Reversion of the Tyrosine Ochre Strain *Escherichia coli* WU3610 under Starvation Conditions Depends on a New Gene *tas*

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ABSTRACT

When 3×10^8 bacteria of the *Escherichia coli tyrA14*(oc) *leu308*(am) strain WU3610 are plated on glucose salts agar supplemented with leucine only, colonies of slow-growing Tyr^+ suppressor mutants begin to appear after about a week and increase in numbers roughly linearly with time thereafter (stationary phase or starvation-associated mutation). From a library constructed from two of these mutants, a clone was obtained that suppressed the tyrosine requirement of WU3610 when present on a multicopy plasmid. The activity was identified to an open reading frame we call *tas*, the sequence for which has homology with a variety of known genes with aldo-keto reductase activity. The activity of *tas* complements the prephenate dehydrogenase dysfunction of *tyrA14* (the chorismate mutase activity of *tyrA* possibly being still functional). A strain deleted for *tas* showed no spontaneous mutation under starvation conditions. Whereas neither tas^+ nor *tas* bacteria showed any increase in viable or total count when plated under conditions of tyrosine starvation at 3×10^8 cells per plate, at lower density ($\sim 10^7$ per plate) tas^+ but not *tas* bacteria showed considerable residual growth. We suggest that the single copy of *tas* present in WU3610 allows cryptic cell or DNA turnover under conditions of tyrosine starvation and that this is an essential prerequisite for starvation-associated mutation in this system. The target gene for mutation is not *tas*, although an increase in the expression of this gene, for example, resulting from a suppressor mutation affecting supercoiling, could be responsible for the slow-growing Tyr^+ phenotype.

IT is more than 30 years since John Drake demonstrated that mutations could accumulate in phage T4 in the complete absence of DNA replication (Drake 1966). A little earlier Ryan had produced evidence that mutations to prototrophy could arise in bacteria starved of a required amino acid in the absence of both cell division (Ryan 1955) and detectable DNA synthesis (Ryan *et al.* 1961). These observations attracted little attention until the publication by Cairns and his colleagues of data leading them to make the controversial suggestion that the mutations that arose under conditions of extreme selection might be restricted to those that would confer a selective advantage, so-called directed or adaptive mutation (Cairns *et al.* 1988). It soon became clear that spontaneous mutation of stationary-phase bacteria, whether these bacteria were starved of an essential nutrient or given an energy source that they normally could not utilize, was in many respects different from spontaneous mutation in growing cells. In particular, there were more mutants than could be accounted for by the amount of DNA replication that was believed to occur, and the spectrum of mutants that arose was different (for review, see Foster 1993).

Escherichia coli WU3610 carries an ochre mutation (*tyrA14*) and an amber mutation (*leu308*). Both of these auxotrophies show mutation under starvation conditions, but reversion to Tyr^+ has been subject to the most study. In cells growing in the presence of tyrosine and assayed under conventional conditions, the Tyr^+ mutations that appear are either reversions at the ochre site (Li *et al.* 1991) or a variety of suppressor mutations at tRNA loci (Osborn and Person 1967). In cells starved of tyrosine, very few of these are seen. Instead, slow-growing mutants begin to appear on plates after about 6 days incubation (Bridges 1994), their number eventually increasing to several hundred per plate. Because of their extremely slow rate of growth it was not practicable to map the mutations responsible, but it was suggested by a process of elimination that they might be transversions at G:C base pairs.

The number of slow-growing prototrophs increased fairly linearly with time once they started to appear, and this suggested that they might reflect the accumulation of DNA damage within the bacteria, in a manner analogous to the accumulation of mutagenic damage within phage T4 during mild heating (Baltz *et al.* 1976; Bingham *et al.* 1976). This damage occurs at G residues and results in G to T transversions (Kricker and Drake 1990). Experiments with related ochre strains defective in various DNA repair pathways indicated that if DNA damage was involved, it was not excisable by nucleotide excision repair and did not require the SOS error-prone pathway. It would most likely be a lesion that caused little distortion of the DNA and that was capable of direct miscoding during polymerization (Bridges 1993).

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This paper is dedicated to John Drake on the occasion of his retirement from the position of Editor of Genetics and to honor his large contribution to the field of mutagenesis.

Such a lesion could in principle give rise to apparently directed mutation because, by mistranscription, it could confer a temporary prototrophic phenotype on the cell and trigger a round of DNA replication during which the mutation could be fixed permanently (Bridges 1994).

A candidate lesion with such properties is 7,8-dihydro-8-oxoguanine (8-oxoG), which is formed in DNA following reaction with various naturally occurring oxidative species within the cell. It can pair with roughly equal facility with cytosine (C) or adenine (A) and can thus give rise to guanine G to T transversions (Wood *et al.* 1990; Shibutani *et al.* 1991; Moriya and Grollman 1993). Bacteria contain two glycosylases to deal with this lesion: MutM protein, which removes 8-oxoG that is paired with C (Boiteux *et al.* 1987, 1990; Michaels *et al.* 1991), and MutY protein, which removes A that is mispaired with 8-oxoG (Nghiem *et al.* 1988; Au *et al.* 1989). Defects in *mutM* and *mutY* result in a substantial increase in the rate of appearance of the slow-growing mutants in WU3610 under starvation conditions, and overproduction of the gene products lowers the rate (Bridges 1995; Bridges *et al.* 1996). It is thus clear that 8-oxoG (or conceivably some similar oxidation product of guanine) is responsible for much of the observed stationary-phase mutation in this strain. The molecular nature of the mutational change, however, has remained unresolved. Caution about the G to T transversion hypothesis has become necessary because it was found that, in a system in which molecular changes could be determined, the presumptive 8-oxoG lesion gave rise not only to G to T transversions but also to a relatively high number of small deletions under starvation conditions (Bridges and Timms 1997).

In the present study we have cloned Tyr^+ activity from slow-growing mutants of WU3610 and have found it to reside in a new gene *tas*. The gene appears to be essential for mutation under starvation conditions in this strain, although it is not itself the mutational target.

MATERIALS AND METHODS

Strains and plasmids: Bacterial strains and their plasmids are shown in Table 1. P1 transductions were performed as described by Miller (1992).

Culture methods: Bacteria were routinely grown overnight in Oxoid (Basingstoke, UK) Nutrient Broth number 2 with shaking at 37°, centrifuged, and resuspended in phage buffer (Boyle and Symonds 1969). Total counts were performed after appropriate dilution using a Weber (Teddington, UK) 'Thoma' counting chamber, and viable counts were made on L-agar plates. Minimal agar plates were made using the salts solution of Davis and Mingioli (1950) supplemented with 0.4% glucose and solidified with 1.5% Difco (Detroit) Bacto agar. Starvation-associated mutation to tyrosine independence was observed when $\sim 3 \times 10^8$ bacteria in phage buffer were spread onto the surface of a minimal agar plate containing 10 μg/ml leucine but no tyrosine. Plates were incubated at 27° to minimize loss of viability (see Bridges 1994). Plasmids were maintained with ampicillin (100 μg/ml), chloramphenicol (150 μg/ml), or kanamycin (50 μg/ml) as appropriate. For phenotype testing, amino acids, where appropriate, were added at 10 μg/ml.

Molecular methods: Standard DNA isolation and plasmid manipulations were as described in Sambrook *et al.* (1989). Two slow-growing tyrosine independent mutants, isolated from day 8 of a starvation-associated mutation experiment, were purified on minimal leucine plates. Single colonies from each were subsequently grown overnight in L-broth at 37° with shaking. Genomic DNA was extracted and digested with *Pst*I. The resulting fragments were ligated into the *Pst*I site in the high-copy-number vector pGEM-3Zf(-). The ligation mixtures were introduced into electrocompetent WU3610 according to the protocol of Dower *et al.* (1988), using an EasyjecT (Flowgen, Litchfield, UK) apparatus and spread onto minimal leucine plates containing ampicillin. The plates were incubated at 27° for 48 hr. Cloned inserts complementing tyrosine auxotrophy were sequenced in both directions, using a kit from United States Biochemical (Cleveland), by primer walking along the 2.2-kb fragment.

For PCR, 1 ml of an overnight culture was centrifuged and washed with 1 ml of distilled water, and the pellet was resuspended in 50 μl of water and placed at 99.9° for 10 min. The solution was centrifuged and the cleared lysate was transferred to a fresh tube. Five microliters was used as a seed for PCR, including PARR buffer (Cambio Ltd., Cambridge, UK), primers at 100 pmol, and dNTPs at 125 μm. The tubes were cycled 30 times, at 94° initially for 5 min and subsequently for 1 min, at 65° for 1 min and at 70° for 1 min. The PCR product was cleaned using the Promega (Madison, WI) DNA cleanup kit according to the manufacturer's protocol.

Sequencing of the chromosomal gene was accomplished using PCR primers SAMPCR1 (ACAAATAAGGTCAGCATCC GGCTGGCC) and SAMPCR3 (CCGGGTGTTCCAGTGATTA CGTCATG). The SAMPCR3 primer was biotinylated at the 5′ end, allowing direct solid-phase sequencing of the PCR product (Hultman *et al.* 1989).

The *tas* gene was subcloned into the low-copy-number vector pACYC184 on a 2-kb *Sph*I/*Bam*HI fragment to produce pART51, with an origin of replication compatible with pKCMT-W. Plasmid pART52 was derived from pKIMP-UAUC by excision of the *Cla*I fragment containing the monofunctional prephenate dehydrogenase (*tyrA*) and replacement by the *tas* gene on a 2-kb *Sph*I fragment with addition of *Sph*I-*Cla*I linkers to each end. The plasmids were introduced into WU3610 or KA12 by electroporation and plated to minimal agar plates containing appropriate amino acids and antibiotics. Plasmid pART47 was generated by digestion of pART36 with *Bst*11071 and *Eco*RV, deleting 822 bp from the *tas* gene. A unique *Bam*HI site was introduced by insertion of a linker (CGCGGATCCGCG) into the resulting blunt-ended DNA fragment. The *Bam*HI fragment from pUC4K, containing the kanamycin-resistance cassette, was inserted into pART47 to produce pART48. The *tas* deletion fragment was subcloned into pMAK705 on a *Kpn*I/*Sph*I fragment to produce pART50, and the deletion allele (del *tas1*::Km) was transferred into the WU3610 chromosome using the method of Hamilton *et al.* (1989).

To probe for *tas* gene amplification, DNA was isolated from the bacteria using the Promega Genomic DNA purification kit according to the protocol supplied by the manufacturer. Five hundred nanograms of DNA was digested with *Pst*I and run on a 1% agarose gel, and the gel was blotted according to the protocol of Southern (1975). The resulting filter was probed with either a labeled 1200 bp PCR product generated using the primers SAMPCR1 and SAMPCR2 (CGCTGTGATCTGGC AGCGCCGTCAT) or, for the control gene *rpoB*, using a 880-

TABLE 1

Strains and plasmids

	Relevant genotype	Source or reference
Strains		
AB1157[a]	*argE3*(oc), *hisG4*(oc)	T. Kato
KA12[b]	Δ(*pheA-tyrA-aroF*)	Kast *et al.* 1996
N3078	*pheA13*::Tn*10*	R. Lloyd
WP2[c]	*trpE65*(oc)	E. M. Witkin
WU3610	*leu308*(am), *tyrA14*(oc)	E. M. Witkin
CM1338	as WU3610 but *pheA13*::Tn*10*	P1(N3078) X WU3610
CM1355	as WU3610 but Δ*tas1*::Km	This work
Plasmids		
pACYC184	Cm^r, p15A origin or replication	New England Biolabs Inc. (Beverly, MA)
pGEM-3Zf(-)	Ap^r, colE1 origin of replication	Promega UK
pKCMT-W	Ap^r, *aroH*	Kast *et al.* 1996
pKIMP-UAUC	Cm^r, *pheC, tyrA*	Kast *et al.* 1996
pMAK705	Cm^r, pSC101 origin of replication^{ts}	Hamilton *et al.* 1989
pUC4K	Ap^r, Km^r geneblock	GIBCOBRL (Gaithersburg, MD)
pART34	as pGEM-3Zf(-), *tas* orientation I	This work
pART35	as pGEM-3Zf(-), *tas* orientation II	This work
pART36	as pART34 (*Kpn*I-*Pst*I)	This work
pART37	as PART34 (*Ssp*I-*Pst*I)	This work
pART38	as pART34 *Eco*RV-*Pst*I)	This work
pART39	as pART34 (*Pst*I-*Eco*RV)	This work
pART40	as pART34 (*Ssp*1-*Bst*11071)	This work
pART42	as pART34 (*Ssp*I-*Hpa*I)	This work
pART47	as pART36 (Δ*Eco*RV-*Bst*11071 insertion *Bam*HI)	This work
pART48	as pART47, insertion of Km^r geneblock	This work
pART50	as pMAK705, insertion of Δ*tas1*::Km	This work
pART51	as pACYC184, insertion of *tas*	This work
pART52	as pKIMP-UAUC, Δ*tyrA* insertion of *tas*	This work

[a] AB1157 additional markers: *leuB6, rpsL31, rfbD1, mgl-51, kdgK51, ara-14, galK2, lacY1, mtl-1, proA2, thi-1, thr-1, tsx-33, xyl-5, SupE44.*

[b] KA12 additional markers: *thi-1, endA1, hsdR17,* Δ*(argF-lac)U169,* Δ*(srlR-recA)306*::Tn*10, SupE44.*

[c] WP2 additional markers: *lon-11, sulA1.*

bp product generated using primers RB1 (CATACCAGTACC AACCAGCG) and RB6 (GCGAAGAAATCGAAGGTTCCG).

RESULTS

Cloning of a Tyr$^+$ determinant: In an attempt to clone the Tyr$^+$ determinant in the slow-growing mutants that arise under conditions of tyrosine starvation, we made a genomic library from two such mutants and isolated several plasmids containing a 2.2-kb *Pst*I fragment of DNA by virtue of their ability to complement the tyrosine auxotrophy of WU3610. When present on a multicopy replicon, the plasmids restored almost a wild-type growth rate on minimal media lacking tyrosine. Sequencing of the two plasmids pART34 and pART35 bearing the same insert, isolated independently but in opposite orientations, revealed that their sequences were identical. There were two potential open reading frames (ORFs) associated with the cloned fragment, only one of which had associated 5′ regions. The other potential coding region began 3 bp in from one terminus of the insert and, disregarding readthrough from plasmid sequences, was unlikely to form a functional gene in this context.

Based on the sequencing results, we constructed a number of deletion plasmids to show that this particular ORF was responsible for the tyrosine independent phenotype (Figure 1). Only plasmids containing the coding region and ~200 bp 5′ to the putative start codon were able to complement tyrosine auxotrophy in WU3610. We therefore concluded that this ORF was responsible for the observed complementation and that it had its own promoter region in the DNA immediately 5′ to the coding region. We have provisionally called this gene *tas* (tyrosine auxotrophy suppressor).

Direct sequencing of the wild-type *tas* gene from the parental strain WU3610 revealed that there was no identifiable difference in sequence within or immediately 5′ to the *tas* coding region compared with the cloned gene. The tyrosine independent phenotype that we observed was therefore because of the presence of the wild-type *tas* gene and is presumed to result from a gene dosage effect conferred by the multicopy plasmid.

We also sequenced two slow-growing Tyr-indepen-

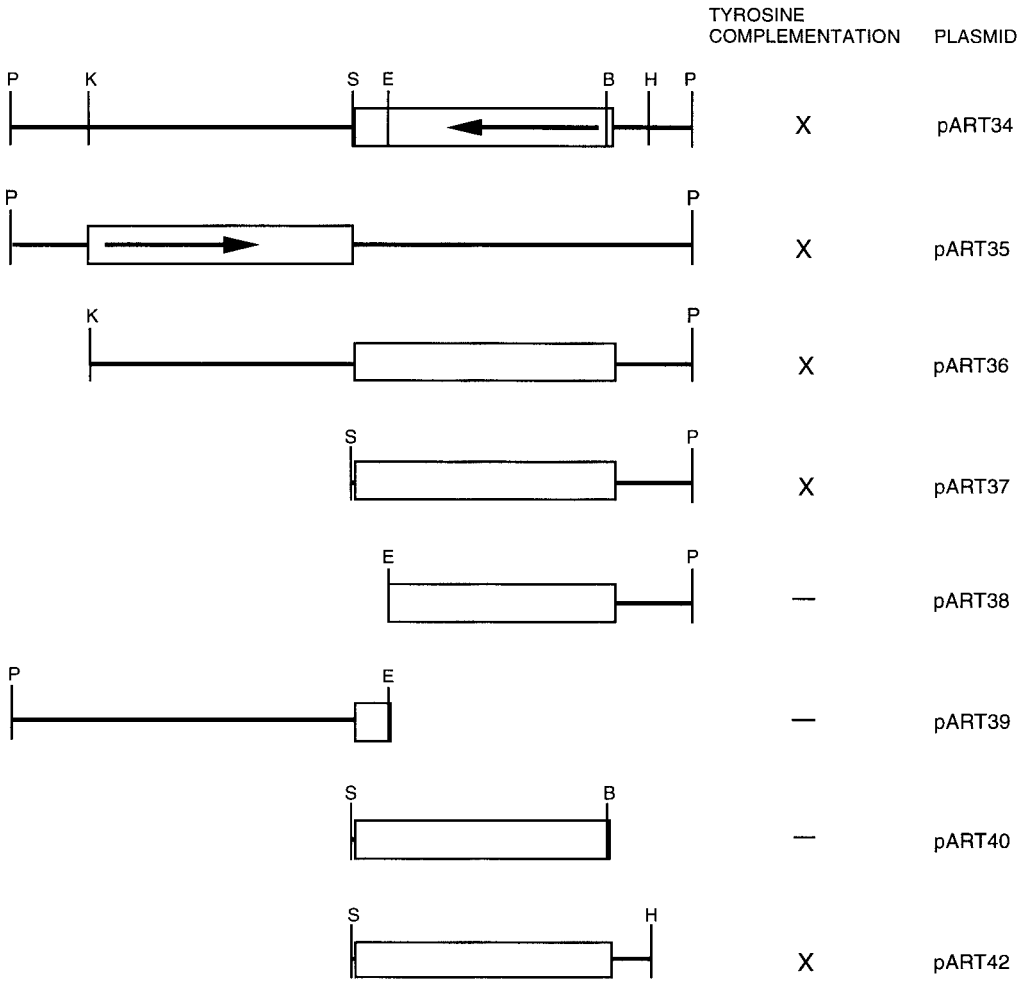

Figure 1.—Construction of plasmids used to identify the ORF suppressing the tyrosine requirement in WU3610. Cloned genomic DNA is indicated by a thick line. The Tas ORF is indicated by an open box with an arrow showing the direction of transcription. Relevant restriction sites are shown: (B) *Bst*11071, (E) *Eco*RV, (H) *Hpa*II, (K) *Kpn*I, (P) *Pst*I, and (S) *Ssp*I. The ability of each plasmid to complement the tyrosine auxotrophy in WU3610 is shown thus: (X) indicates suppression, (−) indicates no suppression.

dent mutants to confirm that the wild-type *tas* gene was present in our mutants, and we found that they also showed no sequence variation compared to wild type.

Recently, the sequence for the entire *E. coli* genome was released; the *tas* fragment was found to be homologous to a sequence contained in the fragment accession number U29581 and was mapped to 64 min at a position of 2978.4 kb. There would appear to be a single base pair difference between our *tas* coding sequence (accession number Y14609) for the region 1929 to 889 bp obtained from a B/r strain and the K12 sequence contained on the database, although it does not result in an amino acid change in the Tas protein.

***tas* complements prephenate dehydrogenase deficiency of *tyrA14*:** The multicopy *tas* plasmid failed to complement a range of other auxotrophies caused by ochre mutations, including *hisG4* and *argE3* in AB1157 and *trpE65* in WP2. The *tas* gene was therefore unlikely to be involved in general ochre suppression. The terminal pathways of tyrosine and phenylalanine biosynthesis are very similar until they branch at chorismate. Each branch proceeds via a bifunctional enzyme encompassing chorismate mutase and prephenate dehydrogenase in TyrA (T-protein) and chorismate mutase and prephenate dehydratase in PheA (P-protein). The terminal pathway of tyrosine biosynthesis is illustrated in Figure 2. Because the multicopy *tas* plasmid failed to complement the phenylalanine auxotrophy in strain N3078 or KA12, it is likely that the gene product is specifically able to complement the tyrosine requirement in *tyrA* strains.

Examination of the Tas protein sequence showed significant homology to several members of the aldo-keto reductase superfamily of enzymes, which catalyze reactions involving carbonyl reduction. The prephenate dehydrogenase step in tyrosine biosynthesis would appear to involve such a reaction. WU3610 contains a fully functional P-protein, the chorismate mutase activity of

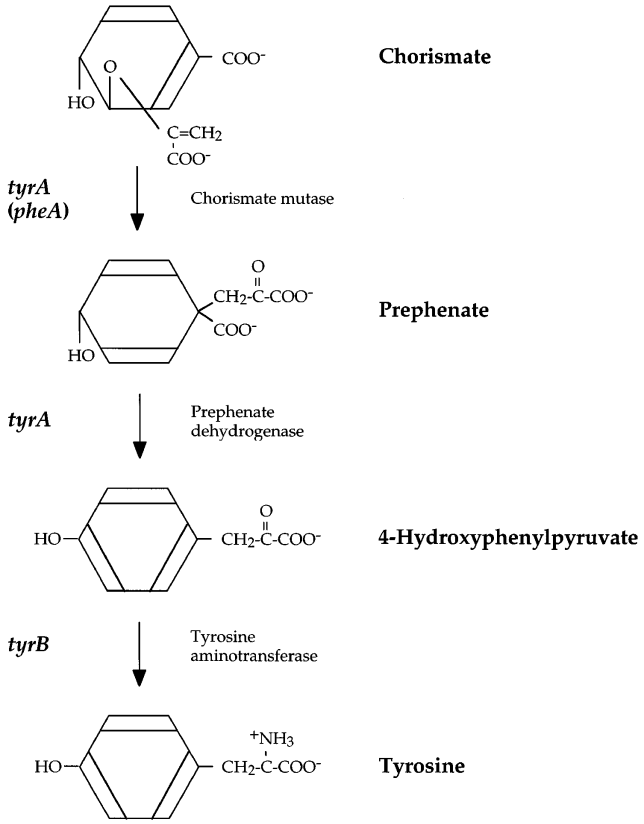

Figure 2.—Terminal pathway of tyrosine biosynthesis (from Pittard 1996). The product of *tyrA* is also known as T protein, and *pheA* specifies an activity of P protein involved in phenylalanine synthesis.

which could complement any possible deficiency in the chorismate mutase of the T-protein in this strain. In addition, there is some evidence to suggest that the T-protein is not deficient in chorismate mutase activity but that it lacks prephenate dehydrogenase (see below). The auxotrophy in WU3610 is therefore almost certainly because of the lack of the prephenate dehydrogenase activity.

We reconstituted the terminal part of the tyrosine biosynthetic pathway using monofunctional enzymes on a two plasmid system (Kast *et al.* 1996) to demonstrate a specific role for *tas* in complementing tyrosine auxotrophy. When plasmids with monofunctional chorismate mutase activity (pKCMT-W) and monofunctional prephenate dehydrogenase and dehydratase genes (pKIMP-UAUC) were combined with *tas*, we showed that *tas* complemented the prephenate dehydrogenase activity in tyrosine biosynthesis but specified no intrinsic chorismate mutase activity of its own (Table 2). The strain KA12 carries a deletion of both the bifunctional *E. coli tyrA* and *pheA* genes; consequently, there is no source of endogenous chorismate mutase activity. In the two plasmid system when *tas* replaced the monofunctional prephenate dehydrogenase *Erwinia herbicola tyrA* gene on pKIMP-UAUC, it provided full complementation of tyrosine auxotrophy in both WU3610 and KA12, in the latter case in the presence of pKCMT-W.

When *tas* was cloned into a lower-copy-number vector, pACYC184, and introduced into WU3610, it allowed a slow rate of growth on minimal plates lacking tyrosine. However, the growth rate was still far greater than that observed for the slow-growing Tyr^+ mutants. Colonies were visible in 2 to 3 days, rather than 5 to 6 days for the mutants, and may be compared with 1 to 2 days when *tas* was on the high-copy-vector pGEM-3Zf(-).

Metabolic studies with the strain CM1338 showed that the *tyrA*14 allele may retain some chorismate mutase activity. When a *pheA13*::Tn*10* allele was transduced into WU3610 and a tyrosine/phenylalanine double auxotroph selected, *tas* still complemented the tyrosine auxotrophy. Because *tas* requires a functional chorismate mutase for complementation to occur, a chorismate mutase activity must be present in the *tyr*/*phe* double mutant. Either the *phe*A::Tn*10* allele retains this activity or the *tyrA14* allele, which we assume to be unchanged by the transduction, provides the necessary function.

Properties of a *tas* deletion strain: To attempt to discover the normal role of *tas*, we constructed strain CM1355, a derivative of WU3610 in which *tas* was deleted. A segment of 822 bp was excised from the cloned gene and replaced with a kanamycin-resistance determinant. The construct was transferred to pMAK705 with a temperature-sensitive origin of replication and used to replace the chromosomal gene. Deletion was verified using primers SAMPCR1 and SAMPCR2. The PCR product from the deletion strain was ~400 bp larger than the wild-type gene because of the presence of the kanamycin gene block.

Growth of CM1355 with the deleted *tas* gene was indistinguishable from that of its parent WU3610 when streaked onto either L-agar or minimal agar containing tyrosine and leucine. Clearly, *tas* is not an essential gene. When tested for the ability to show mutation under conditions of tyrosine starvation, however, there was a very pronounced difference. CM1355 did not show any starvation-associated mutation under the conditions normally used for WU3610 (Figure 3).

There could be three possible reasons for this: (1) CM1355 loses viability faster than WU3610, (2) Tas gene product is necessary for the mutation process, or (3) *tas* is the target gene for stationary-phase mutation in this system. We therefore carried out an experiment in which bacteria were plated onto minimal medium lacking tyrosine at two cell densities, 3×10^8 and 10^6 per plate. At various times after plating the bacteria were washed off, and the number of total and viable bacteria determined. At the higher cell density, both WU3610 and CM1355 behaved similarly; there was no increase in viable count over the first 2 days, and viability then slowly declined similarly (data not shown). The lack of stationary-phase mutation in CM1355 under conditions of tyrosine starvation therefore cannot be ex-

TABLE 2

Complementation of amino acid auxotrophies

Strain	Plasmids	Media					
		MTP	MLT	MT	MP	ML	M
KA12	pKCMT-W	X	nd	—	—	nd	—
	pKIMP-UAUC	X	nd	—	—	nd	—
	pART34	X	nd	—	—	nd	—
	pART51	X	nd	—	—	nd	—
	pART52	X	nd	—	—	nd	—
	pKIMP-UAUC						
	pKCMT-W	X	nd	X	X	nd	X
	pKIMP-UAUC						
	pART34	X	nd	—	—	nd	—
	pKCMT-W						
	pART51	X	nd	—	X	nd	—
	pKCMT-W						
	pART52	X	nd	X	X	nd	X
WU3610	pKCMT-W	nd	X	nd	nd	—	—
	pKIMP-UAUC	nd	X	nd	nd	X	—
	pART51	nd	X	nd	nd	X	—
	pART52	nd	X	nd	nd	X	—

The results from metabolic complementation experiments are shown: X, complementation of the selected phenotype; −, no complementation; nd (not determined), the particular combination of amino acids was not tested. Medium (M) was glucose minimal salts agar supplemented where shown with (L) leucine, (T) tyrosine, and (P) phenylalanine. For strain KA12, plates were additionally supplemented with thiamine at 5 μg/ml and arginine at 10 μg/ml. Chloramphenicol and/or ampicillin were added where required to maintain plasmid selection. Plates were counted when colonies were between 2 and 3 mm in diameter and were incubated for between 2 and 6 days before counting, as some combinations of plasmids were slower growing than others.

plained by a differential viability of the two organisms over the short term. At the lower cell density (Figure 4), WU3610 increased in viable count to a plateau value of 6×10^7 per plate. CM1355, however, did not show this increase in bacterial count when starved for tyrosine, although the residual growth at low cell density was similar to that of WU3610 when starvation was for leucine. This implies that the activity of the *tas* gene is needed for residual growth at low cell density in the absence of tyrosine and suggests that the ability to undergo a very small amount of growth may be necessary if starvation-associated mutation is to occur at the higher cell density normally used. We therefore conclude that one role of *tas* in starvation-associated mutation may be to confer the potential for a small amount of growth in the absence of tyrosine.

The role of *tas* may not be quite as simple as this, however, because we have also failed to observe any slow-growing revertants even when plates were spiked with 0.1 μg/ml tyrosine to stimulate some residual growth or when growth conditions were used as described previously (Bridges 1994). We therefore have considered the third hypothesis above, namely, that *tas* may also be the target gene for mutation. Because no molecular alteration in the sequence of *tas* or its promoter region could be detected in the slow-growing Tyr^+ revertants, we considered the possibility that the number of gene copies is amplified in the revertants. This would be entirely consistent with the fact that *tas* on a multicopy plasmid confers a Tyr^+ phenotype. However, probing of DNA from six slow-growing tyrosine-independent mutants and two fast-growing tyrosine revertants revealed no sign of amplification of the *tas* region compared to a control region (*rpoB*) (results not shown).

DISCUSSION

The results presented above describe a new gene *tas*, which, when present on a multicopy plasmid, is able to suppress the tyrosine requirement of the *tyrA14* strain WU3610, and which, when deleted from the chromosome, prevents the appearance of the slow-growing tyrosine-independent mutants that normally arise during prolonged incubation on minimal plates lacking tyrosine.

The TyrA protein is a bifunctional enzyme and catalyzes two steps in the pathway for tyrosine biosynthesis (see Figure 2). Metabolic studies have shown that the product of the *tyrA14* gene in WU3610 may retain the chorismate mutase activity but not the prephenate dehydrogenase activity characteristic of the wild-type product. Monofunctional derivatives of the T-protein have been produced (Rood *et al.* 1982) showing that the chorismate mutase function can operate without concomitant prephenate dehydrogenase activity. The *tyrA14* allele contains an ochre termination codon correspond-

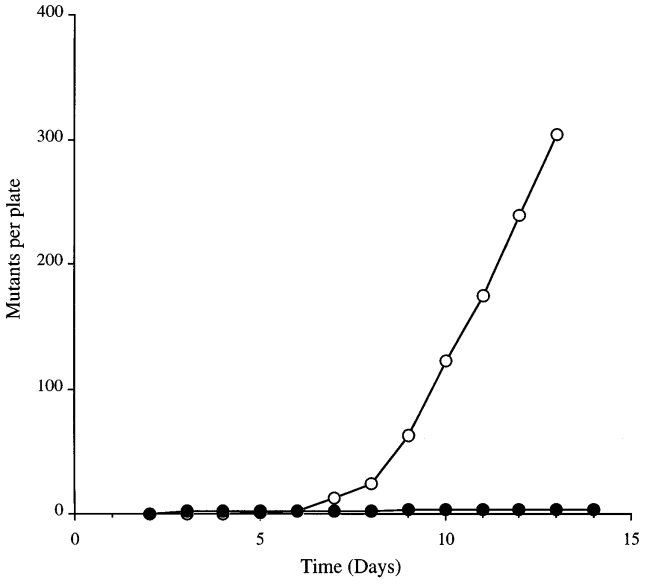

Figure 3.—A representative starvation-associated mutation experiment showing the appearance of slow-growing mutants on glucose minimal salts plates containing leucine at 27°. WU3610 (○) is the parental *tas*$^+$ strain and CM1355 (●) contains the Δ*tas1*::Km allele.

ing to residue 161 (Li *et al.* 1991). Because the chorismate mutase activity has been shown to reside in the N-terminal region of the protein (Hudson and Davidson 1984), the region of the gene translated could specify an active monofunctional protein. Alternatively, chorismate mutase activity may be complemented in *trans* by the fully functional P-protein in WU3610.

Whether or not one or both of these possibilities operate, it is clear that *Tas* on a multicopy plasmid can complement prephenate dehydrogenase deficiency. A database search revealed a 35.5% amino acid identity of *tas* with a putative aldo-keto reductase gene of *Babesia bovis* and limited homology in the region of the active site with the genes for mouse aldose reductase, rabbit aldo-keto reductase, and human alcohol dehydrogenase. There are three regions where the conservation of sequence is marked, the two most obvious being residues 121–134 and 228–238 in *tas* (see Table 3). It is not unreasonable to assume that Tas protein has some small ability to act as a dehydrogenase on prephenate and thus complements the defect in *tyrA*, but the normal function of the gene is unknown. All that can be said is that it is not an essential gene.

The possible objection that *tas* is not acting as a prephenate dehydrogenase but is in some way enabling the bacteria to utilize some complementing substance present as a contaminant in the agar can be countered by two arguments. First, the use of Difco Noble agar, a purer alternative to conventional Difco Bacto agar, did not reduce the yield of starvation-associated mutants (unpublished observation). Second, the growth rate in the absence of tyrosine depends on the number of *tas* gene copies, as shown by the results with the plasmid transformants, and with a high-copy-number plasmid, where the growth rate approaches that seen when tyrosine is supplied.

Previous work on starvation-associated mutation in some bacterial systems has indicated that there may be a requirement for a certain amount of leakiness (Jayar-

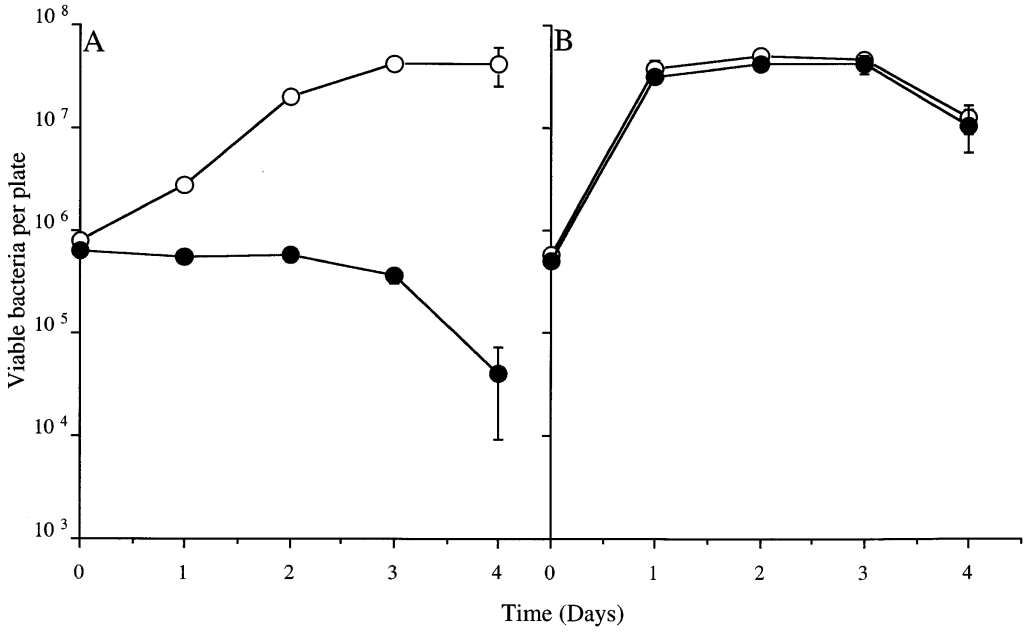

Figure 4.—Viability of WU3610 (○) and CM1355 (●) under starvation conditions followed over 4 days. Plates were inoculated with about 10^6 bacteria, and viabilities were determined from whole plate wash-off experiments. Data points are the means of three or more experiments. (A) Shows viability of strains incubated on glucose minimal salts plates supplemented with leucine. (B) Shows the same strains on glucose minimal salts plates supplemented with tyrosine.

TABLE 3

Homologies between Tas and aldo-keto reductases

Organism	Protein	Amino acid sequence
E. coli	Tas	121 L Q T D Y L D L Y Q V H W P 134
B. bovis	Aldo-keto reductase	42 L N T D Y I D L L Q L H W P 55
Mouse	Aldose reductase	100 L K L D Y L D L Y L V H W P 113
Rat	Aldo-keto reductase	101 L Q L D Y V D L Y I I H F P 114
Human	Alcohol dehydrogenase	106 L Q L E Y L D L Y L M H W P 119
Consensus		L * D Y * D L Y * H W P
E. coli	Tas	228 V E L L A Y S C L G F 238
B. bovis	Aldo-keto reductase	159 I A I L A Y A P L A G 169
Mouse	Aldose reductase	205 I A V T A Y S P L G S 215
Rat	Aldo-keto reductase	211 I V L V A Y S A L G S 221
Human	Alcohol dehydrogenase	214 L E V T A Y S P L G S 214
Consensus		* * A Y S * L G

The two major regions of homology between Tas and members of the aldo-keto reductase superfamily of enzymes. Numbers refer to the position of residues within the proteins. A consensus sequence is indicated where four of the five sequences agree; *signifies a conservative amino acid substitution at this position. The consensus sequence includes residues that are widely conserved throughout the alko-keto reductase superfamily and are diagnostic of membership of this group.

aman 1995; Mittler and Lenski 1992), although a direct quantitative relation between leakiness and mutation rate is doubtful (Galitski and Roth 1996; Prival and Cebula 1996). Leakiness appears not to be involved in other systems (Hall 1990; Foster 1994). In the WU3610 system, although the number of viable bacteria on the plate does not increase, examination of the lawn after 2 or 3 wk showed evidence of population turnover and increase in biomass with microcolonies visible under the microscope (Bridges 1994). The present data show that *tas*$^+$ bacteria, but not those carrying a deletion of *tas*, can grow on minimal agar lacking tyrosine when plated at low cell density, although we do not know why the cell number levels off at $\sim 6 \times 10^7$ bacteria per plate (Figure 4). If we assume that the leakiness conferred by *tas* also occurs at higher cell density even though it is too little to result in measurable cell growth, it is reasonable to postulate that this property is also the reason why *tas*$^+$ but not *tas*$^-$ bacteria show starvation-associated mutation. Nevertheless, even under conditions where failure to undergo leaky growth should not have been a problem, we still failed to find any evidence for slow-growing revertants in the strain carrying the *tas* deletion, leading us to wonder whether *tas* may be required not only for the formation but also for the continued viability of the slow-growing revertants.

Although a metabolic role of *tas* may explain its requirement for starvation-associated mutation in this system, might not *tas* also be the target gene at which the mutations occur? This was, after all, the rationale of the cloning experiment that led to its isolation. A simple point mutation in *tas* is excluded by the absence of any detectable difference between the sequence of the *tas* region in WU3610 and a number of slow-growing Tyr$^+$ mutants. We have considered, however, the possibility that the mutation responsible is not a sequence change but an amplification of the *tas* region. We have obtained data with a related strain that deletions are prone to occur in starved cells (Bridges and Timms 1997); other chromosomal rearrangements, including duplications, might well be associated with this condition. Duplications are by no means uncommon in *E. coli* (for review, see Anderson and Roth 1977). If the population that we plate onto minimal plates was to contain a proportion of cells with duplication of the *tas* region, these bacteria would be expected to have a selective advantage and grow into microcolonies. Within these microcolonies further duplications could occur until a cell was produced capable of giving rise to a visible colony [*cf.* the results of Tlsty *et al.* (1984) with a *lacI-Z* fusion strain]. The mutations that arise under starvation conditions would then be likely to include some that existed as *tas* duplications at the time of plating and that underwent further amplification on the plate, as well as some in which the first duplication arose on the plate. However, we have been unable to demonstrate any amplification of the *tas* gene in slow-growing Tyr$^+$ revertants. It must therefore be concluded that the *tas* gene is not the mutational target in this system and that the identity of the real target gene remains to be revealed.

Because both a mutation in *tas* and an amplification of its sequence have been ruled out, the possibility may be considered that there is an increase in *tas* expression, because of, for example, an alteration in supercoiling. Such an alteration could be because of a mutation in a gene controlling supercoiling or, conceivably, because of a more local sequence change affecting superhelicity in the neighborhood of *tas*. We estimate that an increase

in expression of no more than twofold would be sufficient to confer the slow-growing Tyr^+ phenotype. Unfortunately, such an increase in expression under the given experimental conditions cannot be detected currently. Other types of suppressor mutation could be envisaged, for example, in tRNA, in ribosomal protein genes, or in a gene affecting mRNA stability. All would be anticipated to interfere with the growth rate and would be consistent with the slow-growing phenotype observed (although to a less extreme extent), even on L-agar where the tyrosine requirement is not limiting. Moreover, a cell carrying such a suppressor allele on a multicopy plasmid would probably not be viable, which could explain why we failed to pick it up from the gene library made from slow-growing revertants.

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