Levels of control exerted by the Isc iron–sulfur cluster system on biosynthesis of the formate hydrogenlyase complex

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The membrane-associated formate hydrogenlyase (FHL) complex of bacteria like Escherichia coli is responsible for the disproportionation of formic acid into the gaseous products carbon dioxide and dihydrogen. It comprises minimally seven proteins including FdhF and HycE, the catalytic subunits of formate dehydrogenase H and hydrogenase 3, respectively. Four proteins of the FHL complex have iron–sulfur cluster ([Fe–S]) cofactors. Biosynthesis of [Fe–S] is principally catalysed by the Isc or Suf systems and each comprises proteins for assembly and for delivery of [Fe–S]. This study demonstrates that the Isc system is essential for biosynthesis of an active FHL complex. In the absence of the IscU assembly protein no hydrogen production or activity of FHL subcomponents was detected. A deletion of the iscU gene also resulted in reduced intracellular formate levels partially due to impaired synthesis of pyruvate formate-lyase, which is dependent on the [Fe–S]-containing regulator FNR. This caused reduced expression of the formate-inducible fdhF gene. The A-type carrier (ATC) proteins IscA and ErpA probably deliver [Fe–S] to specific apoprotein components of the FHL complex because mutants lacking either protein exhibited strongly reduced hydrogen production. Neither ATC protein could compensate for the lack of the other, suggesting that they had independent roles in [Fe–S] delivery to complex components.

Together, the data indicate that the Isc system modulates FHL complex biosynthesis directly by provision of [Fe–S] as well as indirectly by influencing gene expression through the delivery of [Fe–S] to key regulators and enzymes that ultimately control the generation and oxidation of formate.

INTRODUCTION

Dihydrogen is an electron donor that has had a major impact on the evolution of life on this planet. The reverse reaction, dihydrogen production, is used by many heterotrophic microorganisms as a means of removing excess reducing equivalents when an exogenous electron acceptor is unavailable. These reactions are catalysed by hydrogenases. The enterobacterium Escherichia coli produces hydrogen gas during fermentative growth to offset the acidification of its cytoplasm by formic acid, a major product of glucose oxidation under anaerobic conditions (Sawers et al., 2004). The reaction is catalysed by a large membrane-associated complex termed formate hydrogenlyase (FHL) that disproportionates formic acid into CO₂ and H₂. The catalytic components of the FHL complex comprise a selenocysteine- and bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor-dependent formate dehydrogenase (FDH-H) encoded by fdhF (Zinoni et al., 1986; Boyington et al., 1997), and a [NiFe]-hydrogenase, Hyd-3, which is encoded by the hyc operon (Sauter et al., 1992). The complex also has two integral membrane subunits (HycC and HycD) and three additional subunits, HycB, F and G, which have a function in electron transfer between the catalytic subunits (Fig. 1). An enzyme complex similar to FHL from the archaeon Thermococcus onnurineus has recently been shown to be a formate-driven proton pump (Kim et al., 2010); however, whether the FHL complex of E. coli is also involved in energy conservation remains to be determined.

The biosynthesis of the FHL complex in E. coli is absolutely dependent on formate (Rossmann et al., 1991), which is generated during fermentative growth by the glycol radical enzyme pyruvate formate-lyase (PFL) (Knappe & Sawers, 1990). PfB synthesis is induced under anaerobic conditions
and this induction is under the dual control of the iron–sulphur cluster [Fe–S]-containing FNR transcription factor and the redox-sensing ArcAB two-component system (Sawers & Böck, 1988; Sawers & Suppmann, 1992).

The large subunit of [NiFe]-hydrogenases contains the hetero-bimetallic [NiFe] active site that usually has one CO and 2 CN⁻ ligands bound to the iron atom. The maturation of the [NiFe]-centre requires the action of a specific set of proteins and at least one of these, the HypD protein, is an [Fe–S]-containing protein (Blokesch et al., 2004; Watanabe et al., 2007), although its precise role in the process is still unclear (Böck et al., 2006). A hypD mutant also fails to synthesize an active FHL complex (Sawers et al., 2004; Böck et al., 2006; Forzi & Sawers, 2007).

Electron transfer within the FHL complex requires [Fe–S] and four of the FHL complex components are [Fe–S] proteins (Sauter et al., 1992). HycG is the small subunit of Hyd-3 and has a single [4Fe–4S]. The large subunit of FDH-H, FdhF, also has a [4Fe–4S] (Boyington et al., 1997), while the small subunit of FDH-H, HycB, is predicted to have four [4Fe–4S]. A further ferredoxin-like subunit HycF is also predicted to have multiple [4Fe–4S] (Böhm et al., 1990). The involvement of [Fe–S] in the biosynthesis and activity of the hydrogen-evolving FHL complex is therefore extensive (Fig. 1). While a considerable amount of information is available concerning the biosynthesis of the metallocentres of Hyd-3 (for reviews, see Böck et al., 2006; Eitinger & Mandrand-Berthelot, 2000; Forzi & Sawers, 2007) and of Fdh-F (Sawers et al., 2004), virtually nothing is known about the incorporation of the [Fe–S] into the protein components of the FHL complex.

There are two central systems, referred to as Isc (iron–sulfur cluster) and Suf (sulfur mobilization), which are involved in [Fe–S] biosynthesis in bacteria and eukarya (Ayala-Castro et al., 2008; Johnson et al., 2005; Py & Barras, 2010). In E. coli the Suf system is thought to play an important role in [Fe–S] biosynthesis, particularly during aerobic growth or under oxidative stress conditions, while the Isc system has a function under non-stress conditions (Takahashi & Tokumoto, 2002; Py & Barras, 2010). Both systems comprise components dedicated to [Fe–S] assembly and to the subsequent delivery of the pre-formed cluster to the ultimate apoprotein acceptor (Py & Barras, 2010). In the E. coli Isc system IscU is a central assembly protein,
while the phylogenetically related A-type carrier (ATC) proteins IscA and ErpA are believed to deliver [Fe–S] to specific target apoproteins (Loiseau et al., 2007; Vinella et al., 2006). The IscA and ErpA proteins were recently shown to be involved in biosynthesis of the hydrogen-oxidizing [NiFe]-hydrogenases (Pinske & Sawers, 2012a) and the anaerobic formate–nitrate respiratory pathway of E. coli (Pinske & Sawers, 2012b). The Suf system is not essential for [Fe–S] delivery to these anaerobic enzymes. While the results of these studies have advanced our knowledge on the biosynthesis and delivery of [Fe–S] to anaerobic respiratory pathways, we know essentially nothing about the importance of these [Fe–S] biosynthetic systems for fermentative pathways. Therefore, in this study we wished to address the potential role of the Isc and Suf system is essential for hydrogen production by the FHL complex and reveal that ErpA and IscA have distinct roles in complex assembly.

**METHODS**

### Strains, plasmids and growth conditions.

All bacterial strains and plasmids used in this study are listed in Table 1.

For the preparation of overnight cultures E. coli was grown aerobically in Erlenmeyer flasks filled to maximally 10% of their volume with either LB (Miller, 1972) or TGYEP medium, pH 6.5 (Begg et al., 1977). Cultures were incubated on a rotary shaker (250 r.p.m.) at 37°C. Anaerobic growths were performed at 37°C in sealed bottles filled with anaerobic growth medium under a nitrogen gas atmosphere. When required, the growth medium was solidified with 1.5% (w/v) agar. All growth media were supplemented with 0.1% (v/v) SLA trace element solution (Hormann & Andreesen, 1989). The antibiotics chloramphenicol, kanamycin and ampicillin, when required, were added to the medium at the final concentrations of 12.5 µg ml⁻¹, 50 µg ml⁻¹ and 100 µg ml⁻¹, respectively. Where indicated, L-arabinose was added to the growth medium to 0.2% (v/v).

**Phage transduction and strain construction.** The ΔiscU::KanR allele from JW2513 (Table 1) was transduced into MC4100 using phage P1kc-mediated transduction (Miller, 1972). The KanR cassette of the MC4100 (ΔiscU::KanR) transductant was removed by transforming the strain in question with pCP20 encoding an Flp-recombinase (Cherepanov & Wackernagel, 1995). Mutants were subsequently tested for sensitivity to kanamycin and the strain constructed was called CP1244 (ΔiscU).

Lambda phage constructs were introduced into λatt as described (Simons et al., 1987).

**Subcellular fractionation and determination of enzyme activities.** Cells from anaerobic cultures grown to mid-exponential growth phase (OD600 of approximately 0.8) were harvested by centrifugation at 4000 g for 10 min at 4°C, resuspended in 2–3 ml of 50 mM MOPS buffer pH 7.0 and lysed on ice by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and cell debris were removed by centrifugation for 15 min at 10 000 g at 4°C and the supernatant was used as the crude cell extract. Membrane and soluble fractions were prepared as described (Ballantine & Boxer, 1985). Samples designated cells in Fig. 4 indicate that whole cell samples were collected by centrifugation and either the cell pellets were resuspended directly in SDS sample buffer for Western blot analysis or they were treated with 4% (v/v) Triton X-100 for 30 min on ice prior to being loaded directly onto non-denaturing polyacrylamide gels as described.

**Table 1. Strains, plasmids and phages used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype*</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>F⁻ araD139 Δ(argF-lac)U169 pts25 deoC1 relA1 flbB5301 rplL150⁻</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>DHP-F2</td>
<td>MC4100 ΔhysE 59-629AA; ECK2707</td>
<td>Paschos et al. (2002)</td>
</tr>
<tr>
<td>CP477</td>
<td>As MC4100 but ΔiscA::KanR; ECK2525</td>
<td>Pinske &amp; Sawers (2012b)</td>
</tr>
<tr>
<td>CP1223</td>
<td>As MC4100 but ΔsufA::CmR; ECK1680</td>
<td>Pinske &amp; Sawers (2012b)</td>
</tr>
<tr>
<td>CP971</td>
<td>As MC4100 but ΔhycA::KanR</td>
<td>Pinske et al. (2011)</td>
</tr>
<tr>
<td>CP967</td>
<td>As MC4100 but ΔiscA::KanR ΔhyAI::CmR</td>
<td>This study</td>
</tr>
<tr>
<td>LL401</td>
<td>MG1655 conditional araA::ermA</td>
<td>Loiseau et al. (2007)</td>
</tr>
<tr>
<td>LL402</td>
<td>MG1655 ΔerpA::CmR⁻</td>
<td>Loiseau et al. (2007)</td>
</tr>
<tr>
<td>DV1151</td>
<td>MG1655 ΔerpA::CmR⁻ ΔiscA</td>
<td>Vinella et al. (2009)</td>
</tr>
<tr>
<td>JW2513</td>
<td>BW25113 ΔiscU::KanR; ECK2526</td>
<td>† This study</td>
</tr>
<tr>
<td>CP1244</td>
<td>As MC4100 but ΔiscU</td>
<td>Zinoni et al. (1986)</td>
</tr>
<tr>
<td>FM911</td>
<td>As MC4100 but ΔfdhF srl::ATn10 recA56</td>
<td>Hesslinger et al. (1998)</td>
</tr>
<tr>
<td>RM220</td>
<td>As MC4100 but ΔpflB ΔpflA</td>
<td>Sauter &amp; Sawers (1990)</td>
</tr>
<tr>
<td>234M1</td>
<td>As MC4100 but ΔpflA</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>perpA</td>
<td>pBluescript SK(+) containing erva in BamHI and EcoRI site; AmpR⁺</td>
<td>Pinske &amp; Sawers (2012b)</td>
</tr>
<tr>
<td>pscA</td>
<td>pBluescript SK(+) containing icsA in BamHI and EcoRI site; AmpR⁺</td>
<td>Pinske &amp; Sawers (2012b)</td>
</tr>
<tr>
<td><strong>Phage</strong></td>
<td>ΔfdhF⁻ ΔlacZ</td>
<td>Falke et al. (2010)</td>
</tr>
</tbody>
</table>

*Allele numbers are given for single gene mutants and refer to the K-12 nomenclature.
†National BioResources Project (NIG, Japan): E. coli (Baba et al., 2006).
(Pinske et al., 2012). Protein concentration of subcellular fractions was determined (Lowry et al., 1951) with BSA as standard. Hydrogenase activity was measured according to Ballantine & Boxer (1985) and benzyl viologen-associated formate dehydrogenase activity according to Sawers et al. (1985), except that the buffer used was 50 mM MOPS, pH 7.0. The wavelength used in the hydrogenase enzyme assay was 578 nm and an $E_{600}$ value of 8600 M$^{-1}$ cm$^{-1}$ was assumed for reduced benzyl viologen. One unit of activity corresponded to the oxidation of 1 μmol of hydrogen min$^{-1}$. Experiments were performed minimally three times, and each time, enzyme assays were performed in triplicate. Data are presented with standard deviation of the mean.

Quantitative determination of FHL activity was performed as described (Pinske & Sawers, 2010).

**Determinant of formate concentration in the culture supernatant.** The concentration of formate in the culture supernatant was determined using a Formate Assay kit (Sigma-Aldrich) and assays were performed exactly as described by the manufacturer. Assays were carried out in 96-well plates and after completion of the reaction the absorbance at 450 nm was determined using a Tecan Sunrise Plate-Reader (Tecan GmbH, Salzburg, Austria). Concentrations were determined using the XFluor4 software. Assays were performed minimally three times independently and each sample was analysed in triplicate.

**Polyacrylamide gel electrophoresis and immunoblotting.** Aliquots of 25–50 μg of protein from the indicated subcellular fractions were separated by SDS-PAGE (PAGE) using 7.5 or 10 % (w/v) polyacrylamide (Laemmli, 1970) and transferred to nitrocellulose membranes as described (Towbin et al., 1979). Antibodies raised against HygC (1:3000; Sauter et al., 1992), FdhF (1:5000; Sauter et al., 1992), HygC (1:3000; a kind gift from A. Böck, Munich Germany), PfIB (1:3000; Suppmann & Sawers, 1994) and GroEL (1:5000; Enzo Life Sciences) were used. Secondary antibody conjugated to horse-radish peroxidase was obtained from Bio-Rad. Visualization was done by the enhanced chemiluminescence reaction (Stratagene).

Non-denaturing PAGE was performed using 7.5 % (w/v) polyacrylamide gels pH 8.5 and included 0.1 % (w/v) Triton X-100 in the gels and running buffer (Ballantine & Boxer, 1985). Samples (25 μg of protein) were incubated with 5 % (w/v) Triton X-100 prior to application to the gels. Hydrogenase activity staining was done as described in Ballantine & Boxer (1985), except that the buffer used was 50 mM MOPS pH 7.0.

**RESULTS**

The [Fe–S] assembly protein IscU is essential for formation of an active FHL complex

An iscU mutant has no detectable hydrogen:BV oxidoreductase activity (Table 2), which is in agreement with previous studies (Pinske & Sawers, 2012a). Quantitative assessment of the hydrogen-evolving activity of the iscU mutant CP1244 revealed that it produced no hydrogen (Table 2). Introduction of plasmid pRKISC (carrying ORF1–iscR–iscS–iscU–iscA–hscB–hscA–fdx–ORF3) (Nakamura et al., 1999) into the iscU mutant restored both total hydrogenase activity and hydrogen production (Table 2).

The hydrogen:BV oxidoreductase activity of Hyd-1, Hyd-2 and Hyd-3 can be visualized after non-denaturing PAGE followed by activity staining under an atmosphere of 100 % hydrogen (Pinske et al., 2012). This method was used to assess the ability of pRKISC to restore the activity of Hyd-1 through Hyd-3 to the iscU mutant CP1244 (Fig. 2). Activity bands corresponding to Hyd-1, Hyd-2 and Hyd-3 could be clearly observed in extracts derived from the wild-type MC4100. To verify that Hyd-3 was correctly assigned, an extract derived from strain RM220 (ApIB) grown anaerobically lacked the formate-inducible Hyd-3 activity band (Fig. 2a). The activity band migrating more slowly than Hyd-3 is due to a hydrogen-oxidizing activity associated with the respiratory formate dehydrogenases FDH-N and FDH-O (Soboh et al., 2011) and this is particularly apparent in the ApIB strain. While the iscU mutation abolished not only the hydrogenase activity of Hyd-1, Hyd-2 and Hyd-3 but also that of FDH, a mutation in the hypF gene (Paschos et al., 2002) only affected hydrogenase activity (Fig. 2a). Introduction of plasmid pRKISC into strain CP1244 (ΔiscU) restored the activities of all five oxidoreductases, albeit the activity of Hyd-3 was slightly weaker than that observed for the wild-type (Fig. 2a). To demonstrate equal loading of the lanes, subsequent to hydrogenase activity determination the gel was stained with Coomassie brilliant blue (Fig. 2b).

**Both IscA and ErpA are required for hydrogen production**

Introduction of either an iscA mutation or an erpA mutation reduced FHL activity by minimally 85 % (Table 3). Combining the iscA and erpA mutations in strain DV1151 (Vinella et al., 2009) abolished FHL activity, yielding the same FHL phenotype as the control strains DHP–F2 (ΔhypF) and CP971 (ΔhycA-I) (Table 3). These findings are also in accord with the lack of detectable total hydrogenase activity in DV1151 reported previously (Pinske & Sawers, 2012a). Both IscA and ErpA therefore appear to be essential for hydrogen evolution in fermentatively growing E. coli.

**Table 2. Effect of iscU mutation on total hydrogenase and FHL specific activities**

<table>
<thead>
<tr>
<th>Strain (genotype)*</th>
<th>Hydrogenase [U (mg protein)$^{-1}$]**</th>
<th>FHL [mU (mg protein)$^{-1}$]**</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100 (wild-type)</td>
<td>2.5 ± 0.35</td>
<td>112 ± 12</td>
</tr>
<tr>
<td>DHP–F2</td>
<td>0.02</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(ΔhypF)</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP1244</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ΔiscU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP1244 + pRKISC</td>
<td>2.85 ± 1.10</td>
<td>110 ± 11</td>
</tr>
</tbody>
</table>

*Cells were grown anaerobically in TGYEP to OD$_{600}$ 0.4.
**The mean and sd of at least three independent experiments are shown.
Introduction of a ΔsufA allele into MC4100 reduced the Hyd-3-dependent FHL activity by half (Table 3), suggesting a minor degree of overlap in function between the Isc and Suf systems with respect to FHL activity. Transformation of a plasmid encoding SufA into the ΔsufA mutant resulted in extremely poor anaerobic growth, and therefore it was not possible to determine FHL activity of the complemented strain.

Transformation of a plasmid (piscA) carrying the iscA gene into the iscA mutant CP477 restored FHL activity to roughly 65% of the level of the wild-type MC4100 (Table 3). It was noted, however, that introduction of the empty vector pBluescript into MC4100 reduced FHL activity by 30% (data not shown), suggesting that pRKISC indeed complemented the phenotype. On the other hand, growth of strain LL401, in which expression of the erpA gene is under the control of the arap promoter (Loiseau et al., 2007), in the presence of arabinose restored FHL activity to only 50% of wild-type (Table 3). Growth of the wild-type in the presence of arabinose reduced FHL activity by 10–15%, which might account in part for the lack of full complementation of FHL activity in the erpA mutant after growth in the presence of arabinose.

Multicopy iscA could not compensate for the absence of erpA in restoring FHL activity in strain LL401, nor did introduction of the plasmid into DV1151 (Δisca ΔerpA) recover hydrogen production (Table 3). Together, these results indicate that while ErpA and IscA are both essential for synthesis of a functional FHL complex, apparently these proteins have at least some non-redundant functions in FHL complex maturation.

**FDH-H activity depends on both IscA and ErpA**

No measurable FDH-H enzyme activity could be detected in extracts of either strain CP477 (Δisca) or LL401 (erpA) (Table 4). Transformation of CP477 (Δisca) with piscA restored FDH-H activity, but only to approximately 50% of the wild-type level, suggesting perhaps that the effect of the plasmid backbone on FHL activity (see above) might be caused by the reduction in FDH-H activity (see Table 3). FDH-H enzyme activity could be fully restored to the conditional erpA mutant LL401 by either supplementing the growth medium with L-arabinose or transforming the strain with perpA (Table 4).

**The iscU mutant has lower levels of the FdhF polypeptide**

Formate is essential for expression of the fdhF gene and the hyc operon, which encodes the structural components of the FHL complex (Rossmann et al., 1991). To examine whether the FdhF polypeptide was synthesized in strain

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**Table 3. FHL activity of mutants lacking the A-type carrier proteins IscA and ErpA**

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Specific hydrogen-evolving activity [mU (mg protein)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No plasmid</td>
</tr>
<tr>
<td>MC4100 (wild-type)</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>DHP-F2 (ΔhypF)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP971 (Δhyca-I)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP477 (ΔiscA)</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>LL401 (conditional erpA)</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>LL401†</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>DV1151 (Δisca erpA)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP1223 (ΔsufA)</td>
<td>15 ± 5</td>
</tr>
</tbody>
</table>

*Cells were grown anaerobically in TGYEP to OD_{600} 0.8.
†The mean and SD of at least three independent experiments is shown.
‡The activity was measured after growth in the presence of 0.2% (w/v) L-arabinose.
The ability to generate formate intracellularly is compromised by an *iscU* mutation

To determine whether the reduced level of the FdhF polypeptide in the *iscU* mutant was due to reduced formate production, the concentration of formate in the culture medium was determined enzymically (see Methods). The formate concentration for MC4100 (wild-type) was 1.03 mM, for the *pflB* mutant RM220, it was 0.12 mM and for CP1244 (*AiscU*), it was 1.33 mM. This indicates that the amount of formate produced by cultures of CP1244 was similar to that of the wild-type.

It was not possible to determine the intracellular level of formate accurately in cells of MC4100 or CP1244 by the enzymic method and therefore expression of a formate-dependent *fdhF-lacZ* transcripational fusion was analysed to determine whether intracellular formate levels were significantly different between the strains (Table 5). Analysis of β-galactosidase enzyme activity after anaerobic growth of the wild-type and the *iscU* mutant in TGYEP medium revealed that expression of the *fdhF* gene was reduced nearly 10-fold in the *iscU* mutant compared to the wild-type. This is a similar low level of *fdhF-lacZ* expression to that observed in a mutant lacking active PflB (Birkmann et al., 1987). Growth of the *iscU* mutant in the presence of 5 mM sodium formate restored expression of *fdhF-lacZ* to 70% of the wild-type level, strongly suggesting that the reduction in *fdhF* expression in the *iscU* mutant was due to a reduction in intracellular formate concentration. Reintroduction of the *iscU* gene on plasmid pRKISC restored *fdhF-lacZ* fusion expression to wild-type levels (Table 5). The high standard deviation of the β-galactosidase activity in the plasmid-bearing strain might suggest strong variation in the intracellular formate levels.

Previous studies had shown that formate-inducible *fdhF* gene and *hyc* operon expression in a mutant unable to synthesize PflB required addition of minimally 5 mM formate to the growth medium (Birkmann et al., 1987; Rossmann et al., 1991). Therefore, we analysed FdhF polypeptide levels in a mutant (strain 234M1) which synthesized PflB but was unable to activate the enzyme. This revealed that no FdhF polypeptide could be detected unless minimally 5 mM exogenous sodium formate was supplied to the culture (Fig. 3c).

Membrane association of the HycG small subunit of Hyd-3 is compromised in *erpA* and *iscA* mutants

The FHL complex is located on the cytoplasmic side of the membrane and consists of seven proteins, of which four (HycB, HycF, HycG and FdhF) have at least one [4Fe–4S] cluster. Previous studies were performed to analyse the subcellular localization of key enzymes using Western blotting. Western blots were probed with antibodies raised against PflB because in the presence of 5 mM formate, the FdhF polypeptide can be readily visualized by Western blotting (Sauter et al., 1990). The presence of the active form of PflB required addition of minimally 5 mM sodium formate or not (Fig. 3a, right panel).

Intracellular formate is generated mainly by PflB (see Fig. 1), which has to be converted to its active form by the radical-SAM enzyme PflA. PflA has an essential [Fe–S] cluster (Knappé & Sawers, 1990). The presence of the active form of PflB can be readily visualized by Western blotting with antibodies raised against PflB because in the presence of oxygen a specific scission of the radical-bearing species of the PflB polypeptide occurs at amino acid position 733–734, resulting in a C-terminal truncation of the polypeptide chain. Extracts derived from fermentatively grown MC4100 (wild-type), RM220 (ΔpflB ΔpflA) and CP1244 (ΔisCU) all showed the double band characteristic of the radical-bearing PflB species (Fig. 3b), which indicated that PflB was active, even in the *iscU* mutant. However, the extract derived from CP1244 revealed reduced (approx. two-fold based on densitometric evaluation) levels of PflB polypeptide compared to wild-type (Fig. 3b).

<table>
<thead>
<tr>
<th>Strain (genotype)*</th>
<th>Specific FDH-H activity [U (mg protein)−1]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100 (wild-type)</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>BW25113 (wild-type)</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>CP477 (<em>AiscA</em>)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CP477 + piscA</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>LL401 (conditional <em>erpA</em>)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LL401 + L-arabinose‡</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>LL401 + perpA</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>CP1223 (<em>AnufA</em>)</td>
<td>0.14 ± 0.12</td>
</tr>
</tbody>
</table>

*Cells were grown anaerobically in TGYEP to OD₆₀₀ 0.8.
†The mean and SD of at least three independent experiments are shown.
‡The activity was measured after growth in the presence of 0.2% (w/v) L-arabinose.
The importance of the Isc system for hydrogen evolution

complex components. It is known that association of HycE with HycG, the large and small Hyd-3 subunits, respectively, occurs only after insertion of the active site into HycE (Magalon & Böck, 2000) and presumably also only once

the HycG protein has received its [Fe–S]. Subcellular fractionation studies demonstrated that HycG was membrane-associated in both MC4100 and the sufA mutant CP1223 after fermentative growth with glucose (Fig. 4a).

Analysis of the soluble and membrane fractions derived from an extract of DHP-F2 (ΔhypF) failed to detect any HycG antigen and thus acted as a negative control for the experiment. The small subunits of Hyd-1 and Hyd-2 are likewise undetectable in strain DHP-F2 (Pinske & Sawers, 2012a). Subcellular fractionation of extracts derived from CP477 (ΔiscA) and LL401 (erpA) revealed an altered distribution of HycG compared with that seen in MC4100. The bulk of the polypeptide was in the soluble fraction and not membrane-associated (Fig. 4a). While a small amount of HycG was membrane-associated in CP477, no HycG was observed in the membrane fraction of the erpA mutant. This finding suggests that the association of HycG with the FHL complex was compromised in the iscA and erpA mutants and it was not the amount of HycG per se that was limiting. Reintroduction of the iscA gene on a plasmid into CP477 resulted in an overall increase in abundance of HycG but also the protein was associated with the membrane, consistent with restoration of FHL activity to the mutant (see Table 3).

Table 5. Effect of an iscU mutation on the expression of a chromosomally encoded fdhF-−lacZ fusion

<table>
<thead>
<tr>
<th>Strain and genotype*</th>
<th>β-Galactosidase activity (Miller units)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>MC4100 ΔfdhF−−lacZ</td>
<td>312 ± 42</td>
</tr>
<tr>
<td>CP1244 ΔiscU ΔfdhF−−lacZ</td>
<td>39 ± 21</td>
</tr>
<tr>
<td>CP1244 ΔfdhF−−lacZ+pRKISC</td>
<td>344 ± 120</td>
</tr>
</tbody>
</table>

*Cells were grown anaerobically in TGYEP to OD₆₀₀ 0.4–0.6.
†Activity was determined according to Miller (1972) and data for the mean and SD of at least three independent experiments is shown.
The FdhF polypeptide does not associate efficiently with the membrane fraction in erpA and iscA mutants

With the aid of antibodies it was possible to examine the localization of the FdhF polypeptide (which is the catalytic subunit of the FDH-H activity) in the various ATC mutants (Fig. 4b). The 80 kDa FdhF polypeptide was distributed evenly between the soluble and membrane fractions in MC4100, the ΔhypF mutant DHP-F2, and the sufA mutant CP1223. This result indicates that FdhF can associate with the membrane even in the absence of a matured Hyd-3 enzyme, e.g. strain DHP-F2. FdhF has been proposed to be loosely associated with the membrane-associated FHL complex (Sawers et al., 1985; Axley et al., 1990). Crude extracts derived from these strains also exhibited similar FDH-H activity to each other (Table 4).

Analysis of the subcellular distribution of the FdhF polypeptide in the iscA mutant CP477 revealed that there was approximately two–three-fold less polypeptide present than in MC4100, but nevertheless, there was significantly more than in CP1244 (AiscU) (compare with Fig. 3a). The bulk of FdhF in extracts of strain CP477 was mainly localized in the soluble fraction but was still weakly detectable in the membrane (Fig. 4b). In the erpA mutant, FdhF was only detectable in the soluble fraction (Fig. 4b).

The 20.5 kDa HycF polypeptide has similarity to ferredoxin-like proteins (Böhm et al., 1990). In contrast to either the FdhF or HycG FHL complex components, Western blot analysis of HycF revealed that it remained tightly associated with the membrane fraction in MC4100 and in the iscA and erpA mutants (Fig. 4c).

DISCUSSION

The importance of both Isc assembly and delivery proteins for hydrogen production

It was recently shown that the Isc system is essential for biosynthesis of functionally active hydrogen-oxidizing [NiFe]-hydrogenases 1 and 2, as well as the enzymes of the anaerobic formate–nitrate respiratory pathway in E. coli (Pinske & Sawers, 2012a, b). In this study, we have shown that the isc system is also essential for fermentative hydrogen production by E. coli. It was anticipated that a mutant unable to synthesize IscU would be devoid of FHL activity because we could show previously (Pinske & Sawers, 2012a) that an iscU mutant had no detectable hydrogen : BV oxidoreductase activity. This is because it lacks a functional HypD enzyme, which is essential for maturation of all [NiFe]-hydrogenase large subunits (Böck et al., 2006; Forzi & Sawers, 2007), as well as because it fails to deliver [Fe–S] to the respective small subunit (Pinske & Sawers, 2012a). A somewhat unanticipated finding of this study, however, was that an iscA mutant retained some FHL complex activity (see Table 3). In contrast, a mutant lacking the other Isc-dependent [Fe–S]- trafficking protein, ErpA (Loiseau et al., 2007), had essentially no FHL activity. This suggests that IscA and ErpA have different functions with respect to FHL complex maturation. This conclusion is also supported by the fact that overproduced ErpA could not restore FHL activity to an iscA mutant, nor vice versa could overproduced IscA functionally replace ErpA (data not shown). Clearly, there is a strong dependence on ErpA for the delivery of [Fe–S] to the FHL complex components.
It was notable that FDH-H activity required both ATC proteins. This was somewhat surprising because the FdhF component of the FHL complex is essential for hydrogen production, but nevertheless 15% FHL activity was retained in an icsA mutant although little to no FDH-H activity could be detected in the mutant (see Table 4). We currently have no obvious explanation for this result. FDH-H enzyme activity is notoriously difficult to determine (Sawers et al., 1985; Axley et al., 1990), and this possibly accounts for this paradoxical result. Whether both IscA and ErpA play a role in delivery of the [Fe–S] to the FdhF polypeptide or whether they are both required for bis-MGD-cofactor biosynthesis remains to be determined.

Mis-localization of two [Fe–S]-containing subunits of the FHL complex in ATC mutants

Previous studies have shown that one reason for the lack of activity of the hydrogen-oxidizing hydrogenases in icsA and erpA mutants is the absence of the small subunit, which is presumed to be more rapidly degraded if it fails to receive its complement of [Fe–S] (Pinske & Sawers, 2012a). None of the [Fe–S]-containing polypeptides of the FHL complex was reduced in abundance in the ATC mutants, except FdhF, which was reduced approximately two–three-fold in the icsA mutant. In contrast, however, membrane association of the FdhF and HycG proteins, presumably with the core complex, was absolutely dependent on the presence of ErpA and was also strongly dependent on IscA. Presumably the lack of association of FdhF with the core FHL complex was due to either aberrant [Fe–S] insertion into HycB, the small subunit of FdhF, or incomplete maturation of FdhF itself, or both. We have shown recently that the other two related formate dehydrogenases FDH-N and FDH-O both showed aberrant maturation in an icsA mutant (Pinske & Sawers, 2012b), probably reflecting defective cofactor insertion. The HycB component of the FHL complex was not analysed in this study so no conclusions can be drawn regarding whether it was stable or membrane-associated in the ATC mutants.

It is perhaps noteworthy that the putative [Fe–S]-containing HycF protein retained a tight association with the cytoplasmic membrane fraction in the ATC mutants, despite not being predicted to be a membrane protein (Bohmen et al., 1990). Therefore either HycF receives its [Fe–S] from a source other than IscA or ErpA [an icsA iscA double mutant also showed membrane-associated HycF (data not shown)], or its apoprotein still associates with the membrane, even in the absence of [Fe–S]. Recent studies revealed that the atypical carrier protein NfuA (Py et al., 2012) has an important role in delivering [Fe–S] to key aerobic enzymes and it appears to receive its [Fe–S] from either the Isc or the Suf assembly proteins. Perhaps, therefore, this carrier also delivers [Fe–S] clusters to discrete enzymes of anaerobic metabolism, including HycF, even in the absence of a fully functional Isc system.

The Isc system, formate, PflB and FNR

Expression of the anaerobically inducible focA-pflB operon is regulated by FNR and the ArcAB two-component system (Sawers & Bock, 1988; Sawers & Suppmann, 1992). Significantly, an fnr mutation fails to abolish anaerobic induction completely. Here, we demonstrated that an icsU mutant synthesizes less PflB, presumably due to limited activity of the [Fe–S]-containing FNR transcription factor. Mettert et al. (2008) have shown that FNR activity is reduced by up to 60% in the absence of a functional Isc system. Limiting the amount of PflB lowers the intracellular formate concentration, thus hindering expression of the formate-dependent fahF gene and hyc operon and ultimately FHL synthesis (Birkmann et al., 1987; Rossmann et al., 1991). Despite exhibiting a two-fold reduction in PflB levels, the icsU mutant CP1244 nevertheless was able to excrete formate into the growth medium at levels similar to those found in the wild-type. Accumulation of extracellular formate by the icsU mutant was anticipated because the cells lack functional formate dehydrogenase enzymes. Notably, however, fahF^{−1}lacZ expression data clearly indicated that the icsU mutant had reduced levels of intracellular formate suggesting that a mechanism exists to ensure that formate does not accumulate intracellularly during growth with glucose. This finding supports the recent demonstration that a selC mutant (which lacks all FDHs) accumulates formate in the growth medium (Beyer et al., 2013).

Together, these findings, coupled with the incomplete maturation of the [Fe–S]-containing FHL subunits, mean that the Isc system affects both the transcriptional and post-translational regulation of FHL biosynthesis. How these various levels of regulation impact on FHL biosynthesis is summarized in Fig. 1.

That FNR retains some activity in an Isc− strain (Mettert et al., 2008), together with the fact that the [Fe–S]-containing radical-SAM enzyme PflA maintains at least partial function in an icsU mutant, strongly suggests that a further system to ensure [Fe–S] biosynthesis probably exists in E. coli. For certain apoprotein substrates, this might be partially compensated by the Suf system, as has been suggested (Mettert et al., 2008); however, it might also indicate that a further system exists as back-up for Isc to ensure key anaerobic [Fe–S]-containing proteins are synthesized (Py et al., 2012). Future experiments will be required to identify which of these possibilities ensures anaerobic [Fe–S] assembly and delivery in the absence of the Isc system. Moreover, precisely how IscU, IscA and ErpA function to ensure maturation of the modular hydrogenases, formate dehydrogenases and nitrate reductases of E. coli will be a major focus of future studies.

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