Ferrous iron oxidation and rusticyanin in halotolerant, acidophilic '*Thiobacillus prosperus*'

James Le C. Nicolle,[†] Susan Simmons,[‡] Stephan Bathe[§] and Paul R. Norris

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Correspondence

Paul R. Norris

P.R.Norris@warwick.ac.uk

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The halotolerant acidophile '*Thiobacillus prosperus*' was shown to require chloride for growth. With ferrous iron as substrate, growth occurred at a rate similar to that of the well-studied acidophile *Acidithiobacillus ferrooxidans*. Previously, the salt (NaCl) requirement of '*T. prosperus*' was not clear and its growth on ferrous iron was described as poor. A subtractive hybridization of cDNAs from ferrous-iron-grown and sulfur-grown '*T. prosperus*' strain V6 led to identification of a cluster of genes similar to the *rus* operon reported to encode ferrous iron oxidation in *A. ferrooxidans*. However, the '*T. prosperus*' gene cluster did not contain a homologue of *cyc1*, which is thought to encode a key cytochrome *c* in the pathway of electron transport from ferrous iron in *A. ferrooxidans*. Rusticyanin, another key protein in ferrous iron oxidation by *A. ferrooxidans*, was present in '*T. prosperus*' at similar concentrations in cells grown on either ferrous iron or sulfur.

INTRODUCTION

Salt (NaCl) tolerance in ferrous-iron-oxidizing acidophiles is of considerable interest in the context of potential biomining operations where chloride is present in the ore or where only saline process water might be readily available. *Acidithiobacillus ferrooxidans*, the most intensely studied acidophilic, ferrous-iron-oxidizing bacterium, is not tolerant of salt and many strains are totally inhibited by 1% (w/v) NaCl (Razzell & Trussell, 1963; Brock, 1975). The salt-tolerant, iron- and sulfur-oxidizing '*Thiobacillus prosperus*' (Huber & Stetter, 1989) was isolated from close to marine hydrothermal vents of Vulcano (one of the Aeolian Islands), but has been the subject of little work beyond its original description. The original isolate was proposed as the 'type strain', but because it has not been formally named, this designation is inappropriate. It is

referred to in this paper as '*T. prosperus*' DSM 5130 in order to distinguish it from other isolates of the species. Its presence in the genus *Thiobacillus*, which is a member of the β -Proteobacteria (Kelly & Wood, 2000), would be incompatible with its phylogenetic position in the γ -Proteobacteria (Goebel *et al.*, 2000; Meyer *et al.*, 2007).

The mechanism of ferrous iron oxidation by proteobacteria has been extensively studied only with *A. ferrooxidans*. The genes proposed to encode the major components involved in electron transfer from ferrous iron to oxygen comprise an operon encoding an outer-membrane protein of unknown function, two cytochromes *c*, rusticyanin and a cytochrome oxidase *aa₃* (Appia-Ayme *et al.*, 1999). Rusticyanin, a periplasmic, small blue copper protein, has also been the focus of much work concerning the structural basis of its acid stability and high mid-point redox potential (e.g. Botuyan *et al.*, 1996; Kanbi *et al.*, 2002). Examination of six strains of *A. ferrooxidans* showed that three of them contained two rusticyanin isozymes, types A and B (Sasaki *et al.*, 2003). Type A was present in all the strains. This paper describes a gene cluster in '*T. prosperus*' that is similar to the *rus* operon of *A. ferrooxidans*.

METHODS

Organisms and growth conditions. Strains of '*Thiobacillus prosperus*' were isolated from a shallow, acidic pool by the shore of Baia di Levante, Vulcano, as described previously (Simmons & Norris, 2002), and from acidic, geothermal, shoreline sediments of Palaeochori Bay, Milos, in the Aegean Sea. Single colonies were obtained from ferrous-iron-supplemented enrichment cultures by

[†]Present address: Green Biologics Ltd, Unit 45A, Milton Park, Abingdon, Oxfordshire OX14 4RU, UK.

[‡]Present address: National Blood Service, Southmead Road, Bristol BS10 5ND, UK.

[§]Present address: Institut für Ingenieurbiologie und Biotechnologie des Abwassers, Universität Karlsruhe (TH), Am Fasanengarten, 76128 Karlsruhe, Germany.

Abbreviations: DP, difference product; mRDA, micro-representational difference analysis; SD, Shine–Dalgarno.

The GenBank accession numbers for the partial 16S rRNA gene sequences of '*T. prosperus*' strain V6 and '*T. prosperus*' (DSM 5130) are EU653290 and EU653291, respectively. The GenBank accession number for the sequence of the genomic DNA region containing the strain V6 *rus*-operon-like gene cluster is EU653292.

serial dilution and plating on ferrous-iron-supplemented medium solidified with Phytigel (Sigma). Strain V6 (one of the isolates from Vulcano) and '*Thiobacillus prosperus*' (DSM 5130) were grown at 35 °C and *Acidithiobacillus ferrooxidans* (ATCC 23270^T) was grown at 30 °C in a medium which contained MgSO₄·7H₂O (0.4 g l⁻¹), (NH₄)₂SO₄ (0.2 g l⁻¹), K₂HPO₄ (0.1 g l⁻¹) and FeSO₄·7H₂O (10 mg l⁻¹). The initial pH was adjusted with H₂SO₄ to pH 1.7 for growth on ferrous iron and to pH 3 for growth on sulfur. Sodium chloride was added as required to test its effect on growth. Growth substrates were ferrous iron (50 mM iron supplied as FeSO₄·7H₂O, 13.9 g l⁻¹) or elemental sulfur (5 g l⁻¹). The effects of yeast extract and potassium tetrathionate on growth were investigated using inocula from cultures grown under identical conditions to those under test. Potassium tetrathionate (0.5 mM) was used to meet a requirement for a reduced sulfur source for growth of strain V6 and '*T. prosperus*' DSM 5130 on ferrous iron (see Results). Ferrous-iron-grown cultures were harvested by centrifugation when 50 % of the substrate was oxidized and sulfur-grown cells were harvested when the pH reached about 1.9. Cells were washed by resuspension in acidified water (pH 1.7) and then in deionized water prior to storage of pellets at -80 °C. Ferrous iron oxidation was determined by titration of residual ferrous iron. One-millilitre samples from cultures were added to 1 ml 5 % (w/v) sulfuric acid and titrated against standard ceric sulfate (Fisher Scientific) using 1,10-phenanthroline-ferrous-complex solution (Fisher Scientific) as the end-point indicator. Cell numbers and biomass volume were determined by orifice electrical flow impedance using a CellFacts Particle Size Analyser (CellFacts Instruments).

Cell fractionation, SDS-PAGE and spectrophotometry. Strain V6 and *A. ferrooxidans* cells were resuspended in water acidified to pH 2 with sulfuric acid and were lysed by sonication. Cell debris was removed by centrifugation at 15 000 g for 10 min. Cell membranes were pelleted by centrifugation at 48 000 g for 30 min, washed by resuspension and recentrifuged. Supernatant and membrane fractions were analysed by SDS-PAGE (15 %, w/v, acrylamide) and staining with *o*-dianisidine for haem or with Coomassie blue. Optical spectra of membrane and soluble fractions were determined at pH 6.5 and pH 2 respectively at room temperature and with a Hewlett Packard 8452A spectrophotometer. Ammonium persulfate and sodium dithionite were used as oxidant and reductant respectively.

Micro-representational difference analysis (mRDA). Total RNA was extracted from '*T. prosperus*' strain V6 using TRIzol reagent following the manufacturers' instructions (Invitrogen). mRDA was carried out as described previously (Becker *et al.*, 2001; Bathe & Norris, 2007). Difference products 1 (DP1) and 2 (DP2) were obtained from two rounds of subtractive hybridization with tester and driver cDNAs from ferrous-iron-grown cells and from sulfur-grown cells respectively. DNA fragments from DP2 were cloned using the TOPO-TA cloning kit (Invitrogen) after separation by electrophoresis. Extended sequences of selected DNA fragments were obtained following inverse PCR with RNA-free chromosomal DNA, which was digested with a number of restriction enzymes and recircularized at a concentration of 5 ng µl⁻¹ for PCR templates. BLAST searches were made of GenBank databases (Altschul *et al.*, 1997) and the *A. ferrooxidans* genome (available from The Institute for Genomic Research website: <http://www.tigr.org>).

Reverse transcription (RT-PCR). The ImProm-II reverse transcription system (Promega) was used for first-strand cDNA synthesis for RT-PCR. This PCR used cDNA corresponding to 50 ng RNA in a 25 µl reaction volume with 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer (Table 1) and 0.02 U µl⁻¹ of Platinum Taq DNA polymerase (Invitrogen). Between initial denaturation at 94 °C for 2 min and a final 5 min at 72 °C, there were 30 cycles comprising 0.5 min at 94 °C, annealing for 1 min at approximately 2 °C below

Table 1. Oligonucleotide primers used for RT-PCR

Oligonucleotides are named with respect to the ORF in which they bind and their orientation (f or r). Positions refer to the sequence NCBI GenBank EU653292.

Name	Sequence (5'-3')	Position
cyc2(f)	GGCCAGTACTTCGGCGTTAG	3584-3603
ORF1(r)	CCGCGAAGAAGTTCTGATCC	4338-4357
ORF1(f)	TCACCATCGACACCAAGTCC	4361-4380
coxB(r)	AATGACCACTGCCACTGCTG	5050-5069
coxB(f)	AAGCGCGCAGATCCATACTC	4881-4900
coxA(r)	ATACGCCAATCCCCATGAAC	5673-5692
coxA(f)	GATCTCCATCAACGCCTTCC	7086-7105
coxC(r)	CTTGCGGATTTTTCCTTTCG	7845-7864
coxC(f)	TTGTTCCGACATCGAGTGC	7601-7620
coxD(r)	CAACCGTATGCGGAATGATG	8033-8052
coxD(f)	TCATTCCGCATACGGTTGAC	8035-8054
ctaA(r)	GCGATGAACCATCTCCTTCC	8541-8560
ctaA(f)	CGATCCAGGTAGTGCACAGG	9078-9097
cyoE(r)	CAAACGTAAGCCCCTGGAAC	9796-9815
cyoE(f)	ATTCCTATGCTGCCCGTGAC	10124-10143
rus(r)	ACCTGATTATGGCCCGTCAC	10882-11001

the lower primer melting temperature and extension at 72 °C for 1 min per 1 kbp product length.

Mass spectrometry. Proteins from soluble fractions of strain V6 and *A. ferrooxidans* were separated by hydrophobicity-dependent release from an HPLC column. Tryptic-digest fragments were analysed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with a Q-ToF instrument fitted with an in-line capillary LC system (Waters MS Technologies). Peptide mass fingerprints were analysed using the Mascot v2.1 (Matrix Science) search engine aimed at the NCBI database and an in-house database (consisting of UniProt sequences and the strain V6 genomic fragment sequenced in this work).

Sequencing and nucleotide accession numbers. Partial 16S rRNA genes of '*T. prosperus*'-like isolates were amplified by a PCR with forward (F27, 5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (R1492, 5'-TACGGYTACCTTGTTACGACTT-3') primers. PCR products were sequenced following cloning with the TOPO-TA Cloning kit (Version J) vector (pCR2.1-TOPO) and host *Escherichia coli* strain (Invitrogen). The partial 16S rRNA gene sequences of '*T. prosperus*' strain V6 and '*T. prosperus*' DSM 5130 have been deposited in the GenBank database with accession numbers of EU653290 and EU653291 respectively. The sequence of the genomic DNA region containing the strain V6 *rus*-operon-like gene cluster (see Results) has been deposited with GenBank accession number EU653292.

RESULTS

Isolation and growth of '*T. prosperus*' strain V6

Ferrous-iron-oxidizing '*T. prosperus*'-like isolates were obtained from solid medium on which the colonies were coloured dark brown by the deposition of hydrated ferric oxides or sulfates. 16S rRNA gene sequences were obtained from four isolates whose origins were two different samples

from Vulcano and two different samples from acidic coastal sediments of Milos. These sequences were the same but differed from that of the '*T. prosperus*' DSM 5130 principally in a short sequence (9 nt) of one loop of the molecule (Davis-Belmar *et al.*, 2008).

One of the new isolates from Vulcano (strain V6) was selected for further studies; representative growth curves are shown in Fig. 1. It grew autotrophically with ferrous iron as substrate when the medium was supplemented with tetrathionate, but not in ferrous-iron-containing medium without any supplements. Oxidation of 50 mM ferrous iron was complete with an initial concentration of 1 mM tetrathionate (Fig. 1a). The initial rate of growth-associated

Fig. 1. Growth and ferrous iron oxidation by '*T. prosperus*' strain V6 at 35 °C. (a) Effect of tetrathionate concentration on growth-associated ferrous iron oxidation (a); (b, c) particle number and total volume in the absence (b) and presence (c) of yeast extract.

ferrous iron oxidation was similar with lower concentrations of tetrathionate but the oxidation ceased abruptly when limiting amounts of tetrathionate were provided (Fig. 1a). The rate of ferrous iron oxidation by strain V6 was not affected by yeast extract (Fig. 1b, c), but cells were attached to vessel surfaces in its presence and not free in suspension until about 20 % of the ferrous iron was oxidized (Fig. 1c). Thereafter, cells were released into suspension and the final cell yield was similar to that in the absence of yeast extract. Addition of Tween 20 (final concentration 0.01 %, v/v) before 20 % of the ferrous iron was oxidized released the cells into suspension immediately (data not shown). There was no precipitation of iron during the time-course analyses, so particle counts and particle volumes were derived from cells. Cell counts and the total particle volumes indicated a similar rate of growth in the absence of aggregated cells. The doubling time at the optimum temperature of 36 °C was about 4 h (data not shown).

Growth was optimal with NaCl between 1 % and 2 % (w/v) (Davis-Belmar *et al.*, 2008) and was maintained at a reduced rate through serial cultures with 6 % (w/v) NaCl (data not shown). '*T. prosperus*' strains V6 and DSM 5130 did not grow when NaCl was omitted from the medium. Growth occurred when the NaCl was replaced by MgCl_2 but not by Na_2SO_4 (data not shown). '*T. prosperus*' strain V6 also grew on tetrathionate, elemental sulfur and mineral sulfides (C. Davis-Belmar & P. R. Norris, unpublished data).

mRDA with ferrous iron- and sulfur-grown strain V6

Seven major bands were seen on electrophoresis of difference product 2 (DP2) with ferrous-iron-grown cells as the source of tester cDNA (Fig. 2). BLAST searches with a cloned and sequenced DNA fragment from band A showed that the predicted product had 40 % identity to a conserved, uncharacterized protein from many bacteria, including *Leptospirillum ferrooxidans* and species of *Chloroflexus* and *Vibrio*. The predicted product of a band B gene fragment showed about 50 % identity to cytochrome *bd* ubiquinol oxidases from *Pseudomonas syringae*, several cyanobacteria and *A. ferrooxidans*. Potential coding regions of band D and band E fragments indicated products with similarities only to database hypothetical proteins of various bacteria. A GenBank BLAST search showed that the band F-derived gene product sequence was most similar to those of cytochrome *c* precursors of *Pseudomonas aeruginosa* and *Ralstonia metallidurans*. However, a BLASTX search of the *A. ferrooxidans* genome indicated greater identity (36 % over 63 amino acids) and similarity (50 %) of this product to a cytochrome *c* family protein, the gene for which was not located close to any others encoding redox-active proteins. The predicted protein product of fragments cloned from band G showed identities of about 90 % to DnaK from various proteo-

Fig. 2. Agarose gel electrophoresis of the cDNA amplicon (Amp), and the first (DP1) and second (DP2) difference products from mRDA of ferrous iron- versus sulfur-grown '*T. prosperus*' strain V6 with tester cDNA from iron-grown cells. M, 100 bp ladder.

bacteria. A sequence derived from band C showed 53 % identity (over 56 amino acids) to the *A. ferrooxidans* cytochrome Cyc2, previously indicated as being involved in ferrous iron oxidation (Appia-Ayme *et al.*, 1999). This band C gene was used to initiate a series of inverse PCRs that led to sequencing of 11.8 kb of genomic DNA, mainly downstream of the putative *cyc2* gene.

Genes of the *rus* operon-like gene cluster

In BLAST searches, seven of the ORFs found in the sequenced 11.8 kb of strain V6 DNA showed highest identities and similarities to genes of the *A. ferrooxidans* *rus* operon. These ORFs were preceded by putative Shine–Dalgarno (SD) sequences (Table 2). The second gene in the

A. ferrooxidans operon, *cyc1*, was not found in the strain V6 gene cluster (Fig. 3). Most previous *rus*-operon work has involved *A. ferrooxidans* ATCC 33020 (Appia-Ayme *et al.*, 1999) but the genome sequence of *A. ferrooxidans* ATCC 23270^T was used here for comparison in order to include the putative cytochrome assembly genes *ctaA* and *cyoE*, which have not been described for *A. ferrooxidans* ATCC 33020. These genes precede the *rus* gene in strain V6 and appear relatively unrelated to those of *A. ferrooxidans* ATCC 23270^T, in which they follow the *rus* gene. The strain V6 predicted CtaA amino acid sequence showed most similarity to sequences from various, mostly marine, γ -proteobacteria. The precise start of the gene in strain V6 is not certain, with alternative possible SD regions and start codons. The indicated size of the protein (Table 2) assumes a similar size to the most closely related proteins, which are found in *Nitrococcus* and *Alkalilimnicola* species and have approximately 40 % identity to the strain V6 protein over 340 amino acids. The *A. ferrooxidans* CtaA sequence appears most similar to that of one γ -proteobacterium, *Nitrococcus mobilis*, while the other most related sequences are from acidophilic archaea, including species of *Picrophilus*, *Metallosphaera* and *Sulfolobus*. The strain V6 putative CyoE protein was more closely related to those of various γ -proteobacteria (for example, species of *Pseudomonas*, *Alteromonas* and *Idiomarina*) than to the *A. ferrooxidans* protein (data not shown). The *ctaA* and *cyoE* genes in strain V6 are immediately downstream of four genes that encode subunits of a cytochrome oxidase *aa₃* and which are here named *coxBACD* following the designations used previously with *A. ferrooxidans* (Appia-Ayme *et al.*, 1999).

The predicted Cyc2 protein of strain V6, minus the predicted signal peptides, is slightly smaller than the mature *A. ferrooxidans* protein, with insertions or deletions leaving two unmatched regions (of 5 and 18 amino acids) in the strain V6 sequence and several unmatched, dispersed

Table 2. Putative *rus* operon-like genes and inferred gene products in '*T. prosperus*' strain V6

Gene/inferred protein	Putative SD core...→...start codon	Inferred protein (aa/kDa)	% Identity/similarity to related aa sequences of <i>A. ferrooxidans</i> *
<i>cyc2</i> /cyt. Cyc2	ggagg...n ₅ ...atg	464/49.4	
Mature Cyc2		434/46.5	37/56 (411)
<i>orf1</i> /Orf1, unknown	ggaga...n ₈ ...atg	190/21.0	46/61 (181)
<i>coxB</i> /cyt. <i>c</i> oxidase subunit I	gaggt...n ₈ ...atg	251/28.3	49/65 (249)
<i>coxA</i> /cyt. <i>c</i> oxidase subunit II	agagg...n ₈ ...atg	632/69.8	54/72 (620)
<i>coxC</i> /cyt. <i>c</i> oxidase subunit III	gagga...n ₈ ...atg	189/21.1	31/57 (182)
<i>coxD</i> /cyt. <i>c</i> oxidase subunit IV	ggagg...n ₈ ...atg	72/8.6	32/52 (60)
<i>ctaA</i> /‘haem A synthase’	gcaag...n ₅ ...atg	356/38.5	—
<i>cyoE</i> /protohaem farnesyltransferase	ggaga...n ₇ ...atg	328/35.8	—
<i>rus</i> /rusticyanin	ggagg...n ₅ ...atg	187/19.6	
Mature rusticyanin		155/16.3	50/61 (150)

*Number of residues compared after exclusion of gaps and non-overlapping terminal regions of sequences.

Fig. 3. The *A. ferrooxidans* ATCC 23270^T *rus* gene operon and similar genes in '*T. prosperus*' strain V6. The results of RT-PCRs with primers situated in adjacent ORFs of strain V6 are also shown. Horizontal lines indicate the regions between the primer pairs; the associated triplets of agarose gel lanes indicate the results of the PCR with cDNA derived from ferrous-iron-grown cells (left-hand lanes), with negative controls minus RT in the cDNA synthesis protocol (middle lanes), and with genomic DNA (right-hand lanes).

regions (up to 9 amino acids) in the *A. ferrooxidans* protein. The N-terminal regions of the mature proteins of strain V6 (VPAFARQLGVSCAACHT) and *A. ferrooxidans* (LPSFARQTGWSCAACHT) include sequences (CXXCH) typical of haem-binding sites and share 66 % identity over the first 35 amino acids.

The precise length of an apparent signal peptide in the strain V6 ORF1 sequence could not be predicted, but the identity of the mature protein to that of *A. ferrooxidans* was estimated at about 50 % compared to the 46 % identity of the gene products (Table 2).

PCR products of the expected size were obtained using cDNA and forward and reverse primers (Table 1) designed to amplify intergenic regions of adjacent genes in the strain V6 *cyc2-ORF1-coxB-coxA-coxC-coxD* sequence, indicating that these genes are co-transcribed (Fig. 3). A weak PCR product was obtained with primers linking the *coxD* and *ctaA* genes. There was no evidence for cotranscription of the *cyoE* and *rus* genes. Two potential stem-loop structures (with 14 bp and 11 bp stems) were present after the *rus* gene. The ORFs found immediately upstream and downstream of those indicated for *A. ferrooxidans* and '*T. prosperus*' strain V6 (Fig. 3) were different in each organism, without any obvious link to electron transport. In the case of *A. ferrooxidans*, these were related to a transposase (upstream) and a major facilitator superfamily MFS_1 protein (downstream). In strain V6, the indicated upstream ORF showed similarities to response regulators containing CheY-like receiver and GGDEF domains, and potential ORFs immediately downstream of the *rus* had no database matches.

'*T. prosperus*' rusticyanin

The same numbers of residues occur in the leader sequences (32 amino acids) and the mature Rus proteins

(155 amino acids) of *A. ferrooxidans* ATCC 23270^T (Rus A) and strain V6. However, there is a five-residue extension of the *A. ferrooxidans* mature protein N-terminal sequence and five insertions at various positions in the strain V6 protein (Fig. 4). Overall sequence identity between the mature proteins over 150 paired amino acids is 50 %. The strain V6 mature protein sequence is also 49 % identical to the RusB sequence of *A. ferrooxidans* strain 3865 (Sasaki *et al.*, 2003). The strain V6 and *A. ferrooxidans* proteins show little similarity over the 16 N-terminal residues. The highly conserved C-terminal regions contain three (C138, H143 and M148) of the four ligands of the copper ion (demonstrated with *A. ferrooxidans*). The fourth ligand, H85, is also present in the strain V6 protein.

Fig. 4. An alignment of the predicted mature rusticyanin of '*T. prosperus*' strain V6 and rusticyanin A of *A. ferrooxidans* ATCC 23270^T. Identical regions are boxed and the four copper ligands as determined for the *A. ferrooxidans* protein are in bold type.

Freshly prepared, soluble fractions of *A. ferrooxidans* grown on ferrous iron were pale blue, as described previously, with the colour attributed to rusticyanin (Cobley & Haddock, 1975), whereas those of strain V6 grown on ferrous iron were red. On oxidation, the soluble fractions of both species showed broad absorbance peaks from 570 to 595 nm (Fig. 5a) with the presence of *c*-type cytochromes in the crude fractions probably causing a shift of the absorbance maximum from the 597 nm associated with purified rusticyanin of *A. ferrooxidans* (Cox & Boxer, 1978). Difference spectra of soluble fractions from strain V6 and *A. ferrooxidans* were similar, with principal Soret-, β - and α -absorbance peaks at 419, 522–524 and 551–552 nm respectively (Fig. 5b). Additional absorbance peaks at 440 and 595–597 nm, typical of cytochrome *aa*₃ oxidases, were found with membranes of both bacteria (Fig. 5c). An absorbance peak at 557 nm was specific to the strain V6 membrane fraction. SDS-PAGE of the soluble fractions of strain V6 and *A. ferrooxidans* showed major bands with apparent molecular masses of about 18 and 19 kDa respectively (Fig. 6a). MS/MS peptide analysis of the proteins constituting these bands showed that the sequences of two different peptides (11 and 22 amino acids) from the *A. ferrooxidans* band and two different peptides (13 and 21 amino acids) from the strain V6 band were identical to parts of the amino acid sequences of the respective rusticyanins of these bacteria. These proteins ran with the expected apparent molecular masses (about 16 kDa) when cell extracts were prepared in the presence of β -mercaptoethanol and boiled prior to electrophoresis (C. Davis-Belmar & P. R. Norris, unpub-

lished data). Comparison of soluble fractions from ferrous-iron-grown and sulfur-grown cells of strain V6 showed similar levels of rusticyanin were present during growth on each substrate (Fig. 6b). A haem-staining protein of apparent molecular mass about 15 kDa was present in the strain V6 soluble extract and more abundant in ferrous-iron-grown cells (Fig. 6c). This protein migrated further, with an apparent molecular mass of about 12 kDa, on electrophoresis of boiled preparations.

DISCUSSION

Strain V6 is a new isolate of '*T. prosperus*', with a short, different sequence (9 nt) in one region of a 16S rRNA gene that is otherwise very similar to that of the 'type strain' (Davis-Belmar *et al.*, 2008). However, its growth on ferrous iron did not fit that described previously for the 'type strain'. This was noted as very poor (Huber & Stetter, 1989) compared to *A. ferrooxidans*, or as extremely slow and weak (Huber *et al.*, 1986). The relatively good growth of strain V6 (and '*T. prosperus*' DSM 5130) on ferrous iron in the present study probably resulted from supplementation of the medium with a reduced sulfur source (tetrathionate). A source of reduced sulfur is also necessary for growth and ferrous iron oxidation of some other acidophiles (Norris & Barr, 1985). The effects of temperature on growth of strain V6 and its tolerance of salt were as described previously for '*T. prosperus*' DSM 5130 (Huber & Stetter, 1989). '*T. prosperus*' DSM 5130 was described as growing with 0–3.5 % salt, with highest final cell

Fig. 5. Room temperature, optical spectra of soluble and membrane fractions of '*T. prosperus*' strain V6 (upper spectra) and *A. ferrooxidans* ATCC 23270^T (lower spectra): (a) oxidized soluble fractions (0.7 mg protein ml⁻¹); (b) reduced minus oxidized difference spectra of soluble fractions (0.5 mg protein ml⁻¹); (c) membrane fractions (0.5 mg protein ml⁻¹).

Fig. 6. SDS-PAGE of soluble fractions (10 µg protein per lane) of '*T. prosperus*' strain V6 and *A. ferrooxidans* ATCC 23270^T with Coomassie blue (a and b) or haem staining (c). Growth substrates were ferrous iron (Fe) or sulfur (S). Rusticyanins are arrowed.

concentrations in the absence of added salt (Huber & Stetter, 1989). However, strain V6 showed a requirement for chloride, and '*T. prosperus*' DSM 5130 also could not be maintained in salt-free medium (not shown). The medium given as that usually used by Huber & Stetter (1989) contained about 2.3 g Cl l⁻¹ from various salts, e.g. NH₄Cl (Huber *et al.*, 1986), equivalent to about 0.38 % (w/v) NaCl. Therefore, the slightly slower growth on addition of a further 1 % (w/v) NaCl (Huber & Stetter, 1989) was similar to the influence of the salt concentration seen with strain V6 (data not shown).

Three cytochrome-encoding genes were identified via mRDA with strain V6. The functions of one of the predicted cytochromes *c* and the ubiquinol *bd* oxidase are unknown. The presence of the other cytochrome *c*-encoding gene in a *rus* operon-like gene cluster indicates that its product probably functions like its homologue, Cyc2, in *A. ferrooxidans*. The absence of the *cyc1* gene from the strain V6 *rus* operon does not preclude the presence of a Cyc1-equivalent cytochrome encoded by a gene at a different location. However, the abundance of the small, haem-staining cytochrome in strain V6, an abundance that was much greater in ferrous-iron-grown cells than sulfur-grown cells (Fig. 6), suggests that it could be responsible for the red colour of the strain V6 soluble fraction and have a role in growth on ferrous iron. Multiple cytochromes *c* have been found in *A. ferrooxidans*, including a small cytochrome that was visualized by chemiluminescence, but an abundant small cytochrome that haem stains with *o*-dianisidine was not revealed (Yarzabal *et al.*, 2002).

The most conserved regions of the '*T. prosperus*' and *A. ferrooxidans* rusticyanins, after the C-terminal region, are the proline-rich loop 5 (11 identical consecutive residues)

and loop 9 (eight identical consecutive residues). After the N-terminal region, these are the most flexible regions, with relevance to chain interactions and folding processes (Alcaraz *et al.*, 2005). Proteins that are adapted to an acid environment, such as the extracellular α -amylase (Schwermann *et al.*, 1994) and the extracellularly exposed maltose-binding protein (Schäfer *et al.*, 2004) of *Alicyclobacillus acidocaldarius*, have fewer charged residues exposed on their surface than their non-acidophilic counterparts, compensated by an increase in neutral polar residues. In the case of the strain V6 rusticyanin, there are also reductions in aspartic acid content (six less) and gains in serine (four residues) and asparagine (four residues) in comparison with the *A. ferrooxidans* protein, potentially indicating a reduction in charge density beyond that required for acid tolerance, possibly consistent with adaptation to relative halotolerance and the saline habitats of '*T. prosperus*'. However, in the absence of sequences of rusticyanins of other '*T. prosperus*'-like species and *A. ferrooxidans* strains for comparison, a strain-dependent rather than environment-dependent factor has not been excluded.

The *rus* operon in *A. ferrooxidans* is generally highly expressed during growth on ferrous iron (Quatrini *et al.*, 2006), although some expression also occurs during growth on sulfur (Ramírez *et al.*, 2004), particularly in the early exponential phase of growth (Yarzabal *et al.*, 2004). With strain V6, abundant rusticyanin was present during growth on sulfur. Initial real-time PCR studies (data not shown) indicated that the *rus* and *cyc2* genes appeared the most highly expressed of the genes in the *rus* operon-like cluster and at slightly higher levels (approx. twofold) in sulfur-grown cells than in ferrous-iron-grown cells, while the *coxA* gene, *coxB*, *coxC* and *ORF1* were expressed at higher levels (approx. four- to sevenfold) in cells grown on ferrous iron. In comparison, the expression of *cyc2*, *coxA* and *rus* genes of *A. ferrooxidans* was approximately three, five and eight times higher respectively in ferrous-iron-grown than in sulfur-grown cells (data not shown). The relatively greater expression of the *cyc2* gene during growth on sulfur was unexpected given that the *cyc2* gene in strain V6 was revealed through mRDA with tester cDNA from iron-grown cells. Further work is required to assess the influence of different stages of growth and of sulfur metabolism on the gene expression. The activity of the small, soluble cytochrome of strain V6 that appeared absent in *A. ferrooxidans* also requires study. However, it appears that '*T. prosperus*' and *A. ferrooxidans* could share a similar system for ferrous iron oxidation. There has been speculation that small, blue copper proteins in species of *Ferroplasma* and *Metallosphaera* could have a rusticyanin-like role in ferrous iron oxidation (Tyson *et al.*, 2004; Dopson *et al.*, 2005; Auernik *et al.*, 2008), but the protein in '*T. prosperus*' strain V6 is the first with the similarity to the rusticyanin in *A. ferrooxidans*, and the genetic context, to support the possibility of a similar role for this type of protein in a second organism.

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