

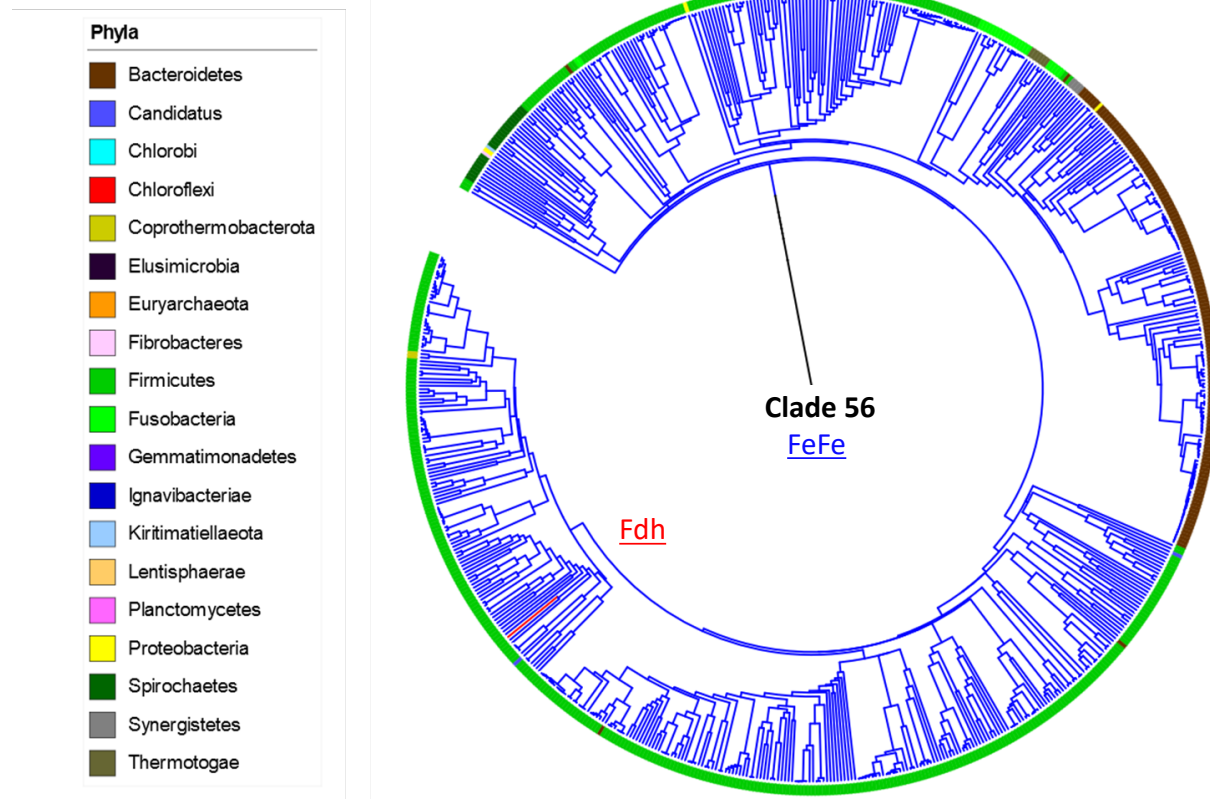
**An Abundant and Diverse New Family of Electron Bifurcating Enzymes With a
Non-Canonical Mechanism**

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Michael W. W. Adams^a

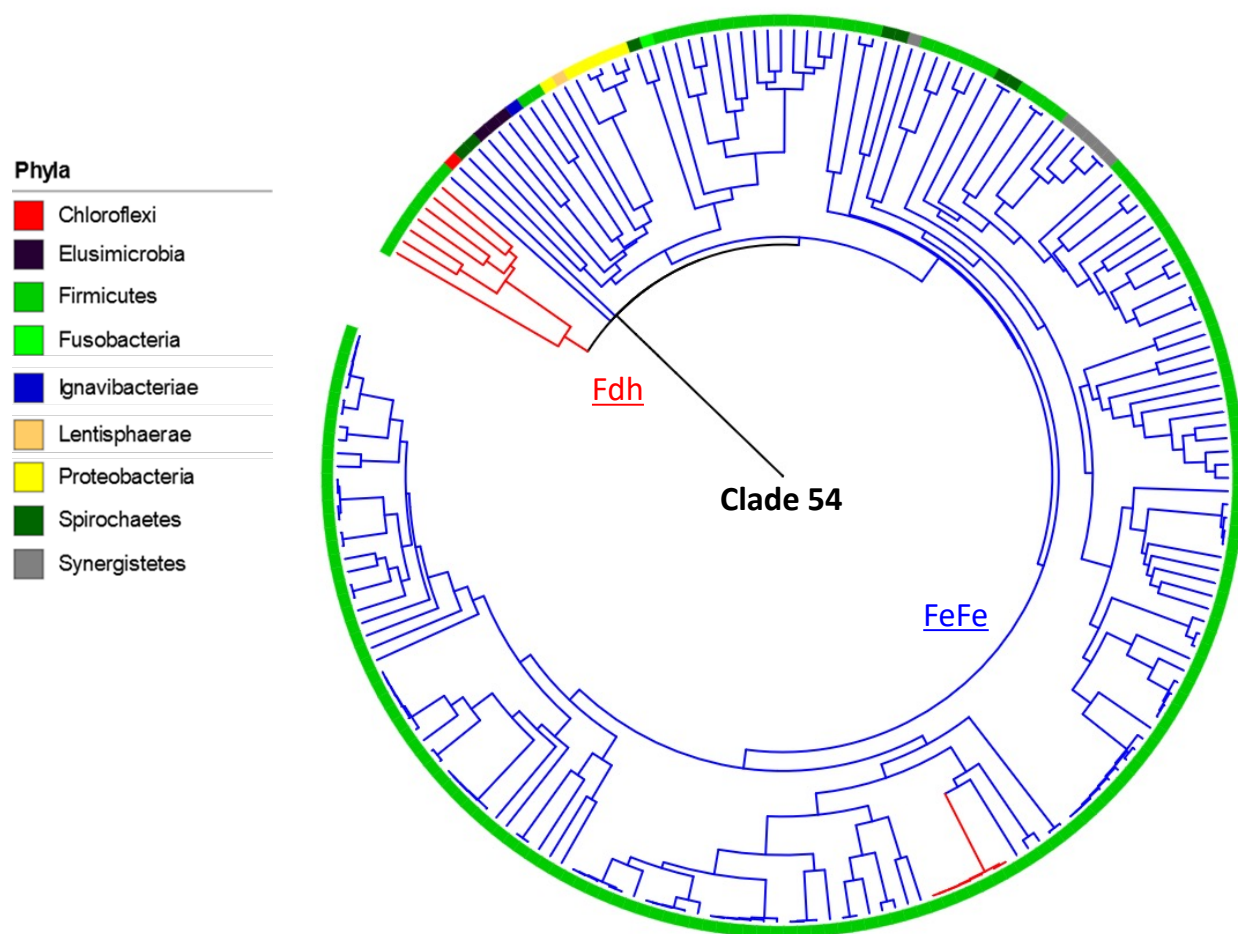
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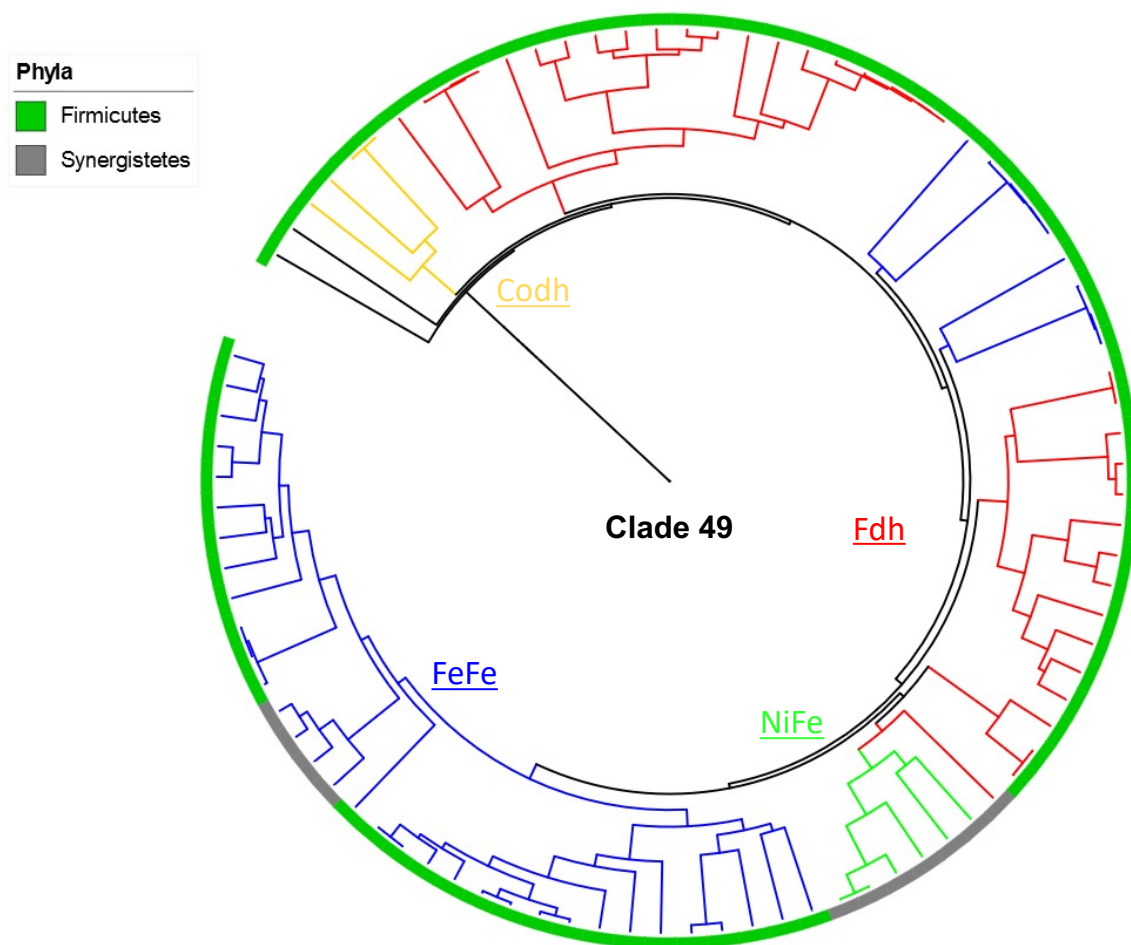
Supplementary Figures 1-10 and Supplementary Table 1



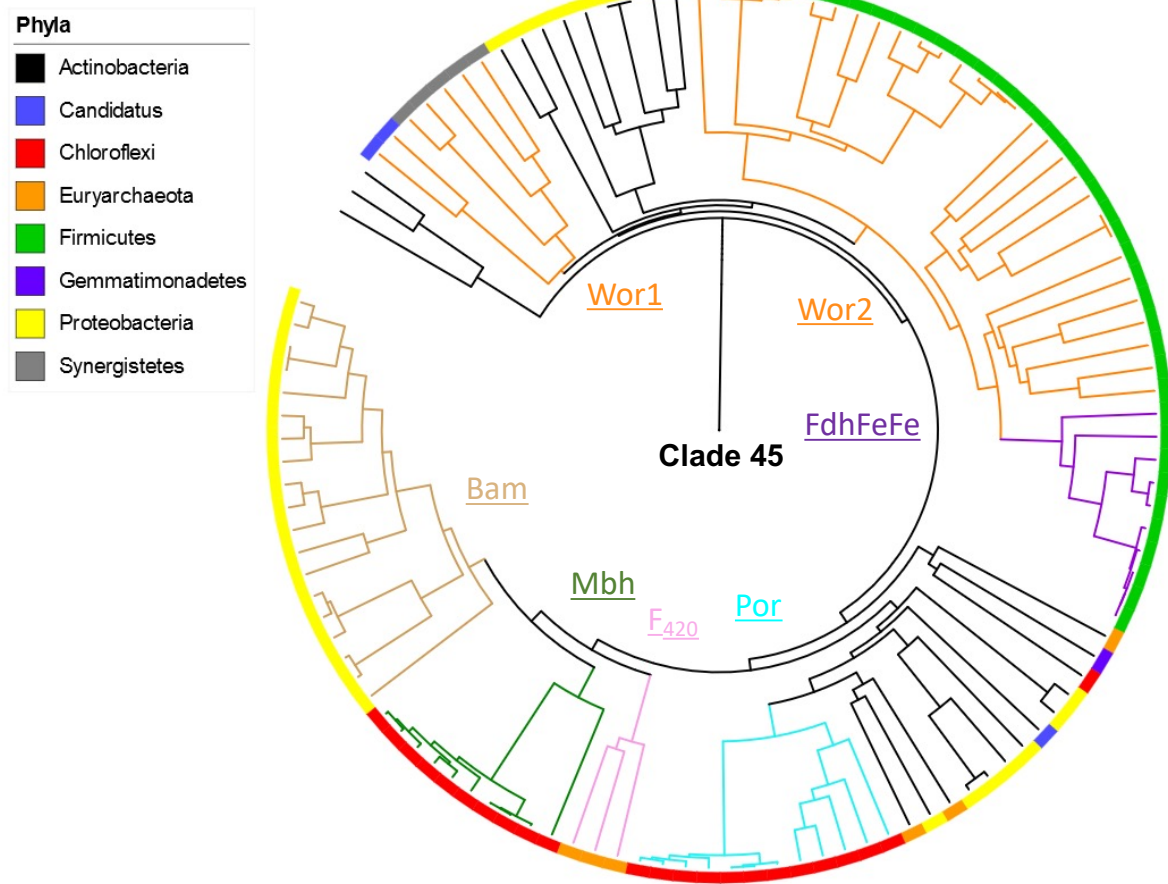
Supplementary Figure 1. Phyla associated with clade 56 of the BfuB phylogenetic tree (see Figure 2). All are FeFe-enzymes (blue) except for a single branch of Fdh (red).



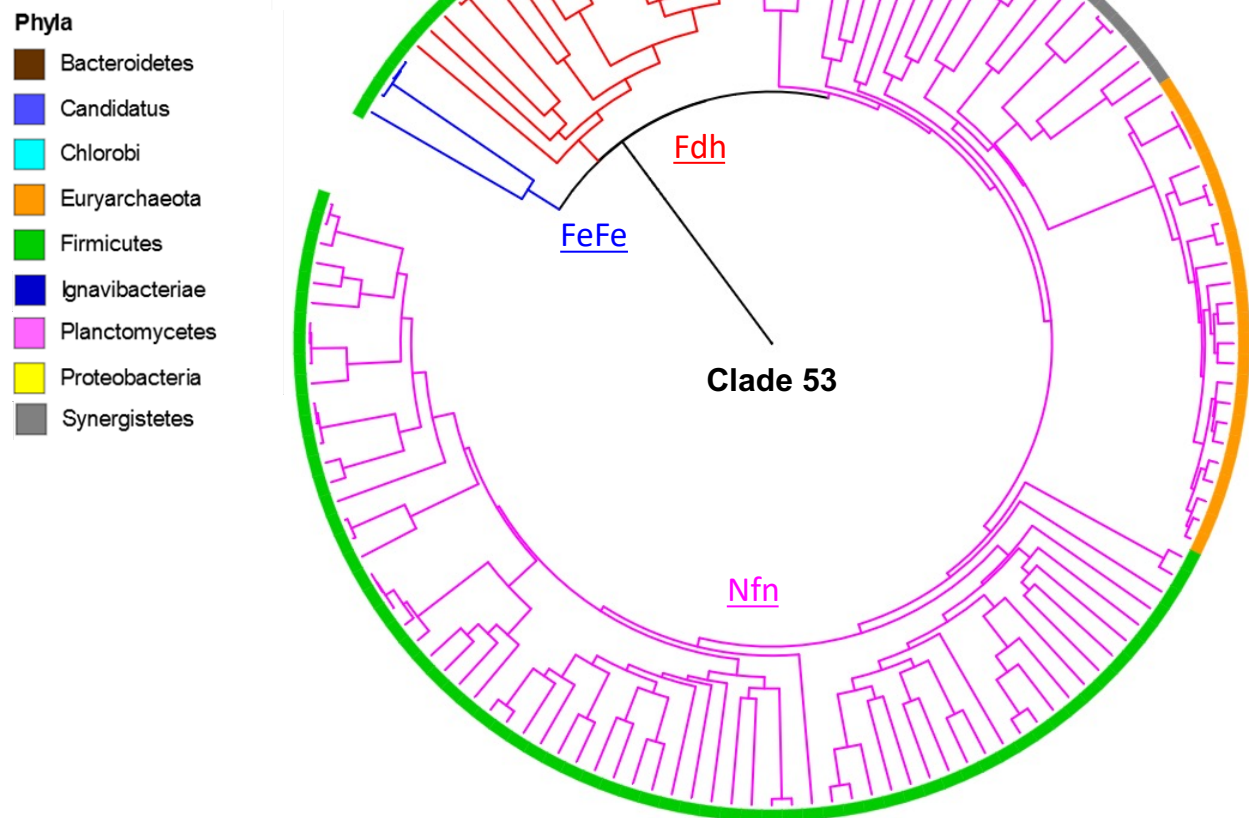
Supplementary Figure 2. Phyla associated with clade 54 of the BfuB phylogenetic tree (see Figure 2). The positions of the Fdh (red) and FeFe (blue) enzymes are indicated.



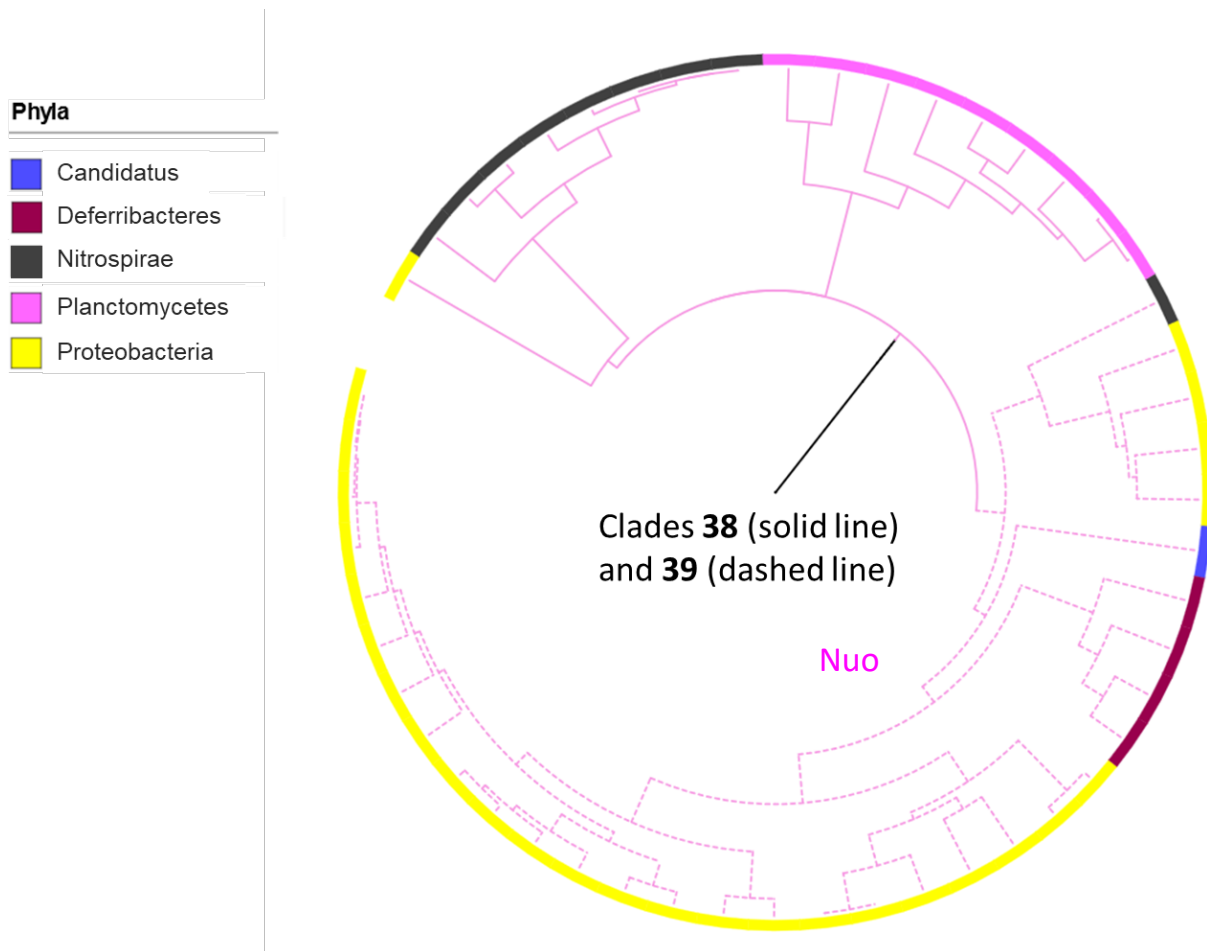
Supplementary Figure 3. Phyla associated with clade 49 of the BfuB phylogenetic tree (see Figure 2). The positions of the Codh (orange), Fdh (red), NiFe (green) and FeFe (blue) enzymes are indicated.



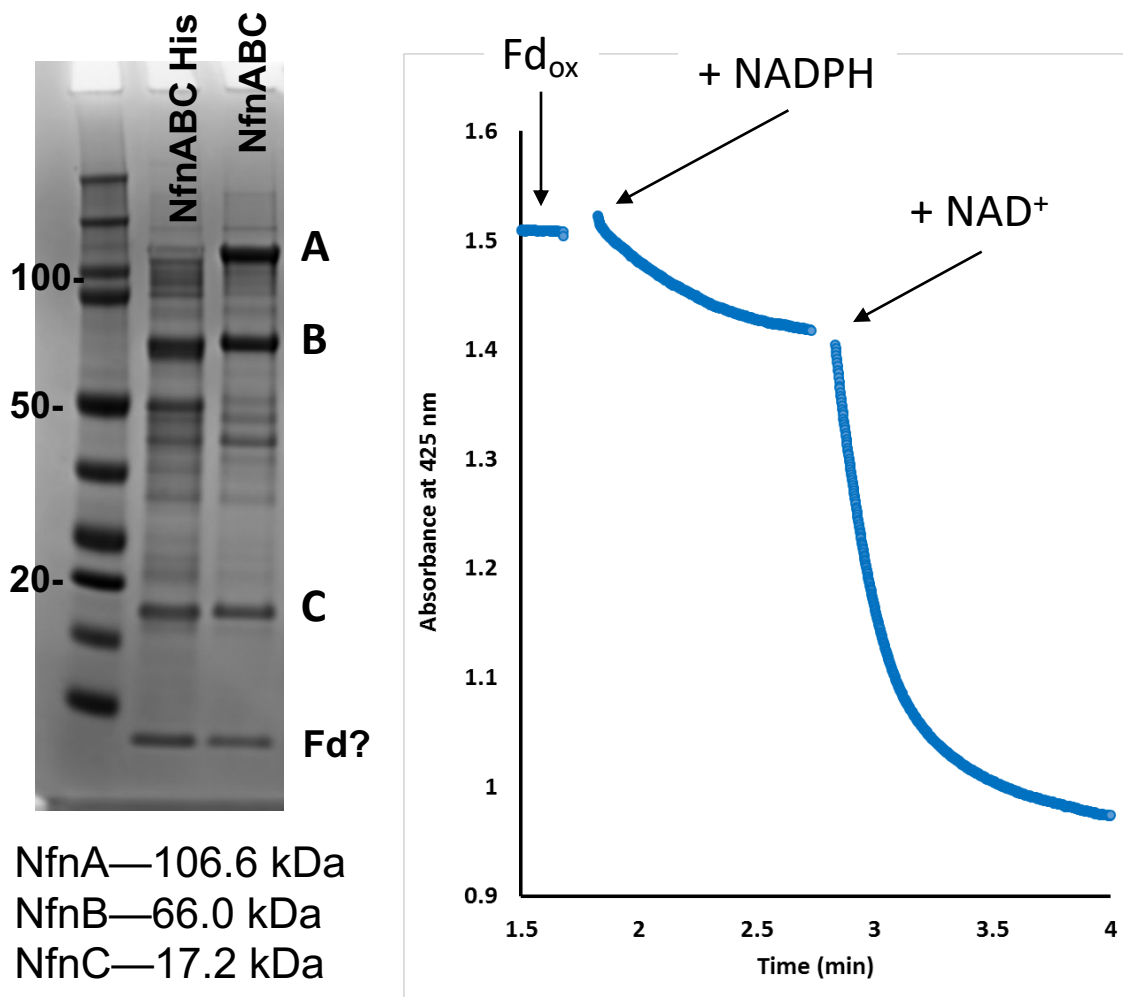
Supplementary Figure 4. Phyla associated with clade 45 of the BfuB phylogenetic tree (see Figure 2). The positions of the Wor1/2 (orange), FdhFeFe (purple), Por (aqua), F₄₂₀ (light purple), Mbh (dark green) and Bam (brown) enzymes are indicated.



Supplementary Figure 5. Phyla associated with clade 53 of the BfuB phylogenetic tree (see Figure 2). The positions of the FeFe (blue), Fdh (red) and Nfn (pink) enzymes are indicated.

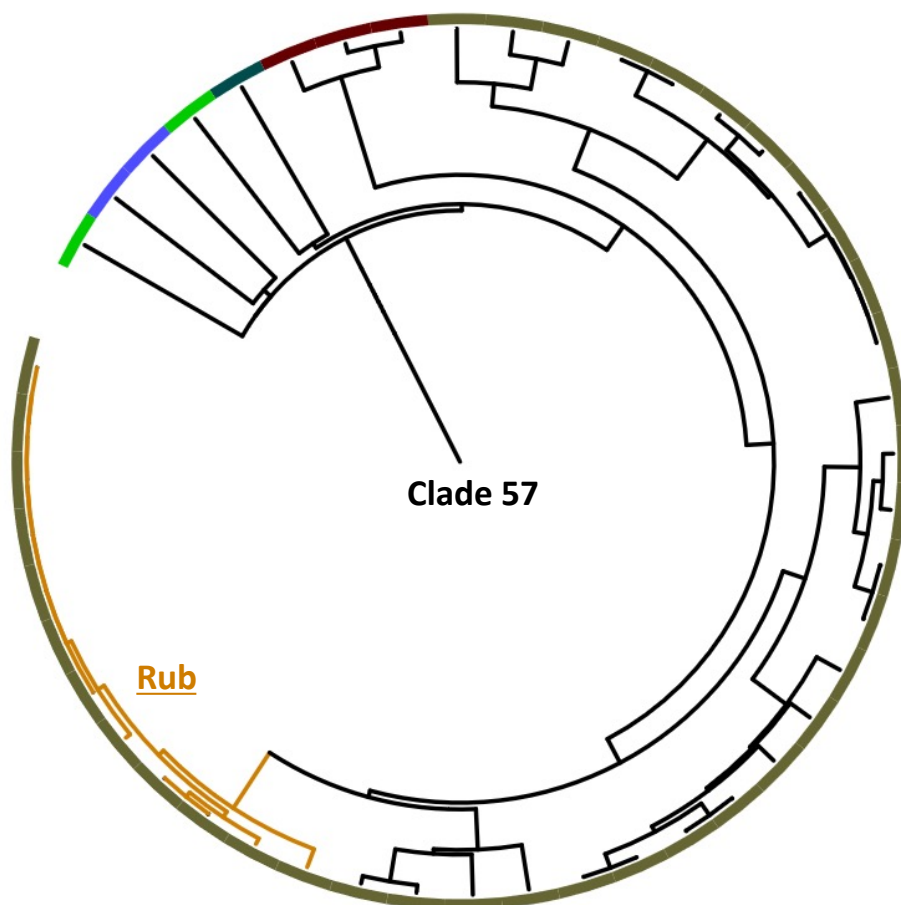


Supplementary Figure 6. Phyla associated with clades 38 (solid line) and 39 (dashed line) of the BfuB phylogenetic tree (see Figure 2). Both clades contain the Nuo-enzyme, separated into Deferribacteres and Proteobacteria (clade 38, 26; Nuo) and Planctomycetes and Nitrospirae (clade 39, 16; Nuo). In addition, clade 38 contains 4 Fdh and clade 39 contains 1 Fdh.



Supplemental Figure 7. *Left:* SDS-PAGE gel of *T. sibiricus* Nfn-Bfu purified from the hyperthermophile *Pyrococcus furiosus* after heterologous expression. NfnA (106.6 kDa), NfnB (66.0 kDa), and NfnC (17.2 kDa) subunits are indicated. Ladder sizes (in kDa) are indicated for select bands. *Right:* Assay progress curve showing bifurcating activity (20 U/mg) of *T. sibiricus* Nfn-Bfu. Fd_{ox} , NADPH, and NAD^+ were added where indicated. The activity only proceeds in the presence of all three substrates.

Phyla	
■	Candidatus
■	Cyanobacteria
■	Dictyoglomi
■	Firmicutes
■	Thermotogae



Supplementary Figure 8. Phyla associated with clade 57 of the BfuB phylogenetic tree (see Figure 2). The positions of the Rub (brown) enzymes are indicated

		B2		B1		B2		B1		B2									
		C438	FeB		C455	C458	C461		C476	FeA		C501		H525	FeB	C531	FeB	C536	
<i>Tm</i>	FeFe	KT	CMVEVARFFLDFTKRES	CGKC	VPC	REGTMQ	-AYNILEKE	THG	KATYEDLKTLEHLSKTIKTASL	CGLGKTAPNPILSTLKLFREEYIA	HHIE	-GE	CP	SGM	CTAF				
<i>Cb</i>	FeFe	DT	CMVDIAKFFLEFTVDES	CGKC	PPC	RIGTRR	-MLEILQKIT	SG	NGTEEDLEKLEELAYSIKDSAL	CGLGQTAPNPVLSTLRYFRDEYEA	HHVKEKR	CPAGAC	KAL						
<i>Ap</i>	Fdh	DT	CMVDVAKFFLEFTQAES	CGKC	VPC	REGTKK	-MLDILTRIT	TEG	KQEGDIEKLQYLGTIKDASL	CGLGQTAPNPVLTTIRYFRHEYE	HHIKDKK	PAGAC	VAL						
<i>So</i>	Nfn	TT	CMVDMARFFLDFTVKES	CGKC	IYC	RIGTKR	-MLEILERIT	T	GEGREGDIEELELSISIKDGS	L	CGLGQTAPNPVLTTIRYFRDEYE	HHIRDKK	CPAKS	CKPL					
<i>Ts</i>	Nfn	DT	CMVDVAKFFLDFTVKES	CGKC	TF	CRLGTR	-MWELLDKIT	TEG	GALEDIEKLEKLAPLVKTGSL	CGLGQTAPNPVLTTLKYFKDEYLA	HHIE	-GR	CPAKV	CKPL					
<i>Mh</i>	F ₄₂₀	DT	CAVDLARYFVDFLCDES	CGKC	VPC	REGLRQ	-MRDILSEIV	AGR	GEAEDLKTLEVAGVMKSASL	CALGR	TAANPVLSTIRHFEEYV	HHIQDQR	CALV	CEQL					
<i>Am</i>	Wor	RS	CMVDVAKYFIDFLVES	CGKC	TPC	REGLKV	-LQKLLHDIT	EG	KGSLQDVGLLED	TAHEL	GKTAL	CGLG	KTAA	NPVLSTLKYFHEEYEA	HHVE	-GY	C	RAGV	CTGL
<i>Aa</i>	Codh	DT	CMVDVARYFLDFTVSES	CGKC	CAP	SEGTRK	-MLDILERIT	OG	NGKQEDLEQLEKLA	EVIKD	TS	L	CGLG	QTAPNPVLSTLKYFRDEYKA	HHVKNK	CPAGV	CKSL		
<i>Dm</i>	Por	DN	CMVEIARYFLSFLEGES	CGKC	I	PCREGVKR	-MRQILEKIT	TEG	EGEEDIEELLEQLSQA	IQDASL	CALGGS	APNPVMSTIKYFRDEYE	HHIRQKT	CPAKE	CKAL				
<i>Dm</i>	Mbh	ET	CMVDIARYFINFLSDES	CGKC	CLP	REGLRQ	-LVDILTRIT	TEG	KGTMDMDTLEDLSGVMSEAC	L	CALGQ	QAPNPVLLTTLKYFRHEYI	HHIKNKH	CEAGV	CKAL				
<i>Gm</i>	Bam	DT	CMVDIARYFIDFLKDES	CGKC	TPC	REGIRQ	-MLAVLTRIT	TVG	KGEGDIEELLELAES	-TGAAL	CALGKS	APNPVLSTIRYFRDEYE	HHIREKK	CPALS	CKEM				
<i>Ca</i>	FdhH ₂	RT	CMVDVTRYFLSFLAES	CGKC	VPC	REGVKR	-MLEILT	DIC	NGDGKEGDIEELLEICSMTSKASL	CSL	GKS	APNPVIASIRYFRDEFE	HHIKNKR	C	RAGV	CKKL			
<i>Am</i>	NiFe	DT	CMVDVAKFFLEFTQRES	CGKC	VPC	REGTKQ	-MLMLQKIT	C	NGE	TMDDLSKLELAHMVKETSL	CGLG	QTAPNPVITIRYFRDEYV	HHIKDKR	CPAKI	CPAL				
<i>Ga</i>	Fdh	DT	CMVDIARFFLDFTVES	CGKC	TPC	REGTKR	-MLELLEKIT	OG	KEMEDLRLSLAETIKSSSL	CGLG	QTAPNPVLSTLKYFRDEYE	HHIKDKK	CPAGAC	QSL					
<i>Gs</i>	Nuo	TT	CMVDVARFFLFTFRMES	CGKC	VPC	RIGLKA	-MLDILERIT	TEG	RQAGDIETLLEMGSTIKKASL	CGLG	QTAPNPILSTIKYFREEYEA	HHNDRR	CP	SNCKEL					
<i>Tm</i>	Rub	DD	CMVDVARFFLEFTVES	CGKC	TPC	REGTKK	-MLEILEKIT	TEG	EGTEEDIEELEKLAHVVKD	TS	L	CGLG	QTAPNPVLSTLRYFRDEYLA	HHVKEKR	CP	SKK	KAL		
<i>Rc</i>	Fdh	TAD	MLKLARFAMEFCAIES	CGT	C	TPCRIGAVR	-GVEVIDRIAAGDA	--	SAMPLD	DL	CL	QTMKLGSL	CALG	GFT	PPYVQSAIRHFPADFPCAREAAE	-----			
<i>Tt</i>	Ngo	RVS	MDAMWNLTRFYAHES	CGKC	TPC	REGVAGFMVNLFAKIGT	QGQEEKDVENLEALLPLIEGRS	FC	PLADA	AVW	PKV	SLRHF	KDQY	LALAREKRPVPRPS	--	L			
<i>Re</i>	Hox	KRD	LLEIVRDHMQFFVEES	CGI	C	VPCRAGNVD	-LHRKVEWVIAGKACQKDLDD	MVSWGALVRR	TSR	CGLG	ATSPKPI	LTLEK	FPET	YQNK	IVRHEG	PLPSFDL			
<i>Sw</i>	Hyd1	SRD	IVNVVARIAKFFEHEHES	CGKC	SPC	REGTKR	-MHEMMERLNAGEGNAED	VELLGR	L	GKVM	SVACL	CGLG	QAAPVLT	TIK	NFNADYQAKFN	-----			

Supplementary Figure 9. Sequence alignment showing coordination of B1 and B2 clusters. Residues highlighted in red coordinate the indicated cluster. The horizontal line separates Bfu family enzymes (top) from homologous non-bifurcating complexes (bottom). The amino acid numbering is based on *A. mobile* NiFe-BfuB. While the three Cys (438, 531 and 536) and one His (525) coordinating the B2 cluster are conserved in all 1,558 Bfu sequences examined, the distribution of residues equivalent to Cys276 was as follows: 1055 Thr, 205 Cys, 195 Val, 55 Ser, 22 Ile, 10 Ala, 5 Leu, 4 Lys, 3 Met, 3 Pro, 1 Asp, 1 Glu and 1 Arg. The two Fe atoms of the B2 cluster are designated FeA (coordinated by two Cys) and FeB (coordinated by two Cys and a His). Abbreviations for organisms and enzymes are given in the text.

		A202 M204	F282
Tm FeFe	VCNGDEGDPGAFMNR	T	KEGAGAFVCGEETALL
Cb FeFe	VCNADEGDPGAYMDRS	R	RLGAGAFVCGEETALM
Ap Fdh	ICNADEGDPGAFMDRS	R	KEGAGAFVCGEETALM
So Nfn	VCNADEGDPGAFMDRS	K	KKGAGAFVCGEETALI
Ts Nfn	VCNADEGDPGAFMDRN	R	KEGAGAFVCGEETALI
Mh F420	IVNCDEGDPGAFMDRA	H	HRGAGAFVSGESTALM
Am Wor	IANGDEGDPGAFMDRS	C	CKGGGAFVCGESSALM
Aa Codh	ICNADEGDPGAFMDRS	R	KEGAGAFVCGEETALI
Dm Por	ICNCDEGDPGAFMDRS	N	NRGGGAFVCGESTALM
Dm Mbh	LVNCDEGDPGAFMDRS	H	HEGAGAFVSGESSALM
Gm Bam	IVNADEGDPGAFMDRA	H	HMGAGAFVCGESSALM
Ca FdhH2	VCNGDEGDPGAFMDRS	V	VRGGGAFVCGESTALM
Am NiFe	ICNADEGDPGAFMDRS	R	KEGAGAFVCGEETALM
Ga Fdh	LCNADEGDPGAFMDRS	R	RLGAGAFVCGEETALI
Gs Nuo	ICNADEGDPGAFMDRS	K	KKGAGAFVCGEETALM
Tm Rub	VCNADEGDPGAFMDRS	R	RIGAGAFVCGEETALM
Rc Fdh	VCNVDEGDSGSFADRM	R	RVGAGAYVCGEETSLI
Tt Nqo	ICNADESEPGSFKDRY	H	HRGAGAYICGEETALM
Re Hox	ICNADEGEPTGTFKDRV	Q	QMGAGAYICGDESALI
Sw Hyd1	VCNADEGEPTGYKDRI	R	RMGGGAYVCGEESALI
	NADH Binding Domain		FMN Binding Domain

Supplementary Figure 10. Conservation of the residues within the FMN and NADH binding domains in BfuB. The amino acid numbering is based on *A. mobile* NiFe-BfuB. Non-bifurcating enzymes are shown below the horizontal line. Abbreviations are given in the text.

Activity	Clade	Type	Subunit Composition	Third Reaction	E° (mV)	Dominant Phyla	# of Members
Codh	49	2	Codh-Bfu <u>ABC</u> DEE	$\text{CO} + \text{H}_2\text{O} \rightleftharpoons \text{CO}_2 + 2 \text{H}^+ + 2\text{e}^-$	-520	Firmicutes	4
FdhFeFe	45	2/3	FdhFeFe-Bfu <u>ABC</u> DE/FS	$\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$; $\text{HCOO}^- \rightleftharpoons \text{CO}_2 + 2\text{e}^-$	-421; -421	Firmicutes	10
F_{420}	45	1	F_{420} -Bfu <u>ABC</u>	$\text{F}_{420}\text{H}_2 \rightleftharpoons \text{F}_{420} + 2\text{e}^-$	-340	Euryarchaeota	3
Fdh	22, 23, 49, 53, 54, 56	1,2,3	Fdh-Bfu <u>ABC</u>	$\text{HCOO}^- \rightleftharpoons \text{CO}_2 + 2\text{e}^- + \text{H}^+$	-421	Euryarchaeota, Firmicutes, Proteobacteria, Chloroflexi, Synergistetes	82
FeFe	25, 54, 55, 56	1	FeFe-Bfu <u>ABC</u> (D)	$\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$	-421	Firmicutes, Bacteroidetes, Thermotogae	957
Mbh	45	2	Mbh-Bfu <u>ABC</u> DE FGHI	$\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$	-421	Chloroflexi	10
Nfn	53	1	Nfn-Bfu <u>ABC</u>	$\text{NADPH} \rightleftharpoons \text{NADP}^+ + 2\text{e}^-$	-324	Euryarchaeota, Firmicutes	140
NiFe	19,49	3	NiFe-Bfu <u>ABC</u> SL	$\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$	-421	Synergistetes, Euryarchaeota	6
Nuo	38,39	3	Nuo-Bfu <u>ABC</u> DE FGHIJKLMN	$\text{MQH}_2 \rightleftharpoons \text{MQ} + 2\text{e}^-$	-74	Deferribacteres, Proteobacteria Planctomycetes, Nitrospirae	51
Por	35,44,45	2	Por-Bfu <u>ABC</u> DEFG	$\text{Pyruvate} + \text{CoA} \rightleftharpoons \text{CO}_2 + \text{Acetyl-CoA} + 2\text{e}^-$	-500	Chloroflexi, Proteobacteria	12
Rub	57	4	Rub-Bfu <u>RBC</u>	$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightleftharpoons 2 \text{H}_2\text{O}$	+1349	Thermotoga	13
Wor	45	2	Wor-Bfu <u>ABC</u> DE	$\text{RCHO} + \text{H}_2\text{O} \rightleftharpoons \text{RCOO}^- + 3\text{H}^+ + 2\text{e}^-$	-580	Synergistetes, Firmicutes	37

Supplementary Table 1. The types of enzyme within the Bfu family and their characteristics. Characterized and predicted enzymes and their microbial sources are shown in black and red font, respectively.