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Fluorocitrate Resistant Tricarboxylate Transport Mutants of *Salmonella typhimurium*

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Summary. Spontaneous and Tn*10* induced fluorocitrate resistant mutants were isolated and characterized. These mutants were unable to grow on either *cis*-aconitate or DL-isocitrate but were still able to grow slowly on sodium citrate and normally on potassium or potassium-plus-sodium citrate. These mutants were defective in both citrate transport and citrate binding to periplasmic proteins. Tn*10* insertion mutants were unable to produce immunologically detectable amounts of the citrate inducible periplasmic C protein previously shown to bind tricarboxylates.

Using a series of *tct*::Tn*10* directed Hfrs the *tct* locus was accurately positioned at 59 units between *srlA* and *pheA*, but was not cotransducible with either gene. In the absence of P22 mediated cotransduction with 16 adjacent chromosomal markers the *srlA* and *tct* loci were bridged by using a series of *tct* flanking Tn*10* insertions, and by newly isolated and characterized *nalB* mutants. In addition the *hyd* and *recA* loci were located establishing the gene order in this region of the chromosome as: *pheA tct nalB recA srlA hyd cys*. Nitrosoguanidine derived tricarboxylate mutations (Imai 1975) were also mapped within the *tct* locus.

Introduction

Salmonella typhimurium shares with a wide range of bacteria the ability to transport and metabolize citrate, *cis*-aconitate and isocitrate, the three tricarboxylates of the Krebs cycle (Kay 1978). *S. typhimurium* also possesses chemoreceptors for citrate and isocitrate (Kahira 1979) as well as divalent cation-citrate chemoreceptors (Ingolia 1979).

The transport of citrate appears to be a relatively simple system when viewed kinetically in whole cells (Kay and Cameron 1978); however, genetically it appears to be more complicated (Imai 1977). The only transport component identified so far is the tricarboxylate binding C protein (Sweet et al. 1979). The C protein will also bind the toxic analogue 2-fluoro-L-*erythro*-citrate (Ashton et al. 1980) and is presumably at least partly responsible for the toxicity of this lethal analogue.

In this study we systematically characterized a range of fluorocitrate resistant mutants with respect to growth on the Na and K salts of tricarboxylates[1], citrate transport, C protein synthesis and activity, and precisely identify the genetic locus responsible. Since the *tct* locus was found in a relatively "silent" region of the chromosome we introduced a useful Tn*10* flanking insertion technique for filling transduction gaps such as this one and more accurately established the order of genetic markers in the 58–60 unit region of the *Salmonella* chromosome.

1 In the text Na refers to Na^+, K to K^+ and NaK to both Na^+ and K^+

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Materials and Methods

Strains. The strains of *Salmonella typhimurium* LT2 used in this study are listed in Table 1. Bacteriophage strains P22 int_3 HT 12/4 (Kleckner et al., 1975) and P22 # 503 [*am H101* (13^-)] *amN114* (5^-)] were the generous gifts of Dr. John Roth.

Media. Cells were routinely grown in L broth containing 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, 1,000 ml distilled water. Induction of P22 from lysogenic strain NK337 was performed in superbroth containing 50 g tryptone, 30 g yeast extract, 7.5 g NaCl, 3.5 ml NaOH (10 N), 1,000 ml distilled water. Green indicator agar (Chan et al. 1972) containing 25 μg tetracycline per ml was used to select tetracycline-resistant (Tet^R) transductants.

The minimal medium used (Davis) was modified with respect to the composition of K^+ or Na^+ by altering the relative composition of the phosphate salts such that Na medium contained 155 mM Na^+, K medium contained 154 mM K^+ and NaK medium contained 78 mM Na^+ and 77 mM K^+. All carbon sources used were sterilized and added separately to a final concentration of 10 mM. Amino acid supplements were added as required at 20 μg/ml. All incubations were at 37° C unless otherwise indicated.

Chemicals. DL-fluorocitric acid (barium salt) and tricarboxylic acids were obtained from Sigma Chemical Company, [1–5-^{14} C]-citrate was from Amersham.

^{14}C-Citrate Uptake and Transport Experiments. Cells were grown from a nutrient broth inoculum to stationary phase in 100 ml of Na minimal salts medium fortified with both 0.8% nutrient broth and 10 mM Na citrate. Cells were chilled on ice, harvested, and washed three times using the appropriate Na, K, or Na and K-containing minimal medium and then resuspended to an $OD_{650\ nm}$ of 1.05. For the uptake experiments cells were incubated at 37° C for 5 min with shaking, brought to 10 mM α-glycerol-phosphate to energize the cells (Kay and Cameron 1978) and incubated for another 5 min. Finally, Na^+ [1,5-^{14}C]-citrate (10 μM, S.A. 14 mCi mmole^{-1}) was added to initiate the uptake experiment. At appropriate intervals cells were filtered, washed in the same resuspension medium and assayed for radioactivity. For transport experiments cells were poisoned with 0.1 mM DL-fluorocitrate after energization with α-glycerol-phosphate. At the end of all experiments a sample was treated for 10 min with 1 N HCl and assayed for radioactivity to determine losses due to $^{14}CO_2$ evolution.

Periplasmic Proteins. To prepare the crude periplasmic protein fraction, cells were grown either in nutrient broth fortified minimal medium

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Table 1. Strain list

Strain	Genotype or description	Source
SU453	*hisF1009 trpB2 metA22 rpsL201 xyl-1*	K.E. Sanderson
KS3000	*metE61*	K.E. Sanderson
SA263	*purC7 proA46 ilvA405 fla* Str^R	K.E. Sanderson
KS3001	*purI305*	K.E. Sanderson
KS3002	*guaA1*	K.E. Sanderson
KS3003	*glyA10*	K.E. Sanderson
KS3004	*purG301*	K.E. Sanderson
KS3005	*thiH22 ara-9*	K.E. Sanderson
KS3006	*tyr-3*	K.E. Sanderson
KS3007	*pheA5*	K.E. Sanderson
KS3008	*cysD220 ara-9*	K.E. Sanderson
KS3009	*argA51*	K.E. Sanderson
KS3010	*serA6*	K.E. Sanderson
KS3011	*metC88*	K.E. Sanderson
KS3012	*argG72*	K.E. Sanderson
NK337	leu^-_{am} su^+_{II} [P22 $C_{2ts}29$, 12^-_{amNT1} 13^-_{amH101}, int_3, Tn*10*]	J. Roth
TR248	$cysA1349_{am}hisC527_{am}$	J. Roth
TT628	*strA-1 pyrC7*/F'_{114TS} lac^+ *zzf21*::Tn*10*	J. Roth
TT520	*srlA*::Tn*10*	J. Roth
SB1743	*srlA113*	P.E. Hartman
TA2403	*strB362*	B.N. Ames
TA1778	*hisS6331 hisC6330*/F′ his^+	B.N. Ames
KS191-201	Spontaneous FC^R mutants of SU453	This study
KS1-14	Tn*10*-induced mutants of KS3000	This study
KS174-184	Tn*10* insertions flanking *tct* in SU453 (*zfi*-1-9::Tn*10*)	This study
M72 I	Nitrosoguanidine-induced tricarboxylate mutant of SU453	K.E. Sanderson
M188 I	Nitrosoguanidine-induced tricarboxylate mutant of SU453	K.E. Sanderson
M189 II	Nitrosoguanidine-induced tricarboxylate mutant of SU453	K.E. Sanderson
M272	Nitrosoguanidine-induced tricarboxylate mutant of SU453	K.E. Sanderson
M72-1	Nitrosoguanidine-induced tricarboxylate mutant of SU453	K.E. Sanderson
CB724	*hyd*	M. Chippaux
KS315	SU453 (*ΔnalB-recA*)	This study
KS316	SU453 (*Δtct-nalB*)	This study
KS317	SU453 *nal*B	This study
KS318	SU453 *nal*B	This study
SA409	*recA leu197*	K.E. Sanderson
TT98	*trp1013*::Tn*10*	J. Roth

or the same medium to which 5 mM citrate had been added to induce C protein synthesis. At the end of exponential growth cells were cooled, preconditioned with 33 mM NaCl and 33 mM Tris-HCl (pH 7.3), harvested, and osmotically shocked (Willis et al. 1974), in the presence of 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF) to prevent undue proteolysis. The crude shock fluid was dialyzed against water, concentrated by lyophilization, and resuspended in 10 mM Tris-HCl (pH 7.3) to a known protein concentration. The C protein was purified as previously described (Sweet et al. 1979).

Analytical Methods. ^{14}C-Citrate binding by the crude periplasmic protein was assayed by membrane filtration as previously described (Sweet et al. 1979), except that 200 µl of crude shock fluid was incubated with 2 µM [1,5-^{14}C]-citrate and that nitrocellulose filters were previously autoclaved in 10^{-6} M EDTA to reduce non-specific binding.

Ouchterlony immunodiffusion was performed as described before (Sweet et al. 1979) except that the slides were stained with acid fuchsin.

SDS-polyacrylamide gel electrophoresis was also carried out essentially as previously described, primarily on 12% gels (Ames 1974).

Isolation of Spontaneous Fluorocitrate-Resistant (FC^R) Mutants. Clones of strain SU453 were pregrown to saturation in NaK minimal malate medium. 0.1 ml samples were spread on malate minimal agar, and a sterile filter paper disc (0.75 cm diameter) previously soaked with 25 µl fluorocitrate (200 mg/ml) was placed in the centre of each plate. FC^R mutant colonies growing in the zone of inhibition surrounding each disc after 72 h were picked and purified by streaking on nutrient agar. The clones were verified as FC^R by a radial streak test and independent FC^R mutants were retained for study.

Induction of P22 Tc-10. The method is based on that of Kleckner et al. (1975) and J. Roth (personal communication). A single colony isolate of NK337 was grown to 3×10^8 cells/ml at 30° C in 1,500 ml superbroth, the culture was shifted to 39° C and incubated for 3 h to induce lysis, and the remaining cells lysed with chloroform and removed by centrifugation. Since a high proportion of the induced phage particles are deficient in tail parts, tails were prepared separately and added to the particles as follows : TR248 grown in 2 litres L broth to 2×10^8 cells/ml were infected with P22 # 503 at multiplicity of infection (MOI) MOI ~ 5, incubated 35 min, the cells collected by centrifugation and resuspended in 0.85% saline, 1/100 of the original volume. The cells were lysed with chloroform and treated with 10 µg DNAse per ml for 10 min at 25° C. The preparation was centrifuged at 5,000 rpm for 15 min to remove cell debris, and then twice at 20,000 rpm to remove complete phage particles. The supernatant containing the tails was added to the head preparation, incubated for 2 h at 30° C, and the complete phage particles concentrated by high speed centrifugation.

Tn10-Induced Mutagenesis. Recipient strain KS3000 was grown to 5×10^8 cells/ml in L broth, concentrated 10-fold in L broth, infected with P22 Tc-10 (MOI 0.8), incubated for 30 min at 37° C for adsorption, and 0.1 ml samples were plated onto green tetracycline agar plates. After 48 h incubation at 42° C, Tet^R colonies were picked to

fresh green tetracycline master plates and replicated to test for growth on tricarboxylates and dicarboxylates. Mutant strains unable to grow on tricarboxylates were retained for further study. In addition, all the Tet^R transductant colonies (approximately 7,000) were pooled by washing the plates with L broth, and stored at −75° C. The pooled cells were subsequently used to isolate Tn*10* insertions which are co-transducible with, but are not within, the *tct* locus, as described in the results.

Isolation of Nalidixic Acid Resistant Mutants. Overnight broth cultures of independent clones of SU453 were plated (0.1 ml) on BT agar (Hane and Wood 1969) containing 4 μg nalidixic acid per ml. Resistant colonies growing after 24 h were purified by restreaking on the same medium and then checked for survival on BT agar containing concentrations of nalidixic acid from 2–12 μg/ml. Independent mutants showing 60–80% survival at 4 μl/ml and no survival at 8 μg/ml and above were considered to be potential *nalB* mutants and retained for genetic studies.

Phage P22 Preparation and Transduction Experiments. For each preparation, a single plaque of P22 int_3 HT12/4 grown on the donor strain was inoculated into a 250 ml flask containing 30 ml L broth and 0.5 ml of an overnight culture of the same donor strain. The flasks were shaken at 200 rpm at 25° C for 24 h in a New Brunswick shaker. The cells were removed by centrifugation, and the supernatant, containing about 10^{11} pfu/ml, was stored at 4° C over chloroform.

In transduction experiments where tetracycline resistance was the selected marker, donor phage was added to late-exponential phase recipient cells growing in L broth at a MOI of approximately 1–2. The adsorption mixture was incubated at 37° C 30–60 min prior to plating on green tetracycline plates. In experiments where selection was for prototrophy, late-exponential phase recipient cells and donor phage (MOI 1–2) were directly co-plated on selective minimal agar.

Tn10-Directed Hfr Formation and Plate Mating Tests. The method is based on that of Chumley et al. (1979). *tct*::Tn*10* mutants KS1, 2, 3, and 6 were first converted to F′ strains by cross-streaking with F′ strain TT638. The F′ factor in this strain carries Tn*10*, genes for lactose fermentation, and is temperature-sensitive for replication. The strain also carries a chromosomal *pyrC* mutation. Mutant cells infected with the F′ factor were counterselected at 30° C (permissive temperature for F′ replication) on minimal agar supplemented with methionine and with lactose as the sole carbon source. After single cell purification by restreaking on the same medium at 30° C, the mutant F′ strains were streaked out on MacConkey plates and incubated at 42° C, which prevents autonomous F′ replication. The majority of colonies grown at 42° C were white (lac^-), due to curing of the episome, but the rare red (lac^+) colonies are potential Hfr strains resulting from integration of the F′ which is now under chromosomal replication control. These lac^+ colonies were picked and purified by restreaking on MacConkey agar at 42° C prior to testing for their point of origin of chromosome transfer.

Plate mating tests were performed by spreading 0.2 ml overnight auxotrophic F^- strains as a background on unsupplemented minimal glucose agar plates, and spotting loops of late log phase Hfr cultures over the F^- cells. The presence of many prototrophic recombinants after 48 h incubation indicated early gene transfer by the Hfr strain in question.

recA Phenotype. To test the *recA* phenotype transductants were picked onto nutrient agar, incubated at 37° C for 6–7 h, replicated onto a fresh nutrient agar plate and immediately UV irradiated under a 30 watt germicidal lamp (General Electric) at a distance of 36 cm for 60 s. The plates were then incubated overnight. *recA* mutants did not survive irradiation except for the occasional few colonies but wild-type cells grew normally as a patch.

Transduction of hyd^- to hyd^+. The hydrogenase character was tested by a modification of the method of Abou-Joude et al. (1978). Transductants were patched onto benzylviologen containing media, covered with 7 ml of melted agar and incubated anaerobically under H_2 overnight. Patches were scored within 2 min but the purple color remained visible for approximately 5 min.

Imprecise Excisions of the Tn10 Element. Cells (10^9) of KS183 containing a Tn10 insertion close to *nalB* were plated on BT agar containing 5 μg nalidixic acid per ml and incubated for 48 h. Resistant clones were purified by restreaking on the same media and the Tet^S clones detected on green plates – which also were used to detect the $recA^-$ character (Kleckner 1977). Clones were also screened for cysteine auxotrophy, sorbitol utilization and hydrogenase activity to detect the extent of the deletions. Imprecise excisions enriched by ampicillin selection were carried out as described (Kleckner et al. 1979).

Results

Growth Characteristics of Spontaneous Fluorocitrate-Resistant (FC^R) Mutants. Eleven independently isolated spontaneous FC^R mutants of SU453, KS191-201, were studied. Clones were replicated from master nutrient agar plates onto Na, K, and NaK minimal agar plates containing 10 mM tricarboxylates as sole carbon sources, and also onto NaK minimal agar containing 10 mM dicarboxylates (Succinate, fumarate, or L-malate) or 20 mM acetate. Growth was read after 18 and 42 h incubation. All FC^R mutants had identical growth responses, and the results are summarized in Table 2. All mutants differed from the parent strain SU453 in that they showed slower growth on Na citrate, although their growth was normal on K and NaK citrate. More striking, however, was the complete absence of growth of the mutants on K and NaK *cis*-aconitate, and on NaK isocitrate; the parent strain shows normal growth on these media. Neither the parent strain nor the mutants grew on Na *cis*-aconitate or Na isocitrate. All the mutants showed wild-type growth on the NaK succinate, fumarate, malate, and acetate, indicating that the enzymes of the TCA cycle and glyocxylate shunt were functional. These enzymes, as well as those concerned with acetate metabolism [acetokinase (E.C. 3.8.2.1), phosphotransacetylase (E.C. 2.3.1.8)] and gluconeogenesis [NADH and NADPH malic enzymes (E.C. 1.1.1.38 and E.C. 1.1.1.40), phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32)] were measured directly and found to be normal. These results support the hypothesis that FC^R mutants are indeed defective specifically in tricarboxylate transport.

Characteristics of Tn10 Insertion Mutants. Over 7,000 tetracycline-resistant (Tet^R) transductants of parent strain KS3000 were initially screened for altered growth response on Na citrate agar. Fourteen were found to grow more slowly than the parent strain and were classified according to the growth criteria shown in Table 2. KS1, 2, 3, and 6 clearly fell into a class indistinguishable from the spontaneous FC^R mutants KS191–201. This report is primarily concerned with the properties of the FC^R mutants, and studies on the remaining Tet^R FC-sensitive mutants will be reported in a later publication.

For later mapping purposes, it was necessary first to establish that FC-resistance in KS1, 2, 3, and 6 was caused by Tn10 insertion into the *tct* region of the genome, rather than the alternative possibility that the Tn*10* insertion and the mutation to FC-resistance arose from simultaneous independent events in the parent strain. Accordingly, KS1, 2, 3, and 6 (Tet^R tct^-) were each used as donors in transduction experiments with SU453 recipient cells (Tet^S tct^+). Tet^R transductants were selected and all were shown to be now tct^-, i.e. unable to grow on NaK isocitrate and NaK *cis*-aconitate. Thus the Tn*10* insertions in these mutants are in the *tct* locus, and resistance to tetracycline

Table 2. Growth responses[a] of wild type and mutant strains of *S. typhimurium* on various carbon sources[b]

Strain	FC	Citrate				Aconitate			Isocitrate		Tricarballylate		
		Na		K	NaK	Na	K	NaK	Na	NaK	Na	K	NaK
		18 h	42 h										
SU453 (*tct*$^+$)	S	+	+	+	+	−	+	+	−	+	±	+	+
KS191-201	R	−	±	+	+	−	−	−	−	−	±	+	+
KS3000 (*tct*$^+$)	S	+	+	+	+	−	+	+	−	+	±	+	+
KS1, KS2, KS3, KS6	R	−	±	+	+	−	−	−	−	−	±	+	+
M72 (*tct* Group I)	R	−	±	+	+	−	−	−	−	−	±	+	+
M188 (*tct* Group I)	S	−	−	+	+	−	−	−	−	−	±	+	+
M189 (*tct* Group II)	S	+	+	+	+	−	−	−	±	+	±	+	+
M272 (*tct* Group III)	S	+	+	+	+	−	+	+	±	+	−	−	−
M72-1 (*tct* Group IV)	R	−	−	−	−	−	−	−	−	−	±	+	+

Results were scored as: +, full growth; ±, partial growth; −, no growth; S, sensitive to FC; R, resistant to FC

[a] Incubation was 18 h unless indicated otherwise

[b] Concentration of all carbon sources was 10 mM

can be used for direct selection of *tct*$^-$ recombinants in genetic crosses. (Due to the extreme toxicity and expense of fluorocitrate, it is not practicable to routinely incorporate this substance with media for growth tests or for direct selection of *tct*$^-$ recombinants).

Characteristics of Other Tricarboxylate-Negative Strains. Imai (1975) has described four groups of nitrosoguanidine-induced mutants of SU453 which are unable to utilize tricarboxylates. Group I mutants, e.g. M72 and M188, were unable to grow on NaK *cis*-aconitate and NaK isocitrate. On these criteria, therefore, they are indistinguishable from our FC^R mutants. Group II mutants, e.g. M189, grew on NaK citrate and NaK isocitrate but not on NaK *cis*-aconitate; group III mutants e.g. M272, are NaK tricarballylate negative; and group IV double mutants, e.g. M72-1, derived by further nitrosoguanidine mutagenesis of group I mutant M72, are unable to utilize NaK citrate, NaK *cis*-aconitate, or NaK isocitrate. We have further characterized these mutants by testing for growth on Na^+ and K^+ tricarboxylates and for FC-resistance, and the results of these tests are also shown in Table 2. Of all these mutants, only M72 and its derivative double mutants M72-1 were FC^R. Surprisingly, group I mutant M188 was FC sensitive although it closely resembled our FC^R mutants in its growth responses on carboxylic acids under all conditions tested.

Citrate Uptake, Transport and Binding. Citrate incorporation into whole cells can be measured by a variety of methods: by the uptake of labelled citrate, but only over very short intervals to reduce the influence of metabolism, by citrate transport into fluorocitrate poisoned cells or into aconitase mutants (Kay and Cameron 1978) and by transport of the non-metabolizable citrate analogue 2-fluoro-L-*erythro*-citrate (Ashton et al. 1980). We have used the first two methods both for ease of operation and in order to compare our results with those of others (Imai 1975), and the results with various mutants appear in Table 3. As described previously (Ashton et al. 1980), the transport of citrate is largely K^+ requiring. Both spontaneous and Tn*10* induced mutants however were unable to take up citrate even in the presence of both Na and K and their periplasmic proteins were unable to effectively bind citrate. The single exception to this pattern was the tct::Tn*10* insertion strain KS1 which exhibited

Table 3. ^{14}C-citrate uptake, transport and binding activities of various transport mutants

Strain	Ionic medium	^{14}C-Citrate			CRM[a]
		Uptake (pmoles min^{-1} mg^{-1})	Transport (nmoles mg^{-1})[b]	Binding (pmoles mg^{-1})	
KS3000	Na	0.2	1.1	140	+
	NaK	11.8	7.3		
KS1	Na	1.3	2.7	91	+
	NaK	1.9	4.2		
KS2	Na	0	0.4	11	−
	NaK	1.0	2.2		
KS3	Na	0	0.4	12	−
	NaK	1.0	2.2		
KS6	Na	0.1	0.8	−[c]	−
	NaK	0.22	1.5		
SU453	Na	0	0.2	103	+
	NaK	0.9	3.2		
KS191	Na	0	0.1	0	−
	NaK	0	0.2		
KS192	Na	0	0	0	−
	NaK	0	0.3		
KS193	Na	0	0	0	−
	NaK	0.2	0.5		
M72	Na	0	0.2	24	−
	NaK	0.2	0.4		
M188	Na	0.1	0.4	0	−
	NaK	0.5	1.8		
M189	Na	0	0.1	14	+
	NaK	0.1	0.3		
M272	Na	0.1	0.1	75	+
	NaK	1.4	3.7		
M72-1	Na	0	0.1	0	−
	NaK	0	0.1		

[a] CRM refers to cross-reacting material to C protein antisera

[b] Measured at 5 min

[c] Not tested

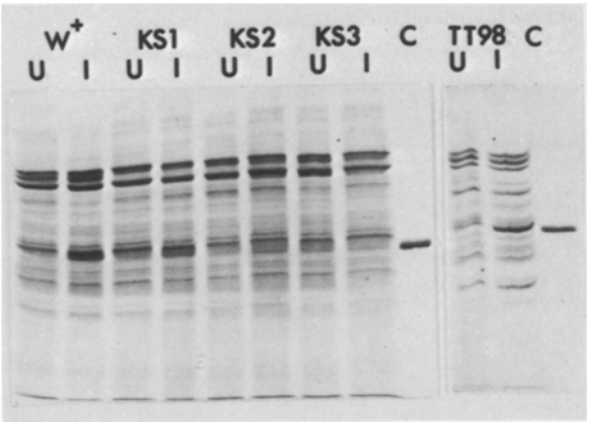

Fig. 1. SDS-Polyacrylamide gel electrophoresis of shock fluids from *S. typhimurium* strains. Cells were grown in complex medium with (*I*) and without (*U*) 5 mM citrate. Strains KS1–4 are *tct*::Tn*10* insertions and TT98 is a *trp*::Tn*10* insertion

both reduced transport and binding. Nitrosoguanidine induced mutant M72 which was FC^R (Table 2) appeared to have a leaky transport and binding activity, however the double mutant M72-1 was virtually devoid of activity. Strain M188 did not exhibit binding activity but was still able to transport citrate albeit at about one-half that of the parent. M189 exhibited only about 10% of the activities of the parent. As expected M272, the tricarballylate defective strain, was transport and binding protein positive.

Periplasmic Proteins. The periplasmic proteins of the various *S. typhimurium* transport mutants were examined by SDS-polyacrylamide gel electrophoresis, for ^{14}C-citrate binding and for cross-reacting material with C protein antibody (Fig. 1). All FC^R mutants, either spontaneous or Tn*10* derived, were unable to produce the C protein (28 K) when induced with citrate, to bind ^{14}C-citrate to the periplasmic fraction or to form detectable cross-reacting material. The single exception to this fixed pattern was the Tn*10* insertion mutant KS1 which appeared to produce some binding activity even in the uninduced state. An even more unusual finding was the abrupt disappearance of a prominent 50 K dalton protein in all FC^R strains (Fig. 1). Control experiments with three unrelated tetracycline Tn*10* insertions indicated that this was not due to the presence of the Tn*10* transposon. One such insertion (TT98) is shown in Fig. 1 for comparison. This 50 K protein is apparently not a percursor to the C protein since it is present in uninduced cells and no cross-reacting activity was detected in the periplasmic fraction from such cells.

Approximate Location of the tct Locus on the Salmonella Chromosome. Time of entry mating experiments (Ashton et al. 1980) showed that the *tct* locus mapped at approximately 58–60 units on the *Salmonella* chromosome (Sanderson and Hartman 1978). However, we were unable to detect cotransduction between *tct* and any of the following genetic markers in the 54–68 unit region of the chromosome: *purC, purI, guaA,* (54U); *strB,* (55U); *hisS,* (56U); *glyA, purG,* (57U); *thiH, tyrA, pheA,* (58U); *srlA,* (59U); *cysD,* (60U); *argA,* (61U); *serA* (63U); *metC,* (66U); *argG,* (68U).

We therefore decided to use the technique of Tn*10*-directed Hfr formation (Chumley et al. 1979) to determine more accurately the location of the *tct*::Tn*10* insertions in FC^R mutants KS1, 2, 3, and 6. After introduction into these mutants of an F′ factor carrying Tn*10*, and subsequent integration of this F′ into the chromosome by recombination between the chromosomal *tct*::Tn*10* and episomal Tn*10* transposons, the resulting Hfr strains will have their point of origin of chromosome transfer within the *tct* region. Accordingly, KS1, 2, 3, and 6 were converted first to F′ and then to Hfr strains as described in Materials and Methods, and these Hfr's were used as donors in plate mating tests with F^- strains with auxotrophic markers in the 54–68 unit region of the chromosome. The presence of many prototrophic recombinants after 48 h incubation indicates early gene transfer by the Hfr in question, thus locating the origin of transfer, and hence the *tct* locus, between known chromosomal markers.

The results of these tests showed that several independently-isolated Hfr clones of each strain transferred *srlA* as the proximal marker and *pheA* as the distal marker. The *tct* locus was therefore positioned between *srlA* and *pheA* on the *Salmonella* chromosome. The direction of transfer was the same in each Hfr strain, indicating that each original mutant arose from insertion of Tn*10* with the same orientation ('A') in the chromosome (Chumley et al. 1979).

Isolation of Tn10 Insertions Cotransductible with tct. The finding that the *tct* locus is between the *srlA* and *pheA* loci but cotransducible with neither indicates that the distance between these two outside markers is probably greater than the 1 unit indicated on the *Salmonella* map (Sanderson and Hartman 1978). Since the availability of easily selected cotransducible outside markers is important for future fine structure genetic studies, we set out to isolate Tn*10* insertion mutants in which the transposon was cotransducible with, but not itself within, the *tct* region. Such insertions may also show cotransduction with known chromosomal markers in the region and hence could be used to close the transduction gap between *srlA-tct-pheA*.

In order to isolate cotransducible Tn*10* insertions, transducing phage P22 int_3 HT12/4 was grown on the pooled Tet^R cells from the Tn*10* mutagenesis experiment described in the methods, and used to transduce the spontaneous FC^R mutant KS191 (Tet^S tct^-) to tetracycline resistance. The transductants were replicated onto NaK isocitrate to check for any colonies which had been cotransduced to tct^+. Eleven such Tet^R tct^+ colonies were isolated from over 50,000 Tet^R transductants screened. These transductants were purified by restreaking twice on green tetracycline plates, verified as tct^+ (growth on Na citrate, NaK *cis*-aconitate and NaK isocitrate), and given isolation numbers KS174–184.

The cotransduction frequencies between the Tn*10* insertions in strains KS174–184 and the *tct* locus were determined as follows: preparations of P22 int_3 HT12/4 grown on each strain were used as donors to KS191 (Tet^S tct^-), and Tet^R transductants were selected and replicated onto NaK isocitrate agar. The results presented in Table 4 show that the various Tn*10* insertions were between 2–16% cotransducible with *tct*.

Transduction Mapping of the 58–60 Unit Region of the Salmonella chromosome. To test for cotransduction between the Tet^R markers in strains KS174–184 and known chromosomal genes in the region, P22 grown on these strains were used as donors to *cysD, pheA, tyrA,* and *srlA* recipient strains. Tet^R transductants were selected and checked for growth on appropriate selective minimal agar. No cotransduction was observed between any Tet^R marker and *cysD, pheA,* or *tyrA*. However, the insertions in strains KS178, 179, 180, and 183 showed low cotransduction with *srlA* (Table 4). Although the cotransduction frequencies were higher than the background level of spontaneous reversion

Table 4. Percentage cotransduction between Tn*10* insertions in strains KS174-183[a] and the *tct*, *srlA*, and *nalB* loci

Donor phage Tet^R *tct*$^+$ *srl*$^+$ Nal^S	Recipient strain		
	KS1000 (*tct*)	SB1743 (*srlA*)	KS317 (*nalB*)
KS180	2.9 (816)	0.6 (990)	73.5 (283)
KS183	2.3 (621)	1.2 (1,272)	69.0 (458)
KS179	9.1 (2,281)	0.2 (3,447)	59.5 (200)
KS178	11.9 (2,589)	0.07 (2,681)	60.5 (200)
KS176	10.5 (1,637)	0 (1,154)	49.3 (300)
KS174	13.9 (1,041)	0 (1,614)	38.5 (200)
KS182	16.1 (1,000)	0 (1,120)	34.0 (200)
KS177	2.4 (1,203)	0 (1,365)	0 (291)

Figures in parentheses show the total number of Tet^R transductants analyzed per cross

[a] KS175 and KS181 gave low numbers of Tet^R transductants in preliminary crosses and the data is not included

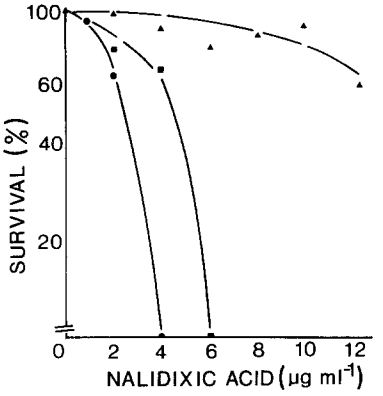

Fig. 2. Nalidixic acid sensitivity of various *S. typhimurium* strains. Wild-type, SU453 (●—●), *nalB*, KS317 (■—■), and *nalA*, KS319 (▲—▲) strains are shown for comparison

of the recipient to *srl*$^+$, they were too low to be used to draw and accurate transduction map.

Isolation of Nalidixic Acid Resistant Mutants. Resistance to low levels (4 μg/ml) of nalidixic acid (Hane and Wood 1979) is determined by the *nalB* locus at approximately 59 units on the *Salmonella* chromosome (Sanderson and Hartman 1978). We decided to isolate *nalB* mutants and check for cotransduction of *nalB* with all markers in the region. Figure 1 described the typical survival characteristics of both presumptive *nalB* and *nalA* mutants and strongly resembles those reported for *E. coli* (Hane and Wood, 1969). Of a total of 7 low level nalidixic acid-resistant mutants studied (described in Materials and Methods), 2 were found to be cotransducible (1.7%) with *srlA* and were therefore presumed to be *nalB* mutants. One of these (KS317) was checked for cotransduction with the Tet^R markers in KS174–184 and the results (Table 4 and Fig. 2) show cotransduction frequencies ranging from 34–74%. No cotransduction was observed between *nalB* and *cysD, tct, pheA*, or *tyrA* (data nor shown).

The combined results of the crosses shown in Table 4 were used to construct the transduction map of the 58–60 U region of the *Salmonella* chromosome as shown in Fig. 3.

It should be noted that only 5 *tct* mutants have been used in the mapping studies described so far – preliminary localization was carried out by spot mating tests using Hfr derivatives of KS1, 2, 3, and 6, and the studies using the cotransducible Tn*10* insertions in strains KS174–184 were done with spontaneous FC^R mutant KS191. The availability of the cotransducible Tn*10* insertions now enabled us to check the approximate location of the *tct* mutations in the remaining spontaneous FC^R mutants and in the various NTG-induced mutants listed in Table 2. P22 grown on Tn*10* insertion strains KS177, 182 and 183 were used as donors to each mutant strain, Tet^R transductants were selected and checked for their ability to grow on NaK isocitrate (or on NaK *cis*-aconitate in crosses with M189 and on NaK tricarballylate in crosses with M272). The contransduction frequencies obtained are shown in Table 5, and establish that the mutations in all the strains investigated, except M272 (tricarballylate negative), are in the same region of the chromosome as the *tct* mutation in KS191. Furthermore, when examined more closely, the mutations of strains M72, M72-1, M188 and 189 cluster near the centre of the region defined by our various FC^R mutants and thus appear to fall within the *tct* locus.

Mapping of the hyd Gene. A variety of Tn*10* insertion strains were used as donor strains in phage crosses with CB724, a hydrogenase deficient strain. No cotransduction was detectable from 200 Tet^R clones derived from strains carrying *tyrA*::Tn*10*, or *tct*::Tn*10*. However the *hyd* gene was found to be 0.9% (328

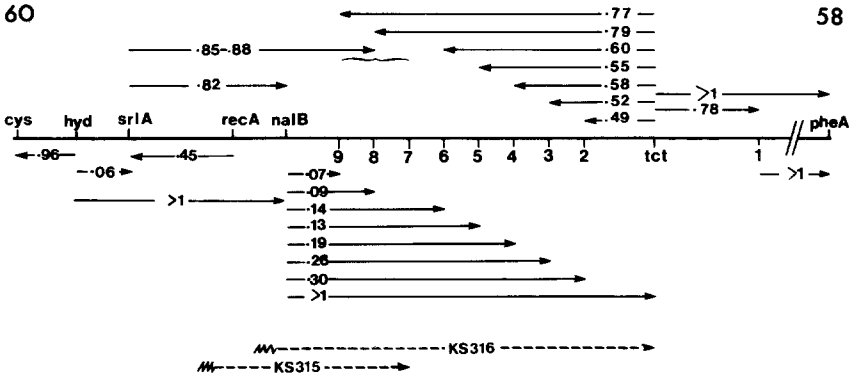

Fig. 3. Map of the 58–60 unit region of the *S. typhimurium* chromosome. The map is *not* drawn to scale and linear distances are indicated as fractions of a P22 phage chromosome length (14). Numbers 1–9 on the chromosome represent independent Tn*10* flanking insertions *zfi* 1–9. The overall distance between 58 and 60 units actually calculates to be >3.4 units. The average distance between the *nalB* and *tct* loci (0.78±0.045) was arrived at by summing and averaging the linear distances between the various insertions and these loci. Deletions are indicated by the broken arrows

Table 5. Percentage cotransduction between Tn*10* insertions in strains KS183, 182 and 177 and various *tct* mutants[a]

Recipient (Tet^S *tct*$^-$)	Donor phage (Tet^R *tct*$^+$) grown on strain		
	KS183	KS182	KS177
KS191	2.4 (378)	21.3 (310)	3.2 (340)
KS192	1.1 (457)	5.5 (715)	4.2 (409)
KS193	0 (397)	4.4 (496)	2.1 (436)
KS194	2.7 (415)	13.4 (397)	1.0 (315)
KS200	0 (593)	3.0 (504)	1.2 (338)
KS201	1.0 (577)	14.1 (765)	2.2 (743)
M72	0.2 (580)	5.1 (568)	1.1 (561)
M188	0 (974)	2.1 (854)	2.0 (587)
M189	0.3 (895)	5.5 (957)	2.0 (1,063)
M272	0 (503)	0 (409)	0 (394)
M72-1	0 (630)	5.4 (701)	1.0 (834)

Figures in parentheses show the total number of Tet^R transductants analyzed per cross

[a] *tct* mutants KS195-199 were P22-resistant and could not be analyzed by P22-mediated transduction

clones) cotransducible with *cysC* and 76.8% (200 clones) cotransducible with *srlA*. Since no cotransduction of the *nalB* locus with *hyd* was found the gene order was assumed to be *srlA hyd cysC*.

Orientation of the recA and nalB Loci. Imprecise excisions of the Tn*10* transposon are known to occur spontaneously at a frequency of approximately 10^{-9} without prior penicillin enrichment (Kleckner 1977). In order to establish the gene order from *nalB-srlA* we selected and screened several spontaneous nalidixic acid resistant mutants arising from the excision of Tn*10* insertion KS183 (Fig. 3). One of these *Tct*$^+$ *Nal*^{R} strains was also *recA*$^-$ but *srl*$^+$ and *cys*$^+$, representing a deletion from the Tn*10* insertion KS183 through *nalB* and *recA* but not as far as *srlA*. This established the gene order as *nalB recA srlA*.

A second imprecise excision was selected from KS3 (*tct*::Tn*10*) which extended from *tct* (negative growth on isocitrate) through *nalB* (resistance to 4 µg nalidixic acid per ml but not as far as *recA* or *srlA*. This confirmed the above gene order and also indicated that no unknown lethal genes exist between *tct* and the *nalB* loci.

Mapping of recA. The precise location of the *recA* gene in *S. typhimurium* has not previously been determined although the gene has been reported to be approximately 20% cotransducible with *srlA* (Kleckner 1979). In order to more accurately position this gene we first transduced strain TT520 (*srlA*::Tn*10*, *recA*$^+$) to *srl*$^+$ using strain SA409 (*recA*$^-$) as a donor and scored the transductants for *recA*$^-$. *recA* was found to be 19.3% (150 clones) cotransducible with *srlA*. In a similar cross using strain KS300– (*cysD*$^-$) and selecting *cys*$^+$ transductants no linkage (200 clones) of *recA* to *cysD* was observed confirming that *recA* was between *srlA* and *nalB*.

Discussion

The transport of tricarboxylates in *S. typhimurium* appears to be a complicated process. It is unusual among transport systems in that it is strongly K^+-dependent with respect to either citrate or fluorocitrate transport (Ashton et al. 1980). The K^+-requirement does not appear to confer substrate specificity to the transport system since K^+ is not preferred over Na^+ for citrate binding to the C protein (Sweet et al. 1979), nor is it required stoichiometrically for fluorocitrate transport (Ashton et al. 1980). Thus it appears to be concerned with the energetics of tricarboxylate transport. The FC^R mutants described here are concomitantly defective in citrate transport as well as growth on citrate, *cis*-aconitate and isocitrate suggesting these three substrates share a common transport component. Another system, apparently citrate specific, appears to be operative since these strains continued to grow, albeit slowly, on Na citrate and normally on K or NaK citrate. Although it is difficult to observe in the citrate uptake or transport experiments it does appear as a NaK fluorocitrate transport or binding system (Ashton et al. 1980).

The most apparent biochemical defect in FC^R strains is the inability to produce the citrate binding C protein found in the periplasm (Sweet et al. 1979). However it is difficult at this time to simply ascribe the inability to produce the C protein to a single genetic defect in these mutants since *cis*-aconitate is neither a competitive inhibitor of binding to the C protein (Sweet et al. 1979) nor of transport into whole cells for either citrate (Kay and Cameron 1978) or fluorocitrate (Ashton et al. 1980) yet FC^R strains are unable to grow on *cis*-aconitate. Furthermore, mutants (M272) can be obtained which are singly defective in the ability to use *cis*-aconitate – perhaps representing a unique *cis*-aconitate transport system. To complicate matters even further we have described here a 50 K dalton periplasmic protein which disappears in all our transport mutants as well as from the NTG induced strains (Imai 1975). This protein is presently under study. It is apparently not under the same control as the C protein since it appears to be constitutive. Neither does it appear to be a percursor since it is immunologically distinct. An outer membrane 81K M.W. protein has been reported in *E. coli* which is inducible by growth in citrate containing media and has been suggested to be the receptor (Frost and Rosenberg 1973) for the citrate-iron complex (Hancock et al. 1976). We do not know as yet whether a similar system exists in *S. typhimurium* or whether the 51 K protein found here is a periplasmic equivalent.

Many of the problems described herein can be elucidated genetically. Thus we have carefully mapped the *tct* locus to the 59 unit region of the *Salmonella* chromosome (Sanderson and Hartman 1978). Also we have more accurately positioned the *hyd* and *recA* loci and further isolated, described and mapped *nalB* mutants of *S. typhimurium* in order to complete our picture of the 59 unit region. This region is unusually barren of appropriate genetic markers, necessitating the use of Tn*10* insertions flanking the *tct* locus.

The mapping of this 59 unit region of the *Salmonella* chromosome in this detail permitted a more accurate estimation of the accurate linear distance of this region using the equation of Kemper (1974) which relates contransduction frequency to the length of P22 phage DNA. From this analysis (Fig. 3) the 58–60 unit region appears to be larger than indicated on the standard *Salmonella* map (Sanderson and Hartman 1978), measuring a minimum of 3.4 units rather than 2. This perhaps explains part of our inability to achieve contransduction within this region.

The flanking Tn*10* insertions made a comparative genetic analysis of the various transport mutants possible. All our FC^R strains were located in the same region. All of the NTG mutants (Imai 1975), with the exception of M272 which was previously located at approximately 1 unit on the *Salmonella* chromosome (Imai 1977), map in the *tct* region also. The relative map position is in agreement with that of others (Imai 1977), that is at approxi-

mately 59 units. However it is difficult at this time to rationalize these data with the variety phenotypes found, unless of course the degree of growth restriction parallels the severity of the mutation. In support of this is the observation that the NTG derived strains are more leaky in their transport ability; lack of support stems from similarly leaky transport observed in the insertion mutants of which one would expect of polar effect of the *tct*::Tn*10* insertion. Even more likely is the existence of multiple citrate transport genes in this locus. However any conclusions regarding these genetic relationships must of necessity await a more detailed analysis of this region which is currently in progress.

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