A connection between iron–sulfur cluster metabolism and the biosynthesis of 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate in *Salmonella enterica*

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Several cellular pathways have been identified which affect the efficiency of thiamine biosynthesis in *Salmonella enterica*. Mutants defective in iron–sulfur (Fe–S) cluster metabolism are less efficient at synthesis of the pyrimidine moiety of thiamine. These mutants are compromised for the conversion of aminoimidazole ribotide (AIR) to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), not the synthesis of AIR. The gene product ThiC contains potential ligands for an Fe–S cluster that are required for function *in vivo*. The conversion of AIR to HMP-P is sensitive to oxidative stress, and variants of ThiC have been identified that have increased sensitivity to oxidative growth conditions. The data are consistent with ThiC or an as-yet-unidentified protein involved in HMP-P synthesis containing an Fe–S cluster required for its physiological function.

INTRODUCTION

The essential cofactor thiamine pyrophosphate (TPP) is formed in bacteria from two independently synthesized molecules, 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) and thiazole monophosphate (THZ-P) (Fig. 1). The pyrimidine moiety is generated as a branch off the well characterized purine biosynthetic pathway, where aminoimidazole ribotide (AIR) is the direct precursor of 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) (Estramareix & Therisod, 1984; Estramareix & David, 1990). Mutations in *thiC*, or its homologues, are the only lesions reported to result in an absolute requirement for the 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) moiety of thiamine (Vander Horn *et al.*, 1993; Zhang & Begley, 1997), suggesting that the complex rearrangement needed to convert AIR to HMP-P is catalysed by a single gene product.

Several results have raised the possibility that a gene product(s) in addition to ThiC is directly or indirectly involved in the synthesis of HMP-P. The conversion of AIR to HMP-P has recently been reconstituted in a cell-free

system (Lawhorn *et al.*, 2004). These experiments utilized extracts of a ThiC-overexpressing strain; purification of ThiC from the extract resulted in complete loss of activity. One explanation for this result is that another protein is required for efficient conversion of AIR to HMP-P. This result could also be explained by loss of a cofactor upon purification or protein instability under the purification conditions. The activity present in extracts was enhanced by addition of pyridine nucleotides and S-adenosylmethionine, but the role and/or requirement of these cofactors in the formation of HMP-P remains unclear (Lawhorn *et al.*, 2004).

Lesions in a number of loci have been identified that result in a conditional requirement for HMP, indicating that a number of cellular processes affect the formation of HMP-P, at least indirectly. Mutations reducing the synthesis of CoA (specifically *panE*) affect the biosynthesis of HMP-P in a flux-dependent way (Downs & Petersen, 1994; Frodyma *et al.*, 2000; Frodyma & Downs, 1998). Under conditions of high flux through the purine biosynthetic pathway, *panE* mutants are prototrophic, but when flux is reduced (i.e. inhibition or elimination of PurF), a thiamine requirement is unveiled. Unlike other mutations that have been described to prevent HMP synthesis if *purF* is absent (*gnd*, *zwf*, *nuo*), the target of the CoA effect has been shown to be the conversion of AIR to HMP-P specifically (Allen *et al.*, 2002).

Lesions in any of five loci (*iscA*, *gshA*, *rseC*, *apbC*, *apbE*) shown to impair the metabolism of iron-sulfur (Fe–S) clusters can also generate a thiamine requirement (Skovran & Downs, 2000; Skovran *et al.*, 2004). Under conditions

Abbreviations: AICARs, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; AIR, aminoimidazole ribotide; AIRs, aminoimidazole riboside; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; ICP-MS, inductively coupled plasma mass spectrometry; THZ, thiazole; THZ-P, thiazole monophosphate; TPP, thiamine pyrophosphate.

A table of primers and a sequence alignment are available as supplementary data with the online version of this paper.

Fig. 1. Purine mononucleotide and thiamine pyrophosphate biosynthetic pathways in *S. enterica*. Relevant intermediates are indicated structurally and enzymes are indicated with the reaction they catalyse. ThiX indicates the possible participation of an unidentified gene product. Abbreviations: PRA, phosphoribosylamine; AIR, 5-aminoimidazole ribotide; HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate; DXP, deoxy-D-xylulose-5-phosphate; TMP, thiamine phosphate. Phosphorylation of AIRs to the intermediate AIR is facilitated by overproducing STM4066 as previously described (Dougherty & Downs, 2003).

where the protein YggX does not accumulate to high levels, a strain lacking any one of the above loci requires both the thiazole (THZ) and pyrimidine moieties of thiamine (Skovran & Downs, 2000, 2003). In this case, the THZ requirement has been shown to result from an oxygen-labile Fe-S cluster in ThiH, a member of the radical SAM protein family (Gralnick *et al.*, 2000; Leonardi *et al.*, 2003; Skovran & Downs, 2000; Sofia *et al.*, 2001). The reason for the HMP requirement in these mutant strains is not clear. Accumulation of the YggX protein restores prototrophy in these mutants. YggX is a small protein implicated in protection from oxidative stress and found to bind Fe(II) *in vitro* (Cui *et al.*, 2006; Gralnick & Downs, 2001). This study was undertaken to explore the connection between Fe-S cluster metabolism and the function of ThiC in the generation of HMP for thiamine synthesis.

METHODS

Bacterial strains, media and chemicals. All strains used in this study are derivatives of *Salmonella enterica* LT2 and are listed with their genotypes in Table 1. MudJ refers to the Mud1734 insertion element (Castilho *et al.*, 1984) and Tn10d(Tc) refers to the transposition-defective mini-Tn10 described by Way *et al.* (1984). No-carbon E medium supplemented with 1 mM MgSO₄ (Davis *et al.*, 1980; Vogel & Bonner, 1956) and glucose (11 mM), glycerol (22 mM), citrate (40 mM) or gluconate (11 mM) was used as a minimal medium. For growth under anaerobic respiration conditions sodium nitrate (25 mM) was provided. Difco nutrient broth

(8 g l⁻¹) with NaCl (5 g l⁻¹) or Luria-Bertani broth were used as rich media. Difco BiTek agar (15 g l⁻¹) was added for solid medium. When present in the media, supplements were provided at the following final concentrations: thiamine, 10 nM or 100 nM; adenine, 0.4 mM; paraquat, 1 µM. 5-Aminoimidazole riboside (AIRs) was provided at the concentration indicated in each experiment. When needed, antibiotics were added to the following concentrations in rich/minimal media: tetracycline, 20/10 µg ml⁻¹; kanamycin, 50/125 µg ml⁻¹; chloramphenicol, 20/4 µg ml⁻¹ and ampicillin, 50/15 µg ml⁻¹. Restriction enzymes and DNA ligase were purchased from Promega. Cloned *Pfu* DNA polymerase was purchased from Stratagene. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICARs) was purchased from Toronto Research Chemicals. AIRs was synthesized from AICARs by the method of Bhat *et al.* (1990) without modification. All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich.

Genetic and molecular biological techniques

Phenotypic analysis. Nutritional requirements were assessed on solid medium with soft agar overlays and by quantification of growth in liquid media. Protocols for each have been previously described (Beck & Downs, 1998; Petersen *et al.*, 1996).

Efficiency of AIR to HMP-P conversion. The use of strain DM7060 (*purG purE stm4068*) and its derivatives to classify the target of defects in HMP synthesis has been described (Dougherty & Downs, 2004). Briefly, this strain is dependent on exogenous AIRs as a source of thiamine. As such, the concentration of AIRs required to satisfy the thiamine requirement of derivatives of this strain is a measure of the efficiency of the conversion of AIR to HMP-P.

Plasmid construction. The gene *thiC* was amplified by PCR using Cloned *Pfu* DNA Polymerase and the appropriate primers (see Supplementary Table S1, available with the online version of this paper). The resulting PCR products were ligated into *Sma*I-digested pSU19. The ligation mixture was transformed into *Escherichia coli* DH5α and the transformants were screened for those with vectors containing inserts. The resulting plasmids were analysed by restriction digestion and confirmed by sequencing (Table 1). For expression of a ThiC-chitin binding domain fusion, the *thiC* gene was subcloned into the *Nde*I and *Sap*I sites of pTYB1.

Site-directed mutagenesis of *thiC*. Four site-directed mutations were constructed in the *thiC* gene encoded on plasmid pMD18 using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) and the appropriate primers (listed in Supplementary Table S1). The presence of the directed mutation was confirmed by sequencing. The resulting plasmids were digested with *Sac*I and *Xba*I restriction enzymes and the appropriate released fragment was gel purified and ligated into pBAD30 (also digested with *Sac*I and *Xba*I), according to the manufacturers' instructions.

Random mutagenesis of pMD26. Random mutagenesis of pMD26 was performed using XL1-Red competent cells (Stratagene). Briefly, pMD26 was transformed into *E. coli* strain XL1-Red, and transformants were inoculated into LB medium. This culture was grown for ~2 weeks, with subculturing every 16–24 h. A sample of culture was taken every 16–24 h from which plasmid DNA was isolated. This protocol resulted in a number of pools of plasmid DNA with varying levels of mutagenesis.

Construction of chromosomal mutations. A deletion of *araCBAD* and an insertion-deletion of *thiC* were constructed using previously described methods (Datsenko & Wanner, 2000). The resulting

Table 1. *S. enterica* strains and plasmids

All strains and plasmids were generated for this study or are part of laboratory stock. Parental plasmids have been previously described: pSU19 (Bartolome *et al.*, 1991), pBAD30 (Guzman *et al.*, 1995). Plasmid pTYB1 was purchased from New England Biolabs.

Strain	Genotype
DM7060	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc)</i>
DM7080	Δ <i>araCBAD</i>
DM7185	Δ <i>araCBAD thiC1137::Kan</i>
DM7293	Δ <i>araCBAD thiC1137::Kan/pMD26</i>
DM7296	Δ <i>araCBAD thiC1137::Kan/pMD29</i>
DM7301	Δ <i>araCBAD thiC1137::Kan/pBAD30</i>
DM7305	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) apbC69::MudJ</i>
DM7329	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) apbE64::MudJ</i>
DM7330	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) gshA102::MudJ</i>
DM7331	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) iscA2::MudJ</i>
DM7332	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) rseC::Kan</i>
DM7708	Δ <i>araCBAD thiC1137::Kan/pMD48</i>
DM7806	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) zxx8077::Tn10d(Cm)</i>
DM7807	<i>purG3111 purE3043 stm4068-6::Tn10d(Tc) zxx8077::Tn10d(Cm) yggX*</i>
DM8000	Wild-type
DM8109	Δ <i>araCBAD thiC1137::Kan/pMD55</i>
DM8819	Δ <i>araCBAD thiC1137::Kan/pMD81</i>
Plasmid	Vector and insert
pMD5	pTYB1; <i>thiC</i> (chitin-binding domain fusion)
pMD18	pSU19(Cm ^R); <i>thiC</i>
pMD26	pBAD30(Ap ^R); <i>thiC</i>
pMD29	pBAD30(Ap ^R); <i>thiC</i> (C-terminal truncation)
pMD48	pBAD30(Ap ^R); <i>thiC</i> (E324K)
pMD55	pBAD30(Ap ^R); <i>thiC</i> (R548D)
pMD81	pBAD30(Ap ^R); <i>thiC</i> (E324K, R548D)

*This strain contains a mutation that eliminates expression of the *yggX* gene and is expected to affect the promoter

deletion of *thiC* was determined not to result in significant polarity as judged by the nutritional requirement (i.e. HMP alone) of the strain.

Expression and purification of ThiC. *E. coli* strain ER2566 (New England Biolabs) was transformed with pMD5, inoculated into 1 l LB containing 50 µg ampicillin ml⁻¹, grown at 37 °C to OD₆₅₀ 0.6, induced with IPTG (0.4 mM) and incubated at 20 °C for an additional 6 h. Cells were harvested by centrifugation and disrupted using a French pressure cell (103 500 kPa). Purification of ThiC was performed using chitin beads (New England Biolabs) according to the manufacturer's instructions. Metal analysis by inductively coupled plasma mass spectrometry (ICP-MS) of the purified protein was performed by the University of Wisconsin-Madison Soil and Plant Analysis Lab. Fe–S cluster reconstitution was attempted by reducing 22 µM ThiC with 5 mM DTT under anoxic conditions, followed by the addition of 180 µM ferrous ammonium sulfate and 180 µM sodium sulfide. The UV–visible spectrum of the protein sample was monitored over the course of 2 h incubation at 25 °C.

Immunoblot hybridization. Western blot analysis was performed according to the method of Harlow & Lane (1999). Protein concentration was determined with the BCA Protein Assay Kit (Pierce).

Polyclonal rabbit antibodies against ThiC were generated at the University of Wisconsin Animal Care Unit. Proteins were visualized by using horseradish peroxidase conjugated to anti-rabbit secondary antibody (Promega) and the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

RESULTS

Efficient Fe–S cluster biosynthesis/repair is required to convert AIR to HMP

Mutations in five loci (*iscA*, *apbC*, *apbE*, *rseC* and *gshA*) have been shown to compromise Fe–S cluster metabolism (Skovran & Downs, 2000; Skovran *et al.*, 2004). In strains that fail to accumulate YggX (these strains do not accumulate any YggX by Western blot analysis due to an unidentified mutation not in the *yggX* coding sequence), lesions in either of these loci cause a requirement for thiamine that reflects a need for both the HMP and THZ moieties (Gralnick *et al.*, 2000; Skovran & Downs, 2000). In the

presence of *yggX* expression these strains are prototrophic (Skovran *et al.*, 2004). However, a *purF* deletion in combination with a lesion in any of the five loci establishes a thiamine requirement that is unaffected by *yggX*, and can be satisfied by the HMP moiety alone (data not shown). Thus YggX accumulation can suppress the HMP requirement caused by these lesions only when PurF is present. This result suggests that either a distinct mechanism of disrupting HMP synthesis exists in the two strain backgrounds, or there is incomplete suppression by YggX such that an HMP requirement is uncovered by reducing flux through the purine biosynthetic pathway.

The use of strain DM7060 (*purG purE stm4068*) and its derivatives to classify the target of defects in HMP synthesis has been described (Dougherty & Downs, 2004). Lesions in *iscA*, *apbC*, *apbE*, *rseC* and *gshA* were transduced into strain DM7060. The resulting five strains and the parental strain (DM7060) were grown in glucose adenine medium with AIRs as the source of HMP. Results from these studies are shown in Table 2 and indicate that in medium containing thiamine, the strains had similar doubling times. At a concentration of 100 nM, AIRs was limiting for HMP synthesis as indicated by the slightly longer doubling time of the parental strain (DM7060) compared to when thiamine was added. At this concentration of AIRs, growth of the mutant strains was severely reduced. Only the *iscA* mutant strain had a measurable growth rate when 100 nM AIRs was provided as the source of HMP. When AIRs was provided in excess, the doubling time of all strains decreased. In the presence of 1 µM AIRs, all but the *rseC* mutant grew with a doubling time similar to wild-type, consistent with transport and phosphorylation of AIRs functioning in all strains. From these data it was concluded that the thiamine requirement caused by mutations affecting Fe–S cluster metabolism resulted from impaired conversion of AIR to HMP. Thus, disruption of Fe–S cluster metabolism joined reduced pantothenate synthesis (Allen *et al.*, 2002; Dougherty & Downs, 2004) as a process that affects the conversion of AIR to HMP.

Superoxide generated by paraquat induces a thiamine auxotrophy in *S. enterica*

Supplementation of bacterial growth media with the herbicide paraquat has been shown to increase the concentration of superoxide radicals (Hassan, 1984). Physiological studies with *E. coli* mutants lacking superoxide dismutase activity have demonstrated that superoxide stress induces auxotrophy for multiple amino acids (Carlioz & Touati, 1986). Specifically, these strains require branched-chain (Kuo *et al.*, 1987), sulfur-containing (Benov *et al.*, 1996) and aromatic amino acids (Benov & Fridovich, 1999). Because all of these studies were conducted with strains auxotrophic for thiamine, a paraquat effect on thiamine biosynthesis would not have been detected. Based on the connection between Fe–S cluster biosynthesis/repair and thiamine biosynthesis described here, the nutritional requirements imposed by superoxide stress were re-examined in *S. enterica*.

As expected, in minimal glucose medium supplemented with 1 µM paraquat, wild-type *S. enterica* (DM8000) had impaired growth (Fig. 2). The inhibition of growth was partially alleviated by the addition of thiamine, indicating that paraquat had compromised thiamine biosynthesis. Complete restoration of growth was accomplished by the addition of branched-chain amino acids to the medium (data not shown), as expected due to the previously described sensitivity of dihydroxy-acid dehydratase to oxidative damage (Flint *et al.*, 1993). Significantly, both THZ and HMP were required to generate the level of growth allowed by thiamine. Neither THZ nor HMP alone alleviated the growth inhibition, indicating that each branch of the pathway independently contained a target for superoxide toxicity. While the specific target of paraquat in the HMP pathway has not been defined, the target in the THZ pathway is thought to be ThiH (Martinez-Gomez *et al.*, 2004).

Consistent with the above finding, expression of YggX increased the efficiency of the conversion of AIR to HMP-P. Strains lacking YggX have been shown to have an increased

Table 2. Mutations that affect Fe–S cluster metabolism reduce the efficiency of the conversion of AIR to HMP-P

Strain	Relevant genotype	Growth in the presence of*:		
		100 nM AIRs	1 µM AIRs	10 nM thiamine
DM7060	Parental	121 ± 8	80 ± 2	85 ± 9
DM7305	<i>apbC</i>	NG	90 ± 2	80 ± 7
DM7329	<i>apbE</i>	NG	97 ± 27	97 ± 9
DM7330	<i>gshA</i>	NG	102 ± 22	84 ± 13
DM7331	<i>iscA</i>	182 ± 37	86 ± 8	70 ± 9
DM7332	<i>rseC</i>	NG	137 ± 18	63 ± 8

*Growth in liquid media was assessed as described previously (Petersen *et al.*, 1996) using no-carbon E medium with 11 mM glucose, 0.4 mM adenine, and the indicated concentration of AIRs or thiamine. Growth is reported as doubling time (min) ± SD (three independent cultures). NG, No growth.

Fig. 2. Growth in the presence of paraquat results in a thiamine requirement for *S. enterica*. Representative growth curves are shown for strain DM8000 grown in minimal glucose medium (filled circles); with 1 μ M paraquat (empty squares); with 1 μ M paraquat and 100 nM thiamine (filled squares); with 1 μ M paraquat, 100 nM THZ, and HMP (filled triangles); with 1 μ M paraquat and HMP (empty triangles), or with 1 μ M paraquat and 100 nM THZ (empty circles).

level of superoxide damage occurring as measured by spontaneous mutation frequency and SoxR reporter activity (Gralnick & Downs, 2001; Skovran *et al.*, 2004). Isogenic strains derived from strain DM7060 (*purG purE stm4068*), differing only at the *yggX* locus, were constructed and growth was monitored with AIRs as the source of HMP. Strains lacking YggX grew more poorly than wild-type when limiting concentrations of AIRs were provided as the source of HMP. This result indicated that these strains convert AIR to HMP-P less efficiently than strains with a wild-type *yggX* locus (Fig. 3), consistent with a target for oxidative stress being involved in the HMP branch of thiamine biosynthesis.

Conserved cysteine residues are required for ThiC function *in vivo*

A simple explanation for the above growth data would be the presence of a labile Fe-S cluster in an enzyme involved in the conversion of AIR to HMP. While no canonical motif for an Fe-S cluster ligand is present in the ThiC sequence, the motif C(S/T)MCXXXXC⁵⁸⁹ near the C-terminus of *S. enterica* is conserved in every ThiC homologue representing over 100 diverse organisms (see Supplementary Fig. S1, available with the online version of this paper). An additional cysteine residue ~90 amino acids from the above motif (C494) is also absolutely conserved. Plasmids containing wild-type *thiC* or one of four mutant alleles, with each of the four cysteines individually changed to alanine, under the control

Fig. 3. A *yggX* null mutation decreases the efficiency of the conversion of AIR to HMP-P. Representative growth curves are shown for strains DM7806 (*purG purE stm4068 zxx8077::Tn10d(Cm)*, filled symbols) and DM7807 (*purG purE stm4068 zxx8077::Tn10d(Cm) yggX*, empty symbols) grown in minimal glucose adenine medium with 100 nM AIRs and 100 nM THZ (circles) or 10 nM thiamine (squares).

of the P_{BAD} promoter were constructed. These plasmids were transformed into a strain of *S. enterica* carrying an insertion-deletion mutation in *thiC*, such that the plasmid-encoded allele was the only source of *thiC*. The remaining genes involved in thiamine biosynthesis were functional on the chromosome. When provided as the only source of ThiC, none of the four mutant proteins supported growth in the absence of thiamine under either aerobic or anaerobic conditions (data not shown). Under these conditions, a plasmid-encoded wild-type allele of *thiC* supported full growth. While not conclusive, these data are consistent with these cysteine residues being involved in coordinating an Fe-S cluster in ThiC.

There is, as yet, no biochemical evidence for the presence of an Fe-S cluster in ThiC. Metal analysis by ICP-MS of ThiC purified under aerobic conditions showed that only very small amounts of iron were associated with the purified protein [$0.11 \text{ mol Fe (mol ThiC)}^{-1}$]. Reconstitution of the putative Fe-S cluster in ThiC under anoxic conditions was unsuccessful; no evidence for coordination of an Fe-S cluster was seen in the UV-visible spectrum of ThiC under these conditions (data not shown).

Sequence analysis of *thiC* homologues indicates distinctions between aerobes and anaerobes

Sequence comparison showed that ThiC homologues from strict anaerobes and cyanobacteria were significantly shorter

than those from aerobes and facultative anaerobes (see Supplementary Fig. S1, available with the online version of this paper). In the former case, the predicted proteins lack up to 150 amino acids at the N-terminus and approximately 30 amino acids at the C-terminus. In addition to these truncations, six residues that appeared to have the same distribution as the truncations between aerobes and anaerobes were identified. These residues include E324K, C363M,

F371P, N405S, I485A and R548D (*Salmonella* numbering; residue in aerobes preceding residue in anaerobes).

Mutant variants of ThiC distinguish structural features required for aerobic function

Plasmids containing wild-type *thiC* or various mutant alleles were constructed and transformed into a *thiC* mutant strain

Fig. 4. Plasmids containing mutant alleles of *thiC* complement a *thiC* mutant under anaerobic, but not aerobic growth conditions. Representative growth curves are shown for different conditions (a–d) and six different plasmids. In each case, a deletion of the *araCBAD* locus and a non-polar insertion in *thiC* are present in the chromosome. Shown are strains DM7293 (Δ *araCBAD thiC1137::Kan/pMD26*(wt), filled diamonds), DM7296 (pMD29(C term truncated), filled squares), DM7301 (pBAD30, filled triangles), DM7708 (pMD48(E324K), filled circles), DM8109 (pMD55(R548D), empty diamonds), and DM8819 (pMD81(E324K, R548D), empty circles). All strains were grown in minimal glycerol medium with 1 mM arabinose; 25 mM sodium nitrate was added to standing cultures. (a) Shaking cultures, no additions. (b) Shaking cultures, 100 nM thiamine. (c) Standing cultures, no additions. (d) Standing cultures, 100 nM thiamine.

as described above. The growth of the resulting strains was monitored under both aerobic and anaerobic respiration conditions and data are shown in Fig. 4. Three components of the sequence that differed in phylogenetic distribution were targeted: (1) N-terminal sequences, (2) C-terminal sequences and (3) single internal residues. A plasmid containing *thiC* encoding an N-terminal truncation (missing residues 2–148) failed to accumulate protein as detected by Western blotting (data not shown) and was not tested further. Strains containing each of the remaining constructs accumulated protein (data not shown). The plasmid encoding a C-terminally truncated (missing residues 599–631) ThiC protein allowed thiamine-independent growth under anaerobic but not aerobic conditions. Site-directed mutagenesis of *Salmonella thiC* encoded on plasmid pMD18 was performed to change each of the six residues discussed above to the corresponding amino acid prevalent in anaerobic organisms. Two of the six mutant alleles (E324K and R548H) allowed thiamine-independent growth under anaerobic but not aerobic conditions (Fig. 4). The other four alleles allowed wild-type growth under all conditions tested (data not shown). A plasmid containing both mutations described above (E324K and R548H) was generated by site-directed mutagenesis. This plasmid failed to complement a *thiC* null mutant under aerobic or anaerobic conditions (Fig. 4).

It was formally possible that the growth above reflected a lower cellular thiamine requirement under anaerobic growth conditions. In this scenario, if the altered proteins were less active, they might provide sufficient thiamine to allow growth under anaerobic, but not aerobic conditions. To address this possibility, the concentration of thiamine required for growth of a *thiC* mutant strain was titrated under both aerobic and anaerobic conditions where glycerol (25 mM) was provided as carbon/energy source and nitrate (25 mM) or oxygen was provided as an electron acceptor. These data from this experiment (Fig. 5) are most consistent with a similar requirement for thiamine under the two conditions used. While absolute yield when cells are grown anaerobically is notably less than aerobically, there was no significant difference in the pattern of growth as a function of thiamine concentration under the two conditions.

DISCUSSION

This work was initiated to better understand the connection between Fe-S cluster metabolism and the biosynthesis of the pyrimidine moiety of thiamine. The data presented here show that strains defective in Fe-S cluster metabolism are compromised specifically in the conversion of AIR to HMP-P. HMP synthesis is sensitive to oxidative stress generated by the presence of paraquat, and YggX accumulation has a positive effect on the efficiency of the conversion of AIR to HMP-P. These data indicate that incomplete suppression by YggX is the most likely explanation for the phenotypic difference observed for the effect of mutations disrupting Fe-S cluster metabolism in different strain

backgrounds (i.e. *purF* vs *purF*⁺). The fact that the presence of YggX affects the efficiency of AIR to HMP conversion when this step is physiologically isolated argues against mechanistic differences between high and low purine flux conditions.

The presence of possible ligands for an Fe-S cluster in ThiC, and the identification of alleles of *thiC* that encode proteins that function under anaerobic but not aerobic growth conditions are consistent with a model in which ThiC requires an Fe-S cluster to be biochemically active. To date, no biochemical evidence has been presented demonstrating the presence of an Fe-S cluster in ThiC. Purified ThiC has not been found to contain significant amounts of iron. It is possible that the putative Fe-S cluster is not stable under the purification conditions that have been used to date, a possibility consistent with the loss of activity upon purification of ThiC (Lawhorn *et al.*, 2004). Reconstitution of a putative

Fig. 5. Assay of thiamine (B1) requirement under aerobic (a) and anaerobic conditions (b). Growth was assayed by measuring OD₆₅₀ after 24 h incubation at 37 °C in minimal glycerol medium with 25 mM sodium nitrate and 1 mM arabinose. Data are shown for DM7080 (Δ *araCBAD*, filled bars) and DM7185 (Δ *araCBAD thiC1137::Kan*, empty bars). Each point is the mean $\pm$ SD of three independent cultures.

Fe-S cluster in ThiC with iron and sulfide under anoxic conditions has also been unsuccessful. This procedure has been successfully used in this laboratory to reconstitute Fe-S clusters in several other proteins. While it is formally possible that ThiC is not able to be reconstituted under these conditions due to some biochemical difference in the protein or cluster, these data may suggest that ThiC does not require an Fe-S cluster for its function. In a different scenario, an as-yet-unidentified protein required for the conversion of AIR to HMP-P may contain an Fe-S cluster. The requirement for a gene product in addition to ThiC for HMP-P synthesis was suggested by, and could explain, recent biochemical data using a cell-free system (Lawhorn *et al.*, 2004) and data from extensive genetic studies (Dougherty & Downs, 2004; Downs & Petersen, 1994; Skovran & Downs, 2000).

The finding that a variant ThiC missing the final 29 amino acids is functional under anaerobic but not aerobic growth conditions leads to the hypothesis that the C-terminal region is required to protect the protein from oxidative damage. This hypothesis is not without precedent. Many organisms use pyruvate:ferredoxin oxidoreductase (PFOR), which contains a thiamine pyrophosphate cofactor and multiple Fe-S clusters, to catalyse the oxidative decarboxylation of pyruvate (Charon *et al.*, 1999). Most of the characterized PFOR enzymes are highly sensitive to oxygen; however, PFOR isolated from *Desulfovibrio africanus* was found to be stable in the presence of oxygen (Pieulle *et al.*, 1995). *D. africanus* PFOR contains a C-terminal extension of approximately 60 amino acids which contains two cysteine residues. Reduction of the protein with dithioerythritol or deletion of the C-terminal extension resulted in a protein with increased sensitivity to oxygen and increased activity (Pieulle *et al.*, 1997). Analysis of the crystal structure confirmed the presence of a disulfide bond between the two cysteines of the C-terminal extension which blocks access to the proximal [4Fe-4S] cluster (Chabriere *et al.*, 1999).

Our current working model is that ThiC contains an oxygen-sensitive component, which may or may not be an Fe-S cluster, required for its function in the conversion of AIR to HMP-P, and that this component becomes more sensitive to oxygen when certain structural features are disrupted. In this model, an additional gene product containing an Fe-S cluster is required for the conversion of AIR to HMP-P. The hypothesized additional gene product (ThiX) required for HMP synthesis has not been identified despite extensive genetic analysis. However, the increasing body of knowledge on this conversion and the connections to other pathways may facilitate the design of successful genetic screens for the identification of *thiX*. The lack of a simple, sensitive activity assay for AIR to HMP-P conversion has hampered biochemical studies of this step of thiamine biosynthesis. The description of *in vitro* reconstitution of HMP synthesis by Lawhorn *et al.* (2004) has provided an important starting point for assay development and extended previous labelling data.

A number of different cellular processes that affect the efficiency of conversion of AIR to HMP-P have now been identified. These data indicate that, *in vivo*, the complex chemical rearrangement that occurs when AIR is converted to HMP-P is affected directly or indirectly by numerous cellular pathways. Analysis of this, and other integrated metabolic pathways will continue to provide insight into mechanisms used to create and maintain a robust and efficient cellular metabolism.

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