide Sequence and Expression in *Escherichia coli* of the *Lactococcus lactis* Citrate Permease Gene

SILKE DAVID,[1] MICHEL E. VAN DER REST,[2] ARNOLD J. M. DRIESSEN,[2] GUUS SIMONS,[1] AND WILLEM M. DE VOS[1]*

Molecular Genetics Group, Netherlands Institute for Dairy Research, P.O. Box 20, 6710 BA Ede,[1] and Department of Microbiology, University of Groningen, 9751 NN Haren,[2] The Netherlands

Received 27 April 1990/Accepted 20 July 1990

The plasmid-encoded citrate determinant of the *Lactococcus lactis* subsp. *lactis* var. diacetylactis NCDO176 was cloned and functionally expressed in a Cit$^-$ *Escherichia coli* K-12 strain. From deletion derivative analysis, a 3.4-kilobase region was identified which encodes the ability to transport citrate. Analysis of proteins encoded by the cloned fragment in a T7 expression system revealed a 32,000-dalton protein band, which correlated with the ability of cells to transport citrate. Energy-dependent [1,5-^{14}C]citrate transport was found with membrane vesicles prepared from *E. coli* cells harboring the citrate permease-expressing plasmid. The gene encoding citrate transport activity, *citP*, was located on the cloned fragment by introducing a site-specific mutation that abolished citrate transport and resulted in a truncated form of the 32,000-dalton expression product. The nucleotide sequence for a 2.2-kilobase fragment that includes the *citP* gene contained an open reading frame of 1,325 base pairs coding for a very hydrophobic protein of 442 amino acids, which shows no sequence homology with known citrate carriers.

As in members of the family *Enterobacteriaceae* (25), the ability to utilize citrate is a useful metabolic characteristic for identifying *Lactococcus lactis* species (6, 34). The citrate-fermenting ability of these gram-negative bacteria appears to be linked to the presence of genetically unstable determinants such as plasmids (13, 14, 18, 32, 33, 37, 38) or transposons (15). The presence of plasmid- or transposon-encoded citrate transport systems enables members of the *Enterobacteriaceae* to utilize citrate as the sole carbon source. In contrast, the citrate-fermenting lactococcal strains, designated *L. lactis* subsp. *lactis* var. diacetylactis (7, 34), require an additional source of metabolic energy for the transport of citrate (5, 12). Although biochemical details of lactococcal citrate metabolism have been the subject of many studies (12, 36, 41), the energetics of citrate uptake are not yet understood. Kempler and McKay (19) demonstrated that the ability to transport citrate was linked to a 7.9-kilobase (kb) plasmid that appears to be present in all citrate-fermenting *L. lactis* strains analyzed. A detailed physical map of one of these citrate plasmids, pCT176, has been reported (10).

In the bacterial species described until now, the ability to grow on citrate is associated with cation-dependent transport systems. Na^+-dependent citrate utilization is found in *Enterobacter aerogenes* (16, 28) and *Salmonella typhimurium*, which also possess a K^+-dependent transport system (1, 18, 40). In *Bacillus subtilis* citrate transport is coupled to magnesium ion transport (2). Cit$^+$ *Escherichia coli* strains contain a citrate permease, which seems to be H^+ dependent (30), whereas two citrate transport systems are present in *Klebsiella pneumoniae*, one being dependent on H^+ (45) and the other being dependent on Na^+ (9). The genes for H^+-dependent citrate transport systems of *E. coli* and *K. pneumoniae* have been isolated, and sequence analysis has shown that they code for related citrate-transport proteins (van der Rest et al., in press).

To assess the characteristics of citrate transport in lactococci, we describe in this paper the cloning, functional expression, and sequencing of the citrate carrier of *L. lactis* NCDO176 in *E. coli*. Additionally, we present an initial characterization of the mechanism of citrate uptake mediated by the lactococcal citrate carrier.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. lactis* subsp. *lactis* var. diacetylactis NCDO176 was the source of the Cit$^+$ determinant in plasmid pCT176 (11). *E. coli* K-12 strain DH1 [F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17*(r$^-$ m$^-$) *supE44 relA1* lambda$^-$] was used for selection of Cit$^+$ transformants. *E. coli* DH1 harboring plasmid pES1 containing the citrate carrier of *K. pneumoniae* (35) was the Cit$^+$ positive control in these experiments. *E. coli* NZ1021 is a derivative of MC1061 (4) carrying plasmid pGP1 (43) and was used in T7 RNA polymerase expression experiments. *E. coli* BL21 (DE3) (42) (F$^-$ *hsdR gal*) (obtained from F. W. Studier) was used for membrane vesicle isolations. *E. coli* cloning vectors pBR328 (39) and pT75 (obtained from S. Tabor and C. C. Richardson) were used to clone the citrate determinant from strain NCDO176.

Media and growth conditions. *E. coli* strains were grown in L-broth (24) with vigorous shaking at 37°C. When appropriate, the medium was supplemented with carbenicillin (100 μg/ml), kanamycin (20 μg/ml), or tetracycline (12.5 μg/ml) or a combination of these antibiotics.

Citrate-positive recombinants of *E. coli* DH1 were selected after overnight incubation on Simmons citrate agar plates (Difco Laboratories).

Cloning of the *citP* gene. CsCl-ethidium bromide density gradient-purified plasmid DNA from *L. lactis* NCDO176 was prepared by the method of Maniatis et al. (24) with minor variations as described previously (6) and was digested to completion with *Eco*RI. The 7.9-kb linearized plasmid band of pCT176 was isolated, inserted into the unique *Eco*RI site of vector pBR328, and transformed to *E. coli* MC1061.

* Corresponding author.

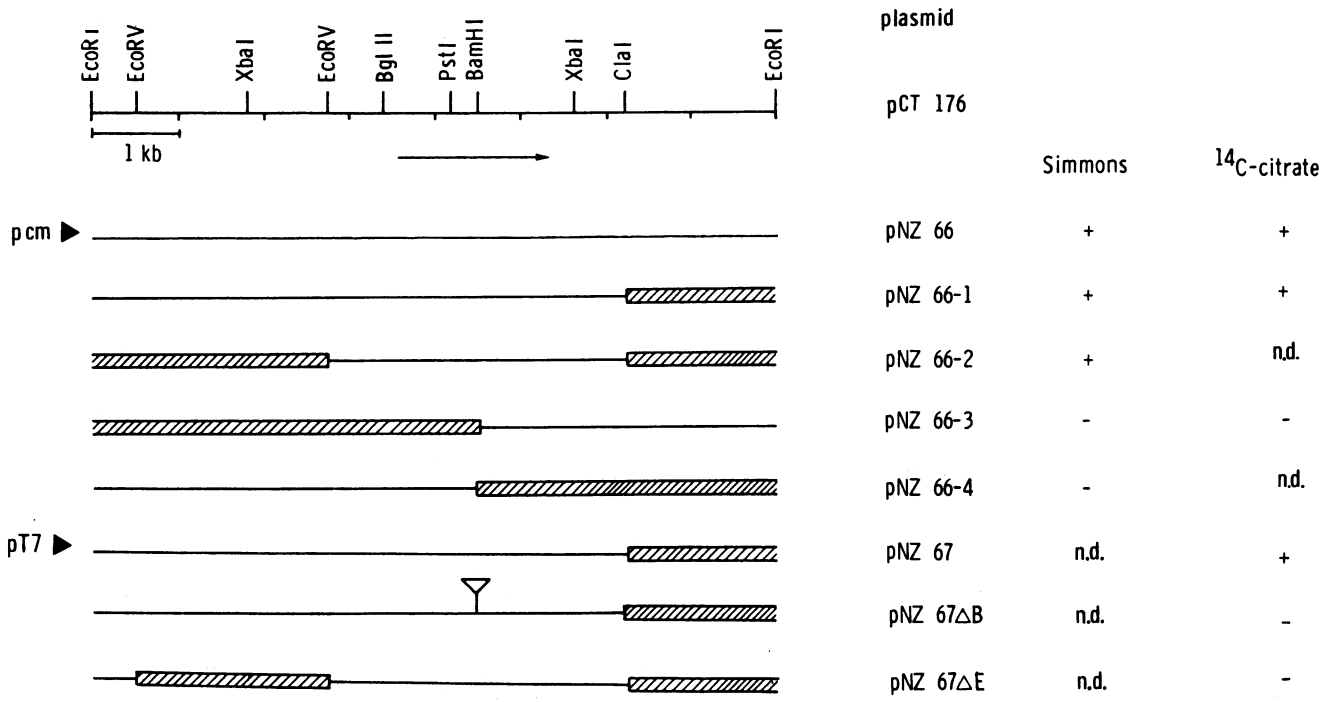

FIG. 1. Physical map and subcloning of pCT176. A partial restriction nuclease map of the citrate plasmid pCT176 and deletion derivatives of the fragment cloned in pBR328 and pT75 are shown. Symbols: →, location and orientation of the citrate permease (*citP*) gene on pCT176; ▨, regions that have been deleted in the cloned fragment. Plasmid pNZ66 contains pCT176 cloned as an *Eco*RI fragment (indicated by the line) into pBR328. The direction of the promoter of the chloramphenicol resistance gene located on pBR328 in front of the cloned fragments is indicated. Plasmid pNZ67 is a derivative of pT75. The 5-kb *Eco*RI-*Cla*I fragment of pCT176 was cloned behind the T7 promoter. The direction of the promoter transcription is indicated. In plasmid pNZ67ΔB the frameshift mutation within the *Bam*HI site of the cloned fragment is indicated. In pNZ67ΔE, an *Eco*RV fragment was deleted from the original *Eco*RI-*Cla*I fragment. The ability to transport citrate which is conferred by these plasmids on *E. coli* DH1 is shown in the right half of the figure and was determined by using Simmons citrate indicator agar plates and by [^{14}C]citrate uptake studies with intact cells. The amount of radioactivity found in citrate-positive cells ranged from 6,000 to 16,000 cpm, whereas in citrate-negative cells less than 3,000 cpm was detected. Symbols: +, citrate uptake or utilization; −, no citrate uptake or utilization; n.d., not determined.

Further subcloning and other DNA manipulations were performed as described by Maniatis et al. (24).

T7 expression experiments. For radioisotope labeling of proteins encoded by pCT176, DNA fragments were cloned in *E. coli* NZ1021 by using pT75 and the expression products were analyzed as described by Tabor and Richardson (43) on sodium dodecyl sulfate-12.5% polyacrylamide gels (21).

Membrane vesicle preparation. Membrane vesicles were prepared by the method of Kaback (17) from exponentially growing cells (A_{600}=0.8 to 1.0) of *E. coli* BL21, containing the appropriate plasmids, after induction of logarithmically growing cells with 400 μM isopropyl-β-D-thiogalactopyranoside for 90 min. Membrane vesicles were suspended in 50 mM potassium phosphate (pH 6.6) and stored in liquid nitrogen.

Transport assays (whole cells and membrane vesicles). We studied the transport of citrate in exponentially growing *E. coli* cells which were washed three times with 50 mM potassium phosphate (pH 5.5) containing 2 mM $MgSO_4$ and resuspended in the same buffer to 10 to 20 mg of total cellular protein per ml. Transport was assayed over 20 min with 9 μM [1,5-^{14}C]citric acid (110 mCi/mmol; The Radiochemical Centre, Amersham, England) at room temperature with samples containing 1 to 2 mg of protein per ml.

Incubation and processing were performed as described by Reynolds and Silver (30) for both whole cells and membrane vesicles, except that for transport studies in membrane vesicles we used 50 mM potassium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (K-PIPES; pH 6.6). Controls were assayed for the transport of L-[U-^{14}C]proline (154.5 mCi/mmol). The energy for citrate transport was supplied by 10 mM ascorbate and 100 μM phenylmethosulfonate (PMS). Protein determinations were performed by the method of Lowry et al. (22).

DNA sequence analysis. The DNA sequence of a 2.2-kb *Bgl*II-*Xba*I fragment of plasmid pCT176 (Fig. 1) was determined by using the method of Sanger et al. (31). Sequence data were analyzed by using PC/Gene, version 5.01 (Genofit, Geneva, Switzerland), nucleic acid and protein analysis programs and the computer facilities of the CAOS/CAMM Center, Nijmegen University, with the National Biomedical Research Foundation (NBRF/PIR) (release 23.0) and SWISS-PROT (release 13.0) data bases.

RESULTS

Cloning and functional expression of the Cit$^+$ determinant in *E. coli*. Cells of a derivative of *L. lactis* NCDO176, lacking the 7.9-kb plasmid pCT176, were unable to take up radioactively labeled citrate, indicating that this plasmid encodes a citrate permease (results not shown). Tetracycline-resistant transformants of *E. coli* DH1(pNZ66) were tested on Simmons citrate agar plates, on which colonies with a Cit$^+$ phenotype have a blue halo around the colonies. As a positive control in these experiments, we used *E. coli* DH1 (pES1), a pBR325 derivative containing the *K. pneumoniae*

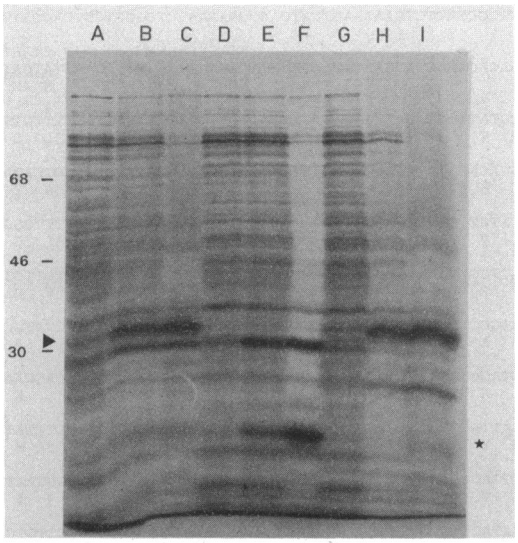

FIG. 2. Expression of the *citP* gene under T7-RNA polymerase control. *E. coli* NZ1012 cells containing plasmids pNZ67 (lanes A to C), pNZ67ΔB (lanes D to F), or pNZ67ΔE (lanes G to I) are shown. Lanes A, D, and G contain noninduced cells; lanes B, E, and H contain 42°C induced cells with no rifampin added; and lanes C, F, and I contain induced cells to which rifampin was added. The arrowhead indicates the position of the induced *citP* gene product. The asterisk indicates the position of the truncated *citP* gene product.

citrate transport gene (35). The pCT176 fragment was found to contain a functional citrate permease gene (*citP*), which was also demonstrated by the ability of transformants to take up citrate (Fig. 1). The Cit$^+$ phenotype was expressed in only one of the two possible orientations of the 7.9-kb *Eco*RI-cut plasmid DNA within the vector, suggesting that the promoter of the citrate permease gene either was not present on the cloned fragment or was not recognized in *E. coli*. The *Eco*RI fragment of pCT176 was further subcloned in pBR328 to narrow down the region encoding the *citP* gene (Fig. 1). Growth of these deletion derivatives in *E. coli* DH1 revealed that a 3.4-kb *Eco*RV-*Cla*I fragment in pNZ66-2 was still capable of conferring the Cit$^+$ phenotype.

T7 expression experiments. To analyze the proteins encoded by the cloned DNA fragments, we made additional constructs in the expression vector pT75. One of these recombinant plasmids, pNZ67 (Fig. 1), contained the 6.4-kb *Eco*RI-*Cla*I fragment of pCT176 under control of the T7 RNA polymerase promoter. One derivative of pNZ67, pNZ67ΔB, containing a frameshift mutation in the *Bam*HI site of the insert, was constructed by cutting with *Bam*HI, filling up the protruding ends with Klenow DNA polymerase, and religating the fragment. A second derivative, pNZ67ΔE, had the 2-kb *Eco*RV fragment deleted from the insert. [^{35}S]methionine-labeled proteins specified by the recombinant plasmids were analyzed. After temperature induction, the presence of a 32-kilodalton (kDa) protein band was visible in preparations of cells harboring pNZ67 or pNZ67ΔE but not pNZ67ΔB (Fig. 2). Also, the cells showing the 32-kDa protein band were able to take up radioactively labeled citrate. Cells containing plasmid pNZ67ΔB were unable to transport citrate (Fig. 1). This strain showed a band of approximately 20 kDa, which was absent in cells containing plasmid pNZ67ΔE or pNZ67ΔB. Strain NZ1021 harboring pNZ67 or pNZ67ΔB showed an additional protein band of approximately 30 kDa, which was absent in cells harboring pNZ67ΔE.

Transport studies in membrane vesicles. Membrane vesicles were prepared from *E. coli* cells carrying pNZ67 or pNZ67ΔB. IPTG induction proved to be a more reproducible and efficient method than temperature for induction of T7 polymerase-dependent citrate transport in vesicle preparations. Plasmids pNZ67 and pNZ67ΔB were transformed to *E. coli* BL21 containing a chromosomally linked T7 RNA polymerase gene under control of the IPTG-inducible *tac* promoter (42). Membrane vesicles of BL21 cells harboring pNZ67 or pNZ67ΔB accumulated proline in the presence of

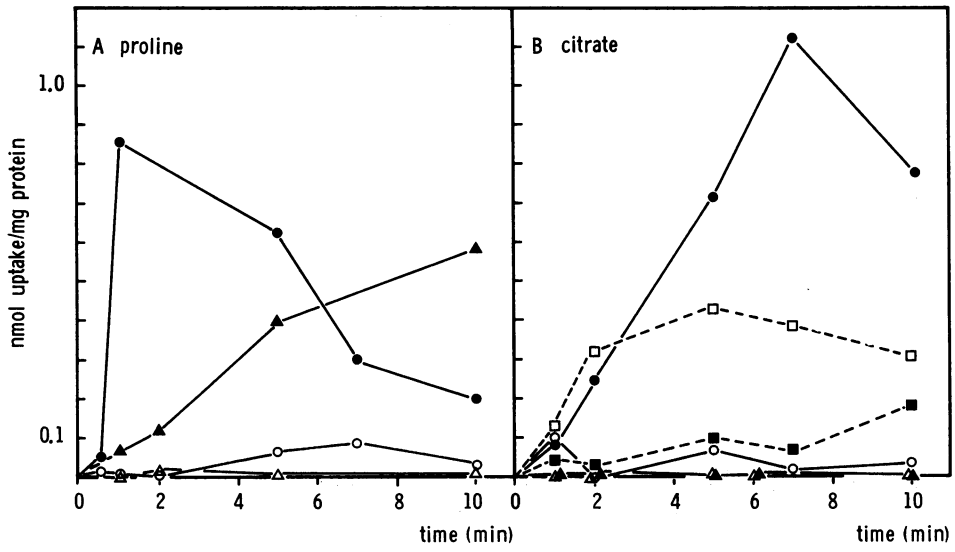

FIG. 3. Uptake studies of [^{14}C]proline (2 μM) (A) and [^{14}C]citrate (4.5 μM) (B) by membrane vesicles of *E. coli* BL21 with ascorbate-PMS as the electron donor (●, ▲, ■) and without ascorbate-PMS (○, △, □). Symbols: ●, ○, uptake of membrane vesicles of strain BL21(pNZ67); ▲, △, uptake of membrane vesicles of BL21(pNZ67ΔB); ■, □, [^{14}C]citrate uptake of ascorbate-PMS-energized membrane vesicles after the addition of valinomycin (□) or nigericin (■). The values are averages of at least two separate experiments.

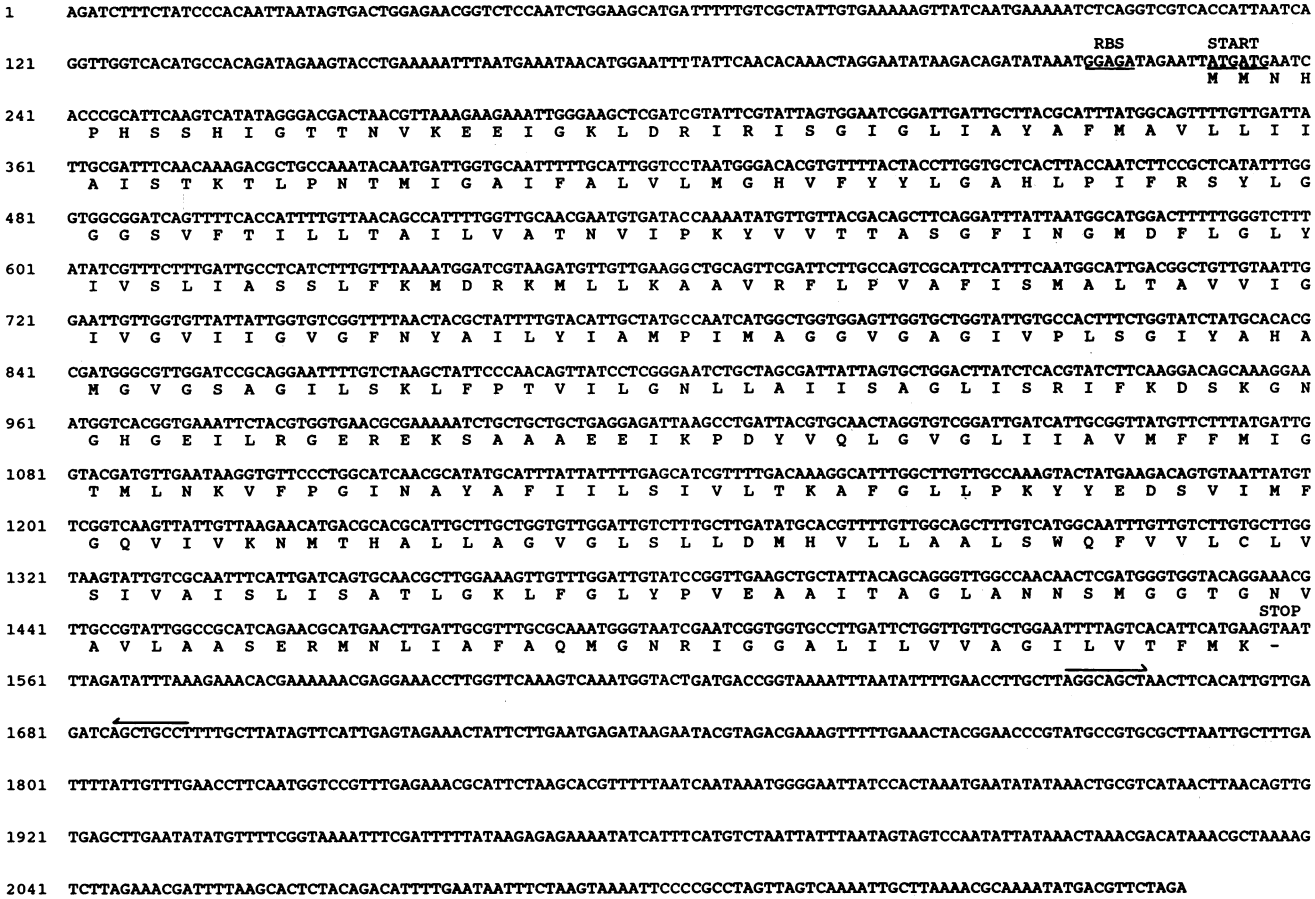

FIG. 4. Nucleotide sequence of the 2.2-kb *Bgl*II-*Xba*I (Fig. 1) fragment containing the *citP* gene and deduced amino acid sequence of the encoded protein. The putative start codons are underlined. A putative ribosome-binding site (RBS) is indicated. Arrows indicate an inverted repeat, which could function as a terminator of transcription.

ascorbate-PMS, as expected (Fig. 3A). The differences in proline uptake between the strains harboring either plasmid pNZ67 or pNZ67ΔB may be attributed to differences in the vesicle preparations. Similar differences were observed with different vesicle preparations of one strain (data not shown). Membrane vesicles from Cit^+ cells, containing plasmid pNZ67, were also able to take up citrate (Fig. 3B). Citrate transport in these vesicles appeared to be driven by the proton motive force (ΔP), since dissipation of the $\Delta\psi$ by valinomycin or of the ΔpH by nigericin inhibited the accumulation of citrate (Fig. 3B).

Nucleotide sequence of the *citP* gene and flanking regions. The nucleotide sequence of a 2.1-kb fragment containing the citrate transport gene (*citP*) was determined (Fig. 4). There was only one open reading frame, from positions 131 to 1557, of sufficient size to encode a 32-kDa protein. The DNA sequence for the open reading frame contained the single *Bam*HI site at position 852. Introduction of a frameshift mutation within this site introduces a stop codon at position 872 and results in the formation of a truncated, nonfunctional protein product (Fig. 2 and 3). The open reading frame for *citP* starts with two adjacent ATG triplets. If the first initiation codon was used (position 259), the gene would encode a protein of 442 amino acids with a calculated molecular weight of 46,645.

Analysis of the deduced amino acid composition of the presumed citrate carrier indicates that this protein is highly hydrophobic, with 12.4% polar and 87.5% nonpolar amino acid residues. The ratio of basic (lysine, arginine, and histidine) to acidic (glutamate and aspartate) residues is 2.1:1 and indicates that *citP* encodes a basic protein with a calculated isolectric point of 9.97. The deduced amino acid sequence of the citrate carrier contains segments of high hydrophobicity that alternate with short hydrophilic segments (data not shown, 20). Both N- and C-terminal ends of the amino acid sequence are more hydrophilic, as is the region between residues 240 and 280.

There was no homology between the *Lactococcus* citrate carrier protein and other known citrate carriers, such as those of *E. coli* (32) and *K. pneumoniae* (45). Also, a search of the NBRF and SWISS data bases with the predicted primary sequence of *citP* failed to detect significant homology to any of the published sequences.

DISCUSSION

We describe the cloning, expression in *E. coli*, and nucleotide sequence of the plasmid-encoded *citP* gene of *L. lactis* NCDO176. Transport of [^{14}C]citrate by whole cells and membrane vesicles of *E. coli* harboring *citP* expressing plasmids was demonstrated.

The *citP* gene product is a protein with a gel electrophoresis rate corresponding to an apparent molecular mass of 32 kDa (Fig. 2). The 32-kDa protein band is probably the *citP*

gene product, and the 20-kDa band visible in strains carrying pNZ67ΔB may represent a truncated derivative of the *citP* gene product. The 30-kDa protein band encoded by pNZ67ΔE may be a second protein encoded by the *Eco*RV fragment that has no apparent function in citrate transport. The results were supported by citrate uptake studies in membrane vesicles of *E. coli* cells carrying *citP*-expressing plasmids (Fig. 3). These studies show that citrate transport is driven by the ΔP. More extensive studies are needed to reveal the nature of the cations symported with citrate and the contribution of the components of the ΔP in the transport process.

The nucleotide sequence of *citP* (Fig. 4) was identified which starts with two ATG codons. At this stage we do not know which initiation codon is actually used. A putative ribosome-binding site (GGAG at position 247), complementary to the 16S rRNA of *E. coli* ($\Delta G°$ of −7.2 kcal/mol [ca. −30.1 kJ/mol], calculated by the method of Tinoco et al. [44]) is present 9 nucleotides preceding the first of the two possible initiation codons. However, regions that are similar to *E. coli* (26) or *L. lactis* (8) consensus promoter transcription initiation sequences were not found. An inverted repeat 97 base pairs downstream of the stop codon at position 1557 showed homology to typical ρ-independent terminators of transcription (29) (Fig. 4). It was also found that in other citrate carrier genes of *E. coli* (32) and *K. pneumoniae* (45), no promoter sequences were present in the region preceding the sequence encoding the citrate carrier. A second open reading frame, located 54 base pairs in front of the citrate carrier gene, is proposed to be necessary for undelayed citrate utilization in *E. coli* (32). In *K. pneumoniae* no such open reading frame has been detected, although the inability to obtain functional expression in some deletion derivatives has been interpreted as evidence for the presence of such an open reading frame (45). There are no indications of a similar structure in *L. lactis*, since deletion of a region upstream of the *citP* gene, such as in pNZ67ΔE, did not show any delayed growth or delayed uptake of labeled citrate into whole cells (Fig. 1 and results not shown).

The hydropathy profile of *citP* resembles those of other membrane-associated proteins. For instance, the citrate transport proteins of both *E. coli* and *K. pneumoniae* also contain a central hydrophilic region as well as hydrophilic N and C termini. The hydropathy profile of a hydrophobic protein may be a good description of the folding structure of the protein (27). The hydrophobic regions of the sequence may well represent membrane-spanning domains. These results strongly suggest that the *L. lactis* citrate permease is an integral membrane protein; this is in agreement with the location of the *citP* expression product in the cytoplasmic membrane.

The molecular mass calculated from the deduced primary sequence of the putative citrate carrier is 46.6 kDa, larger than the molecular mass of 32 kDa estimated from the mobility of the *citP* gene product on a sodium dodecyl sulfate-polyacrylamide gel. Such an aberrant migration on sodium dodecyl sulfate-polyacrylamide gels is well documented for a variety of hydrophobic proteins (3, 23).

The lack of homology between the citrate carriers of gram-negative bacteria and the lactococcal CitP suggests that the *L. lactis* citrate permease belongs to a different class of carriers. The observation that the *citP* gene can functionally complement *E. coli* suggests that all information for citrate transport is contained in its gene product.

ACKNOWLEDGMENTS

We thank B. Poolman for helpful discussions and practical guidance in preparing membrane vesicles and transport studies. We are also grateful to W. N. Konings for critically reading the manuscript.

This work was partly supported by the Programme Committee on Agricultural Biotechnology and the Biotechnology Action Programme of the Commission of European Communities (grant no. BAP-0011-NL).

LITERATURE CITED

1. **Ashton, D. M., G. D. Sweet, J. M. Somers, and W. W. Kay.** 1980. Citrate transport in *Salmonella typhimurium*: studies with 2-fluoro-L-erythro-citrate as a substrate. Can. J. Biochem. **58:** 797–803.
2. **Bergsma, J., and W. N. Konings.** 1983. The properties of citrate transport in membrane vesicles from *Bacillus subtilis*. Eur. J. Bacteriol. **134:**151–156.
3. **Buchel, D. E., B. Grononborn, and B. Muller-Hill.** 1980. Sequence of the lactose permease gene. Nature (London) **283:** 541–545.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **143:**179–207.
5. **Cogan, T. M., and C. Daly.** 1987. Cheese starter cultures, p. 178–249. *In* P. F. Fox (ed.), Cheese: chemistry, physics and microbiology. Applied Science Publishers, London.
6. **David, S., G. Simons, and W. M. de Vos.** 1989. Plasmid transformation by electroporation of *Leuconostoc paramesenteroides* and its use in molecular cloning. Appl. Environ. Microbiol. **55:**1483–1489.
7. **Deibel, R. H., and H. W. Seeley, Jr.** 1983. Family II. *Streptococcaceae*, p. 490–509. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of determinative bacteriology, 2nd ed. The Williams & Wilkins Co., Baltimore.
8. **De Vos, W. M.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46:**281–295.
9. **Dimroth, P., and A. Thomer.** 1986. Citrate transport in *Klebsiella pneumoniae*. J. Biol. Chem. **367:**813–823.
10. **Gasson, M. J., and F. L. Davies.** 1984. The genetics of lactic-acid bacteria, p. 99–126. *In* F. L. Davies and B. A. Law (ed.), Advances in the microbiology and biochemistry of cheese and fermented milk. Applied Science Publishers, London.
11. **Gasson, M. J., S. H. A. Hill, and P. H. Anderson.** 1987. Molecular genetics of metabolic traits in lactic streptococci, p. 242–245. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
12. **Harvey, R. J., and E. B. Collins.** 1962. Citrate transport system of *Streptococcus diacetylactis*. J. Bacteriol. **83:**1005–1009.
13. **Ishiguro, N., K. Hirose, and G. Sato.** 1980. Distribution of citrate utilization plasmids in *Salmonella* strains of bovine origin in Japan. Appl. Environ. Microbiol. **40:**446–451.
14. **Ishiguro, N., C. Oka, Y. Hanzawa, and G. Sato.** 1979. Plasmids in *Escherichia coli* controlling citrate-utilizing ability. Appl. Environ. Microbiol. **38:**956–964.
15. **Ishiguro, N., G. Sato, C. Sasakawa, H. Danbara, and M. Yoshikawa.** 1982. Identification of citrate utilization transposon Tn*3411* from a naturally occurring citrate utilization plasmid. J. Bacteriol. **149:**961–968.
16. **Johnson, C. L., Y.-A. Cha, and J. R. Stern.** 1975. Citrate uptake in membrane vesicles of *Klebsiella aerogenes*. J. Bacteriol. **121:**682–687.
17. **Kaback, H. R.** 1971. Bacterial membranes. Methods Enzymol. **22:**99–120.
18. **Kay, W. W., and M. Cameron.** 1978. Citrate transport in *Salmonella typhimurium*. Arch. Biochem. Biophys. **190:** 270–280.
19. **Kempler, G. M., and L. L. McKay.** 1979. Characterization of plasmid DNA in *Streptococcus lactis* subsp. *diacetylactis*: evidence for plasmid-linked citrate utilization. Appl. Environ. Microbiol. **37:**316–323.
20. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for

displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Maiden, M. C. J., E. O. Davis, S. A. Baldwin, D. C. M. Moore, and P. J. F. Henderson.** 1987. Mammalian and bacterial sugar transport proteins are homologous. Nature (London) **325**: 641–643.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Martin, W. J., and J. A. Washington II.** 1980. *Enterobacteriaceae*, p. 195–219. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
26. **McClure, W. R.** 1985. Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. **54**:171–204.
27. **Nakao, T., I. Yamato, and Y. Anraku.** 1987. Nucleotide sequence of *putP*, the proline carrier gene of *E. coli* K12. Mol. Gen. Genet. **208**:70–75.
28. **O'Brien, R. W., and J. R. Stern.** 1969. Requirement for sodium in the anaerobic growth of *Aerobacter aerogenes* on citrate. J. Bacteriol. **98**:388–393.
29. **Platt, T., and D. G. Bear.** 1983. Role of RNA polymerase, ρ-factor, and ribosomes in transcription termination, p. 123–161. *In* J. Beckwith, J. Davies, and J. A. Gallant (ed.), Gene function in procaryotes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Reynolds, C. H., and S. Silver.** 1983. Citrate utilization by *Escherichia coli*: plasmid- and chromosome-encoded systems. J. Bacteriol. **156**:1019–1024.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Sasatsu, M., T. K. Misra, L. Chu, R. Laddaga, and S. Silver.** 1985. Cloning and DNA sequence of a plasmid-determined citrate utilization system in *Escherichia coli*. J. Bacteriol. **164**: 983–993.
33. **Sato, G., M. Asagi, M. Oka, N. Ishiguro, and N. Terakado.** 1978. Transmissible citrate-utilizing ability in *E. coli* isolated from pigeons, pigs and cattle. Microbiol. Immunol. **22**:357–360.
34. **Schleifer, K. H., and R. Klipper-Baelz.** 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. Syst. Appl. Microbiol. **10**:1–19.
35. **Schwarz, E., and D. Osterhelt.** 1985. Cloning and expression of *Klebsiella pneumoniae* genes coding for citrate transport and fermentation. EMBO J. **4**:1599–1603.
36. **Seitz, E. W., W. E. Sandine, P. R. Elliker, and E. A. Day.** 1963. Distribution of diacetyl reductase among bacteria. J. Dairy Sci. **46**:186–189.
37. **Shinagawa, M., S. Makino, T. Hirato, N. Ishiguro, and G. Sato.** 1982. Comparison of DNA sequences required for the function of citrate utilization among different citrate utilization plasmids. J. Bacteriol. **151**:1046–1050.
38. **Smith, H. W., Z. Parsell, and P. Green.** 1978. Thermosensitive H1 plasmids determining citrate utilization. J. Gen. Microbiol. **109**:305–311.
39. **Soberon, X., L. Covarrubias, and F. Bolivar.** 1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene **9**:287–305.
40. **Somers, J. M., G. D. Sweet, and W. W. Kay.** 1981. Fluorocitrate resistant tricarboxylate transport mutuants of *Salmonella typhimurium*. Mol. Gen. Genet. **181**:338–345.
41. **Speckman, R. A., and E. B. Collins.** 1968. Diacetyl biosynthesis in *Streptococcus diacetylactis* and *Leuconostoc citrovorum*. J. Bacteriol. **95**:174–180.
42. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
43. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
44. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) **246**:40–41.
45. **Van der Rest, H. E., E. Schwarz, D. Oesterhelt, and W. N. Konings.** 1990. DNA sequence of a citrate carrier of *Klebsiella pneumoniae*. Eur. J. Biochem. **189**:401–407.