An electron transfer flavoprotein is essential for viability and its depletion causes a rod-to-sphere change in *Burkholderia cenocepacia*

Ruhi A. M. Bloodworth,1 Soumaya Zlitni,2 Eric D. Brown2 and Silvia T. Cardona1,3

Correspondence
Silvia T. Cardona
Silvia.Cardona@umanitoba.ca

1Department of Microbiology, University of Manitoba, Winnipeg, Canada
2Department of Biochemistry & Biomedical Sciences, McMaster University, Hamilton, Canada
3Department of Medical Microbiology & Infectious Disease, University of Manitoba, Winnipeg, Canada

Essential gene studies often reveal novel essential functions for genes with dispensable homologues in other species. This is the case with the widespread family of electron transfer flavoproteins (ETFs), which are required for the metabolism of specific substrates or for symbiotic nitrogen fixation in some bacteria. Despite these non-essential functions high-throughput screens have identified ETFs as putatively essential in several species. In this study, we constructed a conditional expression mutant of one of the ETFs in *Burkholderia cenocepacia*, and demonstrated that its expression is essential for growth on both complex media and a variety of single-carbon sources. We further demonstrated that the two subunits EtfA and EtfB interact with each other, and that cells depleted of ETF are non-viable and lack redox potential. These cells also transition from the short rods characteristic of *Burkholderia cenocepacia* to small spheres independently of MreB. The putative membrane partner ETF dehydrogenase also induced the same rod-to-sphere change. We propose that the ETF of *Burkholderia cenocepacia* is a novel antibacterial target.

INTRODUCTION

The advent of next-generation sequencing has led to a wealth of genetic knowledge, but the ability to assign phenotypes to these genotypes has lagged. One basic phenotype is whether genes are absolutely required for growth under standard conditions. The search for these so-called essential genes is given added urgency by their potential as novel targets for antibiotics in an era of increasing antibiotic resistance (Cardona et al., 2014, Fang et al., 2005; Gerdes et al., 2006; Juhas et al., 2012). High-throughput screens (HTSs) for essential genes normally involve high-density transposon mutagenesis followed by mapping transposon insertion sites in the population to identify the non-essential genes (Langridge et al., 2009). These screens provide an accurate and cost-effective method to identify essential genes on a genomic scale. However, the confidence of assigning essentiality to a gene depends on the transposon insertion density. At lower densities, non-essential genes may not have insertions due to chance or insertion biases, whilst essential genes may tolerate insertions that do not disrupt required domains. Although this limitation can partially explain why some genes appear to be essential when they encode products of demonstrated dispensability, HTSs for essential genes also uncover novel essential functions for the products of genes with already characterized non-essential homologues in other species.

One example where HTS essentiality results and known function of gene products conflict is that of the electron transfer flavoproteins (ETFs). These soluble FAD-containing homodimeric proteins are distributed across all domains of life, and are responsible for funnelling electrons from dehydrogenases to the membrane-bound respiratory chain of bacteria and mitochondria (Toogood et al., 2007) or to nitrogen fixation in some bacteria (Scott & Ludwig, 2004). ETFs consist of a large (ETF-α) and small (ETF-β) subunit, and based on comparative amino acid sequence analysis, they are traditionally divided into three

Abbreviations: BACTH, bacterial adenylate cyclase two-hybrid; DHFR, dihydrofolate reductase; DIC, differential interference contrast; ETF, electron transfer flavoprotein; HTS, high-throughput screen; MPN, most probable number.

Three supplementary tables and five supplementary figures are available with the online Supplementary Material.
groups, which have different biological functions (Tsai & Saier, 1995). Group I ETFs, found in eukaryotes (Ghisla & Thorpe, 2004) and some bacteria, including Clostridium kluyveri (Li et al., 2008), Paracoccus denitrificans (Roberts et al., 1999) and Bacillus subtilis (Matsuoka et al., 2007), funnel electrons from the oxidation of fatty acids, branched-chain amino acids (Li et al., 2008), and lysine and tryptophan (Roberts et al., 1999) to the electron transport chain (Fig. 1). Group II ETF genes (fixB and fixA) are found in nitrogen-fixing bacteria where they divert electrons from dehydrogenases to nitrogenases, bypassing the electron transport chain (Scott & Ludwig, 2004) (Fig. 1). The only studied group III ETF is found in Escherichia coli where it is required for the anaerobic reduction of carnitine (Walt & Kahn, 2002). Multiple ETFs are present in some bacterial genomes, as in the case of Bradyrhizobium japonicum, which contains both a group I ETF expressed under aerobic conditions and a group II ETF that is expressed during anaerobic growth (Weidenhaupt et al., 1996). Intriguingly, HTSs have identified ETFs as putatively essential in Acinetobacter baylyi (de Berardinis et al., 2008), Caulobacter crescentus (Christen et al., 2011), Porphyromonas gingivalis (Klein et al., 2012), Mycobacterium tuberculosis (Griffin et al., 2011), Sphingomonas wittichii RW1 (Roggo et al., 2013) and Burkholderia pseudomallei (Moule et al., 2014). The existence of ETFs with an essential role is unexpected because nitrogen fixation is non-essential on rich media and metabolism of many carbon sources is expected to bypass ETFs. For example, both NADH and succinate dehydrogenases transfer electrons to ubiquinone without the involvement of ETFs (Fig. 1).

We previously reported a screen for essential genes in Burkholderia cenocepacia KS2-2 (Bloodworth et al., 2013), a clinical isolate recovered from the sputum of a cystic fibrosis patient (Mahenthiralingam et al., 2000). Burkholderia cenocepacia belongs to the Burkholderia cepacia complex, a group of multiple-antibiotic-resistant Gram-negative species (Vandamme & Dawyndt, 2011) known to infect the airways of people with cystic fibrosis (Drevinek & Mahenthiralingam, 2010). In our study, we identified a group I ETF-α-coding gene, etfA, for which conditional expression led to a complete inhibition of growth. This finding, together with the inconsistency of this phenotype with previously described functions, led us to further characterize the effect of depleting one of the three ETFs in Burkholderia cenocepacia. Here, we confirm that the etfBA operon (BCAL2394, BCAL2395) is essential in Burkholderia cenocepacia K56-2. We further demonstrate that cells depleted of the corresponding protein (EtfBA) lose viability and redox potential. These cells also transition from the short rods characteristic of Burkholderia cenocepacia to small spheres. We propose that the lack of aerobic respiration and small-sphere phenotype of cells depleted of EtfBA are related, with the metabolic defect inducing the morphological change.

**METHODS**

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, all strains were grown in Luria–Bertani (LB) media at 37°C supplemented as required with 0.2% (w/v) rhamnose, 100 or 50 μg trimethoprim ml⁻¹ for B. cenocepacia or E. coli, respectively, 50 μg gentamicin ml⁻¹ and 40 μg kanamycin ml⁻¹. Growth

---

**Fig. 1.** A composite figure of electron paths through ETFs. Group I ETFs funnel electrons from a variety of species-specific primary dehydrogenases (additional partners may be present in some species) to the ETF dehydrogenase (ETFDH) from which they enter the electron transport chain at the ubiquinone pool (UQ) (Ghisla & Thorpe, 2004). Group II ETFs divert electrons away from primary dehydrogenases towards nitrogenase reductase. Single knockouts of primary dehydrogenases do not impair symbiotic nitrogen fixation and therefore it is expected that group II ETFs have multiple electron donors, although the only confirmed partner is pyruvate dehydrogenase in Azorhizobium caulinodans (Scott & Ludwig, 2004). The only studied group III ETF is known to be required for the anaerobic reduction of carnitine, although the direction of electron flow is unknown (Walt & Kahn, 2002). NADH dehydrogenases and succinate dehydrogenases transport electrons to the ubiquinone pool independently of ETFs (Annaku, 1988). Arrows represent experimentally demonstrated electron flow. White, black and grey boxes denote primary dehydrogenases, proteins in the ETF I pathway leading to the electron transport chain and proteins in the ETF II pathway leading to nitrogenase, respectively.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant information</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTH101</td>
<td>F−, cya-99, araD139, galE15, galK16, rpsL1 (Strr), hsdR2, mcrA1, mcrB1</td>
<td>Euromedex</td>
</tr>
<tr>
<td>SY327</td>
<td>araD Δ(lac pro) argE (Am) recA56 Rif⋯ nalA λ pir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td><strong>Burkholderia cenocepacia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K56-2 (LMG18683), ET12 lineage, CF isolate</td>
<td></td>
<td>Mahenthiralingam et al. (2000)</td>
</tr>
<tr>
<td>CgyrB</td>
<td>K56-2; rhaP :: BCAL12935; Tp′, rhamnose-dependent etfBA expression</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>CetfBA</td>
<td>K56-2; rhaP :: BCAS0506, deletion of putative ETF dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>Detfdh2</td>
<td>K56-2; Δ BCAS0506, deletion of putative ETF dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>Cetfdh1</td>
<td>K56-2; rhaP :: BCAL1468; Tp′, rhamnose-dependent etfhl1 expression in K56 background</td>
<td>This study</td>
</tr>
<tr>
<td>Cetfdh</td>
<td>K56-2; rhaP :: BCAL1468, Δ BCAS0506, Tp′, rhamnose-dependent etfhl1 expression in Δetfdh2 background</td>
<td>This study</td>
</tr>
<tr>
<td>16-2C5</td>
<td>K56-2; transposon insertion at chr1 : 2944874 reverse strand, upstream from BCAL2676 (petA)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>16-11C6</td>
<td>K56-2; transposon insertion at chr2 : 2944852, upstream from BCAL2677 (putative permease)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>29-3B1</td>
<td>K56-2; transposon insertion at chr1 : 358492, upstream from BCAL0327 (petC)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>30-4C6</td>
<td>K56-2; transposon insertion at chr1 : 33371, upstream from BCAL0028 (RIF1 ATPase)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>77-16C10</td>
<td>K56-2; transposon insertion at chr1 : 2944852, upstream from BCAL2677 (petA)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>78-16D7</td>
<td>K56-2; transposon insertion at chr1 : 3347313, upstream from BCAL3053 (ribB)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td>89-10K20</td>
<td>K56-2; transposon insertion at chr1 : 3745902, upstream from BCAL3420 (accB)</td>
<td>Bloodworth et al. (2013)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSC200</td>
<td>pGpQTP derivative (Flannagan et al., 2007), orfRkC, rhaR rhaS PrhaB e-gfp</td>
<td>Ortega et al. (2007)</td>
</tr>
<tr>
<td>pRB10</td>
<td>pSC200 containing 5′ end of BCAL2935</td>
<td>This study</td>
</tr>
<tr>
<td>pRB18</td>
<td>pSC200 containing 5′ end of BCAL1468</td>
<td>This study</td>
</tr>
<tr>
<td>pAP20</td>
<td>orfQPRBIPDHFR Cm′</td>
<td>Law et al. (2008)</td>
</tr>
<tr>
<td>pAP20-etfBA</td>
<td>pAP20 derivative containing the BCAL2935–BCAL2934 operon</td>
<td>This study</td>
</tr>
<tr>
<td>pAP20-etfB</td>
<td>pAP20 derivative containing the BCAL2934 locus</td>
<td>This study</td>
</tr>
<tr>
<td>pAP20-etfA</td>
<td>pAP20 derivative containing the BCAL2934 locus</td>
<td>This study</td>
</tr>
<tr>
<td>pGPI-SceI</td>
<td>SceI recognition sequence, Tp′</td>
<td>Flannagan et al. (2008)</td>
</tr>
<tr>
<td>pDAI-SceI</td>
<td>DHFR promoter expressing e-ISce-I, Tc′</td>
<td>Flannagan et al. (2008)</td>
</tr>
<tr>
<td>pRB17</td>
<td>Derivative of pGPI-SceI containing the upstream and downstream flanking regions of BCAS0609</td>
<td>This study</td>
</tr>
<tr>
<td>pKT25</td>
<td>p15 ori Km′; vector for N-terminal fusion with T25</td>
<td>Euromedex</td>
</tr>
<tr>
<td>pUT18</td>
<td>ColE1 ori Ap′; vector for N-terminal fusion with T18</td>
<td>Euromedex</td>
</tr>
<tr>
<td>pU18c</td>
<td>ColE1 ori Ap′; vector for C-terminal fusion with T18</td>
<td>Euromedex</td>
</tr>
<tr>
<td>pKT25-zip</td>
<td>Leucine zipper motif cloned into pKT25</td>
<td>This study</td>
</tr>
<tr>
<td>pUT18c-zip</td>
<td>Leucine zipper motif cloned into pU18c</td>
<td>This study</td>
</tr>
<tr>
<td>pUT18-etfA</td>
<td>Derivative of pUT18 containing a truncation of BCAL2934 (missing 12 bp from 3′ end)</td>
<td>This study</td>
</tr>
<tr>
<td>pKT25-etfA</td>
<td>Derivative of pKT25 containing a truncation of BCAL2934 (missing 12 bp from 3′ end)</td>
<td>This study</td>
</tr>
<tr>
<td>pKT25-etfB</td>
<td>Derivative of pKT25 containing BCAL2935 locus</td>
<td>This study</td>
</tr>
<tr>
<td>pKT25-etfB</td>
<td>Derivative of pKT25 containing BCAL2935 locus</td>
<td>This study</td>
</tr>
</tbody>
</table>
(OD₆₀₀) was monitored using a BioTek Synergy 2 plate reader. Growth experiments were inoculated with a 10⁻³ dilution of a 1 OD₆₀₀ culture, whilst viability experiments used a 10⁻² dilution. Strains grown > 24 h under a single condition were diluted 10⁻³ into fresh media every 24 h. All chemicals were purchased from Sigma unless otherwise indicated.

**Molecular biology techniques.** The primers used in this study are listed in Table S1 (available in the online Supplementary Material). DNA ligations and restriction digestions were performed as recommended by the manufacturer (New England Biolabs). PCR amplification was carried out on an Eppendorf Mastercycler ep gradient S thermal cycler using either Taq or HotStarTaq DNA polymerase (Qiagen). PCR conditions were optimized for each primer pair and products were purified using a QIAquick PCR purification kit (Qiagen). DNA sequencing was carried out at the Manitoba Institute of Cell Biology DNA Sequencing Facility. E. coli cells were transformed using the Z-competent buffer kit protocol (Zymo Research). Conjugation into *Burkholderia cepacia* KS6-2 was accomplished by triparental mating (Craig et al., 1989) with *E. coli* DH5α carrying the helper plasmid pRK2013 (Figurski & Helinski, 1979).

**Construction of an unmarked etfdh2 deletion.** An unmarked deletion of *etfdh2* (BCAS0609) was constructed using the method described by Flannagan et al. (2008) with the following modifications. Upstream and downstream flanking regions were amplified using primer sets 482/521 and 485/520, respectively (Table S1). The flanking regions had an overlapping region allowing for amplification of the fusion of the two regions using primers 482 and 485. The fusion product was ligated into pGPI-Scel after digestion with *Xba*I and *Eco*RV (pRB17; Table 1). The resulting suicide plasmid was moved into KS6-2 by triparental mating, and integration was confirmed by PCR with primer set 522/523 for the upstream region and primer set 524/525 for the downstream region. After introduction of pDAI-Scel, loss of the flanking regions was confirmed by PCR with primer sets 522/523 and 524/525. The deletion mutant (Detfdh2; Table 1) was then cured of pDAI-Scel by repeated subculturing and the mutation was confirmed by sequencing the flanking regions.

**Construction of *etfBA* and *etfdh1* conditional mutants.** The 5' ends of *etfB* (BCAL2395) and *etfdh1* (BCAL1468) were amplified using the primer pairs 395/396 and 526/527, respectively (Table S1). After digestion with both *Xba*I and *Nde*I, the 5' fragments were cloned into pSC200 (Table 1) immediately downstream from the rhamnose-inducible promoter. The resulting plasmids pRB10 and pRB1B (Table 1) were moved into KS6-2 and Detfdh2, respectively, by triparental mating. Integration was confirmed by PCR amplification of the plasmid/genome junction using the transposon-specific primer 171, and primers 377 and 552 for PrhaB::etfB and PrhaB::etfdh1, respectively.

**Construction of complementing plasmids.** The putative *etfBA* operon (BCAL2395, BCAL2394) was amplified using primers 395/398 (Table S1), *etfB* with primers 395/397 and *etfdh1* with primers 288/398. After digestion with both *Xba*I and *Nde*I, the fragments were cloned into pAP20 (Table 1) immediately downstream from the *dhfr* (dihydrofolate reductase) promoter. The resulting plasmids (Table 1) were moved into CetfBA by triparental mating.

**Microscopy analysis.** Aliquots of 10 µl cultures were spotted onto glass microscope slides covered with a film of 1% agarose. Images were obtained with an AxioCamMR attached to an Axio Imager Z1 (Carl Zeiss) at ×1000 magnification using bright-field and differential interference contrast (DIC) filters. Bright-field images were exported as TIFF files and image analysis was done in ImageJ (http://image.nih.gov/ij/). Images were thresholded to distinguish cells and backgrounds. Large clumps of cells were excluded from further analysis and attached cells that had completed septation were digitally separated. The integral ‘Analyze Particles’ function of ImageJ was used to determine the major and minor axes of bounding ellipses for each cell, which were in turn used to calculate area and roundness (major/minor axis). Cell area and roundness were compared using the Mann–Whitney sum-rank test (Lehmann, 1998) assuming that the test-statistic *U* is normally distributed given the large sample size.

**Bacterial two-hybrid assay.** Protein–protein interactions were tested using the BACTH (bacterial adenylate cyclase two-hybrid) system (Euromex) according to the manufacturer’s directions. *etfB* and *etfA* ORFs were amplified using primer pairs 445/446 and 447/448, respectively (Table S1). After digestion with both *Xba*I and *Kpn*I, the fragments were cloned into pUT18c and pKT25 to form translational fusions with the *T18* and *T25* fragments, respectively. The plasmids were purified and co-transformed in all combinations into the reporter strain (BTH101; Table 1). Successful co-transformation was confirmed by colony PCR for *etfB* and *etfA*. For each transformant, 10 µl overnight culture was spotted onto LB X-Gal (40 µg ml⁻¹)/IPTG (0.5 mM) plates containing ampicillin and kanamycin. The plates were incubated for 3 days at 25 °C.

**Sensitization index experiments.** Susensions of KS6-2 and mutant colonies in LB were adjusted to OD₆₀₀ 0.1 and diluted 10⁻². Cell suspensions (180 µl) were aliquoted into 96-well plates with 0.12, 0.10–0.08, 0.06, 0.05, 0.04, 0.03 and 0 % (w/v) final concentrations of rhamnose and twofold serial dilutions of the antibiotic to be tested starting with novobiocin at 32 µg ml⁻¹, chloramphenicol at 125 µg ml⁻¹, *S-(3,4-dichlorobenzyl)* isothiourea (A22) at 125 µg ml⁻¹, hydrogen peroxide at 1.1 mM and meropenem at 8 µg ml⁻¹ to a total volume of 200 µl. Plates were then incubated for 16 h at 37 °C with no shaking. The sensitization index for a given rhamnose concentration was calculated as the ratio between the MIC of KS6-2 and the MIC of the mutant.

**Biolog assays.** Biolog plates and accessories were obtained from Biolog. The procedure for growth on PM1 plates was modified as follows. Overnight cultures were grown in LB supplemented with rhamnose and antibiotics as needed. The cultures were washed twice with inoculating fluid for Gram-negative/positive (IF-0 GN/GP) and diluted to a final theoretical OD₆₀₀ 0.001. Rhamnose and antibiotics were added as appropriate. Respiration was measured in LB using reduction of the tetrazolium Biolog redox dye A as a proxy. Reduction of the dye was calculated by subtracting the A₆₀₀ of the cultures with redox dye A from the A₆₀₀ of the cultures without the dye.

**Bioinformatics.** *etfA* and *etfB* amino acid sequences from *Burkholderia* species were downloaded from http://www.burkholderia.com/ (Winser et al., 2008), whilst sequences from other species were retrieved from the National Center for Biotechnology Information. PhyML (Guindon et al., 2010) with the WAG substitution model and the approximate likelihood ratio test was used to reconstruct a maximum-likelihood phylogenetic tree. Sequences were aligned using MUSCLE (Edgar, 2004); regions with more than eight aligned using MUSCLE (Edgar, 2004); regions with more than eight
RESULTS

Burkholderia cenocepacia etfBA operon is essential

Previously, we identified putatively essential genes in Burkholderia cenocepacia K56-2 by screening for rhamnose-dependent growth in transposon mutants after delivery of an outward-facing rhamnose-inducible promoter (Bloodworth et al., 2013). Mutants showing a rhamnose-dependent growth phenotype suggested that the rhamnose-inducible promoter had replaced the native promoter of an essential gene. Mutants of well-known essential genes often showed a reduction in growth, but not complete growth arrest in the absence of rhamnose. In contrast, mutant 77-16C10, in which the transposon had inserted between the etfB and etfA genes of a putative etfBA operon, had no detectable growth in the absence of rhamnose (Fig. 2a). A newly constructed mutant strain with the rhamnose-inducible promoter inserted immediately upstream from the etfBA operon (CetfBA; Table 1) likewise had no measurable growth in the absence of rhamnose. When we complemented CetfBA with the etfBA operon constitutively expressed from a plasmid, the rhamnose-dependent growth phenotype was abolished. Complementation with etfA or etfB alone, however, showed different effects. Expressing etfA from the plasmid during rhamnose-induced expression of the chromosomal copy of etfBA had no effects on the growth phenotype. In contrast, expression of etfB under similar conditions resulted in decreased growth. In the absence of rhamnose, expressing etfB but not etfA abolished the conditional growth phenotype, although not to the growth levels of CetfBA in rhamnose-inducing conditions. We then hypothesized that overexpression of etfB from a plasmid could trigger the formation of EtfB homodimers that were somehow functional. Although most ETFs function as heterodimers, in Clostridium perfringens only EtfA is required for the biosynthesis of dipicolinic acid (Orsburn et al., 2010). To explore the possibility of homodimer formation, we used the BACTH complementation assay (Battesti & Bouveret, 2012). etfB and etfA were cloned into vectors containing the T25 and T18 fragments of Bordetella pertussis adenylate cyclase, respectively. In agreement with the reported heterodimeric nature of ETFs (Chen & Swenson, 1994), co-transformation into the reporter strain demonstrated that EtfA and EtfB interact with each other, but neither interacts with itself (Fig. S1).

Essentiality of ETF is independent of the carbon source utilized

In other organisms, ETFs function as electron shuttles and are required to utilize specific substrates, such as fatty acids...
in Paracoccus denitrificans (Roberts et al., 1999) or carnitine in E. coli (Walt & Kahn, 2002). If ETF has a comparable role in K56-2, the etfBA operon should be essential for the utilization of specific carbon sources and the conditional phenotype should be abolished when CetfBA is grown on an alternative substrate. Whilst this seems unlikely given that the conditional growth phenotype of CetfBA is present in LB, a complex medium, it is not unprecedented. In Bacillus subtilis the glycolytic genes gapA, pgm and eno are required for growth on rich media for as-yet undetermined reasons, but mutants of all three genes are capable of growth on minimal media with malate and glucose as sole carbon sources, and the gapA mutant can grow on glucose alone (Commichau et al., 2013). To investigate growth of CetfBA on different single-carbon sources, we used Biolog PM1 plates, which contain 95 individual carbon sources, including compounds which might require an ETF for metabolism, such as the short-chain fatty acids propionic and acetic acid or the amino acid threonine (Table S3). Cultures of K56-2 and CetfBA grown in LB with rhamnose were washed and resuspended in IF-0 GN/GP media with and without rhamnose. After incubation for 72 h both strains showed at least 15 % of maximum growth in 57 out of 95 carbon sources when supplemented with rhamnose (Fig. 2b, Table S3). However, the mutant was unable to grow on any of these carbon sources in the absence of rhamnose (Fig. 2b), suggesting that the essential function of EtfBA is independent of the utilized carbon source.

**Depletion of EtfBA renders cells non-viable**

The lack of growth of CetfBA in the absence of etfBA expression raised the question of whether growth was merely arrested, but could be restored if etfBA expression resumed, or whether the cells had permanently lost viability. To measure the ability of CetfBA to resume growth, an overnight culture was washed and inoculated into LB media without rhamnose. Aliquots of this culture were then taken at various time and transferred onto LB with rhamnose to determine culturability (Fig. 3a) on both liquid media using the most probable number (MPN) method and on solid media by counting c.f.u. Both methodologies showed that after 30 h without etfBA expression, the number of viable cells fell below the limits of detection (7 MPN ml$^{-1}$ and 1 c.f.u. ml$^{-1}$), indicating that EtfBA depletion had irreversible effects. This loss of culturability was not common amongst conditional expression mutants of essential genes (Table S2) (Bloodworth et al., 2013).

In addition to measuring culturability, we also used the

![Graph showing viability](image)

**Fig. 3.** etfBA expression is required for viability. (a) An overnight culture of CetfBA was adjusted to OD$_{600}$ 0.01. Viability at 0, 6 and 30 h post-inoculation was measured both on LB rhamnose plates by c.f.u. ml$^{-1}$ and in liquid culture using MPN ml$^{-1}$. Error bars represent 95% confidence interval. The horizontal grey line shows the minimum level of detection by MPN (7 MPN ml$^{-1}$); the c.f.u. method could detect 1 c.f.u. ml$^{-1}$.(b) Reduction of Biolog redox dye A was used as a proxy to measure respiration of the conditional expression mutants of gyrB, etfBA and the ATP synthase after inoculation into LB without rhamnose. At 24 h post-inoculation, the cultures were subcultured into new media with rhamnose. Reads were taken every 60 min of four replicates; error bars represent SD. rha, Rhamnose.
**BacLight LIVE/DEAD kit** to measure membrane integrity based on the ability of two dyes to enter the cell. SYTO 9 stains all cells, but propidium iodide is excluded from cells with intact membranes. These experiments showed that although CetfBA lost viability during incubation over 24 h without rhamnose, cells maintained intact membranes (Fig. S2).

Cells depleted of EtfBA were unable to resume growth despite their intact membranes. To examine whether respiration had also stopped, cultures were inoculated into LB containing Biolog redox dye A, which is reduced by cellular respiration forming a purple colour (Tracy et al., 2002). After 24 h, cultures were moved into fresh media supplemented with rhamnose to determine whether respiration resumed. An ATP synthase mutant, used as a negative control for respiration, showed no dye reduction, indicating that interruption of respiration was irreversible (Fig. 3b). Likewise, a gyrB mutant continued to respire regardless of the absence of rhamnose, consistent with GyrB playing no role in respiration. Cells without etfBA expression showed a slight reduction of the dye by 24 h, but respiration did not resume after being switched to inducing conditions, indicating that EtfBA depletion led to an irreversible loss of respiration.

**Lack of ETF causes a rod-to-sphere change in cell morphology that is independent of MreB**

When measuring cell viability, we noticed that even though all cultures were adjusted to the same initial OD_{600}, cultures of CetfBA contained ~3 log fewer viable cells than equivalent cultures of other mutants (Table S2). This lack of correspondence between OD_{600} and c.f.u ml^{-1} suggested a change in CetfBA cell morphology. However, DIC microscopy of cells grown in the presence of 0.2 % rhamnose showed normal morphology (Fig. 4a, lower left). Instead, after 24 h without etfBA expression, cells had transitioned from the normal short-rod morphology of *Burkholderia cenocepacia* cells to small spheres (Fig. 4a, lower right). In rod-shaped bacteria, cell diameter is controlled by the MreB complex, the loss of which is known to trigger a change from rods to spheres (Young, 2010). Treating K56-2 cells with 16 µg A22 ml^{-1}, a known MreB complex inhibitor (Bean et al., 2009), also caused a transition from rods to spheres (Fig. 4a, upper right). However, A22-treated cells appeared to be larger than those depleted of EtfAB. This observation was confirmed quantitatively by fitting a bounding oval to each cell in the microscopy images, and then calculating cell area and roundness (length/width). Both treatment of the K56-2 with A22 and depletion of EtfBA caused a statistically significant shift towards rounder cells (Fig. 5a); however, whilst EtfBA-depleted cells were smaller, treatment with A22 actually increased cell size (Fig. 5b).

To confirm that the effect of depleting ETF was different from MreB depletion, we used A22 in a sensitization index assay, where controlled underexpression of an essential gene results in enhanced sensitivity to growth inhibitors that target the encoded gene product (Cardona et al., 2014). This specific effect upon exposure of small molecules of known function can be used to profile an essential gene of unclear function. We exposed CetfBA and 77-16C10 to the action of A22 and other growth inhibitors, i.e. novobiocin (DNA replication, targets GyrB), chloramphenicol (protein synthesis, targets ribosome), meropenem (cell wall synthesis,
targets penicillin-binding proteins) and hydrogen peroxide (an oxidative stress inducer), at a range of rhamnose concentrations known to include those necessary to produce 30–60 % WT growth (data not shown). We previously showed that this range of growth was necessary to sensitize the mutants to specific growth inhibitors (Bloodworth et al., 2013). When a control mutant underexpressing gyrB was exposed to its binding antibiotic novobiocin, a strong sensitization effect could be observed (Fig. 6), resulting in a sensitization index of 8 (8 × lower MIC than that of the WT). An intermediate sensitization index of 4 could be observed for the gyrB mutant in response to hydrogen peroxide. This was reminiscent of the effect of fluoroquinolone-related antibiotics that target Gyrb and generate reactive oxygen species (Goswami et al., 2006). Expression mutants of lepA, a gene coding for a translation elongation factor (Liu et al., 2011), and lepB, a gene encoding for a signal peptidase (Dalbey & Wickner, 1985), also showed intermediate levels of sensitivity to chloramphenicol, which inhibits translation but does not directly interact with the products of either gene. As in previous studies (Bharat & Brown, 2014; Campbell et al., 2005), sensitization indexes <4 were not considered indicative of sensitization. No hypersensitivity to A22 was observed with either the etfA (sensitization index 2.7) or etfBA (sensitization index 2) mutants, suggesting that the effect of ETF on cell shape is independent of MreB. Interestingly, whilst the etfBA mutant only showed intermediate levels of sensitivity to hydrogen peroxide, the etfA mutant had intermediate sensitivity to both hydrogen peroxide and meropenem. This is not a general response to antibiotics as neither mutant was hypersensitive to either novobiocin or chloramphenicol. Taken together, these data suggest that in Burkholderia cenocepacia ETF triggers a rod-to-sphere change in cell morphology, which is independent of MreB.
ETF dehydrogenase, the putative partner of ETF, also induces a rod-to-sphere change in morphology

Whilst ETFs are known to accept electrons from a variety of partners, in all known cases they donate electrons to corresponding ETF dehydrogenases (Ghisla & Thorpe, 2004; Roberts et al., 1999; Scott & Ludwig, 2004). If the essential function of EtfBA involves passing electrons through to ETF dehydrogenase, then depletion of ETF dehydrogenase should have a similar effect as depleting EtfBA. Burkholderia cenocepacia contains two putative ETF dehydrogenases BCAL1468 (etfdh1) and BCAS0609 (etfdh2) with 99% amino acid identity. As the third chromosome of Burkholderia cenocepacia is dispensable (Agnoli et al., 2012), none of the genes found on the third chromosome, including etfdh2, are likely to be essential. We therefore created a site-directed rhamnose-inducible expression mutant of etfdh1 in the K56-2 (Cetfdh1; Table 1) and Δetfdh2 (Cetfdh; Table 1) backgrounds. In the presence of etfdh2, lack of etfdh1 expression reduced growth to 29% of growth with rhamnose (Fig. S3). In the Δetfdh2 mutant, growth was unaffected as long as etfdh1 was expressed, but fell to 13% in the absence of rhamnose, suggesting that etfdh2 could partially complement etfdh1.

DIC microscopy of the ETF dehydrogenase mutants grown without rhamnose showed that after 48 h cells had undergone a similar change in morphology to depletion of EtfBA (Fig. 4b). Quantification of cell size and shape confirmed that depletion of ETF dehydrogenase resulted in significantly rounder (Fig. 5a) and smaller cells (Fig. 5b). To determine whether this conversion to small round cells is a general result of metabolic disruption, rather than having any relation to ETF function, the morphology of conditional expression mutants of cytochrome c1 as well as E3F3-ATPase were examined. In both mutants cell roundness was unaffected by incubation without rhamnose, whilst cell size actually increased slightly in the ATPase mutant (Figs S4 and S5). These results suggest the morphology seen when EtfAB or ETF dehydrogenase is depleted is not due to a general defect of respiration.

DISCUSSION

Bacteria belonging to the Burkholderia cepacia complex have high levels of intrinsic resistance to current antibiotics (Leitão et al., 2008; Maravić et al., 2012) and disinfectants (Rushston et al., 2013), which warrants the investigation of novel essential genes as possible drug targets. ETFs have been identified as putatively essential in several HTSs and so represent an intriguing case of an essential gene with no known essential functions. We constructed a rhamnose-inducible etfBA expression mutant in Burkholderia cenocepacia K56-2 (CetfBA), and confirmed (through complementation assays) that expression of the operon is required for growth on both rich media and a variety of single-carbon sources. This indicates that whilst traditionally ETF functions in the metabolism of specific carbon sources, the essential function in Burkholderia cenocepacia is carbon-source independent. We did find that high levels of etfB expression alone could partially complement lack of etfBA expression. As etfA is essential in the original transposon mutant (77-16C10) despite native expression of etfB from the original promoter (Bloodworth et al., 2013), the ability of etfB to restore growth of etfBA-depleted cells appears to be dose dependent. However, this effect is not related to homodimerization as EtfB only interacts with EtfA, but not with itself (Fig. S1). One possible explanation is that other EtfA subunits expressed by the paralogous genes BCAM2321 or BCAM1606 (Fig. 7a) are able to bind EtfB with low affinity, and this effect is evidenced when EtfB is overexpressed. In any case, it seems

Fig. 7. Phylogenetic trees of (a) EtfA and (b) EtfB proteins in Burkholderia spp. containing putatively essential ETF genes. Trees were reconstructed based on amino acid similarity; numbers on branches are the support values for each branch; branch length is proportional to the number of substitutions per site. ORFs annotated as being ETFs were included from Burkholderia thailandensis (BTH), Burkholderia pseudomallei (BPS) and Burkholderia cenocepacia (BCA). Known ETFs from E. coli K-12 substrain 3110 (Eco), Paracoccus denitrificans (Pde), Sinorhizobium meliloti 1021 (Sme) and Homo sapiens (Hsa) were used to establish the division into three groups/families (Tsai & Saier, 1995). Genes identified as being putatively essential by high-density transposon mutagenesis are identified with an _E and are bolded in black. Genes examined in this paper are in bold blue.
that optimal function of ETF is observed when *etfA* and *etfB* are expressed at the same level, as expression of *etfB* from both the chromosome and plasmid reduced growth to 65% of WT (Fig. 2a).

In contrast to most of the conditional expression mutants, CetfBA suffered from a severe loss of viability, with the number of viable cells falling below the limits of detection after 24 h (Fig. 2b). The inability of CetfBA to resume growth after depletion of ETF is associated with an irreversible loss of reducing power (Fig. 3b). This is consistent with a central role for EtfBA in respiration, but it is also possible that EtfBA depletion instead causes severe cellular damage, which in turn leads to collapse of respiration. In all known cases, ETFs function by transferring electrons from primary dehydrogenases to a corresponding ETF dehydrogenase (Ghisla & Thorpe, 2004; Roberts et al., 1999; Scott & Ludwig, 2004). In K56-2, lack of *etfdh1* expression leads to a reduction in cell growth (Fig. S3), rather than the complete abrogation of growth seen in CetfBA. As both *etfBA* and *etfdh1* expression in the mutants are driven by the same promoter, the difference in the mutant phenotypes may be due to the length of time required to deplete protein concentration, suggesting that either less Etfdh1 is required or that as a membrane-associated protein it is more stable (Nagata et al., 1998; St John et al., 1979).

In addition to inhibiting cell growth, depletion of either EtfBA or Etfdh results in a change in cell shape from rods to small spheres (Figs. 4 and 5). The uniform spherical cells could be due to arrest of cell growth immediately after cell division. Coupled with the simultaneous arrest of respiration, this suggests a metabolic connection. Growth in nutrient-poor media (Donachie & Begg, 1989; Sargent, 1975) and long-term starvation (Givskov et al., 1994) have been shown to cause a shift to shorter cells. Recently, specific metabolic sensors have been identified in both *Bacillus subtilis* (Monahan et al., 2014; Weart et al., 2007) and *E. coli* (Hill et al., 2013), which couple concentration of UDP-glucose to FtsZ polymerization and hence cell size at division. We propose that in *Burkholderia* species the lack of EtfBA or Etfdh1 may induce a signal for low nutrient availability, triggering early cell division. The lack of a shift to small round cells in electron transport chain mutants (Figs S4 and S5) is consistent with the signal being an as-yet unknown metabolic intermediate rather than energy availability in general.

The confirmation of ETF essentiality in *Burkholderia cenocepacia* and the strong phenotype suggest that it may warrant study in other important human pathogens, especially *M. tuberculosis* and *Burkholderia pseudomallei*. An essential gene screen in *M. tuberculosis* (Griffin et al., 2011) identified its single ETF operon *fixBA* as putatively essential. In contrast, all sequenced *Burkholderia* species have multiple group I ETF operons. Amongst *Burkholderia* species, high-throughput essential gene studies have only been carried out in *Burkholderia pseudomallei* (Moule et al., 2014), a tier 1 select agent and the cause of melioidosis, and *Burkholderia thailandensis* (Baugh et al., 2013), a closely related but far less pathogenic species. In both screens, all the annotated ETF operons contained at least one putatively essential gene and in both species one operon clusters closely with the *etfBA* operon in *Burkholderia cenocepacia* described here (Fig. 7), suggesting a common essential function amongst *Burkholderia* ETFs.

Regardless of the precise nature of ETF essentiality in *Burkholderia cenocepacia*, the loss of viability in cells without *etfBA* expression suggests that ETF would be a strong target for killing *Burkholderia cenocepacia* and potentially other *Burkholderia* species. However, chemical inhibition of bacterial ETFs may be challenging. Inhibition of the human ETF causes metabolic disorders, as fatty acid metabolism is important for energy hungry tissues (Frerman & Goodman, 2001); this makes selective inhibition of the bacterial ETF essential. In addition, ETFs interact with a number of different primary dehydrogenases via a dynamic interface (Frerman & Goodman, 2001), sampling a wide variety of structures before inducing a fit with their partner. This structural flexibility may reduce the possibility of an inhibitor binding to the bacterial but not human ETF. An attractive alternative is the development of antisense RNA silencing of ETF. The *etfBA* conditional expression mutant has a strong lethal phenotype, suggesting that post-transcription inhibition would likewise be bactericidal.

**ACKNOWLEDGEMENTS**

This work was supported by awards from Cystic Fibrosis Canada (315046-352600-2000) and the Natural Sciences and Engineering Research Council of Canada (12500918).

**REFERENCES**


An essential ETF in *Burkholderia cenocepacia*


Edited by: K. Flardh