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

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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 [1,5-¹⁴C]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 *Enterobacteriaceae* (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(rK- 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 pGPI (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 EcoRI. The 7.9-kb linearized plasmid band of pCT176 was isolated, inserted into the unique *EcoRI* site of vector pBR328, and transformed to *E. coli* MC1061.

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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_660nm=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 [1,5-14C]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-14C]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 BglII-XbaI 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*...
E. coli NZ1012 cells containing plasmids pNZ67 (lanes A to C), pNZ67ΔA (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 \textit{citP} gene product. The asterisk indicates the position of the truncated \textit{citP} gene product.

citrate transport gene (35). The pCT176 fragment was found to contain a functional citrate permease gene (\textit{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 \textit{EcoRI}-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 \textit{E. coli}. The \textit{EcoRI} fragment of pCT176 was further subcloned in pBR328 to narrow down the region encoding the \textit{citP} gene (Fig. 1). Growth of these deletion derivatives in \textit{E. coli} DH1 revealed that a 3.4-kb \textit{EcoRV}-\textit{ClaI} fragment in pNZ66-2 was still capable of conferring the Cit$^+$ phenotype.

\textbf{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 \textit{EcoRI}-\textit{ClaI} fragment of pCT176 under control of the T7 RNA polymerase promoter. One derivative of pNZ67, pNZ67ΔB, containing a frameshift mutation in the \textit{BamHI} site of the insert, was constructed by cutting with \textit{BamHI}, filling up the protruding ends with Klenow DNA polymerase, and religating the fragment. A second derivative, pNZ67ΔE, had the 2-kb \textit{EcoRV} fragment deleted from the insert. $^{[35S]}$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 pNZ67ΔB but not pNZ67ΔE (Fig. 1). Also, the cells showing the 32-kDa protein band were able to take up radioactively labeled citrate. Cells containing plasmid pNZ67AB 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 pNZ67AB. Strain NZ1021 harboring pNZ67 or pNZ67AB showed an additional protein band of approximately 30 kDa, which was absent in cells harboring pNZ67ΔE.

\textbf{Transport studies in membrane vesicles.} Membrane vesicles were prepared from \textit{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 \textit{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

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Uptake studies of [\textsuperscript{14}C]proline (2 \textmu M) (A) and [\textsuperscript{14}C]citrate (4.5 \textmu M) (B) by membrane vesicles of \textit{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); ■, □, [\textsuperscript{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.}
\end{figure}
ascorbate-PMS, as expected (Fig. 3A). The differences in proline uptake between the strains harboring either plasmid pNZ67 or pNZ67AB 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 Citr. citratus, 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 (AP), since inhibition of the Δφ 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 BglII-XbaI (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.

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 isoelectric 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 NCD0176. Transport of [14C]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 pNZ67Δp may represent a truncated derivative of the citP gene product. The 30-kDa protein band encoded by pNZ67Δp may be a second protein encoded by the EcoRV 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 (ΔG° 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 p-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 pNZ67Δp, 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 hydrophobic N and C termini. The hydropathic 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.

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