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Ferrous iron oxidation and leaching of copper ore with halotolerant bacteria in ore columns

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ABSTRACT

Growth on ferrous iron of a new isolate of the acidophile *Thiobacillus prosperus* occurred with a substrate oxidation rate similar to that of *Acidithiobacillus ferrooxidans*. As well as similar capacities for iron oxidation, these species were shown to possess similar, but not identical, clusters of genes (the *rus* operon) that encode proteins likely to be involved in transfer of electrons from ferrous iron. Abundant rusticyanin was present in acidified, cell-free extracts of *T. prosperus*. In contrast to these similarities between the species, *T. prosperus* grew at a salt (NaCl) concentration several times that which prevented growth of *A. ferrooxidans*. A mixed culture of halotolerant bacteria maintained continuous ferrous iron oxidation in the presence of 5% w/v NaCl in solution percolating through ore in laboratory columns, and so enhanced ferric iron-dependent solubilization of copper.

1. Introduction

Few species of iron- and sulfur-oxidizing bacteria grow in saline, strongly acidic environments. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* appear inhibited by chloride concentrations well below that found in seawater (Kieft and Spence, 1988; Romero et al., 2003). However, unnamed, sulfur-oxidizing *Acidithiobacillus*-like strains from close to hydrothermal vents of the Aeolian Island of Vulcano in the Tyrrhenian Sea have been grown in acidic, saline media (Gugliandolo and Maugeri, 1993; Simmons and Norris, 2002). These sulfur-oxidizing acidithiobacilli were a major part of a population oxidizing pyrite in the presence of up to 6% w/v salt, where ferrous iron oxidation was carried out by *Thiobacillus prosperus*-like acidophiles (Norris and Simmons, 2004). The halotolerant, iron-oxidizing *T. prosperus* was also isolated from Vulcano (Huber and Stetter, 1989) and possibly similar bacteria have been isolated from marine harbour sediments (Kamimura et al., 1999). Growth of a *T. prosperus* isolate on ferrous iron is described here and it is shown to possess a cluster of genes that suggest its mechanism of iron oxidation is similar to that of *A. ferrooxidans*. The potential application of a halotolerant culture in copper ore leaching is also demonstrated in the context of poor water quality available for heap leaching in some areas where levels of salts (including chloride) inhibit iron-oxidizing acidithiobacilli and leptospirilla.

2. Materials and methods

2.1. Bacteria and culture conditions

T. prosperus strain V6 was isolated from enrichment cultures which were established at 30 °C with samples from a shallow, acidic pool by the shore of Baia di Levante, Vulcano, as described previously (Simmons and Norris, 2002). Strain V6 and *A. ferrooxidans* ATCC 33020 were grown at 30 °C in a mineral salts medium (pH 1.7) which contained (g l^{-1}) $MgSO_4$ $7H_2O$ (0.4), $(NH_4)_2SO_4$ (0.2), K_2HPO_4 (0.1), ferrous iron (13.9 g $FeSO_4$ $7H_2O$, 50 mM ferrous iron). The medium was supplemented with 0.5 mM tetrathionate ($K_2S_4O_6$) for growth of strain V6. The effects of Na^+ and Cl^- on growth were assessed by additions of NaCl, $MgCl_2$ and Na_2SO_4 to the medium as indicated in Results. Cultures were grown in shaken flasks with agitation at 150 rpm. Ferrous iron oxidation was determined by titration of residual ferrous iron with ceric sulfate and phenanthroline/ferrous sulfate as indicator.

2.2. Cell fractionation and denaturing gel electrophoresis

Ferrous iron-grown strain V6 and *A. ferrooxidans* cells were resuspended in water acidified to pH 2 with sulfuric acid and lysed by sonication. Cell debris was removed by centrifugation at 15,000 g for 10 min. Cell membranes were removed by centrifugation at 48,000 g for 30 min and the supernantant fraction was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining and with haem-staining using 3,3′dimethoxybenzidine.

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2.3. RNA and DNA analyses

Ferrous iron-grown cells of *T. prosperus* strain V6 were washed twice by centrifugation and resuspension in acidified water (pH 1.7) and once in distilled water. DNA extraction and manipulations used standard procedures. Amplification of a partial 16S rRNA gene was performed using forward (F27, 5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (R1492, 5′-TACGGYTACCTTGTTACGACTT-3′) primers. PCR products were cloned using *Taq* polymerase and the TOPO TA Cloning Kit (Version J) vector (pCR®2.1-TOPO®) and host *Escherichia coli* strain (Invitrogen). Genes that could be involved in ferrous iron oxidation were sought by micro-representational difference analysis (mRDA) carried out as described previously (Becker et al., 2001; Bathe and Norris, 2007), but in this case with cells of *T. prosperus* strain V6 that were grown on ferrous iron or sulfur. The difference products after rounds of subtractive hybridizations will be described elsewhere (J. Nicolle, S. Bathe and P.R. Norris). A selected mRDA difference product, which was derived from cDNA of ferrous iron-grown cells, was cloned, extended by inverse PCR and sequenced to reveal the genes of interest. The sequence of the genomic DNA region containing the strain V6 *rus*-operon-like gene cluster has been deposited in the GenBank database with accession number EU653292.

2.4. Ore leaching columns

A sample of ore from the Escondida copper mine (Chile) was used. It contained 0.65% w/v copper, 2.82% w/v iron and 2.18% w/v sulfur. Almost half of the copper was present as chalcopyrite, with the remainder principally from chalcocite, covellite and bornite. Pyrite was the main source of iron. Water-jacketed columns were each loaded with 0.7 kg of ore fragments (free of fine particles) with a mean weight of 1.3 g (range 0.9–2.4 g), giving ore columns of approximately 4.5 cm in diameter and 32 cm in height. Columns were maintained at 35 °C. The leaching solutions, reservoirs and tubing leading to columns, but not the ore or columns, were sterilized. Irrigation solution dripped centrally on to the surface of the packed ore fragments at a flow rate of 150 ml day^{-1}, corresponding to a surface application rate of approximately 4 l m^2 h^{-1}. There was no recycling of effluents through columns. Aeration from the base of the columns was at 20 ml min^{-1}. Leaching solution contained $(NH_4)_2SO_4$ (20 mg l^{-1}), $MgSO_4$ $7H_2O$ (40 mg l^{-1}) and K_2HPO_4 (10 mg l^{-1}), adjusted to pH 1.5 with H_2SO_4. The feed to two columns, one of which was inoculated, also contained 2.5% w/v NaCl. The feed to a third column, which was also inoculated, contained 5% w/v NaCl. The column inocula comprised *T. prosperus* strain V6 and salt-tolerant acidophiles from a pyrite enrichment culture which was shown previously (Norris and Simmons, 2004) to contain an uncharacterized species related to *T. prosperus* and two uncharacterized sulfur-oxidizing, halotolerant acidithiobacilli. This mixture was grown on finely ground Escondida ore in shaken flasks before addition to the columns. The columns were flushed with 250 ml leaching solution and percolated with the solution overnight before being inoculated. After these initial procedures, the effluent solutions from all three columns were at pH 1.8. Metals in effluent solutions were measured by atomic absorption spectrophotometry.

3. Results and discussion

3.1. Identification of strain V6 as T. prosperus

Strain V6 and the proposed type strain of *T. prosperus* (DSM 5130) grew on the same substrates and shared similar morphologies, optimum growth temperatures, DNA mol% G+C contents and salt tolerances (data not shown) and have very similar 16S rRNA gene sequences, indicating that they are isolates of the same species. Comparison of the 16S rRNA gene sequence of strain V6 to that of the type strain showed just under 99% identity over 1461 bases (corresponding to the *E. coli* sequence of bases numbering 28–1491). However, a short sequence comprising nine (strain V6) or ten bases (*T. prosperus*) at one loop included eight of the nineteen differences between the whole sequences. Exclusion of these eight differences from the comparison increased the identity to almost 99.5% over 1451 bases.

3.2. Growth of strain V6 and A. ferrooxidans on ferrous iron

Growth of strain V6 and *A. ferrooxidans* was estimated in terms of the maximum doubling times for ferrous iron oxidation (Fig. 1). The quantification of inhibition by chloride did not take into account a time-dependent increase in the severity of the inhibition at the higher concentrations of the ion, which limited the final extent of iron oxidation at these concentrations. Although growth of *T. prosperus* on ferrous iron was originally described as very poor (Huber and Stetter, 1989) compared to that of *Thiobacillus* (now *Acidithiobacillus*) *ferrooxidans*, the rate of growth-associated ferrous iron oxidation by strain V6 was found to be similar to that of *A. ferrooxidans* as long as the medium was

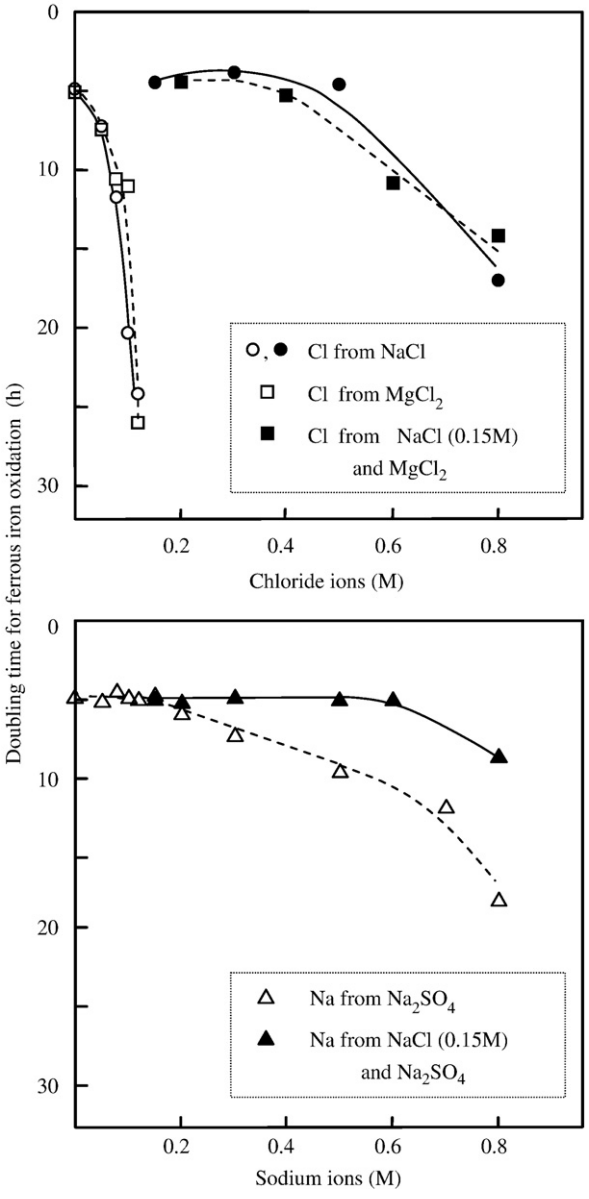

Fig. 1. Effect of chloride and sodium ions on ferrous iron oxidation during growth of *A. ferrooxidans* (open symbols) and *T. prosperus* strain V6 (closed symbols). Some NaCl (0.15 M) was included in the medium for the salt-requiring strain V6 when higher concentrations of Na^+ or Cl^- were tested independently of the NaCl concentration.