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Utilization of Orotate as a Pyrimidine Source by *Salmonella typhimurium* and *Escherichia coli* Requires the Dicarboxylate Transport Protein Encoded by *dctA*

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Mutants deficient in orotate utilization (initially termed *out* mutants) were isolated by selection for resistance to 5-fluoroorotate (FOA), and the mutations of 12 independently obtained isolates were found to map at 79 to 80 min on the *Salmonella typhimurium* chromosome. A gene complementing the mutations was cloned and sequenced and found to possess extensive sequence identity to characterized genes for C4-dicarboxylate transport (*dctA*) in *Rhizobium* species and to the sequence inferred to be the *dctA* gene of *Escherichia coli*. The mutants were unable to utilize succinate, malate, or fumarate as sole carbon source, an expected phenotype of *dctA* mutants, and introduction of the cloned DNA resulted in restoration of both C4-dicarboxylate and orotate utilization. Further, succinate was found to compete with orotate for entry into the cell. The *S. typhimurium dctA* gene encodes a highly hydrophobic polypeptide of 45.4 kDa, and the polypeptide was found to be enriched in the membrane fraction of minicells harboring a *dctA*$^+$ plasmid. The DNA immediately upstream of the deduced −35 region contains a putative cyclic AMP-cyclic AMP receptor protein complex binding site, thus affording an explanation for the more effective utilization of orotate with glycerol than with glucose as carbon source. The *E. coli dctA* gene was cloned from a lambda vector and shown to complement C4-dicarboxylate and orotate utilization in FOA-resistant mutants of both *E. coli* and *S. typhimurium*. The accumulated results demonstrate that the *dctA* gene product, in addition to transporting C4-dicarboxylates, mediates the transport of orotate, a cyclic monocarboxylate.

In *Salmonella typhimurium* and *Escherichia coli*, six enzymic reactions are involved in de novo pyrimidine nucleotide biosynthesis to form UMP, the ordered sequence of the unlinked genes for the enzymes being *carAB* and *pyrBI*, *-C*, *-D*, *-E*, and *-F*. Wild-type cells are not readily permeable to intermediates of the pyrimidine biosynthetic pathway, with the exception of orotate, which is formed from dihydroorotate by the action of dihydroorotate dehydrogenase (*pyrD*). Orotate satisfies the pyrimidine requirement of *carAB*, *pyrBI*, *pyrC*, or *pyrD* mutants and is effective at low to moderate concentrations, provided glycerol is used as the carbon source (29, 39, 40). Accordingly, 5-fluoroorotate (FOA) is a potent growth inhibitor in glycerol minimal medium and has been employed for the selection of *pyrH* (UMP kinase) mutants of both *S. typhimurium* (40) and *E. coli* (15). It has been observed that orotate is growth rate limiting for *carAB* and *pyrBI* mutants in a concentration-dependent manner, and culturing cells at varying orotate concentrations has been used as a means to establish partial pyrimidine starvation for purposes of studying the impact of pyrimidine nucleotide limitation on specific gene expression and general aspects of macromolecular synthesis (3, 5, 27, 36, 38). In contrast, reducing the concentration of uracil serves to lower only the growth yield, not the growth rate (36), and indicates that the transport of orotate into the cell represents a rate-limiting step. Although carbamoylaspartate is not utilized by wild-type cells, carbamoylaspartate (ureidosuccinate)-permeable mutants (*usp*) have been isolated (21, 34), and *pyrB usp* double mutants are able to grow with carbamoylaspartate or orotate as sole pyrimidine source; it has not been determined if a concentration-dependent growth rate pertains to the utilization of carbamoylaspartate.

As part of our continuing research program on pyrimidine metabolism and its regulation, we initiated an investigation on the biochemical and genetic characterization of orotate utilization in *S. typhimurium* with some related studies on *E. coli*. This investigation ultimately led to establishing the involvement of the dicarboxylate transport system in orotate utilization and identifying the product of the *dctA* gene in mediating the entry of orotate into the cell.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. Bacterial strains used were derivatives of either *S. typhimurium* LT2 or *E. coli* K12 and are listed along with their relevant genotypes in Table 1. Plasmid vectors utilized as cloning or expression vehicles are also listed in Table 1.

Media and growth conditions. Lennox broth (6) was used as the complex medium. Minimal medium A has been described previously (7); it contained either 0.2% glucose or 0.3% glycerol or, when tested as carbon sources, C4-dicarboxylates at 0.2%. Unless otherwise indicated, media were supplemented with the following, as required: casamino acids, 0.2%; individual amino acids, 50 μg/ml; thiamine, 2 μg/ml; orotate, 50 μg/ml; carbamoylaspartate, 100 μg/ml; uracil, 25 μg/ml; FOA, 20 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 50 μg/ml; and isopropyl-β-D-thiogalactopyranoside (IPTG), 10 μg/ml. When used, antibiotics were added at the following final concentrations: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 20 μg/ml; kanamycin (Km), 30 μg/ml; and chloramphenicol (Cm), 30 μg/ml. Solid media were prepared by the addition of agar to 1.5%. Cultures were routinely grown at 37°C. Liquid cultures were incubated on a shaker operating at 250 rpm, and growth was monitored by measuring cell turbidity with a Klett-Summerson colorimeter.

Mutagenesis. Chemical mutagenesis was accomplished by treating bacterial cells at a density of 1×10^8 to 2×10^8 cells per ml with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine at 0.1 mg/ml (28). Following mutagenesis, cells were pheno-

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TABLE 1. Bacterial strains and plasmid vectors

Strain	Genotype and relevant properties	Source or reference
Salmonella typhimurium LT2		
AK3108	*zhh-3108*::Tn*10*(Δ*16*Δ*17*) *trpC2 metA22 metE551 his-6165 ilv-452 galE496 fla-66 rpsL120 xyl-404 hsdL6 hsdSA29* Δ*malB*/F′112 (*E. coli*)	SGSC[a]
AK3109	As for AK3108, with the exception *zhh-3109*::Tn*10*(Δ*16*Δ*17*)	SGSC
KR1001	Wild-type LT2	LC[b]
KR1312	Δ*pyrB137*	SGSC
KR1488	Δ*pyrB655 usp-2*	34
KR1562	*metA22 metE55 galE496 rpsL120 xyl-404 hsdL6 hsdSA29*	LC
KR1594	Δ*pyrB655 usp-2 out-11*[c]	This study
KR1596	Δ*pyrB655 usp-2 zhh*::Tn*10* (Tn*10* and *out*$^+$, 80% linked by phage P22 transduction)	This study
KR1647	Δ*pyrB137 usp-2*	This study
KR1649	Δ*pyrB137 usp-2 out-11*	This study
Escherichia coli K12		
BD1854	*minA minB his thi lac rpsL tonA xyl rpsL mtl man*	B. Diderichsen
KUR1349	*araD139* Δ*lacU169 rpsL thi* Δ*pyrB usp-4*	LC
KUR1351	KUR1349 *out-2*	This study
NM522	Δ(*lac-pro*) *supE thi hsd 5*/F′ *proAB*$^+$ *lacI*^{q}ZΔM15	M. Kilstrup
Plasmid vectors[d]		
pBR328	Ap^r Tc^r Cm^r	1
pRAK82	Ap^r (*lacZ* translational fusion vector)	17
pUC18/19	Ap^r	J. Messing
pWSK29	Ap^r	37
pWSK129	Km^r	37

[a] SGSC, Salmonella Genetic Stock Center.
[b] Laboratory collection.
[c] The gene symbol, *out*, is used in reference to orotate utilization and is synonymous with *dctA*.
[d] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

typically expressed for 24 h in minimal medium prior to plating on selective media.

Genetic techniques. Bacteriophage P22HT105/int-201 (10) was used for all transductions with *S. typhimurium*. In some instances, 10 mM EGTA was added to the plating medium to limit lysogeny of transductants. The *usp* marker was transferred by plating the transduced culture of a *pyrB* recipient on glycerol-minimal medium with carbamoylaspartate as sole pyrimidine source. Transfer of an *out* (*dctA*) mutation to a *usp* strain was selected by plating on the same basic medium, but with the addition of FOA. Methods for transposon technology with Tn*10* were as previously reported (27). Conjugations were carried out as described previously (15).

Cloning of the *S. typhimurium out* (*dctA*) gene. A plasmid library of *S. typhimurium* DNA constructed from 8- to 15-kbp *Sau*3AI fragments ligated into the *Bam*HI site of pBR328 (9) was obtained from C. G. Miller. A P22 lysate was prepared on the collection of transformants containing the pBR328 library and used to isolate plasmids containing the *out* locus by transducing KR1594, selecting for Out$^+$ on minimal medium supplemented with orotate and Cm. All other recombinant plasmids containing *S. typhimurium* DNA were initially isolated from *E. coli* and then transferred into restriction-minus *S. typhimurium* KR1562 (16) as an intermediate host before transformation of the final *S. typhimurium* recipient.

DNA techniques. The methods used were primarily adapted from the manual of Sambrook et al. (31). Procedures drawn from this manual include chromosomal and plasmid DNA isolations; restriction endonuclease digestion and ligation of DNA; DNA sequencing of alkaline-denatured templates; transformation; mung bean nuclease treatment; and agarose gel electrophoresis. To sequence the *Bst*EII-*Nru*I fragment of the primary clone, two sets of nested deletion subclones (one pertaining to each DNA strand) were generated by using the Erase-a-Base System (Promega) and with pUC19 as the vector. Sequencing was carried out as a service by the University Core DNA Services, University of Calgary, with an Applied Biosystems 373A automated DNA sequencer. DNA and protein sequence comparison searches utilized the database of GenBank and the BLAST program.

Analysis of plasmid-encoded polypeptides. Plasmids were transformed into the minicell-producing strain BD1854, and isolated minicells were incubated in the presence of [^{35}S]methionine (11). The samples were then fractionated as follows (4): (i) total polypeptides were extracted by incubating the labeled minicells in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol) for 60 min at 37°C; and (ii) the crude membrane fractions were obtained by first treating the minicells with 1% lysozyme and 10 mM EDTA for 30 min at room temperature, followed by centrifugation to pellet the minicells. After resuspension in 30 mM Tris-HCl (pH 8.0), the samples were sonicated and then centrifuged for 60 min at 48,000 × *g* at 2°C. The pellets were resuspended in SDS sample buffer and incubated at 37°C for 60 min prior to electrophoresis. Labeled polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and after the gel was dried, the positions of the labeled bands were located by autoradiography.

Mapping of the transcriptional start site. Total cellular RNA was extracted from exponentially growing cells of KR1312/pKAT206 as described previously (35). Primer extension analysis of the 5′ end of the *out* (*dctA*) transcript was carried out according to Frick et al. (7) with Moloney murine leukemia virus reverse transcriptase and as the primer, a 24-mer oligonucleotide (5′CAGAGA GGTTTTCATAGGGTGTCC3′) complementary to the last 9 nucleotides of the leader mRNA and the first 5 codons (residues 371 to 394; Fig. 2). Extension products were resolved on a 6% polyacrylamide–7 M urea gel alongside a DNA sequence ladder.

Nucleotide sequence accession number. The sequence of the 2,747-bp *Bst*EII-*Nru*I DNA fragment from *S. typhimurium* has been submitted to the EMBL databank and assigned no. X91397. The accession number of the *E. coli* DNA sequence containing the *dctA* gene is U00039.

RESULTS

Growth of *S. typhimurium* on orotate as pyrimidine source. The ability of a pyrimidine auxotroph blocked at the second step of de novo pyrimidine biosynthesis to utilize orotate as sole pyrimidine source in liquid culture was assessed. Table 2 lists the doubling times of KR1312 (Δ*pyrB137*) in minimal medium supplemented with different concentrations of orotate and with either glycerol or glucose as carbon source. With glycerol, growth occurred at a concentration as low as 15 μg/ml, and orotate appeared to be growth rate limiting at concentrations below 75 μg/ml, but above this concentration, the doubling time was similar to that observed with uracil. In contrast, when glucose was the carbon source, orotate at 50 μg/ml or greater was required for growth, and even when it was supplied at 200 μg/ml, the growth rate was less than that with uracil (Table 2).

Isolation of *out* mutants. Mutants defective in orotate utili-

TABLE 2. Growth of *S. typhimurium* on orotate as pyrimidine source[a]

Carbon source	Pyrimidine supplement (μg/ml)	Doubling time (min)
Glycerol	Orotate	
	15	315
	30	135
	45	87
	60	72
	75	57
	100	54
	Uracil 25	54
Glucose	Orotate	
	50	270
	75	228
	100	150
	150	75
	200	60
	Uracil 25	40

[a] KR1312 was grown in minimal medium with the carbon source and pyrimidine supplement indicated.

zation (*out* mutants) were isolated on glycerol minimal medium on the basis of resistance to the analog FOA. The toxicity of FOA depends on initial conversion to 5-fluoroUMP catalyzed by the pyrimidine de novo biosynthesis enzymes, orotate phosphoribosyltransferase (*pyrE*) and OMP decarboxylase (*pyrF*). Hence, *pyrE* and *pyrF* mutants are resistant to FOA and require preformed pyrimidines (e.g., uracil) for growth. However, by using a pyrimidine auxotroph deficient in aspartate transcarbamoylase (*pyrBI*) but permeable to carbamoylaspartate (*usp* mutation) and selecting for resistance to FOA on glycerol medium with carbamoylaspartate present as pyrimidine source, *pyrE* and *pyrF* mutants are precluded.

A chemically mutagenized culture of KR1488 was plated on glycerol minimal medium containing carbamoylaspartate and FOA (20 μg/ml). Several FOA^r isolates were found to have lost the ability to use orotate as pyrimidine source (i.e., Out$^-$). These mutants failed to grow with orotate at 200 μg/ml on either glucose or glycerol, thus showing that in wild-type cells the same uptake system was operative with either carbon source. An isolate, KR1594 (*out-11*), was retained as the representative Out$^-$ strain for use in further studies.

Transposon technology (18) was used to construct a derivative (KR1596) in which Tn*10* and *out* cotransduced at a frequency of 75 to 80%. A lysate prepared on KR1596 was used to transduce the Out$^-$ mutants (12 individual isolates) to Tc^r. In all cases, at least 80% of the transductants acquired the ability to grow with orotate as pyrimidine source.

Mapping of *out*. Time-of-entry experiments and gradient-of-transmission matings with various *S. typhimurium* Hfr strains served to locate the *out* gene and the closely linked Tn*10* insertion near *xyl* on the *S. typhimurium* chromosome (data not shown). Pulsed-field gel electrophoresis of *Xba*I- and *Bln*I-digested chromosomal DNA was used to refine the position of the Tn*10* insertion to approximately 50 kbp counterclockwise of *Xba*I cleavage site 15 (22), thus placing it counterclockwise of *xyl* at 78 to 79 centisomes on the current linkage map (32). As well, transductions with lysates prepared on reference insertion strains, AK3108 (*zhh-3108*::Tn*10*) and AK3109 (*zhh-3109*::Tn*10*), exhibited 15 and 12% cotransduction of *out*$^+$ and the Tn*10* insertion, respectively.

Cloning of the *out* (*dctA*) gene. Putative *out*$^+$ plasmids were isolated from transductants obtained by transducing a pBR328 plasmid library of *S. typhimurium* DNA to KR1594 (*out-11*) selecting for Out$^+$ and Cm^r on minimal glycerol or glucose medium containing orotate at 50 or 25 μg/ml, respectively. The *out*$^+$ plasmid, pKAT202, obtained from the selection on glucose medium harbored a 7-kbp insert, and the plasmid from the glycerol medium, pKEB100, contained an 11-kbp insert (Fig. 1). KR1594/pKAT202 grew on glucose-orotate but not on glycerol-orotate, whereas KR1594/pKEB100 grew on either medium.

From pKAT202 and pKEB100, several plasmid derivatives were constructed in order to localize the *out* gene within the cloned DNA. Original subcloning attempts were based on pUC19 as the vector but were discontinued when it was found that cells harboring pUC19 failed to grow on a medium in which glycerol was the carbon source. Cells containing either of the low-copy-number plasmids, pWSK29 or pWSK129 (37), showed no impairment with glycerol, and these were subsequently chosen as vector systems. Subcloning of the 3.3-kbp *Pst*I$_1$-*Nru*I fragment (Fig. 1) from pKAT202 to pWSK29 re-

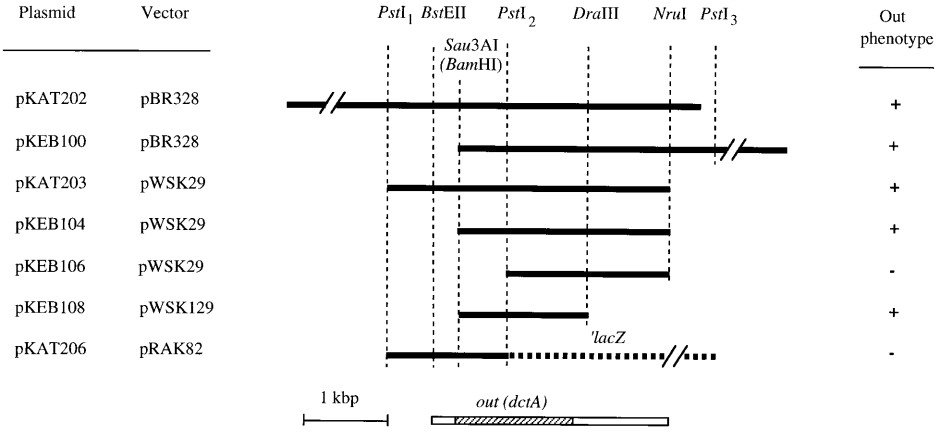

FIG. 1. Structure and properties of specific plasmids. The thick solid line pertains to cloned *S. typhimurium* DNA present in the individual plasmids, and the *lacZ* DNA of pKAT206 is illustrated as a heavy broken line. The positions of various restriction sites are defined by the vertical dotted lines. The fusion of the indicated *Sau*3AI site into a *Bam*HI site (as per pKEB100) generates a *Bam*HI site (shown in parentheses). The abilities of the plasmids to complement the *out* (*dctA*) mutation of KR1594 are listed at the right. The sequenced DNA (see Fig. 2) encompasses the *Bst*EII-*Nru*I fragment shown as a thick open line at the bottom, and the region corresponding to *out* (*dctA*) is indicated by crosshatching.

sulted in complementation of the *out* mutation in KR1594, and this construct was designated pKAT203. Similarly, subcloning of a *Bam*HI-*Nru*I fragment from pKEB100 yielded the complementing plasmid pKEB104. A construct (pKEB106) containing the *Pst*I_2-*Nru*I region from pKEB104 did not complement the *out* mutation, whereas pKEB108, which harbors the *Bam*HI-*Dra*III region, was able to do so. These observations indicated that the *Pst*I_2 site was located within the *out* DNA (Fig. 1).

DNA sequence. The nucleotide sequence of 2,747 bp, encompassing the *Bst*EII-*Nru*I region of the *S. typhimurium* DNA in pKAT203 (Fig. 1), was determined and is presented in Fig. 2. Two major open reading frames (ORFs) were identified: ORF1, residues 380 to 1666, and an apparently truncated gene, ORF2, residues 1886 to 2747. Since only ORF1 is found in the *out*$^+$ pKEB108, this sufficed to establish the corresponding DNA as specifying the *out* gene product. The encoded polypeptide of 428 amino acids has a predicted molecular mass of 45,395 Da, and a hydropathy plot (20) showed it to be highly hydrophobic. A potential ribosome binding site (residues 369 to 373) is located 6 bp upstream of the ATG start codon. Within the upstream region, more than one set of −35 and −10 elements for a putative promoter could be identified with hexamers corresponding to two possible −10 elements beginning at residues 315 and 318 (only the −10 element at residue 318 is shown in Fig. 2; see Discussion). At positions 235 to 256, a sequence resembling a cyclic AMP-cyclic AMP receptor protein complex (cAMP-CRP) binding site is found; it is present in pKAT202 but not in pKEB100, where the 5′ limit is the *Sau*3AI site at position 280. A region of hyphenated dyad symmetry centered at residues 1693 to 1694 is located 12 nucleotides downstream of the TAA stop codon and may represent a signal for termination of transcription.

A search of the protein database at GenBank revealed that there was extensive sequence identity of the Out polypeptide with membrane-bound C4-dicarboxylate permeases encoded by *dctA* in several *Rhizobium* species. Further, the search showed 94% sequence identity to the deduced polypeptide of gene *f428* of *E. coli*, which has been designated *dctA* through comparison with *Rhizobium* species and mapped to 79 min on the *E. coli* chromosome (33).

The *out* (*dctA*) gene product. In order to facilitate expression of *out*, the *S. typhimurium* DNA from pKEB108 (Fig. 1) was subcloned to pUC18 in phase with the *lac* promoter, generating pKAT214. Labeled proteins, total and membrane fractions, from minicells of strain BD1854 harboring pUC18 or pKAT214 were analyzed on SDS-PAGE (Fig. 3). Though the total polypeptide samples show several common bands (e.g., at 31 and 33 kDa), the pKAT214 sample (lane 2) had a faint, somewhat diffuse signal at 34 kDa that was not seen with pUC18 (lane 1). Moreover, this band is highly enriched in the membrane fraction for pKAT214 (lane 4) and absent from the pUC18 control (lane 3).

Mapping of the transcriptional start site. The 1.4-kbp *Pst*I fragment from pKAT203 was subcloned to the translational fusion plasmid, pRAK82, yielding pKAT206 (Fig. 1). Minimal glucose cultures of KR1312/pKAT206 were used as the source of total RNA, and the transcriptional start site was determined for *out* (*dctA*) by primer extension analysis. As shown in Fig. 4, transcriptional initiation was found to occur with GTP at position 327, 7 bp downstream of the last nucleotide of the deduced −10 region (Fig. 2). Also observed was a weaker signal corresponding to a GTP at 11 bp downstream. However, since the constraints dictated by promoter-polymerase interactions appear to define that transcriptional initiation occurs 5 to 9 bp downstream from the −10 region (8), this signal may represent a premature termination product of the reverse transcriptase reaction or a degradation product.

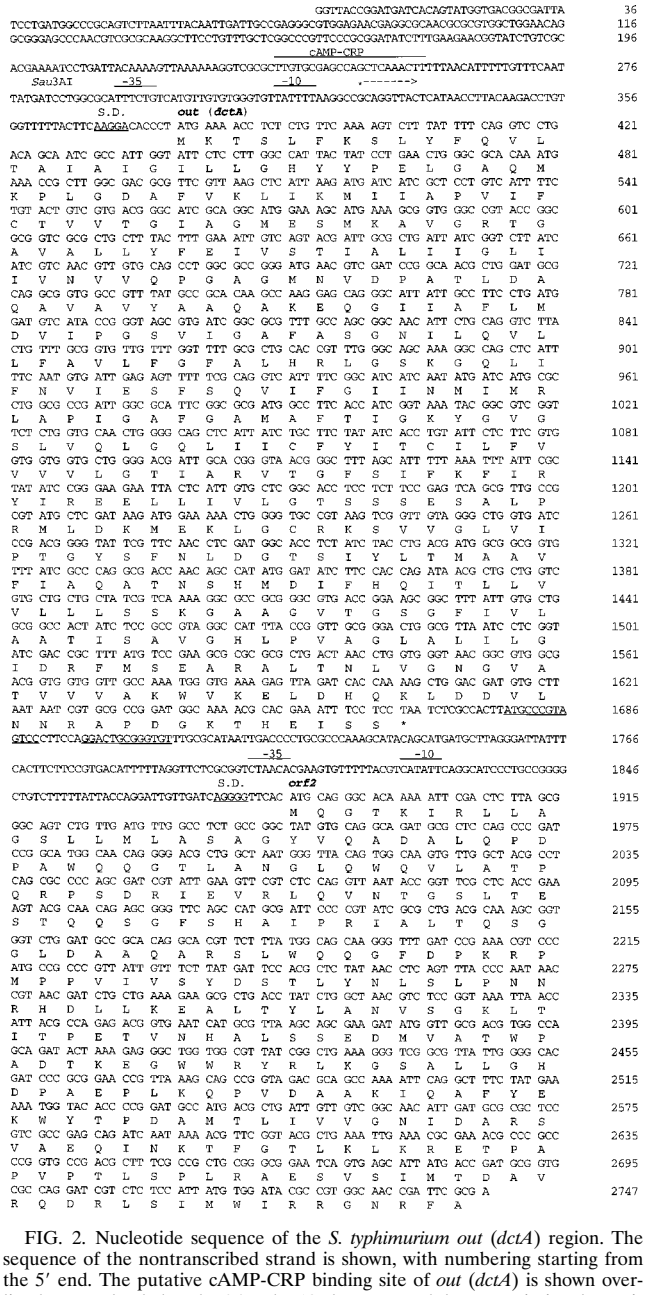

FIG. 2. Nucleotide sequence of the *S. typhimurium out* (*dctA*) region. The sequence of the nontranscribed strand is shown, with numbering starting from the 5′ end. The putative cAMP-CRP binding site of *out* (*dctA*) is shown overlined, as are the deduced −35 and −10 elements, and the transcriptional start is shown by an asterisk followed by a dotted arrow. The inferred Shine-Dalgarno (S.D.) element is underlined, and a region of hyphenated dyad symmetry following the TAA termination codon is also underlined. Putative promoter elements pertaining to *orf2* are similarly depicted. The *Sau*3AI site (residues 280 to 283) corresponds to the 5′ end of the truncated clone in pKEB100.

Cloning of *E. coli dctA*$^+$. A recipient strain, KUR1351 (*out usp pyrB*), was constructed as described above for the *S. typhimurium out* mutants. Transformation of KUR1351 with the *S. typhimurium out*$^+$ plasmids restored the ability to grow with orotate as pyrimidine source.

The lambda Charon 40 clone, Ec30-154 (33), contains a 14.9-kbp *Sau*3AI insert harboring the *E. coli dctA* (*f428*) gene. Analysis of the sequence showed that the *dctA* gene is con-

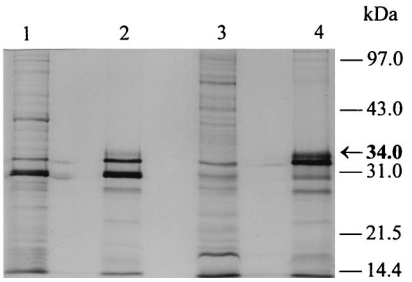

FIG. 3. Analysis of plasmid-encoded polypeptides by SDS-PAGE. Lanes 1 and 2, total polypeptides; lanes 3 and 4, membrane fractions, from minicells of BD1854 transformed with either pUC18 or pKAT214, respectively. Molecular mass markers (values at the right in kilodaltons) were used to estimate the size of the polypeptide products. The *S. typhimurium* DctA polypeptide is indicated by the arrow at 34 kDa.

tained on a 2.5-kbp *Pvu*I fragment. This fragment was cloned into the *Pvu*I site of pBR322 by selecting *dctA*$^+$ transformants of KUR1351 on minimal glucose-Tc with orotate at 25 μg/ml, thus yielding pKAT204. When KUR1351/pKAT204 was plated on glycerol- versus glucose-orotate, growth on the glycerol-based medium was impaired, with only patchy growth being observed after 48 h of incubation, indicating that a high level of the *dctA* product was toxic to the cell.

Upon transfer of pKAT204 to *S. typhimurium* KR1594 (*out-11*), the ability of the cell to utilize orotate was reestablished.

Phenotypic characteristics of *out* (*dctA*) strains. If *out* and *dctA* are identical genes, then the *out* mutants should also be affected in their ability to use dicarboxylates as carbon source, and further, dicarboxylates should be competitors for orotate uptake. Accordingly, KR1649 and KUR1351 were plated on minimal medium with uracil as pyrimidine source and succinate, malate, or fumarate as carbon source; strains KR1647 and KUR1349 were included as the *out*$^+$ (*dctA*$^+$) controls. The *S. typhimurium out* mutant failed to grow on all three media, and though the *E. coli* mutant did not grow on fumarate or malate, it did form small colonies on succinate after 48 h at 37°C.

Further evidence that transport of orotate and the dicarboxylates involves a common carrier was illustrated by the finding that growth of KR1647 and KUR1349 on glycerol-orotate (50 μg/ml) was blocked by the addition of succinate at 200 μg/ml. Additional studies whereby competitors were added to log-phase cultures of KR1647 showed that growth inhibition by succinate (200 μg/ml) occurred almost immediately.

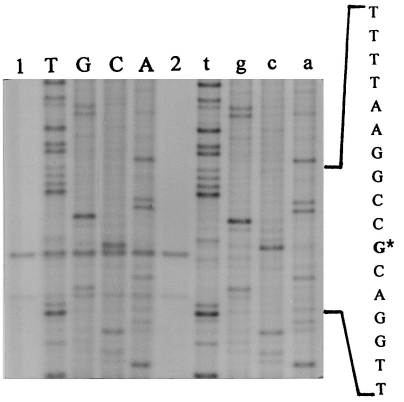

FIG. 4. Transcriptional start site mapping of *S. typhimurium dctA*. Primer extension products synthesized from RNA originating from pKAT206 are shown in lanes 1 and 2. The reverse transcriptase reaction was coelectrophoresed with each sequencing reaction (lanes T, G, C, and A) so that possible shifts in mobility due to differences in reaction components would be obviated; lanes t, g, c, and a pertain to the DNA sequencing reactions alone. The sequencing reaction lanes are labeled as the nontemplate strand to facilitate direct comparison with the mRNA, and the GTP start site is marked by an asterisk.

DISCUSSION

The accumulated evidence supports the conclusion that a gene originally referred to as *out* in relation to orotate utilization as a pyrimidine source is identical to *dctA*, a gene associated with the transport of C4-dicarboxylates. The data consistent with this interpretation include the following: Out$^-$ mutants, obtained by selection for resistance to the analog FOA, are impaired in using succinate, malate, or fumarate as sole carbon source; competition by succinate for orotate uptake; catabolite repression by glucose; genetic and physical mapping of the mutations near *xyl* on the chromosome; and the cloning and sequencing of DNA capable of complementing the mutations.

The *S. typhimurium dctA* mutant failed to grow with any of the three dicarboxylate substrates, whereas the *E. coli* mutant was still capable of slow growth with succinate but did not form colonies on fumarate or malate within 72 h. Since these *dctA* mutants were capable of growth in the presence of 100 μg of FOA per ml, it appears that the alternative mechanism for succinate utilization in *E. coli* lacks affinity for FOA. The ability of *E. coli dctA* mutants to still use succinate at a reduced rate was also observed by Kay and Kornberg in their original study on the *E. coli dct* system (13).

Upon recognition that the *dct* system was apparently involved in orotate uptake, an examination of the competition imposed by known substrates was undertaken. When succinate was added at 200 μg/ml to a culture growing in glycerol-orotate (50 μg/ml), almost immediate cessation of growth occurred. Aspartate can also be transported by the dicarboxylate system (14), but addition of aspartate at 200 μg/ml had a far less dramatic effect, resulting in only a reduced growth rate. This parallels the finding of Vogel et al. (36), who showed that addition of aspartate and glutamate (each at 100 μg/ml) could be used to establish a downshift in pyrimidine supply with *E. coli* cells growing in glucose medium in which orotate served as sole pyrimidine source.

The significantly higher concentration of orotate required to support growth of *S. typhimurium* pyrimidine auxotrophs when glucose is the carbon source (Table 2) is readily explained by a catabolite repressive effect on expression of *dctA*. Inspection of the promoter-regulatory region (refer to Fig. 2) identified a putative cAMP-CRP site encompassing 22 bp, centered 81 bp upstream of the +1 transcriptional start site. Assuming the same +1 transcriptional start point, an identical 22-bp sequence at exactly the same position is also found in *E. coli dctA*. The 22-bp sequence has a 55% match to consensus and contains 11 out of 16 of the most highly conserved residues (19). Previous work by Lo et al. (24) has shown that induction of *dctA* in a *cya* mutant required the presence of both cAMP and succinate. Catabolite repression of *dctA* expression may afford an explanation of why strains harboring pKEB100, but not pKAT202, can grow when glycerol is the carbon source, assuming that overproduction of the DctA polypeptide mediated through the presence of a multicopy *dctA*$^+$ plasmid is toxic for the cell. Since the 5′ end of the cloned DNA in pKEB100 is residue 280 (Fig. 2), it lacks the putative cAMP-CRP binding site that is present in pKAT202. Therefore, rel-

atively lower-level expression of *dctA* from pKEB100 could be expected under conditions of increased cAMP, i.e., in glycerol-grown cells.

The result from the transcriptional mapping lends support to the assignment of the −10 region of the putative promoter for *S. typhimurium dctA*; the GTP start is located 7 bp downstream of the 3′ limit of the assigned −10 region, and this is the most frequently observed separation distance (8). Applying the optimal spacer distance of 17 bp between the −10 and −35 regions, the deduced −35 element matches at only two residues with the consensus sequence, but these are associated with the highly conserved TTG triplet, and poor matching at the −35 region has been observed for a number of other positively regulated genes (8). Assuming the same organization as established for the *E. coli dctA* region (33), the direction of transcription on the chromosome would be counterclockwise.

The *S. typhimurium* DctA polypeptide has a predicted molecular mass of 45.4 kDa. The analysis with minicells showed that DctA was enriched in the membrane fraction, and it exhibited an increase in mobility in SDS-PAGE characteristic of hydrophobic membrane proteins (4). By using the TopPred program (2), the polypeptide is predicted to have 12 membrane-spanning helices in accordance with the consensus proposed for bacterial transport proteins (26).

Comparison of the *dctA* region of *S. typhimurium* with that of *E. coli* showed that the two *dctA* genes are 85% identical at the nucleotide level and the deduced polypeptides have the same chain length (428 amino acids) with 95% amino acid identity. With *Rhizobium* species, the identity at both the nucleotide and amino acid sequence levels is approximately 60%. Downstream of *S. typhimurium dctA*, an ORF beginning at residue 1886 but lacking a termination codon within the cloned DNA was detected. This ORF has sequences indicative of a promoter (see Fig. 2) and presumably is an independently expressed gene. It would appear that it is a truncated homolog of the *E. coli f498* gene encoding a deduced polypeptide of 498 amino acids but of unknown function (33).

Prior to this investigation on orotate utilization, the *dctA* genes of *S. typhimurium* and *E. coli* had not been specifically cloned and characterized, although as part of the analysis of the *E. coli* genome, *dctA* had been identified through sequence similarity of the encoded polypeptide to the *dctA* gene products of *Rhizobium* species (33). Earlier studies by Kay and Kornberg (13, 14) showed that *E. coli* possesses an inducible system specifically involved in the uptake of C4-dicarboxylates; mutants deficient in uptake (*dct* mutants) were isolated by selection for resistance to 3-fluoromalate and the corresponding gene was mapped close to *xyl*. Later work by Lo and Sanwal (25) provided evidence that three genes, *dctA*, *dctB*, and *cbt*, are involved in aerobic transport of C4-dicarboxylates, with *dctA* being located close to *xyl* (80 min) and the other two loci linked to *gal* (17 min). In *S. typhimurium*, Parada et al. (30) and Kay and Cameron (12) isolated and partially characterized mutants defective in the transport of C4-dicarboxylates; these mutants all harbored mutations mapping near *xyl* and were thus classified as *dctA* mutants. These two previous studies and our current study offer no evidence for the existence of a *dctB* locus, which by analogy to *E. coli* (23), if mutated, should result in an inability to transport C4-dicarboxylates and, by logical extension, the transport of orotate as well.

As mentioned above, the *dct* system is inducible by its substrates, and our results here show that orotate, a cyclic monocarboxylate, is also a substrate, but it is unresolved whether the presence of orotate has an inductive effect. Currently, we are using *lacZ* as a reporter gene to assess the regulation of expression of the *dctA* genes of *S. typhimurium* and *E. coli.*

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