

Mutations in *yhiT* enable utilization of exogenous pyrimidine intermediates in *Salmonella enterica* serovar Typhimurium

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Mutants capable of utilizing the pyrimidine biosynthetic intermediates carbamoylaspartate and dihydroorotate for growth were derived from pyrimidine auxotrophs of *Salmonella enterica* serovar Typhimurium LT2. The gain-of-function phenotypes both resulted from mutations in a single gene, *yhiT*, the third gene of a putative four-gene operon, *yhiVUTS*, for which there is no homologous region in *Escherichia coli*. Notably, when a mutant *yhiT* allele was transferred to a pyrimidine-requiring *E. coli* strain, the transformant was then capable of using carbamoylaspartate or dihydroorotate as a pyrimidine source. The operon arrangement of the *yhiVUTS* genes was supported by genetic analyses and studies employing RT-PCR, coupled to the determination of the transcriptional start site using 5'-random amplification of cDNA ends (RACE). Computer-generated predictions indicated that YhiT is an integral membrane protein with 12 putative transmembrane domains typical of bacterial transport proteins. Competition experiments showed that mutant YhiT interacts with the C₄-dicarboxylates succinate and malate, as well as the amino acids aspartate and asparagine. The native function of wild-type YhiT remains undetermined, but the collective results are consistent with a role as a general transporter of C₄-dicarboxylates and other compounds with a similar basic structure.

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INTRODUCTION

Synthesis and repair of DNA and RNA requires a continual supply of deoxyribo- and ribonucleoside triphosphates. In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, these nucleotides can be produced in two ways: either *de novo* from precursors present in the cell as a result of intermediary metabolism, or via defined salvage pathways using preformed purine and pyrimidine compounds arising from endogenous turnover or from exogenous sources.

Uridine 5'-monophosphate (UMP) is the parent compound for the biosynthesis of all other pyrimidine (deoxy)ribonucleotides in the cell. UMP is synthesized *de novo* from carbamoylphosphate and aspartate through a multi-step process involving five enzymes and four intermediate compounds. The order of synthesis and the corresponding genes are: carbamoylaspartate (CAA, or ureidosuccinate; *pyrBI*), dihydroorotate (DHO; *pyrC*), orotate (OA; *pyrD*), orotidine 5'-monophosphate (OMP; *pyrE*), with the final step being the decarboxylation of OMP (*pyrF*) to yield UMP (Fig. 1a).

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Abbreviations: CAA, carbamoylaspartate; DHO, dihydroorotate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; OA, orotate; RACE, random amplification of cDNA ends.

Several proteins or protein complexes that mediate the transport of various pyrimidine compounds across the Gram-negative bacterial envelope in *E. coli* and *S. enterica* serovar Typhimurium have been identified. For example, the non-specific porins OmpC and OmpF, as well as the nucleoside-specific porin Tsx, are involved in the transport of pyrimidine compounds across the outer membrane (Bremer *et al.*, 1990; Hantke, 1976; van Alphen *et al.*, 1978). Inner membrane transport systems for pyrimidine compounds include a uracil permease, UraA (Andersen *et al.*, 1995), a cytosine permease, CodB (Danielsen *et al.*, 1992), and two high-affinity nucleoside transport systems,

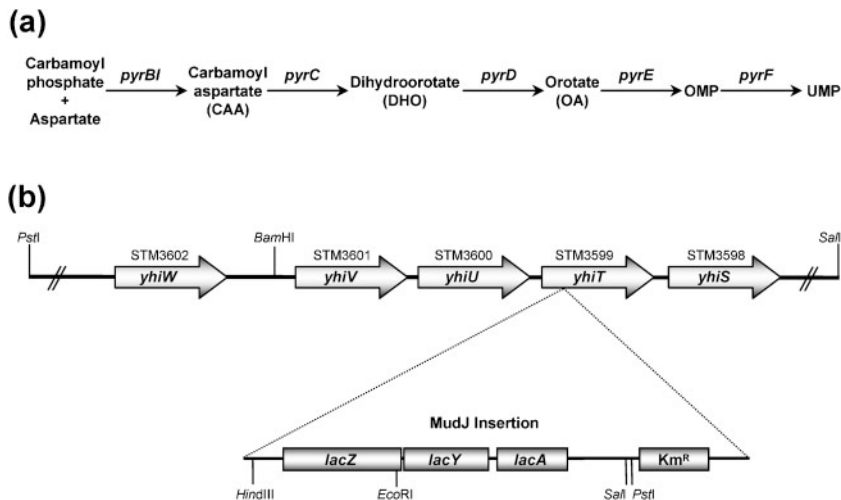


Fig. 1. (a) Pyrimidine biosynthetic pathway. The gene symbol is shown above the arrow for each of the five steps of the pathway. (b) Illustration of the *yhiW*–*yhiVUTS* region of the *S. enterica* serovar Typhimurium chromosome. The STM designations are shown above the corresponding *yhi* gene. The position of the MudJ insertion within *yhiT* of KRM104 is indicated. Also shown are the positions of several key restriction sites used for cloning and subcloning of genomic DNA surrounding the insertion site.

NupC and NupG (Munch-Petersen & Jensen, 1990). Although wild-type *S. enterica* serovar Typhimurium is permeable to pyrimidine nucleosides and nucleobases, it is permeable to only a single pyrimidine intermediate, namely OA. In an earlier study (Baker *et al.*, 1996), we showed that transport of OA was dependent on DctA, an inner-membrane protein with a predicted 12-transmembrane-domain structure that is typical of bacterial transport systems, whose primary function is the transport of the C₄-dicarboxylates succinate, fumarate and malate. Consistent with this role, mutants defective in *dctA* are unable to use these compounds as sole carbon source, and are also incapable of utilizing OA as an exogenous pyrimidine source (Baker *et al.*, 1996).

In this study, we have extended our analyses of the uptake and utilization of pyrimidine intermediates in *S. enterica* serovar Typhimurium. Mutants capable of utilizing exogenous CAA as sole pyrimidine source have been isolated previously (Kelln & Zak, 1980; Legrain *et al.*, 1976; Syvanen & Roth, 1973). These isolates, however, were neither extensively characterized, nor were the genes responsible for the phenotype identified. Here we describe the characterization of the previously isolated *S. enterica* serovar Typhimurium mutants as well as a newly isolated novel mutant capable of using DHO as sole exogenous pyrimidine source. In this report, we show that the utilization of both CAA and DHO is the result of distinct mutations in the previously uncharacterized *yhiT* locus, the third gene in the *yhiVUTS* operon.

METHODS

Bacterial strains and plasmid constructs. Bacterial strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2 or *E. coli* K12, and are listed along with their relevant genotypes in Table 1. Other strains and their construction are described at various points in the text. Plasmid vectors used were: pBR322 (Bolivar *et al.*, 1977), pJQ200 (Quandt & Hynes, 1993), pUC4K (Kokotek & Lotz, 1989), pUC18 (Yanisch-Perron *et al.*, 1985) and pWSK29 (Wang & Kushner, 1991). Plasmids constructed during this study are described in the text in the relevant section.

Genetic nomenclature. The gene symbols *usp-2* (Syvanen & Roth, 1973) and *usp-3* (Kelln & Zak, 1980) were used previously to indicate mutations mediating the use of exogenous ureidosuccinate [preferred name carbamoylaspartate (CAA)] as a pyrimidine source. Accordingly, the phenotype associated with the mutant allele is Caa⁺; for the wild-type it is Caa⁻. Correspondingly, the phenotype associated with the utilization of exogenous dihydroorotate (DHO) is indicated as Dho⁺. The mutation in the parental Dho⁺ isolate described herein, KR1667, was designated *yhi-23*, based on the determined map location. For the corresponding chromosomal DNA region, the currently accepted nomenclature for the *S. enterica* serovar Typhimurium genome was applied (McClelland *et al.*, 2001); therefore, the genes for the four contiguous open reading frames were designated *yhiVUTS*.

Media and growth conditions. Lennox L broth (LB) was the complex medium (Enquist & Sternberg, 1979). The minimal medium, medium A, has been described previously (Kelln *et al.*, 1975). Carbon sources were added at a final concentration of 0.2%, except for glycerol (0.3%). Unless stated otherwise, the final concentrations of supplements ($\mu\text{g ml}^{-1}$) were: uracil, 25; thiamine, 2; individual amino acids, 50; carbamoylaspartate (CAA), 100; dihydroorotate (DHO), 100; orotate (OA), 100; 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (XGal), 50. As required, antibiotics were added at the following final concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 100; chloramphenicol, 15; gentamicin, 20; kanamycin, 120 (minimal medium) or 60 (LB); tetracycline, 20. Solid media were prepared by the addition of 15 g agar l⁻¹. Cultures were grown at 37 °C and liquid cultures were incubated on an air shaker operating at 250 r.p.m. Growth of liquid cultures was monitored by measuring cell turbidity with a Klett–Summerson colorimeter (filter no. 54).

Genetic techniques. Bacteriophage P22HT105/1int-201 (Hughes & Roth, 1984) was used for all transductions with *S. enterica* serovar Typhimurium. EGTA (10 mM) was added to the plating medium to limit lysogeny of transductants. Methods for the creation of MudJ transcriptional fusions and Tn10dTc transposon technology were as reported previously (Gillen & Hughes, 1993; Kleckner *et al.*, 1977). For the isolation of loss-of-function mutants following transposon mutagenesis (e.g. MudJ insertion to create a Dho⁻ derivative from a Dho⁺ strain), the transductants were pooled and grown to stationary phase in a medium containing the required supplement. A sample of washed cells was inoculated into medium without the supplement and used to enrich for the desired isolate by penicillin counter-selection (Miller, 1992). Isolates were screened for the corresponding phenotype and appropriate candidates were then used for genetic

Table 1. Bacterial strains

| Strain | Genotype | Source (reference) |
|---|--|----------------------------------|
| <i>S. enterica</i> serovar Typhimurium LT2 | | |
| KR1312 | <i>pyrB137</i> | LC* |
| KR1485 | <i>pyrC691::Tn10</i> | LC |
| KR1486 | <i>pyrD2266::Tn10</i> | LC |
| KR1562† | <i>metA22 metE55 galE496 rpsL120 xyl-404 (ilv) hsdL6 hsdSA29</i> | LC |
| KR1639 | LT2 wild-type | LC |
| KR1647 | <i>pyrB137 usp-2‡</i> | LC |
| KR1650 | <i>pyrB137 usp-2 dctA1::MudJ</i> | LC |
| KR1654 | <i>pyrB137 dctA1::MudJ</i> | LC |
| KR1667 | <i>pyrB137 usp-2 yhi-23§ dctA1::MudJ</i> | This study |
| KR1692 | <i>pyrB137 yhi-23</i> | This study |
| KRM104 | <i>pyrB137 yhi-24::MudJ</i> | This study |
| KRM106 | <i>pyrB137 pyrC691::Tn10 usp-2 yhi-23 dctA1::MudJ</i> | This study |
| KRM108 | <i>pyrB137 pyrD2266::Tn10 usp-2 yhi-23 dctA1::MudJ</i> | This study |
| KRM115 | <i>pyrB137 yhi-25::Tn10dTc</i> | This study |
| KRM123 | <i>pyrB137 usp-3‡§</i> | This study |
| KRNJK10 | <i>pyrB137 yhi-23 yhiV1::Km</i> | This study |
| KRNJK11 | <i>pyrB137 usp-2 yhiV1::Km</i> | This study |
| KRNJK12 | <i>pyrB137 usp-3 yhiV1::Km</i> | This study |
| KRNJK14 | <i>pyrB137 yhiV1::Km</i> | This study |
| <i>E. coli</i> K12 | | |
| MC1061† | <i>araD139 Δ(araABIOC leu)7697 ΔlacX74 galU galK rpsL hsdR</i> | LC |
| KUR1182 | <i>pyrB lacU169 thi-1</i> | Michaels <i>et al.</i> (1987) |

*LC, Laboratory collection.

†Strain KR1562 was used as an intermediate recipient for plasmid DNA isolated from *E. coli* MC1061, which was used as a general recipient in a range of cloning procedures.

‡§The mutant alleles *usp-2* and *usp-3* were identified in independent investigations by Syvanen & Roth (1973) and Kelln & Zak (1980), respectively. A *usp* mutation imparts the capacity to use exogenous CAA as a pyrimidine source, i.e. the cell is Caa⁺. Strains containing the *yhi-23* allele can also use exogenous DHO (or OA) as a pyrimidine source and are thus Dho⁺. See Methods for further discussion regarding genetic nomenclature.

analyses. Rapid transductional mapping (Benson & Goldman, 1992) was carried out using Kit-22 from the Salmonella Genetic Stock Centre. Genetic recombination of gene fusions and insertional mutations into the *S. enterica* serovar Typhimurium chromosome using suicide vector pJQ200 were carried out as described previously (Quandt & Hynes, 1993).

Mutagenesis. Chemical mutagenesis involved treating the culture at a density of $1-2 \times 10^8$ cells ml⁻¹ with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at 0.1 mg ml⁻¹ (Miller, 1992). Following mutagenesis, cells were cultured for 24 h in appropriately supplemented minimal medium prior to plating on a selective medium.

Disk diffusion assays. KR1667 (*pyrB137 usp-2 yhi-23 dctA1::MudJ*) was grown with either CAA or DHO in liquid minimal medium to

mid-exponential phase and plated onto the same solid medium. A sterile filter paper disk soaked with a test compound was placed on the plate, which was then incubated for 24 h. The presence of a zone of growth inhibition indicated competition for uptake of either CAA or DHO by YhiT.

Uptake and incorporation assays. [5-³H]Orotic acid, 250 µCi (9.25 MBq; specific activity 370–740 GBq mol⁻¹), was purchased from Moravak Biochemicals. Radioactive OA-uptake and incorporation assays were carried out essentially as described previously (Yurgel *et al.*, 2000). KR1667 and KRM106 were grown in medium A with glycerol and OA (25 µg ml⁻¹) to 60 Klett units (OD₆₆₀ 0.24); radioactive OA (3.7 × 10⁴ Bq ml⁻¹) was then added. Samples were taken immediately, then at 15 and 30 min, and subsequently at 30 min intervals for a total of 180 min. The cells were collected by vacuum filtration (0.45 µm pore size; mixed cellulose esters), washed twice with 1 ml medium A salts containing non-radioactive OA (100 µg ml⁻¹), and dried, and the radioactivity was determined by liquid scintillation counting. The effect of CAA on the uptake of radiolabelled OA by KRM106 (and on the corresponding cell growth culture) was examined by adding CAA (200 µg ml⁻¹) after 45 min, followed by sampling of the cultures for 120 min.

DNA techniques. The methods used were primarily adapted from the manual of Sambrook *et al.* (1989). Transfer of plasmids between *E. coli* and *S. enterica* serovar Typhimurium using strain KR1562 as an intermediate in transformations was performed as described previously (Baker *et al.*, 1996). Sequencing was carried out as a service by either the University Core DNA services (University of Calgary, Calgary, AB, Canada) or the Plant Biotechnology Institute, National Research Council, (Saskatoon, SK, Canada). DNA sequence data were compiled and analysed using the Chromas (Version 1.45), DNA Strider (Version 1.3) and Align software packages, the GenBank network BLAST server, and a modified version of the original Targsearch promoter analysis software (Mulligan *et al.*, 1984). Further analysis was carried out using the GenBank ΨBLAST protein server and TopPred II (Version 1.3) (Claros & von Heijne, 1994) protein prediction programs.

RNA isolation and RT-PCR reactions. RNA was isolated as previously described (Mackie, 1989) from exponential-phase cells (KR1312) grown in LB. Samples were treated with DNase I (Invitrogen) at 37 °C for 45 min to remove contaminating DNA prior to reverse transcription reactions. The cDNA encompassing *yhiVUTS* was generated using an oligonucleotide, RAKter1, (5'-GGGTGGGC-GCACAAGCCTGC-3') complementary to nucleotides just downstream of the *yhiS* termination codon. Specifically, RNA (3 µg) was incubated with 2 pmol RAKter1 and 1 mM dNTPs at 65 °C for 5 min. Reverse transcription reactions were assembled according to the manufacturer's instructions and cDNA synthesis was carried out using Superscript II reverse transcriptase (Invitrogen) at 42 °C for 60 min, followed by incubation at 70 °C for 15 min. A 300 ng aliquot of the RT reaction was used in the amplification of various DNA fragments using an Expand High Fidelity PCR System (Roche). Reaction mixtures contained 300 µM dNTPs, 15 pmol of each gene-specific DNA oligonucleotide (see below), 1 × PCR buffer, and 2.6 units of DNA polymerase in a 50 µl reaction volume. Reactions were incubated at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 s, 56 °C for 30 s, and 68 °C for 5 min. Following cycling, reactions were incubated for 7 min at 72 °C and then stored at 4 °C. Reaction aliquots were analysed by agarose gel electrophoresis. Gene-specific oligonucleotide primers for PCR were the common 5' primer, RAKmocD4 (5'-CCTCGCCTGTATCATTGATGGAACC-3'), coupled with one of the 3' primers, RAKmocD2 (5'-GGCGTATTACCTTTG-TGGGAGGCG-3'), RAKmocD3 (5'-CGAGTTCGACCGCACTCAG-CAGGCC-3'), RAKdhp (5'-CGCCTAACCCGCCGACCATCCC-3') or RAKter1 (see above).

5' Random amplification of cDNA ends (RACE). The 5'-RACE kit (Invitrogen, Version 2.0) was used to define the transcriptional start site of *yhiVUTS*. For cDNA synthesis, an oligonucleotide complementary to the 5' end of *yhiV*, RAKmocD1 (5'-CAGACCGACAGT-CAACGCTTTTCGC-3'), was used. The resulting amplified DNA product of 265 bp was cloned in pPCR-Script AMP SK(+) (Stratagene) then sequenced.

In vitro synthesis and cloning of *yhiT* wild-type and mutant (*yhiT23*) alleles. Wild-type and mutant *yhiT* alleles were amplified from plasmids pRSB4 and pRSB3 respectively (see Results) using oligonucleotides 5'-CGGAATTCCTACTCCAGATAACCCAGG-3' and 5'-CCGGATCCTAAGTGGCGTGTCTATAAAAAAC-3', with Phusion High-Fidelity DNA Polymerase from Finnzymes (New England Biolabs) in accordance with the manufacturer's instructions. The 1.3 kb DNA fragments were cloned into the *NruI* site of plasmid pBR322 to generate pCLS41 (harbouring the *yhiT23* mutant allele) and pCLS42 (harbouring the wild-type *yhiT* allele). Both plasmids contained the insert in the same direction with the distal end of the gene proximal to the *tet* promoter of the vector.

RESULTS

Isolation of mutants capable of utilizing pyrimidine intermediates

Two earlier studies described the isolation of *S. enterica* serovar Typhimurium mutants (*usp* mutants) able to utilize CAA as an exogenous pyrimidine source (Kelln & Zak, 1980; Syvanen & Roth, 1973), but characterization of these mutants was not undertaken. In this study, the original *usp-2* and *usp-3* mutations were transduced into a pyrimidine auxotroph, KR1312 (Table 1), by selecting for growth on CAA as sole pyrimidine source. Mutants capable of using dihydroorotate (DHO) as sole pyrimidine source were isolated by treatment of KR1650 (*usp-2 pyrB137 dctA1::MudJ*) with MNNG, followed by selection on minimal medium supplemented with DHO. One Dho⁺ isolate, KR1667, was chosen for further analysis. Based on the results of gene mapping studies (see below), the mutation was designated *yhi-23*.

DHO can spontaneously hydrolyse to produce CAA (R. A. Kelln & J. Neuhard, unpublished results), and therefore the utilization of DHO by KR1667, which harbours the *usp-2* allele, may have been a consequence of CAA utilization arising from the hydrolysis of DHO. To test this possibility, *pyrC691::Tn10* was transduced into KR1667, yielding KRM106, a strain incapable of metabolizing CAA as a pyrimidine source. KRM106, KR1667 and several other strains were re-evaluated for growth on pyrimidine intermediates. KRM106 grew with exogenous DHO (Table 2), confirming that the growth on DHO pertained to the novel gain-of-function mutation, *yhi-23*, and was not simply a consequence of uptake and utilization of CAA arising from hydrolysis of DHO. Notably, both KRM106 and KR1667 grew with exogenous OA (Oa⁺) despite harbouring a disruption in *dctA*. Considering that a functional *dctA* is normally required for utilization of OA (Baker *et al.*, 1996), the growth of these strains on OA indicated an additional capability imparted by the *yhi-23* mutation. The individual

Table 2. Phenotypic characterization of pyrimidine uptake mutants

| Strain (relevant genotype)* | Supplement to growth medium†‡ | | |
|--|-------------------------------|----|-----|
| | CAA | OA | DHO |
| KR1312 | – | + | – |
| KR1647 (<i>usp-2</i>) | + | + | – |
| KRM123 (<i>usp-3</i>) | + | + | – |
| KR1650 (<i>usp-2 dctA1::MudJ</i>) | + | – | – |
| KR1667 (<i>usp-2 yhi-23 dctA1::MudJ</i>) | + | + | + |
| KRM106 (<i>usp-2 yhi-23 dctA1::MudJ pyrC691::Tn10</i>) | – | + | + |

*All strains contained the *pyrB137* deletion mutation imparting pyrimidine auxotrophy.

†The minimal medium contained 0.3 % glycerol as the carbon source. The pyrimidine intermediates were added at 100 µg ml⁻¹.

‡+, Growth after 36 h; –, no growth after 36 h.

usp-2 (Table 2) and *usp-3* (data not shown) mutations, in contrast, did not afford the additional capabilities to use exogenous DHO or OA. Collectively, the results confirmed that the Dho⁺ (and Oa⁺) phenotype was a consequence of the *yhi-23* mutation.

Genetic analysis of *yhi-23*

The *yhi-23* mutation was transduced into KR1312 (*pyrB137*) to yield KR1692, a pyrimidine-requiring strain with no additional mutant alleles associated with the utilization of pyrimidine intermediates. KR1692 was subsequently used to generate KRM115, a strain harbouring a mini-tet (Tc) insertion that resulted in a Dho⁻ phenotype. KRM115 was then used in rapid transductional mapping (Benson & Goldman, 1992), whereby the location of the insertion was determined to be near 78 centisomes on the *S. enterica* serovar Typhimurium chromosome.

To confirm that the Tc-resistance marker of KRM115 was integral to *yhi-23*, the parental Dho⁺ strain, KR1667, was used as the recipient for transductional analysis. Importantly, the resulting transductants were not only Dho⁻, but also Caa⁻. Moreover, transducing the Tc marker into the *usp-2* (KR1647) or *usp-3* (KRM123) strain also resulted in a Caa⁻ phenotype. A number of explanations were consistent with the results, with the simplest interpretations being that the *yhi-23* and *usp* mutations were very closely linked, were in themselves allelic, or that the Tc-resistance element imparted a polarity effect on the expression of both the *usp-2* and *usp-3* alleles.

Cloning and sequencing of DNA harbouring the *yhi-23* mutation

To facilitate the cloning of the DNA region corresponding to *yhi-23* (as well as providing a facile system for gene

expression studies), the MudJ-insertion mutant KRM104 was constructed (see Methods). As observed for the mini-tet insertion, introduction of the MudJ element in *yhi-23* not only resulted in a Dho⁻ phenotype, but simultaneously led to a Caa⁻ phenotype as well. DNA both 5' and 3' to the insertion site was cloned, based on knowledge of restriction sites within the MudJ element (Castilho *et al.*, 1984). More than 4400 bp of genomic DNA sequence encompassing a region corresponding to *yhiVUTS* and flanking DNA was determined, and the analysis established that MudJ was inserted into *yhiT*, the third gene of a putative four-gene operon. (Fig. 1b). Sequence differences between the *yhi-23* mutant DNA and the published wild-type sequence (McClelland *et al.*, 2001) pertained to only two residues, and both were located within *yhiT* (see below). Additionally, these studies confirmed that the cloned DNA was from the 78.5 centisomes region of the *S. enterica* serovar Typhimurium chromosome, as had been approximated by transductional mapping.

Cloning and characterization of wild-type and mutant *yhiT* alleles

A plasmid, pNJK203, containing the Km-resistance cassette from pUC4K inserted into *yhiV* was constructed. The *yhiV1::Km^R* insertion was subcloned into the suicide vector pJQ200 and this construct was used to recombine the insertion in wild-type *S. enterica* serovar Typhimurium, or strains harbouring the various *yhi* mutant alleles. The Km-resistant strains KRNJK14 (*yhiV1::Km^R*), KRNJK11 (*usp-2 yhiV1::Km^R*), KRNJK12 (*usp-3 yhiV1::Km^R*) and

KRNJK10 (*yhi-23 yhiV1::Km^R*) were obtained by this approach (Table 1). A restriction digestion/cloning strategy based on known sequence data enabled the joint cloning of the Km^R marker and DNA encompassing *yhiU* and *yhiT* into pWSK29, yielding plasmids pRSB1 through pRSB4 (Fig. 2a), corresponding to cloned DNA from *usp-2*, *usp-3*, *yhi-23* and wild-type cells, respectively. Transformation of the plasmids containing the individual mutant alleles into KR1312 (*pyrB137*) conferred the ability to utilize CAA (pRSB1, 2 and 3) or CAA and DHO (pRSB3) as sole pyrimidine source, whereas the wild-type *yhiT⁺* plasmid (pRSB4) did not. It was noteworthy that the Km insertion strains (i.e. KNR strains) lost the ability to utilize exogenous pyrimidine intermediates, consistent with a polarity effect due to the insertion of the Km-resistance cassette. Since the plasmids were capable of mediating utilization, their multicopy nature apparently sufficed to overcome the polarity effect of the insertion element in single copy.

Sequence analysis determined that the DNA cloned from the *usp-2*, *usp-3* and *yhi-23* mutants all harboured missense mutations in *yhiT*, confirming that the mutations conferring Caa⁺, or Dho⁺ (and Oa⁺), are allelic, and that they reside within *yhiT*. The *usp-2* allele contained a single mutation resulting in a T89P change, while the *yhiT23* allele harboured the same mutation as well as a mutation conferring a Y383C change. The presence of the T89P alteration in *yhiT23* is consistent with the fact the strain used to select the Dho⁺ mutant contained the *usp-2* mutation. The *usp-3* locus contained two point mutations, leading to G124D and A397T alterations, and thus was

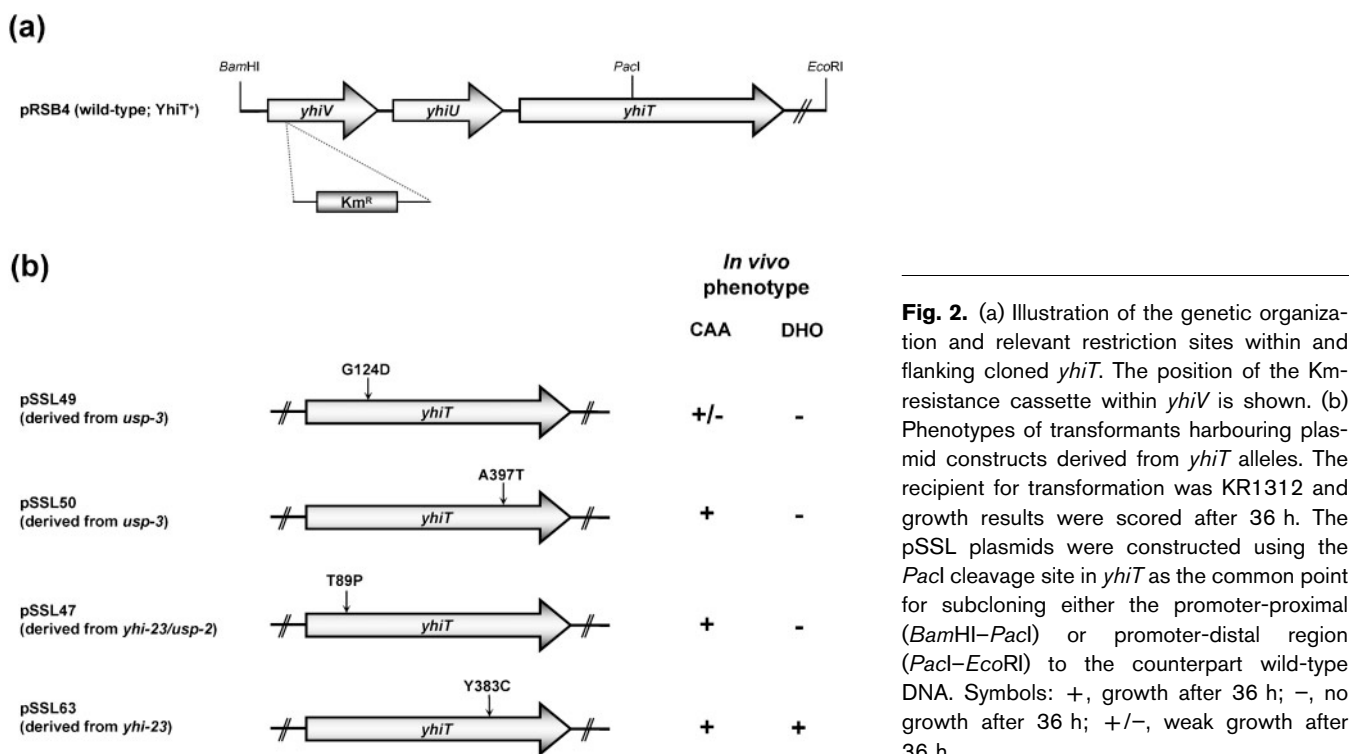


Fig. 2. (a) Illustration of the genetic organization and relevant restriction sites within and flanking cloned *yhiT*. The position of the Km-resistance cassette within *yhiV* is shown. (b) Phenotypes of transformants harbouring plasmid constructs derived from *yhiT* alleles. The recipient for transformation was KR1312 and growth results were scored after 36 h. The pSSL plasmids were constructed using the *PaclI* cleavage site in *yhiT* as the common point for subcloning either the promoter-proximal (*BamHI*–*PaclI*) or promoter-distal region (*PaclI*–*EcoRI*) to the counterpart wild-type DNA. Symbols: +, growth after 36 h; –, no growth after 36 h; +/-, weak growth after 36 h.

distinct from that identified for the *usp-2* (and *yhi-23*) mutation, indicating that the alteration of various amino acid residues within YhiT can result in an ability to utilize CAA.

To determine whether the T89P and Y383 alterations encoded by the *yhiT23* allele exhibited a cooperative effect on DHO utilization, the mutations were separated by subcloning either the proximal or distal DNA region of the *yhi-23* allele with the counterpart region of wild-type DNA (Fig. 2b). As anticipated, the T89P amino acid substitution (pSSL47) rendered the cell Caa⁺, but not Dho⁺. Notably, the Y383C alteration (pSSL63) alone conferred the Dho⁺ phenotype as well as imparting a Caa⁺ phenotype. When the two mutations within the *usp-3* allele were separately analysed, either point mutation, G124D or A397T, conferred a Caa⁺ phenotype (pSSL49 and 50).

Mutant YhiT mediates utilization of pyrimidine intermediates

Inspection of the *E. coli* genome failed to identify any homologous elements to *yhiVUT*, and thus *E. coli* was an ideal test system to determine if the phenotype could be mediated by only the introduction of the cloned DNA. Accordingly, pRSB3 containing the cloned mutant *yhiVUT* region was transformed to KUR1182 (a *pyrB* deletion strain; Table 1) and found to impart a Caa⁺ phenotype. This result was extended by examining the growth of KUR1182 with CAA after introducing a plasmid containing only *yhiT*, either the mutant *yhiT23* allele (pCLS41) or wild-type (pCLS42). The pCLS41 construct imparted a Caa⁺ and Dho⁺ phenotype, whereas the pCLS42 transformant failed to grow with CAA or DHO as a pyrimidine source, demonstrating that the mutant allele alone confers the ability for the cells to grow with exogenous pyrimidine intermediates as a pyrimidine source.

The operon nature of *yhiVUTS*

It was noted that following insertion of the *yhiV1::Km^R* marker to create strains KRNJK10–14, the strains were no longer able to utilize the respective pyrimidine intermediate(s). The foregoing was consistent with a polarity effect on gene expression imposed by the insertion element and where the promoter expressing *yhiT* would be located upstream of *yhiV*. Moreover, while a plasmid harbouring the *yhi-24::MudJ* fusion from KRM104 plus a region of 130 nucleotides directly upstream of *yhiV* maintained expression of the *lacZ* gene within MudJ, deletion of the region upstream of *yhiV* led to the loss of *lacZ* expression, indicative of a functional promoter element having been removed. Further support for the existence of a promoter upstream of *yhiV* was gained by inspection of the sequence, which led to the identification of a putative promoter having a -35 sequence (ATGTCT) and a -10 sequence (AATAAT) separated by 19 nucleotides (Fig. 3a). In contrast, inspection and analysis of the intergenic regions of

yhiVUTS failed to reveal sequence elements characteristic of a promoter element. The presence of a promoter element was confirmed using 5'-RACE, which identified the transcriptional start site to be an adenosine residue, 6 nucleotides downstream of the deduced -10 region and 55 residues upstream of the AUG initiation codon of *yhiV* (Fig. 3a). Together, these findings supported the notion that *yhiVUTS* is an operon, expressed as a single mRNA from a promoter located upstream of *yhiV*. Consistent with this interpretation was the observation that deletion of DNA upstream of the promoter-proximal *Bam*HI site resulted in a twofold reduction in expression from a plasmid-borne *yhiT-lacZ* transcriptional fusion (data not shown).

The prospective operon structure of *yhiVUTS* was directly examined using RT-PCR to determine whether the mRNA products of the four genes could be detected within a single transcript. The cDNA was synthesized from whole-cell RNA isolated from KR1312 using an oligonucleotide complementary to nucleotides directly 3' of the *yhiS* mRNA termination codon (see Methods, Fig. 3b). The cDNA was used as the template for the amplification of various DNA fragments by PCR. Although a PCR product of 4.4 kbp encompassing the entire region defined by *yhiVUTS* was not observed, amplification of 290 bp, 470 bp and 2 kbp DNA fragments was achieved using oligonucleotides complementary to a region directly 5' proximal of the initiation codon of *yhiV* and various regions within the coding region of *yhiV* or *yhiT* (Fig. 3b, lanes 1–3). Importantly, amplification products were not observed when reverse transcriptase was omitted from the cDNA synthesis reaction, or when RNA samples were treated with RNase A prior to cDNA synthesis (data not shown). Notably, the 290 bp, 470 bp and 2 kbp products were generated only in the presence of cDNA that was primed downstream of *yhiS*. Collectively, the data strongly support the interpretation that the *yhiVUTS* genes are organized as an operon and expression occurs from a promoter region located immediately upstream of *yhiV*.

Probing the regulation of expression of *yhiVUTS*

The regulation of *yhiVUTS* expression was analysed by monitoring the expression of *lacZ* from the *yhiT::MudJ* transcriptional fusion in KRM104 following the introduction of mutations in a series of common regulatory loci. The global regulatory genes *cya*, *cra*, *crp*, *oxrA* and *arca* (Tuchi & Lin, 1988; Kolb *et al.*, 1993; Spiro & Guest, 1990), each with an inactivating Tn10 Δ Tc insertion, were introduced into KRM104 by transduction. Expression of *lacZ* in the parental strain, KRM104, was consistently twofold lower when cells were grown in glucose than for glycerol-grown cells (as shown in Table 3), indicating that *yhiVUTS* is a target of catabolite repression. Individual inactivation of the three carbon source regulatory loci *cya*, *crp* and *cra* resulted in a modest lowering of the basal level of *lacZ* expression under either growth condition (data not

latter serving as the control. As shown in Fig. 4(a), incorporation of labelled OA by KR1667 increased with the length of exposure to the compound. In contrast, uptake and incorporation of the radiolabelled substrate did not occur with KR1654. Since OA uptake by the DctA transport system is precluded in both strains, the growth and incorporation of radiolabelled OA in KR1667 resulted from the capability imparted by the *yhiT23* mutation.

Several lines of evidence indicated that the mutant YhiT was able to mediate the uptake of CAA and DHO as well as OA, with the corollary being that CAA (or DHO) should compete for uptake. To test this prediction, exponential-phase cells of KRM106 (a *pyrC* derivative unable to metabolize CAA) were cultured in the presence of radiolabelled OA for 45 min prior to the addition of unlabelled CAA to the medium. The incorporation of OA increased linearly over time in the absence of CAA, whereas the addition of CAA resulted in a rapid and dramatic decrease in OA incorporation (Fig. 4b). The inhibition of

OA uptake and incorporation by CAA demonstrated that transport of these two pyrimidine intermediates was occurring through a shared mechanism in which the mutant YhiT is an integral component.

The uptake associated with the *yhiT23* mutation was further analysed by determining the doubling times for cells growing in the presence of the individual pyrimidine intermediates or combinations thereof. When KRM106 (DctA⁻ and unable to metabolize endogenous CAA) was grown with DHO or OA as the sole pyrimidine source, adding CAA to the growth medium resulted in a dramatic increase in doubling time (Table 4). Similarly, for a strain incapable of metabolizing CAA and DHO (KRM108; Table 1), the addition of either compound to a culture growing with exogenous OA caused a significant increase in the doubling time (Table 4).

Additional characterization of the mutant YhiT system

The affinity of the mutant YhiT system for additional substrates was assessed using a competition disk diffusion assay (see Methods). The compounds analysed included numerous individual amino acids and various dicarboxylates and tricarboxylates. Significant inhibition of growth of KR1667 was observed only in the presence of succinate or malate, while a more modest growth inhibition occurred with aspartate and asparagine. Since the *dctA* mutation in KR1667 precluded transport of succinate or malate by the Dct system, the growth inhibition observed for succinate and malate motivated testing KR1667 for the ability to use succinate and other C₄-dicarboxylates as sole carbon source. KR1667 failed to grow with either succinate or malate as carbon sources, and although these compounds may compete for uptake of OA and DHO by YhiT, if transport is occurring, it is insufficient to meet the metabolic demands of the cell for a carbon source.

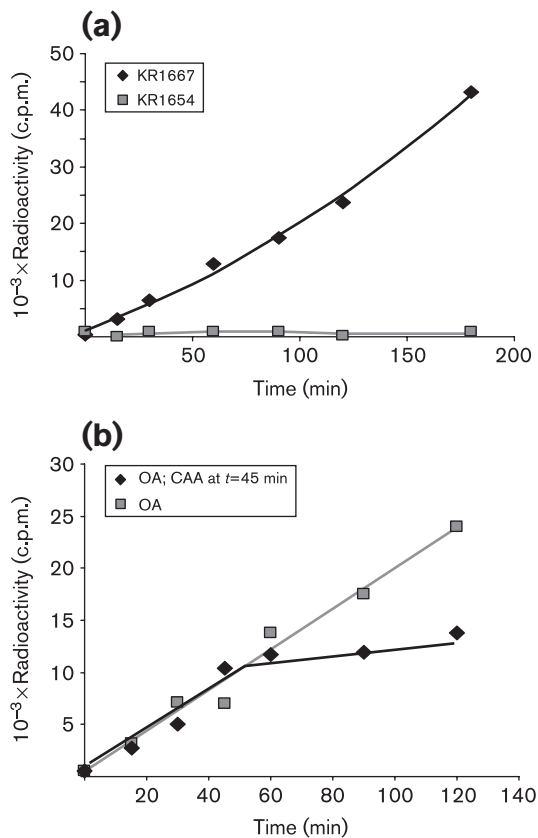


Fig. 4. (a) Uptake of radiolabelled OA by the *yhi-23* mutant, KR1667. Uptake studies were carried out as described in Methods using exponential-phase cells. KR1654 served as the control strain. (b) Effect of CAA on the uptake of OA by KRM106. OA was present at $25 \mu\text{g ml}^{-1}$; after 45 min, CAA was added to $200 \mu\text{g ml}^{-1}$ to one of the two flasks.

Table 4. Doubling times in the presence of different pyrimidine intermediates

| Strain (relevant genotype) | Pyrimidine supplement* | Doubling time (min) |
|------------------------------------|------------------------|---------------------|
| KRM106 (<i>yhiT23 pyrC dctA</i>) | OA | 72 |
| | OA + CAA | 400 |
| | DHO | 68 |
| | DHO + CAA | 400 |
| KRM108 (<i>yhiT23 pyrD dctA</i>) | OA | 60 |
| | OA + CAA | 420 |
| | OA + DHO | 420 |

*Minimal medium contained 0.2% glucose as the carbon source. The pyrimidine intermediates were added at a final concentration of $100 \mu\text{g ml}^{-1}$.

DISCUSSION

In an earlier study we had determined that utilization of OA by wild-type *S. enterica* serovar Typhimurium was dependent on a functional *dctA* gene, encoding the transport protein for C₄-dicarboxylates (Baker *et al.*, 1996). Herein, we have extended the previous work to include an analysis of the utilization of additional pyrimidine nucleotide intermediates, namely CAA and DHO, as exogenous pyrimidine sources. Mutations conferring the Dho⁺ and Caa⁺ phenotypes were found to be closely linked, and cloning and sequencing of the chromosomal region conferring these phenotypes revealed the uncharacterized *yhiVUTS* locus to be the relevant genetic element. Notably, a corresponding region is not found within the genome of the closely related bacterium, *E. coli*, and thus the results are specific to *S. enterica* serovar Typhimurium.

Five ORFs encoding high molecular mass polypeptides are predicted to exist within the analysed genetic region, with one of the five (*yhiW*; STM3602) being well separated from the other four (*yhiVUTS*; Fig. 1b). The first ORF (*yhiV*; STM3601) of the putative four-gene operon is deduced to encode a 36 kDa hydrophobic polypeptide with a conserved phosphosugar isomerase domain commonly observed in glucose phosphate isomerases. *YhiV* shows similarity to gene products involved in the catabolism of sugar–amino acid conjugates, specifically, the conversion of fructosamine 6-phosphates to glucose 6-phosphate and free amino acids in *Bacillus subtilis* (YurP; 58%) (Wiame *et al.*, 2004) and the conversion of mannopine to glutamine and mannose in *Agrobacterium tumefaciens* (MocD; 46%) (Kim & Farrand, 1996). ORF2 (*yhiU*, STM3600) is predicted to encode a 31 kDa hydrophobic polypeptide, which displays 48% similarity to the putative kinase MocE from *A. tumefaciens* (Kim & Farrand, 1996) and 54% similarity to the kinase YurL of *B. subtilis* (Fujita *et al.*, 1986; Wiame *et al.*, 2004).

The DNA coding for ORF3 (*yhiT*, STM3599) harboured the MudJ insertion and, notably, exhibits no significant nucleotide similarity with any sequence currently in the genome database. However, the deduced 47 kDa polypeptide contains 12 transmembrane domains typical of bacterial transport proteins and has similarity to various anaerobic C₄-dicarboxylate transporters (Ullmann *et al.*, 2000), including those from *Bacillus* spp. (~55%), *Erwinia carotovora* (50%) (Bell *et al.*, 2004), and members of the Campylobacterales (~50%). In particular, *YhiT* possesses conserved domains characteristic of the transporters DcuA and DcuB and shows similarity to DcuB of *Bacteroides* spp. (54%) (Kuwahara *et al.*, 2004; Xu *et al.*, 2003), *Mannheimia succiniciproducens* (53%) (Hong *et al.*, 2004) and a variety of Campylobacterales (~46%) (Fouts *et al.*, 2005; Suerbaum *et al.*, 2003), and to DcuA of *Phototrhahdus luminescens* (54%) (Duchaud *et al.*, 2003) and members of the Campylobacterales (~45%) (Fouts *et al.*, 2005; Suerbaum *et al.*, 2003; Tomb *et al.*, 1997). Significantly, *yhiT* harboured the only nucleotide changes observed

between the cloned DNA and published sequences, providing strong evidence that the mutant *YhiT* polypeptides manifest the pyrimidine utilization phenotype.

The final ORF (*yhiS*, STM3598) is predicted to encode a 37 kDa polypeptide with similarity to various prokaryotic asparaginases (Bonthon, 1990). The predicted product exhibits a conserved asparaginase domain with 75% similarity to the L-asparaginase of *Erwinia chrysanthemi* (Minton *et al.*, 1986). Significant similarity is found between *YhiS* and L-asparaginases (I and II) from a variety of Campylobacterales (~60%) and also to the glutaminase-asparaginase enzymes of *Pseudomonas* and *Acinetobacter* spp. (~55%). Notably, *YhiS* displays 58% similarity to AsnB, an L-asparaginase II from *M. succiniciproducens* (Hong *et al.*, 2004) and 41% similarity to the L-asparaginase of *A. tumefaciens* (Wood *et al.*, 2001).

Several lines of evidence support the conclusion that the *yhiVUTS* region of the *S. enterica* serovar Typhimurium chromosome constitutes an operon. First, inspection of the DNA within this region identified a single promoter element upstream of *yhiV*. Second, 5'-RACE analysis confirmed a transcriptional start site at the deduced promoter element (Fig. 3a). Third, a polarity effect on *yhiT* expression as a consequence of insertional inactivation of *yhiV* was observed. Fourth, and most importantly, RT-PCR demonstrated that the *yhiVUTS* genes can be transcribed as a single mRNA (Fig. 3b).

We inferred that the point mutations identified within *yhiT* were solely responsible for the pyrimidine utilization phenotypes since no additional mutations were found in the DNA cloned from the mutant strains. This was confirmed by demonstrating that cloned DNA harbouring the mutant alleles of *yhiT* (pRSB plasmids) imparts the respective Dho⁺ or Caa⁺ phenotype to transformed cells. Furthermore, a plasmid harbouring only the *yhiT23* allele rendered an *E. coli pyrB* mutant capable of growing with CAA or DHO.

The studies on the regulation of *yhiVUTS* expression (Table 3) indicated that catabolite repression was present. Furthermore, a significant increase in expression (approx. fivefold) was observed when cells were grown with citrate as carbon source. Inspection of the DNA region upstream of *yhiV* revealed two weakly conserved cAMP-CRP protein-binding sites, one centred at -70 (AAAtcGgGATCcAGATCgC-ggaT) and one centred at -167 (AAAAGgAaCACTctctcgCg-TTT). A putative Cra protein-binding site located at -189 (AGTGAAAtGATTgA) was also identified, as was another region exhibiting strong conservation as a Fnr protein-binding site centred at -41 (TTGgTTTCCATCtt). However, introducing null mutations into *crp* or *cra* had little or no impact on expression of *yhiVUTS*. Furthermore, no evidence for the involvement of other known regulatory proteins in the control of the operon was observed.

Using the TopPred program (Claros & von Heijne, 1994), T89P and Y383C correspond to amino acid substitutions in

regions of YhiT predicted to be within extracellular loops exposed to the periplasmic space. The effect of the substitutions can be envisioned as altering the substrate-binding affinity of the predicted transport protein for the uptake of additional small molecules found within the periplasm, i.e. pyrimidine intermediates. In contrast, the amino acid substitutions associated with *usp-3* are predicted to be within a region exposed to the cytosol (G124D) and within a transmembrane domain proximal to the cytosol (A397T). Accordingly, these amino acid substitutions would not be expected to alter substrate binding, but, alternatively, may influence substrate release on the cytosolic face, or have more profound effects on the folding and/or function of the YhiT protein.

The results presented here demonstrate that utilization of the three pyrimidine intermediates, CAA, DHO and OA, can arise through gain-of-function mutations associated with a single gene, *yhiT*, resulting in a multifunctional mutant YhiT protein. In addition, the mutant YhiT proteins displayed affinity for succinate, malate and the amino acids aspartate and asparagine. Based on the common structural features of these molecules, we propose that the mutant YhiT protein has affinity for compounds with linear or cyclic four carbon backbones with a carboxyl group present at the C-1 position and a carbonyl group on C-4. One inference from these observations is that wild-type YhiT may function to transport groups of compounds such as C₄-dicarboxylates, assuming that the characterized mutations do not impart a dramatic departure from the function of the native protein. Indeed, the structural similarities between YhiT and DcuA/DcuB are consistent with the hypothesis that wild-type YhiT is involved in the utilization of C₄-dicarboxylates in *S. enterica* serovar Typhimurium, perhaps during anaerobic growth. Investigations aimed at characterizing the native function of YhiT and the regulation of the *yhiVUTS* operon are ongoing.

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