UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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"ANÁLISE DA EXPRESSÃO DE GENES QUE CODIFICAM PROTEÍNAS TRANSPORTADORAS EM *Acidithiobacillus ferrooxidans* E *Acidithiobacillus thiooxidans* NA PRESENÇA DE SULFETOS METÁLICOS"

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Genética e Biologia Molecular, na área de Genética de Microrganismos.

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Campinas
2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

M267a

Madureira, Danielle Jannuzzi
Análise da expressão de genes que codificam proteínas transportadoras em *Acidithiobacillus ferrooxidans* e *Acidithiobacillus thiooxidans* na presença de sulfetos metálicos / Danielle Jannuzzi Madureira. – Campinas, SP: [s.n.], 2008.

Orientadores: Laura Maria Mariscal Ottoboni, Fernanda de Castro Reis.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Acidithiobacillus. 2. Expressão gênica. 3. Sulfetos metálicos. I. Ottoboni, Laura Maria Mariscal. II. Reis, Fernanda de Castro. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

(rcdt/ib)

Título em inglês: Expression analyses of genes that encodes for transporter proteins in *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* maintained in contact with metal sulphides.
Palavras-chave em inglês: Acidithiobacillus; Gene expression; Metal sulphides.
Área de concentração: Genética de Microorganismos.
Titulação: Mestre em Genética e Biologia Molecular.
Banca examinadora: Laura Maria Mariscal Ottoboni, Flavio Henrique da Silva, Wanderley Dias da Silveira.
Data da defesa: 20/02/2008.
Programa de Pós-Graduação: Genética e Biologia Molecular.

Campinas, 26 de junho de 2008

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"Everything should be made as simple as possible, but not simpler"

A. Einstein

Dedicatória

i carry your heart with me(i carry it in my heart)

i carry your heart with me (i carry it in
my heart) i am never without it(anywhere
i go you go, my dear; and whatever is done
by only me is your doing, my darling)
i fear
no fate(for you are my fate, my sweet) i want
no world(for beautiful you are my world, my true)
and it's you are whatever a moon has always meant
and whatever a sun will always sing is you

here is the deepest secret nobody knows
(here is the root of the root and the bud of the bud
and the sky of the sky of a tree called life; which grows
higher than soul can hope or mind can hide)
and this is the wonder that's keeping the stars apart

i carry your heart (i carry it in my heart)
EE Cummings

A minha mãe, saiba que sem você eu não vivo.

Em memória da minha falecida avó.

Agradecimentos

A minha mãe por todo amor, carinho, paciência e incentivo em todos os momentos da minha vida. Ao meu pai por tentar me ensinar que a paciência é uma virtude e que tudo na vida tem um propósito, mesmo quando não conseguimos entender. Aos meus avôs maternos e paternos por tudo o que sou. A minha família por tudo, sem vocês a vida teria sido muito mais vazia e sem graça.

A minha orientadora Laura pela paciência, orientação e estrutura durante esses dois anos.

A Fernanda minha co-orientadora, amiga, irmã, companheira de casa, companheira de viagens e eterna conselheira por estar sempre disposta a me escutar independente dos problemas, da hora e do dia.

Aos membros da banca Profa. Dra. Laura M. M. Ottoboni, Profa. Dra. Maricilda Palandi de Mello e Prof. Dr. Flavio Henrique da Silva por terem aceitado participar deste momento importante na minha vida.

Aos membros da pré-banca Profa. Dra. Laura M. M. Ottoboni, Profa. Dra. Maricilda Palandi de Mello e Prof. Dr. Flavio Henrique da Silva pelas sugestões dadas e pelo incentivo.

A todos os professores que participaram da minha educação. Sem a paciência e a dedicação de todos vocês esse momento não seria tão prazeroso.

Aos meus amigos Bruna, Camila, Cristina, Daniela, Fabiana, Fabio, Fernanda, Flavio, Ilana, Jaime e Juarez, sem vocês a mudança de vida seria quase impossível.

A todos aqueles que participaram desses longos 26 anos de vida, mesmo que passageiramente, vocês me ajudaram a entender o quanto à vida é maravilhosa.

Aos companheiros de bagunça saibam que vocês moram no meu coração. Aos amigos distantes que mesmo longe estão sempre comigo me dando conselhos e me escutando, ou seja, sendo simplesmente amigos.

Aos companheiros do laboratório de Biologia Molecular Ambiental Bruna, Camila, Daniela, Fernanda, Leandro, Lucio, Marcela, Susy e Viviane pelos momentos divertidos que estivemos juntos.

Ao CNPq, a FAPESP e a Companhia Vale do Rio Doce pelo apoio financeiro.

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ABC	ATP Binding Cassette
ATP	Adenosina trifosfato
AAS	Atomic Absorption Spectroscopy
cDNA	DNA complementar
Ct	Critical threshold
COG	Cluster of Orthologous group
DNA	Ácido desoxiribonucleico
Dnase	Desoxiribonuclease
dNTPs	2'-desoxinucleotidios 5'-trifosfato
M	Molar
MATE	Multidrug And Toxic Compounds Extrusion Family
MDR	Multi-drug resistance protein
MFP	Membrane Fusion Protein
MFS	Major Facilitator Super family
mM	Milimolar
mL	Mililitro
µL	Microlitro
mRNA	RNA mensageiro
OMP	Outer Membrane Protein
ORF	Open reading frame
pb	Pares de base
PCR	Polymerase chain reaction
pH	Potencial hidogeniônico
PPI	Protein-protein interaction
RAP-PCR	Random Arbitrarily Primed Polymerase Chain Reaction
RNA	Ácido ribonucléico
RND	Resistance nodulation and cell division
rpm	Rotações por minuto
SMR	Small Multidrug Resistance Family

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RESUMO

Acidithiobacillus ferrooxidans e *Acidithiobacillus thiooxidans* são espécies bacterianas acidofílicas, quimiolitotróficas e mesofílicas, encontradas em ambientes de biolixiviação. *A. ferrooxidans* utiliza íons ferrosos e compostos que contém enxofre como doadores de elétrons enquanto que *A. thiooxidans* utiliza apenas enxofre e compostos contendo enxofre. Ambas as espécies utilizam principalmente o oxigênio como receptor final de elétrons, contudo, podem também crescer em ambientes de anaerobiose. *A. thiooxidans* possui maior resistência ao pH podendo ser encontrada em ambientes com pH variando de 0,5 a 5,0, enquanto que *A. ferrooxidans* é encontrada em pHs que variam de 1,0 a 2,5. Devido a essas características e devido a capacidade dessas bactérias de solubilizar metais, *A. ferrooxidans* e *A. thiooxidans* vem sendo utilizadas em experimentos de biolixiviação. Este processo é utilizado na indústria para recuperação de cobre devido às vantagens oferecidas. Entretanto, pouco se sabe sobre a resposta gênica destas bactérias a presença de sulfetos metálicos e dos metais pesados em solução proveniente do processo de oxidação. Na primeira parte deste trabalho foi analisada a resposta gênica da estirpe de *A. ferrooxidans* LR na presença e na ausência de covelita (CuS) por 24 horas através da técnica de RAP-PCR (*Random Arbitrarily Primed Polymerase Chain Reaction*). Foram obtidos 19 cDNAs com expressão diferencial, dos quais 12 foram confirmados como sendo diferencialmente expressos. Dentre estes, foram isolados sete genes que codificam: proteínas de transporte (AFE_0123, AFE_0989, AFE_0990, AFE_0580, AFE_0671, AFE_2248 e AFE_1149), a proteína diguanilato ciclase, uma proteína de membrana, uma metiltransferase e uma ATPase, todas induzidas na presença da covelita. Apenas o gene *infC*, que codifica um fator de iniciação de tradução tipo 3, teve sua expressão reprimida na presença de covelita. Como sete dos genes diferencialmente expressos pertenciam à classe de transportadores, foram investigadas às modificações químicas presentes no meio de cultura após 24 horas. Análises de absorção atômica mostraram que a quantidade de cobre em solução inicialmente zero passava para aproximadamente 1,13 g/L e medidas de pH mostraram que após este período de tempo houve mudanças de 1,8 para 4,0. Pode-se sugerir que essas alterações químicas sejam responsáveis, pelo menos em parte, pela indução dos genes que codificam proteínas de transporte. Uma análise *in silico* da interação entre proteínas (PPI) relacionadas com transporte, cujos genes foram diferencialmente expressos na presença de covelita e de proteínas pertencentes à mesma categoria

funcional, codificadas por genes que estavam fisicamente localizados nas proximidades dos genes diferencialmente expressos, mostraram que os genes de transporte podem estar envolvidos em diferentes etapas da resposta bacteriana à presença de covelita. A segunda parte deste trabalho teve como objetivo estudar a expressão diferencial, por PCR em tempo real, de duas proteínas do tipo ABC (AFE_0123 e AFE_0125) e uma proteína do tipo RND (AFE_0993) na estirpe de *A. thiooxidans* FG01 na presença de calcopirita ($CuFeS_2$), covelita e pirita (FeS_2) por 24 horas. Para tal, a estirpe foi crescida na presença de enxofre e depois mantida na presença dos sulfetos metálicos por 24 horas. A expressão dos três genes foi induzida na presença de covelita e inalterada na presença de calcopirita. Na presença de pirita, a expressão de dois genes foi reprimida e do gene AFE_0993, permaneceu inalterada. Estes resultados podem ser explicados, pelo menos em parte, pelas quantidades de cobre encontradas em solução na presença de covelita (1 g/L) e na presença de calcopirita (0,07 g/L). Na presença de covelita e calcopirita a medida de pH também sofreu aumento de 1,8 para 4,0 e 2,5, respectivamente. Para investigar se a alteração de pH ou a presença de íons de cobre eram os responsáveis pela indução da expressão desses genes, a bactéria foi mantida na presença de sulfato de cobre (16 mM) e pH 4,0 por 24 horas. Os resultados mostraram que a presença de cobre em solução induz a expressão destes genes, enquanto que a alteração do pH não.

SUMMARY

Acidithiobacillus ferrooxidans and *Acidithiobacillus thiooxidans* are acidophilic, chemolitotrophic and mesophilic bacteria found in bioleaching environments. *A. ferrooxidans* uses ferrous iron and sulphur compounds as an electron donor and *A. thiooxidans* uses only sulphur and sulphur compounds. Both species are aerobic using oxygen as final electron acceptor. However, these bacteria are also able to grow in anaerobic environments. *A. thiooxidans* is able to survive in pHs ranging from 0.5 to 5.0 while *A. ferrooxidans* grows in pHs from 1.0 to 2.5. Due to these characteristics and the ability of these bacteria to solubilize metal sulphides, *A. ferrooxidans and A. thiooxidans* are used in bioleaching. This process has several advantages over the traditional methods and has been used with success in industrial operations to recover mainly copper. However, little is known about the genetic response of these bacteria to the presence of metal sulphides and heavy metal in solution. Therefore, in the first part of this study the *A. ferrooxidans* LR response to covellite was investigated. This bacterium was maintained in contact with covellite for 24 hours and the differentially expressed cDNAs were identified by RAP-PCR (*Random Arbitrarily Primed Polymerase Chain Reaction*) technique. Nineteen cDNAs showed a differential expression and twelve had their differential expression confirmed by real time PCR. Among these cDNAs, seven codified for transporter proteins (AFE_0123, AFE_0989, AFE_0990, AFE_0580, AFE_0671, AFE_2248 and AFE_1149), one codified for a putative diguanylate cyclase, one for a membrane protein, one for a methyltransferase and one for an ATPase. With the exception of *infC* whose expression was down regulated, all the cDNAs had their expression up regulated in the presence of covellite. Since most of the differentially expressed genes are involved in transport, chemical modifications on the culture medium after 24 hours were investigated. The atomic absorption analysis showed that the copper amount in solution was 1.13 g/L and pH changed from 1.8 to 4.0 suggesting that these changes are responsible, at least in part, for the induction of the expression of transport gene. An *in silico* analysis of protein-protein interaction (PPI) between the transport proteins codified by the genes differentially expressed in the presence of covellite and those codified by genes that were physically located around the differentially expressed ones showed that these transport proteins could be involved in different steps of the bacterial response to covellite. The goal of the second part of this study was to analyse the differential expression, by real time PCR, of two ABC

proteins (AFE_0123 and AFE_0125) and one RND protein (AFE_0993) in *A. thiooxidans* FG01 in the presence of chalcopyrite, covellite and pyrite for 24 hours. This strain was maintained in contact with the metal sulphides for 24 hours. The expression of the three genes was up regulated in the presence of covellite and unchanged in the presence of chalcopyrite. In the presence of pyrite two genes had their expression down regulated and in one (AFE_0993) the expression was unchanged. These results can be explained, at least in part, by the quantities of copper found in solution in covellite (1 g/L) and in chalcopyrite (0.07 g/L). In the presence of covellite and chalcopyrite the pH changed from 1.8 to 4.0 and 2.5, respectively. To investigate if the changes in pH or the presence of copper ions in solution were responsible for the induction of the gene expressions, the bacteria were maintained in the presence of copper sulphate (16 mM) and pH 4.0 for 24 hours. The results showed that the presence of copper in solution was the responsible for the up regulation of these genes.

INTRODUÇÃO

1. Biolixiviação

Os métodos convencionais utilizados para a recuperação de metais a partir de minérios são a pirometalurgia e a hidrometalurgia. A pirometalurgia é um processo, no qual altas temperaturas são utilizadas para auxiliar na reação extrativa. Uma grande desvantagem deste processo é a liberação de dióxido de enxofre para o ambiente, o que pode levar à produção de chuva ácida. A hidrometalurgia baseia-se na utilização de um líquido solvente para lixiviar o metal. Apesar do processo utilizado na hidrometalurgia não ser tão agressivo ao ambiente quando realizado em circuitos fechados, quando a solução lixiviante extravasa para o ambiente pode contaminar rios e lençóis freáticos, causando danos ambientais (Bridge, 2000; Alvarado *et al*., 2002).

A biolixiviação, processo no qual os microrganismos estão envolvidos na dissolução de minérios, é uma área multidisciplinar associada à hidrometalurgia, ao processamento de minérios, à química, ao meio ambiente e à microbiologia. Vários metais podem ser recuperados a partir deste processo, como por exemplo, cobalto, ouro, níquel, cádmio, zinco, gálio, manganês, cobre, entre outros (Akcil, 2004).

Muita atenção tem sido dada a biolixiviação, devido ao enorme reservatório de minérios de cobre de baixo teor (teor menor que 0,3% do metal) e devido ao aumento dos regulamentos de proteção ao meio ambiente (Brombacher *et al*., 1998; Demergasso *et al*., 2005; Kinnunen & Puhakka, 2005; Zhang & Fang, 2005). A recuperação dos metais presente nestes minérios é economicamente inviável por métodos convencionais devido ao alto custo das técnicas. Dessa forma, torna-se necessário o aprimoramento de métodos alternativos para o tratamento dos mesmos (Brombacher *et al*., 1998; Dempers *et al*., 2003; Garcia Jr, 1997; Watling, 2006).

O processo biohidrometalúrgico é um método eficiente, simples e ecologicamente correto comparado aos processos convencionais. A principal desvantagem desta técnica na lixiviação de sulfetos metálicos é o tempo que demora quando comparado aos métodos tradicionais (Watling, 2006).

As vantagens desse processo são (Brombacher *et al*., 1998; Dempers *et al*., 2003; Garcia Jr, 1997; Watling, 2006):

- Economia de insumos utilizados, quando comparado a um processo hidrometalúrgico convencional, pois a própria bactéria produz tais insumos a partir de substratos presentes no minério;
- Baixo requerimento de energia, se comparado a um processo pirometalúrgico e mesmo a um processo hidrometalúrgico;
- Baixo investimento de capital inicial e baixo custo operacional, devido à simplicidade das instalações requeridas na biohidrometalurgia;
- Reduzida necessidade de mão de obra especializada na operação;
- Não ocorre poluição atmosférica, pois não ocorre emissão de SO_2, impedindo assim a formação da chuva ácida, como no processo pirometalúrgico.

Os principais fatores que têm sido considerados como variantes significativas do processo de biolixiviação são: fonte e concentração do microrganismo, a concentração, composição e as características físicas do substrato sólido como o tamanho da partícula, sua forma, distribuição e porosidade, a concentração do produto lixiviado e dos produtos finais, assim como, as condições do lixiviado (pH, temperatura, agitação, e outros) (Liu *et al.,* 2004).

2. Sulfetos metálicos

Os sulfetos metálicos são a principal fonte de metais de importância econômica para a indústria mineradora. Os sulfetos podem conter metais como o cobre (calcopirita [$CuFeS_2$] e covelita [CuS]), zinco (esfalerita [ZnS]), níquel (sulfeto de níquel [Ni_2S_3]), prata (sulfeto de prata [Ag_2S]) entre outros.

A lixiviação bacteriana de sulfetos metálicos pode ocorrer de forma direta ou indireta. O mecanismo indireto envolve a ação química dos íons Fe^{3+} e H^+, não sendo necessária a adesão da bactéria à superfície mineral. O mecanismo direto ocorre através do ataque enzimático da bactéria e requer a adesão da bactéria à superfície metálica (Belivaqua *et al.*, 2004; Sand *et al*., 2001).

2.1. Calcopirita

A calcopirita ($CuFeS_2$), mineral inorgânico complexo mais abundante do mundo, é altamente recalcitrante aos processos hidrometalúrgicos (Bevilaqua *et al*., 2002; Qiu *et al.,* 2005; Watling, 2006). A degradação da calcopirita é um processo no qual os íons férricos são os agentes oxidantes e os componentes do sulfeto são oxidados até a formação de enxofre elementar, como pode ser observado na Equação 1 (Bevilaqua *et al*., 2002; Watling, 2006).

$$CuFeS_2 + 4Fe^{+3} \rightarrow 5Fe^{+2} + Cu^{+2} + 2S^0 \qquad \text{(Equação 1)}$$

Além disso, mostrou-se que em paralelo ocorre uma dissolução não oxidativa que contribui para a cinética de dissolução da calcopirita em solução ácida (Equação 2) (Bevilaqua *et al*., 2002; Watling, 2006).

$$CuFeS_2 + 4H^+ \rightarrow Fe^{+2} + Cu^{+2} + 2H_2S \qquad \text{(Equação 2)}$$

No caso da calcopirita as reações contendo íons férricos e ácido sulfúrico liberam produtos que são considerados responsáveis pela lenta dissolução do minério. Os produtos insolúveis formados na superfície do minério são compostos que contêm enxofre e podem ser divididos em quatro fases: a fase sulfeto, a fase de enxofre elementar, a fase do sulfato de ferro e uma fase dissulfeto. Estas quatro fases foram encontradas na lixiviação biótica e abiótica e nas condições de aerobiose e anaerobiose, sugerindo que há um mecanismo comum de oxidação do sulfeto nestas condições (Watling, 2006).

A biolixiviação da calcopirita ainda não foi implementada em escala comercial. Os impedimentos para aplicação e aceitação da biolixiviação da calcopirita não estão necessariamente restritos aos aspectos biológicos (Watling, 2006). Segundo Holmes e Debus (1991) para que uma indústria mineradora tenha real interesse na implementação desta metodologia seria necessário que a biolixiviação possuísse cerca de 20% de vantagens a mais que o processo convencional. Poulter e colaboradores (1999) discutem que a relutância de assumir essa tecnologia é em parte devido às características refratárias da calcopirita, parte devido às

vantagens econômicas e de processos das novas tecnologias de fundição e parte devido aos riscos técnicos da introdução de uma nova tecnologia de processamento.

2.2. Covelita

Covelita (CuS) é um sulfeto de cobre de baixa distribuição mundial mas, apesar disto, tem uma grande importância para obtenção de cobre. Existem poucos trabalhos na literatura sobre a biolixiviação deste sulfeto metálico e, portanto, os mecanismos envolvidos na sua dissolução ainda não estão completamente entendidos (Acar *et al*., 2005; Teixeira *et al.*, 2002).

Walsh e Rimstidt (1986) reportaram diferentes taxas de lixiviação entre covelita *blaubleibender* ($Cu_{1.4}S$) e a covelita normal, sendo a covelita *blaubleibender* de mais fácil lixiviação. A caracterização mineralógica dos sulfetos, como a composição química do sulfeto, é de grande importância, pois este trabalho mostra a importância da identificação mineralógica dos sulfetos utilizados no processo de lixiviação, demonstrando que dependendo da composição química da covelita ela se torna de mais fácil oxidação.

Em estudos para verificar a biolixiviação de covelita por *Acidithiobacillus ferrooxidans* em ambiente de anaerobiose, Donati e colaboradores (1997) observaram que aparentemente a condição de anaerobiose retarda a formação de precipitados de jarosita, os quais retardam a lixiviação deste minério, sugerindo que a biolixiviação de covelita seria mais eficiente nessa condição.

Em estudos realizados por Monteiro e colaboradores (1999) determinou-se que após 20 dias de lixiviação de covelita com *A. ferrooxidans* a quantidade de cobre liberado atingia um platô. Foi observado também que esse limite de lixiviação era atingido somente quando o pH do meio não era controlado, já que o pH aumenta devido ao consumo de ácido por este sulfeto.

Nos experimentos feitos por Olson e Clarck (2001) foi utilizada uma mistura de sulfetos de cobre contendo calcocita, covelita, enargita, luzonita e pirita usando bactérias mesofílicas (25ºC) e termofílicas (50ºC). A biolixiviação utilizando bactérias mesofílicas resultou em 56% de extração do cobre enquanto que a quantidade obtida utilizando bactérias termofílicas foi de 65%. Os autores atribuem este fato à temperatura que seria responsável por um aumento da agitação das moléculas, tornando a reação mais rápida.

Ensaios eletroquímicos realizados por Teixeira e colaboradores (2002) demonstraram que a ação de *A. ferrooxidans* LR sobre a covelita, rompe a camada de enxofre que se forma sobre a superfície do minério, abaixando consideravelmente o potencial eletroquímico e acelerando o processo de oxidação. Acar e colaboradores (2005) observaram que quando uma mistura de sulfetos era utilizada obtinha-se uma melhor biolixiviação com bactérias termofílicas (61 – 63%) do que quando as bactérias eram mesofílicas (12 – 15%), mostrando que aparentemente a melhor biolixiviação da covelita ocorre em ambientes termofílicos.

2.3. Pirita

Pirita (FeS_2) é o sulfeto mais comum na natureza (Tupikina, 2006). A reação de oxidação da pirita, ao contrário do observado na calcopirita e covelita, não altera o pH da solução, pois segue a via tiossulfato (Schippers & Sand, 1999). A equação 3 demonstra a dissolução deste sulfeto por íons férricos.

$$FeS_2 + 14\,Fe^{+3} + 8H_2O \rightarrow 15Fe^{+2} + 2SO_4^{-2} + 16H^+ \qquad \text{(Equação 3)}$$

Em experimentos realizados por Petersen e Dixon (2006) foi observado que em altas temperaturas a interação Fe^{+3}/Fe^{+2} resulta em um baixo potencial operacional o que inicialmente favorece a lixiviação da calcopirita enquanto que em baixas temperaturas a lixiviação da pirita é promovida.

Em experimentos realizados por Tupikina e colaboradores (2006) observou-se que diferentes tipos de pirita eram oxidados pelas mesmas estirpes com diferentes atividades, pois a composição física, química e eletroquímica influencia no crescimento e, portanto na oxidação do minério. Foi verificado também que estas características físico-químicas também influenciam na variação genotípica das estirpes bacteriana, levando à perda ou ganho de plasmídios, alterando assim o perfil plasmidial.

3. Bactérias Mineradoras

Diversas bactérias estão associadas à dissolução de minérios como, por exemplo, *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Acidithiobacillus thiooxidans*, *Metallogenium*, *Acidianus/Sulfolobus* spp., entre outras (Rawlings, 2005; Rohwerder & Sand, 2003).

A utilização de microrganismos acidofílicos e quimiolitotróficos capazes de oxidar ferro e enxofre em processos industriais para remoção de cobre, ouro e urânio de minerais sulfetados é uma tecnologia estabelecida e empregada mundialmente (Valenzuela *et al.*, 2006).

Os microrganismos mais importantes na biolixiviação de minerais são aqueles responsáveis pela produção de ferro III e ácido sulfúrico, necessários para que este processo ocorra. Esses microrganismos possuem muitas características interessantes, dentre elas: i) crescimento autotrófico através da fixação de gás carbônico da atmosfera; ii) utilização de ferro ou de compostos que contenham enxofre (ou ambos) como doador de elétrons, utilizando geralmente para isso, o oxigênio como aceptor final de elétrons; iii) são bactérias acidofílicas capazes de crescer em ambientes ácidos; e iv) são tolerantes a altas concentrações de metais (Irazabal *et al.*, 1997; Liu *et al*., 2004; Rawlings, 2005).

Dumps e *heap leaching*, técnicas utilizadas no processo de biolixiviação, possuem uma composição mineralógica variada e seus diferentes ambientes climáticos representam um habitat microbiológico extremamente complexo. Contudo, poucas bactérias foram isoladas de ambientes de biolixiviação, caracterizadas filogenicamente e fisiologicamente e depositadas em bancos de dados (Rawlings, 2005; Watling, 2006).

Com as novas técnicas moleculares de enumeração e identificação de organismos, é possível agora seguir as mudanças no consórcio microbiano em função do tempo e da localização. Estas metodologias é uma poderosa ferramenta para descrever a biodiversidade e/ ou entender o processo de biolixiviação, além de facilitar a elucidação da natureza da interação microbiana na biolixiviação (Rawlings, 2005; Watling, 2006). Devido ao avanço dessas técnicas, várias bactérias heterotróficas estão sendo identificadas e caracterizadas. Além destas, bactérias termofílicas moderadas e hipertermófilas também têm sido identificadas, pois possuem um papel de grande importância no aumento da eficiência da biolixiviação devido ao aumento da temperatura durante o processo (Rawlings, 2005; Watling, 2006).

O gênero *Acidithiobacillus* tem grande importância na biolixiviação, pois é composto por bactérias acidofílicas, quimiolitotróficas e Gram-negativas que utilizam principalmente o enxofre como doador de elétrons. É composto por quatro espécies: *Acidithiobacillus albertensis, A. caldus, A. ferrooxidans* e *A. thiooxidans*. Algumas espécies deste gênero são mais estudadas entre as bactérias mesofílicas, contudo, outras bactérias também participam do processo de biolixiviação (Kelly & Wood, 2000).

Acidithiobacillus ferrooxidans foi o primeiro microrganismo isolado de ambientes de lixiviação ácida e tem sido objeto de pesquisa desde então. Esta espécie é a única deste gênero que além de utilizar uma variedade de compostos inorgânicos de enxofre como doador de elétrons também utiliza o ferro. Esta bactéria é capaz de oxidar o íon ferroso a íon férrico o que é de grande importância para a biolixiviação, contudo, não é necessariamente a bactéria mais importante neste processo, sendo priorizado atualmente o consórcio microbiano (Rawlings, 2002; Watling, 2006).

A. ferrooxidans cresce preferencialmente em ambientes aeróbios, contudo, é também capaz de crescer utilizando íons férricos como aceptor final de elétrons. Esta bactéria é capaz de crescer relativamente rápido quando comparado aos outros microrganismos que participam do processo de biolixiviação e tem crescimento ótimo em temperaturas entre 20 e 35 ° C e pH entre 1,8 a 2,0 (Rawlings, 2002).

Nos últimos anos vários trabalhos (Quatrini *et al.*, 2006; Ramirez *et al.*, 2004; Yarzábal *et al*., 2004) têm sido realizados com o objetivo de entender o metabolismo energético de *A. ferrooxidans.* Yarzábal e colaboradores (2004) analisaram a expressão dos genes do operon *rus* em células crescidas em meio contendo ferro ou enxofre. Os genes presentes neste operon estão envolvidos na cadeia transportadora de elétrons. Os autores concluíram neste trabalho que o ferro induzia a expressão destes genes. Este resultado já era esperado, pois, estes genes estão envolvidos na oxidação do ferro.

Ramirez e colaboradores (2004) analisaram o proteoma de *A. ferrooxidans* cultivadas em meio contendo ferro, enxofre e sulfetos metálicos como pirita e calcopirita. Eles mostraram que durante o crescimento da bactéria em sulfetos metálicos contendo ferro, como pirita e calcopirita, tanto proteínas reguladas positivamente em ferro quanto em enxofre são sintetizadas, indicando que ambas as vias responsáveis pela geração de energia são induzidas simultaneamente, dependendo da concentração e do tipo dos substratos oxidáveis disponíveis. Recentemente,

Quatrini e colaboradores (2006) analisaram, através de *microarray*, a expressão de genes em células de *A. ferrooxidans* crescidas em meio contendo ferro ou enxofre. Os autores sugeriram que a oxidação do ferro e enxofre pode ser regulada de maneira coordenada.

Acidithiobacillus thiooxidans foi primeiramente isolada em 1922 por Waksman e Joffe (1922) e sua principal característica é a velocidade com que oxida o enxofre (Qiu *et al*., 2005). Esta espécie apresenta crescimento ótimo em temperaturas em torno de 30ºC e pH de aproximadamente 2,0 (Rawlings, 2002), embora cresça bem em temperaturas próximas de 40ºC e valores de pH extremamente ácidos como 0,5 (Neigishi *et al.,* 2005). Em uma investigação de biolixiviação de sulfetos de cobre foi observado que quando o pH atingia valores em torno de 0,7, *A. thiooxidans* era o microrganismo dominante (Válquez & Espejo, 1997). A espécie *A. thiooxidans* também é moderadamente halofílica, sendo que algumas linhagens são capazes de crescer em concentrações de até 2% de NaCl (Kamimura *et al.,* 2005).

A oxidação do enxofre por *A. thiooxidans* é um processo bastante complexo. Diversos trabalhos indicam que esta oxidação requer contato direto entre as partículas de enxofre e a bactéria (Takakuwa *et al.,* 1977; Yasuhiro *et al.,* 1995; Suzuk *et al.,*1999). Takakuwa e colaboradores (1977) analisaram a curva de crescimento de *A. thiooxidans* em meio com enxofre e averiguaram que todas as células estavam ligadas à superfície do enxofre durante o estágio inicial do crescimento, sendo que o número de células livres aumentou com a progressão do crescimento (Takakuwa *et al.*, 1977).

Similarmente, as outras espécies do gênero *Acidithiobacillus, A. thiooxidans* é capaz de desenvolver mecanismos que facilitam a aderência das células a superfícies de sulfetos. Dentre estes mecanismos pode-se citar a secreção de substâncias poliméricas extracelulares (EPS), as quais mediam o contato entre a bactéria e as superfícies metálicas (Bhavaraju *et al.,* 1993). Estudos preliminares mostraram que a adesão envolve alterações na bactéria como: mudanças elétricas, irregularidades na superfície e características da membrana celular (Ohmura *et al.,* 1993).

Esta bactéria também participa do processo de biolixiviação (Chandraprabha & Natarajan, 2006). De acordo com Kelly e Wood (2000) *A. thiooxidans* é uma das populações predominantes em tanques de biolixiviação, alem disso, tem sido discutida a capacidade de *A. thiooxidans* de interagir diretamente com os minérios contendo enxofre, aumentando a taxa de

dissolução do mesmo. A ação dessa bactéria é iniciada pela adsorção das células na superfície do minério (Akcil *et al*., 2006; Ghauri *et al*., 2006; Petersen & Dixon, 2006).

Donati e colaboradores (1996) sugeriram que *A. thiooxidans* provoca a dissolução da camada de enxofre depositada sob os sulfetos metálicos, permitindo a continuação do processo de biolixiviação através da produção de ácido sulfúrico. Esta característica de *A. thiooxidans* é importante na recuperação do cobre a partir da covelita.

4. Metais Pesados

Vários trabalhos relataram a contaminação do meio ambiente por metais, em particular por metais pesados (densidade ≥ 5 g/cm^3), assim como, seu efeito tóxico em uma grande variedade de organismos (Silver, 1996).

Os microrganismos possuem um grande impacto no comportamento e na ciclagem dos metais no ambiente. Este impacto é alcançado por: (i) assimilação / adsorção; (ii) precipitação e dissolução; (iii) oxidação e redução; e (iv) reações de metilação e dealquilação. Em alguns casos essas transformações envolvem algumas mudanças de fase (por exemplo, de solúvel a gasoso) e / ou mudanças de estados de oxidação (Johnson, 2006).

A assimilação / adsorção de metais ocorre, pois estes possuem diversas funções na célula microbiana. A acumulação de metais normalmente envolve um sistema de transporte dependente de energia, onde o metal se liga primeiramente à superfície da célula e depois é transportado ativamente pela membrana da célula. Já a adsorção de metais ocorre tanto em células vivas, através da mediação por processos metabólicos ativos, quanto em células mortas, através de mecanismo físico-químico (Volesky, 2001).

A mobilização ou a imobilização de metais no ambiente pode ser causada por dissolução ou precipitação causada de uma maneira direta ou indireta por microrganismos. Como a maioria dos metais é catiônica e solúvel em ácidos, alguns processos metabólicos como denitrificação, redução de sulfato e metanogênese provocam alcalinização do meio o que pode tornar o metal insolúvel. A precipitação mediada por microrganismos pode ser resultado da formação de hidróxidos, fosfatos, carbonatos e sulfatos insolúveis extracelulares. Além disto, precipitação intracelular também pode ocorrer (Macaskie, 1991; White *et al*., 1995).

Os microrganismos são capazes de realizar transformações no estado de valência dos metais através da catalisação de reações redox. Estas reações envolvem principalmente microrganismos metabolicamente ativos, contudo, também pode ocorrer passivamente quando o metal se liga a sítios reativos de células "em repouso". Exemplos de metais que são passíveis de serem transformados por microrganismos são: ferro, manganês, cromo, cobre, mercúrio, antimônio, ouro, molibdênio, entre outros. Em alguns casos, essas transformações de óxido-redução estão envolvidas na conservação de energia, pois esses metais servem como doadores diretos ou indiretos ou como receptores de elétrons (Johnson, 2006).

A transformação de metais por reações de metilação e dealquilação é realizada por microrganismos aparentemente como um mecanismo de defesa. Ao contrário das outras formas de transformação de metais, a metilação dos metais normalmente libera complexos voláteis, como é o caso do mercúrio (Hughes & Poole, 1989). A alquilação de metais é normalmente considerada insignificante. Sendo que os metais que seguem esta via acabam voltando ao ambiente (Summers & Silver, 1978).

5. Mecanismos de Tolerância e Resistência

Uma importante característica dos microrganismos acidofílicos quimiolitotróficos é a sua tolerância generalizada a altas concentrações de íons metálicos (Dopson *et al*., 2003). O nível de resistência de cada bactéria aos diferentes metais varia de acordo com a estirpe. Provavelmente o fator responsável por esta variação é a adaptação as diferentes concentrações de metais às quais os microrganismos foram expostos (Rawlings, 2005).

A. ferrooxidans é particularmente resistente aos metais pesados e foi descrito como sendo resistente ao cobre (55 g/L), cobalto (30 g/L), níquel (72 g/L), zinco (120 g/L) entre outros. Em um estudo comparativo realizado por Sand e colaboradores (1993), foi observado que *A. ferrooxidans* e *L. ferrooxidans* eram resistentes a praticamente as mesmas quantidades de metais, sendo *A. thiooxidans* mais sensível em comparação a ambas (sensível a menos de 5 g/L de todos os metais testados, menos ao zinco [10 g/L]).

Aparentemente a resistência a íons metálicos em *A. ferrooxidans* varia em função da fonte de energia utilizada. Quando o Fe^{2+} é utilizado *A. ferrooxidans* pode ser até duas mil vezes mais resistentes a íons metálicos do que células que utilizam o tiosulfato. Quando S^0 é utilizado,

valores intermediários de resistência são observados (Tuoniven *et al*., 1971). Silver e Torma (1974) confirmaram estes resultados testando a susceptibilidade de *A. ferrooxidans* ao chumbo, níquel e cobre. Posteriormente, Iwahori e colaboradores (2000) demonstraram que a membrana plasmática de algumas linhagens de *A. ferrooxidans* resistentes ao mercúrio possuía uma atividade mercúrio-redutase ($Hg^{2+} \rightarrow Hg^{0}$) Fe^{2+} dependente. Poucos estudos foram feitos sobre a resistência de *A. ferrooxidans* ao cobre. Linhagens tolerantes ao cobre têm sido obtidas a partir da adaptação a crescentes concentrações deste metal (Das *et al*., 1998). Em um estudo utilizando a estirpe de *A. ferrooxidans* LR, Novo e colaboradores (2000), encontraram três proteínas de membranas que eram induzidas apenas na presença de cobre, sugerindo que essas proteínas poderiam estar participando de uma resposta mais especifica a este metal.

Existem três mecanismos possíveis para a resistência a metais pesados: (a) o metal pode ser transportado por efluxo, (b) os cátions podem ser agrupados em moléculas que possuem grupamento tiol, sendo esses metais classificados como 'sulfur-lovers", (c) alguns metais, como o cobre, podem ser reduzidos a estados menos tóxicos. Entretanto sabe-se que as bactérias utilizam mais de um mecanismo para atingir a resistência e homeostase celular (Nies, 1999; Dopson *et al.*, 2003).

Os mecanismos que conferem resistência a metais em *A. ferrooxidans*, assim como os genes envolvidos no processo, são pouco conhecidos. Genes envolvidos na resistência ao mercúrio foram isolados e seqüenciados por Shiratori e colaboradores (1989). Os genes de *A. ferrooxidans* associados com a resistência a mercúrio diferem de outras bactérias, pois estão localizados no cromossomo e não em plasmídios e também a estrutura do operon apresenta diferenças (Shiratori *et al*., 1989; Inoue *et al*., 1989).

Butcher e colaboradores (2000) clonaram e seqüenciaram genes envolvidos no mecanismo de resistência ao arsênio em *A. ferrooxidans*. Células de *E. coli* transformadas com os genes de *A. ferrooxidans* adquiriram aumento de resistência a arsênio e antimônio.

Dados do genoma de *A. ferrooxidans* e *L. ferrooxidans* sugerem que a resistência a metais é devido a uma combinação de genes que provavelmente estão presentes no cromossomo e de elementos genéticos móveis adquiridos por transferência horizontal (Rawlings, 2005).

Um exemplo de genes presentes no cromossomo da maioria das espécies são os genes de efluxo de arsênio, de cobre, de cádmio-prata e de diversos outros metais. Outro exemplo de mecanismo de resistência que pode estar presente em todas as estirpes de *A. ferrooxidans* é o

mecanismo de polifosfato em relação à resistência ao cobre, pois este mecanismo está relacionado à fisiologia geral da célula (Butcher, Deane & Rawlings, 2000; Alvarez & Jerez, 2004).

As proteínas transportadoras podem ser divididas em duas grandes famílias, primárias e secundárias, baseando-se nas suas características estruturais e bioenergéticas. As proteínas pertencentes à família primária possuem a capacidade de hidrolisar ATP como fonte de energia e, portanto são conhecidas como proteínas ABC (*ATP Binding Cassette*) transportadoras. As proteínas classificadas como secundárias utilizam o gradiente de prótons (ou sódio) como fonte de energia (Márquez, 2005).

Os transportadores do tipo ABC existem em todos os seres vivos e estão envolvidos no transporte de diferentes substâncias, como no efluxo de toxinas, metabólicos e drogas. Normalmente são encontrados na forma de quatro subunidades, sendo duas proteínas integrais de membranas, que podem ser idênticas ou não, e duas subunidades citoplasmáticas responsáveis pela hidrólise de ATP (Higgins, 2001; Márquez, 2005).

Os transportadores secundários podem ser classificados em quarto familias: MFS (*Major Facilitator Superfamily*), RND (*Resistance Nodulation and Cell Division Family*), SMR (*Small Multidrug Resistance Family*) and MATE (*Multidrug And Toxic Compounds Extrusion Family*) (Marquez, 2005).

Os transportadores do tipo MFS pertencem a uma família que transporta açúcares, metabólicos intermediários e drogas. Esses transportadores são divididos em dois grupos de acordo com a quantidade de segmentos transmêmbranicos (12 ou 14). Este tipo de transportador pode ser encontrado em todos os organismos (Saier *et al.*, 1999; Márquez, 2005).

Os transportadores do tipo RND são encontrados principalmente em bactérias Gram-negativas. Sua função é transportar uma variedade de moléculas lipofílicas ou anfifílicas (hidrofílica e lipofílica) e cátions divalentes, sendo também responsáveis pela tolerância a solventes. Em bactérias Gram-negativas podem ser encontrados em associação com outras duas proteínas, MFP (*Membrane Fusion Protein*) e OMP (*Outer Membrane Protein*). Este complexo protéico é capaz de levar um componente tóxico do interior celular diretamente para o meio externo (Tseng *et al.,* 1999; Márquez, 2005).

As proteínas transportadoras do tipo SMR são as menores proteínas de efluxo de drogas, fazendo o efluxo principalmente de drogas catiônicas e lipofílicas. Já as proteínas do tipo MATE são do tipo antiporter de Na^+/drogas (Chung & Saier, 2001; Brown *et al*., 1999; Márquez, 2005).

OBJETIVOS GERAIS

Analisar a expressão gênica diferencial de *A. ferrooxidans* LR e *A. thiooooxidans* FG01 na presença de diferentes sulfetos metálicos.

PARTE I
OBJETIVOS ESPECÍFICOS

- Analisar a expressão gênica diferencial na estirpe de *A. ferrooxidans* LR mantida na presença de covelita e na do ferro por 24 horas.
- Investigar os fatores que influenciaram a indução da expressão de genes que codificam proteínas de transporte (AFE_0123, AFE_0989, AFE_0990, AFE_0580, AFE_0671, AFE_2248 e AFE_1149) na presença de covelita por 24 horas.
- Analisar, através de ferramentas de bioinformática, a interação entre as proteínas de transporte encontradas neste trabalho e diferentes proteínas de transporte encontradas no genoma de *A. ferrooxidans*.

PARTE II
OBJETIVOS ESPECÍFICOS

- Verificar a expressão de três genes (homólogos aos *loci* AFE_0123, AFE_0125 e AFE_0993) que codificam proteínas de transporte na estirpe de *A. thiooxidans* FG01 mantida na presença de calcopirita, covelita e pirita por 24 horas.
- Quantificar o cobre em solução na calcopirita e na covelita, após 24 horas, na presença e na ausência da bactéria e medir o pH na calcopirita, covelita e pirita após 24 horas.
- Analisar o grau de oxidação dos sulfetos metálicos, após 24 horas na presença ou na ausência da estirpe *A. thiooxidans* FG01, por microscopia eletrônica de varredura.
- Verificar a expressão diferencial dos genes que se localizam nos *loci* AFE_0123, AFE_0125 e AFE_0993 na estirpe de *A. thiooxidans* FG01 mantida em pH 4,0 ou em sulfato de cobre (16 mM) por 24 horas.

Capitulo I

***In silico* protein-protein interaction analysis and differential gene expression in response to covellite in *Acidithiobacillus ferrooxidans* LR**

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Running title: Gene expression in *A. ferrooxidans* in response to CuS

Summary

Nineteen differentially expressed genes were identified, using RAP-PCR, in *Acidithiobacillus ferrooxidans* LR in response to covellite (CuS). For this, RNA was isolated from bacterial cells grown on iron (control) and then kept in contact with CuS for 24 hours. Twelve genes had their differential expression confirmed by real-time PCR. All of them, with the exception of *infC*, which codifies for the translation initiation factor IF-3, were up-regulated in the presence of CuS. Seven of the up-regulated genes codify for proteins involved with transport and binding, and the other four genes codify for a putative diguanylate cyclase, a membrane protein, a methyltransferase and an ATPase. Since the majority of the covellite-induced genes were transporter genes. An atomic absorption analysis of the samples showed that the quantity of Cu^{+2} in solution was ~ 1.13 g/L and pH measurements showed that after 24 hours, it changed from 1.8 to 4.0. This way, we can suggest that copper ions and pH may be responsible, at least in part, for the up-regulation of the transporter genes in the presence of covellite. An *in silico* protein-protein interaction analysis of the proteins codified by the seven transporter protein genes as well as proteins from genes of the same functional category, that are physically located around them, showed that besides the correlated function, the transporter genes may act in different steps of the bacteria response to covellite.

Introduction

Acidithiobacillus ferrooxidans is a chemolithoautotrophic bacterium that obtains energy from the oxidation of ferrous iron, elemental sulfur or partially oxidized sulfur compounds (Rawlings, 2002). These bacteria are industrially used in the recovery of metals, especially copper, from low-grade sulfide ores. According to Sand et al. (2001), there are two main mechanisms used to explain the process of oxidation of solid metal sulfides by *A. ferrooxidans*, the direct and the indirect. The direct sulfide biooxidation can be performed only by microbial cells, immobilized on the surface of the sulfide crystal, without any requirement for ferric or ferrous ions (Fowler et al., 2001). In the indirect mechanism the metal sulfides are degraded by a chemical attack of ferric ions and/or protons on the crystal lattice. The mechanism of degradation is determined by the mineral structure. Metal sulfides such as covellite (CuS), sphalerite (ZnS), galena (PbS), and chalcopyrite ($CuFeS_2$), among others, are degradable by ferric ion and proton attack. As a consequence, polysulfide and elemental sulfur are the main intermediates (Sand et al., 2001).

Little is known about the molecular mechanisms used by *A. ferrooxidans* to survive or to obtain energy in the presence of metal sulfides. Ramírez et al. (2002, 2004) analyzed the proteins synthesized by *A. ferrooxidans* when this bacterium grew in the presence of metal sulfides, thiosulfate, ferrous iron and elemental sulfur, by means of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). An exported rhodanese-like protein, which is a thiosulfate sulfur transferase, had its synthesis up-regulated when the bacteria grew in the presence of pyrite, thiosulfate, elemental sulfur, CuS and ZnS and was almost completely repressed by growth on ferrous iron (Ramírez et al., 2002). A set of proteins differentially expressed in the presence of sulfur compounds and ferrous iron was described by Ramírez et al. (2004). In the presence of pyrite and chalcopyrite the proteins up-regulated by sulfur compounds and the ones up-regulated by ferrous iron were synthesized indicating that both energy-generating pathways, sulfur and iron, were induced in the presence of the tested metal sulfides (Ramirez et al., 2004).

The aim of this work was to identify genes differentially expressed in response to covellite in *A. ferrooxidans* strain LR, using RAP-PCR (RNA arbitrarily primed polymerase chain reaction). The differential expression of the isolated cDNAs was confirmed by real time

quantitative PCR and an *in silico* protein-protein interaction analysis was performed for the proteins codified by the transporter genes.

Results and discussion

To characterize the early response of *A. ferrooxidans* LR to covellite, total RNA from cells grown in the presence of iron and from cells grown in the presence of iron and then kept in contact with covellite for 24 hours was analyzed by RAP-PCR. Nineteen differentially expressed cDNAs were identified. They were sequenced and a BLAST search in the *A. ferrooxidans* ATCC 23270 genome (TIGR - www. tigr.org) was conducted. The search results showed that genes from different functional categories are involved in the *A. ferrooxidans* response to covellite. In this work, these genes will be referred by their names or by its locus name as assigned by TIGR on the *A. ferrooxidans* ATCC 23270 genome.

Of the 19 genes identified by RAP-PCR, 12 had their differential expression in the presence of covellite validated by quantitative real-time PCR, as shown in Table 1. All of them, with the exception of the *infC*, which codifies the translation initiation factor IF-3, were up-regulated in the presence of covellite. Seven genes codify for transport and binding proteins, among them, four (*loci* names AFE_0123, AFE_0989, AFE_0990 and AFE_0580) presented the greatest changes in transcript levels as shown in figure 1.

According to the *A. ferrooxidans* genome (TIGR), the genes, whose *loci* names are AFE_0123, AFE_0989, AFE_0990 and AFE_0671, codify a periplasmic peptide ABC transporter, two ABC transporter putative proteins with a permease activity, and an outer membrane protein CzcC which is a component from the cobalt, cadmium and zinc CzcABC efflux complex, respectively.

Table 1 rapPCR products differentially expressed in response to covellite in *A. ferrooxidans* LR

Genes assigned by role category (TIGR)	TIGR annotated function	Locus name by TIGR	Expression in response to covellite
Transport and binding proteins	Peptide ABC transporter, periplasmic peptide-binding protein	AFE_0123	Up-regulated
	ABC transporter, permease protein, putative	AFE_0989	Up-regulated
	ABC transporter, permease protein, putative	AFE_0990	Up-regulated
	Carbohydrate-selective porin, OprB family	AFE_0580	Up-regulated
	Heavy metal efflux pump, CzcA family	AFE_0671	Up-regulated
	Na+/H+ antiporter, putative	AFE_2248	Up-regulated
	Phosphate ABC transporter, permease protein, putative	AFE_1149	Up-regulated
Regulatory functions	Diguanylate cyclase, putative	AFE_1373	Up-regulated
Cell envelope	Membrane protein	AFE_0333	Up-regulated
Unknown function	Methyltransferase, FkbM family domain protein	AFE_0145	Up-regulated
	ATPase, AAA family	AFE_1585	Up-regulated
Protein synthesis: translation factors	Translation initiation factor IF-3	AFE_0490	Repressed

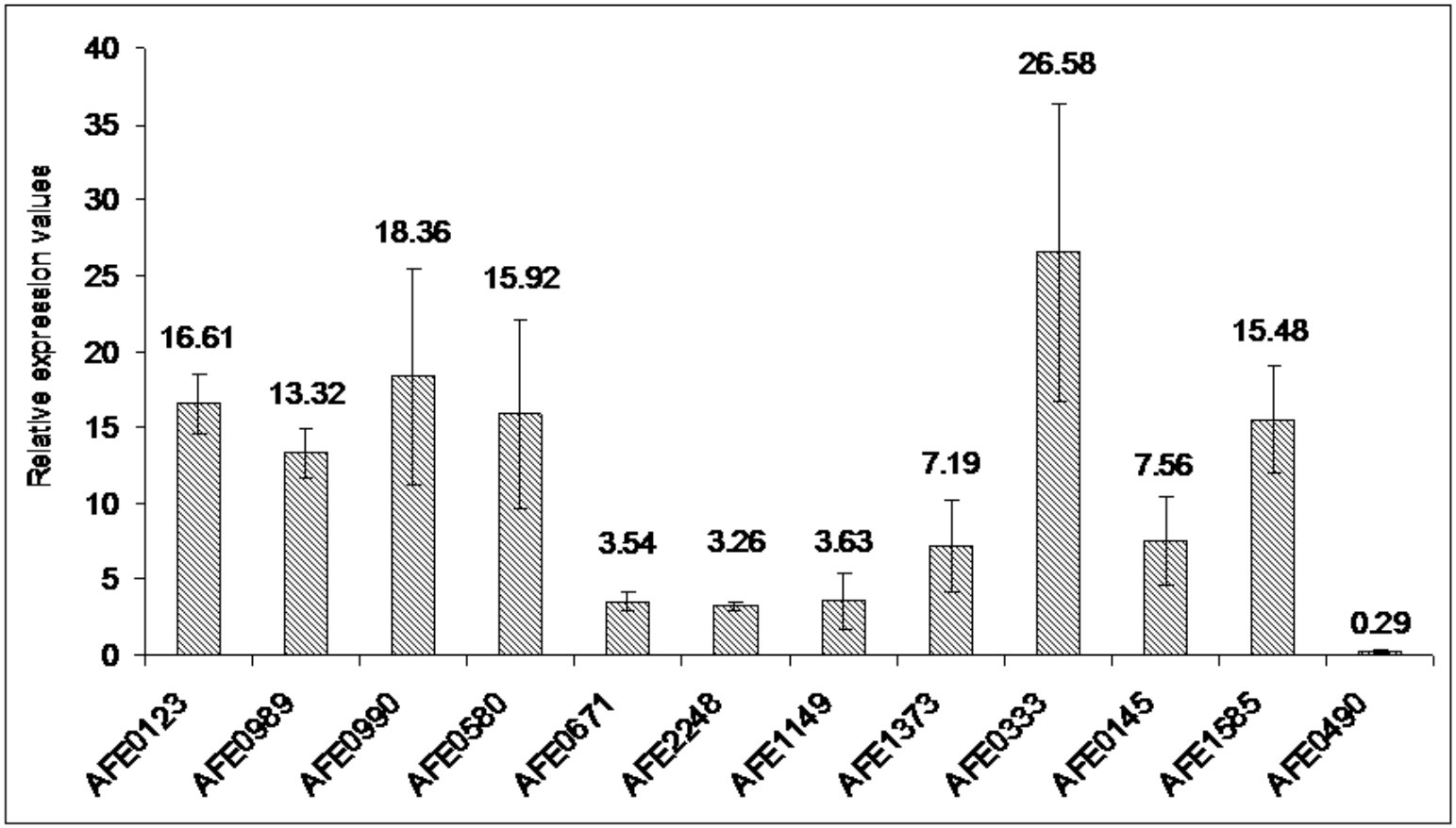

Figure 1. **Quantitative real-time PCR analysis of gene expression in *A. ferrooxidans* LR kept in contact with covellite for 24 hours. The expression of the 12 genes was quantified using a comparative critical threshold ($2^{-\Delta\Delta Ct}$) method. The iron growth condition was defined as the calibrator parameter**

Using the BLASTP algorithm, we found that the periplasmic peptide ABC transporter protein showed similarity with an extra cellular solute-binding protein from family 5 (e-value 3e-97), which is specific for peptides and nickel transport. The proteins codified by this family of genes are involved with transport, chemoreception and may function as a receptor in the initiation of sensory transduction pathways (Tam and Saier, 1993). With respect to the putative ABC transporter permease proteins, there are experimental evidence indicating that their group of proteins may be involved in cellular extrusion of metals, working as ATP-dependent efflux pumps preventing metal accumulation (Achard-Joris and Bourdineaud, 2006; Ambudkar et al., 1999).

The outer membrane component CzcC associates with CzcB, a periplasmic coupling protein, and CzcA, a large inner membrane protein, forming an inside/outside cell channel. The heavy metal efflux pump of cadmium/zinc/cobalt protein belongs to the CzcA family, which is responsible for metal detoxification (Anton et al., 1999; Legatzki et al., 2003).

The high expression of these four genes (*loci* names AFE_0123, AFE_0989, AFE_0990 and AFE_0671) in the presence of covellite may be explained as an attempt of the bacterial cells to survive in the presence of copper, which can be toxic to them. Although the protein codified by the gene at locus AFE_0671 has been reported as a specific transporter for zinc ions (Anton et al., 1999; Legatzki et al., 2003), the gene up-regulation in the presence of covellite may be an indication that this protein can also transport copper ions.

Atomic absorption experiments results showed that, in the presence of covellite, in samples with and without bacteria (control) the amount of Cu^{2+} detected in solution was ~ 1.11 g/L and 1.13 g/L, respectively. These results show that there were no changes in the amount of copper in solution due to the presence of bacteria and that the amount of copper in solution may be responsible for the up-regulation of the transporter genes.

The gene (locus name AFE_0580), highly expressed in the presence of covellite, codifies for an outer membrane carbohydrate-inducible porin (OprB), which belongs to the RND (resistance, nodulation and cell division) family of efflux transporter proteins (Adewoye et al., 1998; Castillo et al., 2007). Quatrini et al. (2007) described this gene for *A. ferrooxidans* as being the *feoP* gene, which belongs to the gene cluster *feoPABC*, involved in ferrous iron uptake transporter. The up-regulation of this gene in the presence of covellite, a copper sulfide with no iron on its composition, can be explained as an attempt of the bacteria to obtain iron.

The gene at locus AFE_2248 codifies for a citoplasmatic membrane protein that transports sodium to the outside and protons to the inside of the cells (each Na^+ transfers $2H^+$) (Melo et al., 2006; Screpanti et al., 2006). This Na^+/H^+ antiporter protein is involved in the maintenance of the intracellular pH, the intracellular Na^+ concentration and cellular volume. To understand the up-regulation of this gene, we must have in mind that covellite follows the polysulfide pathway, that is, it is attacked by protons and this reaction elevates the medium pH from 1.8 to 4.0. As this gene is known to confer resistance to the cell in alkaline pHs (Hunte et al., 2005; Leaphart et al., 2006), its up-regulation may be explained by the higher (4.0) than the optimal medium pH (1.8).

According to TIGR the gene at locus AFE_1149 codifies an ABC phosphate transporter protein that presented similarity (e-value 5e-61) with the PstA protein from *Acidiphilium cryptum*, according to the BLASTP algorithm (Altschul et al., 1997). This hydrophobic protein forms a transmembrane channel and is known to be induced when the phosphate concentration outside the cell is low (Braibant et al., 1996; Ramírez et al., 2004). Ramírez et al. (2004) found a putative phosphate binding protein (PstS) that had its expression induced in *A. ferrooxidans* by the lack of phosphate and is apparently part of the *Pho* regulon in this microorganism. PstS levels decreased when these bacteria grew on sulfur or thiosulfate. Since after 24 hours the pH of the medium increased from 1.8 to 4.0, we can speculate that the up-regulation of the gene at locus AFE_1149 in the presence of covellite is due to the lack phosphate which precipitates in higher pHs.

Since the majority of the differentially expressed genes codify for transport and binding proteins, we analyzed if these proteins show a direct (physical) or an indirect (functional) interaction. In the analysis we included the seven transporter genes as well as genes from the same functional category that were physically located around them in the chromosome map (Table 2). One gene did not produce a hit in the COG database (loci name AFE_0990) and other three did not show protein-protein interaction (loci names AFE_0578, AFE_2248 and AFE_0580). For all the other genes, it was possible to map orthology information in the COG database and to predict a putative protein interaction network (figure 2). The predicted network clearly shows six distinct sub-modules of interaction (Predicted protein-protein interaction [PPI] groups a to f). Interestingly, with the exception of group c, all the other PPI groups included a gene whose expression was induced in the presence of covellite. These results suggest that the transporter genes may have a correlated function

when *A. ferrooxidans* is kept in the presence of covellite and besides that, they might act in different steps of the bacteria response to this copper sulfide.

According to TIGRFAM, the gene at locus AFE_1373 has regions with high similarity with two protein domains: cyclic diguanylate phosphodiesterase (EAL) and diguanylate cyclase (GGDEF) (e-value 2.3e-97 and 6.3e-60, respectively). Proteins with the GGDEF domain also have regulatory motifs and can control the levels of c-di-GMP (Bis-(3'-5')-cyclic dimeric guanosine monophosphate) that controls the properties of cell surface, cell adherence, extracellular matrix synthesis, pili and flagellum formation. The EAL domain is not completely understood however, apparently, it is involved with c-di-GMP degradation (D'Argenio and Miller, 2004). According to Kim and McCarter (2007), the concentration of c-di-GMP in the cell may be involved in the coordination of cell surface molecules production. This way, the up-regulation of the gene at locus AFE_1373 may be an indication of alterations in the cell surface properties in the presence of covellite.

The gene at locus AFE_0333 codifies an integral membrane protein, probably involved in the constitution of the double layer of the cell envelope. The sequence of this protein was compared to others using the BLASTP algorithm and showed a similarity with a hypothetical transmembrane protein from *Gluconobacter oxydans* (e-value 7e-41). The gene at locus AFE_0145 codifies for a DNA methyltransferase (MTase) that belongs to the FkbM family domain protein. Members of this family are characterized by two well-conserved short regions, the first of these regions is found in a large number of proteins outside this subfamily, a number of which have been characterized as methyltransferases (Motamedi et al., 1996). The gene at locus AFE_1585 codifies an ATPase that cleaves ATP (adenosine triphosphate) producing ADP (adenosine diphosphate) and phosphate, liberating energy for further reactions inside the cell. According to TIGR genome annotation, this protein is involved in general biological processes, and belongs to the AAA family (ATPases Associated with various cellular Activities), which often performs chaperone-like functions assisting in the assembly/disassembly of protein complexes (Neuwald et al., 1999).

Table 2. Homology relations between *A. ferrooxidans* genes and orthologous groups (COG). The interaction was assigned by orthology and was able to classify seventeen genes that encode transport and biding proteins. For this, four genes were up regulated in response to covellite and the others are located around them in the chromossomo map. PPI groups indicate the six sub modules of the interactio network.

COG ID	COG description	Locus name by TIGR	TIGR annotated function	PPI group
COG3696	Putative silver efflux pump	AFE_0671	Heavy metal efflux pump, CzcA family	a
COG0845	Membrane-fusion protein	AFE_0669	Efflux transporter, RND family, MFP subunit	a,b
		AFE_0993	Efflux transporter, RND family, MFP subunit	
COG1538	Outer membrane protein	AFE_0668	Outer membrane efflux protein	a,b
COG0841	Cation/multidrug efflux pump	AFE_0992	Transporter, AcrB/AcrD/AcrF family	a,c
COG1131	ABC-type multidrug transport system, ATPase component	AFE_0991	ABC transporter, ATP-binding protein	b
COG0842	ABC-type multidrug transport system, permease component	AFE_0989	ABC transporter, permease protein, putative	b
COG1132	ABC-type multidrug transport system, ATPase and permease components	AFE_0125	ABC transporter, ATP-binding/permease protein	b
COG0444	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	AFE_0121	Peptide ABC transporter, ATP-binding protein	c,d
COG0601	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	AFE_0119	Peptide ABC transporter, permease protein	c,d
COG4608	ABC-type oligopeptide transport system, ATPase component	AFE_0122	Peptide ABC transporter, ATP-binding protein	d
COG0747	ABC-type dipeptide transport system, periplasmic component	AFE_0118	Peptide ABC transporter, periplasmic peptide-binding protein	d
		AFE_0123	Peptide ABC transporter, periplasmic peptide-binding protein	
COG1173	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	AFE_0120	Peptide ABC transporter, permease protein	d,e
COG0581	ABC-type phosphate transport system, permease component	AFE_1149	Phosphate ABC transporter, permease protein, putative	e,f
COG0226	ABC-type phosphate transport system, periplasmic component	AFE_1151	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS	f
COG0573	ABC-type phosphate transport system, permease component	AFE_1150	Phosphate ABC transporter, permease protein PstC	f

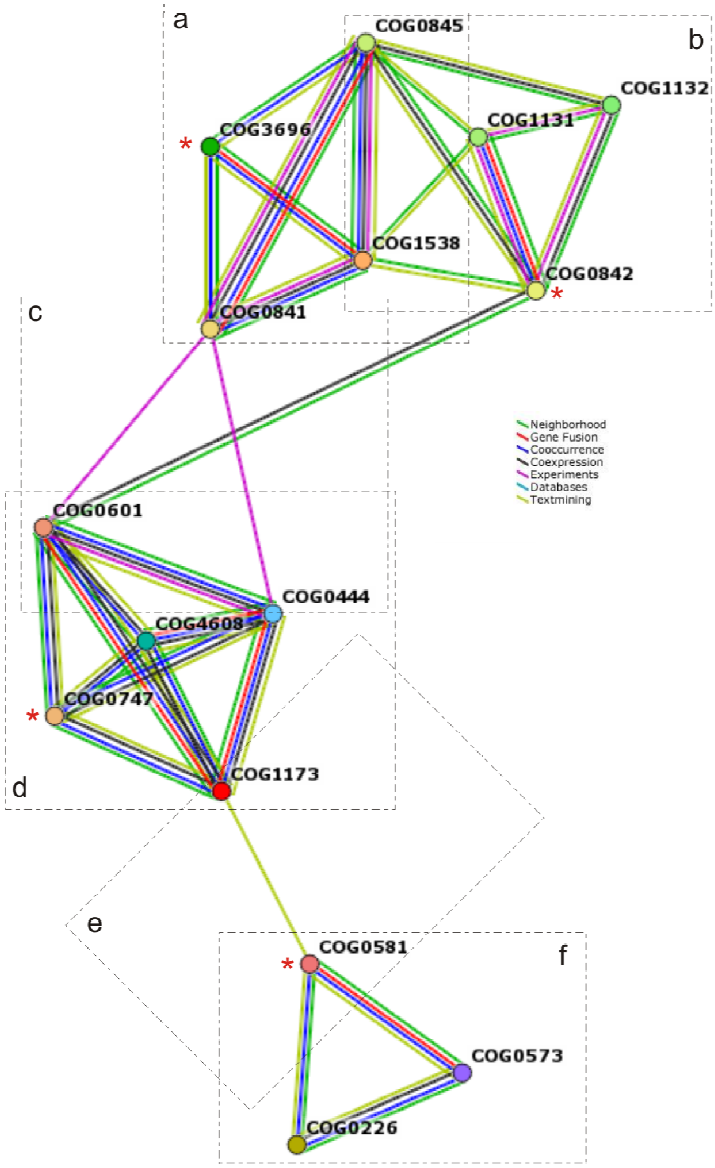

Figure 2. Predicted potein interaction network. The figure shows a putative network of *A. ferrooxidans* transporter and binding proteins codified by genes whose expression was induced in the presence of covellite. The dashed boxes shows clearly distinct groups (a to f) of protein-protein interaction. In the network, links between orthologous group signify the various interaction data supporting the network, colored by evidence type (see color legend in figure), and the asterisk show the proteins whose genes were differentially expressed in this study.

The *infC* gene (locus AFE_0490), which codifies the translation initiation factor 3 (IF-3), was down-regulated in the presence of covellite. IF-3 is involved in protein biosynthesis, a very complex cellular process with many different steps like initiation, elongation and termination (Cousineau et al., 1997). The IF-3 is one of the three factors required for the initiation of this process in bacteria (Liveris et al., 1993), this way the down-regulation of *infC* could be explained by an alteration on the *A. ferrooxidans* protein profile due to the presence of covellite.

In conclusion, this work shows that the majority of the differentially expressed genes in response to covellite in *A. ferrooxidans* LR codified for transport and binding proteins. This fact can be explained in part by the presence of copper ions in solution which can be toxic to the cells. The results obtained by bioinformatics analysis indicates that the transporter proteins analyzed interacts among them. They can be involved in different steps of the cellular response to copper stress, what could be explained by the different expression values observed for their genes.

Experimental procedures

Bacteria and culture conditions

Acidithiobacillus ferrooxidans strain LR, isolated from an effluent of column leaching of uranium ore from Lagoa Real, State of Bahia, Brazil (Garcia, 1991), was used in this work. The cells were grown in T&K liquid medium (Tuovinen and Kelly, 1972) containing (in g/L): $K_2HPO_4.3H_2O$, (0.5); $MgSO_4.7H_2O$, (0.5); $(NH_4)_2SO_4$, (0.5); $FeSO_4.7H_2O$, (33.4), pH 1.8, adjusted with sulfuric acid, at 30°C, 250 rpm. Cells were harvest when 80% of the ferrous iron (Fe^{2+}) was oxidized. The cells were washed in T&K medium and after centrifugation at 10.000 rpm for 5 min, they were resuspended and inoculated in 100 mL of iron-free T&K medium (pH 1.8) in a 250 mL flask containing 2.5 g of covellite (CuS, synthetic covellite with 99% of purity). The inoculum consisted of ~ 2 x 10^{14} cells/mL. Flasks were incubated at 30ºC, 250 rpm for 24 hours. The cells were collected by filtration on Millipore membrane (0.45 μM). They were washed until the complete removal of the metal sulfide and then stored at -70°C until total RNA isolation.

RNA isolation

Total RNA was isolated, as described by Winderickx and Castro (1994), from *A. ferrooxidans* LR cells grown in the presence of iron (control condition) and from cells kept in contact with covellite for 24 hours. The RNA samples were treated with DNAse for 1h at 37°C and then stored at -70°C.

RAP-PCR

The RAP-PCR (RNA arbitrarily primed polymerase chain reaction) experiments were performed as described by Paulino et al. (2002). The arbitrary primers (Operon Technologies) used in the experiments were: OPF9, OPF13, OPF20, OPL03 and OPL12. The RAP-PCR products were cloned into pGEM-T easy vector (Promega) and then sequenced in an ABI Prism 3700 DNA Analyzer (Applied Biosystems). The obtained sequences were compared to the *A. ferrooxidans* ATCC 23270^T genome made available by TIGR – The Institute for Genomics Research (http://cmr.tigr.org). The TIGRFAM (Haft et al., 2003), a tool that supports the automated functional identification of proteins by sequence homology, was also used in the analysis of the proteins codified by the differential expressed genes.

Differential gene expression confirmation by real-time PCR

The differential expression of the RAP-PCR products was confirmed by real-time PCR. For this, RNA was isolated from cells cultured on iron (control) and cells kept in contact with covellite for 24 hours. The RNA was treated with DNaseI AmpGrade (Invitrogen) and used for total cDNA synthesis with the ThermoScript™ RT-PCR System (Invitrogen), according to the manufacture's protocol. The real-time PCR experiments were performed three times, with three independent total RNA and cDNA preparations.

Quantitative PCR was performed with a 7500 real-time PCR System (Applied Biosystems) and threshold cycle (Ct) numbers were determined using the real-time System RQ Study Software v. 1.3.1 (Applied Biosystems). The reaction mixture contained 6.25 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 0.25 µl of the passive reference ROX (Invitrogen), 2 µl of template cDNA (100 ng/µl), 10 µM of each primer, and H_2O added to a final volume of 12.5 µl. Each reaction was performed in triplicate and threshold cycle numbers were averaged. The reaction was cycled with preliminary UDG treatment for 2 min at 50°C and a denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and primer extension at 72°C for

32 s. At the end of each run, the melting curves for the amplicons were measured by raising the temperature slowly from 65°C to 95°C while monitoring fluorescence. The specificity of the PCR amplification was checked by examining the derivative melting curve for T_m, its symmetry and the lack of non-specific peaks. Real-time PCR primers (Table 3) were designed with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3www.cgi) using the *Acidithiobacillus ferrooxidans* ATCC 23270 genome sequence (http://cmr.tigr.org).

The genes relative expression pattern was calculated according to the formula $2^{-\Delta\Delta Ct}$ where ΔΔCt is the difference between the ΔCt from the experimental condition, observed in the presence of covellite, and the ΔCt from the calibrator condition, observed in the presence of iron. The ΔCt was calculated by the difference between the Ct from the experimental gene and the Ct from the constitutive gene (Livak and Schmittgen, 2001). The *alaS*, which codifies for the protein alanyl-tRNA synthetase, was used as the constitutive gene (Yarzábal et al. 2004) in the experiments. The primers for this gene (AlaS13 and AlaS3) are shown in Table 3. The relative expression values higher (induced by covellite) or smaller (repressed by covellite) than 1 were considered differential. The statistical significance of the obtained results was verified by the Student's t test ($n = 3$, $p < 0.05$).

pH and copper ion determination

The pH of the T&K liquid medium, containing 2.5 g of covellite, with and without the bacteria, was measured before and after incubation at 30°C, 250 rpm, for 24 hours. After this period of time, 1 mL of each sample was mixed with 14 mL of nitric acid and the concentration of copper ions (Cu^{2+}) in solution was estimated by Atomic Absorption Spectroscopy (AAS). A ContrAA 300 (Analytik Jena, Germany), equipped with xenon short-arc lamp (XBO 301, 300 W, GLE, Berlin, Germany) as a continuum radiation, was used in the experiments. The atomic line for copper determination was 324.724 nm.

Table 3. Primers used for the real-time PCR experiments.

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length (bp)
AFE_0123	GGGCAGCGTCTTCAGTTTAC	CCAGTCCGCTTTCAACATTT	84
AFE_0145	ACCTGCCTACGCAAGACTGT	GCATGGTCTTTAGCGATGGT	150
AFE_0289	AGCTCAACCGAAGTCTGGAA	CATCGCGATAGAGGAAGAGG	114
AFE_0490	GATGACGCCGACTACCAGAT	CCTTTCTACCCGATTGAGCA	144
AFE_0333	GGTAATGTACGCTGGGCAGT	TGAGCAGTGCAAAGAGGATG	124
AFE_0580	AGATTGGTGGCGAGTACGAC	CTGGATGTCGCCGCTATAAT	119
AFE_0671	GCCCGTAATCTGGTACTCCA	TTGGGCCAGATTCAAAGTTC	150
AFE_0989	CGCAATGGCTGTTAACTGAA	AGGGGAGGAGGCTATAAGCA	128
AFE_0990	GGTGTTATTGTTGGCGCTCT	GTGAAATTGGGAGCGTCTGT	91
AFE_1149	AAAGAACCCATCGGCTACCT	GCAAATGCGAGCATATGAGA	83
AFE_1373	CAGTTACGGGCAGGAGACAT	GAGTAATCGTTCGCTGACACC	111
AFE_1412	GACTATAGACGCCGGACA	AACCAATTTGGCGTACTTCG	133
AFE_1585	ACGATATCATCACCGGGAAA	ATCCCAGTTGTCCTGAATGC	141
AFE_1628	CAACAACAAAAAATGGCAACG	CTGTCGGCGGTACATCAGTA	99
AFE_1684	GTGCGCAGGGAGATTGAA	ACCAGGAGTCGATGATGAG	172
AFE_1751	TCTTTGTGCGCAACTACCAG	GTGGCAAAGGTCGACTCAAT	148
AFE_2248	TTCGCTTTCATCGTCATCTG	ATCCGGAAACGAATATGCTG	133
AFE_2711	CCATGTGACCGTGTACGAAG	ACCCAACACACGCAGGATA	94
AFE_3005	GGTGAGGAGGAGATGAAGGA	CCTTACGGCGAATCAGACTT	229
AlaS13/S3	GTGCCTTTCCCGAACTCACG	TCCTCCAGCAGACTGAGTCC	108

Prediction of protein-protein interactions

Our dataset made to predict protein-protein interactions was composed by the seven *A. ferrooxidans* transporter and binding proteins that were codified by the genes (*loci* names AFE_0123, AFE_0989, AFE_0990, AFE_0580, AFE_0671, AFE_2248 and AFE_1149) whose expression was induced in the presence of covellite, and by fourteen other *A. ferrooxidans* proteins codified by the genes at *loci* AFE_0991, AFE_0992, AFE_A0993, AFE_0118, AFE_0119, AFE_0120, AFE_0121, AFE_0122, AFE_0125, AFE_0578, AFE_0668, AFE_0669, AFE_1150, and AFE_1151. The genes that codify for these fourteen proteins are located around the seven covellite induced genes in the *A. ferrooxidans* ATCC 23270 chromosome map, and all were annotated in the same category by TIGR.

The STRING database (von Mering et al., 2007) was used to predict putative protein interaction networks providing an overview of the physical and functional associations and interactions between proteins. The homology relations between the *A. ferrooxidans* genes and the STRING database was performed by mapping orthology information from COG database. The associations found between COGs were used to build a network view, displaying the proteins as nodes, and different kinds of associations as edges. In this analysis a confidence score of 0.4 was used.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant 2005/51332-7. FCR and CC had fellowships from FAPESP (05/00139-02 and 06/54433-1, respectively). DJM (134062/2006-0), LMMO and OGJ had fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and LFCF had fellowship from Companhia Vale do Rio Doce (CVRD).

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Capitulo II

Transporter genes are differentially expressed in the presence of metal sulphides in *Acidithiobacillus thiooxidans*

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Summary

Acidithiobacillus thiooxidans is involved in bioleaching, a process that has been successfully used in copper recovery. To investigate the expression of three transporters protein genes, two that encodes for ABC transporters proteins (*loci* names AFE_0123 and AFE_0125) and one that encodes for an efflux transporter protein (locus name AFE_0993), *A. thiooxidans* FG01 was maintained in contact with covellite, chalcopyrite and pyrite and the expression was analysed by real-time PCR. For this, *A. thiooxidans* FG01 was cultivated in the presence of sulphur (standard condition) and then maintained in contact with the metal sulphides for 24 hours. The expression patterns of the three genes were up regulated in the presence of covellite and unchanged in the presence of chalcopyrite. In the presence of pyrite, two genes were down regulated and the expression of one (locus name AFE_0993) remained unchanged. These results can be explained, at least in part, by the amounts of copper in solution in the covellite (1 g/l) and chalcopyrite samples (0.07 g/l), which were measured by atomic absorption. pH measurements showed that both chalcopyrite and covellite sequestered H^+ ions, increasing the pH. Scanning electron microscopy analyses of the sulphide samples showed a superficial corrosion in covellite, corroborating with the higher amount of copper found in solution. To investigate if either the pH or the presence of copper ions in solution were responsible for the increased expression of these genes, the bacteria were maintained in contact with copper sulphate or pH 4.0 for 24 hours. The results showed that the presence of copper ions in solution induced the expression of these genes, while the increase in pH from 1.8 to 4.0 did not. These results indicated that the transporters genes analysed were regulated mainly by the presence of copper ions in solution.

Keywords: *Acidithiobacillus thiooxidans*, metal sulphides, transporter protein genes, real time PCR

Introduction

Acidithiobacillus thiooxidans is an acidophilic chemolithotrophic bacterium that obtains energy from the oxidation of elemental sulphur or reduced sulphur compounds. This bacterium is involved in bioleaching, a process used for metal recovery (Chandraprabha and Natarajan, 2006). *A. thiooxidans* is one of the most dominant populations in bioleaching tanks, and recent works have shown the important role of this bacterium in prevent sulphur and jarosite accumulation on metal sulphides (Akcil *et al.*, 2006; Falco *et al.*, 2003; Ghauri *et al.*, 2006; Petersen and Dixon, 2006).

An important characteristic of the acidophilic chemolithotrophs is their general tolerance to high concentrations of metal ions. Apparently *A. thiooxidans* is sensitive to less than 5 g/l of many cations including copper, with the exception of zinc (10 g/l) (Rawlings, 2005). There are three possible mechanisms for heavy metal resistance: (a) the metal can be excluded from the cell by efflux, (b) cations can be segregated into complex compounds by thiol-containing molecules and (c) some metal ions, like copper, may be reduced to a less toxic state ($Cu^{2+} \rightarrow Cu^{+}$). However, most bacteria use a combination of more than one mechanism to achieve resistance and homeostasis (Dopson *et al.*, 2003; Nies, 1999). Little is known about copper resistance in acidophilic bacteria and no studies have been carried out at the molecular level (Barreto *et al*., 2003; Rawlings, 2005).

Experimental evidence indicates that some ATP-binding cassette (ABC) transporter proteins may be directly involved in the cellular extrusion of metals, working as ATP-dependent efflux pumps preventing metal accumulation (Achard-Joris and Bourdineaud, 2006; Ambudkar *et al.*, 1999). This large family of transmembrane proteins binds ATP, and uses the energy produced to drive the transport of various molecules across all cell membranes (Achard-Joris and Bourdineaud, 2006; Holland and Blight, 1999). The ABC-transporters are mainly composed of a membrane domain (MD), providing a transport pathway and an ATPase. The different members of this family can transport a wide range of compounds including ions, large polypeptides and polysaccharides (Holland and Blight, 1999).

In this work, the expression of three transporter protein genes (loci AFE_0123, AFE_0125 and AFE_0993) was investigated by real time PCR in *A. thiooxidans* FG01

maintained in the presence of covellite, chalcopyrite and pyrite. The effect of copper and pH on the expression of these genes was also investigated.

Materials and Methods

Bacterial strains and growth conditions

Acidithiobacillus thiooxidans strain FG01 obtained from a leaching uranium mine heap in Paraná State – Brazil (Garcia Jr, 1991) was used in the experiments. The strain was grown in T&K liquid medium (Tuovinen and Kelly, 1974) pH 1.8, with powdered sulphur (10 g/l) as the energy source, at 250 rpm and 30º C until an O.D. of 0.11 was reached. The cells were collected by centrifugation (10.000 rpm for 10 min), washed and inoculated into 250 ml flasks containing 100 ml of T&K liquid medium pH 1.8, plus 2.5 g of covellite, chalcopyrite or pyrite or 16 mM of copper sulphate ($CuSO_4.5H_2O$) or at pH 4.0. Each flask was inoculated with an *A. thiooxidans* suspension of approximately 5 x 10^{10} cells/ml. After 24 hours the cells were collected by filtration on Millipore membranes (0.45 μM). The cells were washed until the complete removal of the metal sulphide and stored at -70°C for total RNA isolation. All experiments were repeated at least twice.

RNA extraction and cDNA synthesis

Total RNA was isolated as described by Winderickx and Castro (1994) from *A. thiooxidans* FG01 cells grown in the presence of sulphur (standard condition), from cells maintained in contact with covellite, chalcopyrite, pyrite, copper sulphate, and at pH 4.0, all for 24 hours. The RNA samples were treated with DNaseI AmpGrade (Invitrogen, USA) and the treated RNA was used for cDNA synthesis with the ThermoScript™ RT-PCR System (Invitrogen, USA), according to the manufacture's protocol.

Real Time PCR

Quantitative PCR was performed using a 7500 Real Time PCR System (Applied Biosystems, USA), and threshold cycle (Ct) numbers were determined using the Real Time System RQ Study Software v. 1.3.1 (Applied Biosystems, USA). The reactions were carried out in triplicate and the threshold cycle numbers were averaged. The 12.5 μl reaction mixture was prepared as follows: 6.25 μl of Platinum® SYBR® Green qPCR SuperMix-UDG

(Invitrogen, USA), 0.25 µl of the passive reference ROX, 10 µM of each primer and 2 µl of cDNA (100 ng/ µl). The reaction was cycled with a preliminary UDG treatment for 2 min at 50°C and a denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and primer extension at 72°C for 30 s. This was followed by a melting point analysis of the double-stranded amplicons, consisting of 40 cycles of a 1°C decrement (15 s each) beginning at 95°C. This was used to verify the specificity of the Real Time PCR reaction for each pair of primers. Real time PCR primers (Table 1) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3www.cgi) with the *Acidithiobacillus ferrooxidans* ATCC 23270 genome sequence (TIGR, http://cmr.tigr.org), and then used to amplify the genes of interest in *A. thiooxidans* FG01. Before their use in the real time PCR experiments, the synthesized primers were tested. For this, they were used in the amplification of the genomic DNA from *A. thiooxidans* FG01. The amplification products were sequenced and the sequences obtained compared with those from the *A. ferrooxidans* ATCC 23270 genome.

The relative expression of the genes was calculated according to the formula $2^{-\Delta\Delta Ct}$ where ΔΔCt is the difference between the ΔCt from the experimental condition (presence of covellite, chalcopyrite, pyrite, copper sulphate and pH 4.0) and the ΔCt from the standard condition (sulphur). The ΔCt was calculated from the difference between the Ct from the tested genes and the Ct from the control gene (16*S*) (Livak and Schmittgen, 2001; Yarzábal *et al.*, 2004). The genes studied in this work will be referred to by their locus name according to the *A. ferrooxidans* ATCC 23270 genome nomenclature. The statistical significance of the results obtained was evaluated by the Student's t test ($n = 3$, $p < 0.05$).

pH and copper ion determinations

The pH of the T&K liquid medium containing 2.5 g of the metal sulphides covellite, chalcopyrite and pyrite, with and without bacteria, was measured before and after incubation at 30°C, 250 rpm, for 24 hours. After this period of time, 1 ml of each sample was mixed with 14 ml of nitric acid. The concentration of copper ions (Cu^{2+}) in solution was estimated by Atomic Absorption Spectroscopy (AAS) using a ContrAA 300 (Analytik Jena, Germany) equipped with a xenon short-arc lamp (XBO 301, 300 W, GLE, Berlin, Germany) as a continuum radiation. The atomic line for copper determination was 324.724 nm.

Scanning Electron Microscopy (SEM)

For the SEM analysis, T&K liquid medium pH 1.8 containing 2.5 g of the metal sulphides covellite, chalcopyrite or pyrite, with (experimental condition) or without bacteria (control condition), were incubated for 24 hours at 30 °C, 250 rpm. The metal sulphide particles were collected and analysed in a JEOL scanning electron microscope in the backscattered electron mode with a maximum accelerating potential of 15 kV and working distance of 10 mm.

Results and Discussion

Transporter protein gene expression in the presence of metal sulphides

In a recent work with *Acidithiobacillus ferrooxidans* LR, our group observed that the expression of some transporter protein genes increased in the presence of covellite. Three of those genes, locus AFE_0123, AFE_0125 and AFE_0993 according to the *A. ferrooxidans* ATCC 23270 genome made available by TIGR (http://www.tigr.org), were selected to test their relative expressions in the sulphur oxidizing bacteria *A. thiooxidans* FG01, maintained in contact with covellite, chalcopyrite and pyrite for 24 hours. The genes at loci AFE_0123 and AFE_0125 encode for ABC transporter proteins and the one at locus AFE_0993 encodes for an efflux transporter protein. Multiple protein alignments using the database TIGRFAM (http://cmr.tigr.org) showed that the genes at locus AFE_0123, AFE_0125 and AFE_0993 presented conserved region that exhibited similarity with a peptide and nickel transport (PF00496), a multi-drug resistance protein (MDR) (PF00005 and PF00664) and transport proteins that function as MDR and a metal efflux pump (TIGR01730 and PF00529), respectively.

The two MDR genes were chosen because of the evidence that links these proteins to resistance to heavy metals (Achard-Joris and Bourdineaud, 2006; Ambudkar *et al*., 1999), and the peptide and nickel transport gene was chosen because, like nickel, copper is also a divalent metal ion and is part of the composition of two of the metal sulphides used in the experiments. The putative identification of these proteins, given by TIGR, their conserved domains and the primers used to analyse the gene expression, are given in table 1.

Table 1. Primers used in the real time PCR experiments.

Locus name / Gene	Protein: putative identification	Conserved domains	Primer sequences	Amplicon (bp)
AFE_0123	peptide ABC transporter, periplasmic peptide-binding protein	PF00496, COG0747	F - 5'GGGCAGCGTCTTCAGTTTAC3' R - 5'CCAGTCCGCTTTCAACATTT3'	84
AFE_0125	ABC transporter, ATP-binding /permease protein	PF00005 , PF00664, COG1132	F - 5'TGATGGTCAGGACGTCAGAG3' R - 5'TGTCGACCATAACGCACATT3'	120
AFE_0993	efflux transporter, RND family, MFP subunit	TIGR01730 , PF00529, COG0845	F - 5'TGATTCAAAAAGGCCAGCTT3' R - 5'TTGTTGCTGAGCGATTTCAC3'	104
16S	**-**	**-**	F- 5'ACACTGGGACTGAGACACGG3' R - 5'ACCGCCTACGCACCCTTTAC3'	277

F – Forward; R – reverse; bp - base pair.

The expression of the genes from locus AFE_0123, AFE_0125 and AFE_0993 was analysed by real time quantitative PCR using RNA from cells grown in the presence of sulphur (standard condition), and cells maintained for 24 hours in the presence of the metal sulphides. As shown in Fig. 1, the expression of the 3 genes was up regulated in the presence of covellite and remained unchanged in the presence of chalcopyrite. In the presence of pyrite, the genes at locus AFE_0123 and AFE_0125 were down regulated while the expression of the gene at locus AFE_0993 remained unchanged (Fig. 1). To explain these results, the bacteria were maintained for 24 hours in the presence of the metal sulphides, and then the pH was measured and the amount of Cu^{2+} in the medium was determined. Also, to detect any oxidation, the metal sulphides were submitted to SEM analysis. The controls consisted of the T&K liquid medium with the sulphides but without the bacteria.

pH and copper ion determinations and SEM analysis

The pH measurements, made after 24 hours, showed that both chalcopyrite and covellite sequestered H^+ ions, increasing the pH values. In the chalcopyrite samples, the pH increased from 1.8 to 2.52 (samples without the bacteria) and to 2.46 (samples with the bacteria). As expected, the pH changes in the covellite samples were greater than those observed in the presence of chalcopyrite, since chalcopyrite is more recalcitrant to chemical attack. The pH values in the samples with covellite increased from 1.8 to 4.10 (samples without the bacteria) and to 4.08 (samples with the bacteria). Since pyrite does not sequester H^+ ions, the pH changes in the samples with this metal sulphide were not significant. They changed from 1.8 to 1.85 and from 1.8 to 1.93 in the samples with and without the bacteria, respectively.

SEM experiments were performed to observe the degree of sulphide oxidation after 24 hours in samples with and without bacteria. These results are shown in Fig. 2. In sulphide samples without the bacteria (control), a large amount of precipitate was observed. These precipitates may have originated from components in the medium used in the experiments, such as phosphate. In the samples with *A. thiooxidans* FG01 such precipitates were not observed. As expected, some corrosion was observed in the chalcopyrite and covellite samples. The sulphide pattern of oxidation and corrosion in these two metal sulphides was superficial. The same level of oxidation was not observed in pyrite samples.

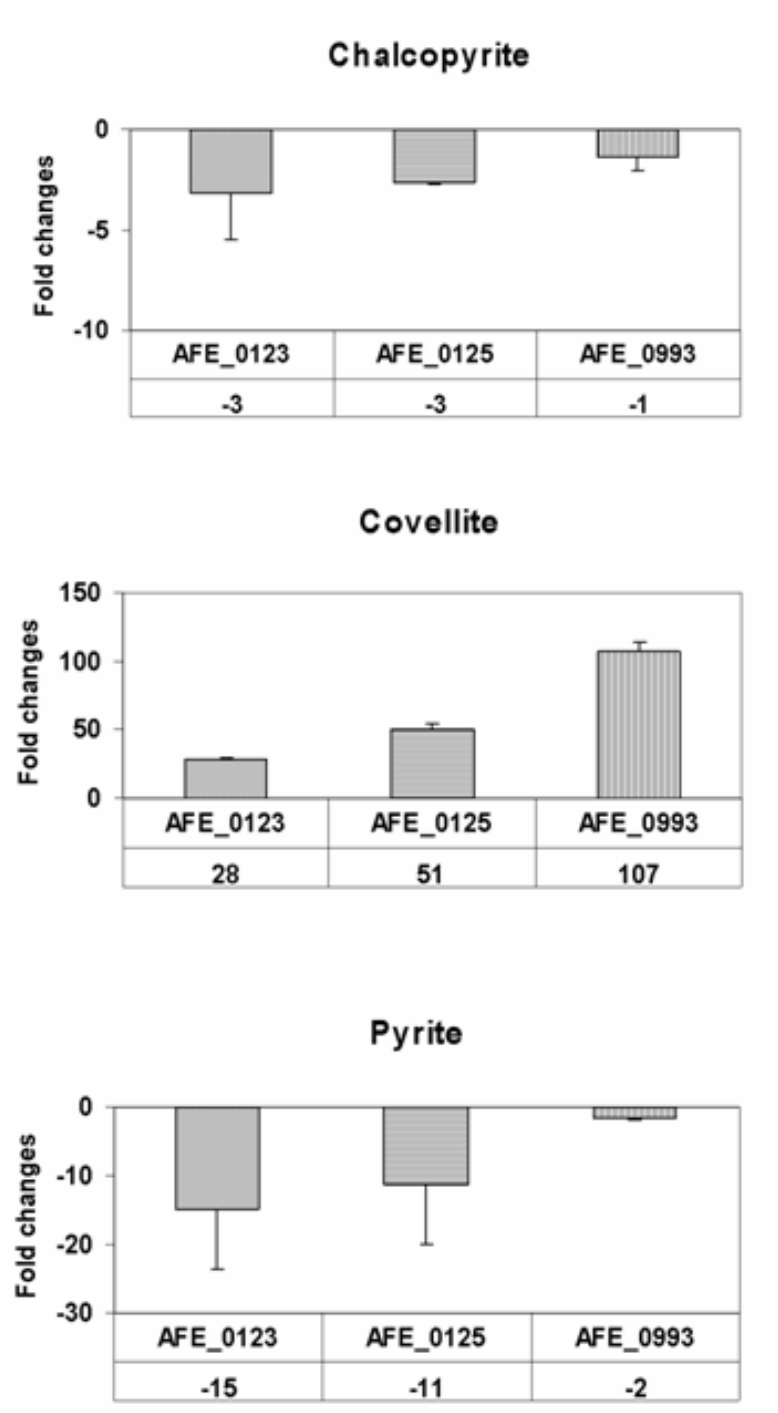

Figure 1. Quantitative real-time PCR analysis of the gene expression in *A. thiooxidans* FG01 maintained in contact with covellite, chalcopyrite and pyrite for 24 hours. The expression of the genes was quantified using a comparative critical threshold ($2^{-\Delta\Delta Ct}$) method. The sulphur growth condition was used as the calibrator parameter.

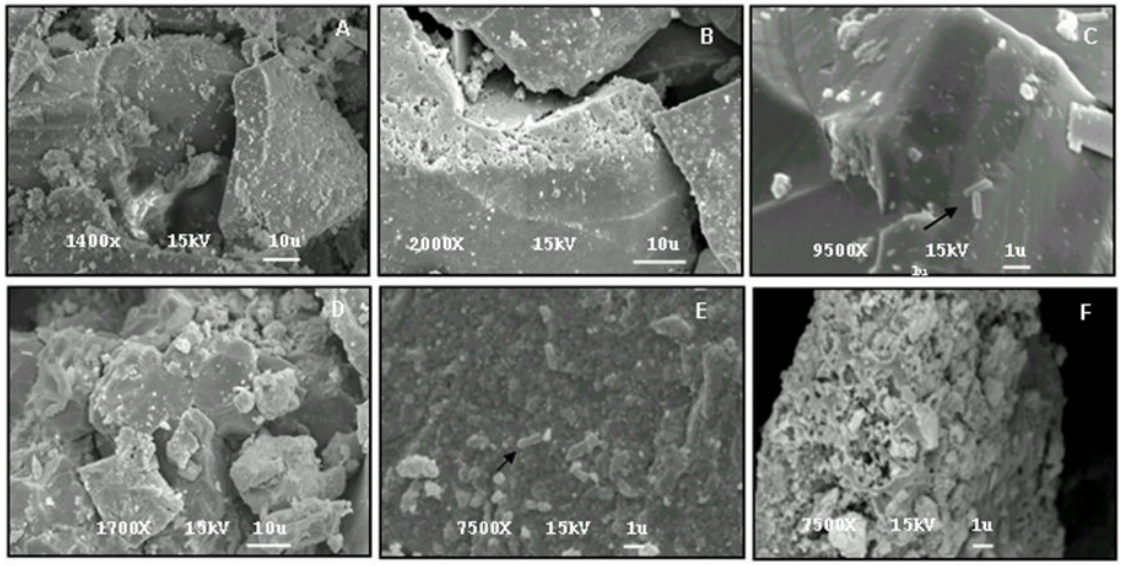

Figure 2: SEM of chalcopyrite and covellite samples after 24 hours in contact with *A. thiooxidans* FG01: Chalcopyrite samples: B) oxidation and C) cells adhered to the sulphide surface. Covellite samples: E) bacterial cells adhered to covellite and F) covellite corrosion. Controls consisted of metal sulphides without bacteria (A and D).

The AAS analysis showed that the amounts of Cu^{2+} in solution in the chalcopyrite samples with and without (control) the bacteria were 0.075 g/l and 0.072 g/l, respectively. In the covellite samples, the amounts of Cu^{2+} were 1.13 g/l in the samples without (control) the bacteria and 1.03 g/l in the samples with the bacteria. These results indicated an insignificant difference in the amounts of Cu^{2+} in the samples with and without the bacteria, although the amounts were approximately 2 folds greater in the covellite samples. Thus one can suggest that the greater amount of Cu^{+2} in the medium could be responsible for the higher expression of the *A. thiooxidans* transporter genes in the presence of covellite. It is known that *A. thiooxidans* can only tolerate 5 g/l of Cu^{+2}, but the mechanism these bacteria use to tolerate these cations is still not understood (Rawlings, 2005). Some studies have tried to explain the resistance of bacteria to heavy metals, but the process is still not completely understood (Rawlings, 2005; Dopson *et al*., 2003; Nies, 1999; Barreto *et al*., 2003).

The pH measurements and AAS results corroborated the fact that covellite and chalcopyrite were soluble in acid solutions, following the polysulphide pathway, while pyrite is insoluble in acid, following the thiosulphate pathway (Schippers and Sand, 1999). This phenomenon explains the greater oxidation observed with the copper sulphides than with the pyrite.

It is known that any kind of imperfection in the sulphide grain, such as cleavages, fractures or gaps, becomes a susceptible area for oxidation and corrosion. Some of these imperfections may come from the sample preparation or from the sample itself (De Leeuw *et al*., 2000; Márquez *et al*., 2006). These flaws probably helped the oxidation process observed in some of the particles.

Despite sample agitation (250 rpm) and the short time of contact (24 hours), it was possible to observe some cells adhered to the chalcopyrite and covellite surfaces. These findings are in agreement with the results obtained by Chandraprabha and Natarajan (2006) in a study with *A. thiooxidans*, which showed that the Cu^{+2} in the chalcopyrite structure did not seem to affect the kinetics of cell adhesion to its surface.

Copper ions are mainly responsible for the transporter protein genes up regulation

To investigate if the copper ions in solution or alterations in pH were responsible for the up regulation of the three transporter genes analysed, *A. thiooxidans* FG01 was grown in the presence of sulphur and the cells were then maintained for 24 hours in the presence of copper or at pH 4.0. The results showed that the presence of copper ions in the solution induced the expression of the three genes, while the increase in pH to 4.0 did not interfere with their expression in a significant manner (Fig. 3).

It is well known that heavy metals like copper are required in trace amounts for bacterial growth, although in excess this metal can be toxic (Teitzel *et al*., 2006). Copper transport and resistance have been extensively studied in bacteria, especially in *E. coli* (Rensing and Grass, 2003) and *Enterococcus hirae* (Solioz and Odermatt, 1995; Solioz and Stovanov, 2003). Teitzel and co-workers (2006) determined the transcriptional profiles of *Pseudomonas aeruginosa* cells adapted to copper and submitted to copper shock. Their results showed that the expressions of genes encoding several active transporter proteins, like two gene clusters homologous with the RND family members, a P-type ATPase, a two component regulatory system and a periplasmic system, were up regulated in the presence of copper, suggesting that these proteins play an important role in copper tolerance, corroborating our results. This way, we can suggest that the transporter proteins codified by the genes analysed in this work have the ability to maintain cellular homeostasis by transporting copper ions to the extracelullar environment. This characteristic may be of great importance for both, survival and the establishment of a bacterial population in changing environmental conditions.

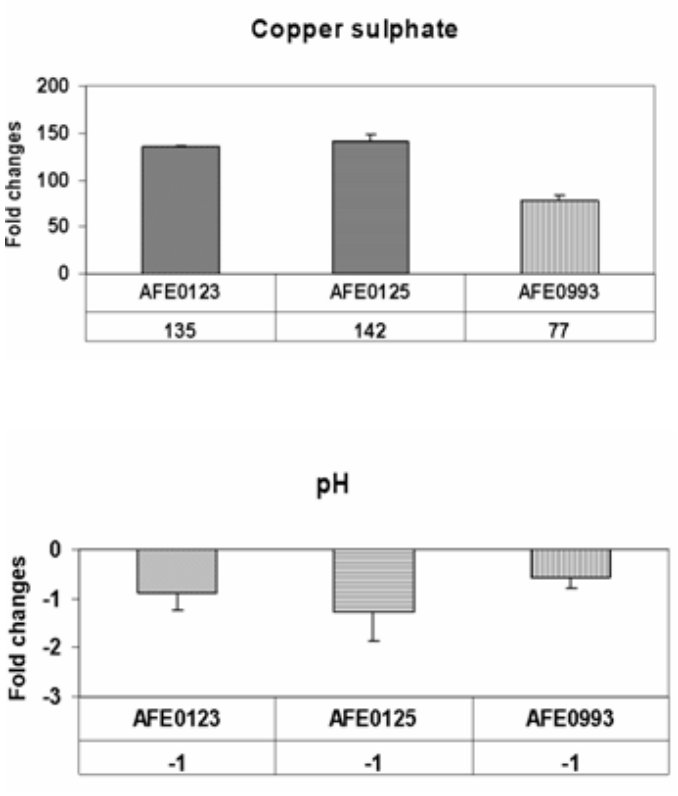

Figure 3. Quantitative real-time PCR analysis of the gene expression in *A. thiooxidans* FG01 maintained in contact with copper sulphate and at pH 4.0 for 24 hours. The expression of the genes was quantified using a comparative critical threshold ($2^{-\Delta\Delta Ct}$) method. The sulphur growth condition was used as the calibrator parameter.

Acknowledgements

This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) grant 2005/51332-7 and Companhia Vale do Rio Doce. DJM (134062/2006-0), OGJ and LMMO received research fellowships from the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq). FCR and CC received fellowships from FAPESP (05/00139-02 and 06/54433-1, respectively).

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CONCLUSÕES

PARTE I

- Doze genes em *A. ferrooxidans* tiveram sua expressão diferencial confirmados por PCR em tempo real na presença de covelita após 24 horas de contato.

- Quatro genes (locus AFE_1373, AFE_0333, AFE_0145 e AFE_1585) de função regulatória foram induzidos na presença de covelita e um teve sua expressão reprimida (locus AFE_0490). A expressão diferencial desses cinco genes pode ser explicada como uma resposta da célula a mudança ambiental

- Sete genes codificavam para proteínas de transporte. Esses genes tiveram sua expressão induzida na presença de covelita, o que talvez possa ser explicado pela presença de cobre em solução. A provável função dessas proteínas seria tentar manter a homeostase da célula transportando o metal pesado para o meio exterior, visto que o cobre é tóxico a mesma.

- A previsão *in silico* da interação entre as proteínas transportadoras induzidas na presença de covelita e, as proteínas transportadoras codificadas por genes que se encontram fisicamente localizadas perto dos genes induzidos, sugerem um possível envolvimento destas proteínas em diferentes passos da resposta ao cobre.

PARTE II

- A indução dos genes (homólogos aos *loci* AFE_0123, AFE_0125 e AFE_0993) na presença de covelita, é principalmente devido à presença de cobre em solução. Este resultado foi confirmado por posteriores analises utilizando sulfato de cobre (16 mM), que mostrou que a indução desses genes era mantida, enquanto que na presença de pH 4,0 a expressão passava a ser inalterada.

- A expressão inalterada dos três genes na presença de calcopirita e do gene locus AFE_0993 na presença de pirita, pode ser explicada pela baixa quantidade ou pela ausência de cobre em solução. A repressão dos outros dos genes (locus AFE_0123 e AFE_0125) na presença de pirita pode por causa da pouca quantidade de enxofre em solução, visto que este sulfeto apresentou pouca oxidação.

- Os sulfetos calcopirita e pirita sofrem oxidação ácida após 24 horas, pois ambos os sulfetos seguem a via polissulfeto de oxidação, ou seja, eles são atacados quimicamente pelo H^+ em solução.

- O sulfeto pirita não apresenta o mesmo nível de oxidação dos outros dois sulfetos, pois segue a via tiossulfato de oxidação, ou seja, não consome os íons H^+ em solução.

- A expressão diferencial destes genes pode ser uma tentativa da bactéria de manter a homeostase celular e estabelecer uma população neste novo ambiente.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de mestrado intitulada **"ANÁLISE DA EXPRESSÃO DE GENES QUE CODIFICAM PROTEÍNAS TRANSPORTADORAS EM *Acidithiobacillus ferrooxidans* E *Acidithiobacillus thiooxidans* NA PRESENÇA DE SULFETOS METÁLICOS"**

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

(x) está inserido no Projeto CIBio (Protocolo n° 03/2003), intitulado

( ) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo n°_____).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (Protocolo n°_____).

Aluno

Orientador

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome: Marcelo Menossi Teixeira
Função: Presidente da CBio