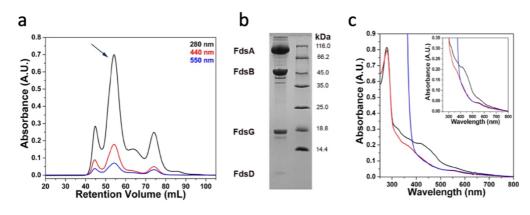
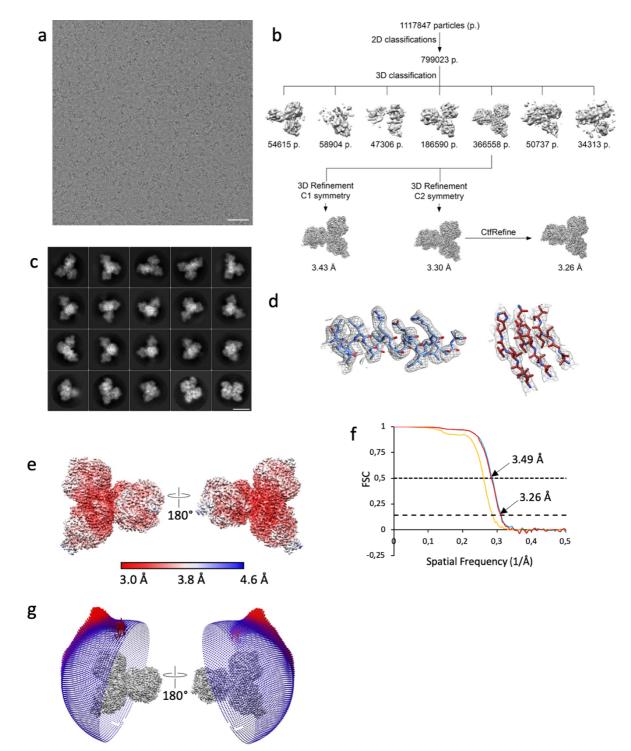
1	Supplementary Information
2	Cryo-EM structures reveal intricate Fe-S cluster arrangement and charging in Rhodobacter
3	capsulatus formate dehydrogenase
4	Christin Radon, Gerd Mittelstädt et al.
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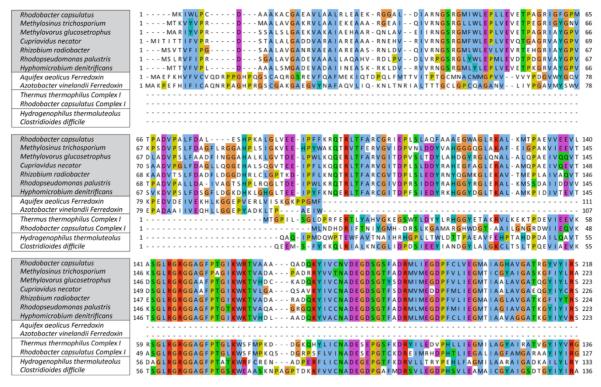


25 Supplementary Figure 1 | Biochemical Characterization of RcFDH. a, Size exclusion 26 chromatography of 90 µM RcFDH performed on a Superdex200pg column in 75 mM potassium phosphate, 10 mM NaN₃, pH 7.5 at 4 °C. The purified fraction corresponding to the holoenzyme 27 28 is represented by the main fraction that eluted at approximately 55.0 mL and is indicated by an 29 arrow. Elution traces represent absorbance wavelengths at 280 nm (black trace), 440 nm (red 30 trace), and 550 nm (blue trace). **b**, 15% SDS-polyacrylamide gel of the purified RcFDH holoenzyme. Gel depicts 46 µg of purified FDH. Subunits corresponding to FdsA, FdsB, FdsG, 31 32 and FdsD are indicated. Reproducible gels could be obtained following at least 3 or more 33 independent purifications. c, UV-visible absorption spectra of as-isolated *Rc*FDH (black trace) 34 that was subjected to immediate reduction with 5 mM sodium formate (red trace), followed by 35 treatment by 2 mM sodium dithionite (blue trace). Inset depicts a zoomed-in view of the features 36 present at 250-800 nm. Spectra were recorded in 100 mM Tris-HCl, 10 mM NaN₃, pH 9.0 buffer under anaerobic conditions in a Coy chamber ($< 10 \text{ ppm O}_2$). 37 38



Supplementary Figure 2 | Cryo-EM analysis of RcFDH. a, A representative cryo-EM 40 41 micrograph of *Rc*FDH data collection. Scale bar represents 500 Å. b, Data processing workflow 42 showing the image processing procedure with the number of particles and the resolution of the reconstruction for every step. c, Representative 2D class averages of various views of RcFDH. 43 44 Scale bar represents 100 Å. d, Mesh representation of the map showing a representative α -helix 45 and β -sheets superimposed on the atomic model. e, Local resolution estimate in Relion. Side 46 views of the density map coloured according to the local resolution estimated by Relion. f, 47 Fourier shell correlation (FSC) plots for the RcFDH EM map with imposed C2 symmetry. The

- 48 resolution at FSC=0.143 and FSC=0.5 are indicated. A comparison of the FSC corrected (red),
- 49 masked (blue) and unmasked map (yellow) is shown. g, Angular distribution of the final
- 50 reconstruction.

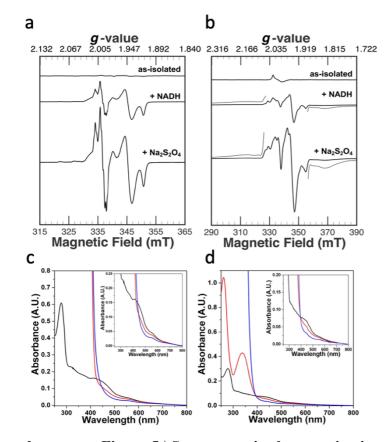


- 53 Supplementary Figure 3 | Multiple sequence alignment of FdsB homologues from NAD+-
- 54 dependent FDHs, Ferredoxins, NADH-quinone oxidoreductases and Hydrogenases

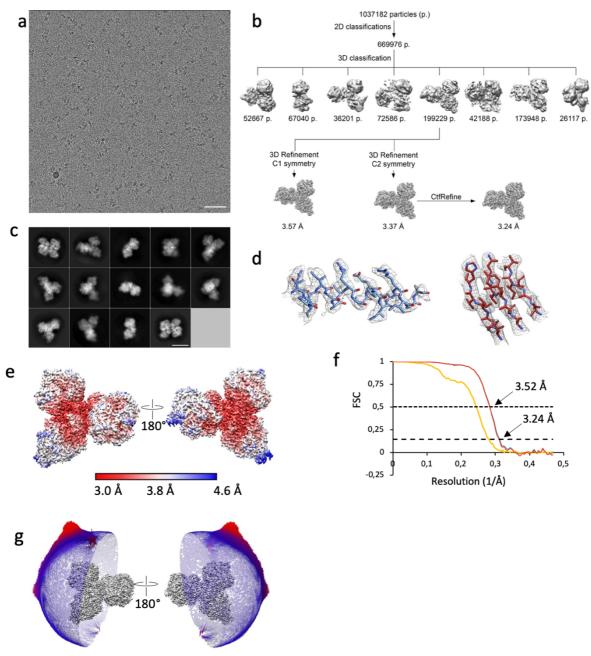


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Methylosinus trichosporium 1 - MS HNTA E K LV KMANQ I G Methylovorus glucosetrophus 1 MN I E H L I TMA NQ I G Cupriavidus necator 1 MK I D N L I TMA NQ I G Rhizobium radiobacter 1 MK I D K L V KMA NQ I G Rhodopseudomonas palustris 1 MS P D R L V YMA NQ I G	** AFFAVQPGD-RA-GPVAAHISENWSAPMRAALLAHVAA-QSPGLDPLVIA 61 AFFAAQRHS-DAVAGTAEHLKKFWDPRMRAGIIAHVEH GGEGLDTVPLQ 65 TFFFSAYPDRELAKNEIVSHIKRFWAHNMRQQLVSHVNEQQGQGLDAIVTD 64 SFFEAMPDREEAVSDIAGHIKRFWEPRMRKALLGHVDAEAGNGLLDIVRE 64 TFFL <mark>SQ</mark> PED-IRVEGVATHINKFWEPRMRKRFFEMVDA-GAGEFLPLVLA 66 AFFR <mark>SQGP</mark> D-KAVPGIAEHLKKFWEPRMRRAIIAHLDA-GGDGLAPEVRA 62 AFFR <mark>SQGPD-KAVPGIAEHLKKFWEPRMR</mark> AIIAHLDA-GGDGLAPLFVE 62
b Val145 Val145 Arg587 Arg333	* * * Rhodobacter capsulatus 385 V C H S P T G Y G L K Q T F G T S A G T Q D F D S V E E T D 414 Methylosinus trichosporium 381 V C H S P T G Y G L S Q A F G T S A G T Q D F D S V E E T D 414 Methylosinus trichosporium 381 V C H S P T G Y G L S Q A F G T S A G T Q D F D S V D S Q 410 Methylosinus trichosporium 381 V C H S P T G Y G L K Q T L G E S A G T Q D F D S V M S D 397 Cupriavidus necator 377 V C H S P T G Y G L S Q A F G T S A G T Q D F D S V E Q S D 412 Rhodopseudomonas palustris 371 V C H S P T G Y G L S T A F G T S A G T Q D F D S V E H A D 400 Hyphomicrobium denitrificans 386 V C H S P T G Y G L S T A F G T S A G T Q D F D S V E H A D 400 Hyphomicrobium denitrificans 386 V C H S P T G Y G L S T A F G T S A G T Q D F D S V E I A D 414 Clostridium carboxidivorans 188 V H S P T V A S L A P T F G T S A G T Q D F D S V E I A D 16 Escherichia coli FDH-N 195 V H G P T V A S L A P T F G R G A M T N H W V D I K N A N 24
d e	
Ad (dreats) Leuise L	Rhodobacter capsulatus 106 R R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Methylosinus trichosporium 101 R R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Methylosorus glucosetrophus 99 R R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Cupriavidus necator 101 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Rhizobium radiobacter 101 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Rhodopseudomonas palustris 101 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Hyphomicrobium denitrificans 104 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Shodopseudomonas complex I 95 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Shodopseudomonas complex I 104 R G V ME L Y I S D H P L D C L T C A A NG D C E L 13 Shodopseudomonas aeruginosa Complex I 88 R S V V E WLMT N H P H D C P V C E E G G N C H L 13 Pseudomonas aeruginosa Complex I 88 R S V V E WLMT N H P H D C P V C E E G G N C H L 13 Pydrogenophilus thermoluteolus 90 R T L L E M L F A E G N H C C P I C E A S G N C E L 13 Labrenzia aggregata 89 R N L V E M L F A E G N H C P I C E A S G N C E L 13 Labrenzia aggregata 82 R A L V E F L F A E G N H C P I C E A

Supplementary Figure 4 | Channel and [4Fe-4S] cluster A4 of RcFDH. a, Multiple sequence 58 59 alignment of FdsD homologues from NAD+-dependent FDHs. Stars indicate conserved 60 phenylalanine residues. **b**, Superimposition of *Rc*FDH (red) and oxidised FdhF (PDB-ID: 1fdo; white) structures at active site. Active site residue Arg 587 and residues Gly³⁹¹ and Gly⁴⁰³ of 61 RcFDH as well as Val¹⁴¹ and Met¹⁵⁷ of FdhF are shown as sticks. The proposed hydrophobic 62 63 channel is indicated in cyan. c, Multiple sequence alignment of FdsA homologues from 64 organisms predicted to harbour FdsD (grey-shaded) or not (white-shaded). Stars indicate 65 conserved glycine residues in NAD+ dependent FDHs which are replaced by valine and 66 methionine, respectively, in other FDHs. d, Dimer interface between the two FdsA subunits. 67 [4Fe-4S] cluster A4 and A4' are shown. Cluster coordinating residues and hydrophobic residues 68 at the dimer interface are shown as stick representation. The distance between sulphur atoms of 69 Cys121 is indicated in Å. e, Multiple sequence alignment of FdsA homologues of several FDH, complex I and NAD+-reducing [NiFe]-hydrogenase family members. Residues that coordinate 70 cluster A4 are indicated with stars. The arrows indicate Leu¹¹⁹ and Leu¹²². 71 72



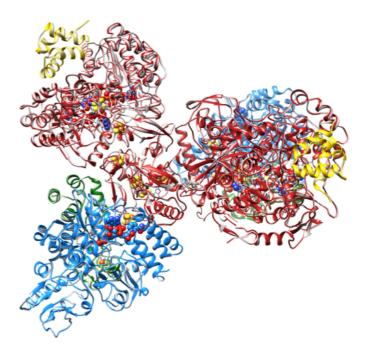
74 Supplementary Figure 5 | Spectroscopic characterization of RcFDH. a, Constant wave Xband EPR spectra of *Rc*FDH recorded at 80 K and **b**, 12 K at a microwave frequency of 9.38 75 76 GHz. Spectra represent as isolated RcFDH treated in the absence or presence of NADH or 77 sodium dithionite. An iron-sulphur cluster signal present in the low- and high-field edges of the 78 NADH-treated and the dithionite-treated signals is partially enlarged at a scale of 5x. c.d. UVvisible spectra of FDH following treatment with NADH. **a** shows the Mo^V and slowly-relaxing 79 Fe-S clusters (as [2Fe-2S] or [4Fe-4S]) visible upon respective treatments. b emphasizes the 80 presence of fast-relaxing Fe-S clusters that are not visible at 80 K, however some spectral 81 82 components (e.g. MoV and Fe-S clusters) are power-saturated. c shows as-isolated FDH (3.5 83 μ M) (black trace) that was subjected to reduction for 5 minutes at ambient temperature with 20 84 mM NADH (red trace), followed by treatment by 2 mM sodium dithionite (blue trace). d shows 85 as-isolated FDH (1.8 μ M) (black trace) that was subjected to immediate reduction at ambient 86 temperature with 40 μ M NADH (red trace), followed by treatment by 2 mM sodium dithionite. 87 Spectra were recorded in 100 mM Tris, 10 mM NaN₃, pH 9.0 buffer under anaerobic conditions 88 in a Coy chamber (< 10 ppm O₂). Insets depict a zoomed-in view of the features present at 280-89 800 nm. The reduction spectra show that the enzyme is only partially reduced by NADH, 90 independent of the NADH concentration used and the time of reduction. The absorbance of the 91 red trace at 340 nm in **d** shows that NADH was not completely consumed during the reduction, 92 also visible in **d** at much higher concentrations of NADH.



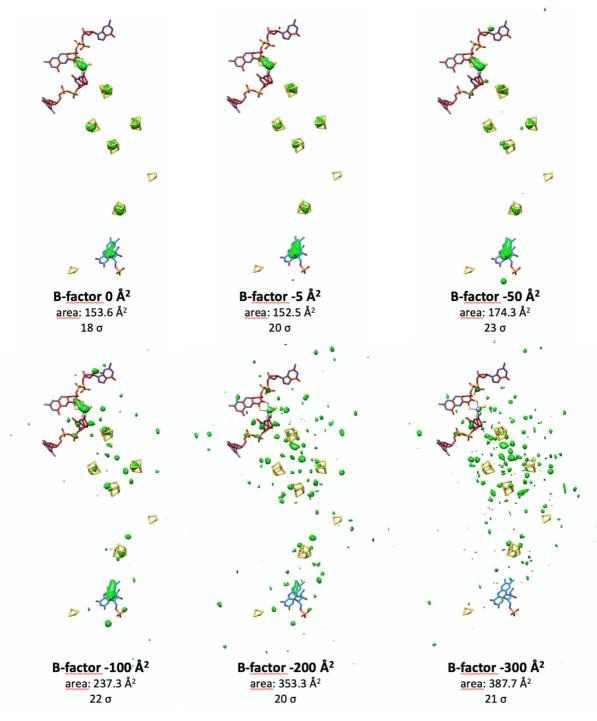
95 Supplementary Figure 6 | Cryo-EM analysis of NADH incubated RcFDH. a, A representative cryo-EM micrograph of *Rc*FDH data collection. Scale bar represents 500 Å. b, 96 97 Data processing workflow showing the image processing procedure with the number of 98 particles and the resolution of the reconstruction for every step. c, Representative 2D class 99 averages of various views of RcFDH calculated from selected particles. Scale bar represents 100 100 Å. d, Mesh representation of the map showing a representative α -helix and β -sheets 101 superimposed on the atomic model. e, Local resolution estimate in Relion. Sideview of the 102 density map coloured according to the local resolution estimated by Relion. f, Fourier shell 103 correlation (FSC) plots for the RcFDH EM map with imposed C2 symmetry. The resolution at

104	FSC=0.143 and FSC=0.5 are indicated	. A comparison of the FSC	corrected (red), masked (blue)
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105 and unmasked map (yellow) is shown. **g**, Angular distribution of the final reconstruction.



- 114
- 115 Supplementary Figure 7 | Overlay of ribbon representation of as isolated *Rc*FDH
- 116 structure and NADH reduced *Rc*FDH structure. As isolated *Rc*FDH structure is shown in
- 117 light grey, NADH reduced *Rc*FDH structure depicted in colour code: FdsA, red; FdsB, blue;
- 118 FdsG, green; FdsD, yellow.
- 119



Supplementary Figure 8 | Influence of B-factor sharpening on difference maps between as isolated and NADH reduced cryo-EM maps of *Rc*FDH. Surface representations of difference maps between as isolated and NADH reduced cryo-EM maps. Cryo-EM maps of as isolated and NADH reduced FDH were B-factor sharpened prior subtraction. For comparison all difference maps were scaled to the same enclosed volume (58.8 Å³). Applied B-factors, the total surface area and sigma values are given for each difference map. Cofactors are shown in stick representation.

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130 Supplementary Tables

131

132 Supplementary Table 1 | Similarity scores (RMSD) of FDH components to closely related

133 structures

RcFDH-		Protein				
chain ^a	pdb ID	name	Homologous protein	r.m.s.d. in $Å^b$	identity ^c	similarity ^c
FdsA	5xf9_B	HoxU	NAD ⁺ -reducing [NiFe]-hydrogenase	1.159 (141)	35%	50%
FdsA	1fdo	FdhF	Formate dehydrogenase H	1.132 (554)	36%	53%
FdsA	3ml1	NapA	Periplasmatic nitrate reductase	1.110 (463)	23%	38%
FdsA	1h0h	FdhA	Tungsten containing formate dehydrogenase	1.184 (398)	21%	38%
FdsA	1kqf	FdnG	Formate dehydrogenase N	1.080 (436)	22%	36%
FdsA	3iam_3	Nqo3	Respiratory complex I	1.195 (305)	29%	44%
FdsB	1m2d	Fdx4	Thioredoxin-like ferredoxin	0.884 (31)	n.d.	n.d.
FdsB	3iam_1	Nqo1	Respiratory complex I	1.099 (311)	35%	48%
FdsG	3iam_2	Nqo2	Respiratory complex I	1.254 (88)	31%	51%
FdsABGD		FdsABGD	Formate dehydrogenase NADH-reduced	0.305 (1659)	100%	100%

^a as isolated

 $^{\text{b}}$ number of pruned Ca atoms in brackets

^c obtained using BLAST 2 sequences

n.d. - not detected

- **Supplementary Table 2** | Amino acid composition of hydrophilic tunnel in *Rc*FDH and *E*.
- *coli* FdhF (PDB-ID: 1fdo)

conserved residues	FdsA-Ser ³⁵⁶	C 109
		Ser ¹⁰⁹
	FdsA-Cys ³⁸²	Cys ¹³⁰
	FdsA-Arg ³⁸⁴	Arg ¹³⁸
	FdsA-His ³⁸⁷	His ¹⁴¹
	FdsA-Arg ⁵⁸⁷	Arg ³³³
	FdsA-Gln ⁵⁸⁹	Gln ³³⁵
	FdsA-Gln ⁵⁹³	Gln ³³⁹
	FdsA-Cys ⁵⁹⁶	Cys ³⁴²
	FdsA-Tyr ⁶⁰⁸	Tyr ³⁵⁶
	FdsA-Arg ⁶³⁵	Arg ³⁸²
	FdsA-Thr ⁹¹⁹	Thr ⁶⁷³
	FdsA-Lys ³⁹⁵	His ¹⁴⁹
	FdsA-His ⁶⁰³	Asp ³⁴
	FdsA-Glu ⁶⁰⁴	Thr ³⁵⁰
exclusive	FdsA-Ser ³⁸⁸	
	FdsA-Ser ⁴⁰¹	
	FdsD-Asn ³³	
		Cys ¹³⁵
		Asp ¹³
		Tyr ⁵⁸⁶

- **Supplementary Table 3** | Amino acid composition of hydrophobic tunnel in *Rc*FDH and *E*.
- *coli* FdhF

	<i>Rc</i> FDH	1fdo
identical	Ala ³⁸³	Ala ¹³⁷
	Leu ³⁹⁴	Leu ¹⁴⁸
	Val ⁵⁹²	Val ³³⁸
	Pro ⁵⁸⁵	Pro ³³¹
Conserved hydrophobic	Gly ³⁹¹	Val ¹⁴⁵
	Gly ⁴⁰³	Met ¹⁵⁷ *
	Phe ⁴⁰⁷	Ile ¹⁶²
	Val ⁴²⁹	Ile ¹⁸³
	Phe ⁴³⁰	Val ¹⁸⁴
	Leu ⁵⁸⁶	Val ³³²
	Phe ⁶⁰¹	Leu ³⁴⁷

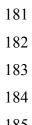
*extend into tunnel

	Sample Preparation Condition	[Spin] (µM)	[Spin]/[FDH]protomer
	as-isolated (12 K)	3	0.03
	+ NADH (12 K)	38	0.38
	+ NADH (80 K)	51	0.51
	+ Na ₂ S ₂ O ₄ (12 K)	131	1.31
	+ Na ₂ S ₂ O ₄ (80 K)	112	1.12
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Supplementary Table 4 | *Rc*FDH spin concentration from EPR spectra

	· 1	-
	FDH as isolated	FDH NADH reduced
	(EMDB-10496)	(EMDB-10495)
	(PDB 6TGA)	(PDB 6TG9)
Data collection and processing		
Magnification	31000	31000
Voltage (kV)	300	300
Electron exposure $(e - / Å^2)$	64	40
Defocus range (µm)	0.5-3.2	0.6-4.4
Pixel size (Å)	0.628	1.07
Symmetry imposed	C2	C2
Initial particle images (no.)	799,023	669,976
Final particle images (no.)	366,573	199,229
Map resolution (Å)	3.26	3.23
FSC threshold	0.143	0.143
Map resolution range (Å)	3.0-4.0	3.0-4.6
Refinement		
Initial model used (PDB code)	de novo	de novo
Model resolution (Å)	3.26	3.24
FSC threshold	0.143	0.143
Model resolution range (Å)	3.0-4.0	3.0-4.6
Map sharpening <i>B</i> factor (Å ²)	167	130
Model composition		
Non-hydrogen atoms	25,324	25,412
Protein residues	3,318	3,318
Ligands	24	26
<i>B</i> factors (Å ²)		
Protein	11.78	19.72
Ligand	9.78	15.60
R.m.s. deviations		
Bond lengths (Å)	0.0007	0.014
Bond angles (°)	0.996	1.161
Validation		
MolProbity score	1.84	2.06
Clashscore	8.35	11.37
Poor rotamers (%)	0.98	0.47
Ramachandran plot		
Favored (%)	94.25	91.97
Allowed (%)	5.45	7.78
Disallowed (%)	0.30	0.24

Supplementary Table 5 | EM data collection, processing and refinement statistics



192 Supplementary Table 6 | Primer sequences used in this study

Primers (5'-3')				
Construct	His6_Csp61_fw	fdsDXho_rv		
pTHfds36	CAGTACATGGGCAGCAGCCATC	GCTCGAGTGGAAACGATGAC		