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"Identificação e caracterização de genes expressos diferencialmente em *Acidithiobacillus ferrooxidans* na presença de sulfetos metálicos"

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

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RESUMO

Acidithiobacillus ferrooxidans é uma bactéria Gram negativa, mesofílica, acidofílica, quimiolitotrófica, capaz de obter energia da oxidação do íon ferroso, enxofre ou compostos reduzidos de enxofre. É um dos principais microrganismos responsáveis pela lixiviação de metais, podendo ser utilizada em processos industriais para obtenção de cobre, urânio ou metais preciosos, a partir de minérios de baixo teor. Na primeira parte deste projeto foi analisada a expressão diferencial de genes, através de RAP-PCR, em células de *A. ferrooxidans* mantidas na presença dos sulfetos metálicos bornita e calcopirita por 24 horas. Dezoito cDNAs com expressão diferencial foram identificados. Esses cDNAs tiveram a expressão diferencial confirmada e caracterizada por PCR em tempo real. Na presença de bornita, nenhum dos genes foi reprimido, e dentre os induzidos estão os envolvidos na síntese de proteínas. Na presença de calcopirita, cinco genes envolvidos no processamento de proteínas foram reprimidos e cinco genes envolvidos no sistema de transporte foram induzidos. A expressão diferencial desses genes na presença dos dois sulfetos de cobre pode ser devido a alterações do pH, a presença de íons de cobre em solução e a limitação de nutrientes. Dentre os genes com expressão mais acentuada na presença de calcopirita, foi encontrado um que codifica uma proteína receptora dependente de TonB. Esta proteína está envolvida no sistema de captação de Fe^{3+}. Na segunda parte deste trabalho foi feita uma análise do genoma de *A. ferrooxidans*. Esta análise mostrou que o gene que codifica a proteína receptora dependente de TonB se encontra agrupado com outros seis genes envolvidos com o sistema de captação de Fe^{3+}. A expressão destes genes foi analisada por PCR em tempo real em células de *A. ferrooxidans* mantidas por 24 horas na presença de bornita e calcopirita. Os resultados mostraram que a expressão dos genes é induzida na presença de calcopirita e inalterada na presença de bornita. Uma possível explicação para isto é a quantidade de ferro que é menor na presença de calcopirita, por ser um minério de difícil oxidação. O sistema de captação de ferro é regulado por uma proteína chamada Fur. Uma análise de bioinformática da região do genoma onde se encontram os genes envolvidos na captação de Fe^{3+} mostrou a existência de três novas regiões de regulação, denominadas de box Fur. Uma análise no banco de dados de domínio conservado (CDD - NCBI) revelou que o receptor dependente de TonB analisado neste trabalho pertence à família de receptores CirA.

SUMMARY

Acidithiobacillus ferrooxidans is a Gram-negative, mesophilic, acidophilic, chemo-lithoautotrophic bacterium that obtains energy from the oxidation of ferrous iron, elemental sulfur and reduced sulfur compounds. *A. ferrooxidans* is one of the most used microorganisms in bioleaching, an industrial process used for the recovery of copper, uranium or gold, from low-grade ores. In the first part of this project the differentially expressed cDNAs were isolated by RAP-PCR from *A. ferrooxidans* cells maintained for 24 hours in the presence of the metal sulfides bornite and chalcopyrite. A total of 18 differentially expressed cDNAs were isolated. The differential expression of the cDNAs was confirmed by real time PCR. The results showed that these genes were not down-regulated in the presence of bornite and among the up-regulated genes were those involved in protein synthesis. In the presence of chalcopyrite, five genes related to protein processing were down-regulated, and another five genes related to transport were up-regulated. The up- and down-regulation of genes in the presence of bornite and chalcopyrite could be due to alterations in the ideal pH, the presence of copper ions in solution and nutrient limitation. Among the genes that were up-regulated in the presence of chalcopyrite, was one that encodes for a TonB-dependent receptor. This protein is part of a system involved in Fe^{3+} uptake. An analysis of the *A. ferrooxidans* genome was performed in the second part of this work and showed that the gene that encodes for the TonB-dependent receptor is clustered with six other genes from the Fe^{3+} uptake system. The relative expression pattern of these genes was investigated by real time PCR in *A. ferrooxidans* cells maintained for 24 h in the presence of bornite and chalcopyrite. The results showed that the expression of the genes is up-regulated in the presence of chalcopyrite and unchanged in the presence of bornite. A possible explanation for this is the amount of iron in solution that was smaller in the presence of chalcopyrite, since this copper sulfide is very refractory. The iron uptake genes are regulated by a protein named Fur. A bioinformatics analysis of the genomic region where these genes were found revealed the existence of three new Fur boxes. An analysis on the Conserved Domain Database (CDD - NCBI) revealed that the TonB-dependent receptor belongs to the CirA family of receptors.

INTRODUÇÃO

Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans é uma bactéria que pertence ao filo Proteobacteria, subgrupo γ-proteobactéria, gênero *Acidithiobacillus* (Kelly & Wood, 2000). O gênero *Acidithiobacillus* compreende as espécies: *Acidithiobacillus albertensis, Acidithiobacillus caldus, Acidithiobacillus ferrooxidans* e *Acidithiobacillus thiooxidans* (Kelly & Wood, 2000). Vários trabalhos mostraram que existe uma grande variabilidade genética e fenotípica dentro da espécie *A. ferrooxidans* (Karavaiko *et al.,* 2003; Waltenbury *et al.,* 2005). Assim sendo, Novo *et al.* (2000) encontraram uma linhagem mais resistente a altas concentrações de metais pesados. Kupka *et al*. (2007) encontraram linhagens que conseguem crescer em temperatura baixas. Chen *et al.* (2007) descreveram linhagens que conseguem crescer em diferentes condições, como diferentes temperaturas, pHs, concentração de metais pesados e quantidades diferentes de enxofre.

Acidithiobacillus ferrooxidans é uma bactéria Gram negativa, não patogênica, quimiolitotrófica, capaz de obter energia da oxidação do íon ferroso, enxofre ou compostos reduzidos de enxofre. Esta bactéria é a única espécie do gênero capaz de utilizar o íon ferroso como fonte de energia. A espécie é anaeróbica facultativa, acidofílica (pH ótimo de crescimento ao redor de 2,0) e mesofílica (temperatura ótima de crescimento ao redor de 30°C) (Rawlings 2002).

Atualmente, o TIGR (*The Institute of Genomics Research* - http://www.tigr.org) disponibiliza uma seqüência do genoma de *A. ferrooxidans* ATCC 23270 com 90,91% dos genes anotados. Dentre os genes anotados, 97,45% codificam proteínas sendo 64,32% com função definida e 23,52% hipotéticos. Esta seqüência serviu como base para a caracterização de genes e *operons* em *A. ferrooxidans* (Yarzábal *et al.*, 2002; Ramirez *et al.*, 2002; Levicán *et al*., 2002). Estes dados também têm sido utilizados para o estudo de alguns aspectos complexos da biologia de *A. ferrooxidans*, tais como os mecanismos de captação, assimilação e metabolismo de enxofre (Valdés *et al.*, 2003) e de dióxido de carbono (Appia-Ayme *et al.*, 2006).

Interesse econômico e ambiental

Acidithiobacillus ferrooxidans possui considerável interesse econômico já que está envolvida na biolixiviação de metais. A biolixiviação é um processo atraente para a recuperação de metais de minérios pobres, cuja extração pelos métodos piro e hidrometalúrgicos convencionais não é economicamente viável (Bosecker, 1997; Garcia Jr., 1997). A biolixiviação requer um baixo investimento inicial e de manutenção, não necessita de mão de obra especializada e, ao contrário do processo pirometalúrgico, não emite SO_2, o agente causador da chuva ácida (Garcia Jr., 1997). A biolixiviação é utilizada para a obtenção de cobre e urânio, sendo este processo responsável por 15% e 13%, respectivamente, da produção mundial destes metais (Brierley & Brierley, 2001).

Entre os minerais sulfetados de cobre, a calcopirita ($CuFeS_2$) é um dos mais abundantes na natureza, e, por esse motivo, existe grande interesse em utilizá-la para obtenção de cobre. Porém, este sulfeto metálico é o mais refratário ao ataque da bactéria (Watling, 2006). Outro sulfeto metálico importante é a bornita (Cu_5FeS_4). Esse sulfeto de cobre existe largamente em depósitos de cobre porfírico junto com a calcopirita, possui elevada porcentagem em peso de cobre e é de fácil corrosão por células de *A. ferrooxidans*.

A biolixiviação pode ocorrer de maneira direta ou indireta, sendo que na forma direta os sulfetos metálicos são oxidados pela ação enzimática das bactérias em contato direto com o sulfeto metálico (Bosecker, 1997; Tributsch, 2001). Na forma indireta, o metabolismo da bactéria produz substâncias lixiviantes que oxidam quimicamente os sulfetos metálicos causando a solubilização. No caso de *A. ferrooxidans*, os agentes lixiviantes produzidos são H_2SO_4 e íons Fe^{+3} (Bosecker, 1997; Tributsch, 2001). Na prática, os dois processos, direto e indireto, ocorrem simultaneamente em uma série complexa de reações químicas. Todavia, existem divergências sobre qual dos dois processos é mais importante para a biolixiviação (Liu *et al.*, 2003; Rohwerder *et al.*, 2003; Lilova *et al.,* 2007).

A biolixiviação indireta pode ocorrer por duas vias: a via do tiosulfato e a do polisulfeto (Schippers & Sand, 1999). A via do tiosulfato ocorre na oxidação de sulfetos metálicos insolúveis em meio ácido, tais como pirita (FeS_2) e molibdenita (MoS_2). Neste processo a solubilização ocorre através do ataque de íons férricos ao sulfeto metálico, tendo o tiosulfato como o principal

produto intermediário e o sulfato como o principal produto final. Nesta via os microrganismos fornecem íons férricos para o ataque oxidativo (Schippers & Sand, 1999; Rawlings, 2005). A via de polisulfeto ocorre em sulfetos metálicos solúveis em ácido como a esfalerita (ZnS), calcopirita ($CuFeS_2$) e galena (PbS). Neste processo a solubilização dos sulfetos metálicos se dá por meio de um ataque de íons férricos e prótons, tendo como principal intermediário o polisulfeto. A função dos microrganismos neste processo é gerar ácido sulfúrico para o ataque de prótons e manter o ferro no estado férrico para o ataque oxidativo ao mineral (Schippers & Sand, 1999; Rawlings, 2005).

Acidithiobacillus ferrooxidans possui também importância ambiental, pois pode ser utilizada na degradação de resíduos químicos provenientes de minerais processados. Os métodos convencionais geralmente apresentam custos elevados e o emprego de *A. ferrooxidans* é uma alternativa econômica e rápida, com benefícios para o meio ambiente (Shiratori & Sonta, 1993; Hubert *et al.*, 1995). Além disso, a capacidade de metabolizar compostos sulfúricos possibilita a utilização de *A. ferrooxidans* em filtros para a eliminação do dióxido de enxofre, um importante poluente ambiental emitido por vários tipos de indústrias (Bonaventura & Johnson, 1997). Lombardi & Garcia Jr. (1999) sugeriram a utilização do processo de lixiviação bacteriana na remoção dos metais tóxicos geralmente encontrados em esgotos municipais. Lopez *et al.* (1999) desenvolveram um sistema para destoxificação de esgotos que utiliza um reator eletroquímico no anodo e culturas de *A. ferrooxidans* no catodo. Kim *et al*. (2005) relataram que células de *A. ferrooxidans* são mais eficientes na remoção de metais pesados quando previamente adaptadas nas condições encontradas no esgoto.

Estudos moleculares em *A. ferrooxidans*

Os estudos moleculares referentes à *A. ferrooxidans* têm se intensificado já que esta bactéria é um dos principais microrganismos envolvidos na biolixiviação de metais de minérios. Assim sendo, Yarzábal *et al. (*2004) analisaram a expressão dos genes do operon *rus*, envolvidos na cadeia transportadora de elétrons, em células crescidas em meio contendo ferro ou enxofre. Os autores concluíram que a expressão destes genes é induzida pelo ferro, o que está de acordo com o fato das proteínas sintetizadas por estes genes estarem envolvidas com a oxidação deste metal.

Ramirez *et al.* (2004) analisaram o proteoma de células de *A. ferrooxidans* cultivadas em meio contendo ferro, enxofre e sulfetos metálicos como pirita e calcopirita. Os autores sugeriram a existência de diferentes vias de utilização de ferro e enxofre. Durante o crescimento das células na presença de sulfetos metálicos contendo ferro, os autores observaram que proteínas das duas vias, ferro e enxofre, foram sintetizadas, indicando que as duas vias responsáveis por gerar energia são induzidas simultaneamente dependendo do tipo e da concentração do substrato que vai ser oxidado.

Recentemente, Bruscella *et al.* (2007) analisaram por PCR em tempo real e *Northern blot* a transcrição dos genes presentes em dois operons, *petI* e *petII*, em células de *A. ferrooxidans* crescidas em meio contendo ferro ou enxofre. Os genes do operon *petI* codificam proteínas do complexo bc_1 que transportam elétrons do ferro para o NAD(P). Os genes do operon *petII* codificam proteínas envolvidas no transporte de elétrons do enxofre para o oxigênio ou Fe^{3+}. Os autores demonstraram que os genes do operon *petI* são transcritos principalmente em células crescidas em meio contendo ferro e os genes do operon *petII* em células crescidas em meio contendo ferro ou enxofre. Quatrini *et al.* (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.

Uma característica marcante de *A. ferrooxidans* é a sua resistência generalizada a altas concentrações de íons metálicos. Isso pode ser atribuído à presença constante de metais no ambiente natural da bactéria, fator que provavelmente determinou a seleção de tipos mais resistentes ao longo da evolução. *A. ferrooxidans* apresenta resistência elevada ao alumínio, zinco, cobalto, manganês, cobre, cromo e urânio (Brierley, 1978; Lundgren & Silver, 1980; Hutchins *et al.*, 1986). Quanto ao nível de resistência, sabe-se que *A. ferrooxidans* é duas a três vezes menos sensível aos efeitos inibitórios dos metais pesados que a maioria das bactérias heterotróficas (Sadler & Trundinger, 1967; Weinberg, 1977).

Embora *A. ferrooxidans* seja resistente a altas concentrações de metais, pouco se sabe sobre os mecanismos moleculares que conferem essa resistência. Shiratori *et al.* (1989) isolaram os genes que conferem resistência ao mercúrio em *A. ferrooxidans*. Esses autores demonstraram a localização cromossomal desses genes em *A. ferrooxidans*, sendo que a inserção no cromossomo pode ter ocorrido por eventos de transposição múltipla.

Os genes envolvidos no mecanismo de resistência ao arsênio em *A. ferrooxidans* foram clonados e seqüenciados por Butcher *et al.* (2000). Células de *E. coli* transformadas com estes genes apresentaram um aumento na resistência ao arsênio e antimônio. Assim como os genes de resistência ao mercúrio, os genes de resistência ao arsênio também estão localizados no cromossomo da bactéria. Segundo Butcher *et al.* (2000), estes genes estão organizados nos operons *arsRC* e *arsBH*.

Aparentemente a resistência a íons metálicos em *A. ferrooxidans* varia em função da fonte de energia utilizada. Quando Fe^{+2} é utilizado, *A. ferrooxidans* pode ser até 2000 vezes mais resistente a íons metálicos do que células que utilizam tiosulfato (Tuovinen *et al.*, 1971). Quando S° é utilizado, valores intermediários de resistência são observados (Tuovinen *et al.*, 1971). Silver & Torma (1974) confirmaram estes resultados testando a susceptibilidade de *A. ferrooxidans* ao chumbo, níquel e cobre. Posteriormente, Iwahori *et al.* (2000) demonstraram que a membrana plasmática de algumas linhagens de *A. ferrooxidans* resistentes ao mercúrio possuía uma atividade mercúrio-redutase ($Hg^{+2} \rightarrow Hg°$) Fe^{+2} dependente. Aparentemente, componentes do sistema de oxidação de íons Fe^{+2}, como as enzimas citocromo c oxidase e rusticianina, estão envolvidas no processo.

Novo *et al.* (2000) demonstraram que o crescimento de células de *A. ferrooxidans* na presença de 200 mM de cobre não foi inibido. Além disso, os autores analisaram a síntese de proteínas em células crescidas na presença de cobre e observaram que o nível de fosforilação das proteínas de membrana foi maior que as do citosol. O maior nível de fosforilação de proteínas de membrana está de acordo com o fato destas proteínas perceberem mudanças no meio ambiente e transmitirem esta informação para proteínas do citoplasma através de eventos de fosforilação (Charter *et al.*, 1995; Kennelly & Potts, 1996; Swanson *et al.*, 1994). Utilizando a técnica de RAP-PCR (RNA *arbitrarily primed polymerase chain reaction*), Paulino *et al.* (2002) identificaram alguns genes expressos diferencialmente em *A. ferrooxidans* na presença de sulfato de cobre. Entre os genes com expressão aumentada estava um citocromo c, reforçando as evidências de um possível papel desta proteína na resistência a íons metálicos em *A. ferrooxidans*, por um mecanismo ainda desconhecido. Felício *et al.* (2003) analisaram mudanças no perfil da síntese de proteínas de membrana e do periplasma de células de *A. ferrooxidans* crescidas na ausência e na presença de 200 mM de cobre. Os autores observaram que uma

proteína de 16,5 kDa tinha a síntese aumentada sugerindo seu envolvimento no mecanismo de resistência ao cobre.

Durante a biolixiviação, *A. ferrooxidans* está sujeita a várias condições adversas tais como mudanças na temperatura e pH, aumento da concentração de íons metálicos no meio e escassez de nutrientes essenciais (Varela *et al.,* 1998). Neste processo, uma considerável quantidade de calor é liberada, devido à natureza exotérmica da oxidação de muitos sulfetos, resultando no aumento da temperatura e podendo levar à inibição ou morte dos microrganismos (Modak *et al.,* 1996). Modak *et al.* (1996) adaptaram uma linhagem de *A. ferrooxidans* à temperatura de 42°C, através de várias subculturas em meio 9K em temperaturas crescentes (de 30°C a 42ºC). Quando os autores voltaram a cultivar a linhagem a 30°C, a tolerância a 42°C foi perdida mostrando que ela era estresse-dependente. Os autores concluíram que a utilização de linhagens adaptadas no processo de biolixiviação deve ser realizada na mesma temperatura a qual a bactéria foi adaptada.

Pouco se sabe sobre os mecanismos moleculares utilizados por *A. ferrooxidans* para responder a mudanças do pH externo. Amaro *et al.* (1991) analisaram o perfil eletroforético de proteínas de *A. ferrooxidans* cultivada em pH 1,5 e 3,5. Quando células cultivadas em pH 3,5 foram transferidas para pH 1,5, observou-se uma alteração na síntese de proteínas incluindo diminuição da síntese da proteína p36 de 36 kDa. Os autores sugeriram que a p36 é uma porina cuja síntese é regulada pelo pH extracelular. Quando células cultivadas em pH 1,5 foram transferidas para pH 3,5 não foram observadas alterações significativas na síntese de proteínas. A exceção foi a proteína p36 que apresentou um aumento na síntese. Assim, de acordo com Amaro *et al.* (1991), os dados obtidos indicam que a resposta ao estresse causado por alterações no pH em *A. ferrooxidans* é mais drástica quando ocorre um aumento na acidificação. Mousavi *et al.* (2007) estudaram o efeito de diferentes valores de pH iniciais (1,25 – 2,25) no processo de biolixiviação do sulfeto metálico esfalerita com *A. ferrooxidans*. Os autores concluíram que o melhor pH inicial foi 1,5. Esses autores sugeriram que não é recomendando a utilização de pH inicial abaixo de 1,5 na biolixiviação, para não afetar o metabolismo da bactéria, e nem acima de 2,0 para não ocorrer a precipitação de íons férricos prejudicando o processo.

Quanto à disponibilidade de nutrientes, a falta de fosfato é um estresse nutricional freqüente para *A. ferrooxidans* e outras bactérias em ambiente de biolixiviação (Varela *et al.,*1998). Segundo Seeger *et al.* (1996), o padrão geral de fosforilação de proteínas é modificado

em *A. ferrooxidans* em condições de privação nutricional de fosfato, incluindo um aumento da fosforilação das chaperonas DnaK e GroEL. Segundo estes autores, as proteínas DnaK e GroEL estão relacionadas com a percepção e regulação da resposta ao estresse em bactérias. Estas proteínas têm maior atividade quando fosforiladas (Seeger *et al.,* 1996).

He *et al.* (2005) analisaram o proteoma de células de *A. ferrooxidans* cultivadas em meio com privação de fosfato e compararam com o proteoma de células crescidas em condições normais. Seis proteínas foram identificadas sendo três (cadeia D da NADH desidrogenase, proteína recombinante recA e RNA helicase) com síntese aumentada em células cultivadas em meio com privação de fosfato e três (fator de transcrição contendo domínio AP2, proteína hipotética e translocase STY3758) com síntese diminuída nesta condição. Os autores sugeriram que a inibição da síntese do fator de transcrição com domínio AP2 pode resultar na inibição da transcrição e na inibição do crescimento de *A. ferrooxidans* em meio com privação de fosfato.

Vera *et al.* (2003) analisaram a expressão de uma proteína induzida em células de *A. ferrooxidans* crescidas em meio com privação de fosfato. Esta proteína apresenta alta similaridade com a proteína PstS, uma transportadora de fosfato do tipo ABC. Após uma análise do genoma de *A. ferrooxidans* os autores sugeriram a existência de um sistema de transporte de fosfato, sistema Pst, e de um regulador Pho similar ao encontrado em *Escherichia coli* e em outras bactérias.

Outro nutriente essencial para *A. ferrooxidans* é o ferro. Embora o ferro seja o metal mais abundante na natureza, ele apresenta baixa solubilidade em condições aeróbicas e pH neutro (Clarke *et al.,* 2001). O ferro tem um papel importante para muitas enzimas redox envolvidas no transporte de elétrons (Braun & Killmann, 1999). Algumas bactérias desenvolveram estratégias para a captação do ferro em ambientes onde este nutriente é escasso. Dentre as estratégias está a síntese e a secreção de sideróforos. Estas moléculas de baixo peso molecular possuem uma alta afinidade com o íon férrico (Schalk, 2004). Bactérias Gram-negativas captam o complexo Fe^{3+}-sideróforo por meio de receptores na membrana externa, utilizando a energia próton motora fornecida pelas proteínas da membrana interna, TonB-ExbB-ExbD (Andrews *et al.,* 2003). No periplasma o complexo Fe^{3+}-sideróforo é captado por uma proteína periplasma-*binding*. Na membrana interna este complexo é transportado por proteínas do tipo ABC (ATP-*binding*

cassette) e permeases. Após a entrada no citoplasma o complexo é desassociado e o Fe^{3+} é reduzido (Andrews *et al.,* 2003).

Quatrini *et al.* (2005) fizeram uma análise de bioinformática do genoma de *A. ferrooxidans* e observaram que esta bactéria possui pelo menos 11 receptores distintos de captação de Fe^{3+} dependente de TonB, além de outras proteínas envolvidas no sistema de captação de ferro. Contudo, *A. ferrooxidans* não possui genes que codificam proteínas envolvidas na síntese destas moléculas. A presença de vários sistemas de captação de Fe^{3+} sugere que *A. ferrooxidans* pode habitar ambientes aeróbicos com pouco ferro e aonde bactérias que sintetizam sideróforos estão (Quatrini *et al.,* 2005). Recentemente, Quatrini *et al.* (2007) fizeram uma análise experimental e de bioinformática no genoma de *A. ferrooxidans* para identificar sítios regulados por Fur. Estes autores observaram que alguns genes envolvidos no sistema de captação de ferro são regulados por Fur.

OBJETIVOS

OBJETIVO GERAL:

O presente trabalho tem por objetivo a identificação e caracterização de genes expressos diferencialmente na presença de sulfetos metálicos em *Acidithiobacillus ferrooxidans* linhagem LR.

OBJETIVOS ESPECÍFICOS:

CAPÍTULO 1

Artigo 1: Gene expression modulation in *Acidithiobacillus ferrooxidans* exposed to chalcopyrite and bornite.

-Identificação através de RAP-PCR de cDNAs expressos diferencialmente na presença dos sulfetos metálicos bornita e calcopirita;

-Clonagem e seqüenciamento dos cDNAs diferenciais;

-Validação/caracterização da expressão diferencial dos cDNAs isolados através de PCR em tempo real.

CAPÍTULO 2

Artigo 2: Ferric iron uptake genes in *Acidithiobacillus ferrooxidans*: *in silico* analyses and differential expression in the presence of copper sulfides.

- Análise da expressão de genes envolvidos no sistema de captação de ferro na presença de calcopirita e bornita.

CAPÍTULO 1

Gene expression modulation in *Acidithiobacillus ferrooxidans* exposed to chalcopyrite and bornite

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ABSTRACT

Acidithiobacillus ferrooxidans is a mesophilic, acidophilic, chemolithoautotrophic bacterium that obtains energy from the oxidation of ferrous iron (Fe^{2+}), elemental sulfur and reduced sulfur compounds. The industrial interest on *A. ferrooxidans* resides on its capacity to oxidize insoluble metal sulfides into soluble metal sulfates, thus allowing the recovery of the desired metals from low-graded sulfide ores. These bacteria are successfully used in bioleaching operations to recover copper from low-grade copper sulfide ores. In the present work, RNA arbitrarily primed PCR (RAP-PCR) was performed to identify cDNAs differentially expressed in *A. ferrooxidans* cells grown in the presence of Fe^{2+} and maintained for 24 hours in contact with the copper sulfides chalcopyrite and bornite. Eighteen cDNAs corresponding to genes with known function were identified and their relative expression pattern was further characterized by real time quantitative PCR. Bornite, a highly soluble copper sulfide, had a mild effect on the expression of the 18 genes analyzed. None of the genes was down-regulated in the presence of bornite and among the few genes up-regulated, it is worth mention *lepA* and *def-2* which are involved in protein synthesis. Chalcopyrite, a very refractory copper sulfide, presented the most significant changes. Five genes related to protein processing were down-regulated, and another 5 genes related to the transport system were up-regulated. The up and down-regulation of these genes in the presence of bornite and chalcopyrite could be due to alterations in the ideal pH, presence of copper ions in solution and nutrient limitations. Gene expression modulation might be important for the *A. ferrooxidans* early response to the presence of copper sulfides.

INTRODUCTION

A serious challenge mankind will be facing in the 21st century is the gradual exhaustion of the earth's mineral resources. Besides, an additional challenge to be pursued is to conciliate efficient industrial operations to environmentally safe procedures. In industrial operations to recover metals from low-grade ores, biohydrometallurgy has emerged as an attractive procedure. It allies low operational cost and energy requirement with ecologically friendly procedures (Rawlings, 2002).

One of the most widely used microorganisms in bio-hydrometallurgical operations is the acidophilic *Acidithiobacillus ferrooxidans*. This mesophilic, chemolithoautotrophic bacterium obtains energy from the oxidation of ferrous iron (Fe^{2+}), elemental sulfur and reduced sulfur compounds such as sulfidic minerals (Rawlings, 2002). The industrial interest on *A. ferrooxidans* resides on its capacity to oxidize insoluble metal sulfides into soluble metal sulfates, thus allowing the recovery of the desired metals from low-graded sulfide ores (Bosecker, 1997).

A. ferrooxidans is successfully used in the biooxidation of low-grade copper sulfide ores to recover copper, which is the most intensively recovered metal in bioleaching operations (Watling, 2006). Chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4) are copper sulfides commonly found in nature. Despite the same elemental composition, they present distinct dissolution characteristics probably due to their unique crystal structures. Bornite is easily solubilized specially in the presence of *A. ferrooxidans* (Bevilaqua *et al.*, 2003), whereas chalcopyrite is more refractory to bacterial and even to chemical leaching, resulting in low dissolution rates (Third *et al.*, 2000). Nonetheless, the economic relevance of chalcopyrite in mining industry still remains, since this ore is the most abundant copper-bearing mineral and, therefore, an important source of copper (Third *et al.*, 2000).

Little is known about the molecular aspects of the bioleaching process. Genomic and proteomic analysis of *A. ferrooxidans* have been focused on the most diverse aspects, including heavy metal tolerance (Novo *et al.*, 2000; Paulino *et al.*, 2002), nutrient starvation (Vera *et al.*, 2003; He *et al.*, 2005) and factors that affect the oxidation of elemental sulfur such as pH (Suzuki *et al.*, 1999). Also, with the advent of *in silico* bioinformatic analysis, information has been obtained about the cellular metabolism including sulfur assimilation (Valdés *et al.*, 2003), iron

homeostasis (Quatrini *et al.*, 2005 and 2007), carbon metabolism (Appia-Ayme *et al.*, 2006) and biofilm formation (Barreto *et al.*, 2005). However, most of these studies were conducted with *A. ferrooxidans* grown in elemental sulfur and/or ferrous iron rather than copper sulfides.

Some proteomic analyses of *A. ferrooxidans* in the presence of copper sulfides have been conducted (Ramirez *et al.*, 2002 and 2004; Felício *et al.*, 2007). This way, a thiosulfate sulfur transferase was shown to be repressed in cells grown in the presence of ferrous iron but greatly induced in cells grown in the presence of covellite, chalcopyrite and other metal sulfides. These results indicate that this protein may have an important role on mineral dissolution and/or sulfur metabolism in *A. ferrooxidans* (Ramirez *et al.*, 2002 and 2004). Rusticyanin, on the other hand, was shown to be down-regulated in the presence of covellite when compared to cells grown in Fe^{2+} (Ramirez *et al.*, 2004). Recently we performed a proteomic approach on *A. ferrooxidans* cells maintained in the presence of copper sulfides (Felício *et al.*, 2007). A ribulose biphosphate carboxylase was among the proteins whose synthesis was repressed in the presence of both chalcopyrite and bornite. Interestingly, an antioxidant protein involved in cellular detoxification was among the proteins induced in the presence of those copper sulfides (Felício *et al.*, 2007). In regard to gene expression in *A. ferrooxidans* cells maintained in contact to copper sulfides, we recently observed that covellite greatly induces transport genes expression (Reis *et al.*, manuscript in preparation).

The present work aimed to identify and evaluate the gene expression pattern on *A. ferrooxidans* cells maintained in contact with chalcopyrite and bornite.

RESULTS AND DISCUSSION

RNA arbitrarily primed PCR (RAP-PCR) was performed to identify cDNAs differentially expressed in *A. ferrooxidans* cells grown in the presence of Fe^{2+} (control condition) and on cells maintained for 24 hours in contact with chalcopyrite or bornite (tested conditions). The cDNAs with the highest levels of induction or repression were cloned and sequenced. The sequences were searched for similarity on the *A. ferrooxidans* ATCC 23270 genome available on the TIGR-CMR database using the BLASTn algorithm. Eighteen cDNAs corresponding to genes with known function were identified and the predicted functional category of each gene was assigned

based on the TIGR-CMR database (Table 1). The relative expression pattern of the 18 selected genes on the tested conditions was further characterized by real time quantitative PCR (RTq-PCR, Table 1 and Figure 1).

The predicted functional categories of the 18 genes identified revealed that chalcopyrite and bornite modulates the expression pattern of genes involved in the transport, protein fate and synthesis, DNA replication and transposition, central metabolism and energy metabolism.

Transport and binding proteins

Among the 18 genes identified, five are related to the transport. These genes are located on *loci Afe 0074*, *Afe 0853*, *Afe 1149* (*pstA* gene), *Afe 1592* and *Afe 3203*. The genes on *loci Afe 0074* and *Afe 3203* were up-regulated in the presence of both copper sulfides. The gene located on locus *Afe 0074* encodes for an ABC-2 type transporter whose substrate is unknown. It is well documented that in bacteria ABC transporters are usually related to virulence, nutrient uptake and the ability to survive in a variety of environments (Linton and Higgins, 1998). The gene located on locus *Afe 3203* encodes for a voltage-gated chloride channel whose gating mechanism is controlled by changes on membrane potential and also by both chloride ions and external and internal pH (Chen *et al.*, 2003; Dutzler, 2004). In enteric bacteria, chloride channels are involved in the extreme acid resistance response (Iyer *et al.*, 2002).

The expression pattern of the other three genes related to transport system were up-regulated in the presence of chalcopyrite but remained unchanged in the presence of bornite. The gene on locus *Afe 0853* encodes for a Na^+/H^+ antiporter involved in the transport of Na^+, and probably other cations, in exchange to H^+ (Padan *et al.*, 2001). Therefore this membrane protein has a primary role in the regulation of cellular cation content and in the intracellular pH homeostasis. In *E. coli* the expression of antiporters is induced by the intracellular Na^+ content and alkaline pH increases this induction (Hunte *et al.*, 2005). Besides being involved with pH homeostasis, a putative role of this antiporter on iron uptake can not be ruled out since according to the TIGR-CMR it can transport iron carrying compounds. *A. ferrooxidans* cells are submitted to pH changes when maintained in medium with copper sulfides (unpublished data), what may explain the up-regulation of genes encoding transporters associated to pH homeostasis, such as the voltage-gated chloride channel (*Afe 3203* locus) and the Na^+/H^+ antiporter (*Afe 0853* locus).

The gene on locus *Afe 1149* encodes for a phosphate ABC transporter with high degree of similarity to the *Acidiphilium cryptum* PstA protein (Score: 237 bits; E-value: $6e^{-61}$; Identities: 143/269, 53%; Positives: 190/269, 70%). The *pstA* gene belongs to the *pst* operon whose genes encode for a high affinity phosphate ABC transporter system. Similarly to the gene that encodes for the ABC-2 type transporter (locus *Afe 0074*), the *pstA* gene was also up-regulated in the presence of chalcopyrite. The up-regulation of these genes may represent an attempt of the cells to overcome the substrate change from ferrous iron (control condition) to copper sulfides. It was shown that besides phosphate starvation, changes in pH also regulate the expression of *pst* operon (Antelmann *et al.*, 2000; Atalla and Schumann, 2003; Fischer *et al.*, 2006). Thus, another possible explanation for the up-regulation of the *pstA* gene in the presence of chalcopyrite may be related to pH changes in the medium when *A. ferrooxidans* cells are incubated in the presence of copper sulfides.

The gene located on locus *Afe 1592* codifies a TonB-dependent receptor related to a high-affinity iron acquisition system mediated by siderophores. The gene on locus *Afe 1592* belongs to a cluster of genes that encodes the components for this iron acquisition system, which includes receptors, periplasmic and inner membrane transporters and energy-transducing proteins (Moeck and Coulton, 1998). The energy required for the active transport of the iron-siderophore complex is provided by the TonB-dependent energy transduction system (Braun, 1995). Ferrous iron is poorly available in the medium containing chalcopyrite, which may explain the up-regulation of this gene in the presence of that copper sulfide. Consistent with our data, previous work has demonstrated that the copies of TonB-dependent receptor proteins increase under conditions of iron limitation (Higgs *et al.*, 2002). Additional data regarding the expression of these genes on a variety of copper sulfides and growth conditions have been investigated and will be reported elsewhere (manuscript in preparation).

Table 1. Functional category and the relative expression pattern of the 18 selected genes characterized by real time quantitative PCR. Statistical significance based on a Student's t-test (*p-value* < 0.05).

Locus/Gene (TIGR)	Protein (TIGR)	Biological Process (Gene Ontology, GO)	Functional Category (TIGR)	Expression Pattern relative to Fe2+ (*fold change – p-value*)	
				Chalcopyrite	Bornite
Afe 0007	Oxidoreductase, short-chain dehydrogenase/reductase family	Metabolic process (GO:0008152)	Unknown function: Enzymes of unknown specificity	**Down-regulated** (0.14x - *p=0.0002*)	**No change** (1x - *p=0.96*)
Afe 0074	ABC-2 type transporter	Transport (GO:0006810)	Transport and binding proteins: Unknown substrate	**Up-regulated** (2.79x - *p=0.0006*)	**Up-regulated** (1.98x - *p=0.01*)
Afe 0225	Choloylglycine hydrolase, putative	Metabolic process (GO:0008152)	Central intermediary metabolism: Other	**Up-regulated** (2.99x - *p=0.01*)	**No change** (0.92x - *p=0.6*)
Afe 0289 (*dnaB*)	Replicative DNA helicase	DNA replication (GO:0006260)	DNA metabolism: DNA replication, recombination, and repair	**No change** (1x - *p=0.71*)	**Up-regulated** (2x - *p=0.009*)
Afe 0467 (*nrdA*)	Ribonucleotide-diphosphate reductase, alpha subunit	2'-deoxyribonucleotide Biosynthesis (GO:0009265)	2'-Deoxyribonucleotide metabolism	**Down-regulated** (0.15x - *p=0.0004*)	**No change** (0.97x - *p=0.85*)
Afe 0853	Na+/H+ antiporter, Putative	Na^+ and H^+ transporter (GO:0006814; GO:0006818)	Transport and binding proteins: Cations and iron carrying compounds	**Up-regulated** (3.9x - *p=0.00002*)	**No change** (1.2x - *p=0.32*)
Afe 0965	Aldehyde dehydrogenase (NAD) family protein	Fermentation (GO:0006113)	Energy metabolism: Fermentation	**Down-regulated** (0.36x - *p=0.01*)	**No change** (1.1x - *p=0.96*)
Afe 1149	Phosphate ABC transporter, permease protein, putative	Phosphate transport (GO:0006817)	Transport and binding proteins: Anions	**Up-regulated** (4.2x - *p=0.02*)	**No change** (1.5x - *p=0.15*)
Afe 1200 (*hflK*)	hflK protein	Negative regulation of Proteolysis (GO:0045861)	Protein fate: Degradation of proteins, peptides, and glycopeptides	**Down-regulated** (0.16x - *p=0.0002*)	**No change** (1.36x - *p=0.96*)
Afe 1321	Membrane protein, putative	Unknown (GO:0000004)	Cell envelope: Other	**Down-regulated** (0.21x - *p=0.03*)	**No change** (1.39x - *p=0.8*)
Afe 1592	TonB-dependent receptor	Transport (GO:0006810)	Transport and binding proteins: Cations and iron carrying compounds	**Up-regulated** (6x - *p=0.002*)	**No change** (1x - *p=0.75*)
Afe 1684 (*lepA*)	LepA	Unknown (GO:0000004)	Unknown function: General	**Down-regulated** (0.7x - *p=0.0002*)	**Up-regulated** (3.9x - *p=0.03*)
Afe 1751	ISAfe2, transposase	Transposition, DNA-mediated (GO:0006313)	Mobile and extrachromosomal element functions: Transposon functions	**No change** (0.91x - *p=0.68*)	**No change** (1.2x - *p=0.88*)
Afe 2698 (*rplP*)	Ribosomal protein L16	Translation (GO:0006412) and ribosome biogenesis (GO:0007046)	Protein synthesis: Ribosomal proteins: synthesis and modification	**Down-regulated** (0.27x - *p=0.002*)	**No change** (0.86x - *p=0.74*)
Afe 2728 (*ribE*)	6,7-dimethyl-8-ribityllumazine synthase	Riboflavin biosynthetic process (GO:0009231)	Biosynthesis of cofactors, prosthetic groups, and carriers: Riboflavin, FMN, and FAD	**No change** (0.85x - *p=0.47*)	**No change** (0.97x - *p=0.82*)
Afe 2902 (*gcp*)	O-sialoglycoprotein endopeptidase	Proteolysis (GO:0006508)	Protein fate: Degradation of proteins, peptides, and glycopeptides	**No change** (1.14x - *p=0.85*)	**No change** (1.1x - *p=0.99*)
Afe 3005 (*def-2*)	Polypeptide deformylase	Protein modification process (GO:0006464)	Protein fate: Protein modification and repair	**Down-regulated** (0.35x - *p=0.006*)	**Up-regulated** (6.1x - *p=0.009*)
Afe 3203	Voltage-gated chloride Channel	Voltage-gated chloride channel (GO:0005247)	Transport and binding proteins: Anions	**Up-regulated** (1.9x - *p=0.04*)	**Up-regulated** (2.7x - *p=0.008*)

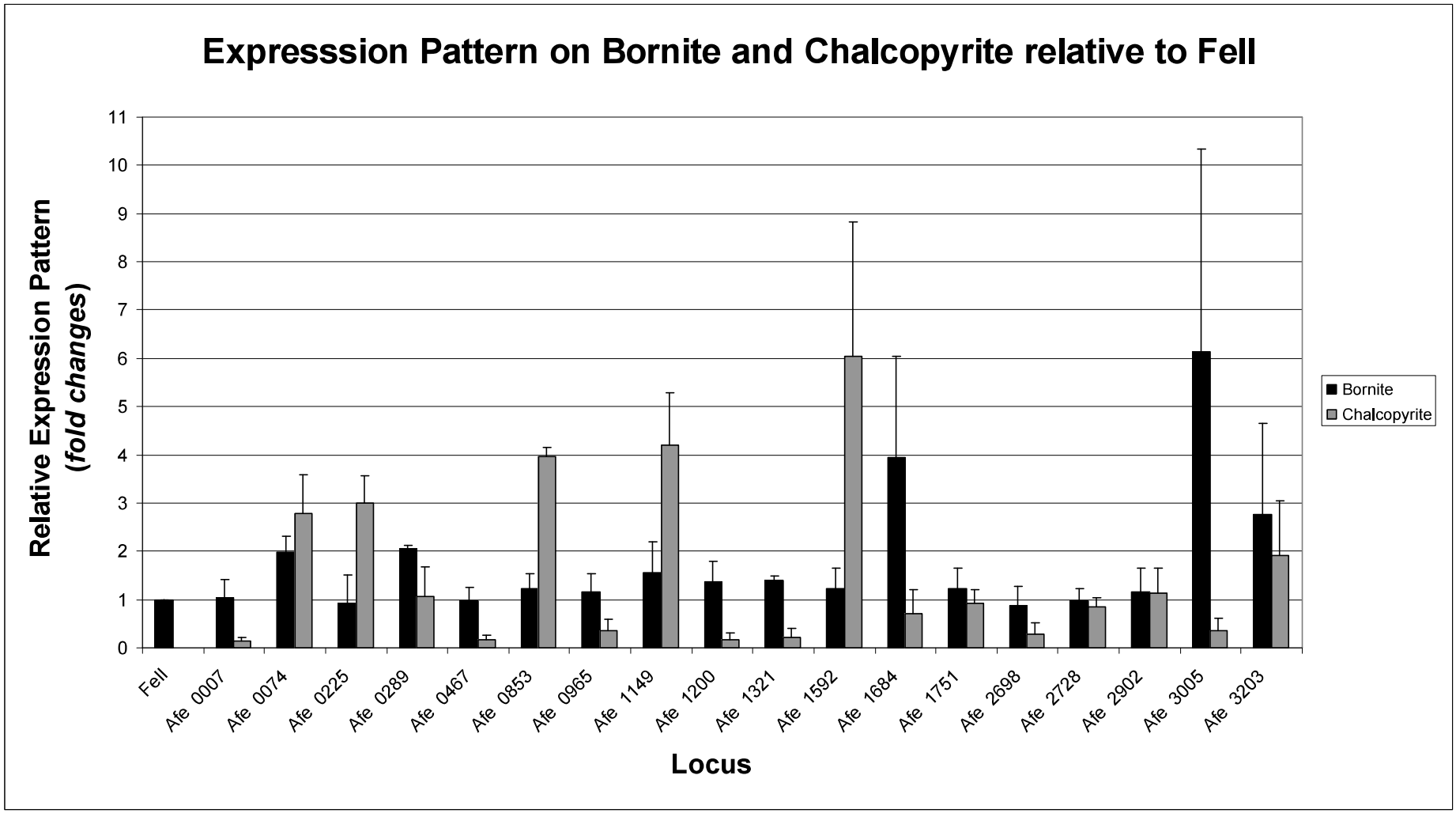

Figure 1. Relative expression pattern of the selected genes in *A. ferrooxidans* cells grown in Fe^{2+} (control) and cells maintained in contact with chalcopyrite and bornite. Values shown are relative to those obtained for cells grown in Fe^{2+}.

Considering the genes described herein, it is tempting to suggest that the expression modulation of the transporter-genes by chalcopyrite and bornite is mediated mainly through two distinct situations in which *A. ferrooxidans* cells are submitted: the nutrients starvation and the changes on pH in a medium containing copper sulfides. The former resulted in the expression of genes related to nutrients uptake, such as the TonB-dependent receptor for iron acquisition and the phosphate ABC transporter for phosphate uptake, whereas the latter resulted in the expression of genes related to pH homeostasis, such as the Na^+/H^+ antiporter and the voltage-gated chloride channel.

Protein processing genes

Six genes related to protein synthesis and fate were identified: *hflK* (*Afe 1200*), the gene located on locus *Afe 1321*, *lepA* (*Afe 1684*), *rplP* (*Afe 2698*), *gcp* (*Afe 2902*) and *def-2* (*Afe 3005*). The expression pattern of *gcp* gene presented no significant difference in chalcopyrite and bornite in comparison to the control cells. On the other hand, the *lepA* and *def-2* genes had their expression levels down-regulated in chalcopyrite and up-regulated in bornite, whereas *hflK*, *rplP* and the gene located on locus *Afe 1321* had their expression down-regulated in the presence of chalcopyrite but unaltered in the presence of bornite.

The *gcp* gene encodes for O-sialoglycoprotein endopeptidase, a protease with high specificity toward cell surface glycoproteins. In *Pasteurella haemolytica* this glycoprotease has been suggested to be a potential virulence factor (Sutherland *et al.*, 1992). However, apparently this is not the case for *A. ferrooxidans* and the unchanged expression of *gcp* gene may be due to its critical role on cell envelope maintenance.

The *lepA* and *def-2* genes encode enzymes related to protein synthesis and maturation, respectively. The *lepA* gene encodes for the GTP-binding protein lepA. Although assigned as an unknown function by the TIGR-CMR database, the lepA protein was recently demonstrated to be an elongation factor during protein synthesis (Qin *et al.*, 2006). The lepA protein, later renamed elongatin factor 4 (EF4), recognizes and reverts imperfectly translocated ribosomes, allowing a proper positioning of the codon in the ribosomal A site and subsequent mRNA decoding. This unique ability of lepA to back-translocate ribosome with erroneous translocated tRNAs ensures

an accurate and efficient protein synthesis (Qin *et al.*, 2006). The *def-2* gene encodes for polypeptide deformylase, a crucial enzyme for bacterial growth (Mazel *et al.*, 1994). Deformylase participates in the post-translational maturation of the nascent protein chain. This deformylation step is necessary for the further removal of the N-terminal methionine by methionine aminopeptidase. Failure in deformylation renders the newly synthesized protein with a more acidic isoelectric point and the accumulation of formylated peptides leads to bacterial growth inhibition (Wang *et al.*, 2006; Bandow *et al.*, 2003).

The *hflK* gene (*Afe 1200*) codifies for hflK, a membrane-bond protein implicated in the regulation of protease activity. The hflK protein interacts with the hflC protein to form the hflKC complex (Saikawa *et al.*, 2004). This complex negatively modulates the proteolytic activity of FtsH, a protease with intrinsic chaperone activity required for proteolytic degradation of unstable proteins (Schumann, 1999). FtsH can also be modulated by the YccA, a protein that can recognize and interact with both FtsH and HflKC complex (Kihara *et al.*, 1998). The gene located on locus *Afe 1321* is assigned in the TIGR-CMR database as a membrane protein with unknown function. However, we believe that this gene encodes for an yccA homologue. A Blastp analysis demonstrated that the product of the gene on locus *Afe 1321* has a high degree of similarity to the *E. coli* YccA protein (Score: 326 bits; E-value: $1.8e^{-31}$; Identities: 74/199, 37%; Positives: 114/199, 57%). The FtsH, HflKC and YccA complexes act against membrane protein substrates and are believed to be responsible for the quality control of cytoplasmic and membrane proteins (Schumann, 1999). The *rplP* gene (*Afe 2698*) is another gene related to protein synthesis whose expression was negatively affected by chalcopyrite. The *rplP* gene encodes for the ribosomal protein L16. This protein has an essential role on the assembly and conformation of the ribosome subunits, thus maintaining the ribosome architecture and the proper orientation of the aminoacyl tRNA binding site in the ribosomal 50S subunit (Nag *et al.*, 1991; Nishimura *et al.*, 2004).

Considering the genes related to protein processing analyzed on the present work, it is interesting to note that chalcopyrite interferes negatively in the protein *turn-over* in *A. ferrooxidans* cells, affecting all aspects that govern the protein fate, including the process of protein synthesis, maturation and degradation. Bornite, on the other hand, has an opposite effect on the expression modulation of genes related to protein processing. The up-regulation of *lepA* and *def-2*, which are respectively involved in protein synthesis accuracy and protein maturation,

seems to indicate that protein synthesis is triggered when *A. ferrooxidans* is maintained in the presence of bornite.

DNA replication and transposition

Three genes with functional category assigned to DNA replication and transposition were identified: the *dnaB* gene (*Afe 0289*), the *nrdA* gene (*Afe 0467*) and the gene located on locus *Afe 1751*. The *dnaB* gene, which encodes the replicative helicase DnaB protein, was the only gene whose expression was up-regulated in response to bornite but unchanged on chalcopyrite. In the replication fork, DnaB helicase unwinds the DNA duplex and allows the DNA primase to synthesize the primers that initiate each Okazaki fragments (van der Ende *et al.*, 1985). Thus, the DnaB helicase has an essential role in DNA replication both on the initiation and elongation process.

The *nrdA* gene (*Afe 0467*) had its expression pattern unchanged in the presence of bornite but down-regulated in the presence of chalcopyrite. The *nrdA* gene encodes for the *alpha* subunit of the ribonucleotide diphosphate reductase (rNDP reductase). The rNDP reductase carries out an essential role on DNA replication and repair since it catalyzes the reduction of ribonucleotide to deoxyribonucleotide (dNTPs), the precursors for DNA synthesis. Besides being the specific enzyme required for dNTPs synthesis, the rNDP reductase is coupled to the DNA replication apparatus in a multienzyme replication complex (Guzmán *et al.*, 2002). As a consequence of the rNDP reductase deficiency, the dNTPs supply is reduced and the replication fork progression is slowed or even arrested, which may lead to DNA double-strand breaks (Guarino *et al.*, 2007). The transcription of the *nrdA* gene is negatively regulated by the NrdR repressor, a protein encoded by the *nrdR* gene. Bioinformatic analysis was employed and allowed us to identify a putative NrdR binding site located in the upstream region of *nrdA*, as previously reported (Rodionov and Gelfand, 2005). In the *A. ferrooxidans* genome the *nrdR* gene is named *ribX* and it is found clustered with the riboflavin biosynthetic genes. The expression pattern of *nrdR* in the presence of copper sulfides was also assessed by RTq-PCR. While the expression of *nrdA* was down-regulated in chalcopyrite, the expression of its repressor, *nrdR* gene, was up-regulated (data not shown). In the presence of bornite, both *nrdA* and *nrdR* presented an unaltered pattern of expression (data not shown).

The expression pattern of the gene located on locus *Afe 1751* presented no significant difference in the presence of chalcopyrite and bornite in comparison to the control cells. This gene encodes for a transposase and belongs to an insertion sequence (IS) element named ISAfe2 (Yates *et al.*, 1988). Transposition of IS elements in the chromosomal DNA of *A. ferrooxidans* has been related to adaptation to different environmental conditions (Kondrat'eva *et al.*, 2005). Previous data have shown that the expression pattern of the gene on locus *Afe 1751* remains unchanged also in covellite when compared to cells grown in Fe^{2+} (Reis, personal communication). Together, these findings indicate that DNA-mediated transposition through ISAFe2 transposase is not triggered in the presence of copper sulfides, as indicated here for bornite, chalcopyrite and covellite. A possible explanation for these results may be due to the short time incubation (24 hours) in the presence of those copper sulfides, especially for a slow growing bacterium like *A. ferrooxidans*.

Energy metabolism

The genes on *loci Afe 0007* and *Afe 0965* are related to the energy metabolism and had their expression pattern unchanged in the presence of bornite but down-regulated in the presence of chalcopyrite. The gene on locus *Afe 0007* (*sdrA1* gene) belongs to the *petI* operon and codifies an oxidoreductase from the short-chain dehydrogenase/reductase family. The *petI* gene cluster encodes redox proteins of the cytochrome bc_1 electron-transfer complex. The cytochrome bc_1 complex is associated to iron oxidation, transferring electron from iron to NAD(P) (Bruscella *et al.*, 2007). The specific function of this oxidoreductase on the bc_1 complex remains unclear, despite the location of *sdrA1* on the same transcriptional unit of the *petI* operon (Bruscella *et al.*, 2007). The down-regulation of *sdrA1* in the presence of chalcopyrite is in accordance with the fact that ferrous iron is poorly available to cells incubated in medium containing chalcopyrite and that the *petI* operon is mainly transcribed in cells grown in iron (Bruscella *et al.*, 2007). The gene on locus *Afe 0965* encodes for an aldehyde dehydrogenase related to the fermentation process. The down-regulation of this gene seems to suggest that the energetic metabolism is affected when *A. ferrooxidans* cells are maintained in the presence of chalcopyrite without ferrous iron as energy source.

Central metabolism

Finally, two genes, out of the 18 genes identified in the present study, are involved with the central metabolism. The gene located on locus *Afe 0225* encodes for a putative choloylglycine hydrolase. There is no reasonable explanation for the up-regulation of this gene exclusively in the presence of chalcopyrite. The *ribE* gene (*Afe 2728*) encodes for 6,7-dimethyl-8-ribityllumazine synthase, a key enzyme that participates in the riboflavin biosynthetic process. Riboflavin is the precursor molecule for the synthesis of two coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both essential in cellular metabolism and redox reactions (Vitreschak *et al.*, 2002). The unchanged expression of *ribE* gene in the presence of copper sulfides emphasizes the crucial role of the product of this gene on the cellular metabolism, as indicated by its functional activity.

In conclusion, the results presented here highlighted the changes on transport system and protein processing in *A. ferrooxidans* cells maintained in the presence of chalcopyrite, as summarized on Table 2. From the results described herein it is possible to pin-point candidate target genes that may be submitted to further investigations. This includes, for instance, those genes encoding transporters associated to phosphate and iron uptake whose expression was greatly induced in chalcopyrite. A mechanistic approach involving molecular manipulation of these target genes in *A. ferrooxidans* cells is underway.

Table 2. Chalcopyrite modulation on the expression pattern of genes related to transport and protein processing.

Modulation	Category	Process	Locus/gene	Protein	Functional activity
Repression	Protein processing	Synthesis	*lepA*	Elongation factor 4 (EF4)	Ensure proper mRNA decoding
			rplP	Ribosomal protein	Ribosome assembly
		Maturation	*def-2*	deformylase	Removal of N-formyl group from the nascent proteins
		Degradation	*Hflk*	Protease modulators	Proteolytic degradation of unstable proteins
			yccA		
Induction	Transport	pH homeostasis	*Afe 0853*	Na^+/H^+ antiporter	Transport of Na^+ in exchange to H^+
			Afe 3203	Voltage-gated chloride channel	Chloride ion transport by a voltage-gated channel
		Heavy metal tolerance or Nutrients uptake	*pstA*	Phosphate ABC transporter	Phosphate and nutrients uptake; Heavy metal tolerance through inorganic phosphates
			Afe 0074	ABC-2 type transporter	
		Iron uptake	*Afe 1592*	TonB-dependent receptor	iron acquisition system mediated by siderophores

EXPERIMENTAL PROCEDURES

Metal sulfides

The metal sulfides used in the experiments were chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4). Research-grade bornite was obtained from Ward´s Natural Science Establishment (Montana, USA) while the chalcopyrite concentrate was obtained from Companhia Vale do Rio Doce (Brazil). Both minerals were ground in a disc mill to 100% <0.2 mm mesh size and sterilized for 20 minutes at 120ºC.

Bacterial strain and growth conditions

The *Acidithiobacillus ferrooxidans* strain LR, originally isolated from a uranium mine effluent in Brazil (Garcia Jr, 1991), was used in the experiments. The cells were grown at 30ºC in salts solution of T&K medium (Tuovinen & Kelly, 1972) comprised of 0.5 g/l of each $K_2HPO_4.3H_2O$, $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$. The pH was adjusted to 1.8 with sulfuric acid and autoclaved

before supplemented with Fe^{2+} (33.4 g/l of $FeSO_4.7H_2O$ in pH 1.8, sterilized by filtration), in a 4 (salts):1 (ferrous sulfate) proportion. Cells were cultured at 30ºC on a rotary shaker at 250 rpm until they reached the late exponential phase of growth (precisely 80% of ferrous iron oxidation, measured by titration with potassium dichromate). At this point, cells were harvested by filtration on Millipore membrane (0.45 μm) and successively washed to eliminate residual ferrous iron from the medium. The pellet was suspended in T&K medium without Fe^{2+} and the cell numbers were determined spectrophotometrically at 595 nm. Approximately 2 x 10^{12} cells/ml were incubated at 30ºC, 250 rpm for 24 hours in 100 ml of T&K medium (without Fe^{2+}) containing 2.5% (w/v) of either chalcopyrite ($CuFeS_2$) or bornite (Cu_5FeS_4). After 24 hours, cells were harvested by membrane filtration (0.45 μm, Millipore) and washed until complete removal of the metal sulfide. Cells grown until 80% of ferrous iron oxidation were used as control. The cells were deep-frozen in liquid nitrogen and stored at -80ºC until total RNA isolation.

RNA isolation

Total RNA was isolated as described by Winderickx and Castro (1994) and Paulino *et al.* (2002). The cells were suspended in a solution containing 1 mM EDTA, 0.1 M LiCl and 0.1 M Tris-HCl and extracted with phenol:chloroform:isoamilic alcohol (25:24:1, v/v/v) containing 10% SDS. The RNA was precipitated at -20°C with 2% potassium acetate pH 5.5 and 100% ethanol, and resuspended in DEPC-treated water. The RNA integrity was checked by agarose gel electrophoresis. RNA samples were submitted to DNase treatment (Invitrogen) for 15 min at 37ºC prior to storage at -80ºC.

RAP-PCR and Sequencing experiments

The RAP-PCR experiments were conducted in duplicate as described by Paulino *et al.* (2002). The following arbitrary primers were used in the experiments: OPF-09, OPF-13, OPF-20, OPL-03 and OPL-12 (Operon Technologies). The differentially expressed RAP-PCR bands were excised and the DNA was eluted from the gel. The DNA was PCR amplified and the PCR products were cloned into pGEM-T *Easy* vector (Promega). At least three clones from each RAP-

PCR product were sequenced using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM 377 Automated DNA Sequencer (Applied Biosystems), according to the manufacturer's recommendations.

Bioinformatic analysis

The sequences of the differentially expressed cDNAs were compared with the *A. ferrooxidans* ATCC 23270 genome available on the TIGR-CMR database (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi), using the BLASTn algorithm (http://tigrblast.tigr.org/cmr-blast/). cDNAs corresponding to genes with known function were identified and the entire coding region of the selected gene was obtained from the TIGR-CMR database. The genes analyzed in this work will be referred by their locus and/or gene names according to TIGR-CMR database's nomenclature. The predicted gene function category was assigned based on the TIGR-CMR database.

Real time quantitative PCR amplification

The relative expression pattern of the 18 selected genes on cells grown in Fe^{2+} (control condition) and cells exposed to either chalcopyrite or bornite (tested conditions) was determined by real time quantitative polymerase chain reaction (RTq-PCR) experiments. The *alaS* gene, a recognized endogenous control that encodes for alanyl tRNA synthetase (Yarzábal *et al.*, 2004), was used as the reference standard. Table 3 lists the specific primers of the 18 genes analyzed in the RTq-PCR experiments. The entire coding region of the selected genes, obtained from the TIGR-CMR database, was used for primer designing. Primers were designed in order to have a melting temperature (T_M) of 60ºC using the web-based program Primer3 v. 0.4.0 (Rozen and Skaletsky, 2000). Prior to the RTq-PCR experiments, the specificity of the primers were confirmed by PCR using genomic DNA isolated from *A. ferrooxidans* strain LR, according to standard procedures.

The RTq-PCR experiments were performed in triplicate, using cDNAs synthesized from RNA samples obtained from at least three independent cultures. The RNA was quantified by

spectrophotometer and used in cDNA synthesis with the *ThermoScript™ RT-PCR System kit* (Invitrogen) as recommended by the manufacturer. RTq-PCR experiments were performed using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen), following the manufacturer's instructions with minor modifications. The reaction mixture consisted of 100 ng of cDNA, 6.25 µl of Platinum® SYBR® Green qPCR SuperMix-UDG, 0.25 µl of ROX reference dye, 1 µl of each primer (5 µM) and distilled water added to a total volume of 12.5 µL. Reactions were submitted to the following thermal cycling conditions: an initial UDG treatment for 2 min at 50°C followed by denaturation for 10 min at 95°C, and 40 cycles consisting of denaturation at 95°C for 15 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 32 s. Subsequently, a dissociation (melting) curve analysis was performed to ensure the specificity of the RTq-PCR amplifications, the absence of primer-dimer artifacts and unspecific PCR products. Data were collected on a 7500 real-time PCR system and processed with the Sequence Detection Software v1.3.1 (Applied Biosystems). The baseline and the threshold parameters were determined by manual analysis settings. The relative expression pattern of the selected genes was calculated based on the comparative critical threshold ($\Delta\Delta C_T$) method, as previously described by Livak and Schmittgen (2001). Briefly, the average threshold cycle (C_T) values for the genes of interest and the endogenous gene were determined on both control (growth on Fe^{2+}) and tested conditions (bornite or chalcopyrite). For a normalization purpose, the delta C_T (ΔC_T) values were then calculated as the difference between the C_T value for the genes of interest and the C_T value for the endogenous gene. Thereafter, the $\Delta\Delta C_T$ value for each gene was determined as the difference between the ΔC_T value obtained from the tested conditions and the ΔC_T value obtained from the control condition (calibrator). Finally, the fold-changes in mRNA levels of the selected genes on the tested conditions, normalized with the endogenous gene and relative to the control condition, was given by the equation $2^{-\Delta\Delta CT}$. Validation experiments using serial dilutions of the cDNA samples were performed, ensuring that the amplification efficiency of the genes of interest and the endogenous gene was approximately equal.

Loci	Forward primer (5'>3')	Reverse primer (5'>3')	Size (bp)
AFE 0007	CCGTTCCACCACCGATAG	CGCATCCAGCCAATCTCT	107
AFE 0074	AAATGCCTTCGCCAACAGT	ACCATGGCCACGAAAATCT	111
AFE 0225	GGCAGAAGTCATGAGTGTTTTG	CGCCATCGGGTAGAAGAA	90
AFE 0289	AGCTCAACCGAAGTCTGGAA	CATCGCGATAGAGGAAGAGG	114
AFE 0467	TTCCTCAAAGTGGTCAACGA	GTGCCAGGTTTCCAGATAGG	90
AFE 0853	GAAATCATCAAACGCGTCAAT	GTACTGATGACGCCGGAATAG	113
AFE 0965	TACATCCAACCTACCCTGCTG	CTTCCGCTTCGTCCTTGA	106
AFE 1149	AAAGAACCCATCGGCTACCT	GCAAATGCGAGCATATGAGA	83
AFE 1200	CCTCCTGCCTTTCCTGGT	CGCAGTACCACGCCTTCT	96
AFE 1321	AGCGGCCTGATCCTTTTT	GCAGCGCCATGAAGAGAT	112
AFE 1592	GTGACTCCATCCAGCCATTT	TATTTCTCCGCTATTGATGACG	96
AFE 1684	GTGCGCAGGGAGATTGAA	ACCAGGAGTCGATGATGAG	172
AFE 1751	TCTTTGTGCGCAACTACCAG	GTGGCAAAGGTCGACTCAAT	148
AFE 2698	GAAGGTGGTACGGAAGAGGTTG	ATCCCATCACCCGTCTACTTAC	97
AFE 2728	GTGATTTTTGGGGTGCTGAC	CAACAACTGCACCATTTCCA	120
AFE 2902	GCGGATACTGGGCATTGA	GGGCAATCTGGCTGAACA	101
AFE 3005	GGTGAGGAGGAGATGAAGGA	CCTTACGGCGAATCAGACTT	229
AFE 3203	CGAAATGACCGGGAGCTA	TCGTGCTGTGCGTTGAAT	120

Table 3. Set of primers of the 18 genes analyzed in the RTq-PCR experiments.

Statistical analysis

The data obtained in RTq-PCR experiments were analyzed by the Student's t-test and the statistical significance of the differential gene expression was defined with a $P\text{-}value < 0.05$.

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CAPÍTULO 2

Ferric iron uptake genes in *Acidithiobacillus ferrooxidans*: *in silico* analyses and differential expression in the presence of copper sulfides

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Abstract

Aims: This work aimed to determine the expression pattern of genes related to the Fe(III) uptake system mediated by siderophores in the iron-oxidizing bacterium *A. ferrooxidans* maintained in contact with copper sulfides.

Methods and Results: The relative expression pattern of seven genes related to the siderophore-mediated Fe(III) uptake system was investigated by real time quantitative PCR (RTq-PCR). For this, cDNAs were synthesized from RNA samples obtained from *A. ferrooxidans* cells maintained for 24 h in contact with the copper sulfides chalcopyrite and bornite. The results showed that bornite has no effect on the expression pattern of the genes while chalcopyrite up-regulate their expression. Among the analyzed genes was a TonB-dependent receptor. An analysis of conserved protein domains revealed that this receptor belongs to the CirA family of receptors. A bioinformatics analysis of the genomic region where the genes investigated in this work were found revealed the existence of three new Fur boxes.

Conclusions: Chalcopyrite triggered the expression of genes related to the siderophore-mediated Fe(III) uptake system probably due to the low amount of ferrous iron in the medium. The presence of three new Fur boxes in the genomic region where these genes are located suggests that they are regulated by Fur.

Significance and Impact of the Study: This study provided further insights on the regulation and expression pattern of genes related to the Fe(III) uptake system in *A. ferrooxidans* cells maintained in contact to copper sulfides.

Keywords: *Acidithiobacillus ferrooxidans*, metal sulfides, real time PCR, Fe(III) uptake system

Introduction

Acidithiobacillus ferrooxidans is a Gram-negative, mesophilic, chemo-lithoautotrophic bacterium that obtains energy from the oxidation of ferrous iron, elemental sulfur and reduced sulfur compounds (Rawlings, 2002). The ability to oxidize insoluble metal sulfides into soluble metal sulfates makes this microorganism of great importance in bioleaching operations to recover copper from low-graded copper sulfide ores such as chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4) (Rawlings, 2002).

The physiology of *Acidithiobacillus ferrooxidans* is unique since this microorganism utilizes iron as energy source and as a nutrient. As an acidophilic microorganism, *A. ferrooxidans* grows in a pH range of 1.5-2.0 (Rawlings, 2002). In this acidic environment *A. ferrooxidans* faces an abundant supply of iron, a situation that requires a coordinated mechanism that regulates the balance of the iron utilized for assimilation in the cellular metabolism and the iron addressed for energy production. For this reason, *A. ferrooxidans* has developed a tightly regulated system to control iron uptake and homeostasis (Quatrini *et al.*, 2005a).

In regard to iron uptake system, *A. ferrooxidans* presents specialized mechanisms based on iron chelators such as siderophores (Quatrini *et al.*, 2005b). Siderophores are low molecular mass compounds with high affinity towards ferric iron (Andrews *et al.*, 2003). The components of the ferric iron acquisition system mediated by siderophores include outer-membrane receptors (OMR), an ATP-binding cassette (ABC) transport system and an energy-transducing system (Quatrini *et al.*, 2005b). *A. ferrooxidans* presents 11 potential Fe(III)-siderophore OMRs, which depend on the energy provided by the energy transduction complex TonB, ExbB and ExbD. The transport of ferric-siderophore from the OMRs into the cytoplasm is mediated by the ABC transport system which consists of a periplasmic iron-binding protein, a permease and an ATP-binding protein (Quatrini *et al.*, 2005b).

Concerning the mechanisms that regulate the iron homeostasis, an iron-responsive transcriptional regulation mediated by the ferric iron uptake regulator (Fur) has been described in *A. ferrooxidans* (Quatrini *et al.*, 2005a). Fur, encoded by *fur* gene, regulates gene expression through binding in regulatory DNA sequences, known as Fur boxes, located in promoter region of the target genes. A putative dual mechanism of action has been proposed, suggesting that Fur

modulates gene expression by either activating genes involved in iron-oxidation energy production or repressing genes involved in iron uptake mainly when the cell faces iron-rich conditions (Quatrini *et al.*, 2005a; Quatrini *et al.*, 2007).

In the present work a cluster of genes related to the siderophore-mediated iron uptake system in *Acidithiobacillus ferrooxidans* was described. The relative expression pattern of these genes was characterized, by real time PCR, in *A. ferrooxidans* cells maintained in contact with bornite and chalcopyrite for 24 h. A search for conserved protein domains was performed to find the family that the TonB-dependent receptor analyzed in this work belongs. Also, bioinformatics tools were used to search for new Fur boxes on the genomic region where these genes are located.

Materials and methods

Copper sulfides

The copper sulfides used in the experiments were chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4). The research-grade bornite was obtained from Ward´s Natural Science Establishment (Montana, USA) while the chalcopyrite was obtained from Companhia Vale do Rio Doce (Brazil). Minerals were grounded in a disc mill to 100% <0.2 mm mesh size.

Bacterial strain and growth conditions

The *Acidithiobacillus ferrooxidans* strain LR (Garcia Jr, 1991) was used in the experiments. The cells were grown in salts solution of T&K liquid medium (Tuovinen & Kelly, 1973) containing 0.5 g l^{-1} of each $K_2HPO_4.3H_2O$, $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$, pH 1.8 adjusted with sulfuric acid. The medium was supplemented with ferrous sulfate (167 g l^{-1}), in a 4 (salts):1 (ferrous sulfate) proportion. The medium was inoculated with bacteria and incubated at 30°C, 250 rpm, until 80% of oxidation of Fe^{2+}, measured by titration with potassium dichromate. Cells were collected and maintained for 24 h in 100 mL of T&K medium (without ferrous sulfate) containing 2.5% (w/v) of the copper sulfides. After 24 h, the cells were harvested by filtration on Millipore membrane (0.45 μm) and washed until complete removal of the copper sulfides. Cells grown in T&K

medium with ferrous sulfate were used as control. The cells were frozen in liquid nitrogen and stored at -80ºC until total RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated as described by Winderickx and Castro (1994) and Paulino *et al.* (2002). Cells were suspended in a solution containing 1 mM EDTA, 0.1 mol l^{-1} LiCl and 0.1 mol l^{-1} Tris-HCl and extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) containing 10% SDS. The RNA was precipitated with 2% potassium acetate pH 5.5 and 100% ethanol at -20°C over night. The RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water. The RNA samples were treated with DNase (Invitrogen) for 15 min at 37ºC and stored at -80ºC. The RNA was quantified in a spectrophotometer and then used to synthesize cDNA with the *ThermoScript*^{TM} *RT-PCR System Kit* (Invitrogen), according to the manufacturer's instructions. The cDNAs were synthesized from RNA samples obtained from at least three independent cultures.

Bioinformatic analysis

A search for genes related to the siderophore-mediated iron uptake system was conducted on the *A. ferrooxidans* ATCC 23270 genome available on the TIGR (www.tigr.org) - Comprehensive Microbial Resource (TIGR-CMR). The genes analyzed in this work will be referred by their locus name according to the TIGR-CMR nomenclature.

To identify novel potential Fur-biding sites, a widely accepted information theoretical approach was adopted (Schneider, 1997; Reents *et al*., 2006). To create the position weight matrix model, a set of 66 experimentally confirmed Fur boxes was used (Quatrini *et al*., 2007). The matrix was used to search the *A. ferrooxidans* genomic region where the siderophore-mediated Fe(III) acquisition system genes were located. A 19 bp sliding window was used in the search. Only windows with scores, in the Fur weight matrix, higher than 7 bits were retained in the analysis. The *A. ferrooxidans* putative Fur-binding sites were aligned and used to create the corresponding sequence logo. A search for similarity of the putative TonB-dependent receptors encoded by *A. ferrooxidans* with known domains of receptors subfamilies was performed on the Conserved Domain Database (Marchler-Bauer *et al*., 2007).

Real time PCR

The relative expression pattern of the genes was determined by real time quantitative PCR (RTq-PCR) analysis. The *alaS* gene that encodes for an alanyl tRNA synthetase was used as the endogenous control (Yarzábal *et al.*, 2004). The primers used in the experiments were designed with the Primer3 program (http://frodo.wi.mit.edu/) using the entire coding region of the selected gene from the *Acidithiobacillus ferrooxidans* ATCC 23270 genome available on the TIGR (www.tigr.org) (Table 1). The specificity of the primers was confirmed by PCR using genomic DNA from *A. ferrooxidans* strain LR.

The experiments were performed on 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 mixtures were done in triplicate with the kit Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), following manufacturer's instructions. After the thermal cycling conditions, a dissociation (melting) curve analysis was performed to ensure the specificity of the amplifications, the absence of primer-dimer artifacts and unspecific amplifications. The gene expression pattern was calculated according to the comparative critical threshold method previously described by Livak and Schmittgen (2001).

Table 1. Primers corresponding to the genes analyzed by real time quantitative PCR.

Loci	Forward primer (5'>3')	Reverse primer (5'>3')	Size (bp)
Afe 1592	GTGACTCCATCCAGCCATTT	TATTTCTCCGCTATTGATGACG	96
Afe 1593	TCCGAGCCTATACCGAGTTG	CAGTCACGGGTTCATCAAGA	105
Afe 1594	CTATACCCAACTTCCGACCATC	AGAAGAATAGGAATCGCCATGA	96
Afe 1595	TCAACCGCCTGTATTCCTTTAT	TCTGGATATCTTTGTCGCTGAA	99
Afe 1596	AGACATTGAGGCGCTATCTTG	AGTCCACGTGCTTTAGTCATTG	115
Afe 1597	TGCCTATGCCCACAATCC	CGGACACATTGACCAGCA	98
Afe 1598	CTTGCCCAGAACCCCTTT	GCCAATTGCTGCTCCAGT	99
Afe 1599	GCTGGTGGTGCTTTTCGT	CCCGGCCTTTTCTGTCTT	104

Statistical analysis

The data obtained in RTq-PCR analysis for the experiments with copper sulfides were analyzed with the Student's t test to determine significant statistical differences.

Results

Cluster of genes related to the siderophore-mediated iron uptake system in *A. ferrooxidans*

In a previous work (Ferraz *et al.*, manuscript in preparation) we have identified a gene (locus *Afe 1592*), encoding for a TonB-dependent receptor related to siderophore-mediated iron uptake system, whose expression was greatly up-regulated in the presence of the copper sulfide chalcopyrite. Bioinformatic analysis on the available *A. ferrooxidans* ATCC 23270 genome (TIGR-CMR) showed that the locus *Afe 1592* is clustered with 6 genes related to the iron acquisition system mediated by siderophores (Figure 1). Besides the gene on locus *Afe 1592*, this cluster comprises the genes on loci *Afe 1593*, *Afe 1594* and *Afe 1595*, that encode the proteins of the ABC transporter system, and the genes on loci *Afe 1597*, *Afe 1598* and *Afe 1599*, that encode the energy-transducing system. A putative gene encoding a hypothetic protein (locus *Afe 1596*) was also found, although a role for this protein on the iron acquisition system it is not clear.

An analysis of the conserved protein domains revealed that the TonB-dependent receptor encoded by the gene on locus *Afe 1592* probably belongs to the CirA receptor family (COG1629, E-value: $4e^{-19}$). Further bioinformatic analyses were performed and allowed us to identify putative Fur boxes upstream the loci *Afe 1593*, *Afe 1594* and *Afe 1599*. Table 2 shows the Fur boxes identified and Figure 2 illustrates their degree of sequence conservation. These new *A. ferrooxidans* predicted Fur boxes are conserved in respect to the *Escherichia coli* Fur box consensus, including the nucleotides known to be critical for Fur binding (Escolar *et al.*, 1998).

Table 2. Putative Fur boxes found upstream loci *Afe 1593*, *Afe 1594* and *Afe 1599*.

Loci	TIGR annotated function	Fur box	Score (bits)
Afe 1593	iron compound ABC transporter, ATP-binding protein	GATGTTGATCATCGTAATG	7.273
Afe 1594	ABC transporter, permease protein, FecCD family	GAAAATGTTTTCCACTGCA	9.161
		GATATCCAGAATTTTTATC	8.176
Afe 1599	MotA/TolQ/ExbB proton channel family protein	GCCAACAATTTTTATTCGA	8.304

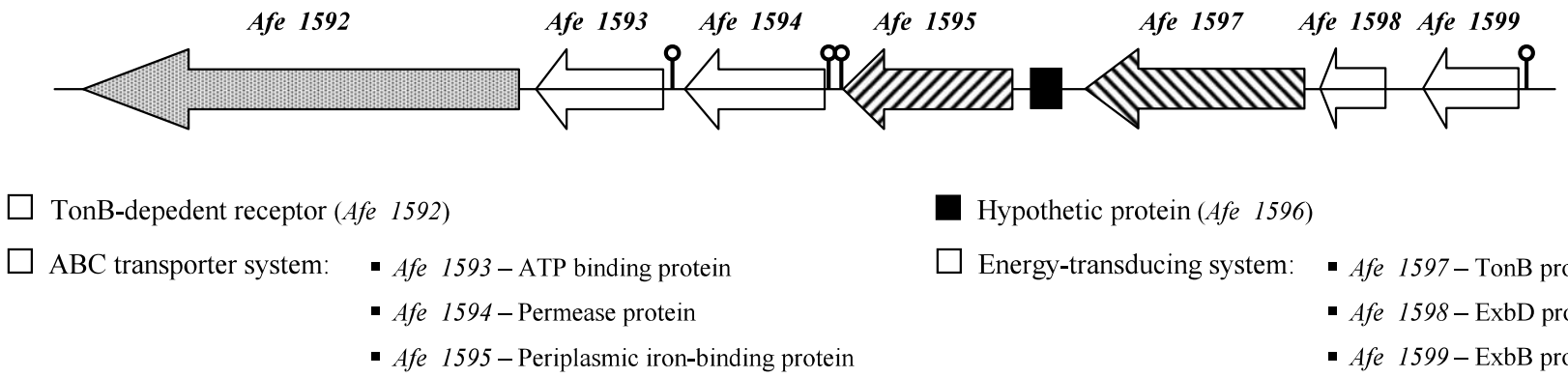

Figure 1. Cluster of genes related to the siderophore-mediated iron uptake system in *A. ferrooxidans* ATCC 23270. This cluster comprises the gene on locus *Afe 1592* that encodes the TonB-depedent receptor, the genes encoding for the ABC transport system (loci *Afe-1593*, *Afe-1594* and *Afe-1595*) and the genes encoding for the energy-transducing system (loci *Afe-1597*, *Afe-1598* and *Afe-1599*). Putative Fur boxes are indicated by stem-loops.

Figure 2. Logo for the putative Fur boxes found in the cluster of genes related to siderophore-mediated iron uptake system in *A. ferrooxidans*. The height of each letter represents the degree of sequence conservation in bits. The letters are sorted with the most frequent on the top.

Gene expression pattern on cells maintained in contact to copper sulfides

Using RTq-PCR experiments, the relative expression pattern of the selected genes was investigated in *A. ferrooxidans* cells maintained for 24 h in contact with chalcopyrite and bornite. The expression pattern of the genes in the presence of bornite presented no significant difference in comparison to the control cells (data not shown). However, in the presence of chalcopyrite these genes were up-regulated as much as 7 folds (Figure 3).

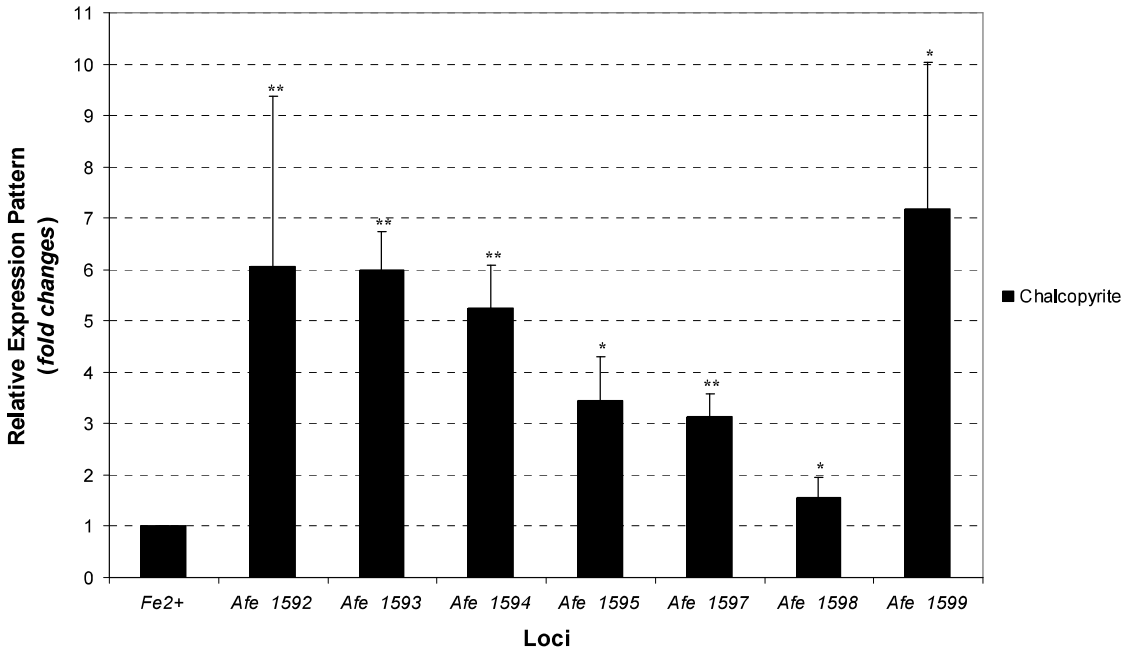

Figure 3. Real time quantitative PCR analysis of *A. ferrooxidans* cells maintained in contact with chalcopyrite. Values are relative to those obtained for cells grown in Fe^{2+} and are given as fold changes. Statistical differences are based on the Student's t-test with $p < 0.05$* and $p < 0.001$**, considered significant.

Discussion

Iron is both a nutrient and an energy source for *Acidithiobacillus ferrooxidans*. For this reason, this iron-oxidizing microorganism has developed tightly regulated mechanisms for iron uptake, regulation and storage. Concerning the uptake system, *A. ferrooxidans* presents a high affinity iron acquisition system mediated by siderophores which includes outer-membrane receptors, ABC transporters and an energy-transducing system (Quatrini *et al.*, 2005b). An intriguing characteristic of the *A. ferrooxidans* genome is the elevated number of TonB-dependent receptor genes despite the apparent absence of putative genes involved in siderophore biosynthesis (Quatrini *et al.*, 2005b).

A search on the *A. ferrooxidans* genome (TIGR-CMR) revealed a cluster of 7 genes related to Fe(III) acquisition system. The identification of genes related to this system organized in a cluster is unique on *A. ferrooxidans*, since other components of this system are found dispersed on the *A. ferrooxidans* genome. The TonB-dependent receptor described herein, encoded by the gene located on locus *Afe 1592*, presents similarity with the CirA receptor. An additional analysis of the conserved protein domains indicated that among the 14 putative TonB-dependent receptors found in *A. ferrooxidans*, 8 belong to the CirA subfamily of receptors. Cir is a family of catecholate siderophore receptor that participates in iron transport and it is also a receptor for colicins and microcins (Griggs *et al.*, 1987). In view of this, the elevated number of receptors for such bacteriocins in *A. ferrooxidans* is somehow unexpected.

Chalcopyrite and bornite are copper sulfide ores commonly found in nature and, therefore, are important sources of copper (Third *et al.*, 2000). Since *Acidithiobacillus ferrooxidans* is one of the most widely used microorganisms in bioleaching operations to recover copper from low-graded copper sulfide ores, we decided to investigate the relative expression pattern of the 7 genes related to the iron uptake system in *A. ferrooxidans* cells maintained in the contact with chalcopyrite and bornite as the sole energy source. The expression pattern observed in the presence of these copper sulfides seems in concordance with the oxidizable properties of each copper sulfide by *A. ferrooxidans* and probably reflects the availability of Fe(II) in the medium. The unaltered pattern of expression in bornite in comparison to the cells grown in Fe(II) is expected since bornite is easily solubilized by *A. ferrooxidans* , thus releasing a sufficient supply

of Fe(II) for the cell energetic metabolism (Bevilaqua *et al.*, 2003). On the other hand, the expression pattern of the genes was greatly up-regulated in the presence of chalcopyrite. A possible explanation for these findings is that chalcopyrite is very refractory to bacterial and even to chemical leaching, resulting in low dissolution rates and a medium with poor energy source.

Since the transcript levels of the genes described in this work ranged from 1.5 to 7.0 folds in the presence of chalcopyrite, we searched for the presence of new regulatory sequences in this genomic region. New putative Fur boxes were identified upstream loci *Afe 1593* and *Afe 1599*. Also, a new Fur box upstream locus *Afe 1594* was identified besides the one previously described by Quatrini et al. (2007). Interestingly, the genes with the highest levels of expression were the ones that harbored Fur boxes in their upstream region.

It is worth to mention that a possible role of copper on the up-regulation of this iron-uptake system in the presence of chalcopyrite can not be excluded. It has been shown that Fur regulator is not exclusively related to iron uptake but seems also related to metal homeostasis in general, such as copper (Quatrini *et al.*, 2007). In fact, these authors suggest that the intracellular levels of both iron and copper might be fine-tuned through Fur-dependent balancing of the uptake and efflux of these metals. As abovementioned, the recalcitrant characteristics of chalcopyrite result in low dissolution rates. In this context, in contrast to the highly soluble bornite, iron and copper are poorly available in the medium containing chalcopyrite, thus resulting in the up-regulation of the genes related to Fe(III) acquisition system which are under the regulation of Fur.

In conclusion, we described here both the regulation and the expression pattern of genes related to siderophore-mediated iron acquisition system in *A. ferrooxidans* cells maintained in contact with copper sulfides. The results provide further insights on Fe(III) uptake mechanisms in *A. ferrooxidans* cells maintained in contact to chalcopyrite, the most abundant copper-bearing mineral.

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CONCLUSÕES

- Foram observadas alterações na expressão gênica em *A. ferrooxidans* na presença de calcopirita e bornita.
- A expressão dos cDNAs que codificam proteínas envolvidas na síntese, maturação e degradação de proteínas foram reprimidas na presença de calcopirita indicando que este sulfeto metálico de difícil oxidação afeta alguns aspectos do processamento de proteínas.
- A expressão dos cDNAs que codificam proteínas envolvidas na homeostase de pH e captação e transporte de nutrientes foram induzidos na presença de calcopirita, indicando uma escassez de nutrientes e mudanças do pH no meio na presença deste sulfeto metálico.
- A expressão dos cDNAs que codificam proteínas envolvidas na síntese e maturação de proteínas, síntese de DNA e homeostase de pH foi induzida na presença de bornita, indicando que este sulfeto metálico de fácil oxidação ativa o processamento de proteínas, a replicação do material genético e mudanças no pH do meio.
- A técnica de RAP-PCR mostrou-se eficaz para análise da expressão diferencial de genes em *A. ferrooxidans* mantida por 24 horas na presença de calcopirita e bornita.
- O cDNA com maior expressão na presença de calcopirita codifica uma proteína receptora dependente de TonB e está agrupada no genoma de *A. ferrooxidans* com 6 outros genes envolvidos no sistema de captação de Fe^{3+}.
- Na presença de calcopirita a expressão dos genes envolvidos no sistema de captação de Fe^{3+} foi induzida, indicando uma escassez de ferro no meio pelo fato deste sulfeto ser de difícil oxidação.
- Na presença de bornita a expressão dos genes envolvidos no sistema de captação de Fe^{3+} foi inalterada, indicando uma disponibilidade de ferro no meio já que este sulfeto é de fácil oxidação.
- Três novos sítios de regulação por Fur foram encontrados entre os genes envolvidos no sistema de captação de Fe^{3+} indicando uma alta precisão na regulação destes genes.
- Uma análise de bioinformática mostrou que o receptor dependente de TonB analisado neste trabalho pertence à família de receptores CirA.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/ tese de mestrado/doutorado intitulada **Identificação e caracterização de genes expressos diferencialmente em *Acidithiobacillus ferrooxidans* 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 EXPRESSÃO DIFERENCIAL DE GENES EM ACIDITHIOBACILLUS FERROOXIDANS SUBMETIDA AO ESTRESSE INDUZIDO POR PH, TEMPERATURA E PRIVAÇÃO DE FOSFATO Nº PROCESSO 03/2003

( ) 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
Função: Presidente da CIBio - CBMEG