Citrate Utilization by *Escherichia coli*: Plasmid- and Chromosome-Encoded Systems

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Citrate utilization plasmids have previously been identified in atypical *Escherichia coli* isolates. A different citrate-utilizing (Cit+) variant of *E. coli* K-12 arose as a consequence of two chromosomal mutations (B. G. Hall, *J. Bacteriol.* 151:269–273, 1982). The processes controlling the transport of citrate in both a Cit+ chromosomal mutant and a Cit+ plasmid system were studied. Both systems were found to be inducible in growth experiments. In transport assays with whole cells, citrate-grown cells accumulated [1,5-14C]citrate at two to three times the rate of uninduced cells. Only the Vmax was affected by induction, and the Km for whole cells remained at 67 μM citrate for the chromosomal strain and 120 μM citrate for the plasmid-conferred system. There was no detectable accumulation of radioactivity with [6-14C]citrate, because of rapid metabolism and the release of 14CO2. Energy-dependent citrate transport was found with membrane vesicles obtained from both the chromosome-conferred and the plasmid Cit+ systems. The vesicle systems were inhibited by valinomycin and carbonyl cyanide m-chlorophenylhydrazone but not by nigericin and monensin. In contrast to whole cells, the vesicle systems were resistant to Hg2+ and showed identical kinetics with [1,5-14C]citrate and [6-14C]citrate. H+ appeared to be important for citrate transport in whole cells and membranes. Monovalent cations such as Na+ and K+, divalent cations such as Mg2+ and Mn2+, and anions such as PO43−, SO42−, and NO3− were not required. The two systems differed in inhibition by citrate analogs.

*Escherichia coli* is typically unable to grow on citric acid as the sole carbon and energy source (16). This inability to grow on citrate is used in the determinative identification of *E. coli* in clinical diagnoses (21).

However, *E. coli* is not totally inert toward citrate (4, 18, 23). Citrate functions in an iron transport system (3) and can be utilized anaerobically in the presence of a cosubstrate that provides reducing power (19).

Atypical *E. coli* capable of utilizing citrate has been isolated from a variety of agricultural, laboratory, and clinical settings (2, 8, 27). In each case, citrate utilization was plasmid conferred (2, 7, 20, 27), and several of these plasmids have been characterized (6, 9, 10). In addition, a complex chromosomal mutation for citrate utilization in *E. coli* was isolated and characterized (5).

The ability of other bacterial species to grow on citrate is associated with cation-dependent transport systems. Citrate utilization by *Enterobacter aerogenes* is Na+ dependent (11, 22). Citrate transport is coupled to magnesium transport in *Bacillus subtilis* (31). *Salmonella typhimurium* possesses two citrate transport systems, *tctI*, which is Na+ dependent, and *tctII*, which is Na+ and K+ dependent (1, 14, 29; W. W. Kay, personal communication). Citrate utilization by *S. typhimurium* requires both membrane transport and a periplasmic protein (28–30).

*E. coli* possesses all of the enzymes necessary for citrate metabolism (17) since citrate is a substrate in the tricarboxylic acid cycle, the major metabolic pathway of aerobically growing cells. Therefore, the inability to transport citrate seemed likely to be the major barrier to the utilization of citrate by *E. coli*. This study characterized the citrate transport systems of plasmid-conferred and chromosome-conferred citrate utilization in *E. coli*.

MATERIALS AND METHODS

**Bacterial strains.** All strains used were *E. coli* K-12 derivatives. Strain WR3081 is strain 2340 lac rpsL containing a naturally occurring 200-kilobase plasmid that confers citrate utilization and resistances to tetracycline and chloramphenicol (2, 20). Strain WR3080 is the isogenic plasmidless control. Strain D21 is HfrC Δ(lacZ)W4680 lacY+ *spc ebgR ebgA−*. Strain D2004 is a spontaneous mutant of D21 able to grow on citrate (5).

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Media and growth experiments. Cells were grown in triethanolamine minimal medium (24) containing 20 mM citrate or 40 mM glycerol as the carbon source. For growth experiments, cells were harvested steriley in the mid-log phase by centrifugation (6,000 × g for 10 min) and suspended in minimal medium with no carbon source. This suspension was used as the inoculum for growth experiments.

Membrane vesicle preparation. Membrane vesicles were prepared by the method of Kaback (12) and Konings and Kaback (15). Vesicles were suspended in 50 mM KPO₄ buffer (pH 6.6) or 50 mM Tris-hydrochloride buffer (pH 7) at a concentration of 5 to 10 mg of protein per ml. One-milliliter samples were frozen in dry ice-ethanol and stored at -70°C until use.

Transport assays. Membrane vesicles were thawed at 37°C and stored on ice. Transport assays were conducted in 50 mM Tris-hydrochloride or KPO₄ buffer at pH 7.0 (unless otherwise specified) and 37°C with constant aeration. Phenazine methosulfate (0.1 mM) plus 10 mM ascorbic acid was used as the energy source. Assays were initiated by the addition of [1,5-¹⁴C]citrate, [6-¹⁴C]citrate, or [¹⁴C]proline to the reaction mixture. Samples were filtered through cellulose nitrate filters (pore diameter, 0.45 μm) and rinsed twice with 3 ml of ice-cold 0.1 M LiCl. Protein was measured by the method of Schaffner and Weissmann (26).

Cells used in whole-cell transport assays were grown in glycerol or in citrate minimal medium. Cells were harvested in the mid-log phase by centrifugation (6,000 × g for 10 min) and suspended in minimal medium at a concentration of 100 Klett turbidity units (no. 54 filter; 0.25 mg [dry weight] per ml). [1,5-¹⁴C]Citrate or [6-¹⁴C]citrate was added at 37°C. Samples were aerated throughout the experiment and filtered as above, except that they were rinsed twice with 5-ml samples of wash solution (24).

Reagent-grade chemicals and deionized water were used in all experiments. [1,5-¹⁴C]citrate, [6-¹⁴C]citrate, and [¹⁴C]proline were obtained from New England Nuclear Corp., Boston, Mass. Carbonyl cyanide m-chlorophenylhydrazone was obtained from Sigma Chemical Co., St. Louis, Mo. Valinomycin, nigericin, and monensin were purchased from Calbiochem, La Jolla, Calif. Cellulose nitrate filters were obtained from Bio-Rad Laboratories, Richmond, Calif. Scintillation counting fluid was obtained from Amersham Corp., Arlington Heights, Ill. All samples were counted in a Tri-Carb liquid scintillation spectrometer obtained from Packard Instrument Co., Inc., Rockville, Md.

RESULTS

The ability of both the chromosome- and the plasmid-derived Cit⁺ strains to grow on citrate was inducible (Fig. 1). Uninduced cells of plasmid-containing strain WR3081 had a 10- to 12-h growth lag upon being shifted from glycerol to citrate minimal medium. Uninduced Cit⁺ chromosome strain D2004 showed a longer lag of 24 to 72 h. Induced cells of both strains grew without lag upon dilution in citrate minimal medium (Fig. 1). The two control strains, D21 and WR3080, showed no growth in citrate minimal medium.

In whole-cell transport assays, citrate-grown cells accumulated [1,5-¹⁴C]citrate at two to three times the rate of uninduced cells (Fig. 2). Only the Yₘₘₙ was affected by induction, and the apparent Kₘ for whole cells remained at ca. 67 μM citrate for strain D2004 and ca. 120 μM citrate for strain WR3081. Table 1 summarizes the kinetics of [1,5-¹⁴C]citrate accumulation in induced whole cells and in membrane vesicles prepared from induced cells. No measurable accumulation of [¹⁴C]citrate was observed with cells of control strains WR3080 and D21. Trans-
port by cells of strains D2004 and WR3081 was inhibited by HgCl₂ and carbonyl cyanide m-chlorophenylhydrazone.

Although both Cit⁺ strains accumulated [1,5-14C]citrate, neither Cit⁺ strain accumulated measurable levels of [6-14C]citrate (Fig. 3) due to rapid metabolism and the release of ¹⁴CO₂ (data not shown; S. McCowen, personal communication).

Citrate transport in right-side-out membrane vesicles (12, 15) of both citrate-utilizing strains was energy dependent. Both the chromosome and the plasmid systems were inhibited by carbonyl cyanide m-chlorophenylhydrazone but, unlike whole cells, not by HgCl₂ (Fig. 4; data not shown). Citrate transport in vesicles was inhibited by valinomycin but not by nigericin or monensin (Fig. 4B), indicating that transport is driven by the membrane potential gradient (ΔѰ) rather than by the pH gradient (ΔpH). In contrast to whole cells, membrane vesicles accumulated [1,5-14C]citrate and [6-14C]citrate with identical kinetics (Fig. 5), indicating that citrate is transported as such. This experiment appears to eliminate the alternative explanation for the results shown in Fig. 3 for whole cells that the [6-14C]citrate might have been metabolized at the cell surface before the uptake of a C₄ or C₅ compound.

Attempts to identify a cation for citrate-cation cotransport ruled out all cations tested except H⁺, Na⁺, K⁺, Mg²⁺, Mn²⁺, Ca²⁺, Li⁺, and Rb⁺ had no effect on citrate accumulation (data not shown). The Kₘ of the citrate transport system of strain D2004 was lowest at low pHs (Fig. 6), supporting the theory that protons are cotransported with citrate. Varying the pH had a similar effect on strain WR3081 (data not shown).

The plasmid- and chromosome-conferred citrate transport systems have different specificities of inhibition by citrate analogs (Table 2). Tricarballylate and cis-aconitate competitively inhibited citrate transport by cells of both strains, whereas isocitrate, trans-aconitate, and DL-fluorocitrate were competitive inhibitors only with strain D2004.

We lack the radioactive substrates to demonstrate whether the competitive inhibitors were also transported. However, strain WR3081 was able to grow on cis-aconitate and tricarballylate as well as on citrate but not on isocitrate or trans-aconitate (data not shown). Thus, the growth experiments suggested that the analogs which competitively inhibited citrate transport for strain WR3081 are also transport substrates. Hall (5) showed that strain D2004 will grow on citrate, isocitrate, cis-aconitate, and trans-aconitate but not on tricarballylate, although tricarballylate was a potent competitive inhibitor of citrate transport with strain D2004 (Table 2).

### DISCUSSION

The inability of whole cells to accumulate measurable amounts of [6-14C]citrate (Fig. 3) led to the proposal by Baron et al. (2) of "metabolic transport" in which the metabolism of citrate (to oxaloacetate) is simultaneous with transport. This mechanism had been suggested earlier for *Aerobacter aerogenes* (25) but has recently been questioned (13). Figure 5 indicates that this is not the case for *E. coli*. The identical kinetics for [1,5-14C]citrate and [6-14C]citrate accumulation by membrane vesicles show that citrate is transported as citrate. The lack of accumulation of [6-14C]citrate by whole cells appears to be due to the rapid intracellular metabolism of citrate and the release of ¹⁴CO₂. The alternative hypothesis, that citrate is metabolized at the cell surface in
whole cells but the complex responsible is disrupted during vesicle preparation, was not tested by our experiments.

Although strains D2004 and WR3081 exhibited long lags upon being shifted into citrate minimal medium (Fig. 1), citrate transport was only inducible by a factor of 3 (Fig. 2). This suggests that there is another barrier to growth on citrate in addition to transport. At present, this additional step or process is not understood. It is even possible (although we consider it unlikely) that citrate transport activity is synthesized constitutively but that some subsequent metabolic step is induced and leads indirectly to the threefold increase in measured transport activity.

The results of the experiments depicted in Fig. 4 demonstrate that citrate transport is driven by \( \Delta \Psi \), the electrical gradient across the membrane, but not by the \( \Delta \text{pH} \) gradient. This finding is somewhat surprising since citrate carries a -3 charge at the pH at which these experiments were conducted. Hence, citrate must be cotransported with more than three monovalent cations to produce a positively charged complex that would move in response to the internally negative membrane potential. The failure to identify any stimulating monovalent or divalent cation other than \( \text{H}^+ \) and the results depicted in Fig. 6 indicate that the cotransported cation is \( \text{H}^+ \).

No other studied citrate transport system has been demonstrated to be proton dependent, although proton-dependent dicarboxylic acid transport systems are known (13). It seems likely that the \( E. \text{coli} \) citrate transport system did not arise from the \( S. \text{typhimurium} \) system (\( \text{Na}^+ \)
TABLE 2. Competitive inhibition of citrate accumulation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (μM)</th>
<th>WR3081</th>
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<tbody>
<tr>
<td>Isocitrate</td>
<td>120</td>
<td>NI(^b)</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>750</td>
<td>250</td>
</tr>
<tr>
<td>trans-Aconitate</td>
<td>325</td>
<td>NI</td>
</tr>
<tr>
<td>Tricarboxylate</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td>DL-Fluorocitrate</td>
<td>200</td>
<td>NI</td>
</tr>
</tbody>
</table>

\(^a\) $K_i$s were determined by Dixon plots of data from determinations of initial uptake rates by washed whole cells. [1,5-14C]citrate was used at concentrations of 25 and 50 μM. The concentration of the analog was varied from 100 to 2,000 μM.  
\(^b\) NI, Not an inhibitor at 500 μM.

and K\(^+\) cotransport (14, 28; Kay, personal communication) or from any other well-characterized system (6, 13). The pattern of substrate specificities for both *E. coli* strains is unlike that of any other studied species. Strain WR3081, which can utilize citrate, cis-aconitate, and tricarboxylate but not isocitrate or trans-aconitate, possesses a specificity pattern common to and unique to plasmid-conferring Cit\(^+\) *E. coli* (6). As previously shown by Hall (5), strain D2004 has a substrate range different from any other species and all other characterized Cit\(^+\) *E. coli* strains. The chromosome- and the plasmid-encoded citrate transport systems described here seem to be sufficiently different from each other that it seems unlikely that they are closely related. The question of their origin remains a mystery.

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LITERATURE CITED