Differential effects of isc operon mutations on the biosynthesis and activity of key anaerobic metalloenzymes in *Escherichia coli*

Monique Jaroschinsky,†‡ Constanze Pinske† and R. Gary Sawers*

**Abstract**

*Escherichia coli* has two machineries for the synthesis of FeS clusters, namely Isc (iron–sulfur cluster) and Suf (sulfur formation). The Isc machinery, encoded by the *iscRSUA-hscBA-fdx-iscX* operon, plays a crucial role in the biogenesis of FeS clusters for the oxidoreductases of aerobic metabolism. Less is known, however, about the role of ISC in the maturation of key multi-subunit metalloenzymes of anaerobic metabolism. Here, we determined the contribution of each iscoperon gene product towards the functionality of the major anaerobic oxidoreductases in *E. coli*, including three [NiFe]-hydrogenases (Hyd), two respiratory formate dehydrogenases (FDH) and nitrate reductase (NAR). Mutants lacking the cytochrome c desulfurase, IscS, lacked activity of all six enzymes, as well as the activity of fumarate reductase, and this was due to deficiencies in enzyme biosynthesis, maturation or FeS cluster insertion into electron-transfer components. Notably, based on anaerobic growth characteristics and metabolite patterns, the activity of the radical-S-adenosylmethionine enzyme pyruvate formate-lyase activase was independent of IscS, suggesting that FeS biogenesis for this ancient enzyme has different requirements. Mutants lacking either the scaffold protein IscU, the ferredoxin Fdx or the chaperones HscA or HscB had similar enzyme phenotypes: five of the oxidoreductases were essentially inactive, with the exception being the Hyd-3 enzyme, which formed part of the H₂-producing formate hydrogenlyase (FHL) complex. Neither the frataxin-homologue CyaY nor the IscX protein was essential for synthesis of the three Hyd enzymes. Thus, while IscS is essential for H₂ production in *E. coli*, the other ISC components are non-essential.

**INTRODUCTION**

Membrane-associated oxidoreductase complexes in aerobic and anaerobic micro-organisms are usually multi-subunit enzymes with a modular architecture [1]. These enzymes minimally comprise a catalytic large subunit, an electron-transferring small subunit and a membrane-anchor subunit. Depending on the function of the respective enzyme complex, the large subunit can be attached to either the outer or the inner side of the cytoplasmic membrane. Common to most of these enzymes is a dependence on iron–sulfur (FeS) clusters, which are among the most ancient cofactors involved in electron-transfer processes [2, 3]. In anaerobic oxidoreductases, FeS clusters function in electron transfer, or as cofactors of the ancillary enzymes required for biosynthesis of the catalytic site cofactor of the oxidoreductases [4]. FeS clusters are also involved in oxygen sensing, for example in the case of the fumarate-nitrate regulator (FNR) transcription factor, which controls the expression of many of the genes encoding these anaerobic oxidoreductases [5]. A further function ascribed to the FeS cluster in radical SAM enzymes is substrate binding and activation [2]. Radical SAM enzymes belong to an ancient family of FeS enzymes with roles in anaerobic DNA synthesis, cofactor biosynthesis and formate metabolism [2].

With very few exceptions, FeS cluster cofactors are found in all species on Earth [6]. Consequently, it is important to understand FeS biogenesis, particularly for anaerobic oxidoreductases, because many bacteria live in anaerobic environments, and because the *in vivo* analysis of FeS cluster biogenesis in relation to anaerobic oxidoreductases is relatively poorly understood.

It is well established that three dedicated mechanisms for FeS biogenesis in bacteria exist: Isc (iron-sulfur cluster) [7], Suf (sulfur formation [8]) and components of the Nif
(nitrogen fixation [2]) system. However, not all bacteria have a simultaneous coding capacity for all three systems. While the Nif system is, in the main, dedicated to the assembly of FeS clusters in proteins specifically associated with nitrogen-fixing bacteria, the Isc and Suf systems are more broadly distributed in the bacterial kingdom [9]. The Suf system is widespread in bacteria, but is mainly functional under iron and oxidative stress conditions and will not be considered further here because it is not involved directly in the biogenesis of the anaerobic oxidoreductases described in this study [10–12]. In contrast, the Isc system is found in many proteobacterial genera and has been particularly well studied in *Escherichia coli*. Isc is required for the biosynthesis of active enzymes of aerobic metabolism [13], and recent studies have analysed the role of some components of the ISC system in the biogenesis of the enzymes for anaerobic metabolism, particularly the [NiFe]-hydrogenases (Hyd) in *E. coli* [10, 12, 14].

The key proteins of the Isc system are encoded by the *iscR-SUA-hscBA-fdx-iscX* operon (Fig. 1) [15]. FeS clusters are formed on a backbone complex comprising the cysteine desulfurase, IscS, and the scaffold protein, IscU, upon which a [2Fe-2S] cluster is assembled [16]. The completed FeS clusters are then transferred to IscA, which is classified as an A-type carrier (ATC) protein. IscA and its homologue ErpA deliver the completed FeS clusters to the apo-enzyme substrates [10, 11, 17, 18]. Ferredoxin, encoded by *fdx*, interacts specifically with IscS [19, 20], where it acts as one of the electron donors required to facilitate reduction of the sulfane sulfur (S^2\-), derived from cysteine, to sulfide (S^2\-). It also facilitates the reductive coupling of two [2Fe-2S] clusters formed on IscU to yield a [4Fe-4S] cluster [19]. HscA and HscB are homologues of Hsp70 and its co-chaperone and they regulate the ATP-dependent transfer of FeS clusters to acceptor proteins [21–23]. The other components of the ISC machinery have an ancillary function. IscR is a transcriptional regulator that is responsive to the cellular demand for FeS clusters [24], while the precise role of IscX remains unclear. Recent evidence suggests, however, that IscX assists the frataxin homologue CyaY, a putative candidate for iron delivery in FeS cluster biogenesis [25]. Nonetheless, an *iscX* mutant lacks a clear phenotype in aerobically growing *E. coli* cells [15].

In this study, our aim was to analyse the effects of in-frame deletions in individual genes of the *isc* operon on Hyd enzyme function, and on the activities of the two molybdoseleno formate dehydrogenases (FDH) O and N, along with nitrate reductase (NAR). Together, FDH-N and NAR comprise an anaerobic respiratory pathway that allows *E. coli* to respire with nitrate [26, 27].

Hyd enzymes allow many anaerobic micro-organisms to use H\_2 as an energy source, where the multi-subunit Hyd enzyme usually forms a membrane-associated complex delivering electrons to the quinone pool. Alternatively, these enzymes function in the reverse direction to produce H\_2 gas as a means of off-loading excess reducing equivalents, which accumulate during anaerobic heterotrophic metabolism [28]. While the catalytic subunit of these Hyds lacks a FeS cluster, the synthesis of the NiFe(CN)\_6CO cofactor in the active site of these enzymes requires at least one FeS enzyme (HypD) to enable its biosynthesis [29, 30]. The small subunit, on the other hand, has up to three FeS clusters and these govern whether the enzyme’s primary physiological function is in proton reduction or H\_2 oxidation [30].

*E. coli* has the coding capacity for four Hyd, but synthesizes three under standard anaerobic laboratory conditions [30, 31]. Recent studies demonstrated that mutants lacking the scaffold protein IscU, IscA or Fdx proteins had only low hydrogenase activity. Analysis of the H\_2-oxidizing Hyd-1 and -2 enzymes in these mutants revealed that they lacked a small subunit, possibly as a result of enhanced metal-free apo-protein degradation, and hence were enzymatically inactive [10]. The [NiFe]-cofactor of the large subunit of Hyd-1 was also incompletely matured, probably due to impaired HypD activity [10]. Mutants deleted in the *iscA* and *erpA* genes retained some activity of the H\_2-producing Hyd-3 in the formate hydrogenlyase (FHL) complex [12]. These results indicate that IscA and IscU have different influences on Hyd biosynthesis.

IscA and ErpA are also important for biosynthesis of the formate–nitrate respiratory pathway, comprising the FDH-N
and NAR multi-subunit oxidoreductases [11]. However, there is no information about the role of any other Isc components, either in Hyd enzyme biosynthesis or in that of the respiratory FDH and NAR enzymes. Therefore, in this study we performed a systematic analysis of the effects of deleting each of the isc operon genes on the activities of six anaerobic oxidoreductase complexes and on their biosynthesis. Our study provides the first detailed description of enzyme phenotypes that can be used to assess the in vivo roles of individual Isc components in anaerobic FeS biogenesis.

METHODS

Strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Strains were grown routinely at either 30 °C or 37 °C on LB-agar plates [32]. All growth experiments were performed at 37 °C as anaerobic standing liquid cultures in stoppered bottles, unless otherwise indicated. The growth medium in the experiments was either M9 minimal medium (containing ×1 M9 salts [32], including 2 mM MgSO$_4$, 0.1 mM CaCl$_2$, 3 μM thiamine hydrochloride and trace element solution SL-A [33]) and 0.4% (w/v) glucose or tryptone yeast extract phosphate (TYEP) medium [10], including 0.4% (w/v) glucose, as indicated in the appropriate figure legends. When required for strain or plasmid selection, ampicillin (100 μg ml$^{-1}$), chloramphenicol (12 μg ml$^{-1}$) or kanamycin (50 μg ml$^{-1}$) were added to the growth medium. Where indicated, M9 minimal medium was supplemented by the addition of all amino acids to a final concentration of 0.2% (w/v). When used, nitrate was added to a final concentration of 5 mM.

Construction of strains

To construct the strains listed in Table 1, mutant alleles were introduced into E. coli wild-type MC4100 using the method of P1kc-mediated phage transduction after amplifying the phage on the appropriate donor strains, as described [34]. When necessary, the Kan$^R$-cassette introduced into the MC4100 strains as part of the mutated allele was removed by transforming the strain with pCP20 encoding a Flp recombinase, as described [35]. Mutants were subsequently tested for sensitivity to kanamycin.

Determination of formate-hydrogenlyase activity

Quantitative determination of FHL complex activity was performed exactly as described [36]. Hydrogen production was measured by gas chromatography using whole cells from 100 ml cultures grown in M9 minimal medium or TGYEP medium.

In order to test semi-quantitatively for the ability of plasmid-encoded genes to complement hydrogen production, strains were grown anaerobically in TGYEP medium for 14 h and the absolute hydrogen content in the head-space of the tubes was determined using gas chromatography as described [37]. Briefly, the H$_2$ content of the 10 ml gas phase of a TGYEP overnight culture was measured by sampling 200 μl in a Shimadzu GC-2010. The system was equipped with a packed column (Shin Carbon Micropacked column ST80/100). The carrier gas was N$_2$ with a flow of 13.9 ml min$^{-1}$, the injector was kept at 140 °C, the column was maintained at 110 °C and the TCD detector was maintained at 150 °C and 40 mA.

Preparation of cell-free extracts and determination of enzyme activities

Cells from anaerobically grown cultures were harvested at an OD$_{500}$ of approximately 0.6 by centrifugation at 4000 g for 10 min at 4 °C. Cells were either frozen and stored at −20 °C or suspended directly in 1% (v/v) of the culture volume of 50 mM MOPS buffer pH 7.0. Cells were lysed at 4 °C by sonication (30 W power for 5 min with 0.5 s pulses) and unbroken cells and cell debris were removed by centrifugation for 15 min at 10 000 g and 4 °C. The supernatant was used as the cell-free crude extract. Total hydrogenase enzyme activity was measured according to [38], FDH-O and -N were determined according to [39], NAR activity was determined according to [40] and FRD activity was determined according to [41]. All assays were performed in 50 mM MOPS buffer, pH 7.0. One unit of activity was defined as the oxidation of 1 μmol of substrate per min and assays were performed in triplicate with at least three biological replicates. Determination of β-galactosidase enzyme activity was carried out as described [34]. Protein concentration of crude extracts was determined as described [42], with bovine serum albumin as standard.

Determination of enzyme activity after non-denaturing polyacrylamide gel electrophoresis

Non-denaturing PAGE was performed using 7.5% (w/v) polyacrylamide gels (pH 8.5), which included 0.1% (w/v) Triton-X100, exactly as described [38]. Samples of cell-free crude extract (usually 25 μg of protein, as indicated in the figure legends) were incubated with 5% (w/v) Triton X-100 prior to application to the gels. Gels were stained for Hyd, FDH-N/O or NAR enzyme activities as described [38, 39]. In all cases, the buffer used was 50 mM MOPS pH 7.0 and the gas atmosphere was 100% hydrogen or 100% nitrogen for the determination of Hyd and FDH/NAR activity, respectively [10, 11].

Organic acid analysis

Analysis of organic acids in culture supernatants was performed as described [37]. Cells were grown anaerobically in M9-glucose medium as described above for 14 h and cells were removed by centrifugation at 15 000 g for 15 min at 4 °C. The cell-free supernatants were then passed through a 0.22 μm sterile filter and 20 μl was applied to an Aminex HPX-87H (300 7.8 mm) ion-exchange column attached to a Hitachi Elite LaChrom HPLC apparatus. The flow-rate was 0.5 ml min$^{-1}$ and 5 mM sulfuric acid was used as the mobile phase. Organic acid retention peaks were recorded using EZChrom software and quantified by comparison with the absorption of known amounts of organic acid standards [37].
Polyacrylamide gel electrophoresis and immunoblotting

In general, western blot analysis was performed using aliquots of 50 µg of protein derived from cell-free crude extracts, unless stated otherwise. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5, 10 or 12.5 % (w/v) gels [43]. Transfer of the polypeptides from the gels to nitrocellulose membranes was performed as described [44]. Antibodies raised against Hyd-1 and Hyd-2 (1 : 20 000; a kind gift from F. Sargent), PfB (1 : 3 000; [45]), FNR (1 : 10 000; a kind gift from J. Green) and NAR (1 : 10 000; a kind gift from A. Magalon) were used. Secondary antibody conjugated to horseradish peroxidase was obtained from Bio-Rad. Visualization was performed by enhanced chemiluminescent reaction (Stratagene). Where indicated, the relative intensity of the protein

<table>
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<th>Strains</th>
<th>Genotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
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<td>DHP-F2</td>
<td>Like MC4100 but ΔhypF</td>
<td>[69]</td>
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<tr>
<td>BW25113</td>
<td>F- Δ(aradD-arab)567 ΔlacZ4787(rrnB-3) λ- rph-1Δ(rhA2-rhB)568 hsdR514</td>
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</tr>
<tr>
<td>MC4100RSN</td>
<td>MC4100 Δ narG::lacZ KanR</td>
<td>[72]</td>
</tr>
</tbody>
</table>

**Plasmids**

- pJET1.2: Blunt-end cloning vector, containing placUV5 promoter; Amp
- piscS: pJET1.2 containing iscS; Amp
- psufS: pJET1.2 containing sufS; Amp
- phscB: pJET1.2 containing hscB; Amp
- phscA: pJET1.2 containing hscA; Amp
- pfdx: pJET1.2 containing fdx; Amp
- piscX: pJET1.2 containing iscX; Amp

*National BioResources Project (NIG, Japan): E. coli [70].
amount from immunoblots was quantified using ImageJ from the National Institutes of Health [46].

RESULTS AND DISCUSSION

Of the isc operon gene products, only the cysteine desulfurase IscS is essential for hydrogen production

A set of strains, each carrying a defined deletion mutation in each gene of the isc operon, was prepared (see Table 1 and the Methods section). Anaerobic growth of these strains in M9-glucose minimal medium revealed growth rates (μ) for the wild-type MC4100 of 0.48 h⁻¹ (Fig. S1a, available in the online Supplementary Material). The hscB and iscU mutants had similar growth rates of 0.34 h⁻¹, while the iscS mutant grew more slowly (μ=0.17 h⁻¹). Remarkably, the fdx mutant grew most slowly (μ=0.1 h⁻¹) and exhibited an unusual form of diauxic growth, possibly caused by growth on the casamino acids (Fig. S1a). Similar growth profiles for all mutants were observed in TGYEP rich medium (Fig. S1b). These data indicate that the lack of ferredoxin has a much more pervasive effect than the lack of cysteine desulfurase IscS.

The effect of these individual isc operon mutations on the ability of the respective strains to produce hydrogen gas catalysed by the Hyd-3-dependent FHL complex was examined after anaerobic growth in M9-glucose minimal medium (Fig. 2a).

Analysis of whole cells revealed that while the wild-type MC4100 had an activity of 38 mU mg⁻¹ of protein, the hypF deletion mutant DHP-F2 had no measurable H₂-producing activity. A mutation in the gene encoding the negative regulator of operon expression IscR resulted in an activity increase relative to the wild type of approximately 10%. This was not an unexpected result based on the fact that IscR is a negative regulator of the expression of the isc, hya and hyb operons [24, 47]. Like the negative control DHP-F2, the iscS mutant was also devoid of H₂-producing (FHL) activity (Fig. 2a). Cells of mutants with deletions in iscA, hscB, iscU or fdx exhibited activities in the range of 20–25% that of the wild-type MC4100. Under these growth conditions, mutation of the iscA gene, encoding the FeS cluster carrier protein IscA, retained an activity of approximately 50% of that of the wild-type, which is significantly higher than the 16% previously reported after growth in rich medium [12]. These data suggest that reliance on H₂ production is possibly stronger during growth in minimal medium.

The IscX protein has been suggested to have a regulatory role in FeS cluster biogenesis, and deletion of the iscX gene reduced H₂ production by approximately 40% (Fig. 2a). It should be noted that the hydrogenase phenotypes of the isc operon mutants could be complemented by introduction of the corresponding missing gene on a plasmid (Fig. S2).

CyaY is a frataxin homologue that binds iron with low affinity [48, 49]. CyaY also interacts directly with IscS [50] and has been suggested to act as a delivery system for the Fe ions in FeS clusters. Because it can also form a complex with IscU [51] and exhibits interplay with IscX [25], it has been suggested to function by modulating FeS cluster trafficking. Mutation of the cyaY gene failed to show any negative phenotype with regard to H₂ production (Fig. S3a). This result suggests that a further protein (or further proteins) to deliver iron for anaerobic ISC-dependent FeS biosynthesis must exist. YggX is such a candidate protein because it has been proposed to function as an alternative route for iron delivery [52]. YggX is a small protein that, like CyaY, binds iron with low affinity [53], and it has been proposed to function in either the delivery of iron for FeS cluster biosynthesis or the repair of oxidatively damaged clusters [52, 54]. We therefore constructed two strains to test the effects of a yggX mutation: one strain has a deletion in yggX and the other lacks both the cyaY and yggX genes. Both strains were analysed for the effects of these mutations on hydrogen production, as determined by measuring H₂ in the head-space after overnight (18 h) fermentative growth (Fig. S3a). Neither the yggX nor the cyaY yggX double mutant showed significantly reduced levels of H₂ production by the FHL complex.

Assessment of hydrogenase enzyme activities in the isc gene mutants

In vitro assay of total hydrogenase enzyme activity can be determined in cell-free extracts using H₂ as the electron donor and benzyl viologen (BV) as the electron acceptor [38]. This measurement determines the combined contributions of Hyd-1, Hyd-2 and Hyd-3 to cellular hydrogen-oxidizing activity. As anticipated, a strain lacking cysteine desulfurase, encoded by iscS, lacked hydrogenase activity and thus had a phenotype similar to the negative control DHP-F2 (ΔhypF) (Fig. 2b). Surprisingly, the iscU mutant also exhibited low total hydrogenase activity, suggesting that this activity was nevertheless sufficient to account for the low FHL activity observed in the strain (see Fig. 2a). All of the other mutants revealed total hydrogen-oxidizing activity that was in a similar range of between 10 and 35% of wild-type activity (Fig. 2b). The activity of the extracts derived from the hscA, hscB and fdx mutants was in a similar range, equating to approximately 10–15% of wild-type activity (Fig. 2b). Both the iscX and cyaY mutants exhibited total hydrogen-oxidizing activity of roughly 35% of wild-type activity. These latter findings demonstrate a clear function for CyaY in hydrogen oxidation.

To gauge the qualitative effect of the different isc operon gene mutations on the activity of the three Hyd enzymes, a hydrogenase activity assay was performed after the separation of protein complexes by native-PAGE (Fig. 3a). Four controls were used in this experiment. The wild-type strain MC4100 (positive control) revealed strong activity bands due to the hydrogen-oxidizing Hyd-1 and Hyd-2 enzymes, a less intense activity band due to Hyd-3 and a very weak hydrogen-oxidizing activity attributable to a side activity of FDH-N/O [39]. The first negative control was DHP-F2 (ΔhypF). Of the two further negative controls, one (labelled ΔhyE) lacked the catalytic large subunit of Hyd-3 of the FHL complex, and the second was a mutant (ΔselB) that lacked all selenoenzymes, thus

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identifying the activity due to FDH-N/O. The selB mutant had a strongly reduced Hyd-3 activity due to destabilization of the FHL complex caused by the lack of the FDH-H catalytic component of the complex [55].

The iscR mutant showed slightly more intense Hyd-1 and Hyd-2 activity bands (Fig. 3a), which was expected due to the de-repression of the respective structural operons hya and hyb[47]. Extracts derived from the iscS, iscU, iscA, hscB, hscA and fdx mutants failed to reveal any clear hydrogenase-related activity band. Note that the phenotypic consequences of individual isc gene mutations on hydrogenase activity could be complemented by transformation with a plasmid encoding the corresponding missing gene (Fig. S3b), indicating that none of the deletion mutations had polar effects on downstream genes. The low level of Hyd enzyme activity in these isc operon mutants was unlikely to be due solely to transcriptional effects, because the FeS-containing transcription factor FNR has little impact on hya (encoding Hyd-1) and hyb (encoding Hyd-2) operon expression [14, 56], strongly suggesting that the main effects of the isc gene mutations were on the level of enzyme maturation.

Cultures grown anaerobically in M9-glucose minimal medium supplemented with all amino acids (see the Methods section for details) revealed stronger hydrogenase activity bands after native-PAGE (Jaroschinsky, Pinske and Sawers, unpublished observations). Analysis of the different isc operon mutants after growth with amino acid supplementation revealed that extracts derived from the iscU, hscB, hscA and fdx mutants all had a similar hydrogenase activity phenotype, whereby Hyd-3 activity was clearly observed, along with the hydrogen-oxidizing activity associated with FDH-N/O (Fig. 3b). Surprisingly, supplementation of amino acids to the growth medium also restored some Hyd-1 and Hyd-2 enzyme activity to the iscA mutant.

In contrast to what was observed with Hyd activity in the iscS, iscU, iscA, hscB, hscA and fdx mutants, extracts from the iscX (Fig. 3b), cyaY and yggX (Fig. 3c) mutants revealed clearly discernible activity bands for Hyd-1, Hyd-2 and Hyd-3.

Hyd-1 and Hyd-2 catalytic subunits are differentially processed in the isc mutants

Findings from an earlier study [14] showed that in a fdx mutant the catalytic subunit of Hyd-2 was processed, indicating successful insertion of the NiFe(CN)_2CO cofactor [29], while the catalytic subunit of Hyd-1 remained unprocessed. This explains the absence of Hyd-1 enzyme activity...
in extracts of the fdx mutant. Both Hyd-1 and Hyd-2 enzymes lacked their associated FeS cluster-containing small subunit, explaining why Hyd-2 activity was absent [14]. To assess the possible causes of the lack of Hyd-1 and Hyd-2 enzyme activity in the other isc operon mutants, the processing status of HyaB, the catalytic subunit of Hyd-1, and the presence of HybO, the small subunit of Hyd-2, were analysed by western blotting (Fig. 4). The results for HyaB clearly show that while the iscS mutant lacked both processed and unprocessed forms of HyaB, extracts derived from the iscU, hscB and hscA mutants only had the unprocessed species, as observed for the extract of the fdx mutant as well as the negative control hypF (Fig. 4a). The sample derived from the iscA mutant showed processed HyaB subunits, congruent with the activity observed in Fig. 3(c). All of the remaining mutants that were analysed (iscR, iscX and cyaY; Fig. 4a) showed processed HyaB, again in accord with the presence of Hyd-1 enzyme activity (compare to Fig. 3). Quantitation of the levels of the catalytic subunit (HyaB) of Hyd-1 (Fig. 4a) revealed that while the iscX and cyaY mutants have similar protein levels to the wild-type, the iscA, hscB and hscA mutants retained between 40 and 60 % of the protein level while the fdx mutant retained approximately 30 % and the iscS and iscU mutants retained 7 and 15 %, respectively (Fig. S4a). We do not currently understand why there is such a strong reduction in HyaB polypeptide levels in the iscS, iscU and fdx mutants; however, due to the fact that FNR is not the main regulator of hya operon gene expression [14, 56], we assume that the HyaB polypeptide is subject to enhanced degradation in these mutant backgrounds.

Immunological analysis of the Hyd-2 large (HybC) and small subunit (HybO) species revealed that, while clearly present in lower abundance than the wild-type, all mutants showed the presence of the processed species of the catalytic subunit (Fig. 4b). In contrast, the Hyd-2 small subunit, HybO, could not be detected in extracts of the iscA, hscB, hscA or fdx mutants. This strongly suggests that the absence of Hyd-2 enzyme activity in these mutant backgrounds was due to a lack of the electron-transferring small subunit. We have shown previously that if the HybO subunit is absent either through deletion of the hybO gene or through degradation of the subunit when the FeS clusters cannot be inserted [10], then no hydrogen:BV oxidoreductase activity can be measured for this enzyme.

Taken together, the results presented so far demonstrate that IscS is essential for all hydrogenase enzyme activity in the E. coli cell. The iscU, fdx, hscA and hscB mutants all exhibited very similar hydrogenase phenotypes, whereby they lacked hydrogen-oxidizing enzyme function. This finding is in accord with the proposed strong overlap in the biochemical functions of all four gene products during FeS cluster biosynthesis [9]. Importantly, while mutations in these genes also reduced overall FHL complex activity, none of these genes was essential for hydrogen production. Moreover, while the Hyd-2 catalytic subunit, HybC, was present in its processed form, indicating cofactor insertion [29], that of Hyd-1, HyaB, was not. These data clearly indicate that HypD, which carries a [4Fe-4S] cluster that is essential for hydrogenase maturation function [29], was at least partially active in the iscU, fdx, hscA and hscB mutants. The fact that HyaB was not processed in the mutants indicates that differential maturation of all three hydrogenase large subunits occurs, despite the HybC subunit receiving its cofactor, as indicated by the observed processed form of the subunit. As has been observed previously, the maturation of Hyd-3 takes precedence over the maturation of the other hydrogenases during iron limitation [57]. Two possible explanations for the lack of HyaB processing in the mutants are conceivable: the less likely explanation is that a further step in the
The maturation of HyaB requires a functional FeS protein, which is dependent on the ISC system; alternatively, and more plausibly, when limiting levels of active HypD are present [10], the cofactor–scaffold complex might interact more efficiently with HybC than with HyaB during cofactor insertion, resulting in preferential processing of HybC.

**Analysis of other anaerobic iron–sulfur cluster-containing oxidoreductases**

Three major respiratory oxidoreductases requiring FeS clusters for both their biosynthesis and function are nitrate reductase (NAR) and the FDH-O and FDH-N isoenzymes [11, 26, 39]. All three enzymes are abundant and active in anaerobic nitrate-grown cells; all three also require the molybdenum cofactor for their activity [11, 40]. Measurement of NAR enzyme activity in extracts derived from some of the isc operon mutants was determined after anaerobic growth with nitrate as the electron acceptor (Table 2). The results revealed that NAR activity was barely detectable in the ∆iscS, hscB, hscA and ∆fdx mutants when compared with wild-type MG1655. Each mutation could only be partially complemented by introduction of the missing gene on a plasmid (Table 2). Verification that NAR activity was absent.

![Western blot analysis of H2-oxidizing enzymes Hyd-1 and Hyd-2.](image)

<table>
<thead>
<tr>
<th>Strain*</th>
<th>A5 Nar (U mg protein⁻¹)</th>
<th>% Nar of parental</th>
<th>A5 FDH-N (U mg protein⁻¹)</th>
<th>% FDH-N of parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>1.23±0.68</td>
<td>100</td>
<td>0.25±0.17</td>
<td>100</td>
</tr>
<tr>
<td>∆iscS</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
<td>0.04±0.04</td>
<td>15</td>
</tr>
<tr>
<td>∆iscS+piscS</td>
<td>0.27±0.04</td>
<td>26</td>
<td>0.26±0.23</td>
<td>99</td>
</tr>
<tr>
<td>∆hscB</td>
<td>0.03±0.01</td>
<td>3</td>
<td>0.03±0.02</td>
<td>12</td>
</tr>
<tr>
<td>∆hscB+phscB</td>
<td>0.33±0.18</td>
<td>31</td>
<td>0.18±0.08</td>
<td>82</td>
</tr>
<tr>
<td>∆hscA</td>
<td>0.01±0.01</td>
<td>1</td>
<td>0.03±0.02</td>
<td>13</td>
</tr>
<tr>
<td>∆hscA+phscA</td>
<td>0.69±0.28</td>
<td>60</td>
<td>0.32±0.21</td>
<td>135</td>
</tr>
<tr>
<td>∆fdx</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
<td>0.04±0.05</td>
<td>12</td>
</tr>
<tr>
<td>∆fdx+pfdx</td>
<td>0.14±0.03</td>
<td>13</td>
<td>0.07±0.04</td>
<td>44</td>
</tr>
<tr>
<td>∆iscX</td>
<td>0.73±0.26</td>
<td>66</td>
<td>0.30±0.17</td>
<td>127</td>
</tr>
<tr>
<td>∆iscX+piscX</td>
<td>0.61±0.20</td>
<td>57</td>
<td>0.24±0.18</td>
<td>100</td>
</tr>
</tbody>
</table>

*Strains were grown anaerobically in TGYEP medium supplemented with 1 % (w/v) sodium nitrate.

**Table 2. Nitrate reductase and respiratory formate dehydrogenase enzyme activity in various isc mutant strains**
in the \textit{iscS} mutant, and was strongly reduced in the \textit{hscB}, \textit{hscA} and \textit{fdx} mutants, was provided by the electrophoretic separation of membrane protein complexes in native-PAGE followed by specific activity staining (Fig. S5). An \textit{iscX} mutant retained NAR enzyme activity levels that were similar to those observed in the wild type (Tables 2 and S5). Western blotting analysis with antiserum raised against \textit{E. coli} NAR revealed that the level of the catalytic NarG subunit of the NAR enzyme was significantly reduced in the \textit{iscA}, \textit{hscA}, \textit{hscB} and \textit{fdx} mutants, while NarG was absent in extracts derived from the \textit{iscS} mutant (Fig. 5a). Quantitative analysis of the western blot shown in Fig. 5a revealed that the level of NarG was reduced approximately 10-fold in the \textit{iscA}, \textit{hscA}, \textit{hscB} and \textit{fdx} mutants compared with the wild-type (Fig. S4b). Because expression of the \textit{narGHJI} operon encoding NAR is dependent on FNR [27] and the synthesis of an active FeS cluster-containing FNR is dependent on Isc [58], the markedly reduced levels of NAR in the \textit{isc} operon mutants could have resulted from incompletely active FNR. A western blot performed with extracts derived from the \textit{isc} mutants revealed that the level of FNR was unaltered in all mutants (Fig. S6). Incorporation of the FeS cluster into FNR is, however, compromised in \textit{iscS} mutants [13], explaining the lack of detectable NarG polypeptide in the \textit{iscS} mutant (Fig. 5a). Analysis of the expression levels of a single-copy \textit{narG-lacZ} transcriptional fusion in the \textit{fdx} mutant after growth in M9 minimal medium supplemented with 20 mM nitrate revealed a \(\beta\)-galactosidase enzyme activity of 336±38 Miller units, corresponding to a 7.5-fold reduction compared with the expression level of the fusion in the wild-type strain MC4100 (2529±218 Miller units). Reintroduction of the \textit{fdx} gene into the \textit{fdx} mutant restored \textit{nar} operon expression to approximately 50 % the value of the wild-type (1274±420 Miller units). These data indicate that reduced expression of the \textit{nar} operon only partially explains why NAR activity was not completely restored by the \textit{fdx} gene (see Table 2), and suggests that an imbalance in either molybdenum cofactor biosynthesis or the FeS insertion could also account for the low enzyme activity recovery.

The combined activity of the highly related FDH-O and FDH-N enzymes can be determined in a dye-based enzyme assay [39]. The FDH-O enzyme allows \textit{E. coli} cells to couple formate oxidation to either oxygen or nitrate reduction [39, 59]. Analysis of this activity in extracts of the different mutants revealed a similar pattern to that observed for NAR enzyme activity (Table 2); activity was strongly reduced, or absent, in \textit{isc}, \textit{hsc}, \textit{fdx} and \textit{hscA} mutants. Expression of the \textit{fdnGHI} operon encoding FDH-N is also FNR-dependent [27]. In contrast to what was observed for NAR, complementation with the missing gene from the \textit{isc} operon restored total FDH-N/O enzyme activity to levels close to those observed for the wild-type; again the exception was \textit{fdx}, where FDH-N activity was restored to only approximately 50 % of the level of the wild-type (Table 2). Finally, activity staining for FDH-N/O after native-PAGE confirmed these findings (Fig. S5). These results demonstrate that the \textit{isc} operon gene products are required for gene expression, as well as for maturation of active respiratory NAR and FDH enzymes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Western blot analysis of NarG and pyruvate formate-lyase. Samples of crude extracts (25 µg protein) derived from cells grown to the exponential phase in M9 glucose minimal medium supplemented with nitrate were separated by denaturing SDS-PAGE (7.5 % w/v polyacrylamide) and, after transfer to nitrocellulose membrane, the polypeptides were challenged with an antiserum raised against formate dehydrogenase (FDH). The asterisk indicates the migration position of a non-specifically cross-reacting polypeptide that acted as a protein loading control. (a) Samples of crude extracts (25 µg protein) derived from cells grown to the exponential phase in M9 glucose minimal medium were separated by denaturing SDS-PAGE (10 % w/v polyacrylamide) and challenged with an antiserum raised against PflB. The migration positions of the full-length and oxygenolytically cleaved polypeptides are indicated on the right-hand side of each panel. The migration positions of the molecular mass markers are indicated on the left-hand side of the figure. MC4100 represents the wild-type; for details of the other strains shown see Table 1.}
\end{figure}
Activity of the radical-SAM enzyme PflB-activase is independent of the ISC system

A further enzyme whose synthesis is partially dependent on FNR is pyruvate formate-lyase (PflB) [60]. In fnr mutants, PflB enzyme levels are reduced approximately five-fold [60]. Analysis of PflB by western blotting reveals two polypeptides, which correspond to the full-length protein of 85 kDa and a polypeptide of 83 kDa, which lacks 26 amino acids from the C-terminus. The loss of the C-terminal peptide is due to oxygenolytic cleavage of the active, radical-bearing enzyme species, but can be used as an indicator of active enzymes in cells prior to lysis [61]. The oxygen-sensitive radical is located on a glycyl residue at amino acid position 734 on the polypeptide chain, and this radical is specifically introduced by the FeS cluster-containing radical-SAM enzyme PflA [61]; note that expression of the pflA gene is constitutive under both aerobic and anaerobic growth conditions [62]. Western blot analysis of extracts derived from anaerobically grown MC4100 cells with anti-PflB antiserum mainly revealed the faster-migrating polypeptide, indicating the presence of the active radical-bearing PflB enzyme in the cells upon exposure to O$_2$ at the time of cell lysis (Fig. 5b). In contrast, an extract from a pflA mutant, lacking PflA, only revealed the slower-migrating species, indicating that an inactive radical-free enzyme was present in the cells at the time of cell lysis. An extract derived from the iscS mutant revealed a five-fold reduction in total PflB polypeptide levels (Figs 5b and S4c), which correlates well with the reduction in PflB levels observed in an fnr mutant [60]. Importantly, this PflB polypeptide in extracts of the iscS mutant migrated at the position of oxygenolytically cleaved PflB [61], suggesting that the enzyme present in these cells was activated by PflA. Extracts derived from all the other isc operon mutants analysed by western bloting mainly revealed the faster migrating polypeptide, consistent with the presence of active PflB (Fig. 5b and S7).

These findings suggest that even in the absence of IscS, a catalytically active FeS cluster can be inserted into PflA [63]. To test whether PflB was indeed active in these cells, thus demonstrating that the activase PflA was active, we determined the levels of formate in the medium after anaerobic growth in M9-glucose minimal medium of the wild-type MC4100, a pflB mutant DH201 [64] and the iscS mutant (Fig. 6). The results reveal that, after quantification of the peak areas, the supernatant of the iscS mutant contained 11.9±0.6 mM formate, which corresponds to approximately 70% of the level of formate produced by the wild-type (16.8±0.4 mM). In contrast, a pflB mutant produced only 0.3 mM of formate. As PflB is the principal route of formate production by fermentatively growing E. coli cells [30], these results clearly demonstrate that the radical-SAM enzyme PflA is active and capable of introducing the glycyl radical into PflB.

It is notable that the profile of organic acids produced by the iscS mutant revealed a peak of increased intensity corresponding to fumarate (Fig. 6), suggesting impaired activity of fumarate reductase (FRD) in the mutant. Measurement of FRD activity in extracts derived from the iscS mutant indeed revealed that no enzyme activity was detectable, while extracts of the wild-type MC4100 had an activity of 0.77±0.05 µmol for fumarate-reduced min$^{-1}$ mg protein$^{-1}$. Extracts derived from the frdA mutant CP887 (Table 1) also showed no measurable FRD activity. Due to the fact that expression of the frd operon is also FNR-dependent [65], the dependence on a functional IscS enzyme for FRD activity is likely to be also the result of impaired FNR function in the iscS mutant.

Conclusions

Here, we demonstrate that the activities of seven major anaerobic oxidoreductases show significant differences in their dependence on various components of the ISC FeS cluster biosynthetic machinery. The exception to this diversity is the fact that all seven enzymes (Hyd-1, Hyd-2, Hyd-3, FDH-N, FDH-O, FRD and NAR) are completely dependent on the cysteine desulfurase, IscS. The reliance on IscS reflects a similar dependence on this enzyme for several oxidoreductases of aerobic metabolism. This is presumably due to the central importance of cysteine desulfurase as the sulfur donor for the FeS proteins involved in transcriptional control (FNR), cofactor biosynthesis and electron-transfer function.

Surprisingly, IscS is apparently not essential for FeS delivery to the radical-SAM enzyme PflB-activase (PflA), suggesting that there might be another route for FeS assembly that can be used for at least a subset of this enzyme class. This alternative route is unlikely to be via the Suf system because suf mutants are unaffected in either PflB activity or their hydrogen metabolism [10]. Moreover, multi-copy sufS on its own could not complement the anaerobic metabolic defects caused by an iscS mutation on FHL complex activity (Fig. S1). Despite the marked dependence on IscS shown by so many anaerobic enzymes, an iscS mutant still grew comparatively well in both minimal and complex medium (see Fig. S1). This finding indicates that cysteine desulfurase is expendable for basal anaerobic growth during fermentation, which has potentially important evolutionary implications for this mode of metabolism.

The proposed close functional relationship between Fdx and the Hsp-70 homologue HscA and its co-chaperone HscB [9] was substantiated for all of the anaerobic enzymes studied here. Importantly, however, along with the IscU scaffold, all three are non-essential for FHL complex and NAR activities. Despite the activity of this enzyme complex being reduced by around 70%, it is still clearly functional in mutants individually lacking these ancillary proteins. Considering that at least eight [4Fe-4S] clusters are present in the active FHL [30, 66], and there are at least two FeS proteins, MoaA and HypD, necessary for molybdenum and [NiFe]-cofactor biosyntheses, respectively [29, 67], considerable capacity for FeS cluster synthesis is maintained in the absence of key Isc components, including the scaffold IscU.
How this is achieved is an important question that will require further study.

Finally, despite the fact that a mutant lacking both the fra-taxin homologue CyaY and the postulated Fe\textsuperscript{2+} trafficking protein YggX was reduced in H\textsubscript{2}-oxidizing Hyd-2 activity, neither protein was essential for anaerobic FeS cluster biosynthesis.

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Conflict of interest

All authors declare that there are no conflicts of interest.

Ethical statement

Neither animals nor humans were used in the experiments described.

References


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