Starvation Survival Response of *Mycobacterium tuberculosis*

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The ability of *Mycobacterium tuberculosis* auxotrophs to survive long-term starvation was measured. Tryptophan and histidine auxotrophs did not survive single-amino-acid starvation, whereas a proline auxotroph did. All three auxotrophs survived complete starvation. THP-1 cells were also able to restrict the growth of the tryptophan and histidine auxotrophs.

Mycobacterium tuberculosis is exposed to low or restricted nutrient concentrations in the host and is able to survive these conditions for long periods of time (2). Several studies suggest that certain amino acids are not available within the compartment of the macrophage in which the bacteria reside, since leucine, tryptophan, and arginine auxotrophs do not survive or multiply in macrophages (1, 5, 6, 13). I investigated the ability of three auxotrophs and one wild-type strain to survive different starvation conditions (Table 1). The proline auxotroph (Tame 2) has been described previously (9). The unmarked tryptophan and histidine auxotrophs were constructed by homologous recombination. Suicide (nonreplicating) delivery vectors were constructed with a rapid cloning system (11); the central 0.3-kbp *Not*I fragment internal to *trpD* was deleted, and the central 0.7-kbp *Pst*I fragment of *hisDC* was deleted. A two-step procedure to replace the wild type with the unmarked, deleted gene was carried out (11). The expected genotype of the mutants was confirmed by Southern blotting, and both these strains showed the expected auxotrophy (data not shown).

We had previously shown that a tryptophan auxotroph is severely attenuated in both macrophage and mouse models of infection (13), suggesting that the organisms are sequestered in an intracellular compartment from which they cannot obtain tryptophan. We do not know if clearance of the bacteria is caused by death due to tryptophan starvation or by active killing of the bacteria by the macrophage. In order to address this question in vitro, I examined the survival ability of the tryptophan auxotroph on withdrawal of tryptophan in axenic culture.

Bacteria were subjected to single-amino-acid starvation (Middlebrook 7H9 liquid plus 10% oleic acid-albumin-dextrose-catalase [OADC; Becton Dickinson] and 0.05% [wt/vol] Tween 80 without amino acid supplementation) and complete starvation (no nutrients, i.e., sterile distilled water). Lysis was measured by monitoring the optical density at 600 nm (OD_{600}), and viability was measured by determining the numbers of CFU per ml on Middlebrook 7H10 agar containing 10% (vol/vol) OADC supplemented with 40 μg of amino acid per ml as required (Fig. 1). Both the wild type and tryptophan auxotroph grew normally in supplemented medium (Middlebrook 7H9 liquid containing 10% OADC, 0.05% [wt/vol] Tween 80, and 40 μg of amino acid per ml). There was no difference in their ability to survive complete starvation. The cultures in water showed an immediate drop in OD_{600} to below the limit of detection and an accompanying log reduction in CFU. There was no further death over 7 weeks. The fact that these bacteria have a remarkable ability to survive complete starvation for long periods of time suggests that there must be a coordinated response to the lack of nutrients which changes their physiology from actively growing cells to persistent (nongrowing) cells. In the nonsupplemented medium, the wild-type strain grew normally; however, the tryptophan auxotroph showed an unusual phenotype. Although the OD_{600} remained stable, indicating that the cells did not lyse, there was a 2-log reduction in cell viability over the course of the 7-week experiment. Whatever response the bacteria made to complete starvation was not induced solely in response to tryptophan depletion.

I repeated the survival experiment over a longer time scale to determine if this pattern of survival would be maintained (Fig. 2). Over 15 weeks, the OD_{600} of the tryptophan auxotroph remained constant in nonsupplemented medium, while the OD_{600} in water rapidly declined in the first week. Again, this did not mirror the viability counts. The wild type showed a small (<1 log) reduction in viability after extended stationary-phase growth (17 weeks) in 7H9 medium, while the tryptophan auxotroph lost viability over this time scale with a 4-log reduction at 13 weeks and a complete loss of viability (approximately 8 log) by 17 weeks. In contrast, there was no difference in the two strains' ability to survive in water, with both strains losing less than 0.5-log viability over 17 weeks. I also determined whether the tryptophan auxotroph could be recovered from the nonsupplemented medium by subculturing into supplemented liquid medium, but no growth occurred after repeated subculture, thus indicating that the cells were nonviable and nonculturable (data not shown).

In order to determine if the inability to survive amino acid starvation was a general feature of auxotrophs, I studied the histidine and proline auxotrophs in the same system. The histidine auxotroph behaved in a manner similar to that of the tryptophan auxotroph (Fig. 3). Prolonged survival of complete starvation (i.e., over 14 weeks) was seen after an initial log reduction in viability, whereas deprivation of histidine led to an

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Genotype	Source or reference
M. tuberculosis strain			
H37Rv	Wild type	$trpD^+ proC^+ hisDC^+$	ATCC 25618
Tame 2	Proline auxotroph	$trpD^+ proC\Delta::hyg\ hisDC^+$	9
Tame 5	Tryptophan auxotroph	$trpD\Delta\ proC^+ hisDC^+$	This study
Tame 24	Histidine auxotroph	$trpD^+ proC^+ hisDC\Delta$	This study
Plasmid			
pSM128	Promoter probe vector, *lacZ* reporter gene, *Sm*		4
pTRIP5	0.2 kbp upstream of *trpECBA* operon in pSM128		This study
pTRIP7	0.2 kbp upstream of *trpD* gene in pSM128		This study
pTRIP10	0.8 kbp upstream of *trpE2* gene in pSM128		This study
pHIP5	0.52 kbp *Sma*I upstream of *hisD* in pSM128		This study

approximate 8-log reduction in viability (no viable bacteria left) after only 8 weeks; this was a much more rapid loss of viability than that exhibited by the tryptophan auxotroph. In marked contrast, the proline auxotroph did not lose any viability in nonsupplemented media over 15 weeks (Fig. 4). Survival of the proline auxotroph in water was the same as that for the other strains. Thus, the ability of auxotrophs to survive starvation is dependent on the particular amino acid that they require. Histidine and tryptophan auxotrophs cannot withstand individual amino acid deprivation, whereas a proline auxotroph can.
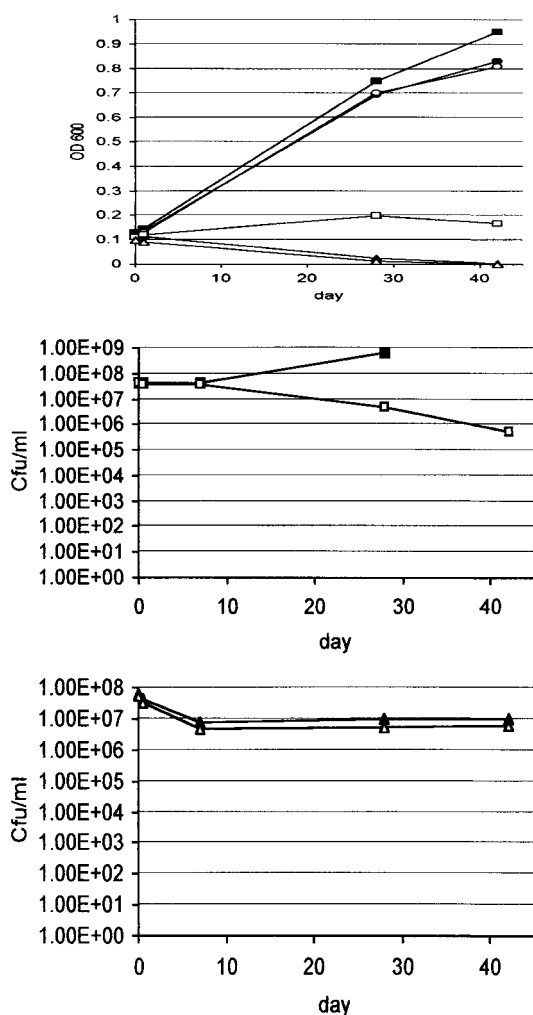

FIG. 1. Short-term starvation survival of the tryptophan auxotroph. The tryptophan auxotroph and wild-type strains were inoculated into the indicated media to an OD_{600} of 0.1 and incubated at 37°C. OD_{600} readings (A) and CFU (B and C) were measured over 42 days. Filled shapes, wild type; open shapes, tryptophan auxotroph; squares, nonsupplemented 7H9; circles, tryptophan-supplemented 7H9; triangles, water. Cultures were assayed in duplicate with a variation of less than 10%. Note that no wild-type CFU were measured at day 42.
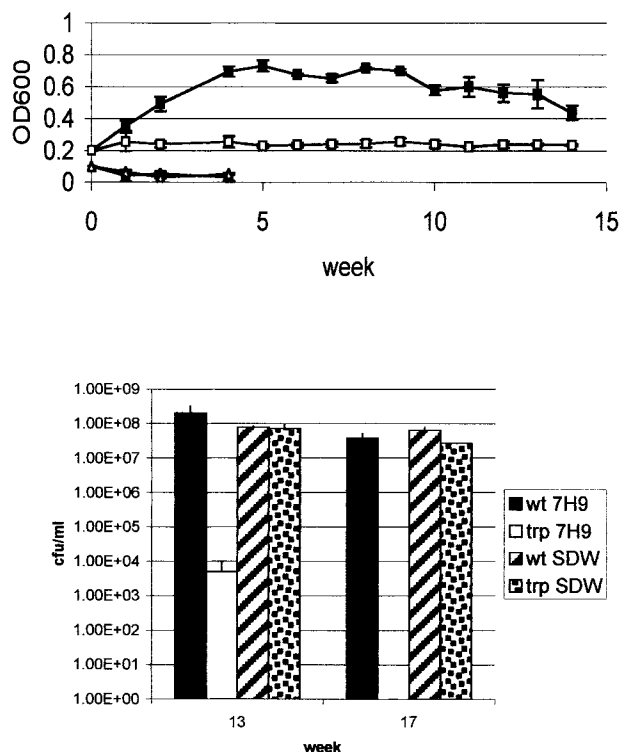

FIG. 2. Long-term starvation survival of the tryptophan auxotroph. The tryptophan auxotroph and wild-type strains were inoculated into the indicated media to an OD_{600} of 0.2 and incubated at 37°C. OD_{600} readings (A) and CFU (B) were measured over 17 weeks. Filled shapes, wild type; open shapes, tryptophan auxotroph; squares, nonsupplemented 7H9; triangle, water.
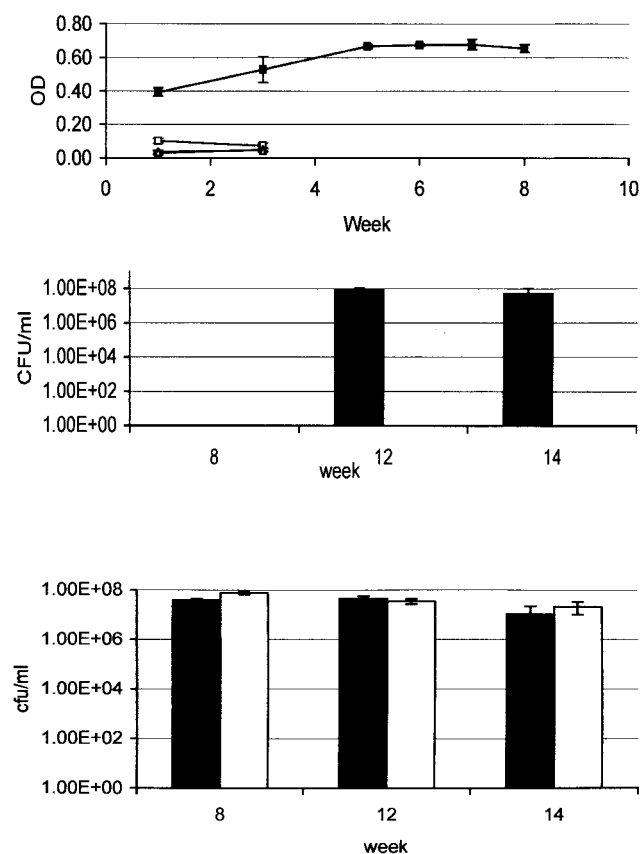
FIG. 3. Long-term starvation survival of the histidine auxotroph. The histidine auxotroph and wild-type strains were inoculated into the indicated media to an OD_{600} of 0.1 and incubated at 37°C. OD_{600} readings (A) and CFU (B and C) were measured over 14 weeks. Filled shapes, wild type; open shapes, histidine auxotroph; squares, non-supplemented 7H9; triangles, water. Panel B depicts results for which nonsupplemented 7H9 was used; panel C depicts results achieved with water. Note that no wild-type CFU were measured at week 8.

The possibility that amino acids were biologically available from the bovine serum albumin in the OADC supplement was considered unlikely, as none of the auxotrophs were able to grow without individual amino acid supplementation. I also repeated the experiment in a chemically defined medium (1.5 g of K_2HPO_4 per liter, 0.5 g of KH_2PO_4 per liter, 0.5 g of $MgSO_4$ per liter, 0.5 mg of CaCl per liter, 0.1 mg of $ZnSO_4$ per liter, 0.1 mg of $CuSO_4$ per liter, 50 mg of ferric chloride per liter, and 30 mM NH_4SO_4) with and without OADC. Survival was assessed after 12 weeks. As was the case for Middlebrook media, the histidine and tryptophan auxotrophs showed a significant decrease in viability, whereas the proline auxotroph and wild-type strains remained viable, thereby confirming that the bovine serum albumin does not directly affect the viability of these strains.

Previously, both the proline auxotroph and a different (marked) tryptophan auxotroph had been shown to be attenuated to different degrees in murine macrophages and mice (13). The histidine auxotroph had not previously been assayed in any model of virulence. I used the human THP-1 cell line in order to quickly determine whether the two new mutants were attenuated. THP-1 cells were infected at a multiplicity of infection of 1 as described previously (8) (Fig. 5). As before, the tryptophan auxotroph showed attenuation in this model, and a reduction in bacterial numbers over 10 days was observed. The histidine auxotroph was attenuated to a similar degree. The proline auxotroph showed the same growth profile as the wild type. These results mirror the starvation results in that the tryptophan and histidine auxotrophs could not survive amino acid starvation, whereas the proline auxotroph could. These findings led to the hypothesis that the intracellular environment does not contain certain amino acids but does contain other nutrients, as the bacteria did not enter the same state of persistence that they did in the complete-starvation model.

In many bacteria, including *M. tuberculosis*, the stringent response is activated in response to nutrient starvation, including amino acid deprivation (12). This response is mediated by intracellular levels of (p)ppGpp and the RelA protein, resulting in the downregulation of most genes and the upregulation of a specific subset of genes. This subset includes amino acid
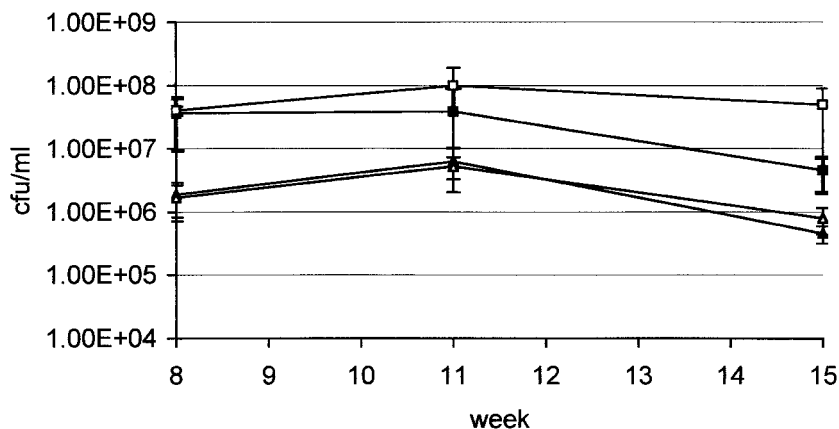

FIG. 4. Long-term starvation survival of the proline auxotroph. The proline auxotroph and wild-type strains were inoculated into the indicated media to an OD_{600} of 0.1 and incubated at 37°C. CFU were measured over 15 weeks. Filled shapes, wild type; open shapes, proline auxotroph; squares, nonsupplemented 7H9; triangles, water.
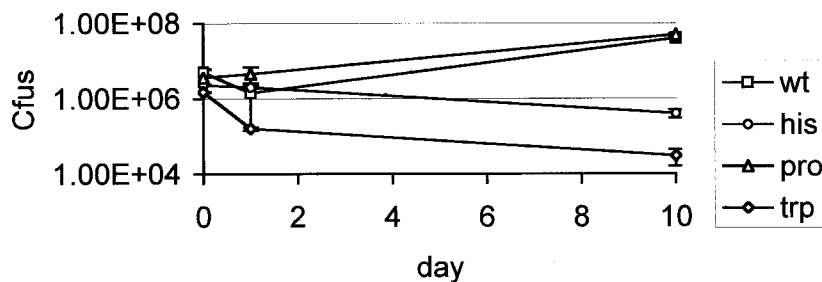
FIG. 5. Intracellular survival. THP-1 cells were infected at a multiplicity of infection of 1, and the number of intracellular viable bacteria was measured over 10 days. Results are given as the means of triplicate wells. wt, wild type.

biosynthesis genes and proteases, including the histidine operon (3). If this response occurred in the histidine auxotroph, then all of the amino acids recycled from other proteins could be directed into making histidine biosynthetic enzymes, possibly resulting in uncontrolled expression. This could also account for the same phenotype in the tryptophan auxotroph. In order to address this possibility, I looked at the promoter activity of the three *trp* loci and the *his* operon. If runaway

Strain	Wild-type	Wild-type	Histidine auxotroph
Supplement	None	Histidine	Histidine
pSM128	1.7 ± 0.3	ND	0.6 ± 0.1
pHIP5 (*hisD*)	80 ± 20	40 ± 37	137 ± 9

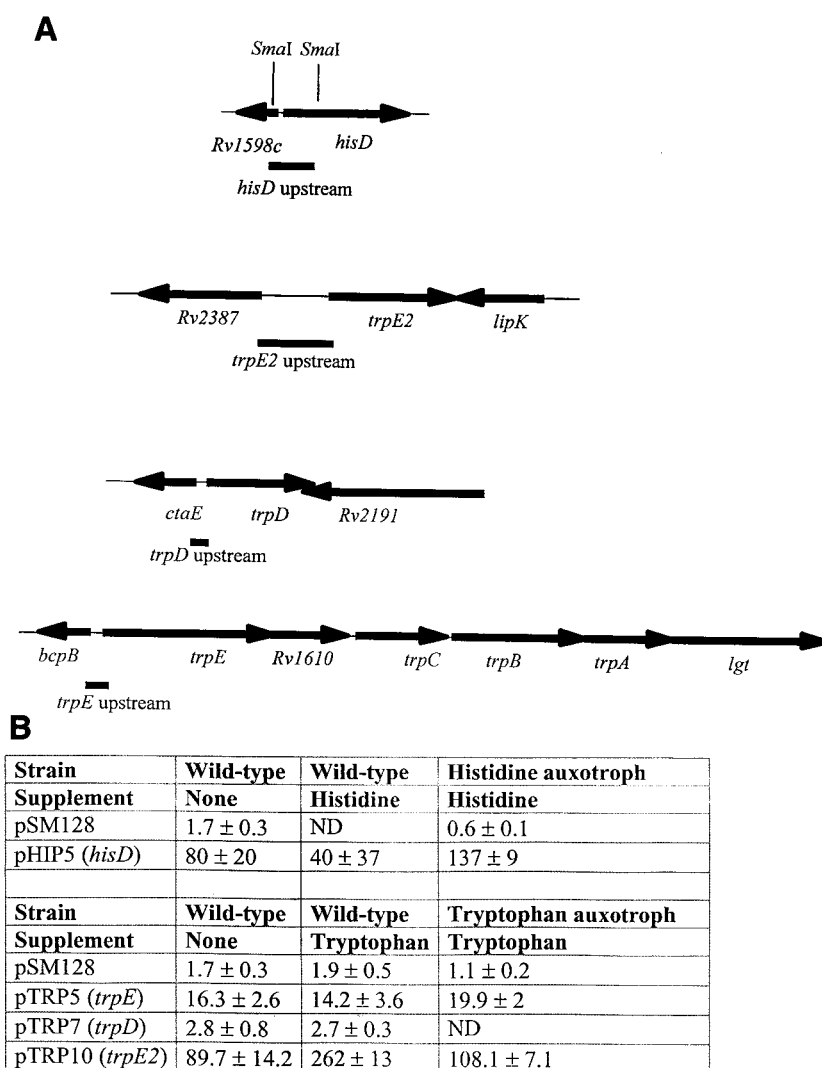| Strain	Wild-type	Wild-type	Tryptophan auxotroph
Supplement	None	Tryptophan	Tryptophan
pSM128	1.7 ± 0.3	1.9 ± 0.5	1.1 ± 0.2
pTRP5 (*trpE*)	16.3 ± 2.6	14.2 ± 3.6	19.9 ± 2
pTRP7 (*trpD*)	2.8 ± 0.8	2.7 ± 0.3	ND
pTRP10 (*trpE2*)	89.7 ± 14.2	262 ± 13	108.1 ± 7.1

FIG. 6. Promoter activity analysis. (A) Constructs to assay promoter activity. The indicated regions upstream of each gene were cloned into the promoter probe vector pSM128. (B) Promoter activity. The plasmids depicted in panel A were transformed into the wild-type and auxotrophic strains. Transformants were grown in 10 ml of medium with the indicated supplement and assayed for beta-galactosidase activity. Each value represents the mean ± standard deviation for three independent transformants, each assayed in duplicate. ND, not determined.

transcription were occurring, then we would expect to see the transcription of these operons being controlled by the appropriate amino acid. The upstream regions of the three *trp* loci were PCR amplified and subcloned into the promoter probe vector pSM128. The 0.5-kbp *Sma*I fragment upstream of the *his* operon was subcloned from pHIS1 into pSM128. Plasmids were electroporated into wild-type and auxotrophic strains, transformants were selected on 20 μg of streptomycin (and amino acid where required) per ml, and promoter activity was assayed (Fig. 6). None of the promoters were repressed by the relevant amino acid and, counterintuitively, the *trpE2* promoter was upregulated twofold by the addition of tryptophan. The plasmid pSM128 is an integrating vector which occurs in one copy per cell, so the lack of transcriptional control is not due to a copy number effect. In *Streptomyces* spp., the main *trp* operon is regulated by growth phase rather than by tryptophan availability (7). The apparent induction of *trpE2* may therefore result from an increased growth rate upon the addition of tryptophan rather than from a direct effect of the amino acid. The histidine operon was not controlled by histidine, although there was a slightly higher level of promoter activity in the *hisDC* mutant strain. Thus, the enzymes of these two biosynthetic pathways are not controlled transcriptionally in response to amino acid availability. This may reflect a general pattern in *M. tuberculosis* in which amino acid biosynthesis genes are constitutively expressed, and the finding meshes with the previous observation that the *impA* gene resides within the *his* operon because of its constitutive nature (10).

In conclusion, both auxotrophic and wild-type strains of *M. tuberculosis* are able to survive long periods of complete starvation. However, histidine and tryptophan auxotrophs are unable to survive single-amino-acid starvation. These results have implications for live, attenuated vaccine development in situations in which the persistence of auxotrophic strains is assumed to be required for generation of protective immunity and for drug development scenarios for which drugs that target bacteria in nongrowing states are required. The results presented here demonstrate that *M. tuberculosis* has a different response to different types of starvation and that models of persistence involving starvation may need to be refined.

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