



The NT-26 cytochrome c_{552} and its role in arsenite oxidation

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Abstract

Arsenite oxidation by the facultative chemolithoautotroph NT-26 involves a periplasmic arsenite oxidase. This enzyme is the first component of an electron transport chain which leads to reduction of oxygen to water and the generation of ATP. Involved in this pathway is a periplasmic c -type cytochrome that can act as an electron acceptor to the arsenite oxidase. We identified the gene that encodes this protein downstream of the arsenite oxidase genes (*aroBA*). This protein, a cytochrome c_{552} , is similar to a number of c -type cytochromes from the α -*Proteobacteria* and mitochondria. It was therefore not surprising that horse heart cytochrome c could also serve, *in vitro*, as an alternative electron acceptor for the arsenite oxidase. Purification and characterisation of the c_{552} revealed the presence of a single heme per protein and that the heme redox potential is similar to that of mitochondrial c -type cytochromes. Expression studies revealed that synthesis of the cytochrome c gene was not dependent on arsenite as was found to be the case for expression of *aroBA*.

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1. Introduction

The soluble forms of arsenic that can be used by microbes for growth are the trivalent arsenite (H_3AsO_3) and pentavalent arsenate ($\text{HAsO}_4^{2-}/\text{H}_2\text{AsO}_4^-$) [1]. Arsenite (As^{III}) can be used as an electron donor for respiratory processes with either oxygen or nitrate as electron acceptors, and arsenate (As^{V}) can serve as a terminal electron acceptor with a variety of electron donors [1]. Both arsenite and arsenate are toxic to most forms of life; arsenate inhibits ATP synthesis and arsenite inactivates proteins by binding to sulfhydryl groups.

The arsenite-oxidising bacteria that have been isolated to date are phylogenetically distant with mesophilic representatives in the α -, β - and γ -*Proteobacteria* and thermophilic representatives in the *Thermus/Deinococcus* lineage [1]. They

can either gain energy from arsenite oxidation [2–5] or have been proposed to oxidise the arsenite for detoxification [6–12]. Chemolithoautotrophic arsenite oxidation where oxygen is used as the terminal electron acceptor, arsenite as the electron donor and carbon dioxide as the sole carbon source has only been described for organisms isolated from gold mines [2,4,5].

NT-26, a member of the α -*Proteobacteria*, was isolated from the Granites gold mine, Northern Territory, Australia [4]. It grows both chemolithoautotrophically and heterotrophically with arsenite as the electron donor. The arsenite oxidase (Aro) from this organism has been purified and partially characterised and the *aro* genes cloned and sequenced [13]. Direct catalytic (arsenite oxidation) electrochemistry of Aro has been achieved [14]. The NT-26 Aro is very similar to the arsenite oxidases of *Alcaligenes faecalis* and NT-14 (both heterotrophic members of the β -*Proteobacteria*) and consists of two heterologous subunits; the larger one containing the molybdenum (Mo)-catalytic site and a [3Fe–4S] cluster (AroA) and a smaller subunit (AroB) containing a Rieske-type [2Fe–2S] cluster [13,15,16].

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All known arsenite oxidases belong to the DMSO reductase family of molybdoenzymes which consists of a diverse group of enzymes that catalyse a variety of oxygen-atom transfer reactions [17,18]. Characterisation of the *A. faecalis* arsenite oxidase shows that the large subunit contains a Mo site, consisting of a Mo atom coordinated by two pterin-dithiolene [molybdopterin guanosine dinucleotide (MGD)] ligands [19]. However, unlike what is seen in other members of the DMSO reductase family, in the *A. faecalis* arsenite oxidase the Mo atom is not coordinated to the protein by an amino acid ligand [20].

NT-26 can oxidise arsenite both chemolithoautotrophically and heterotrophically. Heterotrophic arsenite oxidation is thought to involve arsenite oxidase and a periplasmic electron acceptor. This acceptor has been shown in *in vitro* experiments to be a *c*₅₅₁-type cytochrome in NT-14 [16] or a *c*-type cytochrome and azurin in *A. faecalis* [15]. Nothing is known of the electron transport chain involved in chemolithoautotrophic arsenite oxidation.

Here we report for the first time the purification and characterisation of a periplasmic *c*-type cytochrome from NT-26 which can act as an electron acceptor to the NT-26 Aro. The role of this protein in arsenite oxidation is examined by characterising a mutant and examining its expression profile.

2. Materials and methods

2.1. Chemicals

Eastman AQ 29D polymer (28% w/v) was a gift from Eastman Chemical Company (Tennessee, USA). All other reagents (electrolytes, buffers, etc.) were of analytical purity. MilliQ water was used for all experiments.

2.2. Growth of NT-26

NT-26 was grown aerobically with shaking at 28 °C either chemolithoautotrophically in minimal salts medium (MSM) with 5 mM arsenite as the electron donor or heterotrophically with 0.04% yeast extract with and without 5 mM arsenite (final pH of medium is 8) [4]. For growth experiments, cultures were grown for 24 h and inoculated (5%) into the experimental medium (200 mL). Samples were taken periodically, and either total cell numbers or optical density (600 nm) was determined [4]. Portions of the samples were also taken for arsenic analyses [4]. For DNA isolations NT-26 was grown as previously described [13].

2.3. Cloning and sequencing of the *cytC* gene

A NT-26 *Hind*III genomic library which was used for detecting the *aroA* and *aroB* genes [13] was also used for identifying the *cytC* gene. Sequencing was performed at the Australian Genome Research Facility (Brisbane, Queensland, Australia) with an ABI Prism 210 capillary DNA sequencer and genetic analyser. Database searches were performed by using BlastP [21] at the NCBI web site. The *cytC* gene sequence has been deposited in GenBank under accession number AY345225.

2.4. Expression of the *cytC* gene

Reverse transcriptase (RT)-PCR was used to assess transcription of the *aroB*, *aroA*, *cytC* and *moeA1* genes that form part of the arsenite oxidase gene cluster. NT-26 was incubated under three different growth conditions until late logarithmic phase: (1) chemolithoautotrophically with 5 mM arsenite, (2) 5 mM arsenite and 0.04% yeast extract and (3) 0.04% yeast extract. RNA was extracted using the RNeasy Protect Bacteria kit (Qiagen) and at least one DNase (Qiagen)

treatment performed according to the manufacturer's instructions. The primers used to detect expression of *aroB*, *aroA*, *cytC* and *moeA1*, respectively (Note: the start and/or stop codons of each gene are italicised) were AroBF 5'-GCCTGCAGATGTCACGTTGTCAAACATGGTGC-3' (binds to nucleotides 376–401); AroAF 5'-GCCTGCAGATGCGCTTCAAACGTCACATC-3' (binds to nucleotides 916–937) and AroAR 5'-GCCTGCAGTCAAGCCGAC-TGGTATTCT-3' (binds to nucleotides 3453–3434); CytCF (binds to nucleotides 3542–3564) 5'-GCGAATTCATGCGGAAACTGTTTTTCGATCG-3' and CytCR (binds to nucleotides 3925–3949) 5'-GCGAATTCATGCGGGGTGCT-GAATGTCTTGAG-3' (recognition sequence for *Eco*R1 is underlined); MoeA1R 5'-GCAAGCTTTCACATTGCGAACGGTTCGA-3' (binds to nucleotides 5297–5277). The primers used for amplification of the *cytC* gene were CytCF (binds to nucleotides 3542–3564) and CytCR (binds to nucleotides 3925–3901) described above. AroBF and AroAR were used to test for cotranscription of the *aroB* and *aroA* genes, AroAF and CytCR for cotranscription of the *aroA* and *cytC* genes, and CytCF and MoeA1R for cotranscription of the *cytC* and *moeA1* genes. The RT-PCR experiments were performed using the Access RT-PCR system (Promega) according to the manufacturer's instructions. The reactions consisted of 50–100 ng RNA, 100 ng of each primer and 10% DMSO. The RT-PCR reactions consisted of an initial RT step done at 45 °C for 45 min followed by the PCR 94 °C 2 min (1st cycle only) and 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 68 °C for *x* min (where *x* is 1 min/kb of DNA) and a final extension at 68 °C for 7 min. After each RNA isolation, confirmation that no contaminating DNA was present in the sample was done by PCR using the CytCF and CytCR primers as above but omitting the RT step and using Tfl DNA polymerase (Promega).

2.5. Mutagenesis of the *cytC* gene

Mutagenesis of the *cytC* gene was carried out by targeted gene disruption as described previously for *aroA* [13]. Briefly, the entire gene was amplified by PCR using the two primers CytCF and CytCR described above. The PCR product (384 bp) was digested with *Eco*RI and *Stu*I (cuts at the 3'-end of the gene) resulting in a 289 nt fragment which was cloned into the suicide plasmid pJP5603 [22] at the *Eco*RI and *Sma*I sites, respectively. One mutant was chosen for further study and insertion of the plasmid into the *cytC* gene was confirmed by Southern hybridisation.

The mutant was tested for its ability to oxidise arsenite chemolithoautotrophically and heterotrophically. Growth experiments were performed with two replicates on at least two separate occasions. For testing the specific activity of the Aro, the mutant was grown in 2 L of batch culture in MSM containing 5 mM arsenite and 0.04% yeast extract. Total cell extracts were prepared as described previously [4].

2.6. Heterologous expression and purification of the cytochrome *c*

The *cytC* gene without the leader sequence was amplified by PCR and cloned downstream and in frame with the *pelB* leader sequence and upstream of the His-tag sequence of pET-22b+(Novagen) in the *Nco*I and *Xho*I sites (sites are underlined). The PCR primers were: Forward 5'-GCCCATGGATGAGAG-CAACGCGGAAAAGGGCGCGG-3' and Reverse 5'-GCCCTCGAGCGGGGT-GCTGAATGTCTTGAGG-3'. For expression BL21 DE3 (pLysS) (Promega) (pEC86, *cytC*-pET22b+) liquid cultures were grown at 30 °C in LB broth supplemented with Ap (100 µg/mL) and Cm (60 µg/mL) and a 1:10,000 dilution of a trace element solution [23]. An overnight culture (30 mL) was used to inoculate 900 mL LB (in 1 L flask) and the culture was incubated for 4 h at 30 °C and 180 rpm. Plasmid pEC86 containing the *E. coli* cytochrome *c* maturation genes (*ccm*) was generously provided by Prof. L. Thöny-Meyer [24]. For induction of protein expression 20 µM IPTG (final concentration) was added and the cultures were then incubated overnight. All cell pellets were bright pink and about 0.9 g (wet weight) of cells were obtained/L medium.

Cells were chilled on ice, harvested by centrifugation and the pellet suspended in 20 mM morpholine ethanesulfonate (MES) (pH 5.5) buffer (optimum buffer and pH of the NT-26 Aro; 4), homogenized and passed three times through a French Press (Thermo Electron) at 14,000 psi. Cell debris was removed by centrifugation at 30,000×g for 30 min at 4 °C. The supernatant was loaded onto a SP Sepharose Fast Flow cation exchange column (1.6 × 8 cm) (GE Healthcare). The column was washed with 70 mM NaCl containing MES buffer,

and the cytochrome was eluted using a 0.07–0.45 M NaCl gradient [7 column volumes (CV)] in 50 mM MES (pH 5.5). Fractions containing the cytochrome were pooled and the buffer replaced with 50 mM potassium phosphate/ 0.5 M NaCl (pH 7.4). The protein was loaded onto a 5 mL His-Trap column (GE Healthcare) and eluted using a 0–0.5 M imidazole gradient over three CV. Fractions containing the cytochrome were pooled and contained protein with a purity of >96%. If further purification was desired, samples were concentrated using ultrafiltration (Amicon Ultra, MWCO 10 kDa) and then loaded onto a Superdex 75 (16/60) gel filtration column (GE Health Care) previously equilibrated with 20 mM Tris–HCl/150 mM NaCl (pH 7.8). Fractions containing the protein were pooled and the NaCl removed by dialysis against 20 mM Tris–HCl (pH 7.8) at 4 °C overnight.

2.7. Purification of the arsenite oxidase

The NT-26 arsenite oxidase was prepared as previously described [13] and stored at –70 °C as separate 10 µL aliquots at a concentration of 23 µM.

2.8. Spectroscopic and analytical techniques

Arsenite oxidase activity was determined by measuring the reduction of the artificial electron acceptor 2,4-dichlorophenolindophenol (DCPIP) (0.3 mM) at an absorbance of 600 nm (ϵ of DCPIP at 600 nm is 23 mM⁻¹ cm⁻¹) in 50 mM MES (pH 5.5).

The oxidised and reduced states of the purified *c*₅₅₂, horse heart cytochrome *c* (Sigma Aldrich) and *Pseudomonas aeruginosa* azurin (Sigma) were recorded with a UV absorbance wavelength spectrum (nm) using a Cary 100 UV-Visible double beam spectrophotometer (Varian). The proteins were first oxidised with potassium hexacyanoferrate(III) (ferrocyanide) (Sigma). Residual ferro- and ferricyanide was removed using a PD-10 desalting column (GE Health Care) previously equilibrated with 50 mM MES (pH 5.5). The oxidised absorption spectra of the *c*₅₅₂ and horse heart cytochrome *c* were recorded in 50 mM MES (pH 5.5) with 8 µM and 8.5 µM protein, respectively and 2.5 mM arsenite. Reduction of the cytochrome was initiated by the addition of the NT-26 Aro (140 nM in the case of the *c*₅₅₂ assay and 11.5 nM in the case of the horse heart cytochrome *c* assay). The oxidised absorption spectrum of the azurin was recorded in 50 mM MES (pH 5.5) at various concentrations (1.2 µM, 4.6 µM, 9.2 µM, 16.2 µM and 32 µM). Reduction of the azurin was attempted by the addition of NT-26 Aro (0.01 µM and 0.02 µM) and 2.5 mM arsenite.

Tryptic digests were carried out in 20 mM ammonium bicarbonate, pH 7.9 for 20 h at 37 °C with a porcine trypsin (sequencing grade, Promega) to protein ratio of 1:20 according to the manufacturer's instructions. Samples were analysed using an Applied Biosystems Voyager DE STR 4316 MALDI-TOF mass spectrometer (Voyager™ 5.1 software with data explorer). Sinapic acid and α -cyano-4-hydroxycinnamic acid were used as matrices in sample preparation of whole protein and peptides, respectively. Matrices were mixed with samples in a 1:1 ratio.

Heme content was determined in alkaline pyridine solution [25]. Denaturing and nondenaturing PAGE was performed according to Laemmli [26]. Gels were stained using Coomassie Brilliant Blue or heme-dependent peroxidase activity was detected [27]. N-terminal sequencing was performed as described previously [28].

Protein concentrations were determined using the 2D Quant Kit (Ge Healthcare Biosciences) or the Bradford reagent [29].

2.9. Cyclic voltammetry

All measurements were made with a Bioanalytical Systems BAS100B/W electrochemical workstation. The working electrode was a glassy carbon disk (see below), a Pt wire counter electrode and a Ag/AgCl (3 M NaCl) reference electrode were used. Eastman AQ 29D polymer (28% w/v) was diluted 1 : 20 v/v and 10 µL of this solution was dropped onto an inverted glassy carbon electrode (previously polished with 6 µm and 1 µm alumina powder) and allowed to dry at room temperature to a film. The electrochemical solution contained 50 mM MES/50 mM KCl (pH 5.5). The NT-26 *c*₅₅₂ solution (ca. 70 µM) was diluted 4 : 15 v/v with buffer to give a volume of ca. 0.5 mL. The polymer coated glassy carbon electrode was inserted into the protein solution

and allowed to stand for approximately 1 h prior to measurement to allow the protein to accumulate in the polymer coating. Voltammograms were recorded in the presence of dioxygen but at a potential sufficiently high that oxygen reduction was not a problem.

2.10. Redox potentiometry

Redox potentiometry was performed on 1.3 mL of a 4 µM cytochrome *c*₅₅₂ solution (in 50 mM MES buffer, pH 5.5) in the presence of the mediators Fe (NOTA), Fe(EDTA)⁻ and [Co((Me₃N)₂sar)]⁵⁺ (50 µM each) as described previously [30]. The titration was performed at 25 °C within a small volume 1 cm pathlength spectrophotometer cell equipped with a trough to accommodate a small magnetic stirring flea which was driven by a Variomag electronic stirrer. The titrants were Na₂S₂O₄ (reduction) and K₂S₂O₈ (oxidation) both added as ca. 5 mM solutions in small aliquots (0.5–2 µL) to avoid large jumps in solution potential. The potential was measured with a combination Pt-Ag/AgCl electrode attached to a Hanna Instruments 8417 Meter. The electrode was calibrated with quinhydrone ($E_{m,7} + 86$ mV vs. Ag/AgCl) prior to use. After equilibration (ca. 10–15 min) the solution potential was measured *in situ* and the UV-vis spectrum was measured with an AnalytikJena Specord 210 instrument. The heme redox potential was obtained by fitting the change in absorbance at 550 nm as a function of potential to the Nernst equation as described previously [30].

3. Results

3.1. Cloning and sequence analyses of the *cytC* gene

An open reading frame (ORF) was identified in the arsenite oxidase gene cluster downstream of *aroB* and *aroA* (i.e. 88 nucleotides downstream of *aroA* stop codon) and was designated *cytC* (Fig. 1), as it was found to be similar to various *c*-type cytochromes (see below). Downstream of the *cytC* gene (384 bp) is an ORF, designated *moeA1*, whose putative protein is similar to the Mo cofactor biosynthesis protein MoeA. All four genes appear to be transcribed in the same direction. The only putative promoter (consensus sequence is TGGCACX₅TTGCW) [31] was identified upstream of *aroB* (Fig. 1). All four genes contain putative ribosome binding sites upstream of their respective start codons. No transcription terminator sequences were identified.

The *cytC* gene encodes a putative protein (*c*₅₅₂) with a protein mass of 13,507 Da (pI 8.81) before processing. The sequence contains a single CX₂CH heme binding motif at the N-terminus

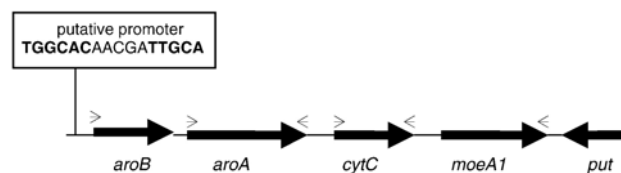


Fig. 1. Arrangement of the NT-26 arsenite oxidase gene cluster. Upstream of *aroB* is a putative promoter (consensus sequence is TGGCACX₅TTGCW). Positions of the *aroB* and *aroA* genes are indicated. These genes encode the subunits of the arsenite oxidase (4). Transcribed in the same direction and downstream of *aroA* is the *cytC* gene. A fourth ORF was identified, *moeA1*, whose putative product is similar to the Mo cofactor biosynthesis protein MoeA. Downstream of this gene is a putative gene transcribed in the opposite direction that encodes a putative phosphonate uptake transport (Put) protein. Each primer binding site is indicated by an arrow. From left to right the primers are: AroBF, AroAF, AroAR, CytCF, CytCR and MoeAIR (see Materials and methods). Amplification direction of each primer is indicated by an arrow.

suggesting that a single heme is incorporated into the apoprotein. It contains a putative Sec-(general secretory pathway) dependent leader sequence as expected [32]. Cytochromes are exported across the cytoplasmic membrane in an unfolded state using the Sec pathway. In general, once exported, they are folded following incorporation of the heme cofactor. Using the SignalP program [33] the *c*₅₅₂ has a predicted signal peptide cleavage site between residues 20 and 21 (MA ↓ ES), and the predicted molecular mass of the processed protein is 11,402 Da. It does not contain any other hydrophobic regions indicative of transmembrane domains and therefore is assumed to be periplasmic.

The *c*₅₅₂ protein shared significant sequence similarities to a number of *c*-type cytochromes from the α -Proteobacteria and mitochondria. The highest sequence identities of the *c*₅₅₂ were with a putative *c*-type cytochrome (96% identity) from the arsenite-oxidising bacterium, *A. tumefaciens* [34] (Acc. no. ABB51926.1), a putative *c*-type cytochrome in *Aurantimonas* sp. SI85-9A1 (73% identity) (Acc. no. ZP-01227689.1) and a number of diheme SoxD proteins (in the range of 60–70% identity) and to a lesser extent mitochondrial *c*-type cytochromes.

3.2. Expression of the *cytC* gene

To determine whether the genes in the arsenite oxidase gene cluster (*aroB*, *aroA*, *cytC* and *moeA1*) are in the one operon RT-PCR experiments were performed (Fig. 2). Total RNA was isolated from NT-26 grown under three different conditions [i.e. MSM containing 1) arsenite, 2) arsenite and 0.04% yeast extract and 3) 0.04% yeast extract] until late log phase. Transcripts of the *cytC* gene were detected when NT-26 was grown under all three growth conditions (lanes 2–4). This was also found to be the case for *moeA1* (data not shown). Confirmation that the *cytC* gene and *moeA1* are part of the same transcriptional unit was also obtained (lane 5). Transcripts of both *aroA* and *aroB* were detected only when NT-26 was grown in the presence of

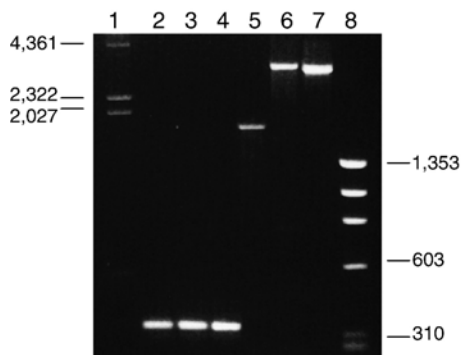


Fig. 2. RT-PCR analysis of the arsenite oxidase gene cluster. RNA was isolated from late logarithmic growth phase NT-26 cultures. The sizes of the molecular weight markers (lanes 1 and 8) are shown. Lanes 2–4 all show amplification of the *cytC* gene from NT-26 cultures grown in MSM containing arsenite, arsenite plus yeast extract and 0.04% yeast extract alone, respectively. Lane 5 shows successful co-amplification of the *cytC* and *moeA1* genes when grown in MSM containing arsenite and yeast extract. Lane 6 shows co-amplification of *aroB* and *aroA* when grown in MSM containing arsenite and yeast extract. Lane 7 shows the co-amplification of the *aroA* and the *cytC* genes when NT-26 was grown in MSM plus arsenite and yeast extract.

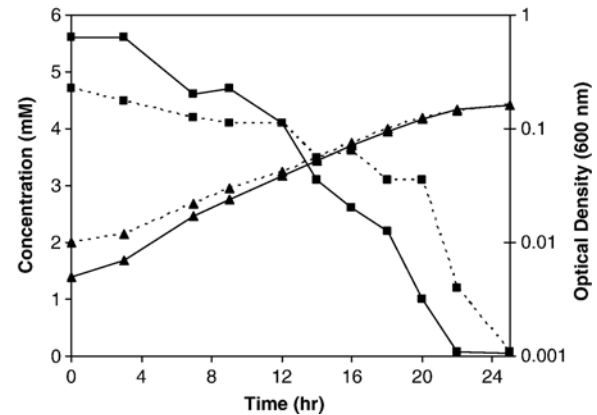


Fig. 3. Growth of NT-26 and NT-26 *cytC* mutant in a minimal salts medium containing arsenite (5 mM) and yeast extract (0.04%). NT-26 wild-type, solid lines and NT-26 *cytC* mutant, dashed lines. Arithmetic plot: ■, concentration of arsenite. Logarithmic plot: ▲, optical density.

arsenite (data not shown). Confirmation that *aroA* and *aroB* are transcribed together was also obtained (lane 6). Interestingly, confirmation that *aroA* and the *cytC* gene are transcribed together was obtained only when NT-26 was grown in the presence of arsenite (lane 7). These results suggest that apart from the putative promoter upstream of *aroB*, there may be an additional promoter upstream of the *cytC* gene that allows expression of the *cytC* and *moeA1* genes when NT-26 is grown in the absence of arsenite.

No PCR products were obtained when the RT step was omitted and only the Tfl DNA polymerase was used, confirming that no DNA contamination was present in the samples (data not shown).

3.3. The effect of a mutation in the *cytC* gene on arsenite oxidation and arsenite oxidase activity

The *cytC* gene was mutated by targeted gene disruption and the effects of this mutation on arsenite oxidation and arsenite oxidase activity were observed. NT-26 contains only a single copy of the *cytC* gene per genome as determined by Southern hybridisation (data not shown). When grown both chemolithoautotrophically and heterotrophically with arsenite as the electron donor the NT-26 *cytC* mutant continued to oxidise arsenite. A reproducible effect was observed on both growth (generation times for wild type and mutant were 4.2 ± 0.1 h and 4.5 ± 0.05 h, respectively) and the rate of arsenite oxidation when grown in a MSM containing 5 mM arsenite and 0.04% yeast extract (Fig. 3). The arsenite oxidation rate in the mutant is significantly slower in the first 22 h of growth and after 22 h 1.2 mM arsenite still remains in the culture medium compared to 0.06 mM for the wild type (Fig. 3). The same was observed when both the wild type and mutant were grown chemolithoautotrophically in a MSM containing 5 mM arsenite and $\text{CO}_2/\text{HCO}_3^-$ as the sole carbon source (data not shown). When grown in the absence of arsenite in a MSM containing 0.04% yeast extract alone no effect on growth of the *cytC* mutant was observed. These results suggest that although *cytC* does contribute to arsenite oxidation it is not essential.

As expected, there was no difference in arsenite oxidase activity (using DCPIP as the artificial electron acceptor) between the NT-26 wild type and the *cytC* mutant when grown in the MSM containing 5 mM arsenite and 0.04% yeast extract (for periplasmic extracts 0.067 and 0.064 μmol of arsenite oxidised min^{-1} mg of protein $^{-1}$, respectively).

3.4. Purification and characterisation of recombinant *c*₅₅₂

The *c*₅₅₂ was successfully heterologously expressed in *E. coli* and purified. The identity of the purified recombinant *c*₅₅₂ was confirmed by mass spectrometry and N-terminal sequencing (Fig. 4). The N-terminus of the recombinant protein was 'MDESNA', as expected, and with the exception of the N-terminus all expected tryptic peptides with masses >500 Da could be identified using MALDI-Tof (Fig. 4). The recombinant protein contained 0.98 ± 0.05 heme groups/molecule as determined by alkaline hemochrome spectra, which is consistent with the presence of a single CX₂CH motif in the gene sequence. Recombinant *c*₅₅₂ existed in both a monomeric and a dimeric form as judged by native PAGE and size exclusion chromatography. The apparent molecular masses of these two protein forms were 15.5 and 28 kDa using a calibrated size exclusion column. The apparent molecular mass of the

monomeric *c*₅₅₂ was also determined using native PAGE and was found to be 14.8 kDa. The molecular mass of monomeric recombinant *c*₅₅₂ was also determined using MALDI-Tof and found to be 13327 ± 7 Da, which is in very good agreement with the theoretical value of 13329 Da for the recombinant protein including one heme group (616.5 Da). CD spectra of both the monomeric and dimeric forms of recombinant *c*₅₅₂ indicated that the proteins were fully folded, and that the formation of the dimer led to small changes in protein conformation (data not shown).

The absorption spectrum of the ferric oxidised *c*₅₅₂ showed maxima at 410 nm in the Soret region and a diffuse α band at 531 nm (Fig. 5A). Ferric *c*₅₅₂ was reduced to its ferrous form in the presence of NT-26 Aro and arsenite as illustrated in Fig. 6A (α -552 nm, β -523 nm and Soret-417 nm). The addition of either Aro or arsenite alone did not reduce the cytochrome (data not shown).

Ferricyanide-oxidised horse heart cytochrome *c* could also accept electrons from the NT-26 Aro (Fig. 5B). The ferric heme spectrum showed a broad α band at 531 nm and Soret maximum at 409 nm. The cytochrome was reduced upon addition of the NT-26 Aro and arsenite (α -550 nm, β -520 nm and Soret-415 nm). The addition of NT-26 or arsenite alone did not result in reduction of the cytochrome (data not shown).

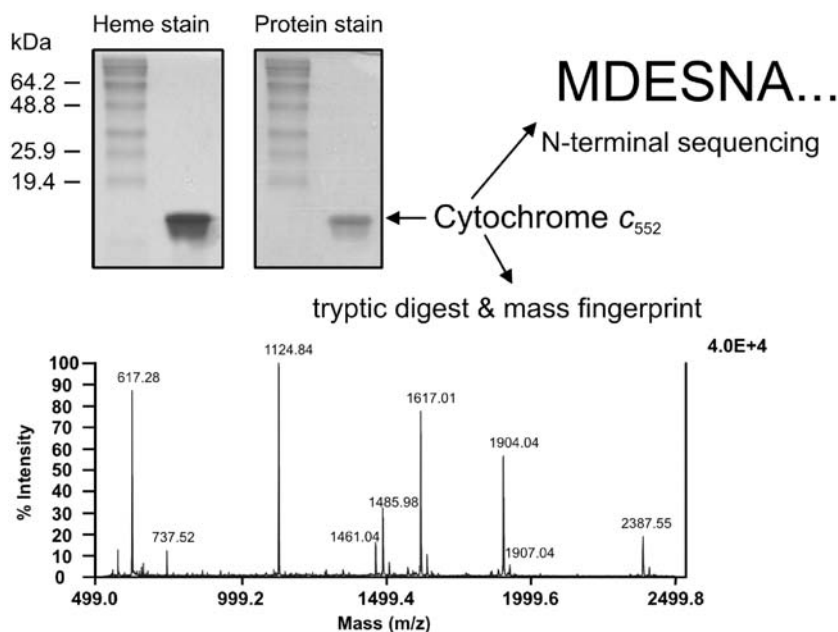


Fig. 4. Characterisation of recombinant *c*₅₅₂ by SDS-PAGE and mass spectrometry. Top panel: 12.5% SDS-polyacrylamide gel of recombinant *c*₅₅₂. Lanes were stained for heme-dependent peroxidase activity and protein as indicated. Bottom panel: mass fingerprint of *c*₅₅₂ following tryptic digestion. All fragments with masses >500 Da could be identified with the exception of the N-terminus (see below).

Theoretical fragment mass (<i>m/z</i>)	Amino acid numbering and sequence of fragment
617.28	aa 7–12, GAWFK
737.52	aa 83–89, MAFAGLK
1124.84	aa 28–38, VGPENGLIGR
1461.04	aa 90–102, KPEDVADVIAFYK
1485.98	aa 40–53, VAGVEGFNYSAPFK
1617.01	aa 105–117, TFSTPLEHHHHHH
1904.04	aa 14–27, CAACHAVGDGAANK+ 616.5 Da (heme group)
2387.55	aa 92–104, AEEGVWVDEVHLYLANPK

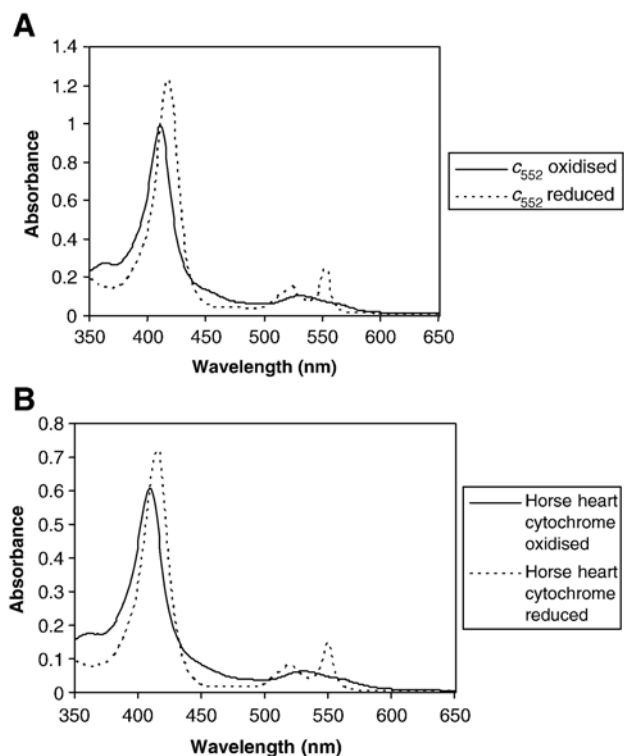


Fig. 5. Oxidised and reduced spectra of c_{552} and horse heart cytochrome. (A) Oxidised and reduced spectra of the purified c_{552} in the presence and absence of NT-26 Aro. (B) Oxidised and reduced spectra of horse heart cytochrome c in the presence and absence of NT-26 Aro.

Unlike in the case of *A. faecalis*, azurin (or pseudoazurin) was not detected in the periplasm of NT-26 using UV/vis spectroscopy when it was grown with arsenite (data not shown). Moreover, *Pseudomonas aeruginosa* azurin which could serve as an electron acceptor to the *A. faecalis* arsenite oxidase (15) was not reduced in the presence of the NT-26 Aro

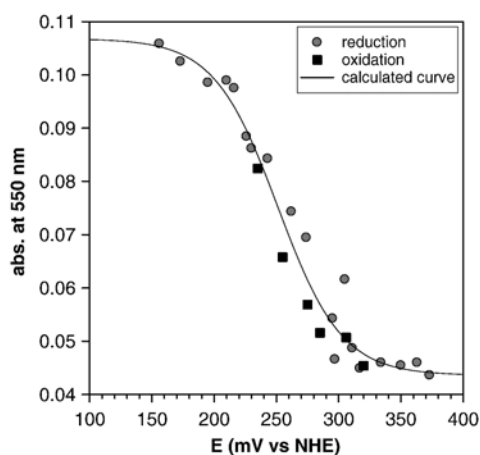


Fig. 6. Redox titration of the c_{552} (4 μ M, pH 6.6) showing absorption at 550 nm (from ferrous cytochrome) as a function of solution potential. The solid line shows the Nernstian curve for a single electron transfer at a potential of $+251 \pm 5$ mV vs. NHE. The reductive and oxidative directions of the titration are indicated by circles and squares respectively. See Materials and methods for other conditions.

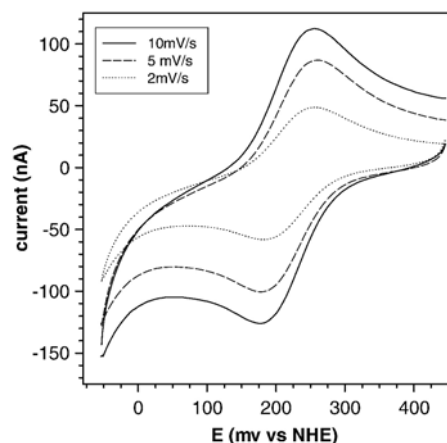


Fig. 7. Cyclic voltammograms of c_{552} (20 μ M) at sweep rates of 2, 5 and 10 mV s^{-1} (pH 7.1). See Materials and methods for other conditions. The averaged potential of the reductive (negative) and oxidative (positive) peaks is $+217$ mV vs. NHE.

and arsenite; a maximum in the absorbance spectrum at 628 nm indicative of fully oxidised azurin was observed despite varying the azurin concentration (data not shown).

3.5. Determination of the c_{552} redox potential

Two approaches were taken to determine the $\text{Fe}^{\text{III/II}}$ redox potential of the c_{552} . Redox potentiometry (an equilibrium method) was employed and the change in absorbance at 550 nm (characteristic of the ferrous cytochrome) as a function of potential is shown in Fig. 6. No hysteresis was apparent as is evident from superposition of the oxidative (squares) and reductive (circles) titrations. A midpoint potential of $+251 \pm 5$ mV vs. NHE was determined at pH 6.5. This potential is similar to other c -type cytochromes such as that from horse heart [35–37] and consistent with the ability of both cytochromes to accept electrons from the Aro.

The heme redox potential was also pursued using direct electrochemistry with the technique of cyclic voltammetry. There are many different ways of conducting direct electrochemistry with proteins but most require specially modified electrodes to avoid denaturing with electrode surface fouling and to facilitate fast electron transfer. The method used here was adapted from previous work on horse heart cytochrome c where a glassy carbon electrode was coated with a permeable layer of Eastman AQ-29D polymer [38]. The polymer is a polyester sulfonic acid and at the pH investigated here contains sulphonate groups capable of attracting proteins with high isoelectric points, as is the case here.

Clear reductive and oxidative currents were observed (Fig. 7) and the average of the peak potentials gave the formal potential under these conditions ($+217$ mV vs. NHE) at pH 6.5. This potential is slightly lower than that determined potentiometrically. One possible reason for this difference is an electrostatic influence of the negatively charged polymer which may lower the apparent redox potential, but it should be noted that the difference between the potentials determined potentiometrically and voltammetrically is rather small.

The voltammetric peak currents increase linearly with the square root of the sweep rate which is characteristic behaviour for a diffusion controlled process [39]. On the basis of previous work with the same working electrode it seems that the c_{552} is capable of diffusing through the polymer film albeit slowly. Higher sweep rates ($>10 \text{ mV s}^{-1}$) gave more distorted voltammetric profiles. It is notable that the protein had to be equilibrated with the polymer-coated working electrode for at least 1 h. During this period it is apparent that the protein permeates the polymer and it is this partially entrapped protein that is electroactive. Similar observations have been made before with the same electrode preparation [38].

4. Discussion

This study is the first to describe the involvement of a c -type cytochrome in chemolithoautotrophic arsenite oxidation. A c -type cytochrome gene was identified in the NT-26 arsenite oxidase gene cluster downstream of *aroBA* and was designated *cytC*. The encoded protein is periplasmic, with a sequence 96% identical to the putative c -type cytochrome of the arsenite-oxidising strain of *A. tumefaciens*. It is also related to various α proteobacterial c -type cytochromes, the heme 2 domains of SoxD proteins and mitochondrial cytochrome c . The c_{552} is phylogenetically distant from (sharing only 20.5% sequence identity) AoxD, a putative c -type cytochrome in the arsenite oxidase gene cluster of the arsenite-oxidising β -*Proteobacterium Herminiimonas arsenicoxydans* sp. str. ULPAs1 [40,41].

Although the NT-26 arsenite oxidase gene cluster (Fig. 1) has the same structure as that of the arsenite-oxidising *A. tumefaciens* [see [34]] it is regulated differently. In *A. tumefaciens* the *aroB*, *aroA*, c -type cytochrome and *moeA* genes are transcribed as a single unit in the presence and absence of arsenite (note: in the absence of arsenite no transcript was detected until late log phase) [34]. The expression studies above showed that in NT-26 *aroB* and *aroA* were transcribed only in the presence of arsenite; and no Aro activity was detected in its absence [4]. Expression of the NT-26 *cytC* gene, however was not dependent on induction by arsenite. Therefore, it appears that there are two promoters in the NT-26 arsenite oxidase gene cluster: one upstream of *aroB* and another upstream of the *cytC* gene.

Examination of the *cytC* mutant shows that this gene contributes to, but is not essential for, arsenite oxidation by NT-26. There must therefore be another protein (e.g. another c -type cytochrome) that can act as an alternative electron acceptor to the Aro in NT-26. This protein is unlikely to be an azurin (or even pseudoazurin) as none was detected in the periplasm when NT-26 was grown with or without arsenite and NT-26 Aro with arsenite did not reduce *P. aeruginosa* azurin.

The $\text{Mo}^{\text{VI/IV}}$ redox potential of *A. faecalis* arsenite oxidase is reported [42] to be 292 mV vs. NHE (pH 5.9) while the [3Fe–4S] cluster and Rieske center potentials are ca. 260 mV and 130 mV, respectively. It is known [15] that the *A. faecalis* arsenite oxidase (in the presence of arsenite) can reduce *P. aeruginosa* azurin (E_m 307 mV) [43] but not horse heart

cytochrome c (E_m 248 mV) [37]. Here we have found the exact opposite with the NT-26 Aro, where the stronger oxidant azurin cannot be reduced by the Aro but horse heart cytochrome c can. Therefore, the marked differences in reactivity between the *A. faecalis* arsenite oxidase and the NT-26 Aro and various possible electron acceptors are clearly unrelated to redox potentials, but instead may be indicative of matched or mismatched non-covalent intermolecular forces between the various enzyme–electron partner combinations.

Here we have described for the first time the *in vivo* and *in vitro* involvement of a c -type cytochrome in arsenite oxidation by NT-26. This protein contributes to, but is not essential to, arsenite oxidation and arsenite is not essential for its synthesis. NT-26 must therefore contain at least one other protein that can serve as an electron acceptor to the Aro. We are presently trying to identify this protein(s) using a combination of molecular genetic and genomic approaches. Elucidation of the arsenite oxidation electron transport chain will allow us to determine how organisms like NT-26 gain energy from arsenite oxidation and whether any of these components share similarities to other metabolic proteins.

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