Heterologous production in *Wolinella succinogenes* and characterization of the quinol:fumarate reductase enzymes from *Helicobacter pylori* and *Campylobacter jejuni*

Mauro MILENI*, Fraser MacMILLAN†, Christos TZIATZIOS‡¹, Klaus ZWICKER§, Alexander H. HAAS*, Werner MÄNTELE‡, Jörg SIMON|| and C. Roy D. LANCASTER*²

*Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, Max-von-Laue-Str. 3, 60438 Frankfurt am Main, Germany, †Institute of Physical and Theoretical Chemistry, J.W. Goethe University, Marie-Curie-Str. 11, 60439 Frankfurt am Main, Germany, ‡Institute of Biophysics, J.W. Goethe University, Max-von-Laue-Str. 1, 60438 Frankfurt am Main, Germany, §Department of Medicine, Institute of Molecular Bioenergetics, Gustav Embden Centre of Biological Chemistry, J.W. Goethe University, Theodor-Stern-Kai 7, Haus 25B, 60590 Frankfurt am Main, Germany, and ||Institute of Molecular Biosciences, J.W. Goethe University, Marie-Curie-Str. 9, 60439 Frankfurt am Main, Germany

The ϵ -proteobacteria *Helicobacter pylori* and *Campylobacter jejuni* are both human pathogens. They colonize mucosal surfaces causing severe diseases. The membrane protein complex QFR (quinol:fumarate reductase) from *H. pylori* has previously been established as a potential drug target, and the same is likely for the QFR from *C. jejuni*. In the present paper, we describe the cloning of the QFR operons from the two pathogenic bacteria *H. pylori* and *C. jejuni* and their expression in *Wolinella succinogenes*, a non-pathogenic ϵ -proteobacterium. To our knowledge, this is the first documentation of heterologous membrane protein production in *W. succinogenes*. We demonstrate that the replacement of the homologous enzyme from *W. succinogenes* with the heterologous enzymes yields mutants where fumarate respiration is fully functional. We have isolated and characterized the heterologous QFR enzymes. The high quality of the enzyme preparation

enabled us to determine unequivocally by analytical ultracentrifugation the homodimeric state of the three detergent-solubilized heterotrimeric QFR enzymes, to accurately determine the different oxidation–reduction ('redox') midpoint potentials of the six prosthetic groups, the Michaelis constants for the quinol substrate, maximal enzymatic activities and the characterization of three different anti-helminths previously suggested to be inhibitors of the QFR enzymes from *H. pylori* and *C. jejuni*. This characterization allows, for the first time, a detailed comparison of the QFR enzymes from *C. jejuni* and *H. pylori* with that of *W. succinogenes*.

Key words: *Campylobacter jejuni*, *Helicobacter pylori*, heterologous gene expression, membrane protein purification, quinol:fumarate reductase, *Wolinella succinogenes*.

INTRODUCTION

Since the discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease by Marshall and Warren [1], who were very recently awarded the Nobel Prize for Medicine [2], this bacterium has been studied intensively. *H. pylori* inhabits approx. 50% of the world population [3]. It colonizes the mammalian stomach, causing peptic ulcers, gastric atrophy, gastric MALT (mucosa-associated lymphoid tissue) lymphoma [4], and it is associated with the development of gastric adenocarcinoma, the world's second leading cause of cancer-related death [5]. *Campylobacter jejuni* can colonize the mucosal surfaces of the intestinal tracts, oral cavities or urogenital tracts, and is established as the most common etiological factor for the bacterial food-borne diarrhoeal disease [6], also known as 'traveller disease'. *C. jejuni* is responsible for gastroenteritis, the GBS (Guillain–Barré syndrome) [7] and, recently, it has also been found associated with the IPSID (immunoproliferative small intestinal disease) [8], a MALT lymphoma. These two bacteria of known genomic sequences [9,10] are microaerophilic, Gram-negative, flagellate species that are members of the ϵ -sub-

class of the proteobacteria [11,12]. Although chemotherapies for the eradication of these species are currently available, the development of more efficient, inexpensive drugs is needed in order to cope with the drawbacks of the therapies, especially for the treatment of *H. pylori* [13], and with the continuous emergence of antibiotic resistances.

The QFR (quinol:fumarate reductase) has been considered to be a potential drug target for *H. pylori* eradication [14]. More recently, this consideration has been strongly supported by the finding that the QFR from *H. pylori* is essential for the colonization of murine stomach [15]. Therefore it is likely that inhibitors of this enzyme would be effective antibiotics, and in analogy this could also apply to the QFR of *C. jejuni*. The QFR couples the reduction of fumarate to succinate with the oxidation of menaquinol to MK (menaquinone) [16], and is a member of the SQOR (succinate:quinone oxidoreductase) membrane protein superfamily, which also comprises the complex II of aerobic respiration [17]. The QFR is a key enzyme of the fumarate respiration [18], the most widespread type of anaerobic respiration. Although QFR activity from *C. jejuni* and *H. pylori* bacterial lysates has been detected in several studies [14,19], these measurements

Abbreviations used: BV, benzyl viologen; cat, chloramphenicol acetyltransferase; cw-EPR, continuous-wave EPR; DMN, 2,3-dimethyl-1,4-naphthoquinone; DMNH₂, 2,3-dimethyl-1,4-naphthoquinol; MALT, mucosa-associated lymphoid tissue; MB, Methylene Blue; MK, menaquinone; QFR, quinol:fumarate reductase; SQOR, succinate:quinone oxidoreductase; SQR, succinate:quinone reductase.

¹ Present address: Technical Education Institute of Larissa, 411 10 Larissa, Greece.

² To whom correspondence should be addressed, at Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, P.O. Box 55 03 53, 60402 Frankfurt am Main, Germany (email Roy.Lancaster@mpibp-frankfurt.mpg.de).

The *frdCAB* sequence from pCatCj4 reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ628040.

of the homologously produced QFR performed on cell homogenates or scarcely purified samples [20] were characterized by very low specific enzymatic activities (see [21] for a review).

The QFR enzymes from ϵ -proteobacteria are so-called B-type [17] SQOR enzymes, and consist of three subunits: the flavo-protein subunit A, containing a FAD cofactor; the iron-sulphur subunit B, containing one [2Fe-2S], one [4Fe-4S] and one [3Fe-4S] cluster; and one hydrophobic transmembrane anchor (subunit C), containing two haem groups. The three-dimensional crystal structure at 2.2 Å (1 Å = 0.1 nm) resolution of this QFR type from *Wolinella succinogenes* was published in 1999 [22]. The QFR operons of *C. jejuni* [9] and *H. pylori* [23] have a structure identical with that found in *W. succinogenes* [24,25], consisting of three concatenated genes in the order *frdC*, *frdA* and *frdB*. Even though the three species are all phylogenetically rather close, the amino acid sequence homology between homologous and heterologous proteins is still relatively low (the membrane anchors show only ~50% homology). Like the two pathogen species introduced above, *W. succinogenes* is an anaerobic ϵ -proteobacterium, which has been extensively studied and in particular used as a suitable organism for investigations on fumarate respiration. A QFR deletion strain (Δ *frdCAB*) of this species [26] could not grow by fumarate respiration.

Here, we present the first successful large-scale heterologous overproduction of the QFR enzymes from *H. pylori* and *C. jejuni* in the anaerobic non-pathogenic bacterium *W. succinogenes*. Subsequently, these membrane protein complexes from ϵ -proteobacteria have been purified, characterized and compared.

EXPERIMENTAL

Biological materials and chemicals

H. pylori 26695 genomic DNA and a clinically isolated strain of *C. jejuni* were kindly provided by Dr Stefan Bereswill (see the Acknowledgments section). Oligonucleotide primers were purchased from ThermoHybaid (Ulm, Germany). Taq polymerase for long-template preparative PCR was purchased from Roche (Expand Long Template PCR System). Taq polymerase GoldStar (Eurogentec) was used for short template analytical PCR. Restriction and modification enzymes were obtained from Fermentas (St. Leon-Rot, Germany). All cloning steps were performed in *Escherichia coli* JM110 and XL1-Blue MRF' Kan supercompetent cells (Stratagene, La Jolla, CA, U.S.A.). Genomic DNA preparation from *W. succinogenes* was performed using the DNeasy Tissue kit (Qiagen, Hilden, Germany). For the production of the heterologous enzymes, the pFrddcat2 (where cat is chloramphenicol acetyltransferase) vector template and a *W. succinogenes* *frdCAB* deletion mutant strain (Δ *frdCAB*) [26] were used. *W. succinogenes* was grown in minimal or rich medium [addition to the minimal medium of Brain Heart Infusion (Difco)] with formate (electron donor) and fumarate or nitrate (terminal electron acceptors) as described elsewhere [27,28]. Kanamycin and chloramphenicol (GERBU Biochemical Mart, Gaiberg, Germany) were added when required at a concentration of 25 and 12.5 mg/l respectively in *W. succinogenes* cultures. The concentration of these antibiotics was doubled (50 and 25 mg/l) in *E. coli* cultures. The *W. succinogenes* QFR has been purified from the wild-type strain DSMZ 1740.

Construction of *W. succinogenes* strains

Table 1 denotes the strains, plasmids and primers used in the present study. The heterologous expression of *frdCAB* loci from *C.*

jejuni or *H. pylori* in *W. succinogenes* is based on the restoration of an intact *frdCAB* operon in the genome of the deletion mutant *W. succinogenes* Δ *frdCAB* [26]. In the genome of this mutant, the complete *frdCAB*-coding region had been replaced by the kanamycin resistance gene, resulting in inability of the cells to grow by fumarate respiration. The *frdCAB* operon was restored in the genome of *W. succinogenes* Δ *frdCAB* by integration of plasmid pFrddcat2 via homologous recombination between the *frd* promoter present both on the plasmid and on the genome of the deletion mutant. The resulting strain showed wild-type properties in terms of fumarate respiration and fumarate reductase activity [26]. In order to replace the *W. succinogenes* *frdCAB* locus on pFrddcat2 by the corresponding genes from *C. jejuni* or *H. pylori*, an *frdCAB*-lacking fragment of pFrddcat2 as well as fragments containing the respective *frdCAB* loci were amplified by PCR. The primer pairs used contained suitable restriction sites at their 5'-ends (Table 1). The vector fragment (5.27 kb) comprised the *frd* promoter and the *frdC* start codon followed by a ClaI restriction site on one end as well as the *frdB* stop codon followed by an AvrII restriction site on the other end. The *frdCAB* coding regions from *C. jejuni* and *H. pylori* were amplified by PCR using genomic DNA or whole cells as template and primer pairs with appropriate restriction sites at their 5'-ends. The PCR products contained the entire *frdC*, *frdA* and *frdB* genes excluding the *frdC* start codon and the *frdB* stop codon. Ligation of the restricted vector with one of the restricted *frdCAB* fragments yielded plasmids pCatCj4 and pCatHpG8 respectively. Due to the cloning procedure, two codons immediately downstream of the *frdC* start codon (encoding Ile and Asp residues) and two codons at the 3'-end of *frdB* (encoding Pro and Arg residues) were introduced. Apart from these modifications, DNA sequencing of the *frdCAB* operon in pCatHpG8 confirmed that the nucleotide sequence was identical with that of the genome sequence of *H. pylori* strain 26695 [10]. The *frdCAB* operon in pCatCj4 was derived from a clinical isolate of *C. jejuni* and was found to differ by 84 nt from the *frdCAB* sequence of *C. jejuni* NCTC11168 [9], although only five amino acids were found to be different after translation. The determined *frdCAB* sequence from pCatCj4 was deposited in the data banks (EMBL nucleotide sequence accession no. AJ628040). Transformation of *W. succinogenes* Δ *frdCAB* with derivatives of pFrddcat2 and Southern-blot analysis were carried out as described previously [26]. Transformants were selected in the presence of kanamycin and chloramphenicol on agar plates containing formate and nitrate as energy substrates. The resulting mutants were named *W. succinogenes* HpGM31 and *W. succinogenes* CjM11 (Table 1).

Expression and purification

The large-scale preparation QFR consisted of a culture of *W. succinogenes* in 60 litres of formate/fumarate rich anaerobic medium. After inoculation with 240 ml preculture, the culture was incubated for approx. 12–15 h at 37 °C until the late exponential phase. The cells were harvested by centrifugation and enzyme isolation was performed as described in [29], except that DEAE CL-6B was replaced by a DEAE-Sepharose chromatography and that the elution gradient was 0–300 mM NaCl. Pressure dialysis (Amicon) using a membrane with a cut-off of 100 kDa enabled us to concentrate the protein and to reduce the NaCl concentration. Protein concentration of small volumes was performed on 2.5 ml capacity and 100 kDa cut-off Centriscart micro-concentrators (Sartorius, Goettingen, Germany). For analytical ultracentrifugation analysis, a further purification step was introduced so as to improve purity and homogeneity of the sample by performing a gel filtration on a TSK-GEL G4000SW column

Table 1 Strains, plasmids and oligonucleotide primers used in the present study

Strain, plasmid or primer	Relevant properties, usage, nucleotide sequence (5'→3')	Reference
Bacterial strains		
<i>W. succinogenes</i> DSMZ 1740	Wild-type strain	[59]
<i>W. succinogenes</i> Δ <i>frdCAB</i>	Fumarate reductase operon (<i>frdCAB</i>) deletion mutant	[26]
<i>W. succinogenes</i> HpGM31	Strain expressing the <i>frdCAB</i> locus from <i>H. pylori</i>	The present study
<i>W. succinogenes</i> CjM11	Strain expressing the <i>frdCAB</i> locus from <i>C. jejuni</i>	The present study
<i>H. pylori</i> strain 26695	Wild-type strain	[10]
<i>C. jejuni</i>	Clinical isolate, University of Freiburg	The present study
<i>E. coli</i> JM110 and XL1-blue MRF' Kan	Strain used for cloning and plasmid propagation	[60]
Plasmids		
pFrdcat2	Derivative of pSC101. <i>E. coli</i> low-copy number vector containing the <i>W. succinogenes</i> <i>frdCAB</i> genes including <i>frd</i> promoter and terminator sequences. Used for restoration of the <i>frdCAB</i> locus in <i>W. succinogenes</i> Δ <i>frdCAB</i>	[26]
pCatHpG8	Derivative of pFrdcat2 containing the <i>frdCAB</i> genes from <i>H. pylori</i> strain 26695 instead of <i>frdCAB</i> from <i>W. succinogenes</i>	The present study
pCatCj4	Derivative of pFrdcat2 containing the <i>frdCAB</i> genes from a clinical isolate of <i>C. jejuni</i> instead of <i>frdCAB</i> from <i>W. succinogenes</i>	The present study
Primers		
pFrdcat2 Fw	CCCCTAGG TAA ATCTCCTGGAGCGGGTCTCCC Forward primer used for amplification of the pFrdcat2 vector fragment; contains an <i>A</i> vrII restriction site (underlined) and the <i>frdB</i> stop codon (in boldface)	The present study
pFrdcat2 Rv	CCATCGAT CAT CTGTTCCCTGTGCAGTATT Reverse primer used for amplification of the pFrdcat2 vector fragment; contains a <i>Cl</i> al restriction site (underlined) and the <i>frdC</i> start codon (in boldface, complementary sequence)	The present study
<i>H. pylori</i> Fw	CCATCGATCAACAAGAAGAGATTATAGAGGGT Forward primer used for amplification of the <i>frdCAB</i> locus from genomic DNA of <i>H. pylori</i> strain 26695. The <i>Cl</i> al restriction site is underlined	The present study
<i>H. pylori</i> Rv	CCCCTAGGCGGGCTTTTACCCACTTCAACATCC Reverse primer used for amplification of the <i>frdCAB</i> locus from genomic DNA of <i>H. pylori</i> strain 26695. The <i>A</i> vrII restriction site is underlined	The present study
<i>C. jejuni</i> Fw	CCATCGATCGTGAGCTTATCGAAGGTTATTGG Forward primer used for amplification of the <i>frdCAB</i> locus from <i>C. jejuni</i> cells. The <i>Cl</i> al restriction site is underlined	The present study
<i>C. jejuni</i> Rv	CCCCTAGGTTTATTCTTTGAGCGACAAGTTGTC Reverse primer used for amplification of the <i>frdCAB</i> locus from <i>C. jejuni</i> cells. The <i>A</i> vrII restriction site is underlined	The present study

(60 cm × 21.5 mm; TOSOH Bioscience, Stuttgart, Germany) with Äkta purifier 10 (Amersham Biosciences, Freiburg, Germany). The concentrated samples were shock-frozen in liquid nitrogen and stored at -77°C until needed.

Analytical ultracentrifugation

The purified QFRs from *C. jejuni*, *H. pylori* and *W. succinogenes* used in the analytical ultracentrifugation experiments were prepared as described above. For sedimentation velocity experiments, the samples were diluted to a final concentration of 0.35 mg/ml using the nitrogen-saturated buffer (0.01 % *n*-dodecyl- β -D-maltoside, 0.1 % *n*-decyl- β -D-maltoside, 20 mM Hepes, pH 7.3, and 2 mM malonate) [29]. Sedimentation velocity experiments were performed in a Beckmann Optima XL-A analytical ultracentrifuge in combination with an An50-Ti rotor and Epon double sector cells of 12 mm path length. Rotor speed was 35 000 rev./min and rotor temperature 4°C . The sample volume was 350 μl . The absorption profiles were recorded at 415 nm, with a radial increment of 0.02 mm, at time intervals of 3 min and with two averages per scan. The program *sedfit* was used for data analysis used [30]. The experimental $A(r,t)$ data were evaluated using the method for direct boundary Lamm equation modelling (i) for continuous sedimentation coefficient distributions [c(s)-method] and (ii) for discrete non-interacting components. The partial specific volume, v , of QFR in aqueous buffers, corrected for protein-bound prosthetic groups, was calculated from its amino acid composition as described by Durchschlag [31] as 0.730 ml/g. The densities and viscosities of the buffer were calculated using the software *sednterp* [32]. The effective molar mass,

$M_{\text{eff,c}} = M_c(1 - v_c \cdot \rho_0)$, of the protein–detergent complexes was calculated from their s and D values using the Svedberg equation.

Protein characterization

Detection of covalently bound FAD was carried out on an SDS polyacrylamide gel containing 5–10 μg of protein. After electrophoresis, the gel was washed for 10 min in 10 % acetic acid and irradiated with UV light [33]. The concentration of haem *b* in the sample has been determined as described earlier [molar extinction coefficient ($\epsilon_{565-575}$) $23.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$] [34]. The total protein concentration was measured with the BCA (bicinchoninic acid) assay (Pierce Biotechnology). SDS/PAGE (12.5 % polyacrylamide) was carried out using Coomassie Blue for staining.

Enzymatic activities

The samples containing isolated QFR were diluted to a concentration of 0.5–1 mg/ml in a nitrogen-saturated buffer, and subsequently incubated at 37°C for 30 min prior to the assay. Volumes of 2–10 μl were added to the assay mixture. All enzymatic assays were performed in nitrogen-saturated 25 mM potassium phosphate buffer at pH 7.3. Three different kinds of enzymatic assays were performed at 37°C in 0.5-cm path-length degassed quartz cuvettes by monitoring photometrically, first, the oxidation of dithionite-reduced BV (benzyl viologen) (ϵ_{546} $19.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) radical by fumarate ('BV assay') [35], secondly, the reduction of MB (Methylene Blue) (ϵ_{578} $17.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) by succinate ('MB assay') [36], and thirdly, the oxidation of DMNH₂ (2,3-dimethyl-1,4-naphthoquinol) ($\epsilon_{270-290}$ $15.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) by fumarate ('DMNH₂ assay'). Whereas the first two assays

are independent of the membrane-integral subunit C, the 'DMNH₂ assay' represents a total activity assay, and is subunit C-dependent. Reduction of DMN (2,3-dimethyl-1,4-naphthoquinone) was performed either by NaBH₄ ('BH₄-DMNH₂ assay') [29,33] or by a coupled reaction with DT-diaphorase (EC 1.6.99.2) and NADH ('DT-DMNH₂ assay') [37]. In the latter case, enzymatic activity is determined indirectly by measuring NADH disappearance at 340 nm, and the maximum velocity appears double if compared with the 'BH₄-DMNH₂ assay'. The assay mixture contained approx. 400 μ M NADH (ϵ_{340} 6.29 mM⁻¹ · cm⁻¹), 1 mM fumarate and 20 μ g/ml rat liver DT-diaphorase.

Inhibition effects of oxantel, thiabendazole and omeprazole were accurately measured using the quinol-regenerating coupled reaction with DT-diaphorase, so that long and stable enzyme kinetics could be measured. With this method, the inhibitor was added 20–30 s after the catalytic reaction was started, so that pre-inhibition activities could be compared at each trial. The enzymatic assays were carried out in the presence of the inhibitor and with a minimum of six different substrate concentrations. DMNH₂ regeneration by DT-diaphorase activity was measured, and proved to be faster than the QFR enzymatic activity at any time and under any condition. As a further experimental control, the DT-diaphorase enzymatic activity was measured with and without the presence of the inhibitors, so that any unforeseen inhibition effect was prevented. One activity unit (U) is defined as 1 μ mol of product produced per min. All spectrophotometer measurements were performed with an Agilent 8453 UV-visible Spectroscopy System (Agilent Technologies, Böblingen, Germany).

Oxidation–reduction ('redox') titration by EPR

The titration of the redox states at pH 7.3 of the FAD and the iron–sulphur clusters of QFR from *W. succinogenes*, *C. jejuni* and *H. pylori* was performed essentially as described by Dutton [38]. A solution of purified protein (60–75 μ M) was stirred at 298 K in an anaerobic reaction vessel. The following redox mediators were added to the protein solution: TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine), phenazine-methosulphate, MB, menadione, resorufin, indigotrisulphonate, 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, phenosafranine, BV, and methyl viologen, resulting in a final concentration of 35 μ M of each. The redox potential of the solution was monitored by a redox microelectrode (Mettler-Toledo GmbH, Giessen, Germany) and adjusted to selected values by addition of small aliquots of a 50 mM sodium dithionite solution. At appropriate redox potentials, 80 μ l aliquots were anaerobically transferred into an argon-flushed EPR tube, frozen in an isopentane/methylcyclohexane mixture (5:1, v/v) at approx. 120 K and stored in liquid nitrogen. The degree of reduction of individual redox centres was monitored by cw-EPR (continuous-wave EPR) spectroscopy under non-saturating microwave conditions. A Bruker EPR spectrometer E500 or ESP300 (Bruker-Biospin) was used equipped with a continuous flow liquid helium cryostat (Oxford Instruments) set at 10 and 50 K for iron–sulphur clusters and FAD respectively. Experimental conditions: microwave frequency 9.43 GHz; microwave power 2 mW; modulation amplitude 2 Gauss. The results from the titrations for the iron–sulphur clusters were plotted and the curve was fitted using the standard Nernst equation. FAD redox midpoint potentials were determined using a double Nernst equation. From the experimental conditions, an accuracy of approx. ± 10 mV can be estimated. Due to overlapping signals arising from FAD and mediators, data obtained from the FAD titrations were normalized by subtracting the EPR intensities generated by a titration of a protein-free solution of buffer and mediators. The yield of FAD was determined by calculating the ratio of the integrated areas

of the maximum intensity peak obtained from FAD and the fully reduced iron–sulphur cluster S1.

Haem redox titration by VIS spectroscopy

The QFR sample in 100 mM potassium phosphate buffer (pH 7) containing 100 mM KCl and 1.0 mM *n*-dodecyl- β -D-maltoside was concentrated to approx. 1.0 mM by using a 100 kDa Microcon filtration cell (Amicon). The redox titrations at pH 7.0 were performed as described in [39] in an ultrathin-layer spectroelectrochemical cell (optical path length is ~ 10 μ m) which was designed for UV–visible and IR spectrometry [40]. Difference spectra were obtained by applying an initial potential at which the two haems (b_P and b_D) were either fully reduced or fully oxidized. The titration curves were generated on the basis of the redox dependence of the amplitudes of the Soret and α -band, independently. To obtain values for the midpoint potentials of haem b_H and haem b_L , iterative fitting of a calculated Nernst function was performed [39]. The error in the determination of the midpoint potentials can be estimated to be ± 10 mV.

RESULTS AND DISCUSSION

Molecular biology/genetics

The clones obtained with the transformation of the Δ *frdCAB* deletion mutant strain of *W. succinogenes* with the constructs pCatCj4 and pCatHpG8, containing the *C. jejuni* and *H. pylori* *frdCAB* operons respectively, were able to grow on rich and minimal media using fumarate as the sole terminal electron acceptor. Two clones were chosen for expression and named HpGM31 and CjM11, containing the *C. jejuni* QFR and *H. pylori* QFR operons respectively. Plasmid integration into the correct genomic locus was further verified by species-specific PCR and by Southern-blot analysis using PCR-synthesized labelled probes complementary to the upstream and downstream regions of recombination (not shown). Both mutants contain a single copy of the *frdCAB* locus on the genome that replaces the genuine *frdCAB* operon of *W. succinogenes* wild-type cells.

Properties of *W. succinogenes* strains expressing *frdCAB* operons from *C. jejuni* or *H. pylori*

The mutants *W. succinogenes* CjM11 and HpGM31 express the respective *frdCAB* operons from *C. jejuni* and *H. pylori* under the control of the *W. succinogenes* *frd* promoter. In contrast with the parental strain *W. succinogenes* Δ *frdCAB*, strains CjM11 and HpGM31 grow by fumarate respiration, albeit at slightly longer doubling times compared with the wild-type (Table 2). Nevertheless, the cell yield for growth by fumarate respiration was found to be identical in the three strains. Apparently, after a correct transcription and translation of the heterologous operon, the protein complex could be correctly folded and delivered into the cytoplasmic membrane. Furthermore, biochemical and biophysical characterization proved that all of the six cofactors (the haem *b* groups, the iron–sulphur clusters and the FAD) were correctly assembled into the enzyme. It was surprising that these enzymes are so efficiently interchangeable, implying also an efficient interplay between the heterologous enzymes and the contingent-associated chaperones. Table 2 shows the specific fumarate reductase activities measured in cell fractions of the strains using various enzyme activity assays.

Protein chemistry

The two operons were heterologously expressed in *W. succinogenes* for a large-scale membrane protein preparation of the

Table 2 Doubling times and specific fumarate reductase activities of *W. succinogenes* strains

The cells were grown by fumarate respiration either in minimal medium or in minimal medium supplemented with 1.3% Brain Heart Infusion broth [26].

	<i>W. succinogenes</i> strain		
	DSMZ 1740	CjM11	HpGM31
Doubling time (h)			
In minimal medium	2.0	2.3	3.0
In supplemented medium	1.0	1.2	1.9
Specific fumarate reductase activity (units/mg of protein)			
Succinate → MB			
Cell homogenate	0.7	0.2	0.8
Membrane fraction	1.4	0.4	1.1
Soluble fraction	≤ 0.03	≤ 0.02	≤ 0.02
DMNH ₂ → fumarate			
Cell homogenate	2.3	1.2	2.4
Membrane fraction	3.2	2.1	3.4
Soluble fraction	≤ 0.1	≤ 0.1	≤ 0.1

H. pylori and *C. jejuni* QFR. In order to properly characterize these two membrane protein complexes, large amounts of highly pure and stable enzyme are required. The adopted procedure of expression and isolation enabled us to obtain, after gel filtration, up to 100 mg of *C. jejuni* QFR and 150 mg of *H. pylori* QFR.

As monitored with the 'MB assay', the specific activity of the cell homogenates was 0.3 unit/mg for *C. jejuni* (total volume 233 ml, starting from 72 g cells) and 0.8 unit/mg for *H. pylori* (255 ml, 105 g cells). The same assay performed on the cytosolic fraction after the first ultracentrifugation of the membranes gave negligible values, demonstrating that the QFR is stably attached to the membrane and the enzyme integrity is maintained even during mechanical disruption of the cells. After purification, the specific partial activity (MB assay) of the protein sample increased to up to 7.3 units/mg for *C. jejuni* and 12.3 units/mg for *H. pylori*. Although such partial specific activities are usually quoted for the characterization of preparations of these enzymes, a more relevant determination of the quality of the enzyme purification procedure can be accomplished by monitoring the specific activity of DMNH₂ oxidation by fumarate (see Table 3). In addition, the protein purity can be assessed by SDS/PAGE (Figure 1). Three bands represent the three subunits FrdA, FrdB and FrdC from the two QFRs with no major contaminations and appropriate stoichiometric ratios, as inferred from the respective band intensities. Based on the amino acid sequences, the molecular masses of the *H. pylori* QFR subunits A, B and C are 80.2, 27.6 and 28.8 kDa respectively. The main cause of the significantly different molecular masses of the respective A subunits are two insertions of 22 and 29 amino acid residues of unknown functional significance in the C-terminal half of the *H. pylori* sequence. The corresponding values for *C. jejuni* QFR subunits are 73.8, 27.5 and 30.3 kDa respectively. As demonstrated by Uden et al. [33], the QFR hydrophobic subunit C appears smallest in the SDS/PAGE experiment. Together with a general improvement, the gel filtration step mainly permitted us to discard an unknown contaminant protein of approx. 55–60 kDa.

When the *H. pylori* QFR sample was subjected to gel filtration, the specific activity decreased dramatically. This may reflect, for instance, the loss of tightly bound phospholipids, which may affect the catalytic efficiency of subunit C. This last hypothesis arises from two main observations: the protein appears as a unique and homogeneous peak in the gel filtration chromatogram, proving that the enzyme is still assembled as a complex; and the

Table 3 Purification profile based on the total specific activity as determined with the 'DT-DMNH₂ assay'

IEF, isoelectric focusing.

	× 10 ⁻³ Total activity (units)	Specific activity (units/mg)	Protein yield (%)	Purification factor
<i>C. jejuni</i> QFR				
Cell homogenate	12.9	1.2	100	1.0
Triton X-100 homogenate	16.3	2.0	126	1.7
Triton X-100 extract	14.3	3.4	111	2.8
Anion-exchange chromatography	4.2	7.3	33	6.1
IEF	1.6	9.3	12.4	7.8
Gel filtration	0.78	9.7	6.0	8.1
<i>H. pylori</i> QFR				
Cell homogenate	40.9	2.4	100	1.0
Triton X-100 homogenate	43.8	3.8	107	1.6
Triton X-100 extract	43.0	6.0	105	2.5
Anion-exchange chromatography	11.7	10.4	29	4.3
IEF	3.9	12.2	10	5.1
Gel filtration	0.5	2–3	~1.3	~1

Figure 1 SDS/PAGE of the *C. jejuni* and *H. pylori* QFR samples during purification

C. jejuni (lanes 1–4) and *H. pylori* (lanes 5–8) QFR samples of 3 µg (lanes 1, 3, 5 and 7) and 6 µg (lanes 2, 4, 6 and 8) applied after isoelectric focussing (lanes 1, 2, 5 and 6), and gel filtration (lanes 3, 4, 7 and 8) purification steps. Molecular mass standards (L) are indicated in kDa.

partial activity measured with the 'MB assay' indicates that the hydrophilic subunits are not adversely affected.

Analytical ultracentrifugation

The oligomeric state of QFR from all three organisms, *C. jejuni*, *H. pylori* and *W. succinogenes*, was studied by sedimentation velocity experiments in combination with Lamm equation fitting. The experiments were performed without density matching for the bound detergent. This procedure, as demonstrated previously by us [41] and others [42], has been shown to be at least as useful in determining the states of association of membrane protein complexes as the results from equilibrium sedimentation. Figure 2 shows the analyses of sedimentation velocity experiments on the three QFR species, based on a continuous distribution model for *s* values in the range between 0.5 and 20 S. The *v* value for the mixed decylmaltoside/dodecylmaltoside micelles was calculated assuming a weight ratio of the two detergents of 10:1. This led to *v* = 0.794 ml/g. All *c(s)*-distributions give an excellent fit to the experimental data, exhibiting the presence of a well-defined sharp peak at approx. 8 S. In addition, the presence of small amounts of material with higher and lower sedimentation coefficients is suggested (Figures 2A and 2B). Since the relative area under a

Figure 2 Sedimentation velocity analysis on QFR from *C. jejuni* (A, B), *H. pylori* (C) and *W. succinogenes* (D)

Experimental sedimentation velocity distributions (A) of the enzyme at different times (symbols) and best fit distributions calculated using solutions of the Lamm equation based on the model of continuous size distribution (solid lines). For clarity only every fourth data set is shown. (B–D) Best-fit sedimentation coefficient distribution $c(s)$.

peak in the $c(s)$ -distribution corresponds to the relative loading concentration of the respective species, we conclude that most of the material is in the single peak at approx. 8 S. The area under this peak accounts for approx. 90 % of the total amount of protein in the sample for QFR from *C. jejuni* and from *H. pylori*, and for approx. 97 % for QFR from *W. succinogenes*.

The peak at approx. 8 S is clearly resolved, which suggests homogeneity of the respective component. The experimental $A(r,t)$ data were therefore analysed using solutions of the Lamm equation for a small number of discrete non-interacting species [30]. For QFR from the first two bacteria, we have used terms for four discrete components for the calculation of sedimentation and diffusion coefficient of the approx. 8 S peak, with starting s values identical with those found by the $c(s)$ -method. In case of QFR from *W. succinogenes*, the experimental sedimentation velocity data were fitted assuming the presence of a single component. The fits were of very good quality. The results found for the main component of QFR from all three organisms were similar: the s and D values found for QFR from *C. jejuni* were 7.82 S and $2.15 \times 10^{-7} \text{ cm}^2/\text{s}$ respectively, those for QFR from *H. pylori* were 7.97 S and $2.14 \times 10^{-7} \text{ cm}^2/\text{s}$ respectively, and those for QFR from *W. succinogenes* were 7.50 S and $1.93 \times 10^{-7} \text{ cm}^2/\text{s}$ respectively. We obtained an effective molar mass ($M_{\text{eff,c}}$) of $83000 \pm 9000 \text{ g/mol}$ for the first complex, $85000 \pm 9000 \text{ g/mol}$ for the second and $88000 \pm 7000 \text{ g/mol}$ for the last one. It should be noted that the relatively large uncertainty of approx. 10 % in determining $M_{\text{eff,c}}$ has its origin mainly in the uncertainty of the D value, which could be varied in the analysis by approx. 10 % without significant increase in the R.M.S.E. (root mean square error) of the fit [43]. These results indicate that the approx. 8 S component represents the same state of association of QFR from either organism.

The oligomeric state of QFR for the main peak in the $c(s)$ -distribution was determined by the method described in [41].

Assuming that the QFR from *C. jejuni* is in a monomeric state with $M_1 = 132\,400 \text{ g/mol}$, the calculated amount of protein-bound detergent was 1.75 g per g of protein, using the v and $M_{\text{eff,c}}$ values given above. The corresponding amounts for QFR from *H. pylori* and from *W. succinogenes* were found to be 1.82 and 2.0 g/g respectively. Such high amounts of detergent bound by the enzyme are certainly not reasonable [44]. In addition, according to the crystal structure analysis of the enzyme [22], only one of the three protein subunits (subunit C with molecular mass 30 kDa), corresponding to less than one-quarter of the molecular mass, is membrane-embedded. Thus the amounts of the bound detergent given above, which refer to the whole molecule, should be multiplied by a factor of approx. 4, to correspond to that part of the dimeric QFR that is integrated into the membrane. Consequently, the assumption that QFR is in a monomeric state cannot be correct. Under the assumption that the main fraction of the enzyme is a dimer, the protein-bound detergent was calculated to be 0.21, 0.25 and 0.34 g/g of protein for QFR from the three organisms. These values are relatively low, nevertheless they are consistent with the range of values observed for other membrane proteins [44]. On the other hand, a trimeric or higher oligomeric state of QFR can be ruled out, since, even in absence of bound detergent, the calculated $M_{\text{eff,c}}$ value for a trimer would be much higher than that calculated from s and D . We conclude that in their detergent-solubilized states, the QFR enzymes from *C. jejuni*, from *H. pylori* and from *W. succinogenes* are all present as homodimers of heterotrimers. This observation is important, considering the variability of the homo-oligomeric state throughout the superfamily of SQORs. For instance, the QFR from *E. coli* [45] and mammalian SQR (succinate:quinone reductase) enzymes [46,47] appear to be homomonomers, whereas the SQR from *E. coli* apparently is a homotrimer [48]. Further work is required to determine whether these diverse structural arrangements also reflect mechanistic variations.

Figure 3 Electrochemical redox titration of the haem groups

(A) Haem titration of the *C. jejuni* QFR. (B) Haem titration of the *H. pylori* QFR. The black and dashed lines are indicating the midpoint potential of the distal haem (haem b_D) and proximal haem (haem b_P) respectively. Difference spectra have been performed in both directions, from reduction to oxidation and vice versa.

Enzymatic characterization: cofactor analysis and redox midpoint potentials

To prove that the haem groups have been inserted correctly into the transmembrane subunit C, haem b content was determined by measuring the absorption difference ΔAbs at 565–575 nm of the reduced and oxidized samples. The haem to protein (monomer) stoichiometric ratio was determined to be 2:1. The redox midpoint potential of the haem b groups at pH 7.0 was determined by analysing electrochemically induced absorbance difference spectra at the wavelengths of 428 nm (Soret-band) and 561 nm (α -band). Two titrating groups, i.e. haem b_P and haem b_D , can be perfectly fitted with a double Nernst function in each of the curves (Figure 3). Within an error of 5 %, the two haems contributed equally to the total change in absorbance. Taking the average value of reductive and oxidative titrations, the fitted Nernst functions yielded midpoint potentials of $E_{M,b_D} = -129$ mV and $E_{M,b_P} = +1$ mV for *C. jejuni* QFR and $E_{M,b_D} = -106$ mV and $E_{M,b_P} = +8$ mV for *H. pylori* QFR. Behaviour of the α -band and Soret-band yielded analogous titration curves and very similar values for the midpoint potentials. In accordance with the determined haem redox midpoint potentials (E_M), DMNH₂ ($E_M = -75$ mV) was demonstrated to reduce only one of the two haem groups, as is well known for *W. succinogenes* QFR (see e.g. [39]). Redox midpoint potentials of the three iron–sulphur clusters S1, S2 and S3 at pH 7.3 were determined by cw-EPR spectroscopy

Figure 4 Redox titration of the FAD prosthetic group and Fe-S clusters performed with EPR spectroscopy

(A) FAD of *H. pylori* QFR. (B) S1 of *C. jejuni* QFR. (C) S2 of *W. succinogenes* QFR. (D) S3 of *C. jejuni* QFR. Right side: EPR spectra performed at 10 K (Fe-S) and 50 K (FAD). Intensity amplitudes were measured as indicated by the arrows. Left side: intensity amplitudes are plotted against the environmental potential; the fitting of the measured data was performed with the Nernst equation (Fe-S) or double Nernst equation (FAD). AU, arbitrary units.

(X-band) as a function of potential (Figure 4), and are listed in Table 4.

The fluorescence associated with QFR subunit A on an SDS/polyacrylamide gel illuminated with UV light (see Supplementary material at <http://www.BiochemJ.org/bj/395/bj3950191add.htm>) ascertained that the prosthetic group FAD of both produced QFRs is covalently linked to the enzyme, in analogy to *W. succinogenes* QFR, where this occurs through an 8 α -[N^ε-histidyl]-linkage to the isoalloxazine ring [22,49]. Redox midpoint potentials of FAD at pH 7.3 were determined by measuring the cw-EPR signal of the flavin semiquinone (radical state of FAD; Figure 4). In order to determine the two half-wave potentials, the yield of the FAD semiquinone state is required. The latter resulted to be 9, 12 and 7% for *W. succinogenes*, *C. jejuni* and *H. pylori* respectively. Since native quinone substrates are known to co-purify together substoichiometrically with membrane proteins in general [50] and with ϵ -proteobacterial

Table 4 Oxidation–reduction midpoint potentials (E_M) of all QFR cofactors from *W. succinogenes*, *C. jejuni* and *H. pylori*

The titration of the haem groups was performed at pH 7.0, whereas iron–sulphur clusters and FAD were titrated at pH 7.3. The assignment of the 'high-potential haem' to b_P and of the 'low-potential haem' to b_D follows the assignment of Haas and Lancaster [61] for *W. succinogenes* QFR.

Cofactor	<i>W. succinogenes</i> QFR E_M (mV)	<i>C. jejuni</i> QFR E_M (mV)	<i>H. pylori</i> QFR E_M (mV)
FAD	−125	−101	−70
[2Fe-2S]	−112	−5	+26
[4Fe-4S]	−340	−235	−260
[3Fe-4S]	−61	+42	+33
Haem b_P	−9	+1	+8
Haem b_D	−152	−129	−106

QFR specifically at a ratio of approx. 0.2 molecules per monomer [51] and since the EPR signal signature from $FAD^{\bullet-}$ is quite similar to that generated by a naphthosemiquinone, such as the native MK-6, any possible misinterpretation of the obtained signals was avoided by re-performing the redox titration in the presence of a 10-fold molar concentration of MK-4. Since the yield of radical signal obtained did not change, it was concluded that the assignment to FAD is correct. Furthermore, three-pulse ESEEM (electron spin-echo envelope modulation) experiments clearly reveal the interaction of ring-nitrogen nuclei of the flavin ring with the unpaired electron, which is also quite similar to that seen previously with other flavin radicals [52,53] (results not shown). The low radical yield agrees well with the mechanism of fumarate reduction by a hydride transfer, thus the transfer of one proton, and two concerted transferred electrons from the FAD prosthetic group [18].

The redox midpoint potentials of the cofactors contained in the QFR from *W. succinogenes*, *C. jejuni* and *H. pylori* have been presented graphically (see Supplementary material), as measured with the enzymes in their detergent-solubilized states. A comparison of previously determined redox midpoint potentials of detergent-solubilized *W. succinogenes* QFR [39] with those of the membrane-bound enzyme [34] indicates significant differences only in the case of the low-potential haem, where the respective value is approx. 50 mV lower in the membrane-bound form of the enzyme. It appears reasonable to assume that the properties of two other enzymes are affected in a similar manner, so that any conclusions based on any differing properties of the detergent-solubilized enzymes apply equally to the respective membrane-bound forms. Although the data obtained previously [54] for the Fe-S centres in the *W. succinogenes* QFR are in reasonable agreement with the data obtained in the present study, the redox midpoint potential attributed to the FAD was questionable. Thus the signal assigned by Unden et al. [54] to the flavin semiquinone

could only be detected and measured as characteristic shoulders at both sides of a major peak arising from the redox mediators. Most importantly, instead of the bell-shaped titration curve, the signal persisted and even increased at low potentials, giving a maximal yield of 19 % and half-maximal amplitude at −120 mV. A similarly shaped titration curve was obtained by poisoning the enzyme with the succinate/fumarate couple, giving, in this case, a redox midpoint potential of −20 mV and a maximal yield of 41 %. In contrast with these quoted experiments, the titrations of the FAD prosthetic group of all the three QFRs that were performed here resulted in clear bell-shaped curves ($n = 2$), which could then be correctly fitted with a double Nernst equation for redox midpoint potential determination. Only relatively small differences are detected between the midpoint potentials of the cofactors from the QFRs of *H. pylori* and *C. jejuni*. However, these potentials were significantly higher than those of *W. succinogenes* (in the case of the Fe-S centres, ~100 mV higher). This may reflect differences in their physiological environments *in vivo*, as these bacteria inhabit quite different niches. For instance, the *H. pylori*'s habitat has an extremely low pH, although the cytoplasmic pH is close to neutrality [10]. Hence, the midpoint potential of the cofactors, and especially those in proximity to the periplasm, would be most likely increased because they are affected by a lower pH (redox-Bohr effect). On the other hand, the *W. succinogenes* species inhabits bovine rumen, which provides a very low environmental redox potential [55] and a pH close to neutrality. The low values of the midpoint potentials of the *W. succinogenes* QFR cofactors, and especially of the iron–sulphur clusters, might hence be ascribed to this highly reducing environment. Thus the functionality of the cofactors is evidently optimal when their redox midpoint potentials are tuned and balanced with the environmental potential found in the rumen. Future work will be directed towards determining which structural features of these enzymes differentially tune the redox midpoint potentials of the cofactors to their observed values.

Enzymatic activity

Total and partial activities of the isolated QFR enzymes belonging to three different ϵ -proteobacteria were calculated and are listed in Table 5. Whereas the partial activity measured with the 'BV assay' is far higher than the one measured with the 'MB assay' in the *W. succinogenes* and *C. jejuni* QFR enzymes, this phenomenon is not observed in the case of *H. pylori* QFR. Furthermore, the partial activities of the *W. succinogenes* QFR as determined by the MB and BV assays are marked by much higher values compared with the other two species. Possibly, this reflects different MB and BV binding site accessibilities in the different enzymes. More importantly, comparing the total enzymatic activity by the 'DT-DMNH₂ assay', the three enzymes do not show remarkable

Table 5 Specific enzymatic activities, catalytic-centre activities, Michaelis constants and inhibitor K_i values of the isolated QFR from *W. succinogenes*, *H. pylori* and *C. jejuni* after IEF

The K_i and K_M values are calculated based on the 'DT-DMNH₂ assay'.

	MB assay		BV assay		DT-DMNH ₂ assay V_{max}		DMNH ₂ K_M (mM)	Fumarate K_M (mM)	Oxantel K_i (mM)	Thiabendazole K_i (mM)	Omeprazole K_i (mM)
	Specific enzymatic activity (units/mg)	Catalytic centre activity (s ^{−1})	Specific enzymatic activity (units/mg)	Catalytic centre activity (s ^{−1})	Specific enzymatic activity (units/mg)	Catalytic centre activity (s ^{−1})					
<i>W. succinogenes</i>	22.5	50	154	340	14.7	32	0.08	–	–	–	–
<i>C. jejuni</i>	4.1	9	41	91	9.3	21	0.06	0.1	0.38	0.96	1.96
<i>H. pylori</i>	12.3	29	15	35	12.2	28	0.05	0.1	0.42	1.35	–

differences: the *C. jejuni* QFR exhibits a $V_{\max}$ of 9.3 units/mg, the *H. pylori* QFR of 12.3 units/mg and the *W. succinogenes* QFR of 14.7 units/mg. As pointed out earlier, the 'DT-DMNH₂ assay' method enhances the $V_{\max}$ values by a factor of 2 if compared with the 'BH₄-DMNH₂ assay'. Previous determinations of specific activities of *W. succinogenes* QFR ('MB assay': 28.8 units/mg; 'BH₄-DMNH₂ assay': 7.4 units/mg [39]; 'BV assay': 180 units/mg [35]) perfectly match the results presented here. For all three QFR enzymes, both 'DMNH₂ assays' performed on proteoliposomes containing QFR (M. Mileni and C.R.D. Lancaster, unpublished work) yielded results identical with those obtained for the respective detergent-solubilized enzymes, thus indicating that the results reported here are also relevant for the membrane-bound forms of the enzymes.

The Michaelis constants (K_m) for the quinol substrate (DMNH₂) were found to be in the order of 0.05–0.10 mM, thus similar to the value (0.1 mM) previously determined for *W. succinogenes* QFR [56]. However, the K_m for fumarate was rather lower than the one found in the literature [21]. To assess the enzymatic stability, activity assays were performed after 10 days of incubation of the detergent-solubilized QFRs at 4 °C, and showed a decrease of only approx. 20 %.

Characterization of inhibitors

In the past years, a number of compounds have been already suggested as inhibitors of QFR from *C. jejuni* and *H. pylori* species [14,19,57]. Unfortunately, some of them, such as metronidazole, nizatidine, morantel and TTFA (thenoyltrifluoroacetone), could not be tested with the available assay methods due to the (prohibitive) high absorbance at the wavelengths monitored (270 or 340 nm). In contrast with the previous measurements, where K_i (inhibition constant) values were estimated in non-purified samples and/or by NMR spectroscopy [14,19,57], in the present study we assigned precise K_i values based on activity measurements of the pure enzymes as monitored by UV spectroscopy. The K_i values (listed in Table 5) reveal that the order of inhibitory effect is oxantel > thiabendazole > omeprazole. However, they are rather weak inhibitors, and are probably not useful as potential drugs. The effects of these three inhibitors were also tested on partial activities ('BV assay' and 'MB assay') of *C. jejuni* and *H. pylori* QFRs. In the former enzymatic assay, oxantel was the only inhibitor that clearly affected the function of the hydrophilic subunits of the two enzymes ($IC_{50} \sim 0.2$ – 0.3 mM). The 'MB assay' was rather sensitive to the addition of DMSO (the inhibitor solvent) and, except for oxantel, where inhibition was unequivocal ($IC_{50} \sim 0.1$ mM), thiabendazole and omeprazole showed questionable effects.

With the use of Lineweaver–Burk plots (Figure 5), we could also assign the type of inhibition exerted by these three compounds. Omeprazole, which has no effect on the *H. pylori* QFR, has a competitive inhibitory effect on the *C. jejuni* QFR, thus it binds to the quinol-binding site. Curiously, the thiabendazole exerts a non-competitive (or allosteric) inhibition effect in the *C. jejuni* QFR, while in the *H. pylori* QFR the inhibition is competitive. In the 'BV assay', this compound does not affect either of the two enzymes, thus this inhibitor is likely to bind to the hydrophobic transmembrane subunit C of the QFR, seemingly close to the quinone-binding site. The *H. pylori* QFR inhibition by oxantel was formerly analysed on broken cells by monitoring fumarate concentration with NMR spectroscopy [14] and it was predicted to bind competitively at the fumarate-binding site with an inhibition constant equal to approx. 60 μ M. Whilst the binding location found in our results does not contradict this previous characterization, our experiment on the isolated enzyme shows a

Figure 5 Lineweaver–Burk plot of *C. jejuni* (A) and *H. pylori* (B) QFR activity

The circles and the solid line (linear regression) represent QFR activity without inhibitors. The squares and the broken line represent QFR activity in presence of 300 μ M oxantel. The 'diamond' symbols and the broken line represent QFR activity in the presence of 2 mM thiabendazole. The '+' symbols and the broken line represent QFR activity in presence of 2 mM omeprazole.

markedly lower inhibitory effect. The results obtained here on the inhibition of QFR by oxantel show that it exerts an uncompetitive inhibition at the quinol-binding site; thus this inhibitor binds to a site other than the active site, but only when the substrate is bound. Inhibition by oxantel is also observed in the partial activity assays, proving that it is binding or affecting the hydrophilic subunits of the enzyme. One possible explanation could be that oxantel binding imposes a structural change that is perceived by the quinol oxidation site. In other words, although the oxantel is presumably binding close to the fumarate-binding site [14], i.e. rather far from the quinone site, it is able to decrease the affinity for the quinone. However, we can also at present not exclude that oxantel binds to multiple sites in the protein (e.g. one close to the fumarate-binding site and one close to the quinone site). Earlier studies of *C. jejuni* QFR activity in the presence of oxantel and thiabendazole have shown a much lower inhibition (IC_{50} of 6 and 70 mM respectively) [19].

Conclusions

We demonstrate here for the first time that the non-pathogenic bacterium *W. succinogenes* is a well-suited host for heterologous membrane protein production. Whereas previous enzymatic isolation from these two species was characterized by low yields of QFR of comparatively low purity [21], this novel expression

system allowed us to establish a large-scale preparation set-up for the production of active QFR from the pathogenic species *C. jejuni* and *H. pylori*. Furthermore, previous attempts at heterologous expression of the *W. succinogenes* *frdA* and *frdB* genes in *E. coli* did not result in the synthesis of functional proteins [25]. The established genetic system for *W. succinogenes* allowed efficient production of functional fumarate reductase complexes from related pathogenic ϵ -proteobacteria. *W. succinogenes* appears to be an ideal host for production of proteins that are subject to post-translational enzyme maturation processes as the biogenesis enzymes involved might be interchangeable between the closely related ϵ -proteobacteria of the genera *Wolinella*, *Helicobacter* and *Campylobacter*. Further advantages of using *W. succinogenes* are its known genome sequence [58] and its rapid growth to high cell densities in minimal media. This enables fast and cost-effective production of cell quantities sufficient for large-scale protein purification. Future genetic work will aim at the optimization of vector-based expression of genes encoding other metalloproteins from pathogenic ϵ -proteobacteria that may become realistic antimicrobial targets.

The mutation in the Δ *frdCAB* strain could be complemented by the expression of the heterologous *H. pylori* or *C. jejuni* *frdCAB* operons, and fumarate respiration was efficiently restored by using a fully functional QFR membrane-protein complex. The relative simplicity of working with *W. succinogenes* and its high yield of expression enabled us to obtain large amounts of pure and stable QFR from *C. jejuni* and *H. pylori*. The SDS/PAGE and the gel filtration chromatogram (not shown) prove that these enzyme preparations are of high purity and homogeneity, and allowed the determination of the oligomeric state of the complexes and the different redox midpoint potentials of all prosthetic groups, as well as the characterization of three known specific inhibitors.

The work described in the present paper provides the basis for further functional and structural studies of these membrane-protein complexes and for the screening of new and highly effective inhibitors. Current eradication therapies for *H. pylori* are non-specific, expensive, and occasionally ineffective due to the antibiotic resistances and often produce severe side effects [13]. As pointed out above, the QFR enzymes from *H. pylori* and presumably also from *C. jejuni* are potential drug targets. These enzyme preparations have already proven to be suitable for protein crystallization (M. Mileni and C.R.D. Lancaster, unpublished work), further evidence of high enzyme stability. Well-diffracting crystals, leading to a high-resolution crystal structure, would be helpful for a better understanding of the structure-function relationships of this protein superfamily, and for obtaining highly efficient inhibitors, for example by structure-based drug design.

This work was supported by the DFG (Deutsche Forschungsgemeinschaft) Sonderforschungsbereich 472 'Molecular Bioenergetics', a grant from the German Federal Ministry of Education, Science, Research and Technology framework programme ('BMBF-Verbundprojekt') on the 'Proteome-wide Analysis of Membrane Proteins' (ProAMP), and the Max-Planck-Gesellschaft. M.M. is supported by the International Max Planck Research School on the Structure and Function of Biological Membranes. We thank PD Dr Stefan Bereswill, then of Institut für Medizinische Mikrobiologie und Hygiene, Abteilung Mikrobiologie und Hygiene, Universitätsklinikum Freiburg, Freiburg, Germany, for providing *C. jejuni* cells and *H. pylori* genomic DNA. C.T. is grateful to Professor Dieter Schubert (Institute of Biophysics, J.W. Goethe University) for helpful discussions. We thank Professor U. Brandt and Professor T. Prisner (J.W. Goethe University) for use of their EPR facilities and Professor H. Michel (Max Planck Institute of Biophysics) for the use of departmental facilities.

REFERENCES

- Marshall, B. J. and Warren, J. R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1**, 1311–1315.
- Enserink, M. (2005) Physiology or medicine: triumph of the ulcer-bug theory. *Science* **310**, 34–35.
- Covacci, A., Telford, J. L., Del Giudice, G., Parsonnet, J. and Rappuoli, R. (1999) *Helicobacter pylori* virulence and genetic geography. *Science* **284**, 1328–1333.
- Cover, T. L. and Blaser, M. J. (1999) *Helicobacter pylori* factors associated with disease. *Gastroenterology* **117**, 257–261.
- Peek, Jr, R. M. and Blaser, M. J. (2002) *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer* **2**, 28–37.
- Butzler, J. P. (2004) *Campylobacter*, from obscurity to celebrity. *Clin. Microbiol. Infect.* **10**, 868–876.
- Chowdhury, D. and Arora, A. (2001) Axonal Guillain-Barre syndrome: a critical review. *Acta Neurol. Scand.* **103**, 267–277.
- Lecuit, M., Abachin, E., Martin, A., Poyart, C., Pochart, P., Suarez, F., Bengoufa, D., Feuillard, J., Lavergne, A., Gordon, J. I. et al. (2004) Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. *N. Engl. J. Med.* **350**, 239–248.
- Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S. et al. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature (London)* **403**, 665–668.
- Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A. et al. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature (London)* **388**, 539–547.
- Solnick, J. V., O'Rourke, J. L., Vandamme, P. and Lee, A. (2003) The genus *Helicobacter*. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community* (Dworkin, M., ed.), 3rd edn, Springer-Verlag, New York.
- Wassenaar, T. M. and Newell, D. G. (2001) The genus *Campylobacter*. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community* (Dworkin, M., ed.), 3rd edn, Springer-Verlag, New York.
- Ge, Z. (2002) Potential of fumarate reductase as a novel therapeutic target in *Helicobacter pylori* infection. *Expert Opin. Ther. Targets* **6**, 135–146.
- Mendz, G. L., Hazell, S. L. and Srinivasan, S. (1995) Fumarate reductase: a target for therapeutic intervention against *Helicobacter pylori*. *Arch. Biochem. Biophys.* **321**, 153–159.
- Ge, Z., Feng, Y., Dangler, C. A., Xu, S., Taylor, N. S. and Fox, J. G. (2000) Fumarate reductase is essential for *Helicobacter pylori* colonization of the mouse stomach. *Microb. Pathog.* **29**, 279–287.
- Kröger, A. (1978) Fumarate as terminal acceptor of phosphorylative electron transport. *Biochim. Biophys. Acta* **505**, 129–145.
- Lancaster, C. R. D. (2002) Succinate:quinone oxidoreductases: an overview. *Biochim. Biophys. Acta* **1553**, 1–6.
- Lancaster, C. R. D. (2004) Structure and function of succinate:quinone oxidoreductases and the role of quinol:fumarate reductases in fumarate respiration. In *Respiration in Archaea and Bacteria* (Zannoni, D., ed.), pp. 57–85, Kluwer Scientific, Dordrecht, The Netherlands.
- Smith, M. A., Mendz, G. L., Jorgensen, M. A. and Hazell, S. L. (1999) Fumarate metabolism and the microaerophily of *Campylobacter* species. *Int. J. Biochem. Cell Biol.* **31**, 961–975.
- Birkholz, S., Knipp, U., Lemma, E., Kroger, A. and Opferkuch, W. (1994) Fumarate reductase of *Helicobacter pylori* – an immunogenic protein. *J. Med. Microbiol.* **41**, 56–62.
- Lancaster, C. R. D. and Simon, J. (2002) Succinate:quinone oxidoreductases from ϵ -proteobacteria. *Biochim. Biophys. Acta* **1553**, 84–101.
- Lancaster, C. R. D., Kröger, A., Auer, M. and Michel, H. (1999) Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 Å resolution. *Nature (London)* **402**, 377–385.
- Ge, Z., Jiang, Q., Kalisiak, M. S. and Taylor, D. E. (1997) Cloning and functional characterization of *Helicobacter pylori* fumarate reductase operon comprising three structural genes coding for subunits C, A and B. *Gene* **204**, 227–234.
- Körtner, C., Lauterbach, F., Trippier, D., Unden, G. and Kröger, A. (1990) *Wolinella succinogenes* fumarate reductase contains a dihaem cytochrome *b*. *Mol. Microbiol.* **4**, 855–860.
- Lauterbach, F., Körtner, C., Albracht, S. P., Unden, G. and Kröger, A. (1990) The fumarate reductase operon of *Wolinella succinogenes*: sequence and expression of the *frdA* and *frdB* genes. *Arch. Microbiol.* **154**, 386–393.
- Simon, J., Gross, R., Ringel, M., Schmidt, E. and Kröger, A. (1998) Deletion and site-directed mutagenesis of the *Wolinella succinogenes* fumarate reductase operon. *Eur. J. Biochem.* **251**, 418–426.
- Bronder, M., Mell, H., Stupperich, E. and Kröger, A. (1982) Biosynthetic pathways of *Vibrio succinogenes* growing with fumarate as terminal electron acceptor and sole carbon source. *Arch. Microbiol.* **131**, 216–223.

- 28 Lorenzen, J. P., Kröger, A. and Unden, G. (1993) Regulation of anaerobic respiratory pathways in *Wolinella succinogenes* by the presence of electron acceptors. *Arch. Microbiol.* **159**, 477–483
- 29 Lancaster, C. R. D. (2003) Crystallization of *Wolinella succinogenes* quinol:fumarate reductase. In *Membrane Protein Purification and Crystallization: A Practical Guide* (Hunte, C., Schägger, H. and von Jagow, G., eds.), 2nd edn, pp. 219–228, Academic Press, San Diego
- 30 Schuck, P. (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys. J.* **78**, 1606–1619
- 31 Durchschlag, H. (1986) Specific volumes of biological macromolecules and some other molecules of biological interest. In *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., ed.), pp. 45–128, Springer-Verlag, Berlin
- 32 Laue, T. M., Shah, B. D., Ridgeway, T. M. and Pelletier, S. L. (1992) Computer-aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J. and Horton, J. C., eds.), pp. 90–125, The Royal Society of Chemistry, Cambridge
- 33 Unden, G., Hackenberg, H. and Kröger, A. (1980) Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of *Vibrio succinogenes*. *Biochim. Biophys. Acta* **591**, 275–288
- 34 Kröger, A. and Innerhofer, A. (1976) The function of the *b* cytochromes in the electron transport from formate to fumarate of *Vibrio succinogenes*. *Eur. J. Biochem.* **69**, 497–506
- 35 Unden, G. and Kröger, A. (1986) Reconstitution of a functional electron-transfer chain from purified formate dehydrogenase and fumarate reductase complexes. *Methods Enzymol.* **126**, 387–399
- 36 Kröger, A., Dorner, E. and Winkler, E. (1980) The orientation of the substrate sites of formate dehydrogenase and fumarate reductase in the membrane of *Vibrio succinogenes*. *Biochim. Biophys. Acta* **589**, 118–136
- 37 Grivnikova, V. G., Gavrikova, E. V., Timoshin, A. A. and Vinogradov, A. D. (1993) Fumarate reductase activity of bovine heart succinate-ubiquinone reductase. New assay system and overall properties of the reaction. *Biochim. Biophys. Acta* **1140**, 282–292
- 38 Dutton, P. L. (1978) Redox potentiometry: determination of midpoint potentials of oxidation–reduction components of biological electron-transfer systems. *Methods Enzymol.* **54**, 411–435
- 39 Lancaster, C. R. D., Gross, R., Haas, A. H., Ritter, M., Mantele, W., Simon, J. and Kröger, A. (2000) Essential role of Glu-C66 for menaquinol oxidation indicates transmembrane electrochemical potential generation by *Wolinella succinogenes* fumarate reductase. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13051–13056
- 40 Moss, D., Nabadryk, E., Breton, J. and Mantele, W. (1990) Redox-linked conformational changes in proteins detected by a combination of infrared spectroscopy and protein electrochemistry: evaluation of the technique with cytochrome *c*. *Eur. J. Biochem.* **187**, 565–572
- 41 Tziatzios, C., Schubert, D., Schuck, P., Lancaster, C. R. D., Gennis, R. B. and Barquera, B. (2004) The state of association of the Na⁺-translocating NADH: quinone oxidoreductase in detergent solution: an ultracentrifugation study. *Prog. Colloid Polym. Sci.* **127**, 48–53
- 42 Musatov, A., Ortega-Lopez, J., Demeler, B., Osborne, J. P., Gennis, R. B. and Robinson, N. C. (1999) Detergent-solubilized *Escherichia coli* cytochrome *bo*₃ ubiquinol oxidase: a monomeric, not a dimeric complex. *FEBS Lett.* **457**, 153–156
- 43 Schuck, P. (1998) Sedimentation analysis of noninteracting and self-associating solutes using numerical solutions to the Lamm equation. *Biophys. J.* **75**, 1–10
- 44 Moeller, J. V. and le Maire, M. (1993) Detergent binding as a measure of hydrophobic surface area of integral membrane proteins. *J. Biol. Chem.* **268**, 18659–18672
- 45 Iverson, T. M., Luna-Chavez, C., Cecchini, G. and Rees, D. C. (1999) Structure of the *Escherichia coli* fumarate reductase respiratory complex. *Science* **284**, 1961–1966
- 46 Huang, L. S., Borders, T. M., Shen, J. T., Wang, C. J. and Berry, E. A. (2005) Crystallization of mitochondrial respiratory complex II from chicken heart: a membrane–protein complex diffracting to 2.0 Å. *Acta Crystallogr. D Biol. Crystallogr.* **61**, 380–387
- 47 Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M. and Rao, Z. (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* **121**, 1043–1057
- 48 Yankovskaya, V., Horsefield, R., Tornroth, S., Luna-Chavez, C., Miyoshi, H., Leger, C., Byrne, B., Cecchini, G. and Iwata, S. (2003) Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* **299**, 700–704
- 49 Kenney, W. C. and Kröger, A. (1977) The covalently bound flavin of *Vibrio succinogenes* succinate dehydrogenase. *FEBS Lett.* **73**, 239–243
- 50 Gast, P., Michalski, T. J., Hunt, J. E. and Norris, J. R. (1985) Determination of the amount and the type of quinones present in single crystals from reaction center protein from the photosynthetic bacterium *Rhodospseudomonas viridis*. *FEBS Lett.* **179**, 325–328
- 51 Mileni, M., Haas, A. H., Mantele, W., Simon, J. and Lancaster, C. R. D. (2005) Probing heme propionate involvement in transmembrane proton transfer coupled to electron transfer in dihemeric quinol:fumarate reductase by ¹³C-labeling and FTIR difference spectroscopy. *Biochemistry* **44**, 16718–16728
- 52 Martinez, J. I., Alonso, P. J., Gomez-Moreno, C. and Medina, M. (1997) One- and two-dimensional ESEEM spectroscopy of flavoproteins. *Biochemistry* **36**, 15526–15537
- 53 Hung, S. C., Grant, C. V., Peloquin, J. M., Waldeck, A. R., Brit, R. D. and Chan, S. I. (2000) ESEEM studies of succinate:ubiquinone reductase from *Paracoccus denitrificans*. *J. Biol. Inorg. Chem.* **5**, 593–602
- 54 Unden, G., Albracht, S. P. J. and Kröger, A. (1984) Redox potentials and kinetic properties of fumarate reductase complex from *Vibrio succinogenes*. *Biochim. Biophys. Acta* **767**, 460–469
- 55 Waghorn, G. C. (1991) Electronegativity and redox potential of rumen digesta *in-situ* in cows eating fresh lucerne. *N.Z. J. Agric. Res.* **34**, 359–362
- 56 Lancaster, C. R. D., Sauer, U. S., Gross, R., Haas, A. H., Graf, J., Schwalbe, H., Mantele, W., Simon, J. and Madej, G. (2005) Experimental support for the 'E-pathway hypothesis' of coupled transmembrane e⁻ and H⁺ transfer in dihemeric quinol:fumarate reductase. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 18860–18865
- 57 Chen, M., Jensen, B., Zhai, L., Colding, H., Kharazmi, A., Kristiansen, J. E. and Andersen, L. P. (2002) Nizatidine and omeprazole enhance the effect of metronidazole on *Helicobacter pylori* *in vitro*. *Int. J. Antimicrob. Agents* **19**, 195–200
- 58 Baar, C., Eppinger, M., Raddatz, G., Simon, J., Lanz, C., Klimmek, O., Nandakumar, R., Gross, R., Rosinus, A., Keller, H. et al. (2003) Complete genome sequence and analysis of *Wolinella succinogenes*. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11690–11695
- 59 Simon, J., Gross, R., Klimmek, O. and Kröger, A. (2000) In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community* (Dworkin, M., ed.), 3rd edn, Springer-Verlag, New York
- 60 Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119
- 61 Haas, A. H. and Lancaster, C. R. D. (2004) Calculated coupling of transmembrane electron and proton transfer in dihemeric quinol:fumarate reductase. *Biophys. J.* **87**, 4298–4315

Received 18 October 2005/15 December 2005; accepted 21 December 2005

Published as BJ Immediate Publication 21 December 2005, doi:10.1042/BJ20051675