Azorhizobium caulinodans electron-transferring flavoprotein N electrochemically couples pyruvate dehydrogenase complex activity to N₂ fixation

John D. Scott and Robert A. Ludwig

INTRODUCTION

Among diazotrophic and endosymbiotic bacteria, N₂ fixation activity is notably thermostable, typically showing little or no activity at or above 37 °C. Moreover, among legumes indigenous to temperate climates, symbiotic N₂ fixation activity is often impaired at or above 30 °C. Yet legume endosymbionts of the family Rhizobiaceae include Azorhizobium caulinodans, whose N₂ fixation activities both in culture and in symbiosis are remarkably thermostable in this temperature range and above. To what is owed this highly unusual thermostability?

To study this question, we isolated and characterized A. caulinodans thermostable point mutants unable to fix N₂ at 42 °C were isolated and mapped to three, unlinked loci; from complementation tests, several mutants were assigned to the fixABCX locus. Of these, two independent fixB mutants carried missense substitutions in the product electron-transferring flavoprotein N (ETFₙ) α-subunit. Both thermolabile missense variants Y238H and D229G mapped to the ETFₙα interdomain linker. Unlinked thermostable suppressors of these two fixB missense mutants were identified and mapped to the lpdA gene, encoding dihydrolipoamide dehydrogenase (LpDH), immediately distal to the pdhABC interlocus. These two suppressor alleles encoded LpDH NAD-binding domain missense mutants G187S and E210G. Crude cell extracts of these lpdA double mutants showed 60–70 % of the wild-type PDH activity; neither fixB lpdA double mutant strain exhibited any growth phenotype at the restrictive or the permissive temperature. The genetic interaction between two combinations of lpdA and fixB missense alleles implies a physical interaction of their respective products, LpDH and ETFₙ. Presumably, this interaction electrochemically couples LpDH as the electron donor to ETFₙ as the electron acceptor, allowing PDH complex activity (pyruvate oxidation) to drive soluble electron transport via ETFₙ to N₂, which acts as the terminal electron acceptor. If so, then the A. caulinodans PDH complex activity sustains N₂ fixation both as the driving force for oxidative phosphorylation and as the metabolic electron donor.

Azorhizobium caulinodans thermolabile point mutants unable to fix N₂ at 42 °C were isolated and mapped to three, unlinked loci; from complementation tests, several mutants were assigned to the fixABCX locus. Of these, two independent fixB mutants carried missense substitutions in the product electron-transferring flavoprotein N (ETFₙ) α-subunit. Both thermolabile missense variants Y238H and D229G mapped to the ETFₙα interdomain linker. Unlinked thermostable suppressors of these two fixB missense mutants were identified and mapped to the lpdA gene, encoding dihydrolipoamide dehydrogenase (LpDH), immediately distal to the pdhABC interlocus. These two suppressor alleles encoded LpDH NAD-binding domain missense mutants G187S and E210G. Crude cell extracts of these lpdA double mutants showed 60–70 % of the wild-type PDH activity; neither fixB lpdA double mutant strain exhibited any growth phenotype at the restrictive or the permissive temperature. The genetic interaction between two combinations of lpdA and fixB missense alleles implies a physical interaction of their respective products, LpDH and ETFₙ. Presumably, this interaction electrochemically couples LpDH as the electron donor to ETFₙ as the electron acceptor, allowing PDH complex activity (pyruvate oxidation) to drive soluble electron transport via ETFₙ to N₂, which acts as the terminal electron acceptor. If so, then the A. caulinodans PDH complex activity sustains N₂ fixation both as the driving force for oxidative phosphorylation and as the metabolic electron donor.
complexes. While inherent rates of ETF-mediated soluble electron transfer processes are slow, ETF activity nevertheless facilitates the overall cellular oxidation-reduction balance. In eukaryotic mitochondria, conceptually, ETF couples to membrane respiration various oxidations carried out by matrix (soluble) flavoprotein dehydrogenases. Itself a matrix protein, ETF primarily serves as an electron acceptor for fatty acid oxidation, re-oxidizing a set of four (long-, medium-, short- and branched-chain) fatty acyl-CoA dehydrogenases. In turn, reduced mitochondrial ETF donates electrons to ETF:ubiquinone oxidoreductase (ETF-QO), a peripheral mitochondrial membrane activity, and thence to membrane respiration (Goodman et al., 1994).

In the Rhizobiaceae, a family of microaerophilic bacteria, a specialized ETFN facilitates both symbiotic (Corbin et al., 1983; Earl et al., 1987) and free-living (Donald et al., 1986) N₂ fixation by acting as the electron-transfer intermediary. In electrochemical terms, N₂ fixation is a highly reductive (one equivalent of) H₂. Dinitrogenase complex activity is thereby shuttles reducing equivalents via soluble electron carriers to the dinitrogenase complex, sustaining N₂ fixation. Thus, PDH complex activity facilitates N₂ fixation and thence to membrane respiration (Goodman et al., 1994).

In bacteria and eukaryotes, NAD⁺ normally serves the PDH complex as the electron acceptor under aerobic physiological conditions. However, as we report here, in microaerobic A. caulinodans ETFN and the pyruvate dehydrogenase (PDH) complex, specifically its dihydrolipoamide dehydrogenase (LpDH) component. The PDH complex, which oxidizes pyruvate to acetyl-CoA, includes three catalytic centres: PDH (E1), dihydrolipoamide S-acetyltransferase (E2) and LpDH (E3). LpDH is a member of the pyridine-nucleotide disulfide oxidoreductase superfamily and carries a single, tightly bound FAD prosthetic group. In bacteria, typically, LpDH is encoded by several paralogous genes, each of which map to tightly linked gene sets encoding the various 2-oxoacid (pyruvate, 2-oxoglutarate and 2-oxoisovalerate) dehydrogenase complexes.

In bacteria and eukaryotes, NAD⁺ normally serves the PDH complex as the electron acceptor under aerobic physiological conditions. However, as we report here, in microaerobic A. caulinodans ETFN and the pyruvate dehydrogenase (PDH) complex, specifically its dihydrolipoamide dehydrogenase (LpDH) component. The PDH complex, which oxidizes pyruvate to acetyl-CoA, includes three catalytic centres: PDH (E1), dihydrolipoamide S-acetyltransferase (E2) and LpDH (E3). LpDH is a member of the pyridine-nucleotide disulfide oxidoreductase superfamily and carries a single, tightly bound FAD prosthetic group. In bacteria, typically, LpDH is encoded by several paralogous genes, each of which map to tightly linked gene sets encoding the various 2-oxoacid (pyruvate, 2-oxoglutarate and 2-oxoisovalerate) dehydrogenase complexes.

**METHODS**

**Bacterial strains, culture methods and mutagenesis.** An A. caulinodans library carrying random, single, vector-insertion (Vi) mutants (Donald et al., 1985; Donald et al., 1986) was screened for thermolabile N₂ fixation-defective strains at 42 °C as the restrictive and 30 °C as the permissive condition in defined N₂ fixation (NIF) salts medium (0.4% potassium succinate, 50 mM potassium phosphate pH 6.4, 1.25 mM magnesium sulfate, 300 μM potassium nicotinate, 0.5 mM calcium chloride, 1 μg NaMoO₄ ml⁻¹, 1 μg FeCl₃ ml⁻¹). For outgrowth under permissive conditions, A. caulinodans strains were propagated in defined minimal medium (ORS-MM; 0.4% potassium succinate, 10 mM potassium phosphate pH 6.3, 1 mM magnesium sulfate, 0.5 mM calcium chloride, 16 μg nicotinate ml⁻¹, 1.0 μg pantothenate ml⁻¹, 0.2 μg biotin ml⁻¹). For liquid batch culture experiments, test strains were first cultured in rich GYPC medium (0.4% d-glucose, 0.2% yeast extract, 10 mM potassium phosphate pH 6.3, 0.2% salt-free casein acid hydrolysate). Culture growth was monitored spectrophotometrically (OD₆₀₀). To measure N₂-dependent growth, A. caulinodans strains were first cultured in liquid ORS-MM under air sparge to late-exponential phase (OD₆₀₀ 0.7). Bacterial cells were recovered by centrifugation, washed twice and diluted to ~1×10⁶ cells ml⁻¹ (OD₆₀₀ 0.05) in NIF medium; cell samples were placed in stoppered serum vials and again cultured at 30 °C under sparge with O₂/CO₂/N₂ (1%:1%:98%). For N₂-dependent colony growth tests, NIF medium was solidified with acid-washed (1 M HCl) agarose and plates were incubated in sealed jars at 30 °C under continuous sparging with CO₂/O₂ (0.1%:1.0%).

Thermolabile N₂ fixation-defective mutants were also isolated after ethyl methanesulfonate (EMS) mutagenesis, followed by repetitive enrichment. A. caulinodans 57100 (wild-type) was cultured in GYPC medium at 30 °C to mid-exponential phase (5×10⁶ cells ml⁻¹; OD₆₀₀ 0.25). Cells were recovered by centrifugation, washed with EB (0.075 M potassium phosphate pH 6.3, 1.5 mM ammonium sulfate, 0.8 mM magnesium sulfate, 0.05 mM calcium chloride), recovered by centrifugation and resuspended four-fold concentrated in EB. EMS was added to 0.25% (w/v), and cultures were incubated with vigorous shaking for 30 min at 30 °C (~1% survival). Mutagenized cells were recovered by centrifugation, washed with GYPC, centrifuged again, resuspended 100-fold-diluted in GYPC and cultured overnight at 30 °C with vigorous shaking. To screen for N₂ fixation-defective missense mutants, washed, EMS-mutagenized, outgrown cell samples (1×10⁶ cells ml⁻¹; OD₆₀₀ 0.05) were induced for 4 h at 42 °C as described above for isolation of null mutants. Following this, Timentin (0.1 mg ml⁻¹), a proprietary mixture of ticarcillin and β-clavulanate, was added and cultures were incubated for 6 h at 42 °C; continuous sparging with a mixture of O₂/CO₂/N₂ (1%:1%:98%) was maintained. In control experiments, viable cell counts showed ~0.1% survival, measured as colony-forming ability on GYPC, after this treatment. Surviving cells were recovered by centrifugation and subcultured overnight at 30 °C in ORS-MM. Enrichments were repeated for a total of four cycles. Surviving cells were first plated on ORS-MM at 42 °C and subsequently replica-plated on NIF medium plates incubated at either 30 or 42 °C for 14 days in sealed jars under continuous sparging with a mixture of O₂/CO₂/N₂ (1%:1%:98%); temperature-sensitive mutants were identified and characterized further (see Results).

**Molecular cloning and DNA sequencing.** The nucleotide sequence for the A. caulinodans lpdA gene located on recombinant plasmid pSKC42, derived from recombinant pλPh4A, was determined by standard techniques (Sambrook et al., 1989). Identification of the gene product was made by BLASTP search versus the...
San Diego Supercomputer Center non-redundant protein database and confirmed by multiple PIMA algorithm alignments (Smith & Smith, 1990).

**Dinitrogenase assay by acetylene reduction activity.** *A. caulinodans* N2 fixation was measured in culture by acetylene reduction and N2-dependent growth. Cells were grown to late-exponential phase in ORS-MM supplemented with 0.1 mM nicotinate, harvested, washed twice, resuspended (4 × 10^8 cells ml⁻¹) in NIF medium, placed in stopped serum vials and sparged with O2/CO2/Ar (3%: 1%:96%) for 8 h at 30°C. Vials were then injected with 0.2 atm acetylene (substrate) freshly generated by hydration of calcium carbide, and assayed for ethylene production versus time using GC (Donald et al., 1985).

**PDH activity.** Mid-exponential phase cultures in ORS-MM were isolated and cell-free extracts were prepared by ultrasonication. Extracts were then cleared by centrifugation (1 h at 100,000 g) and assayed immediately or, for determination of kinetic constants, further purified by immunoprecipitation. Cleared extracts were made by addition of 1% Nonidet P40 (NP40), 0.05% sodium deoxycholate and 10 mM PMSF, and treated for 1 h at 4°C with 1% polyonal antisera prepared against purified Mycoplasma pneumoniae PDH-B (Dallo et al., 2002); 5% protein-A agarose suspension (Boehringer Mannheim) was then added and samples were incubated for a further 3 h at 4°C with slow tumbling. Samples were precipitated by centrifugation (1 min at 15,000 g), washed/precipitated twice for 20 min at 4°C in 1 vol. (original extract) of a mixture of 50 mM Tris/Cl pH 7-4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 1 mM PMSF, and again precipitated by centrifugation. Purified PDH was eluted by resuspension and overnight incubation with 50 mM Tris/Cl pH 7-4, 0.5% NaCl, 0.1% NP40 and 0.05% sodium deoxycholate at 4°C with slow tumbling. After centrifugation (1 min at 15,000 g) at the supernatant containing eluted PDH was dialysed against a mixture of 0.1 M Tris/Cl pH 7-5, 10 mM MgCl2, 0.1 mM CoA and 1 mM DTT at 4°C. Protein concentrations were measured by standard Bradford assays. PDH activity at 30°C was inferred as pyruvate-dependent reduction of NAD+ measured as spectrophotometric absorbance at 339 nm. Reactions comprised 0.1 M Tris/Cl pH 7-5, 10 mM MgCl2, 0.12 mM CoA, 6 mM DTT, 1:2 mM NAD+ and supernatant fractions of cell-free extracts (500 μg total protein), and were initiated with 10 mM pyruvate. Pyruvate-dependent cytochrome reduction was assayed by ferricyanide reduction. Batch cultures were grown on ORS-MM supplemented with 0.2% potassium succinate, 0.1% monosodium glutamate and 0.1 mM potassium nicotinate; early-exponential phase cultures were harvested and washed; cell pellets were resuspended and lysed by ultrasonication. Crude cell-free extracts were then used to measure pyruvate-dependent reduction of ferricyanide at 30°C measured as spectrophotometric absorbance at 420 nm. Reactions contained 0.1 M Tris/Cl pH 7-5, 10 mM MgCl2, 0.07 mM potassium ferricyanide and added crude cell-free extract (100 μg total protein), and were initiated with 20 mM pyruvate.

**Sesbania rostrata nodulation tests.** *S. rostrata* seedlings were germinated aseptically and grown on sterile, defined medium under nitrogen limitation (Kwon & Bevers, 1992). Three-week-old plants, some 50 cm in height, were inoculated with the desired *A. caulinodans* strain between first and second stem internodes, for which region stem-nodule development is synchronized (Donald et al., 1986). Starting 6 days post-inoculation, maturing stem-nodules were excised and tested for N2 fixation activity by acetylene reduction. Thereafter, nodules were recovered, crushed and homogenized (Polytron); cell-free supernatants were analysed for leghaemoglobin measured as spectrophotometric absorbance at 540 nm. All developmental nodulation tests were done in triplicate.

**RESULTS**

*A. caulinodans* fixes N₂ at high temperature (42°C) yet lacks novel genes specifically conferring thermostable N₂ fixation

Mature *S. rostrata* plants nodulated with *A. caulinodans* 57100 (wild-type isolate) and cultivated in defined nutrient salts limited for available-nitrogen reproducibly grew faster at 37°C than at 30°C when given saturating light intensities and a 12 h photoperiod. Enhanced growth required inoculation of seedlings with *A. caulinodans* 57100. In pure cultures, *A. caulinodans* 57100 dinitrogenase complex rates were undiminished at 37°C when compared to 30°C; at 42°C, rates were approximately 40% as measured by acetylene reduction. When *A. caulinodans* 57100 was cultured with defined media in which N2 was the predominant nitrogen source, growth yields corroborated with dinitrogenase activities (results not presented). To help understand (this unusual) thermostable N₂ fixation, two searches were carried out in parallel and screening was done in several steps. In the first search, a strain library of *A. caulinodans* 57100 derivatives carrying single-copy, random Vi2021 vector-insertions was used (Donald et al., 1985); in the second search, a strain library of chemically mutagenized *A. caulinodans* 57100 derivatives was used.

In both searches, batch cultures were replicated onto solid, defined, nitrogen-limited (NIF) medium (see Methods) and duplicate plates were incubated for 5 days at 30 or 42°C. Duplicate plates were then superimposed and opaque (dense) colonies specific to plates incubated at 30°C were identified. Candidate strains were isolated, purified and re-tested for growth both in liquid and on solid defined media. Six, nominally independent candidates from the first (insertion mutant) search, strains 60501–60506, were individually retested for growth on solid NIF medium; while all strains showed absolutely no growth at the restrictive temperature, they showed only limited growth at the permissive temperature. At the restrictive temperature, all six candidates grew indistinguishably from the wild-type on NIF medium supplemented with 10 mM ammonium. Despite repeated searches of the random insertion mutant library, no candidates that both retained wild-type N₂-dependent growth at the permissive temperature and completely lacked growth at the restrictive temperature were identified.

Strains 60501–60506 were subjected to direct molecular (vector-insertion) cloning as described previously (Donald et al., 1985), and recombinant plasmids for each mutant were obtained (Table 1; see Methods). These plasmids (pDRN60501 to pDRN60506) were used as DNA hybridization probes against a battery of recombinant λ phages carrying wild-type *nif* loci (Donald et al., 1986). All six plasmids hybridized generally with phage λ*nif*3 DNA and specifically to a 3·0 kb BglII DNA fragment carrying *nifK* and *nifE* DNA homology. DNA sequencing analysis using as primer a specific IS50 deoxyyligonucleotide sequence of the
insertion element allowed delineation of precise insertion points in the mutants (Pauling et al., 2001). Comparative DNA sequencing analyses versus the wild-type identified all six strains as carrying distal \textit{nifE} insertions (results not presented).

To measure dinitrogenase activity by acetylene reduction assay, the six \textit{nifE} mutants were batch-cultured in liquid defined salts medium (ORS-MM) containing 10 mM ammonium at 30°C to early-exponential phase, pelleted, washed and physiologically shifted to N-limited (NIF) medium (Donald et al., 1985) under constant sparging with O\textsubscript{2}/CO\textsubscript{2}/N\textsubscript{2} (1 % : 1 % : 98 %). The N\textsubscript{2} fixation-induced cultures were then divided; subcultures were incubated at 30 or 42°C and at various times culture samples were removed for dinitrogenase activity assays as measured by acetylene reduction (see Methods). Among the \textit{nifE} null strains, absolute rates for acetylene reduction activities at the permissive temperature (30°C) were approximately 20% those of wild-type strain 57100. And, in all six cases, residual, relative, fractional acetylene reduction activities (activity at 42°C : activity at 30°C) were similar to that of the wild-type. By contrast, control \textit{nifD}228 null strain 60228 showed no detectable acetylene reduction activity at either the restrictive or the permissive temperature. Therefore, when assayed by acetylene reduction, dinitrogenase activities of \textit{nifE} insertion mutants did not show relative thermolability. Thus, from the insertion mutant screen, novel \textit{A. caulinodans} gene(s) specifically required for high-temperature (42°C) N\textsubscript{2} fixation were not identified. As this insertion mutant search was exhaustive, \textit{A. caulinodans} seemingly lacks genes specifically required for high-temperature (42°C) N\textsubscript{2} fixation.

\textbf{Isolation of \textit{A. caulinodans} \textit{N\textsubscript{2}} fixation temperature-sensitive (Nifts) thermolabile point mutants}

Taking a more subtle approach, in a second genetic screen, we sought Nifts thermolabile point mutants arising from ethyl methanesulfonate mutagenesis of \textit{A. caulinodans} 57100 (see Methods). Ethyl methanesulfonate-mutagenized cultures were outgrown in rich medium, shifted to N\textsubscript{2} fixation-dependent liquid growth conditions at the restrictive temperature (42°C) and subjected to a penicillin-type enrichment scheme (see Methods). After four growth/
selection cycles, enriched cells were duplicate-plated and tested for specific, defective N$_2$-dependent growth at 42 °C as discussed above. Candidates were retested for wild-type N$_2$-dependent growth at 30 °C; identified strains were presumed to carry novel Nif$^+$ alleles. To help categorize new Nif$^+$ mutations, candidates were subjected to genetic complementation tests. To each candidate, recombinant plasmids pNSN8, pNSN11, pMSM15, pNSN18, pNSN21, pNSN31 and pNSN41 (Table 1) were introduced by electroporation; these recombinant plasmids carry previously identified A. caulinodans wild-type nif loci (Donald et al., 1986). Each of the 37 newly identified Nif$^+$ mutants was complemented by a particular pNSN plasmid at the restrictive temperature (results not presented). Therefore, no novel nif gene loci were uncovered.

Two Nif$^+$ mutants, strains 62106 and 62111 (Table 1), were complemented to wild-type by pNSN21, which carries wild-type N$_2$ fixation locus-2, including the fixABCX operon (Donald et al., 1986). The fixAB product encodes ETF$_N$; A. caulinodans recombinant ETF$_N$ has been purified and shown to comprise an FAD-containing heterodimer (results not presented). Residues of both ETF$_N$ subunits show approximately 40% identity and 60% conservation when compared with canonical ETF, both bacterial and eukaryotic. Within the Rhizobiaceae, the ETF$_N$ family is even more highly conserved; residues of both subunits typically show 70–80% identity and 90% conservation (Arigoni et al., 1991; Weidenhaupt et al., 1996). ETF mediates electrochemical coupling of multiple flavoprotein dehydrogenases (McKean et al., 1983). In the Rhizobiaceae, while ETF$_N$ functions specifically in N$_2$ fixation (Donald et al., 1986; Arigoni et al., 1991), the presumed flavoprotein dehydrogenases with which ETF$_N$ electrochemically interacts are not known.

To refine map positions for these Nif$^+$ mutations, strains 62106 and 62111 were subjected to recombination tests. Plasmids pVi60101 (FixA$^+$B$^+$) and pVi60113 (FixB$^+$C$^+$X$^+$) were introduced into strains 62106 and 62111 by electroporation, and cells were plated onto NIF medium at 42°C. Thermostable Nif$^+$ recombinants were obtained in the presence of both plasmids for both mutants, implying that strains 62106 and 62111 both carried fixB mutations. Accordingly, the fixB gene sequences of both strains were determined (see Methods) and compared to that of the wild-type (Arigoni et al., 1991). Both fixB temperature-sensitive alleles encoded distinct single-codon substitutions; the fixB238 missense allele of strain 62106 comprised an ETF$_N^{238}$ Y238H substitution, whereas the fixB229 missense allele of strain 62111 comprised an ETF$_N^{229}$ D229G substitution (Table 1). In the rhizobial ETF$_N^{238}$ family, residues Y238 and D229 are highly conserved. Presumably, both missense alleles yield thermolabile ETF$_N$ variants, which fold aberrantly and are possibly impaired in flavoprotein–flavoprotein interactions (see Discussion).

**Extragenic suppressors of A. caulinodans fixB temperature-sensitive mutants map to Ipda**

Spontaneous, thermostable derivatives of strains 62106 and 62111 were selected after four successive subcultures in liquid NIF medium at 42°C. Subcultures were plated onto solid NIF medium at 42°C and wild-type, opaque colonies were identified, isolated and re-tested for both colony morphology on the same medium and diazotrophic growth in liquid culture at the restrictive temperature (see Methods). To analyse the fixB alleles of each thermostable derivative, primer pairs diagnostic both for wild-type and mutant nucleotide sequences were used in PCR amplification experiments (see Methods). Using 17 nt oligodeoxynucleotide primers specific for both fixB temperature-sensitive alleles, as well as a primer pair representing wild-type fixB, each candidate strain yielded a single, diagnostic PCR amplification product corresponding to either the wild-type or a parental fixB temperature-sensitive missense allele (see Methods). From these diagnostic PCR results, nine of 12 independent, thermostable derivatives of strain 62106 were assigned as true wild-type revertants; three of the 12 derivatives retained the parental fixB mutant allele and thus carried some extragenic suppressor mutation(s). For strain 62111, seven of its 12 derivatives were wild-type revertants, and five of the 12 carried extragenic suppressors. In repeated growth tests with NIF medium in both liquid batch cultures and on solid media, all suppressor strains appeared fully wild-type at the restrictive temperature (42°C).

While initial hypotheses about the nature of such suppressor strains were not supported by experimentation, we were aided in the analysis of these strains by a serendipitous discovery. In unrelated work, we had analysed thermolabile derivatives of A. caulinodans strain 62004 that lacked PDH complex activity (Pauling et al., 2001). Unwittingly, two laboratory staff had given identical strain numbers to, in one case, thermolabile 62004 derivatives and, in the other, thermostable revertant/suppressor 62106 and 62111 derivatives. By happenstance, the latter strains were cultured and analysed for PDH activity in crude cell extracts. All but two strains showed wild-type PDH activity, measured as pyruvate-dependent reduction of NAD$^+$ (see Methods). After re-numbering the collection, these two strains were correctly labelled as thermostable 62106 and 62111 derivatives. Upon retesting, these fixB temperature-sensitive suppressor strains, renumbered 62202 and 62223 (Table 1), again showed approximately 60 and 70% of the wild-type PDH activity, respectively (Table 2). As evidenced by diagnostic PCR amplification and DNA sequencing, strains 62202 and 62223 retained a parental fixB temperature-sensitive missense allele and thus carried extragenic suppressor mutation(s).

Crude cell extracts of strains 62202 and 62223 were also tested for membrane respiration-linked pyruvate oxidation activity, measured as pyruvate-dependent reduction of cytochrome c, coupled to artificial electron acceptors (see
Table 2. PDH activities in *A. caulinodans* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>PDH activity (NAD⁺-dependent)*</th>
<th>PDH activity (cytochrome c-dependent)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 °C</td>
<td>42 °C</td>
</tr>
<tr>
<td>57100</td>
<td>0-30</td>
<td>0-31</td>
</tr>
<tr>
<td>62004R</td>
<td>&lt;0-01</td>
<td>&lt;0-01</td>
</tr>
<tr>
<td>62202</td>
<td>0-18</td>
<td>0-19</td>
</tr>
<tr>
<td>62223</td>
<td>0-23</td>
<td>0-23</td>
</tr>
</tbody>
</table>

*Activity defined as µmol pyruvate-dependent NADH generated (mg protein)⁻¹ min⁻¹.
†Activity defined as µmol pyruvate-dependent ferrocyanide generated (mg protein)⁻¹ min⁻¹.

Methods). As measured by cytochrome c-dependent assay, pyruvate oxidation activity was present at essentially wild-type levels in both cases (Table 2). Therefore, PDH activity in strains 62202 and 62223 was partially defective.

To strengthen this inference, *fixB* temperature-sensitive parental strains 62106 and 62111 were dosed with extra copies of the wild-type *pdh* operon and tested for thermostable *N₂* fixation. Plasmid pTROY13 (Ludwig, 1987), which carries a functional, recombinant *E. coli* lamB gene, was introduced into *fixB* temperature-sensitive strains 62106 and 62111 by intergeneric bacterial conjugations, using *E. coli* SM10 as donor (Table 1). Because this lamB gene encodes a trimeric porin, localized to outer membranes, and which mediates maltodextrin transport, and because the LAMB porin gratuitously serves as a receptor for coliphage λ (Randall-Hazelsau & Schwartz, 1973; Schwartz, 1975), diverse Gram-negative bacteria expressing/exporting a recombinant LAMB are efficiently infected by DNA packaged as coliphage λ (Ludwig, 1987). Liquid batch cultures of *A. caulinodans* strains 62106(pTROY13) and 62111(pTROY13) were infected at a high m.o.i. (∼100 cell⁻¹) with recombinant *lpdH*C4 carrying the wild-type *A. caulinodans* *pdh* operon (Pauling et al., 2001), and *N₂* fixation activity was induced by physiological shift (see Methods). When tested 4 h post-infection at the restrictive temperature (42 °C), *lpdH*C4-infected strains 62106(pTROY13) and 62111(pTROY13) reproducibly showed increased acetylene reduction activity, albeit not at fully wild-type level; in the absence of added recombinant *lpdH*C4, no increases in acetylene reduction activity at 42 °C were noted (results not presented). Therefore, both *fixB* temperature-sensitive strains were partially suppressed by obtaining additional copies of the wild-type *pdh* operon; phenotypic changes were presumably gene dosage effects. Moreover, this phenotypic change was similar to that obtained with thermostable, extragenic suppressors mapping to the *pdh* operon.

*A. caulinodans* strain 62004R, which carries a null *pdhB* allele and is absolutely defective for PDH complex activity, shows pronounced growth and *N₂* fixation phenotypes when tested in culture (Pauling et al., 2001). Accordingly, suppressor strains 62202 and 62223 were subjected to various physiological tests in liquid batch cultures at 30 °C for comparison to both Pdh⁻ control strain 62004R and wild-type strain 57100. For the suppressor strains, growth phenotypes and *N₂* fixation activities in aerobic and microaerobic conditions showed either very subtle or no significant differences. Likewise, while both strains carried *fixB* temperature-sensitive mutations, no significant growth differences at either the permissive or the restrictive temperature were observed.

To characterize extragenic suppression precisely, *pdh* operon nucleotide sequences from thermostable suppressor strains 62202 and 62223 were determined (see Methods). In both suppressor strains, the *pdhB*, *pdhD* and *pdhC* genes proved identical to those of wild-type strain 57100. Therefore, nucleotide sequencing was extended distal to the *pdhABC* genes. Several hundred nucleotides downstream of *pdhC*, a 1-4 kbp ORF (GenBank accession no. AY331184) was identified. From multiple alignments of the translated product, this ORF was conclusively identified as that encoding LpDH, the third (E3) component of the PDH complex. For comparison, nucleotide sequences for the *lpdA* gene in suppressor strains 62202 and 62223 were also obtained (see Methods). For strains 62202 and 62223, single point mutations in the *lpdA* coding sequence were identified and confirmed by double-stranded nucleotide sequencing analyses. When parental thermolabile strains 62106 and 62111 were similarly analysed both strains carried the wild-type *lpdA* allele.

The distinctive *lpdA* point mutations carried by suppressor strains 62202 and 62223 resulted in single-codon substitutions of the inferred translated product LpDH, 472 residues in length. In strain 62202, the LpDH product carried a G187S substitution, whereas in strain 62223, LpDH carried an E210G substitution (Table 1). Among the *γ-Proteobacteria*, the LpDH gene family is highly conserved. Indeed, atomic resolution crystal structures for both *Azotobacter vinelandii* LpDH (PDH complex) and *Pseudomonas putida* LpDH (2-oxoisovalerate dehydrogenase) are virtually superimposable (Mattevi et al., 1991, 1992). Accordingly, when the *Azorhizobium caulinoi"dans* LpDH sequence was superimposed on the *P. putida* LpDH structure, residues 187 and 210 both mapped to
the NAD\(^+\)-binding domain and, as a result, the missense suppressor variants quite possibly adversely affected NAD\(^+\) binding.

To test this possibility, active PDH complex was purified from mid-exponential phase, aerobic batch cultures of wild-type, 62202 and 62223 grown in defined medium with L-malate as the energy source (see Methods). Values of \(K_m\) (apparent) for NAD\(^+\) binding were measured for each PDH complex preparation under standard assay conditions (see Methods). Wild-type PDH complex yielded an apparent \(K_m\) (NAD) value of 0.025 mM; 62202 PDH complex yielded an apparent \(K_m\) (NAD) value of 0.13 mM; 62223 PDH complex yielded an apparent \(K_m\) (NAD) value of 0.10 mM. Thus, the PDH complex activities comprising LpDH variants G187S and E210G showed four- to fivefold diminished NAD\(^+\)-binding affinities. Moreover, wild-type PDH activity was strongly inhibited by the addition of 10 \(\mu\)M NADH. Such inhibition is likely to be allosteric. Therefore, in microaerobic wild-type \(A.\ caulinodans\) cultures, in which steady-state NADH-to-NAD\(^+\) ratios approach unity (Pauling et al., 2001), in vivo PDH activity might then be NADH-inhibited.

In summary, distinct \(lpdA\) alleles were found to suppress distinct \(fixB\) thermolabile alleles, allowing \(N_2\) fixation. As this extragenic suppressor analysis yielded a gene-for-gene interaction between \(A.\ caulinodans\) \(fixB\) and \(lpdA\), physical interaction of the respective gene products ETF\(_N\) and LpDH is implicit. Presumably the ETF\(_N\)-LpDH physical interaction mediates electron transfer between these two flavoproteins. Thermolabile ETF\(_N\) variants most likely possess decreased affinity for LpDH; suppressor LpDH variants compensate with decreased affinity for NAD\(^+\).

**DISCUSSION**

In both symbiotic \(N_2\)-fixing and diazotrophic bacteria, contiguous \(fixAB\) genes encode heterodimeric ETF\(_N\). In \(A.\ caulinodans\), single \(fixA\) or \(fixB\) null mutants are phenotypically \(Nif^-\) in culture but are otherwise wild-type. Thus, ETF\(_N\) is specifically required for dinitrogenase complex activity (Donald et al., 1986). In the \(Rhizobiaceae\), \(fixAB\) reside in a highly conserved \(fixABCX\) operon (Corbin et al., 1983; Earl et al., 1987; Dusha et al., 1987; Arigoni et al., 1991). As assigned by gene order, \(fixA\) encodes ETF\(_N\)\(\beta\) and \(fixB\) encodes ETF\(_N\)\(\alpha\). The ETF family shows the strongly conserved folded structures representative of the pyridine-nucleotide disulfide oxidoreductase superfamily. In bacteria and eukaryotes, the ETF heterodimer tightly but non-covalently binds a single FAD and a single 5'AMP, the latter an evolutionary vestige of the conserved pyridine-nucleotide site typical of this superfamily. However, the ETF family lacking both an active disulfide and its participating cysteine residues otherwise conserved among this superfamily. In electrochemical studies, ETF shows both one- and/or two-electron transfer to/from various flavoprotein dehydrogenases. In eukaryotic mitochondria, principal electron donors to ETF include the acyl-CoA dehydrogenases of fatty-acid catabolism. However, as the \(Rhizobiaceae\) do not actively catabolize aliphatic fatty acids for use as an energy source, ETF\(_N\) must serve as an electron acceptor to other flavoprotein dehydrogenases.

In eukaryotic mitochondria, a peripheral, membrane-associated ETF:ubiquinone oxidoreductase (ETF-QO) serves as the principal electron acceptor to ETF, which then couples mitochondrial oxidations by soluble (matrix) flavoprotein dehydrogenases to membrane respiration (Goodman et al., 1994). However, in the \(Rhizobiaceae\), ETF\(_N\)-dependent membrane respiration characteristic of ETF-QO activity has not been observed. From primary sequence homology, the FIXC family is a highly conserved flavoprotein distantly related to ETF-QO yet lacking both hydrophobic and amphipathic transmembrane \(\alpha\)-helices. Thus, the FIXC family is unlikely to be stably membrane-associated. In the \(Rhizobiaceae\), the tightly linked \(fixX\) gene encodes a typical, small, soluble [4Fe-4S]ferredoxin (Arigoni et al., 1991). In diverse microaerophilic \(N_2\)-fixing bacteria, because the \(fixAB\) gene set is both highly conserved and invariably tightly linked, FIXC likely comprises an ETF: ferredoxin oxidoreductase. If so, then a plausible (soluble) electron transfer pathway is

\[
\text{ETF}_N \rightarrow \text{ETF} \rightarrow \text{ferredoxin oxidoreductase} \rightarrow \text{ferredoxin} \rightarrow N_2\ase \rightarrow N_2
\]

What flavoprotein(s) serve as the electron donor to ETF\(_N\)? In the \(Rhizobiaceae\), the trail blazed by genetic analysis of \(N_2\) fixation abruptly ends; despite saturation mutagenesis experiments, no \(Nif^-\) mutant representing a putative electron donor to ETF\(_N\) has been identified. This result leaves two possible explanations, which are not mutually exclusive: (i) redundancy and (ii) pleiotropy. In support of the former, if ETF\(_N\) functions to recruit multiple electron donors for \(N_2\) fixation, any single genetic defect in a particular ETF\(_N\) electron donor might not necessarily render a phenotype. In support of the latter, there might exist a specific ETF\(_N\) electron donor in which mutations would yield multiple growth defects and not a simple \(Nif^-\) phenotype.

Consistent with pleiotropy, we have here described a gene-for-gene interaction between the \(A.\ caulinodans\) \(fixB\) and \(lpdA\) genes. This genetic interaction implies physical interaction between the respective products ETF\(_N\)\(\alpha\) and the LpDH parologue comprising the E3 component of the PDH complex. This physical interaction presumably facilitates electron transfer. If the PDH complex functions as electron donor to ETF\(_N\), then pyruvate oxidation is the ultimate metabolic source of reducing equivalents for dinitrogenase complex activity (Fig. 1). Indeed, \(A.\ caulinodans\) strain 62004 carrying the null \(pdhB4\) mutation shows a conditional \(Nif^-\) phenotype. However, the PDH complex also serves as the driving force for oxidative phosphorylation as generally required for \(N_2\) fixation (Pauling et al., 2001).
Might the PDH complex serve N₂ fixation in both capacities?

In aerobic culture, the PDH complex LpDH (E₃) component uses NAD⁺ as an electron acceptor. During microaerobic N₂ fixation, both NAD⁺ and ETFₐ might kinetically compete to re-oxidize the FADH₂ centre of LpDH. Is this kinetic competition plausible? In the Rhizobiaceae, N₂ fixation is optimally adapted to true microaerobic conditions. When shifted from fully aerobic conditions, A. caulinodans microaerobic cultures show 10-fold increases in steady-state in vivo NADH-to-NAD⁺ ratios, approaching unity (Pauling et al., 2001). The archetype E. coli LpDH activity, a freely reversible enzyme, shows quite similar standard mid-point potentials for both the dihydrolipoamide and NADH two-electron electrochemical half-cell reactions (Koike et al., 1960). From equilibrium considerations, in vivo LpDH activity should theoretically tend to exhibit less regulation as the intracellular NADH-to-NAD⁺ ratio approaches unity. However, NADH is itself a powerful allosteric inhibitor of E. coli LpDH activity (Koike et al., 1960). From genome considerations, in vivo LpDH activity should theoretically tend to exhibit less regulation as the intracellular NADH-to-NAD⁺ ratio approaches unity. However, NADH is itself a powerful allosteric inhibitor of E. coli LpDH activity (Koike et al., 1960). Similarly, added 10 μM NADH yields strong inhibition of purified A. cauliodans PDH activity. Thus, in A. cauliodans microaerobic cultures, LpDH activity might be allosterically NADH-inhibited, possibly affecting its response to either NAD⁺ or ETFₐ as oxidant. As the fixABCX operon encoding ETFₐ is derepressed by FIXK, itself specifically produced in response to microaerobic culture (Kaminski et al., 1991), ETFₐ would necessarily then be more abundant.

From comparisons of stably folded structures, several inferences may be drawn from the thermolabile ETFₐ mutants and their suppressor LpDH mutants. In Paracoccus denitrificans, the ‘housekeeping’ ETF has a folded structure highly conserved with that of mammalian ETF (Herrick et al., 1994). ETFₐ folds into two globular domains, one of which binds FAD, the second acts to form a flexible hinge, stabilizing flavoprotein–flavoprotein interactions; ETFβ folds into a single domain, which binds 5’-AMP. If rhizobial ETFₐ folds similarly, then the thermolabile mutations ETFₐ Y238H (fixB238) and ETFₐ D229G (fixB229) both map to the ETFₐ interdomain loop, in which single residue substitutions would be unlikely per se to affect stable ETFₐ folding. Rather, the observed thermolability of these ETFₐ variants might be owed to weakened flavoprotein–flavoprotein interactions, presuming the ETFₐ interdomain loop were to help stabilize such interactions.

What of the suppressor LpDH single residue variants? From genome analyses, α-Proteobacteria typically have three or more dispersed, paralogous lpd genes encoding LpDH isoforms. Discrete lpd genes map to loci encoding pyruvate, 2-oxoglutarate and 2-oxoisovalerate dehydrogenase complex activities. (In the α-Proteobacteria, an unlinked, fourth lpd gene typically encodes an exported, periplasmic LpDH activity replete with its own lipoamide-anchor domain.) These three oxoacid dehydrogenase complexes share a conserved reaction mechanism. Atomic resolution crystal structures for both Azotobacter vinelandii LpDH (PDH complex) and P. putida LpDH (2-oxoisovalerate dehydrogenase complex) are virtually superimposable (Mattevi et al., 1991, 1992). Rhizobiaceae LpDH isoforms show strong primary sequence homology with both P. putida LpDH (PDH) and A. vinelandii LpDH (2-oxoisovalerate dehydrogenase). Indeed, the atomic resolution crystal structure of P. putida LpDH complexed with substrate NAD⁺ is

---

**Fig. 1.** A. cauliodans electron-transfer pathways in microaerobic culture in support of N₂ fixation. NAD⁺ and ETFₐ kinetically compete to re-oxidize the PDH complex. Membrane electron (e⁻) transport (oxidative phosphorylation) yields ATP; soluble electron transport to N₂ consumes ATP.
physiological condition that represses N₂ fixation. In energy source results in steady-state nitrogen excess, a isoleucine and valine, the use of these amino acids as defined avidly catabolizes the branched-chain amino acids leucine, An obvious model for the sensitive suppressors include both lpdA187 and lpdA210 alleles which yield LpDH single-residue substitutions G187S and E210G. Both substitutions affect conserved residues of the NAD⁺-binding domain, as defined for Azotobacter vinelandii LpDH. Indeed, as evidenced here, these substitutions yield variant LpDH products with four- to fivefold decreased NAD⁺ affinities.

An obvious model for the lpdA–fixB gene-for-gene interaction follows from these inferences: ETF₅₆₇ Y238H and ETF₅₆₇ D229G both suffer decreased ETF₅₆₇–LpDH affinity; LpDH G187S and LpDH E210G both show decreased NAD⁺–LpDH affinity. The phenotypic effects of these decreased affinities are thus compensatory.

Other flavoprotein dehydrogenases might conceivably serve as electron donors to ETF₅₆₇. Parsimoniously, our results simply correlate certain ETF₅₆₇ loss-of-function alleles with certain LpDH (PDH) gain-of-function alleles. (In formal genetic parlance, ‘gain-of-function’ – gain of property ‘B’ – may fortuitously result from loss of property ‘A’.) Given ETF’s general role of facilitating the oxidation–reduction balance among multiple flavoprotein dehydrogenases, and given that Azorhizobium caulinodans possesses multiple (including PDH, 2-oxoglutarate dehydrogenase and 2-oxoisovalerate dehydrogenase) flavoprotein dehydrogenase complexes, might not other flavoproteins donate electrons to ETF₅₆₇? The rhizobial LpDH (2-oxoglutarate dehydrogenase) gene family carries an additional anchor domain conserved among peripheral membrane proteins. Quite possibly, this domain links rhizobial 2-oxoglutarate dehydrogenase complex activity directly to membrane-bound quinones as electron acceptor, precluding any interaction with ETF₅₆₇. Regarding the 2-oxoisovalerate dehydrogenase complex, inferences are problematic. While A. caulinodans avidly catabolizes the branched-chain amino acids leucine, isoleucine and valine, the use of these amino acids as defined energy source results in steady-state nitrogen excess, a physiological condition that represses N₂ fixation. In A. caulinodans, cognate 2-oxoacids (products of amino-transferase activities) of leucine, isoleucine and valine are taken up very poorly and sustain little or no culture growth.

In an analogous fashion, Klebsiella pneumoniae, a microaerobic diazotroph unrelated to the Rhizobiaceae, employs a specific flavodoxin-dependent pyruvate oxidoreductase activity as a metabolic electron donor for N₂ fixation (Hill & Kavanagh, 1980). Notably, this pyruvate:flavodoxin oxidoreductase is completely absent from the Rhizobiaceae.

In collaborative experiments, A. caulinodans recombinant ETF₅₆₇ has been purified and its active FAD centre has been isolated. However, electrochemical properties of purified ETF₅₆₇, including operative mid-point oxidation–reduction potentials, are markedly perturbed by the addition of other α-proteobacterial pyridine-nucleotide disulfide oxidoreductase flavoproteins, rendering any conclusions problematic. Quite possibly, given the ‘flexible-hinge’ folding model, flavoprotein–flavoprotein complex formation might precipitate large conformational changes in ETF, substantially altering its operative electrochemical oxidation–reduction potential (Byron et al., 1989; Jones et al., 2000).

From purely kinetic considerations, ETF’s participation in soluble electron-transfer pathways would seem of dubious merit. As higher-order complex formation lacks both theoretical and experimental support, ETF presumably forms only binary complexes with other flavoprotein dehydrogenases, alternately serving as electron acceptor and electron donor. If so, in vivo electron-transfer rates would reflect successive, soluble flavoprotein–flavoprotein formation/dissociation steps, and apparent rate constants would then be higher-order with respect to ETF concentrations. Among (evolutionarily) modern organisms, and given these kinetic constraints, N₂ fixation, with its exceedingly low kₐ₅ value (~ 10⁻² s⁻¹), is perhaps one of the very few metabolic processes accepting of ETF₅₆₇ as an obligate electron-transfer intermediary.

What of the unusual capacity of A. caulinodans to carry out N₂ fixation at a relatively high temperature (42°C)? In various diazotrophic bacteria, transcriptional regulation in general, and the NifA transcriptional activator in particular, seem to exhibit thermolability (Zhu & Brill, 1981). In the present study with A. caulinodans, thermolabile nifA mutants were absent. Rather, thermostable nifE null mutants were obtained. Yet, while nifE null mutants have been identified in various diazotrophic bacteria (MacNeil et al., 1978; Evans et al., 1988), a thermostable phenotype was not noted. NifE is purported to be important for biogenesis of the iron–molybdenum cofactor, itself required for dinitrogenase complex activity (Roberts et al., 1978; Imperial et al., 1987). Indeed, in the present study, A. caulinodans null mutants showing absolute thermolability for N₂ fixation were not identified. While such ‘negative results’ must be cautiously interpreted, multiple, independent nifE mutants with a partially defective phenotype were indeed isolated, it is tempting to regard this search as exhaustive. If so, then A. caulinodans lacks particular gene(s) specifically required for high temperature N₂ fixation and likely possesses an orthodox nif gene set whose alleles are collectively adapted to relatively thermostable N₂ fixation.

ACKNOWLEDGEMENTS

This work was supported by grants from the US National Science Foundation and the US National Institutes of Health to R.A.L.

REFERENCES


