

## *Salmonella enterica* Strains Lacking the Frataxin Homolog CyaY Show Defects in Fe-S Cluster Metabolism In Vivo

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**In *Salmonella enterica*, the *isc* operon contains genes necessary for the synthesis of Fe-S clusters and strains lacking this operon have severe defects in a variety of cellular processes. Other cellular loci that impact Fe-S cluster synthesis to a lesser extent have been described. The *cyaY* locus encodes a frataxin homolog, and it is shown here that lesions in this locus affect Fe-S cluster metabolism. When present in combination with other lesions, mutations in *cyaY* can result in a strain with more severe defects than those lacking the *isc* locus.**

Iron sulfur (Fe-S) clusters are a component of various cellular proteins and have diverse roles in metabolism (reviewed in references 14, 15, and 17). Significant progress has been made in identifying the components and the biochemistry involved in the synthesis of Fe-S clusters (reviewed in reference 25). Many of these components are structurally and functionally conserved throughout all kingdoms of life, emphasizing the similarity of this process throughout biology (22, 40).

The small protein frataxin was first identified as the missing protein in patients with Friedreich's ataxia, a progressive cardio- and neurodegenerative disease resulting from abnormal iron homeostasis and oxidative damage (5–8, 27, 36). Subsequently, frataxin was suggested to have a role in Fe-S cluster assembly, due to the cooccurrence of frataxin with the Isc Fe-S cluster assembly proteins (22, 37) and the finding that loss of the frataxin homolog in *Saccharomyces cerevisiae* resulted in phenotypes indicative of defects in Fe-S cluster assembly (1, 12, 16).

Biochemical, structural, and biophysical studies with purified protein have determined that proteins of the frataxin family have an affinity for iron (4, 26, 28, 38). In particular, Bou-Abdallah et al. demonstrated that under anaerobic conditions, the bacterial ortholog CyaY forms a tetramer and binds two ferrous ions per monomer with weak affinity (2).

To our knowledge, there has been a single report addressing the in vivo consequences of a *cyaY* mutation in a prokaryotic system (24). The authors concluded that loss of CyaY did not detectably alter the metabolism of the cell (24) and suggested that CyaY in bacteria may have a different function than in eukaryotes. An alternative interpretation is that the function(s) of CyaY can be performed by other gene products in the cell. In our work on metabolic integration, a number of loci with weak and possibly indirect effects on the metabolism of Fe-S clusters in *Salmonella enterica* were identified (35). The defects associated with lesions in these genes are often detectable only in the presence of other lesions and/or under nonstandard

growth conditions. This study addresses the possibility that lesions in *cyaY* could result in detectable phenotypes if present in combination with other specific lesions. We demonstrate that *cyaY* mutations in *S. enterica* can result in severe metabolic defects, which are exacerbated or only detected when other mutations are present.

**Loss of CyaY affects Fe-S cluster metabolism in *S. enterica*.** An insertion mutation conferring kanamycin resistance was constructed in the *cyaY* gene of *S. enterica* by linear replacement followed by appropriate confirmation (11). Strains defective in up to three loci (*apbC*, *yggX*, and *cyaY*) were generated. YggX is a small protein (91 amino acids) implicated in the protection of the cell from oxidative stress (20) and has recently been shown to affect the consequences of other lesions impacting Fe-S cluster metabolism (35) and weakly bind iron (10). ApbC is a member of the MinD protein family (29, 34) and is implicated in Fe-S cluster metabolism in the three domains of life (23, 31, 33).

In several studies, the activity of succinate dehydrogenase (SDH), an Fe-S enzyme, has been used to assess the status of Fe-S cluster biosynthesis/repair (32–34). Table 1 shows that a *cyaY* lesion had no significant effect on the SDH activity in the strain (strain DM7644 versus strain DM8000). When both *cyaY* and *yggX* were defective (DM7643), SDH activity was significantly decreased, dropping to the level caused by a lesion in the *isc* locus (DM7220) (35). As previously reported (20), strains lacking only *yggX* were indistinguishable from the wild-type parent (data not shown). The addition of an *apbC* mutation to either the single (*cyaY*) or double (*cyaYyggX*) mutant had no further effect on the level of SDH (DM7642 and DM7641). Thus, by this assay, a lesion in *cyaY* was additive with a *yggX* lesion, and the resulting strain was unaffected by a lesion in *apbC*.

**Thiamine auxotrophy is exacerbated by lesions in *cyaY*.** A class of mutant loci that indirectly inhibit thiamine synthesis via disruption of Fe-S cluster metabolism has been described previously (19, 34). In general, the thiamine auxotrophy of these mutant strains requires loss of the *yggX* locus and can be eliminated by addition of iron to the medium (35). Strains lacking *cyaY*, with or without a *yggX* mutation, had no detectable requirement for thiamine. However, under conditions where the *apbCyggX* double mutant was prototrophic (i.e., with excess iron in the medium), a lesion in *cyaY* eliminated

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TABLE 1. Loss of CyaY activity impacts the activity of the Fe-S cluster enzyme SDH<sup>a</sup>

Strain	Relevant genotype	SDH activity <sup>b</sup>	Relative activity <sup>c</sup>
DM8000	Wild type	34,840 ± 159	1.00
DM7220	<i>iscS</i>	10,804 ± 102	0.31
DM7644	<i>cyaY</i>	32,645 ± 271	0.93
DM7643	<i>cyaY yggX</i>	8,537 ± 232	0.24
DM7642	<i>apbC cyaY</i>	31,809 ± 113	0.90
DM7641	<i>yggX apbC cyaY</i>	9,679 ± 250	0.27

<sup>a</sup> Strains were grown in nutrient broth to an optical density at 650 nm of 0.5. Crude cell-free extracts were generated by sonication as previously described (35). In all cases, loci indicated for relevant genotypes were inactivated by insertion.

<sup>b</sup> SDH activity was measured as described previously (34) and is reported as a specific activity ( $\Delta A_{600}/\text{min}/\text{mg}$  protein [average  $\pm$  standard deviation]).

<sup>c</sup> Relative activity was obtained by dividing the activity of the indicated strain by the activity of the wild-type parent (DM8000).

growth in the absence of thiamine. A representative experiment in Fig. 1 shows that the combination of three lesions results in a strict requirement for thiamine. These data showed that only the triple mutant was defective in thiamine synthesis, implying that the function of just one of the three loci was needed for prototrophic growth in this medium. One interpretation of these results is that the CyaY protein is required to allow iron to suppress the lesion in *yggX*. This scenario is consistent with the general roles proposed for each of these proteins in the cell and the finding that each can interact with iron (2, 10).

**Sensitivity to paraquat is increased by *cyaY* mutations.** Strains compromised in Fe-S cluster metabolism have increased sensitivity to the redox cycling compound paraquat (35). Data from

TABLE 2. Increased sensitivity to paraquat caused by a *cyaY* mutation<sup>a</sup>

Strain	Relevant genotype	Final OD <sub>650</sub> in NB containing paraquat at <sup>b</sup> :		
		0 $\mu\text{M}$	40 $\mu\text{M}$	80 $\mu\text{M}$
DM7226	Wild type	0.97 $\pm$ <0.01	0.92 $\pm$ 0.01	0.92 $\pm$ 0.03
DM7225	<i>yggX</i>	0.98 $\pm$ 0.01	0.74 $\pm$ 0.12	0.25 $\pm$ 0.01
DM7644	<i>cyaY</i>	1.03 $\pm$ 0.01	0.93 $\pm$ 0.02	0.74 $\pm$ 0.06
DM7307	<i>apbC</i>	0.97 $\pm$ 0.01	0.73 $\pm$ 0.04	0.62 $\pm$ 0.01
DM7642	<i>apbC cyaY</i>	1.00 $\pm$ 0.01	0.37 $\pm$ 0.01	0.33 $\pm$ 0.01
DN7306	<i>yggX apbC</i>	0.98 $\pm$ <0.01	0.35 $\pm$ 0.01	0.32 $\pm$ <0.01
DM7643	<i>yggX cyaY</i>	1.00 $\pm$ 0.01	0.34 $\pm$ 0.01	0.31 $\pm$ 0.02
DM7641	<i>yggX apbC cyaY</i>	1.00 $\pm$ 0.01	0.22 $\pm$ 0.01	0.19 $\pm$ 0.01

<sup>a</sup> Overnight cultures grown in rich media were subcultured with 150  $\mu\text{l}$  added to 5 ml nutrient broth (NB) containing 0, 40, or 80  $\mu\text{M}$  paraquat as indicated. Cultures were incubated with shaking at 37°C, and growth was monitored by measurement of optical density at 650 nm (OD<sub>650</sub>) with a Bausch and Lomb Spectronic 20. The final OD<sub>650</sub> reported was after 24 h, since absorbance for all strains had reached a plateau by that time. In all cases, loci indicated for relevant genotypes were inactivated by insertion.

<sup>b</sup> Values reflect the averages ( $\pm$  standard deviations) of two independent cultures.

growth experiments in Table 2 show that in the absence of paraquat all cultures reach a similar final density but that with  $\geq 40$   $\mu\text{M}$  paraquat in the medium the strains reach different growth densities. The *cyaY* mutation alone did not significantly affect the sensitivity of a strain to paraquat, but in combination with a lesion in *yggX*, it resulted in dramatically increased sensitivity. An *apbC* mutation also increased the sensitivity of the *cyaY* strain to paraquat. The data in Table 2 indicated that when any two of the three loci tested (*apbC*, *yggX*, and *cyaY*) were defective, a similar increase in sensitivity to paraquat occurred. Lesions in all three loci further decreased growth of the strain when  $\geq 40$   $\mu\text{M}$  paraquat

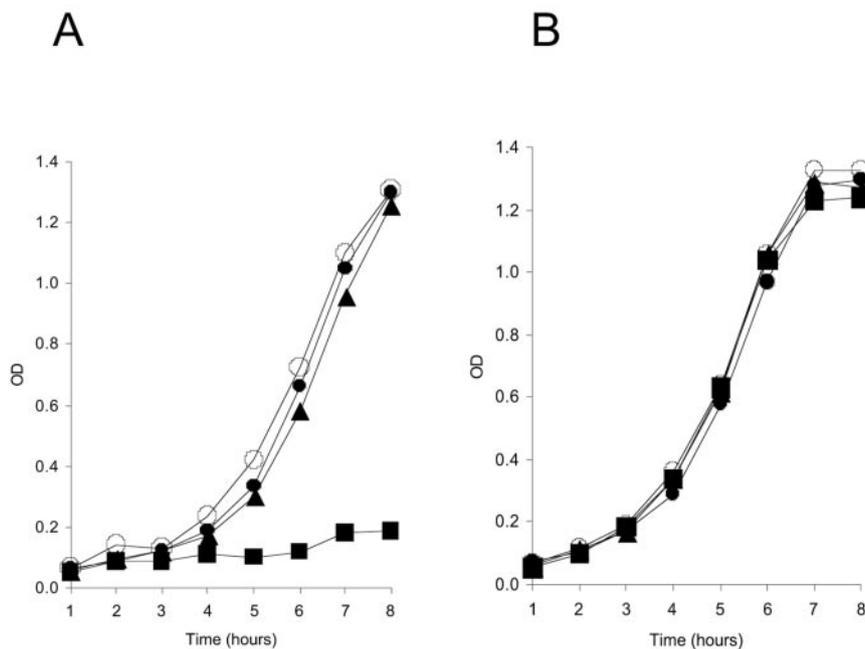


FIG. 1. The *yggX apbC cyaY* mutant has a stringent thiamine requirement. Overnight nutrient cultures were subcultured and grown at 37°C with shaking, as described in reference 35. Growth of strains was monitored over time by measurement of absorbance (optical density [OD] at 650 nm) with a Bausch and Lomb Spectronic 20. Strains DM7226 (wild type) (●), DM7306 (*apbC yggX*) (▲), DM7644 (*cyaY*) (○), and DM7641 (*apbC yggX cyaY*) (■) were grown in (A) minimal medium supplemented with adenine (5 mM) and ferric chloride (20  $\mu\text{M}$ ) or (B) the same medium as described for panel A but with thiamine (50  $\mu\text{M}$ ).

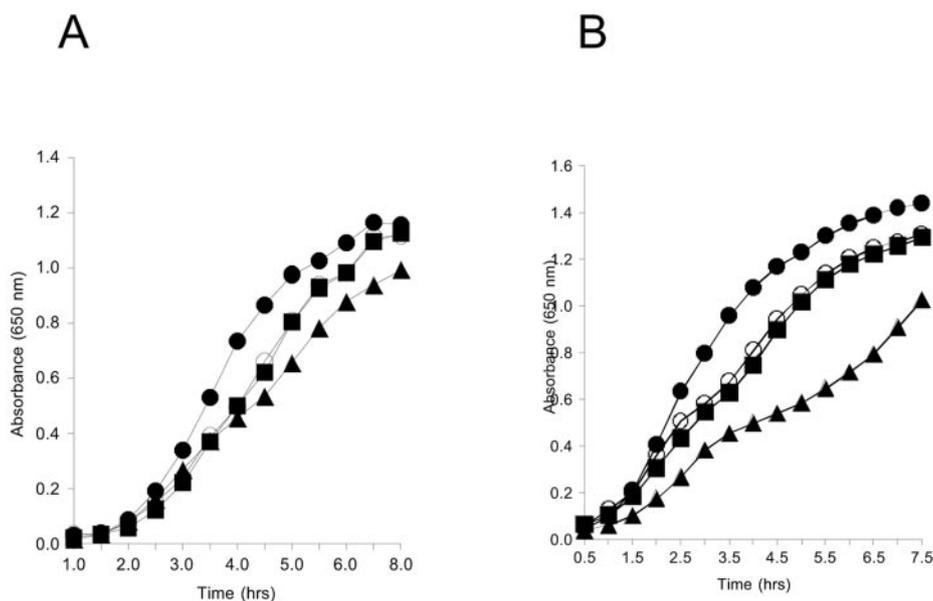


FIG. 2. Distinct growth pattern of a *yggX apbC cyaY* mutant in rich medium. Strains were grown and subcultured as described in the legend for Fig. 1. Growth in (A) nutrient broth and (B) Luria broth is shown for strains DM7641 (*apbC yggX cyaY*) (▲), DM7642 (*apbC cyaY*) (○), DM7643 (*yggX cyaY*) (■), and DM7644 (*cyaY*) (●). hrs, hours.

was present. These results were consistent with a partially overlapping function of the YggX, CyaY, and ApbC proteins in the processes that impact sensitivity to superoxide.

**Growth results detect a complex phenotype caused by a *cyaY* lesion.** To quantify an apparent growth defect of strains lacking *cyaY*, *yggX*, and an additional locus (*gshA*, *apbC*, or *apbE*), growth in both nutrient broth and Luria broth (LB) was monitored. From these growth studies, the following conclusions could be made: when grown in Luria broth, and to a much lesser extent in nutrient broth, the triple mutants grew with a pattern distinct from that of the parental strains. A representative experiment is shown in Fig. 2. As illustrated, the severity of the defect was dependent on the number of mutations present in addition to the *cyaY* lesion. No growth defect was detected with a strain lacking both *apbC* and *yggX*, emphasizing the contribution of the *cyaY* lesion. Addition of various carbon sources to the medium (glucose, gluconate, or Casamino Acids) eliminated the unusual growth pattern, while addition of others (citrate, succinate, or fumarate) had no effect. A negative effect of acetate on the cell growth in LB was reminiscent of strains lacking NADH:ubiquinone oxidoreductase (EC 1.6.99.3; NADH dehydrogenase complex I) (30). A comparison of growth patterns of a strain lacking *nuo* (DM4489 [9]) and the triple mutant (*cyaY yggX apbC*) on a number of rich media proved that the growth patterns, while not identical, showed several similarities (data not shown).

The NADH dehydrogenase complex I (NDH-I) is a 14-subunit complex that contains nine Fe-S clusters of various arrangements (18, 21) and is the product of genes in the *nuo* locus (13). Enzyme assays tested whether the severity of the growth phenotype observed with LB correlated with reduced NDH-I activity. Data from a representative experiment are shown in Table 3, indicating that strains lacking *cyaY* had a reduced level of NDH-I activity. Under these conditions, a

strain lacking *nuo* had undetectable activity (data not shown). However, the data eliminated a simple correlation between this activity and growth phenotype. An *isc* mutant (DM7220) did not have the distinct growth pattern of the triple (*apbC cyaY yggX*) mutant (data not shown), and yet the two strains had similarly low levels of NDH-I activity. Rather, the *isc* mutant had a reduced growth rate, as previously noted (32, 34). The strain with the second-most-severe defect in activity (*apbC yggX*) had no growth defect. Conversely, double mutants that lacked *cyaY* and one of the other loci of interest (e.g., *apbC*) showed an intermediate growth defect (Fig. 2) yet had enzyme activity similar to that of the *cyaY* mutant, which had no detectable growth defect.

TABLE 3. Multiple lesions in combination with a *cyaY* mutation reduce Nuo activity<sup>a</sup>

Strain	Relevant genotype	Nuo activity <sup>b</sup>	Relative activity <sup>c</sup>
DM8000	Wild type	23,508 ± 136	1.00
DM7220	<i>iscS</i>	3,990 ± 397	0.17
DM7644	<i>cyaY</i>	11,420 ± 518	0.48
DM7642	<i>apbC cyaY</i>	13,631 ± 810	0.58
DM7643	<i>cyaY yggX</i>	17,790 ± 513	0.76
DM5986	<i>yggX apbC</i>	6,146 ± 1239	0.26
DM7641	<i>yggX apbC cyaY</i>	3,375 ± 213	0.14

<sup>a</sup> Strains were grown in nutrient broth to an optical density at 650 nm of 0.5. Crude cell-free extracts were generated by sonication as has been described previously (35). The activity of the *iscS* strain reflects the level of cluster synthesis maintained in a strain lacking the major Fe-S cluster biosynthetic operon. All loci listed for relevant genotypes were inactivated by insertion.

<sup>b</sup> Nuo activity was measured as described previously, by using D-amino-NADH to distinguish it from the cytoplasmic activity encoded by the *ndh* gene (39), and is reported as a specific activity ( $\Delta A_{600}/\text{min}/\text{mg}$  protein [average ± standard deviation]).

<sup>c</sup> Relative activity was obtained by dividing the activity of the relevant strain by the activity of the wild-type parent.

On tryptone swarm plates, strains lacking *nuo* fail to form the inner growth band that reflects chemotaxis to aspartate (30). Relevant strains were inoculated into tryptone swarm agar as described in reference 30. After incubation at 37°C for ~5 h, of the seven strains tested, only DM4489 (*nuo*) was unable to form the interior ring of growth (data not shown). This result, consistent with the above findings, indicated that the defect in *nuo* was not the cause of the growth pattern detected for strains lacking *cya* in combination with other loci. Thus, a growth phenotype that is specific for a *cyaY* lesion and is amenable to genetic analysis was identified.

**Endogenous Fe(II) levels are not altered in *cyaY* mutants.** Yeast mutants lacking the frataxin homolog Yfh1 displayed a 10-fold increase in mitochondrial iron levels (1), while analysis of *Escherichia coli cyaY* mutants showed no aberrant accumulation of endogenous iron (24). Transcription of the Fur reporter *entB* (as measured by  $\beta$ -galactosidase activity from a *lac* fusion) should reflect the amount of Fe(II) that the cell senses as available for cellular processes (reviewed in reference 3). A *lac* fusion in the *entB* gene was used to monitor Fur activity. When monitored for various stains, transcription of the *entB* fusion was unaffected by the presence of CyaY (data not shown). While not definitive, this result is consistent with the finding that in *E. coli* loss of *cyaY* did not alter the endogenous levels of Fe(II) (24).

**Conclusions.** Similarly to the report of Li et al. with *E. coli* (24), these studies failed to define a significant phenotypic consequence of eliminating the *cyaY* locus in an otherwise wild-type strain. However, when strains multiply defective in loci involved in Fe-S cluster metabolism are used, phenotypes specific to *cyaY* lesions emerge. Here it was demonstrated that strains lacking *cyaY* in combination with one or two other loci are more defective than the parental strains when a number of parameters are monitored. The three loci tested (*apbC*, *yggX*, and *cyaY*) appear to interact differently, depending on the process being monitored, as judged by the different synergies displayed for different phenotypes.

By surveying the defects of various multiple mutant strains, we have defined conditions in which a single mutation (i.e., *cyaY*) can make a measurable difference in phenotype (i.e., when the parental background is *apbC yggX*). This result provides a phenotype that can be attributed to the lesion in the *cyaY* locus. As such, this work lays a foundation for future genetic and biochemical efforts to tease apart the physiological role of CyaY in the context of other gene products involved in Fe-S cluster metabolism in *Salmonella enterica*.

Results herein are consistent with a complex integration of the three loci investigated, and possibly others, in fine-tuning Fe-S metabolism of the cell. These data illustrate the synergy of complex cellular systems such as Fe-S cluster homeostasis and emphasize the need to consider multiple interacting loci when performing genetic and biochemical studies to dissect the system.

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