Three multihaem cytochromes c from the hyperthermophilic archaeon *Ignicoccus hospitalis*: purification, properties and localization

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Three different multihaem cytochromes c were purified from cell extracts of the hyperthermophilic archaeon *Ignicoccus hospitalis*. One tetrahaem cytochrome, locus tag designation Igni_0530, was purified from membrane fractions together with the iron–sulfur protein Igni_0529. Two octahaem cytochromes, Igni_0955 and Igni_1359, were purified from soluble fractions but were also present in the membrane fraction. N-terminal sequencing showed that three of the four proteins had their signal peptides cleaved off, while results were ambiguous for Igni_0955. In contrast, mass spectrometry of Igni_0955 and Igni_1359 resulted in single mass peaks including the signal sequences and eight haems per subunit and so both forms might be present in the cell. Igni_0955 and Igni_1359 belong to the hydroxylamine dehydrogenase (HAO) family (29 % mutual identity). HAO or reductase activities with inorganic sulfur compounds were not detected. Igni_0955 was reduced by enriched *I. hospitalis* hydrogenase at a specific activity of 243 nmol min⁻¹ (mg hydrogenase)⁻¹ while activity was non-existent for Igni_0530 and low for Igni_1359. Immuno-electron microscopy of ultra-thin sections showed that Igni_0955 and Igni_1359 are located in both *I. hospitalis* membranes and also in the intermembrane compartment. We concluded that these cytochromes might function as electron shuttles between the hydrogenase in the outer cellular membrane and cellular reductases, whereas Igni_0530 might be part of the sulfur-reducing mechanism.

INTRODUCTION

*Ignicoccus hospitalis* is an autotrophic hyperthermophile growing optimally at 90 °C (Paper et al., 2007), which uses H₂ oxidation with elemental sulfur as electron acceptor as the only pathway of cellular energy conversion (sulfur/hydrogen autotrophy; Stetter et al., 1986). It is also the host of the small symbiotic archaeon *Nanoarchaeum equitans* (Paper et al., 2007). *I. hospitalis* has an unusual ultrastructure: the coccoid cells have an outer cellular membrane (OCM) and an inner membrane enclosing a space densely contrasted in transmission electron micrographs (cytoplasm), where the DNA and ribosomes are located. The two membranes enclose a wide and weakly stained intermembrane compartment (IMC) (Huber et al., 2012) containing well-contrasted vesicles (Junglas et al., 2008). Protein composition and function of the IMC are unknown. The OCM is energized and contains the archaeal ATP synthase and also hydrogenase and sulfur reductase (SR) required for chemiosmotic energy conversion (Huber et al., 2012; Küper et al., 2010).

*I. hospitalis* belongs to the Desulfurococcales order of the Crenarchaeota, which comprises some of the most thermophilic micro-organisms known, including *Pyrodictium* spp.,

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**Abbreviations:** CV, column volumes; DDM, dodecyl maltoside; GPC, gel permeation chromatography; HAO, hydroxylamine dehydrogenase (oxidoreductase); IMC, intermembrane compartment; (M)CC (multihaem) cytochrome c; OCM, outer cellular membrane; PSR, polysulfide reductase; SR, sulfur reductase; TMH, transmembrane helix.

One supplementary table and seven supplementary figures are available with the online version of this paper.

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which also thrive by sulfur/hydrogen autotrophy, and *Pyrolobus fumarii*, which grows by hydrogen oxidation with other inorganic electron acceptors (Blöchl et al., 1997; Stetter, 1982, 2006). A hydrogenase/SR complex purified from *Pyrodictium abyssi* (Dirmeier et al., 1998) gave rise to the heterologous antiserum used for the detection of the enzymes in the OCM of *I. hospitalis* (Küper et al., 2010). The SR complex consisted of nine subunits including cytochromes *b* and *c* and a nickel–iron hydrogenase. The 30 kDa cytochrome *c* (CC) was reduced after incubation with H₂ (Dirmeier et al., 1998). In an earlier study, a different and smaller CC had been identified coupling hydrogenase and SR in the related species *Pyrodictium brockii*; however, sulfur reduction also depends on the presence of quinones (Pihl et al., 1992). The genes encoding hydrogenase, SR and cytochromes are not known in either of the *Pyrodictium* species; however, these reports showed that CCs play an important role in the electron transport chains of these sulfur-reducing archaea.

Archaeal CCs have not attracted much attention to date, because many of the well-studied lineages, including the *Sulfolobales*, seem to be devoid of these proteins and use abundantly available quinones and ferredoxins as electron transfer agents (Laska et al., 2003; Schäfer, 2004; Teixeira et al., 1995; Thauer et al., 2008; Thurl et al., 1986). Still, there are several reports in the literature, including the bioinformatic identification of CCs and their biosynthesis proteins (Allen et al., 2006; Bertini et al., 2006; Meyerdierks et al., 2010; Sharma et al., 2010). Most of the biochemical evidence has come from haloarchaea. Cytochromes *c* and/or *bc₁*-like complexes were found in cell extracts of *Halofexs volcanii* and *Halobacterium salinarum* identified by the characteristic absorption around 552 nm in the reduced state (Cheah, 1970a, b; Lanyi, 1968; Sreeramulu, 2003; Ku¨hn & Gottschalk, 1983; Wang et al., 2011). Two CCs were enriched from *Natronomas pharaonis*. One soluble cytochrome *c₅₅₀* had an apparent molecular mass of 75 kDa while another isolated from the membrane was part of a heterodimeric cytochrome *bc* complex (Scharf et al., 1997). Other CCs were identified in *Methanospirillum* spp. (Kamlage & Blaut, 1992; Kühn & Gottschalk, 1983; Wang et al., 2011). N-terminal sequences and mass spectrometry data are not available, and so identification of the genes was not possible. Recently, a cytochrome *bc* complex was purified from the hyperthermophilic crenarchaeon *Aeropyrum pernix*, also belonging to the *Desulfurococcales*. The monohaem cytochrome *c₅₃₃* is a protein of 190 amino acids encoded in the gene locus APE_1719.1 (Kabashima & Sakamoto, 2011).

In this work, we aimed to identify electron transfer components that might link hydrogenase and SR in *I. hospitalis*. We had previously tried to isolate both enzymes; however, we have so far been unsuccessful concerning SR (M. Forth, C. M. Eckert, U. M. Küper, H. Huber and A. Kletzin, unpublished). *I. hospitalis* extracts and even cell pellets are red, indicating high concentrations of CCs. We show here that the organism contains at least three different CCs. The proteins were purified and Igni_0955 seems to act as an electron acceptor for the hydrogenase of the organism. We also show that the two presumably soluble octaahem CCs not only are attached to both membranes but also are found in the IMC.

**METHODS**

**Strain and culture conditions.** *I. hospitalis* Kin4/I DSM 18386 (Deutsches Sammlung für Mikroorganismen und Zellkulturen) was grown anaerobically at 90 °C in 0.5 × SME (synthetic seawater) medium at 90 °C and pH 5.5 with elemental sulfur as electron acceptor and a gas phase consisting of H₂/CO₂ (250 kPa; 80:20 v/v; Paper et al., 2007). Large-scale fermentations were carried out in an enamel protected fermenter (300 l, HTE Bioengineering) at the fermentation plant at the University of Regensburg. After growth, cells were harvested by centrifugation at 17000 g (continuous flow centrifuge Z61; Padberg).

**Preparation of cell extracts.** Frozen *I. hospitalis* cells were thawed at room temperature and resuspended in 5 ml lysis buffer (g cells)⁻¹ [wet mass; buffer composition 25 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF]. Cells were disrupted by sonication (Branson Sonifier 250, level 3, microtip 3 mm, 40% duty cycle). Cell debris was removed by centrifugation at 2400 g for 10 min in conical centrifuge tubes (Universal 320 R; Hettich). The supernatant was centrifuged at 100 000 g (Beckman 45Ti rotor) for 30–45 min. This supernatant (soluble extract) was dialysed three times against the same buffer and stored frozen until use. The pellet of the ultra-centrifugation (membrane fraction) was resuspended in 2.5 ml (g cells)⁻¹ in washing buffer containing 50 mM Tris/HCl pH 7.5, 10% (w/v) glycerol and 1 mM PMSF. After an additional ultracentrifugation step using the same conditions as described above, the pellet was resuspended in solubilization buffer containing 50 mM Tris/HCl pH 7.5, 10% (w/v) glycerol and 1 mM PMSF. After an additional ultracentrifugation step using the same conditions as described above, the pellet was resuspended in solubilization buffer containing 50 mM Tris/HCl pH 7.5, 10% (w/v) glycerol and 1 mM PMSF. The membrane fraction was solubilized in an ultrasonic bath at 0 °C for 45 min, followed by another ultra-centrifugation step as above. This supernatant was used for purification of membrane-bound proteins.

**Protein purification.** Protein purification was performed with an Akta Purifier 10 GE (GE Life Sciences). The cytochromes were detected by following the absorbance at 280 and 410 nm; the purity of cytochrome fractions was assessed by the OD₄₁₀/OD₂₈₀ ratio.

**Protein purification from solubilized membrane extracts.** The solubilized membrane fraction from 10.7 g *I. hospitalis* cells (87 mg protein) was applied to a 1 ml Q Sepharose column equilibrated with a buffer composed of 25 mM AMPSO buffer pH 9 (3-(1,1-Dimethyl-2-hydroxyethyl)amino)-2-hydroxypropanesulfonic acid; Roth, Karlsruhe; pH adjusted with NaOH), 10 mM MgCl₂, and 0.05% DDM. The column was equilibrated with the same buffer, with NaCl added to give specific conductivities of 5.5, 9.0 and 16.5 mS cm⁻¹. This was followed by a linear gradient to 1 M NaCl over five column volumes (CV) in the same buffer. The flow-through was reapplied to the same column and step-wise eluted with a 12 mS cm⁻¹ buffer following a similar elution gradient. The cytochrome fraction (6.3 mg) was dialysed against lecithin equilibration buffer (20 mM Tris/HCl pH 7.4, 1 mM MnCl₂, 1 mM CaCl₂, 500 mM NaCl, 0.05% DDM) and applied to a 5 ml HiTrap Con A 4B lectin column (GE Life Sciences). The proteins were eluted with a linear gradient over 10 CV (elution buffer 20 mM Tris/HCl pH 7.4, 300 mM methyl-α-D-glucoside, 500 mM NaCl, 0.05% DDM). The cytochrome-containing fractions (1.5 mg protein) were pooled, dialysed against the lecithin equilibration buffer and reapplied to the Con A column in order to improve the purification.
results. The eluted fractions were pooled, followed by dialysis against gel permeation chromatography buffer (GPC; 20 mM Tris/HCl pH 8.2, 150 mM NaCl, 0.05 % DDM), concentrated to 1.1 ml in volume and applied to a Superdex 200 26/60 GPC (GE Life Sciences) column run with the same buffer. The GPC column was calibrated with lysozyme, ovalbumin, BSA, katalase, aldolase and Blue Dextran 2000 run separately in the same buffer. The dimer and trimer peaks of ovalbumin and BSA were included in the calibration.

For hydrogenase enrichment, 14.7 mg solubilized membrane protein was loaded on a 1 ml concanavalin A column (GE Life Sciences) equilibrated with a buffer consisting of 20 mM Tris/HCl pH 7, 0.5 M NaCl 0.05 % DDM, 1 mM CaCl₂ and 1 mM MnCl₂. The column was eluted with an increasing gradient of 0.5 M methyl-α-D-glucoside (Acros Organics) in the same buffer. The hydrogenase-active fraction had a specific activity of 7 U (mg protein)⁻¹ with benzyl viologen as electron acceptor.

**Cytochrome purification from soluble extracts.** Two slightly different methods were applied to purify Igni_0955 and Igni_1359 from the soluble *I. hospitalis* extracts. A 145 ml volume of the solution, containing 443 mg total protein, was used for each of the purification procedures.

For Igni_1359 purification, the soluble fraction was applied to a 330 ml DEAE Sepharose fast-flow column (GE Life Sciences). After washing with 1.5 CV equilibration buffer, a linear salt gradient of 3 CV and 0–100 % DEAE elution buffer (20 mM Tris/HCl, pH 7.4, 1 M NaCl) was applied. All red fractions were pooled (pool α; 176 ml, 153 mg protein), again dialysed against the same equilibration buffer, and reapplied to the column. After washing with equilibration buffer, a salt gradient of 5 CV and 0–50 % elution buffer was applied, followed by step-wise elution of the remaining protein. The main 410 nm absorbing protein peak eluted around 130 mM NaCl. The red fractions were pooled and dialysed overnight against QS equilibration buffer (20 mM Tris/HCl, pH 7.4). The solution (pool β; 462 ml, 103 mg) was applied to a 30 ml Q Sepharose Fast Flow column (GE Healthcare) and initially eluted with a steep pH gradient (1 CV) of QS elution buffer I (QSI; 10 mM citric acid adjusted to pH 5.5 with 1 M Tris base solution). This was subsequently followed by 3 CV 50 % QSI and a linear gradient of 50–76 % QSI (3.5 CV). The remaining proteins were eluted with 3 CV DEAE elution buffer. Cytochrome Igni_0955 (calculated isoelectric point 8.2) eluted during the plateau phase (pool δ; 9 ml, 1.9 mg protein), while ~90 % of Igni_1359 (IEP=7.1) eluted during the second gradient (pool γ; 18 ml, 11.7 mg protein). About 10 % of Igni_1359 eluted during the final 1 M NaCl step. Pool γ, with the highest apparent purity judged by SDS gel electrophoresis, was concentrated to approximately 1.5 ml and applied to a Superdex 200 26/60 GPC column. The column was developed using QSI buffer with 150 mM NaCl. The cytochrome eluted in two 9 ml fractions, α and δ, with 2.3 and 1.5 mg of protein, respectively.

The Igni_0955 fraction δ was separated by GPC using the same conditions, but the yields were low (~150 μg). Therefore, a similar purification procedure was utilized with slightly changed conditions. The same volume of soluble extract was separated on the DEAE Sepharose column with the same buffers but with a flatter initial gradient (0–30 % DEAE elution buffer in 5 CV). Most of the red protein fractions eluted around 141 mM NaCl. The pooled cytochrome-containing fractions (pool ρ; 396 ml, 122 mg) were dialysed against QS equilibration buffer and applied to the same column. The proteins were eluted with a gradient of 1 CV 0–50 % QSI, followed by a plateau of 3 CV 50 % QSI. The second gradient, from 50–100 % QSI, was 3.5 CV, followed by 2 CV DEAE elution buffer. Five adjacent Q Sepharose fractions with the highest apparent purity were pooled (pool ν; 45 ml, 3.2 mg), concentrated and separated by GPC using the conditions described above. The corresponding Igni_1359-containing fractions τ (18 ml, 2.3 mg protein) and ν (18 ml, 7.1 mg protein) were treated accordingly. The pools were concentrated as described above and applied to the GPC column under the same conditions. Igni_0955 eluted in two fractions (χ and χ'; 9 ml each; 1.7 and 1.3 mg of protein, respectively).

**Ferredoxin purification and quinone extraction.** In order to detect putative ferredoxins, brown protein fractions from the soluble extract were collected and analysed by SDS-PAGE for the presence of significant amounts of small proteins (≤10 kDa). Alternatively, chromatography fractions not used for further cytochrome purification were precipitated with five volumes of ice-cold acetone (Mortenson, 1964), incubated on ice for 2 h and subsequently centrifuged for 30 min at 16 000 g. The pellets were dissolved in SDS sample buffer. The supernatants were evaporated to dryness in a SpeedVac; the resulting pellets were resuspended in SDS sample buffer.

Both types of samples were boiled prior to electrophoretic analysis.

Quinones were extracted from solubilized membrane fraction for 15 min at 37 °C with vigorous shaking with an equal volume of a 1:1 mixture (v/v) of petroleum ether (40–60 °C fraction) and methanol. Subsequently, 0.5 volume of acetone was added to the mixture and shaken again for 15 min at 37 °C. The mixture was incubated on ice for 10 min and centrifuged for 3 min at 2000 g and 4 °C. The organic supernatant was transferred to a new tube, and the water phase was re-extracted with the same volume of solvents and centrifuged as above. The combined organic fractions were evaporated to dryness under a nitrogen stream. The dry residues were redissolved in ethanol and subjected to UV/vis spectroscopy. Thin-layer chromatography was performed with HPTLC silica gel plates 60 F254 (Merck) and 10 % diethyl ether in hexane as the mobile phase.

**Polyacrylamide gel electrophoresis.** Denaturing SDS-PAGE (SDS-PAGE) of the soluble cytochromes was performed with Tris-glycine buffers (10 % acrylamide) (Laemmli, 1970). The membrane-bound cytochrome was analysed in 10 % Tris-Tricine gels (Schagger & von Jagow, 1987). Gels were stained with either colloidal Coomassie blue (Rotilblue; Roth) or silver (Blum et al., 1987). In-gel haem staining was performed according to Francis & Becker (1984). Non-denaturing gradient gels (3–12 %) were purchased from Serva and run with SDS-free Tris-glycine buffer (Laemmli, 1970). Pre-stained molecular mass markers were from Thermo Scientific (cat. no. SM0671).

**Antisera.** Polyclonal antisera directed against Igni_0955 and Igni_1359 were raised in rabbits by Seqlab. Rabbits were immunized three times each with approximately 250 μg protein in polyacrylamide gel slices. The protein bands were excised from the gels and disinfected with 70 % ethanol prior to delivery.

**Electron microscopy.** For electron microscopy analysis, fresh cells were cultivated, high-pressure frozen and freeze-substituted in 95 % acetone, 0.5 % glutaraldehyde, 0.5 % uranyl acetate and 5 % water as described (Rachel et al., 2010). After freeze substitution fixation, samples were embedded in Epon (Fluka). For localization of proteins on ultra-thin sections, the primary antisera directed against Igni_0955 and Igni_1359 were used without further purification. For detection, secondary antibodies coupled to ultra-small gold particles were made visible by silver enhancement. For digital imaging, a slow-scan CCD camera (type 0124; TVIPS) attached to a CM12 transmission electron microscope operated at 120 kV was used (FEI).

**Analytical procedures.** The protein concentration was determined with the bicinchoninic acid kit from Novagen (Merck KGaA) (Smith et al., 1985) with BSA for calibration. UV/vis spectra were recorded in a Beckman DU640 spectrophotometer with the protein in the as-isolated state, and after reduction with either dithionate, dihydroerythritol, or ascorbic acid (all at 5 mM concentration). The purity of protein fractions was calculated from the A410/A280 ratio in the oxidized, as-isolated state.
RESULTS

CCs in *I. hospitalis* cell extracts

Total cell extracts of *I. hospitalis* Kin4/I were red and gave the characteristic signature of CCs in reduced UV/vis spectra, with broad features at 552–525 nm (x peak), 524 nm (β) and 419–421 nm (γ or Soret peak; not shown). Haem-stained SDS gels of extracts showed that at least three different proteins were responsible for the coloration (Fig. 1a), later identified as the gene products of the ORFs Igni_1359, Igni_0955 and Igni_0530 of the genome sequence. The apparent molecular masses were 70, 64 and 24 kDa, respectively. The 24 kDa band appeared only in membrane extracts, while the other two proteins appeared in both cytoplasmic and membrane extracts. Strong bands of high-molecular-mass aggregates appeared at the top with positive signals in haem staining. They were characteristic for the cytochromes because they did not disappear in urea-containing SDS gels (not shown) or in subsequent purification steps (Fig. 2).

Igni_0530 purification from membrane fraction and properties

Igni_0530 is a 216 aa protein containing four of the characteristic CxxCH haem-binding motifs typical for CCs and a predicted C-terminal TMH in addition to a canonical signal sequence; these predictions suggest membrane localization (Table S1, available in the online Supplementary Material). Two attempts were made to purify Igni_0530 from solubilized membrane fractions. The fraction with the highest purity (GPC2; Fig. 1b) showed two protein bands in addition to high-molecular-mass aggregates. The N-terminal sequence of the 24 kDa protein was YTPAYPCTY, corresponding to aa 28–36 of Igni_0530 and confirming the cleavage of a signal peptide (Fig. S1). This protein and the aggregates reacted positively in haem staining. A second protein band, with an apparent mass of 38 kDa, co-purified with Igni_0530, gave the N-terminal sequence LGYVYEEGTV, which corresponds to aa 21–31 of Igni_0529. (Fig. 1b, Table 1). The ORFs Igni_0530–0528 form an operon-like arrangement in the genome (Fig. S2). The Igni_0529 protein is homologous to iron–sulfur subunits of membrane-bound molybdopterin oxidoreductases with four predicted [4Fe-4S] clusters and a twin-arginine protein translocation motif (Table S1, Fig. S2). Igni_0528 is a transmembrane protein of the NrfD family with ten predicted TMHs (PFAM family PF03916). In GPC, Igni_0530 eluted in one peak with a molecular mass ≥440 (Table 1), suggesting the formation of a high-molecular-mass complex. Separation of the complex with non-denaturing gels did not yield any defined bands (not shown). The fractions of the other purification (CC and GPC; Fig. 1b) had an additional band with ~64 kDa mass and haem staining and migration behaviour identical to Igni_0955 (see below). The visible absorption maxima of the fraction with the highest purity were within the range typical for CCs (Table 1, Fig. 3).

Multihaem cytochromes c from *I. hospitalis*

Haem quantification with pyridine haemochrome spectra was performed according to published procedures (Takaichi & Morita, 1981). A calibration curve was recorded in triplicate with horse heart CC in a concentration range of 8–40 μM.

N-terminal amino acid sequencing of the soluble cytochromes was performed by Edman degradation after transfer of the proteins to a PVDF membrane with semi-dry blotting according to standard procedures. The N-terminal amino acid sequencing of the membrane-bound cytochrome was determined directly from the reddish band in an unstained PVDF membrane, while the other proteins were sequenced after Coomassie staining of the membrane.

Mass spectrometry of the soluble cytochromes. Cytochrome solution (100 μl, containing 18.6 μg of Igni_0955 or 25.6 μg of Igni_1359) was purified and de-salted with a ZipTip C4 (Millipore) and eluted with 10 μl 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile/water. The protein solution was mixed 1:1 (v/v) with the matrix, consisting of 2,5-dihydroxybenzoic acid. One microlitre of the mixture was loaded on the target. After evaporation of the solvent, the samples were analysed with MALDI-TOF (Bruker Autoflex III Smartbeam; Bruker AXS). BSA was used for calibration.

Enzyme assays. Igni_0955 and Igni_1359 were tested for enzyme activities in 100 mM sodium phosphate buffer, pH 6.4. Tetrathionate and thioulate reductase assays were performed in anaerobic cuvettes under N2 atmosphere with 5 mM Ti(III) citrate-reduced benzyl viologen as electron donor (Zehnder & Wuhrmann, 1976). Changes in absorbance were measured at 578 nm and 80 °C with 0.9 μg Igni_0955 or with 1.3 μg Igni_1359. The enzyme reaction was started by addition of 1 mM substrate. Controls included enzyme without substrate and substrate alone. Hydroxylamine dehydrogenase (HAO) activity was measured at 80 °C with three different electron acceptors, ferricyanide (2.5 mM, 420 nm), 2,6-dichloroindophenol (0.1 mM, 600 nm) and horse heart CC (20 μM, 550 nm). Hydrazine was also used as a potential substrate with the same electron acceptors; however, the background reaction at 80 °C was too high for resolution of any enzyme activity.

*I. hospitalis* hydrogenase activity was measured photometrically by benzyl viologen (BV) reduction with gaseous hydrogen in anaerobic cuvettes at 578 nm and 80 °C (5 mM BV in 100 mM sodium phosphate buffer pH 6.4 with 0.05% dodecyl maltoside). Reduction of the cytochromes by hydrogenase was measured at 423 nm without BV under otherwise identical conditions with 2.4 μg ConA-enriched hydrogenase. Different amounts of the purified cytochromes served as electron acceptors.

Bioinformatics procedures. All *I. hospitalis* amino acid sequences were retrieved from the GenBank database in FASTA format, reformatted by removal of the field delimiters and subsequently searched for the motif CxxCH at the 3oFS server (x representing any amino acid in the 3oFS algorithm; http://www.dkfz.de/maa2/3oFS/3oFS.html) (Seiler et al., 2006). Hit sequences were reformatted into FASTA format and analysed for transmembrane helices and signal sequences at the appropriate servers: SOSUI, http://harriarg.nagahama-i-bio.ac.jp/sosui/ (Hirokawa et al., 1998); SignalP4.0, http://www.cbs.dtu.dk/services/SignalP/ (Petersen et al., 2011). Sequences with one or more CxxCH motifs were defined as putative CCs if they had a predicted signal sequence and/or an N-terminal transmembrane helix (TMH). The identified protein sequences were analysed for similarities with Blast and/or PSIBLAST at the NCBI Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Structure predictions were performed using Phyre2 and 1-Tasser; the alignment of the resulting models versus template was done using TM-align at the 1-Tasser server (http://shanglab.ccmmb.med.umich.edu/TM-align/).
**Fig. 1.** SDS gels of total *I. hospitalis* extracts and of Igni_0530 purification. (a) 10 % Tris-Tricine SDS gel (Schägger & von Jagow, 1987) with cell extracts; left, haem staining; right, subsequent colloidal Coomassie staining; M, prestained marker proteins (molecular masses in kDa); Cyt, soluble fraction; W, wash fraction after first ultra-centrifugation (supernatant); Mem, membrane fraction. (b) Tris-glycine SDS gel (Laemmli, 1970) of the last steps of two separate Igni_0530 tetrahaem cytochrome c purifications from the membrane fraction and identification of the proteins; left, haem staining; right, subsequent colloidal Coomassie staining; M, marker proteins; CC, fraction after second concanavalin A column; GPC, fraction after gel permeation chromatography from the first purification; GPC2, fraction after GPC from the second purification.

**Fig. 2.** Purification of the octahaem cytochromes Igni_0955 (a) and Igni_1359 (b) from the soluble fraction. (a) Haem- and subsequently Coomassie-stained 10 % Tris-glycine SDS gel (Laemmli, 1970) with enrichment fractions of Igni_1359; M, prestained marker proteins (in kDa). Histogram depicts the enrichment calculated from the OD_{410}/OD_{280} ratios from UV/vis spectra divided by the ratio of the soluble extract (0.26); Greek letters designate the fractions described in Methods. (b) Same as in (a) but for Igni_0955.
BLAST searches with Igni_0530 and a multiple alignment showed the presence of similar proteins (\(\geq 40\%\) identity) in the related crenarchaeotes Pyrolobus fumarii and Hyperthermus butylicus [multihaem cytochrome c (MCC) sequence cluster no. 85 defined by Sharma et al. (2010)]. The sequence comparisons also showed that cytochromes of cluster no. 102 share distant similarity and conservation of four putative axial haem histidine ligands (Fig. S1).

Attempts at modelling Igni_0530 using the Phyre2 and I-Tasser servers did not give significant results in terms of a reasonably defined model or a structural similarity to known proteins (not shown).

### CC purification from the soluble fraction

The two major CCs, which were identical to the gene products of the Igni_0955 and Igni_1359 ORFs, were purified from soluble extracts in two separate but similar purification procedures (Fig. 2, Table 1). The bulk of the red fraction (\(\geq 90\%\)) eluted in a single peak from a DEAE Sepharose column developed with a salt gradient. The CCs were separated using a Q Sepharose column developed with a gradient of decreasing pH corresponding to the different calculated isoelectric points (Table S1). GPC was the final purification step, yielding almost homogeneity (Fig. 2).

The enrichment factors were calculated from the OD_{410}/OD_{280} ratios in the oxidized, as-isolated state (Fig. 2). The ratios were 4.2 for the purified Igni_0955 protein and 0.26 for the soluble extract, resulting in an enrichment factor of 16.3 (Fig. 2a). The OD_{410}/OD_{280} ratio of Igni_1359 was 3.0; the maximal enrichment was 11.6 (Fig. 2b). From the protein yield, the enrichment factors estimated protein losses; we calculated that the fractions of Igni_0955 and Igni_1359 were approximately 1.4 and 3\%, respectively, of the total soluble protein.

### Properties of Igni_0955

The deduced amino acid sequence of Igni_0955 corresponds to a protein with 551 aa, including a predicted signal sequence and eight haem-binding motifs (Table S1). Several attempts at N-terminal amino acid sequencing of the Igni_0955 monomer and oligomer bands gave either no results at all or ambiguous results as in the preparation shown in Fig. 2a: [(\(Mq\))(gA)(kv)(Dmnk)(tdE)(ea)(A)(dN)(Te)] (hits for each position in parentheses; capitals denote identity with amino acid sequence). This did not confirm the protein identity unambiguously; however, the amino acid positions 23–31 were identified, suggesting either

<table>
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<th>Property</th>
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<td>6.5–6.9</td>
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<tr>
<td>Absorption maximum (nm)</td>
<td>UV/vis spectroscopy</td>
<td>408</td>
<td>–</td>
<td>410</td>
<td>409</td>
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<tr>
<td>(\gamma)-Peak (Soret, oxidized)</td>
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<td>–</td>
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<tr>
<td>(\gamma)-Peak (Soret, reduced)</td>
<td>417</td>
<td>–</td>
<td>523</td>
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<tr>
<td>(\beta)-Peak</td>
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<td>–</td>
<td>555</td>
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<tr>
<td>(\alpha)-Peak</td>
<td>551</td>
<td>–</td>
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<td>554</td>
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<tr>
<td>(A_{410/280}) purified protein</td>
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<td>NDILL</td>
<td>4.2</td>
<td>3.0</td>
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<td>Maximal enrichment</td>
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<td>NDILL</td>
<td>16.3</td>
<td>11.6</td>
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<tr>
<td>(e_{280}) (M(^{-1}) cm(^{-1}))</td>
<td>UV/vis spectroscopy</td>
<td>31 860</td>
<td>63 830</td>
<td>96 830</td>
<td>169 140</td>
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<tr>
<td>without signal sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e_{\text{Soret oxidized}}) (M(^{-1}) cm(^{-1}))</td>
<td>UV/vis spectroscopy</td>
<td>NDILL</td>
<td>NDILL</td>
<td>310 600</td>
<td>324 200</td>
</tr>
<tr>
<td>(e_{\text{Soret reduced}}) (M(^{-1}) cm(^{-1}))</td>
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<td>NDILL</td>
<td>NDILL</td>
<td>206 600</td>
<td>216 700</td>
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<tr>
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<td>NDILL</td>
<td>–</td>
<td>38 900</td>
<td>21 000</td>
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*616 g mol\(^{-1}\).
†Including 4 × [4Fe-4S] clusters.
‡ND, not determined.
§Absent.
||The final protein concentrations were too low for reproducible and robust values.
N-terminal processing of the signal peptide or an alternative translational start site at M23. When analysed with high-resolution MALDI-TOF mass spectrometry, Igni_0955 gave two peaks of 33.05 (double ionization) and 66.16 kDa (single ionization; Fig. S3), corresponding to the molecular mass of the subunit including signal peptide and eight haem moieties (calculated mass 66.32 kDa; Table 1). The protein was embedded in a dihydroxybenzoic acid matrix; other matrices or ESI-q-TOF did not give any signals beyond background. Pyridine haemochrome spectra gave a ratio of 7.5 haems per monomer (Table 1). The absorption maxima (Fig. 3, Table 1) were rather broad, presumably because the maxima of the individual haems in the protein vary.

Igni_0955 had apparent molecular masses of 64 kDa in SDS gels for the monomer band (Fig. 2a) and of 111–121 kDa in GPC, which would correspond to a dimer (not shown). High-molecular-mass bands in SDS gels presumably correspond to dimer, trimer and oligomer formation, as they gave a response in haem staining (Fig. 2a). In non-denaturing gels, the protein smeared in aggregates in the range 800 ± 300 kDa (not shown).

**Properties of Igni_1359**

The amino acid sequence of the Igni_1359 protein is 638 aa length including signal sequence and eight haem-binding motifs (Table S1). The apparent molecular mass of the monomer was 70–72 kDa in SDS gels (Fig. 2a); in addition there was an oligomer band of ~190 kDa. A 25 kDa protein was visible as the sole contaminant of this preparation. In GPC, the major peak corresponded to a molecular mass of 160–164 kDa. SDS-PAGE and GPC results point to a dimeric or trimeric quaternary structure. Additional minor peaks in GPC corresponded to molecular masses of 211 and 401 kDa. In non-denaturing gels, Igni_1359 showed two bands of approximately 500 and 600 kDa. After excision and separation of the gel-embedded protein in an SDS gel, the same pattern of one monomer and one oligomer band was reproduced (not shown).

N-terminal amino acid sequencing of the monomer and oligomer bands (Fig. 2b) gave identical results, DVSSMAKLQ, corresponding to aa 22–30 of the amino acid sequence. The N terminus of the 25 kDa protein (MgQIPLIG) was identical to that of Igni_0459 except for position 2. The protein is annotated as an alkyl hydroperoxide reductase in GenBank. It is unclear whether it has a functional relevance for the CCs reported here. MALDI-TOF mass spectrometry of Igni_1359 yielded similar results to those described above for Igni_0955. Two mass peaks were observed of 38.84 (double ionization) and 77.62 kDa (single ionization; Fig. S3), again corresponding to the mass of the subunit including signal peptide and eight haem moieties (calculated, 77.96 kDa; Table 1). Pyridine haemochrome spectra gave a ratio of 6.7 haems per monomer; the observed lower value is probably a result of contamination. The absorption maxima were similar but not exactly identical to those of Igni_0955 (Fig. 3, Table 1).

**The soluble cytochromes are electron acceptors of the hydrogenase in vitro**

The activity of the *I. hospitalis* hydrogenase was measured with all three cytochromes as electron acceptors. In order to enrich for hydrogenase activity, 14 mg solubilized membrane protein was separated aerobiocly on a concanavalin A column and eluted with increasing concentrations of methyl-α-D-glucoside. We used 2.4 μg protein of the

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**Fig. 3.** UV/vis spectra of the three purified cytochromes c. Oxidized, as-isolated spectra; reduced, dithionite-reduced spectra; Red-Ox, reduced−oxidized difference spectra; insets, enlargement of the difference spectra around the α and β peaks.

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**Igni_0955 and Igni_1359 are localized in all cellular membranes**

Immunoelectron microscopy of ultra-thin *I. hospitalis* sections using α-Igni_0955 and α-Igni_1359 antibodies showed a localization of both CCs in both membranes of *I. hospitalis* but not in the cytoplasm. They were also present in the IMC, possibly attached to or enclosed in the vesicles trafficking between both membranes, although under the conditions used for optimal labelling, the vesicles remained almost invisible (Fig. 5).

**Igni_0955 and Igni_1359 belong to the HAO family of octahaeem cytochromes**

The two soluble cytochromes Igni_0955 and Igni_1359 share 29% identity in a global amino acid alignment. BLAST searches showed that they belong to the HAO family of MCCs (cluster no. 12; Kern et al., 2011; Klotz et al., 2008; Sharma et al., 2010). The protein backbones can be modelled into the existing 3D structure of the *Nitrosomonas europaea* HAO (PDB identifier 1FGJ) with a root mean square deviation of 1.58 (Igni_0955) and 0.85 Å (Igni_1359; not shown); however, some of the haem moieties could not be included without clashes of atoms overlapping with amino acids. This and a multiple sequence alignment of both *I. hospitalis* cytochromes with 68 HAO example sequences showed that five out of seven axial histidine ligands are conserved and that the identification of the remaining ligands requires additional structural information (Fig. S5). A phylogenetic dendrogram based on these 97 sequences (Fig. S6) showed that the HAO family splits into two major subclusters as already shown in earlier studies (Kern et al., 2011; Klotz et al., 2008). The *Ignicoccus* MCCs Igni_0955 and Igni_1359 group within subcluster 1 (Figs S5 and S6), while the known HAO and hydrazine dehydrogenases fall with in subcluster 2. Among Archaea, highly similar proteins are found in *H. butylicus* and *Pl. fumarii* (both Desulfurococcales) and *Ferroglobius placidus* (*Archaeoglobales*), and one more distantly similar protein is found in *Methanococcoides burtonii*. The major difference between the paralogous MCCs Igni_0955 and Igni_1359 (and their respective orthologues in other species) is an insertion of approximately 100 aa between the haem-binding CxxCH motifs nos 3 and 4, which explains the size difference between both *Ignicoccus* HAO-type cytochromes (Fig. S5).

**Ferredoxins and quinones**

Three small ferredoxins (<100 aa) are encoded in the *I. hospitalis* genome, when counting only those that are not part of obvious operons (e.g. oxoacid: ferrodoxin oxido-reductase genes):Igni_0189, Igni_0812 and Igni_1238. We tried to follow the purification of ferredoxins by their characteristic brown colour and absorbance in the range between 400 and 430 nm. However, only one brown band appeared during column chromatography. It did not contain any small protein (<12 kDa) as judged from

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**Fig. 4.** Hydrogenase activity with Igni_0955 as electron acceptor. The enzyme assay was performed in an anaerobic cuvette at 80 °C under hydrogen atmosphere with 36 μg Igni_0955 and 2.4 μg hydrogenase-enriched membrane fraction (addition at arrow); absorption was followed at 423 nm. For assays with lower amounts of Igni_0955, controls and Igni_1359, see Fig. S4.
SDS-PAGE results (not shown). We also tried to enrich for small, acetone-soluble proteins after precipitation of the majority of proteins in 80% acetone. The major protein in the supernatant had an apparent molecular mass of 10–12 kDa in SDS-PAGE. It did not have any coloration and was identical to Igni_0174, annotated as the nucleic acid-binding protein AlbA (not shown).

We also tried to extract quinones from the *I. hospitalis* membrane fraction; however, no yellow organic extract was obtained in several attempts with different solvents and different batches of cells. The extracts were separated via an HPLC column but the fractions did not exhibit spectra typical for quinones. It was concluded that the quinone concentration is below the limits of detection (not shown).

**Genes for CCs and maturation proteins in the *I. hospitalis* genome**

Apart from Igni_0530, Igni_0955 and Igni_1359, three other putative CCs can be identified in the *I. hospitalis* genome sequence. Igni_1130 encodes another octahaem protein with 526 aa and a C-terminal TMH in addition to the signal sequence (Table S1). It belongs to the large tetrathionate reductase family of MCCs (cluster no. 10; Sharma et al., 2010). The only archaeal homologues are encoded in the *H. butylicus* genome and in several uncultivated samples (not shown). A root mean square deviation of 0.81 Å resulted when Igni_1130 was modelled against the *Shewanella oneidensis* tetrathionate reductase (1SP3; not shown). Most of the distal haem ligands could be identified in alignment and model. In this study no protein was detected with a band of the predicted size in SDS gels, and *I. hospitalis* is not known to grow on tetrathionate due to its instability under the growth conditions.

A survey for additional CC genes in the *I. hospitalis* genome resulted in the identification of two mutually similar proteins, each with a single CxxCH motif and a signal sequence (Igni_1052 and Igni_0759; Table S1). Pair-wise identities were 49% in positions 1–162 of the proteins, while the remaining parts were dissimilar, resulting from a predicted C-terminal TMH in Igni_1052. Again, significantly similar sequences were found in *Pl. fumarii* (Pyrfu_1333) and *H. butylicus* (Hbut_1376) (both include a C-terminal TMH), and more distantly similar sequences in several bacteria (Fig. S7). A conserved histidine residue was identified as a putative distal haem ligand. Modelling of the proteins gave ambiguous results (not shown). Neither of the proteins was detected in SDS-PAGE of *I. hospitalis* extracts (Fig. 1, and other SDS gels not shown).

(M)CCs require maturation proteins for the covalent attachment of the haem group via thioether bonds to the cysteine side chains within CxxCH. BLAST searches showed that homologues of the bacterial system I (also termed cytochrome *c* maturation system, CCM) (Sanders et al., 2010; Stevens et al., 2011) were present in the *I. hospitalis* genome (*ccmB*, Igni_0093; *ccmC*, Igni_0047; *ccmF*, Igni_0314; *ccmA*, *ccmG* and *ccml* are not suitable because they belong to large protein families, leading to numerous non-specific hits) (Allen et al., 2006). Homologues of other CC maturation proteins were not found. The results suggest that *I. hospitalis* uses at least some of the *ccm* gene products for CC maturation.

**DISCUSSION**

CCs seem to play an important role in the metabolism of *I. hospitalis*, demonstrated by the red coloration and the high...
percentage of CCs in cell extracts. We showed here that at least three MCCs are present in significant amounts and that three additional CCs are encoded in the genome.

Igni_0955 and Igni_1359 purified from the soluble extract are mutually similar in sequence and biochemical properties: they show 29% amino acid sequence identity, both contain eight haems per subunit, and they have subunit masses between 64 and 73 kDa. The difference in size results from an insertion between haem-binding motifs 3 and 4 in Igni_1359 (Fig. S5). N-terminal sequencing of Igni_1359 gave the clear result that the first 21 amino acids were cleaved off. More difficult was the interpretation of the N-terminal sequencing of Igni_0955: in most attempts, the N terminus was blocked, while weak and ambiguous signals appeared occasionally. Nevertheless, some of the N-terminal amino acids were correctly identified, but the results must be regarded with care. Similarly, sequencing of the 64 kDa CC band of membrane extracts was not successful (Fig. 1b, lanes CC and GPC). Its running behaviour was identical to Igni_0955 but its identity is not finally confirmed. In contrast to the sequencing results, the subunit masses of both Igni_1359 and Igni_0955 were almost identical to the respective calculated masses of the uncleaved holoproteins including eight haems. In consequence, the proteins might exist either in different forms in *I. hospitalis* or with an as-yet- unidentified post-translational modification.

In spite of having purified Igni_0955 from the soluble extract, a significant portion remained in the solubilized membrane fraction. In fact, it was difficult to separate Igni_0530 from a band with running behaviour identical to Igni_0955 (see Fig. 1b, lanes CC and GPC, and above). This observation has implications for the interpretation of the immuno-labelled electron microscopy images. Both with Igni_0955 and with Igni_1359, the inner and OCMs were strongly stained, with additional signals in the IMC (Fig. 5). These signals are weaker and not evenly distributed as expected for a soluble protein. Their distribution could even point to an attachment to membrane vesicles, although the resolution is not good enough to distinguish between the vesicle-bound and soluble states. The immuno-labelled electron micrographs and the biochemical results suggest that Igni_0955 and Igni_1359 might be bound rather loosely to the membrane by protein–protein rather than protein–membrane interactions. The results also suggest that both CCs are easily detached during purification so that a large part ends up in the soluble fraction.

We also showed that Igni_0955 can act in vitro as electron acceptor for the single *I. hospitalis* hydrogenase (Igni_1366-69), but we could not show any intrinsic enzyme activity of Igni_0955 or Igni_1359. They belong to a family of octahaem MCCs that is dominated by nitrogen cycle enzymes like HAO (working in either direction) and the closely related hydrazine dehydrogenases (Hanson et al., 2013; Klotz et al., 2008; Shimamura et al., 2007). We had previously found the four hydrogenase subunits in membrane extracts and we had measured sulfur reductase activity but not identified the proteins (M. Forth, M. E. Eckert and A. Kletzin, unpublished). The main reductive power during growth of *I. hospitalis* comes from hydrogenase-mediated H2 oxidation (Küper et al., 2010), but most of the reducing equivalents (and ATP) are required for CO2 fixation and the biosynthesis of biomolecules. A significant fraction of the enzymes involved in these processes is expected to be present in the electron-dense inner compartment (Huber et al., 2012). The activity of hydrogenase with Igni_0955 (and to a lesser degree, Igni_1359) as electron acceptors and their presence close to both membranes led us to the hypothesis that electron transfer from H2 to various intracellular reductases might be one of the functions of the CCs, especially since we did not find any quinones as primary electron acceptors of the hydrogenase. The *I. hospitalis* ferredoxins, other candidates for primary electron acceptors, should be confined to the inner compartment of the cells, as they do not have twin-arginine signal peptides. Therefore, they should not be in direct contact with the hydrogenase. In many studies of anaerobic bacteria, MCCs were described as versatile acceptors or relays of electrons for distribution to downstream redox complexes (Grein et al., 2013; Matias et al., 2002), and so we should like to suggest a similar function for the soluble but membrane-attached *I. hospitalis* MCCs.

MCCs have also been implicated for a role in reduction of elemental sulfur in a number of different studies. For example, the *Desulfovibrio* tetrahaem cytochrome c5 has sulfur-reducing capabilities (Cammack et al., 1984; Fauque et al., 1979). The most convincing evidence for the participation of CCs in sulfur reduction came from the *Pd. abyssi* hydrogenase/sulfur reductase complex (see Introduction) (Dirmeyer et al., 1998). The Igni_0530 CC, co-purified here with the iron–sulfur protein Igni_0529, is encoded together with the membrane protein Igni_0528 in an operon-like arrangement. Igni_0528 is a very hydrophobic protein with ten predicted TMHs, which is probably the reason why it did not co-purify with the other two subunits: the solubilization might have been incomplete (Laska et al., 2003). The operon arrangement is reminiscent of the sulfur or polysulfide reductases (SR/PSR; Fig. S2). These are molybdenum-containing proteins consisting of a large molybdopterin subunit (DMSO reductase family) and FeS and NrfD-type subunits (Hedderich et al., 1998), as for example in the mesophilic *c*-proteobacterium *Wolinella succinogenes* (Krafft et al., 1995). Igni_0529 and Igni_0528 are clearly homologous to these FeS and NrfD subunits, respectively. If one assumes that Igni_0530 replaces the molybdopterin subunit, this novel CC could be part of an SR, especially since sulfur-reducing CCs have been described before (Cammack et al., 1984; Fauque et al., 1979). The molybdopterin SR/PSR enzymes belong to the DMSO reductase family. Three of these proteins are encoded in *I. hospitalis*: Igni_1379, Igni_0802 and Igni_1101. None of them was identified in our study, and none of them has high similarity to the known SR/PSR from...
Acidianus ambivalens or W. succinogenes (Krafft et al., 1995; Laska et al., 2003). Taken together, our results presented here could suggest that Igni_0530, Igni_0529 and Igni_0528 might form an important part of the hitherto hypothetical sulfur-reducing complex.

Summarizing our conclusions, we propose a model predicting that the abundant cytochromes Igni_0955 and Igni_1359 might function as an electron distributor similar to the Desulfovibrio high-molecular-mass MCC (Grein et al., 2013), while ferredoxins are electron carriers in the cytoplasm. This does not preclude additional functions of the MCCs in catalysis, for which we do not yet have any evidence. The membrane-bound Igni_0530 tetrahaem CC might be part of the sulfur-reducing mechanism.

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Multihaem cytochromes c from l. hospitalis


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