Cloning and Nucleotide Sequence of the Gene (citC) Encoding a Citrate Carrier from Several Salmonella serovars*

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The nucleotide sequence of the citC coding for the citrate carrier in several Salmonella serovars has been determined, and the amino acid sequence of the carrier protein was deduced. The predicted citrate carrier from Salmonella pullorum and Salmonella enteritidis consists of 446 amino acids with a molecular weight of 47,621, whereas that from Salmonella dublin is the same 446 amino acids with the slightly different molecular weight of 47,591, because 1 amino acid residue was substituted. The predicted proteins are highly hydrophobic (69% nonpolar amino acids). The hydropathy profile suggests that the proteins are composed of 11-12 hydrophobic membrane-spanning segments with two hydrophilic cores in the middle of the protein sequence. No homology in the nucleotide and amino acid sequences was found in the molecular structures of citA, citP, and tctI genes. The citC-coding citrate transport activity is Na+-dependent and specific for citrate only. The transcript from the citC gene was not detected in the total RNA from several Salmonella serovars except S. dublin in Northern blot analysis, although the promoter of the citC genes appeared to be functional in Escherichia coli and Salmonella typhimurium strains using the lacZ fusion assay. These results suggested that the citC gene-coding citrate carrier is probably a TctIII system such as that identified previously in S. typhimurium.

Citrate utilization has been used as an important marker for characterization of bacteria, especially in clinical microbiology, where this characteristic is made use of in medical diagnostic tests. Most species of the Enterobacteriaceae are able to utilize citrate either aerobically or anaerobically as a sole carbon source. Escherichia coli is typically unable to utilize citrate as the sole source of carbon and energy; therefore, this inability is useful for distinguishing E. coli from other enterobacteria. However, naturally occurring citrate-utilizing (Cit+) E. coli strains have been widely isolated from animal and human sources (1, 2), and this ability was shown to be controlled by a plasmid (3-5). Recently, the plasmid-borne citrate utilization operon, which consists of citA and citB genes, has been cloned and sequenced independently in two laboratories (6, 7). In two genes, the citA gene encoding a 431-amino acid polypeptide and the citB gene coding for a 379-amino acid polypeptide, citA was found to be an essential gene required for citrate utilization in E. coli (7, 8). This citA protein was identified as a hydrophobic membrane protein that mediates the transport of citrate across the cytoplasmic membrane via a proton-citrate symport system (7, 9).

Among the enterobacterial strains in which the citrate transport system has been studied, Salmonella typhimurium has been well characterized. S. typhimurium is reported to possess three citrate transport systems, TctI, which is Na+-dependent and a periplasmic binding protein-dependent, TctII and TctIII, which are also Na+-dependent (10). The tctI operon encoding the TctI system has been cloned and partially sequenced (11, 12), but the other two transport systems (TctII and TctIII) have not been thoroughly studied at molecular level.

Our previous study (13) showed that there was a DNA region that was homologous to the plasmid-derived citA gene in the chromosomal DNA of Salmonella serovars revealed by Southern blot hybridization. To isolate and characterize the DNA regions hybridized with the plasmid-borne citA gene, we cloned the DNA region from the S. typhimurium chromosome to a vector plasmid using Cit+ ability as a selection marker in an E. coli K-12 derivative. In a previous study (14), we isolated the Cit+ determinant, which had a structure similar to the plasmid-derived cit operon (citA and citB genes) from S. typhimurium, and determined its nucleotide sequence. The Salmonella citA gene sequence appeared to encode a 434-amino acid protein which showed 94% identity with those of Tn3411 (6) and Citrobacter amalonaticus (15) and 69% identity with the Cit+ carrier of Klebsiella pneumonaeiae (16). However, the Salmonella citA-coding citrate transport system seemed to be different from the three known citrate transport systems (TctI, TctII, and TctIII), because it was not Na+-dependent (10). We further continued to isolate the Cit+ determinant from several Salmonella serovars and cloned two kinds of cit genes from Salmonella strains in E. coli. One of them corresponded to the Salmonella citA gene which was reported previously (14), whereas the other cit gene (designated as citC) differed in molecular structure from the known citrate transport genes.

To assess the characteristics of the new citrate transport system from several Salmonella serovars, we describe herein the cloning, functional expression, and DNA sequencing of the citrate carrier from Salmonella strains in E. coli K-12. We present the molecular structure of a new citrate transport...
Nucleotide Sequence of Citrate Carrier Gene (citC)

Isolation of Two Kinds of cit Genes from Several Salmonella Strains— Chromosomal DNAs from five Salmonella serovars, S. typhimurium, S. enteritidis, S. abortusequi, S. dublin, and S. pullorum were digested with enzyme PstI. The resulting DNA fragments were ligated with PstI-digested pBluescript and transformed into E. coli XL1-blue to select Cit+ colonies on Simons citrate agar. A total of 13 Cit+ colonies were obtained from three Salmonella serovars, S. enteritidis 1074 and 1078, S. dublin 35, and S. pullorum Narl and 1037, but no positive colony was found from S. typhimurium LT-2 and S. abortusequi 96 and 1141. The recombinant plasmids prepared from the Cit+ transformants were digested with various enzymes, and their molecular structures were analyzed. They were classified into two groups: one group possessed a 5.2-kb PstI DNA fragment and the other had a 3.0-kb PstI DNA fragment. The 5.2-kb DNA fragment showed a structure similar to that of the citA gene from S. typhimurium (14) and identity with the cit operon (citA and citB genes) of Tn3411 (6, 8) in Southern blot analysis (data not shown), but the 3.0-kb DNA fragment was distinct from those of the tctl operon and the citA gene from S. typhimurium. Therefore, a new functional citrate-utilizing gene within a 3.0-kb DNA fragment was tentatively designated as the citC gene and used as described below.

As shown in Fig. 1, A and B, two (citA and citC) genes obtained in this study were found by Southern blot analyses to be widely conserved in several Salmonella serovars. However, there was no DNA region homologous to both citA and citC genes on the E. coli chromosome.

Subcloning of the Cit+ Determinant and Growth Property—To examine the location of the citC gene in the 3.0-kb DNA fragment, three representative plasmids, pOH61, pOH1074, and pOH353 from S. pullorum 1037, S. enteritidis 1074, and S. dublin 35, respectively, were used for subcloning. All three plasmids showed the same restriction map. The map of the 3.0-kb DNA fragment in pOH61 and its derivatives are presented in Fig. 2. From the deletion analysis of pOH61 derivatives, the citC gene appeared to be located in the 1,730-bp internal HindIII-BamHI DNA region. The growth properties of E. coli SG11 carrying pOH61 were examined in Tanaka minimal medium containing sodium citrate or potassium citrate as the sole carbon source. E. coli SG11 cells with

Fig. 1. Hybridization of the 32P-labeled citA DNA region (the 450-bp Ddel fragment) (A) and 32P-labeled citC DNA region (the 490-HindIII-Sall fragment) (B) to PstI digests of chromosomal DNAs from several Salmonella serovars. Chromosomal DNAs for hybridization analyses were digested with PstI and transferred to nitrocellulose filters. Lane 1, S. typhimurium LT-2; lane 2, S. enteritidis 1074; lane 3, S. enteritidis 1078; lane 4, S. abortusequi 96; lane 5, S. abortusequi 1141; lane 6, S. dublin 26; lane 7, S. dublin 35; lane 8, S. pullorum Narl; lane 9, S. pullorum 1037; lane 10, E. coli SG11. Sizes of the hybridizing bands are calculated using standard markers (λ HindIII digests) and indicated in kilobase pairs.
pOH61 (citC) were able to grow on only citrate in the presence or absence of Na+, but not on other tricarboxylic acids such as cis-aconitate, trans-aconitate, isocitrate, and tricarballylate. This substrate specificity was different from that of the Salmonella citA gene (14), but coincided with the TctIII system found previously in S. typhimurium (10). All known citrate transport systems except the citA-coding system of S. typhimurium have a lag-time before growth (10). A similar long lag-phase was observed with the Salmonella citA gene (14), but coincided with the TctIII system found previously in S. typhimurium (10). All known citrate transport systems except the citA-coding system of S. typhimurium have been reported to be dependent on Na+ and have a lag-time before growth (10). A similar long lag-phase was observed with S. enteritidis and S. dublin, from which the citC gene has been isolated (data not shown). Although E. coli SG11 cells with pOH61 showed a lag-phase in the growth on citrate, the lag-time was shorter than that observed in cells with pHD1 or pCUT103 (Salmonella citA gene; Ref. 14) (data not shown).

Citrate Transport and Na+ Dependence—The citrate transport activity of E. coli SG11 with or without Cit+ plasmids was investigated. E. coli SG11 strains carrying Cit- plasmids pOH61 and pOH353 from S. pullorum and S. dublin, respectively, could constitutively transport citrate in spite of induction by the citrate (Fig. 3), whereas E. coli could not transport citrate. The transport activity of E. coli strain SG11 possessing the deletion plasmid pOH62 was much lower than that of E. coli SG11 with pOH61. This lower activity may be due to the lack of either its own promoter or N-terminal amino acids in the citC gene (Fig. 3), because the N-terminal nucleotides of the citC gene in pOH62 were found by DNA sequencing analysis to be deleted during the subcloning (below and Fig. 6). The expression of the citC gene in pOH62 seemed to be controlled by the promoter activity of the vector plasmid.

To examine the substrate specificity of the citrate carriers, competitive inhibition tests using tricarboxylic acid cycle-related intermediates were done. As shown in Fig. 4, it was immediately apparent that there was no significant interference in E. coli SG11/pOH61 and SG11/pOH353 strains from various tricarboxylic acid-related substrates. In contrast, the transport activity encoded by pCUT103 (the citA gene) which is isolated from S. typhimurium (14) was completely inhibited by two tricarboxylic acids, cis-aconitate and tricarballylate (Fig. 4). Therefore, the citC-coding citrate carrier appeared to be specific for citrate, and its specificity was different from that of the Salmonella citA gene.

Since the citrate transport activity of the three Tct systems found in S. typhimurium LT-2 is markedly stimulated by sodium ions (25), the effect of Na+ on the citC-coding citrate transport was examined. Fig. 5 shows the effect of the external Na+ concentration on the transport of citrate in E. coli SG11 strains carrying pOH61 and pOH353. Maximal stimulation was attained at 10 mM NaCl in E. coli SG11/pOH353, but the citrate transport activity of E. coli SG11/pOH61 was dependent on the concentration of sodium ions (Fig. 5). It thus appeared that citrate transport activity encoded by the citC gene was dependent on sodium ions. In these experiments, the total concentrations of NaCl and KCl were kept constant at 100 mM so that there would be no osmotic difference.

**Nucleotide Sequence of the citC Gene and Deduced Amino Acid Sequence**

The nucleotide sequence of the citC gene in plasmid pOH61 and deletion derivatives of the fragment cloned in pBluescript (A) and hydropathy profile of the amino acid sequence of the citC gene (B). A, the black boxes indicate the cloned regions. The ability of citrate utilization, which is conferred by the deletion derivatives in E. coli SG11, is shown at the right of the figure and was determined by using Simmons citrate agar plates. The location and direction of the citC gene are indicated by the open-arrow box. Vertical lines indicate the cleavage sites of the following endonucleases: B, BamHI; H, HindIII; P, PstI; S, Sall. B, hydropathy was calculated according to Kyte and Doolittle (28) with a span of 11 residues.

**Fig. 2. Restriction endonuclease map of the citC gene in plasmid pOH61 and deletion derivatives of the fragment cloned in pBluescript (A) and hydropathy profile of the amino acid sequence of the citC gene (B). A, the black boxes indicate the cloned regions.**

**Fig. 3. Transport of citrate in E. coli SG11 with or without plasmids.** Cells were (O) or were not (C) induced with 10 mM sodium citrate. Transport of citrate was measured in E. coli SG11 carrying the indicated plasmids.

**Fig. 4. [14C]Citrate transport activity and competitive inhibition by several tricarboxylic acids and succinate.** E. coli strains carrying plasmids used in this study are indicated. Several substrates for inhibition were added at a final concentration of 5 mM before the addition of [1,5-14C]citric acid and incubated for 1 min at 25°C. Samples were taken and measured for citrate transport activity.
The amino acid sequence is shown in Fig. 6. The putative citrate carrier indicates that the carrier is highly hydrophobic (31% polar, 69% nonpolar). This citrate carrier contains 62 charged amino acid residues (27 acidic residues and 35 basic residues), suggesting that the citC-encoding citrate carrier is a basic protein. There was no homology between the citC-coding citrate carrier protein and the other known citrate carriers, such as those of E. coli (6, 7), S. typhimurium (12, 14), K. pneumoniae (16), and Lactococcus lactis (27). A search of NBRF and SWISS data bases with the predicted primary sequence of citC failed to detect significant homology with any of the published sequences.

Hydropathy Profile of the Citrate Carrier—Hydropathy analysis of the citrate carrier was carried out by the method of Kyte and Doolittle (28). The hydropathy profile calculated with an 11-residue span is shown in Fig. 2B. This profile shows that the citrate carrier contains 11-12 hydrophobic segments with two hydrophilic cores in the middle of the protein sequence. This unique profile suggests that the citrate carrier encoded by the citC gene is probably a membrane protein.

Recently, Maiden et al. (29) pointed out that the Arg-X-

Acid Sequence of the Citrate Carrier—The nucleotide sequences of 2.1-kb DNA fragments between the external HindIII sites and the PstI sites of three plasmids, pOH61, pOH353, and pOH1074, were determined. The nucleotide sequence of the 2.1-kb DNA fragment in pOH61 is presented in Fig. 6 and compared with that in pOH353. The nucleotide sequence of the 2.1-kb fragment in pOH1074 was the same as that of pOH353, except that one T nucleotide at nucleotide position 155-157 and 161-163 were preceded by the sequence GTG.

The open reading frame from the ATG codon at position 161-163 is shown above the arrows. This profile shows that the citrate carrier contains 11-12 hydrophobic segments with two hydrophilic cores in the middle of the protein sequence. This unique profile suggests that the citrate carrier encoded by the citC gene is probably a membrane protein.
Gly-Arg-Arg motif is conserved in the hydrophilic regions of several H+-sugar cotransporters of mammalian and bacteria. In fact, this motif was found in the citrate carrier sequence of citA genes from E. coli (6, 7), S. typhimurium (14), and K. pneumoniae (16), but was not observed in the protein sequences of the citC gene reported here or in the citP gene from L. lactis (27). In contrast, Deguchi et al. (30) reported that the unique alignment of 5 amino acid residues (Gly42-Ala82-X-X-X-X-Leu87-X-X-X-Gly91-Arg92) was commonly conserved in four Na+ symport carrier proteins, the glutamate carrier and proline carrier of E. coli and the Na+/glucose co-transporters of rabbit and human intestines. They proposed that this consensus motif may play an important role in cation recognition or binding in the Na+/solute symport reaction. However, although the citC-coding citrate transport system was Na+-dependent, this consensus motif was not found in its protein sequence (6). These results may indicate that the Na+ binding and coupling mechanism of the citC-coding citrate carrier is different from that of the other carriers. Moreover, unique kinetics of the citrate transport activity of the citC gene in different Na+ concentrations was observed in E. coli SG11 strains carrying pOH61 and pOH353 (Fig. 5). This difference in affinities for Na+ of the citrate transport may be interpreted as being due to a difference between the carrier conformations outside and inside the membrane, because 1 amino acid was changed in the N terminus of the citC gene in pOH353 (Fig. 6). Since there was a report (31) showing that cation specificity mutations of the gene of S. typhimurium were located in the N and C termini, the N-terminal part of the citC-coding citrate carrier may be related to the binding site for coupling cations.

Expression of the citC Gene in Salmonella Strains—To examine whether the citC gene is indeed transcribed in several Salmonella species, we performed Northern blot analysis. Fig. 7A shows that two bands (1.7 and 1.4 kb) could be detected in RNAs from E. coli SG11 carrying pOH61 and from S. dublin 35 with a citC gene probe. However, no band was detected in total RNA samples of S. typhimurium, S. abortus-equii, S. enteritidis, and S. pullorum strains. No message of the citC gene was observed in most of Salmonella strains except S. dublin 35 in this study (Fig. 7A). In contrast, when hybridized with the Salmonella galE probe (32) as a control, three (4.2, 2.5, and 1.4 kb) messages were detected in the total RNA from all samples, although two RNA samples, one from E. coli SG11/pOH61 and one from S. abortus-equii, were too small or too degraded for DNA-RNA hybridization (Fig. 7B). No transcript corresponding to the citA gene was detected in the RNA samples from several Salmonella strains in Northern blot analysis (data not shown). This evidence may suggest that the promoter activities of both citA and citC genes are either weak at undetectable levels or negatively regulated by in cis or in trans elements.

Construction of cit-lacZ Fusions and Measurement of Promoter Activities—To study the regulation of transcription of the two genes (citA and citC), we measured the promoter activities of two activities using the lacZ-fusion system (17) and the effect of citrate as an inducer on β-galactosidase expression. The 790-bp Nael fragment of pHD1 (14) carrying the Salmonella citA promoter region and the 176-bp HindIII fragment of pOH61 carrying the citC promoter region were digested with BglI and XhoI, and the shortened DNA fragments were ligated with the EcoRI linker at both ends, followed by cloning into the pCRII site of pREP747-10. Two lacZ-fusion plasmids, pOH50 and pOH51, carrying the citA and citC promoter regions, respectively, were obtained. The nucleotide sequence of the promoter region in each DNA insert fragment was confirmed to be in a translation frame by DNA sequencing. These plasmids were transformed to both E. coli MC1061 and S. typhimurium LB5010 strains lacking the intact lac operon on the chromosome, and the activity of the promoters in the inserts was measured by expression of β-galactosidase in the presence or absence of citrate (Table I). The β-galactosidase activity of E. coli MC1061 with pOH51 (the citC gene promoter) grown in L broth was about 8-fold that of pOH50 (the citA gene promoter). When E. coli MC1061 cells with these lacZ-fusion plasmids were incubated in M9-glycerol medium, the β-galactosidase activity of the cells with pOH51 was about 4-fold that of pOH50. These β-galactosidase activities were not influenced by the presence of citrate when cells had been grown in either L broth or M9-glycerol medium (Table I). From these results, we suspect that citrate is not an inducer for transcription initiation of the citA and citC genes and that the transcriptional level of the citC promoter is higher than that of the citA promoter.

In an attempt to determine whether citA and citC genes are inherently regulated in Salmonella strains, we transformed

![Fig. 7. Northern blot analysis of citC gene expression in several Salmonella serovars. Total bacterial RNAs were denatured with glyoxal and run in 1% agarose gel. The separated RNAs in the gel were transferred to nitrocellulose filters. The RNA was hybridized with a 32P-labeled citC probe (the 490-bp HindIII-Sall fragment) (A) and subsequently rehybridized with a 32P-labeled galE probe (the 2.3-kb ClaI fragment) (B). Lane 1, E. coli SG11 carrying pOH61 and from S. dublin 35 with a citC gene probe. However, no band was detected in total RNA samples of S. typhimurium, S. abortus-equii, S. enteritidis, and S. pullorum strains.](image-url)

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<tr>
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Values are means from at least two independent determinations.
the two lacZ-fusion plasmids to S. typhimurium LB5010. The β-galactosidase activity of the citC promoter in pOH51 was generally higher than that of the citA promoter in pOH50. The level of β-galactosidase activity in Salmonella strains with pOH51 was slightly higher than that in E. coli with the plasmid, although the activity of pOH50 in Salmonella strains was almost the same as that in E. coli cells (Table I). However, there was no direct evidence suggesting that the transcriptional activities of citA and citC genes were negatively regulated in Salmonella strains. Table I shows that the promoters of both citA and citC genes were functional in E. coli and Salmonella strains, in both the presence and the absence of citrate.

Kay et al. (10) reported that at least three separate tricarboxylate transport systems (TctI, TctII, and TctIII) are present in S. typhimurium, among which TctI is the major transport system and is expressed, whereas the other systems are normally not expressed. The results on the citC gene obtained in this study support the hypothesis proposed by Kay et al. (10). However, the question of why the transcripts from two cit genes (citA and citC) were normally not detected in several Salmonella strains in Northern blot analyses remains unsolved. Judging from the evidence of the substrate specificity (Fig. 4), cation dependence (Fig. 5), and lower expression of the citC-coding system seems to be a TctIII system, such as that previously found in S. typhimurium and its function is yet unknown.

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