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Avaliação do perfil transcricional de genes relacionados à absorção e homeostase de ferro em *Paenibacillus riograndensis* SBR5

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Resumo

Para as bactérias, assim como a maioria dos organismos vivos, o ferro é um elemento essencial em muitos processos celulares. Embora o ferro seja abundante na crosta terrestre, ele não está prontamente disponível para os organismos. Para garantir a demanda e evitar a toxidez, os organismos desenvolveram vários mecanismos fisiológicos para lidar com mudanças na disponibilidade de ferro. Essa regulação fisiológica refinada é alcançada, principalmente, por mudanças na expressão de genes relacionados ao transporte e metabolismo do ferro. Apesar da sua importância, o metabolismo do ferro ainda é pouco compreendido em bactérias, especialmente em bactérias Gram-positivas. O gênero *Paenibacillus* contém mais de 30 espécies anaeróbicas facultativas, formadoras de endósporos, neutrofílicas, periflageladas heterotróficas, com baixo teor de C+G e Gram-positivas. A linhagem SBR5 de *P. riograndensis* foi isolada da rizosfera de *Triticum aestivum* (trigo), cultivado no Rio Grande do Sul, Brasil. Fixa nitrogênio e produz ácido-3-indol-acético, duas características importantes para bactérias promotoras do crescimento vegetal. Neste trabalho, culturas de *P. riograndensis* foram submetidas a condições de suficiência e deficiência de ferro. Surpreendentemente, *P. riograndensis* mostrou-se muito resistente à deficiência de ferro. O sequenciamento de RNA (RNA-seq) foi a metodologia utilizada para elucidar os mecanismos globais envolvidos na resistência de SBR5 à deficiência de ferro. Por esse método observou-se que a deficiência de ferro causou diversas mudanças na expressão gênica. Dos 150 genes diferencialmente expressos, 71tiveram a sua expressão induzida e 79 foram reprimidos. Oito genes cuja expressão foi pelo menos duas vezes maior ou menor na condição limitante, quando comparada à suficiência de ferro, foram escolhidos para análise por RT-qPCR, para validar os dados obtidos com RNA-seq. Em geral, a maioria dos genes apresentou o mesmo padrão de expressão após 24 h. Os resultados sugerem que, durante a deficiência de ferro, as bactérias expressam vários genes relacionados à absorção de nutrientes, a fim de obter todas as moléculas necessárias para manter os principais processos celulares. Porém, quando o ferro se torna altamente limitante e não existem mais condições adequadas para o crescimento exponencial, a bactéria começa a expressar, de forma antecipada, genes relacionados à formação de esporos, a fim de resistir a essa adversidade. Outro resultado importante foi que a metodologia escolhida se mostrou adequada para a descoberta de novos genes e a técnica de RT-qPCR foi eficiente na validação dos dados obtidos por RNA-seq e pode ser utilizada como alternativa para organismos em que não existem microarranjos disponíveis.

Abstract

For bacteria, as most living organisms, iron is an essential micronutrient to many cellular processes. Although iron is abundant in crustal, it is not readily bioavailable for organisms. To ensure demand and avoid toxicity, organisms have developed several physiological mechanisms to address changes in iron availability. This tight physiological regulation is achieved mainly through the differential expression of genes related to iron uptake and metabolism. Despite its importance, iron metabolism is still poorly understood in microorganisms, especially in Gram-positive bacteria. The genus *Paenibacillus* contains more than 30 species of facultative anaerobic, endospore-forming, neutrophilic, periflagellated heterotrophic, and low C+G Gram-positive bacilli. The *Paenibacillus riograndensis* SBR5 strain was isolated from *Triticum aestivum* (wheat) rhizospheres in Rio Grande do Sul, Brazil. It fixes nitrogen and produces indol-3-acetic-acid, two important plant growth promoting factors. In this work we submitted *P. riograndensis* cultures to iron sufficiency and deficiency conditions. Surprisingly, *P. riograndensis* was very resistant to iron starvation. RNA-sequencing (RNA-seq) was the technology used to elucidate the global mechanisms involved in SBR5 iron-starvation resistance. Through this methodology we observed that iron deficiency caused several changes in gene expression. From 150 differentially expressed genes, 71 were up- and 79 were down-regulated. Eight genes for which expression was at least twice as high (induced) or twice as low (repressed) in the iron-limited conditions compared with iron-sufficient conditions were chosen for RT-qPCR analysis to validate the RNA-seq data. In general, most genes exhibited the same pattern of expression after 24 h. Our results suggest that during iron deficiency, the bacteria express several genes related to nutrient uptake to obtain all of the molecules necessary to maintain major cellular processes. However, once iron becomes highly limiting and is no longer able to sustain exponential growth, the bacteria start to express genes related to the sporulation process in the anticipation of spore formation as a way to resist this stress. Another important result was that RNA-seq methodology was suitable to the discovery of new genes and RT-qPCR is a good technique to validate RNA-seq data, especially for organisms for which microarrays are not available.

Sumário

1. Introdução

1.1. Absorção e homeostase de ferro em bactérias

O ferro é um elemento essencial para quase todos os organismos vivos, exceto para bactérias ácido-láticas, que usam manganês e cobalto em seu lugar. Em condições fisiológicas, ele está presente nas formas de íon ferroso (Fe^{+2}) e íon férrico (Fe^{+3}). Por causa deste potencial redox, o ferro é um íon bastante versátil, participando de vários processos fisiológicos, como fotossíntese, respiração, ciclo do ácido tricarboxílico, transporte de oxigênio, regulação gênica, biossíntese e reparo de DNA, entre outros (Krewulak e Vogel, 2008). Neste contexto, centros de ferro/enxofre (Fe/S) são cofatores de diversas proteínas envolvidas nos processos acima citados (Roche *et al.*, 2013).

Devido a sua reatividade, o ferro é geralmente sequestrado em proteínas hospedeiras, como a transferrina, a lactoferrina e a ferritina. Portanto, a concentração celular do íon férrico é muito baixa para os micro-organismos sobreviverem utilizando apenas o ferro livre, tendo em vista que, nas células, grande parte do ferro se encontra nas proteínas hospedeiras citadas anteriormente. Os micro-organismos vencem esta limitação nutricional obtendo ferro extracelularmente, da lactoferrina ou da transferrina, de hidróxidos férricos precipitados ou intracelularmente da hemoglobina. Isto acontece por dois mecanismos: através da utilização de quelantes, chamados sideróforos, ou pela aquisição de proteínas carreadoras, como a transferrina ou a lactoferrina, mediada por receptores. A entrada de ferro por meio da lactoferrina, transferrina, hemoglobina e sideróforos foi identificada em bactérias Gram-negativas e Gram-positivas. Em Gram-negativas, a membrana externa funciona como uma barreira de permeabilidade. Transferrina, lactoferrina, hemoglobina e a maioria dos complexos ferro-sideróforo excedem o tamanho das porinas, que constituem canais aquosos que permitem a difusão de pequenas moléculas hidrofílicas através da membrana externa das bactérias Gram-negativas (Cowan *et al.*, 1992). Portanto, essas moléculas requerem transportadores

específicos para entrar no espaço periplásmico. Todos esses processos dependem de um receptor na membrana externa - PBP (*Periplasmic Binding Protein*) – e de um transportador na membrana interna – ABC (*ATP-Binding Cassete*) (Krewulak e Vogel, 2008).

As bactérias Gram-positivas, que não possuem membrana externa, apresentam uma parede celular composta de mureína, polissacarídeos e ácido teicoico e mais uma parede celular composta de proteínas, sendo isso, tudo o que separa o citoplasma bacteriano do meio externo. A entrada de ferro em bactérias Gram-positivas acontece por meio de proteínas ancoradas na membrana similares às PBP de bactérias Gram-negativas, existindo, também, proteínas similares aos transportadores ABC na parede celular interna. O aumento no número de sequências genômicas de bactérias Gram-positivas tem permitido a identificação de genes que codificam transportadores de ferro relacionados aos transportadores de ferro de bactérias Gram-negativas. Porém, ainda existe pouco conhecimento acerca deste assunto em bactérias Gram-positivas (Krewulak e Vogel, 2008).

Em excesso, o ferro é tóxico para as células. Portanto, é necessária uma regulação bastante controlada para garantir a demanda e evitar a toxidez, sendo que mudanças na disponibilidade de ferro causam mudanças na expressão de genes relacionados ao metabolismo do ferro (Rudolph *et al.*, 2006).

Os sideróforos são quelantes necessários para os micro-organismos absorverem ferro do ambiente. Além disso, são necessários, também, receptores na membrana externa bacteriana, os quais possuem alta especificidade pelos complexos ferro-sideróforos. Em *Escherichia coli* são conhecidos os receptores FepA (para ferro-enteroquelina), FecA (para ferro-citrato) e Fhu (para ferro-hidroxamato) (Rudolph *et al.*, 2006). Em muitas bactérias Gram-negativas, as proteínas TonB, ExbB e ExbD formam um complexo que fornece a energia necessária para o transporte dos ferro-sideróforos

através dos receptores citados anteriormente. A proteína TonB está associada às membranas interna e externa, sendo que grande parte dela ocupa o espaço periplásmico. Uma vez no espaço periplásmico, o complexo ferro-sideróforo é transportado para o citoplasma por um transportador ABC (Rudolph *et al.*, 2006). Dentro da célula, o ferro é reduzido e integrado ao metabolismo celular; o sideróforo é exportado (Braun e Hantke, 2011).

Fur (*Ferric uptake regulator*) é uma metaloproteína dimérica, caracterizada por apresentar atividade repressora dos genes regulados por ferro (Ernst *et al.*, 1978; Hantke, 1981). Fur liga-se a sítios específicos no DNA, chamados Fur-*box*, que se localizam nas regiões promotoras de genes envolvidos, por exemplo, na homeostase de metais e na resposta ao estresse oxidativo, reprimindo a transcrição destes genes (Rudolph *et al.*, 2006).

1.2. *Paenibacillus riogradensis*

Os bacilos são um grupo grande de micro-organismos caracterizados pela formação de esporos. O gênero *Paenibacillus*, pertencente à família Paenibacillaceae, pode ser encontrado associado a plantas ou livremente em solos, sendo que várias espécies deste gênero possuem propriedades interessantes para a utilização como inoculantes na produção de grãos. Dessas propriedades, destacam-se a fixação biológica de nitrogênio, a produção de fitormônios, que auxiliam o crescimento das plantas (auxinas e citoquininas), a produção de substâncias antimicrobianas e de sideróforos (Beneduzi *et al.*, 2010).

A linhagem SBR5 de *P. riograndensis* foi isolada da rizosfera de *Triticum aestivum* (trigo), cultivado no Rio Grande do Sul, Brasil. É Gram-variável, móvel, anaeróbica facultativa e apresenta boas características de PGPR (*Plant Growth Promoting Rhizobacteria*): fixa nitrogênio, produz substâncias antimicrobianas e ácido-3-indol-acético (Beneduzi *et al*. 2010).

O genoma de *P. riograndensis* foi sequenciado pelo nosso grupo utilizando a tecnologia Illumina. O conteúdo G+C de *P. riograndensis* (55,1%) é similar ao de *P. polymyxia* (54,58%). O genoma da linhagem SBR5 de *P. riograndensis* é composto por um cromossomo circular de aproximadamente 7.370.000 de pares de bases. Quase 70% das ORFs (*Open Reading Frame*) possuem função putativa anotada. Foram encontrados vinte e três genes relacionados à fixação de nitrogênio e vários genes envolvidos na resistência a antibióticos. É importante ressaltar que foram encontrados vários genes relacionados ao metabolismo de ferro: seis genes envolvidos com a absorção de sideróforo, genes *fhu BGD*, que codificam transportadores do tipo ABC, além de proteínas ligantes ao ferricromo. (Beneduzi *et al.* 2011). Embora no trabalho realizado por Beneduzi *et al.* (2010) tenha sido encontrado genes relacionados à absorção de sideróforos, ensaios posteriores não detectaram atividade de produção de sideróforos nem genes relacionados à sua biossíntese (Beneduzi *et al.*, 2011).

1.3. Esporulação

Algumas bactérias respondem a adversidades ambientais formando endósporos, células metabolicamente inativas e resistentes (Higgins e Dworkin, 2012). A formação de endósporos em *Bacillus subtilis* é conhecidamente ativada por estresses nutricionais como deficiência de carbono, nitrogênio e, em alguns casos, fósforo (Errignton, 2010). A formação do endósporo envolve um programa complexo de diferenciação celular coordenada por uma cascata de fatores sigma, além da indução do gene Spo0A, que regula diretamente a transcrição de muitos genes, entre eles diversos fatores de transcrição (Fawcett *et. al.*, 2000). Depois da iniciação, há a formação do septo que gera assimetria e, quando fixada essa assimetria, é gerada uma célula contendo o precursor do esporo e a precursora da célula-mãe (fase II) (Lewis *et al.*, 1994). Na fase III, acontece a translocação do cromossomo através do septo polar (Bath *et al.*, 2000). Nas fases IV e V acontece a fixação do cromossomo no polo da célula (Hempel *et al.*, 2008). Por fim, na

fase VI, ocorre a maturação e liberação do endósporo e a morte programada da célula-mãe (Errington, 2010). Embora a esporulação seja um mecanismo bem estudado em *B. subtilis*, há poucos estudos acerca deste assunto em outras espécies da família Bacillaceae.

1.4. Metodologias modernas de sequenciamento de RNA

As novas tecnologias de sequenciamento, denominadas de tecnologias de sequenciamento de nova geração, começaram a ser comercializadas em 2005 e estão evoluindo rapidamente. Todas essas tecnologias promovem o sequenciamento de DNA em plataformas capazes de gerar informação sobre milhões de pares de bases (pb) em uma única corrida. Dentre as novas plataformas de sequenciamento, duas já possuem ampla utilização em todo o mundo: a plataforma 454 FLX (Roche) e a Solexa (Illumina). Outros dois sistemas de sequenciamento que começam a ser utilizados são a plataforma da *Applied Biosystems*, denominada *SOLiD System*, e o *HeliscopeTrue Single Molecule Sequencing* (tSMS), da Helicos. Todas essas novas plataformas possuem como características comuns um poder de gerar informação muitas vezes maior que o sequenciamento de Sanger, com uma grande economia de tempo e custo por base para o sequenciamento.

1.4.1. Plataforma SOLiD (***Sequencing by Oligonuclotide Ligation and Detection***)

No sistema SOLiD (Mckernan *et al*. 2006), diferentemente dos demais processos, a reação de sequenciamento é catalisada por uma DNA ligase, e não por uma DNA polimerase. O DNA alvo é mecanicamente fragmentado em um sonicador em fragmentos de 60-90 pb, para as bibliotecas de *tags* únicas, ou 1-10 Kb, para as bibliotecas de *tags* duplas (*mate-pair*). Os fragmentos de 60-90 pb são diretamente ligados a adaptadores universais (P1 e P2) em ambas as extremidades. Já nas bibliotecas *mate-pair*, a fragmentação resulta na produção de um contínuo de fragmentos de 1 a 10 Kb, que são visualizados em gel para seleção da faixa de tamanho de interesse. Uma vez

selecionados, os fragmentos são ligados aos mesmos adaptadores P1 e P2, mas são circularizados e clivados com uma enzima de restrição que reconhece seu sítio no adaptador e cliva adiante, liberando fragmentos formados por 27 bases de uma região, mais a sequência dos adaptadores e mais 27 bases adicionais de outra região que está separada da primeira pela distância utilizada no intervalo de seleção dos fragmentos.

Uma corrida no SOLiD leva cerca de cinco dias, havendo apenas 1% de erro, sendo que estes podem ser detectados e removidos, pois os dados gerados possuem um *score* de qualidade. A esta rapidez se agrega, também, a capacidade de ler milhares de *reads* (sequências individuais) de 25 a 35 pares de base por corrida, gerando arquivos que podem variar entre 6 a 10 GB (Chu e Corey, 2012). A alta eficiência e sensibilidade da plataforma, aliadas à possibilidade de analisar 320 amostras distintas em uma única corrida, tornaram a plataforma SOLiD destinada principalmente aos estudos de transcritômica (Cloonan *et al.*, 2008; Passalacqua *et al.*, 2009; Tang *et al.*, 2009).

2. Justificativa

Tendo em vista que *P. riograndensis* é um micro-organismo recém descoberto e apresenta boas características promotoras de crescimento vegetal, ele se torna interessante para a agricultura. Desta forma, estudos sobre o metabolismo e a expressão gênica de *P. riograndensis* podem ser extremamente úteis. Além disso, pouco se sabe sobre a homeostase de ferro em bactérias Gram-positivas, portanto, a geração de conhecimento acerca deste assunto também é de grande valor para a microbiologia de um modo geral.

3. Objetivos

3.1. Objetivos gerais

Avaliar a expressão diferencial de genes de *P. riograndensis* nas condições de deficiência e suficiência de ferro, gerando dados para um melhor entendimento sobre o controle da absorção e do metabolismo de ferro em nessa bactéria.

3.2. Objetivos específicos

1. Observar o comportamento de *P. riograndensis* em meio suficiente e deficiente em ferro.

2. Sequenciar o transcriptoma de *P. riograndensis* multiplicado em meio suficiente e deficiente em ferro.

3. Validar os dados do sequenciamento do transcriptoma por meio de RT-qPCR quantitativo, selecionando os genes diferencialmente expressos e com maior probabilidade de estarem envolvidos no metabolismo de ferro.

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Transcriptome analysis reveals that sporulation is used as an iron deficiency resistance mechanism in *Paenibacillus riograndensis* SBR5

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Abstract

Despite its importance for growth and cell division, iron metabolism is still poorly understood in microorganisms, especially in Gram-positive bacteria. In this work, we used RNA-sequencing technology to elucidate the global mechanisms involved in iron-starvation resistance in *Paenibacillus riograndensis* SBR5, a potential plant growth promoting bacterium. Iron deficiency caused several changes in gene expression, and 150 differentially expressed genes were found: 71 genes were over-expressed and 79 genes were under-expressed. Eight genes which the expression was at least twice as high or twice as low in the iron-limited condition compared with iron-sufficient condition were chosen for RT-qPCR analysis to validate the RNA-seq data. In general, most genes exhibited the same pattern of expression after 24 h of *P. riograndensis* growth under iron-limiting condition. Our results suggest that during iron deficiency, the bacteria express several genes related to nutrient uptake when they start to grow to obtain all of the molecules necessary to maintain major cellular processes. However, once iron becomes highly limiting and is no longer able to sustain exponential growth, the bacteria start to express genes related to the sporulation process in the anticipation of spore formation as a way to resist this stress.

Key words: iron deficiency; *Paenibacillus* genus; sporulation; gene expression.

1. Introduction

The genus *Paenibacillus* contains more than 30 species of facultative anaerobic, endospore-forming, neutrophilic, periflagellated heterotrophic, and low C+G Gram-positive bacilli [1]. *Paenibacillus* species are widely distributed and play important roles in microbial communities [2]. This genus also contains species capable of promoting plant growth [3]. The *Paenibacillus riograndensis* SBR5 strain was isolated from *Triticum aestivum* rhizosphere in Rio Grande do Sul, Brazil. It fixes nitrogen and produces indol-3-acetic-acid, two important plant growth promoting factors [4]. The genome sequence of the *P. riograndensis* SBR5 strain reveals that it is composed of one circular chromosome of 7,893,056 base pairs [5-6]. It contains 6,705 protein coding genes, 87 tRNAs and 27 rRNAs. The rRNA genes are organized in nine individual operons (*rrnA, rrnB, rrnC, rrnD, rrnE, rrnF, rrnG, rrnH* and *rrnI*) located in different regions of the genome [6].

Iron is essential to many cellular processes, and it is required by most bacteria for growth and cell division [7]. Under physiological conditions, iron can be found in its reduced ionic ferrous form (Fe^{+2}) or its oxidized ferric form (Fe^{+3}). Its redox potential makes this metal extremely versatile when incorporated into proteins, and it participates in numerous biological processes [8]. Iron/sulfur (Fe/S) clusters play an important role in many of these processes, including respiration, central metabolism, DNA repair and gene regulation [9].

Although essential, iron in excess is toxic because it can generate reactive hydroxyl radicals through Fenton chemistry [10]. Therefore, to ensure demand and avoid toxicity, organisms have developed several physiological mechanisms to address changes in iron availability. This tight physiological regulation is achieved mainly through genes related to iron uptake and metabolism, such as those genes involved in iron transport, siderophore production and/or reception, and transcription factors [11].

Some bacteria respond to nutrient limitation by forming an endospore, which is a metabolically dormant and environmentally resistant cell [12]. Endospore formation involves a complex cellular differentiation program coordinated by a cascade of sigma factors that temporally and spatially regulate the expression of genes required for endospore formation [13]. *Bacillus subtilis* cells are induced to differentiate into spores by carbon, nitrogen and phosphorus starvation [14].

Up to now, little is known about the relationship between iron starvation and spore formation in Gram-positive bacteria. In this study, we applied the RNA-sequencing (RNA-seq) technology to *P. riograndensis* SBR5 strain exposed to iron-limiting condition to better understand iron homeostasis and to identify genes that were induced during this stressful situation. The results obtained indicate that the *P. riograndensis* SBR5 strain uses spore formation as a survival mechanism to resist this stress situation.

2. Materials and methods

2.1 Bacterial strain, growth conditions and experimental design

The *P. riograndensis* SBR5 strain was grown in King B medium [15] for 24 h at 37°C at 150 rpm. After reaching an OD_{600} value of 0.1, 100 μl of this culture was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of sufficient-iron medium (SIM - control) or limited-iron medium (LIM – treatment) as described by Raza & Shen [16] and were incubated for 24 h at 37°C at 150 rpm. All solutions were prepared with ultrapure water, and the glassware used for the experiments was washed five times with ultrapure water to prevent residual iron contamination. Three replicates were generated for each iron condition. Growth curve analysis was performed by collecting liquid samples every 4 h for a 24 h period, and the OD_{600} values were measured using a T70 + UV/VIS Spectrometer (PG Instrument). Three milliliters of each culture were collected at each time point and kept at -80°C for subsequent RT-qPCR analysis. An identical and independent experiment was performed with two replicates for the construction of libraries for the RNA-seq experiment. For this experiment, samples (3 ml) were collected after 24 h of growth and subjected to RNA extraction and sequencing.

2. 2 RNA isolation, mRNA enrichment, cDNA synthesis and sequencing

Total RNA was extracted from bacterial cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was verified by electrophoresis on a 1% agarose gel. After the samples were treated with DNase I (Invitrogen), the concentration and purity were determined by spectrophotometer readings at 260 nm and 280 nm using a NanoDrop 2000 (Thermo Scientific). To remove ribosomal RNA, total RNA was processed using a MICROB Express Bacterial mRNA Enrichment Kit (Ambion from Life Technologies), precipitated with ethanol and suspended in nuclease-free water. cDNA libraries were constructed using a Whole Transcriptome Analysis Kit (Life Technologies) followed by full-scale emulsion PCR according to the supplier’s instructions. Two libraries

obtained for each iron condition were sequenced by a SOLiD 4 sequencer (Life Technologies), generating a total of four libraries. Deep sequencing was performed at Universidade Federal do Paraná (Curitiba, PR, Brazil).

2.3 Bioinformatics analysis

After removing the adaptors and trimming 15 bp off of the 3'-end, the sequences were obtained, compared and mapped to the *P. riograndensis* SBR5 genome database located on the Aramis platform (http://aramis.genopar.org/bacteria/PR/) using CLC Genome Workbench 5.5 software. Library normalization and analysis were performed using the RPKM method as described by Mortazavi *et al.* [17]. The cut-off values for designating a gene as differentially expressed included a change in transcript levels of at least 1.5-fold and an adjusted *P*-value of equal or less than 0.05. Sequences were classified according to the Pfam database (http://pfam.sanger.ac.uk/), and COG function. When appropriate, the BLASTP tool (http://blast.ncbi.nlm.nih.gov/) was used to infer gene function according to protein domain analysis (Table S1). Transcriptome sequence data have been submitted to the National Center for Biotechnology Information (NCBI) databank for SRA archiving (GEO accession number GSE66816).

2.4 Quantitative RT-PCR analysis

Genes most likely to play an important role in iron homeostasis were chosen to validate the RNA-seq data using quantitative RT-PCR analysis (RT-qPCR). Total RNA was extracted from bacterial cells using TRIzol reagent (Invitrogen). The samples were treated with DNAse I (Invitrogen). cDNA synthesis was performed with the M-MLV RT Kit (Invitrogen) in the presence of RNase OUT (Invitrogen) using Random Hexamer primers (Invitrogen). All of these procedures were performed according to the manufacturer's instructions. Primers (Table 1) for selected genes and for 16S rRNA, which was used as a control gene, were designed using OligoPerfect Designer (Life Technologies, available at http://tools.lifetechnologies.com/). All primers were designed to amplify 90-190 bp

fragments and to exhibit similar Tm values (60 ± 2°C). Primer specificity was determined by matching the primers with genome sequences and by the presence of unique peaks in the melting curves obtained by qPCR.

For RT-qPCR, the first strand of cDNA synthesized from each sample was diluted 50 times. Reaction conditions included an initial denaturation step of 5 min at 94°C followed by 40 cycles of 15 s at 94°C, 20 s at 60°C and 20 s at 72°C. Samples were held for 2 min at 60°C to allow annealing of the amplified products and then subsequently heated from 60 to 99°C with a ramp of 0.3°C/s to provide the denaturation curves of the amplified products. RT-qPCR reactions were carried out using a StepOne Plus Real-Time PCR System (Applied Biosystems) in a final volume of 10 μl containing 5 μl of each reverse transcribed sample (diluted 50 times), 1 μl of 10 X PCR buffer, 0.6 μl of 50 mM $MgCl_2$, 0.05 μl of 10 mM dNTPs, 0.4 μl of 10 μM primer pairs, 2.125 μl of water, 1.0 μl of SYBR green (1:10,000, Molecular Probes), and 0.025 μl of Platinum Taq DNA Polymerase (5 U μl^{-1}, Invitrogen). Gene expression was evaluated using a modified $2^{-\Delta CT}$ method [18]. The equation $Q0_{target\ gene}/Q0_{16S} = [(Eff_{16S})Ct_{16S}\ /\ (Eff_{target\ gene})\ Ct_{target\ gene}$, where Q0 corresponds to the initial amount of transcripts, was used for normalization. PCR efficiency was calculated for each individual amplification plot using LinRegPCR software [19]. Means were compared by Tukey HSD test. Each data point corresponds to three true biological replicate samples.

2.5 Spore visualization

Sufficient and deficient *P. riograndensis* SBR5 iron cultures were harvested after 48 h of incubation. Slides were stained using green malachite and safranin according to Schaeffer & Fulton [20]. Cells were visualized at Zeiss Axio Imager D2 microscope in an augmentation of 1,000 times.

3. Results

3.1 Growth curve analysis

P. riograndensis cultures subjected to sufficient or limiting iron conditions demonstrated a similar growth rate during the first 8 h of incubation (Fig. 1). However, between 8 and 12 h, the cultures subjected to iron-sufficient condition exhibited a higher growth rate when compared to those subjected to iron-deficient condition. Interestingly, cultures grown in iron-deficient medium showed a fast growth rate between 12 and 16 h of incubation and reached OD_{600} values similar to those of cultures subjected to iron-sufficient concentration. After this period (16 h), all cultures reached the stationary phase.

3.2 RNA-Seq analysis

A hundred and fifty differentially expressed genes were identified, including 79 under-expressed and 71 over-expressed genes (Fig. 2, Tables 2 and 3). Data concerning the coverage, numbers of unigenes and amount of reads for each library are presented in Table 4. In general, for both conditions (sufficient and limited), the average coverage was around two-fold with libraries S1 and S2 (representing sufficient iron condition) presenting higher values for the numbers of unigenes and the amount of reads than libraries L1 and L2 (representing limited iron condition). Genes related to several functions were differentially expressed: RNA processing and modification, energy production and conversion, cell cycle control and mitosis, amino acid metabolism and transport, nucleotide metabolism and transport, carbohydrate metabolism and transport, coenzyme metabolism; lipid metabolism, translation, transcription, replication and repair cell wall, membrane and envelop biogenesis, post-translational modification, protein turnover and chaperone functions, inorganic ion transport and metabolism, secondary structure, signal transduction; function unknown and signal transduction (Fig. 2).

3.3 RT-qPCR analysis and RNA-Seq validation

Eight genes in which expression was at least twice as high (induced) or twice as low (repressed) under iron-limiting condition relative to iron-sufficient condition were chosen for RT-qPCR analysis to validate the RNA-seq data. In general, after 24 h of *P. riograndensis* growth under iron-limiting condition most of the genes exhibited similar pattern of expression in the RT-qPCR and RNA-seq analyzes. The gene *0109.0003* likely encodes a major facilitator superfamily transporter (MFS) and exhibits considerably high expression 12 h after being subjected to iron limiting condition. The expression of this gene was similar for both cultures at 16 and 20 h. However, at 24 h, the expression of gene *0109.0003* in cultures subjected to iron-sufficient condition was higher than its expression in iron-deficient cultures (Fig. 3a). Similar expression patterns were found for both the *spoIIIAH* and *fecE* genes, although for the latter gene, the peak of over-expression under iron-limiting condition occurred at 16 h of growth relative to its growth under iron-sufficient condition (Fig. 3b and c). The *spoIIIAH* gene encodes the stage III sporulation protein AH, and the *fecE* gene encodes an iron chelating uptake ABC transporter. Each of these three genes exhibited a under-expressed pattern after 24 h of growth under iron-limiting condition as demonstrated by the RNA-seq analysis. y*utM*, which codes for an iron-cluster assembly accessory protein was only expressed in sufficient iron condition (Fig. 3d). Among those genes that were over-expressed after 24 h of growth under iron-limiting conditions, all four of the genes tested by RT-qPCR (*spoVT, 0116.0006, 0117.0004* and *0012.0015*) exhibited a similar pattern of expression that was statistically different from the control samples (Fig. 3e, f, g and h). According to the similarities between their predicted protein sequences and those sequences obtained from databanks, the *spoVT* gene codes for a stage V sporulation protein T and it is a positive and negative transcriptional regulator of sigma G-dependent genes. It may provide a mechanism of feedback control that is important for spore development [21]; gene *0116.0006* codes for a DNA-binding protein; gene *0117.0004* codes for a regulatory protein from the ArsR family; and gene *0012.0015*

codes for a WhiA N-terminal domain that regulates sporulation proteins in Gram-positive bacteria [22]. As observed for the under-expressed genes above, the genes over-expressed in the RT-qPCR analysis corroborated the results obtained by the RNA-seq experiment.

4. Discussion

As previously mentioned, iron is an essential micronutrient for all organisms, and its concentration in cells must be tightly controlled. In this work, we demonstrated that the *P. riograndensis* SBR5 strain was able to grow under iron-deficient condition. Because biochemical and physiological changes are necessary for an organism to resist iron deficiency and because these changes are coordinated by differential patterns of gene expression, we decided to investigate, via RNA-seq and RT-qPCR methodologies, which genes *P. riograndensis* expresses to overcome this situation.

The similar growth behavior of *P. riograndensis* under the two iron tested conditions was not a surprise, as a similar result was observed by Raza & Shen [16] for the *P. polymyxa* SQR-21 strain. Therefore, the SBR5 strain must have some resistance mechanism that allows it to proliferate under iron-limiting conditions. In the search for genes that might respond to SBR5 growth under iron-deficient condition, we observed high expression levels after 16 h of growth under iron-starvation condition for the *fecE* gene, which encodes an Fe^{+3}-siderophore transporter. However, even though Beneduzi *et al.* [5] reported siderophore production during the first SBR5 evaluation, genes related to siderophore synthesis were not found in its genome [6, 23]. Subsequent assays indicated that the *P. riograndensis* SBR5 strain is not able to produce siderophores (unpublished data). These data agree with the results obtained by our RNA-seq analysis. Because *fecE* was highly induced during the first 16 h of growth, the SBR5 strain must use siderophores produced by other species in the soil. Non-siderophore-producing strains belonging to the *Campylobacter* genus were demonstrated to utilize exogenous siderophores as an iron source [24]. This characteristic seems to be common in soil bacteria and was also demonstrated by Jurkevitch *et al.* [25] for several rhizospheric soil bacteria. However, this strategy has no advantage for the bacterium *in vitro* because there are no other species present to release siderophores. This observation could explain why SBR5 was not able to

remain in the exponential growth phase after 16 h and why *fecE* expression was significantly reduced after 24 h of growth despite *fecE* induction at the beginning of the growth curve.

According to the RT-qPCR data, gene *0109.0003* was highly induced by iron deficiency after 12 h of growth, indicating that the putative MFS transporter encoded by this gene may be substantially able to stimulate SBR5 growth. MFS transporter proteins are widely distributed and transport a variety of small molecules, such as sugar, drugs, organic and aromatic acids, nucleosides and organic or inorganic ions [26]. Pao *et al.* [26] also demonstrated that citrate is transported by MFS proteins, and Bhatt & Denny [27] demonstrated that a strain of *Ralstonia solanacearum* uses citrate as an iron chelator. This may be a useful route for the acquisition of iron by SBR5 under iron-limiting conditions. More studies are required to define the molecules that are transported by the product of the *0109.0003* gene and its role in iron deficiency maintenance in the SBR5 strain.

Gene *0117.0004* was also identified by RNA-seq analysis as a differentially expressed gene in the SBR5 strain under iron-limiting conditions. The predicted protein encoded by this gene has a domain similar to the ArsR family of negative regulators of gene transcription involved in metal efflux [28]. In addition, these proteins could take part in general stress responses. For example, Dahlsten *et al.* [29] recently suggested the involvement of *arsR* gene products in the cold tolerance mechanism of *Clostridium botulinum.* It is possible that the *0117.0004* gene product may act as a repressor of iron-efflux genes during stress caused by iron limitation.

Sporulation is a known resistance mechanism for several abiotic stressors, including carbon, nitrogen and phosphorus starvation [14]. In *Clostridium acetobutylicum*, cells compromised by sporulation also induced the expression of iron transporter genes and genes related to energy production [30]. Additionally, SBR5 over-expressed several genes involved in energy metabolism (Table 3) because sporulation is a long and

extensive energy consuming process [13]. We observed that when SBR5 is grown under iron-deficient condition, its sporulation cycle began at approximately 12 h of growth and reached its final stages at approximately 24 h. *spoIIIAH* induction after 12 h of growth under iron-limiting condition demonstrated that SBR5 is already compromised for sporulation at the beginning of its growth; *spoVT* induction upon iron starvation after 24 h of growth indicates that the bacterium is in the final stages of sporulation. These results were corroborated by the finding that the sporulation factor gene *0012.0015* was highly induced after 24 h of growth under iron limiting condition. To our knowledge, this is the first report in the literature describing sporulation induction by iron deficiency.

While most metabolic activities in sporulating cells decrease, Rivas-Castillo *et al.* [34] suggested the importance of detecting, repairing and tolerating DNA damage to complete the sporulation process and to maintain DNA integrity. DNA maintenance is achieved mainly by DNA repair processes and is very active in sporulating cells [35]. However, these processes depend on DNA repair enzymes and several DNA metabolism enzymes that rely on Fe/S clusters, including those involved in DNA repair [9]. Under iron-limiting conditions, cells face obstacles to produce Fe/S cluster dependent proteins and are impaired for DNA repair. In our RNA-seq experiment, we observed several genes that were under-expressed after iron deprivation, including *0106.0002* and *0128.0002,* which encode products that are putatively involved in DNA replication, recombination and repair, and *yutM*, the product of which is putatively involved in Fe/S cluster assembly (Table 2), only had its expression detected in iron-sufficient condition (Fig. 3d). This result indicates that, under iron deprivation, SBR5 must have other genes expressing proteins involved in DNA repair. To overcome this stress, sustain DNA integrity and complete sporulation, SBR5 needs to adopt an alternative strategy. In this context, the *0116.0006* gene, which encodes a product exhibiting homology to the poorly characterized YbaB/EbfC family, was also identified by the RNA-seq analysis. The YbaB/EbfC family is widely distributed among

bacteria, and its members are able to bind DNA [36]. Jutras *et al.* [37] observed that EbfC/YbaB is a histone-like protein that binds to DNA throughout the genome, and Wang *et al.* [38] demonstrated that a protein belonging to this family is involved in DNA damage protection in *Deinococcus radiodurans*. Therefore, we propose that the *0116.0006* gene product performs a crucial role in protecting DNA because iron is not an available source to generate other proteins related to DNA repair under this adverse condition.

In conclusion, the combination of RNA-seq and RT-qPCR methodologies was a successful strategy allowing for the identification of several genes whose expression was affected by iron starvation, most of which are yet uncharacterized. The methodologies also exhibited good reproducibility because similar results were obtained through independent experiments. Our most important finding was the discovery that the *P. riograndensis* SBR5 strain uses sporulation as a mechanism to survive iron deficiency. Up to now, it was thought that bacteria first acquired all nutrients necessary to grow and, when nutrients became scarce, bacteria starts the sporulation cycle [12, 14]. Importantly, our results suggest that bacteria senses iron deficiency from the beginning and start to express genes related to nutrient uptake and genes related to the initial steps of sporulation. Once iron becomes highly limiting, the bacteria start to express genes related to the final steps of sporulation process in anticipation of spore formation as a way to resist to this stress. To corroborate this conclusion we observed a higher number of spores in SBR5 cultures submitted to iron deficiency when compared with cultures submitted to iron sufficiency (Supplementary Fig. 1).

5. Conflict of interest

No conflict of interest declared.

6. Ackowledgements

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8. Legends to figures

Fig. 1. Growth curves of *P. riograndensis* grown in sufficient-iron medium (SIM) or limited-iron medium (LIM). Each point represents three biological replicates ± standard error.

Fig. 2. Functional classes distribution of genes differentially expressed between low- and sufficient-iron conditions according to COG classification (A: RNA processing and modification; C: Energy production and conversion; D: Cell cycle control and mitosis; E: Amino acid metabolism and transport; F: Nucleotide metabolism and transport; G: Carbohydrate metabolism and transport; H: Coenzyme metabolism; I: Lipid metabolism; J: Translation; K: Transcription; L: Replication and repair; M: Cell wall/membrane/envelop biogenesis; O: Post-translational modification, protein turnover, chaperone functions; P: Inorganic ion transport and metabolism; Q: Secondary structure; R: General function prediction only; S: Function unknown; T: Signal transduction).

Fig. 3. Differential gene expression levels of *P. riograndensis* grown in sufficient-iron medium (SIM – control) or in limiting-iron medium (LIM – treatment) obtained from samples collected at 12, 16, 20 and 24 hours. (a) *0109.0003*, (b) *spoIIIAH*, (c) *fecE*, (d) *yutM* (e) *spoVT*, (f) *0116.0006*, (g) *0117.0004*, (h) *0012.0015,*. Values are the averages of three samples ± standard error. Statistical differences were assessed by Tukey test and differences in means are represented by different letters.

Table 1. Primers used for RT-qPCR experiments.

Gene	Primer sequences (5' – 3')	Product lenght bp
0117.0004	For: TACCAGCTATGCCTGTGACG Rev: GCGGAGAATGTAGAGCCTTG	109
0116.0006	For: CAAAAAGCTGCTCTCGATCC Rev: CATTATTCGCCAGCTCTTCC	128
fecE	For: CAAGGTATCCCTGGCTGAAA Rev: CACGGTGAAGTCAAAGCTCA	109
0109.0003	For: TTCTCTTCCTGCTTGGTGCT Rev: ATCGATTGCAGCGAGAAAGT	112
spoIIIAH	For: AGCCGCAACAGAAGACAAGT Rev: TTTCTGCTGTAACCGCATTG	98
spoVT	For: TGATCACCGACAGAGACAGC Rev: CTTGCGGTTTTCCATGACTT	107
0012.0015	For: GCCAACCTCAACAAGACCAT Rev: ATATCCGGGTGTGCCAGT	139
yutM	For: ATATGTTCCTTCGTCTTGGTGTAAC Rev: GATATCATCTTTGCTAACGACAACC	140
16S rRNA	For: CACGTGTAGCGGTGAAATGC Rev: ACTTCGGCACCAAGGGTATC	184

Table 2. Genes under-expressed in limited-iron conditions relative to sufficient-iron conditions. Bold font indicates genes chosen for RT-qPCR analysis.

Identity	*P-value*	Fold change	Function
perR	0.0001	-1.6639	Transcription regulator
0100.0005	0.0001	-70.3358	Hypothetical protein
sigY	0.0015	-7.8203	RNA polimerase sigma factor
0100.0018	0.0018	-4.1206	Thioredoxin fold
0101.0011	0.0037	-7.5703	Hypothetical protein
0103.0005	0.0061	-3.2429	Peptidase
0103.0007	0.0063	-6.9899	Hypothetical protein
amyC	0.0067	-5.4013	Sugar ABC transporter
0105.0005	0.0068	-2.0089	ABC-type sugar transport system
0105.0009	0.0071	-3.0521	NAD dependent epimerase
0106.0002	0.0084	-4.9283	Hypothetical protein
fecE	**0.0091**	**-8.3816**	**Iron chelate uptake ABC transporter**
0109.0003	**0.0098**	**-2.5281**	**Major Facilitator Superfamily**
mrdA	0.0104	-2.9090	Cell wall/membrane/envelope biogenesis
yutJ	0.0137	-3.0695	Pyridine nucleotide-disulphide oxidoreductase
thiH	0.0140	-2.5822	Thiamine biosynthesis enzyme
yutM	**0.0140**	**-2.2160**	**Iron-sulfur cluster assembly accessory protein**
0109.0024	0.0145	-3.7598	Methyltransferase domain
yumC	0.0155	-2.5826	Pyridine nucleotide-disulphide oxidoreductase
yqfD	0.0170	-9.1537	Putative stage IV sporulation protein YqfD
phoH	0.0171	-2.9338	PhoH family protein
dnaG	0.0184	-3.1443	DNA primase
sigA	0.0184	-1.7161	RNA polimerase sigma factor rpoD
0112.0029	0.0201	-1.6730	Subtilase family
0115.0006	0.0212	-2.2084	Sulfatase
0117.0012	0.0255	-1.9014	Bacterial extracellular solute-binding protein
0117.0014	0.0272	-1.6943	Binding-protein-dependent transport system inner membrane component
0119.0006	0.0287	-18.4656	Acetyltransferase family
0011.0005	0.0304	-4.0544	Carbon-nitrogen hydrolase
0124.0003	0.0308	-2.3110	Sugar ABC transporter
0124.0022	0.0338	-2.5259	Histidine kinase
0126.0005	0.0356	-2.7955	Glyoxalase-like domain
0126.0012	0.0374	-2.2602	Binding-protein-dependent transport system inner membrane component
0126.0021	0.0395	-2.7623	Response regulator receiver domain
0126.0022	0.0395	-21.2184	Glycosyl hydrolase family
0128.0001	0.0399	-4.5183	Glycosyl hydrolase family
0128.0003	0.0401	-2.2555	Histidine kinase
ald	0.0409	-9.1654	Alanine dehydrogenase
0128.0012	0.0416	-2.0584	Cadherin-like beta sandwich domain
0128.0018	0.0419	-2.3212	Hypothetical protein
0128.0019	0.0420	-2.0793	Glycosyl transferase family involved in cell wall biogenesis
0128.0002	0.0425	-1.7620	Probable transposase
0128.0023	0.0426	-1.9081	SWIM zinc finger
0128.0028	0.0434	-4.7455	Binding-protein-dependent transport system inner membrane component

argE	0.0446	-2.9875	Peptidase family
0012.0019	0.0492	-2.1898	Hemerythrin cation binding domain
0135.0003	0.0514	-2.6413	FtsX-like permease family
ygjM	0.0516	-3.3653	NADH oxidase family
spoIIIAA	0.0525	-1.6476	Stage III sporulation protein AA
spoIIIAD	0.0527	-2.0777	Stage III sporulation protein AD
spoIIIAH	**0.0535**	**-2.6810**	**SpoIIIAH-like protein**
0143.0002	0.0584	-1.6068	Prenyltransferase-like
0145.0001	0.0594	-2.4390	ABC transporter substrate binding protein
mrsF	0.0637	0.0000	ABC transporter substrate binding protein
0147.0029	0.0640	-1.6456	Hypothetical protein
queA	0.0644	-2.9300	Queuosine biosynthesis protein
0153.0015	0.0764	-2.1299	Beta-glucosidase
0153.0029	0.0798	-5.9647	PA14 domain
0153.0003	0.0799	-2.7196	Endo-beta-glucanase
0153.0031	0.0801	-1.5387	Nucleotidyl transferase
0153.0033	0.0805	-3.2368	UDP-glucose 6-dehydrogenase
0153.0037	0.0812	-3.6877	Glycosyl transferase family 2
0153.0038	0.0813	-2.2657	Polysaccharide biosynthesis protein
0153.0043	0.0817	-5.0030	Priming glycosyltransferase
gtaB	0.0818	-2.0911	Glycosyl hydrolase family
0015.3005	0.0835	-1.6551	Polysaccharide deacetylase family sporulation protein PdaB
0153.0051	0.0837	-4.3029	Glycoside hydrolase
0156.0003	0.0851	-2.8538	Binding-protein-dependent transport system
0156.0004	0.0853	-5.3799	Binding-protein-dependent transport system
0157.0015	0.0878	-2.4004	PTS beta-glucoside transporter subunit IIABC
licT	0.0879	-3.1253	Transcriptional antiterminator
uppP	0.0890	-2.0472	UDP pyrophosphate phosphatase
0157.0034	0.0902	-3.0449	ABC transporter
0162.0006	0.0936	-7.7978	Unknown function
0162.0009	0.0942	-2.9824	Serine dehydratase subunit alpha
0164.0005	0.0952	-1.8008	ThiF family
0164.0013	0.0958	-1.7121	50S ribosome-binding GTPase
0164.0015	0.0967	-8.1222	Methyltransferase small domain
0164.0017	0.0973	-2.772	Cell cycle protein

Table 3. Genes over-expressed in limited-iron conditions relative to sufficient-iron conditions. Bold font indicates genes chosen for RT-qPCR analysis.

Identity	*P-value*	Fold change	Function
0105.0001	0.0076	7.5390	Hypothetical protein
yqxD	0.0184	3.9810	Hypothetical protein
0137.0018	0.0546	12.3600	Hypothetical protein
0112.0004	0.0161	2.1930	Yqey-like protein
yqfG	0.0173	3.4060	16S rRNA maturation RNase YbeY
0115.0014	0.0223	4.9590	Nucleoside-diphosphate sugar epimerase
0011.0002	0.0296	4.0310	Hypothetical protein
0012.0015	**0.0478**	**2.8730**	**WhiA N-terminal LAGLIDADG-like domain**
0124.0006	0.0310	10.7650	Hypothetical protein
0112.0027	0.0195	2.5380	Bacterial regulatory proteins gntR family
0116.0006	**0.0231**	**44.4700**	**YbaB/EbfC DNA-binding family**
0117.0004	**0.0238**	**2.6140**	**Bacterial regulatory protein ArsR family**
0135.0006	0.0517	4.1020	MarR family
spoIIIAB	0.0525	2.8560	Stage III sporulation protein AB
spoVT	**0.0575**	**6.1450**	**Stage V sporulation protein T**
0101.0004	0.0030	2.1730	Sugar ABC transporter permease
0115.0008	0.0212	6.9660	RNA polymerase. sigma-24 subunit. ECF subfamily
asnB	0.0052	2.3020	Asparagine synthase
rpmE	0.0228	2.0640	Ribosomal protein L31
rph	0.0278	5.9430	3' exoribonuclease family
pgpA	0.0280	2.2190	Phosphatidylglycerophosphatase A
tpiA	0.0466	1.8720	Triosephosphate isomerase
ycsK	0.0472	2.2760	GDSL-like Lipase/Acylhydrolase family
0012.0017	0.0489	2.3170	ATPase protein family
0012.0023	0.0500	4.2090	Tetratricopeptide repeat
0012.0025	0.0507	2.0610	Histidinol phosphatase
0128.0032	0.0445	1.6280	Enoyl-(Acyl carrier protein) reductase
prs	0.0505	2.4720	N-terminal domain of ribose phosphate pyrophosphokinase
hisF	0.0508	13.7870	Histidine biosynthesis protein
hisA	0.0509	2.7350	Histidine biosynthesis protein
lysC	0.0522	2.0900	Aspartate kinase
0147.0011	0.0620	3.9918	Sulfate transporter
0147.0012	0.0621	1.8244	6-phosphogluconolactonase
0147.0013	0.0622	4.5274	6-phosphogluconolactonase
0147.0014	0.0624	14.3301	Membrane protein
yhaQ	0.0640	1.5876	Cell wall surface anchor family protein
bofC	0.0658	3.2254	Forespore regulator of the sigma-K checkpoint
0149.0017	0.0668	8.2219	Hypothetical protein
0149.0036	0.0695	2.0524	Hypothetical protein
0149.0037	0.0696	3.0613	LysR family transcriptional regulator
0151.0001	0.0699	4.4659	Membrane protein
0151.0002	0.0700	3.6876	Permease membrane protein TerC
hesA	0.0726	2.0254	Protein HesA, molibdopetrin biosynthesis
0153.0004	0.0732	2.7535	dGTP triphosphohydrolase

0153.0011	0.0748	2.0570	Molibdate ABC transporter
0153.0012	0.0773	4.5486	Hypothetical protein
0153.0013	0.0779	2.8964	Ferritin-like domain
0153.0014	0.0806	2.3598	Glicolsyl-transferase
0153.0015	0.0813	3.2344	Hypothetical protein
0153.0016	0.0815	4.1395	Acetyl-mannosamine transferase
0153.0017	0.0817	3.6303	Glicosyl-transferase
0153.0018	0.0822	2.1432	Capsular polysaccharide biosynthesis protein
0153.0019	0.0838	6.9436	Endonuclease superfamily
0153.0020	0.0839	2.9223	Putative ATPase subunit of terminase (gpP-like)
0153.0021	0.0842	1.8535	ABC transporter
0153.0022	0.0846	2.9067	LacI family transcriptional regulator
0153.0023	0.0857	2.3781	Bacterial extracellular solute-binding protein
0153.0024	0.0859	3.2466	Glycosyl hydrolases family 32 N-terminal domain
0153.0025	0.0869	8.9209	Radical SAM superfamily
0153.0026	0.0878	1.6001	Beta-glucosidase
0153.0027	0.0881	3.0226	Putative ABC transporter ATP-binding protein YknV
0153.0028	0.0908	1.5232	DNA-binding response regulator
0153.0029	0.0915	1.8571	Fe-S cluster assembly
0153.0030	0.0918	2.3039	Cysteine desulfurase
0153.0031	0.0933	7.0404	Short chain dehydrogenase
0153.0032	0.0940	2.2833	Serine dehydratase beta chain
0153.0033	0.0954	1.5821	Bacterial regulatory proteins, tetR family
0153.0034	0.0955	1.8856	Phospholipase
0153.0035	0.0968	1.7698	Swarming motility protein SwrC
0153.0036	0.0987	1.5558	Pterin binding enzyme
0153.0037	0.0990	2.4303	7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase
0153.0038	0.0991	1.8567	Dihydrouridine synthase

Table 4. RNA-seq experiment quality data of libraries. S1 (sufficient iron condition 1), S2 (sufficient iron condition 2), L1 (limited iron condition 1) and L2 (limited iron condition 2).

Library	Coverage	Number of Unigenes	Number of Reads
S1	1.66	311,425	10,029,718
S2	2.59	521,788	8,648,538
L1	2.35	408,985	9,273,447
L2	1.66	298,135	8,648,538

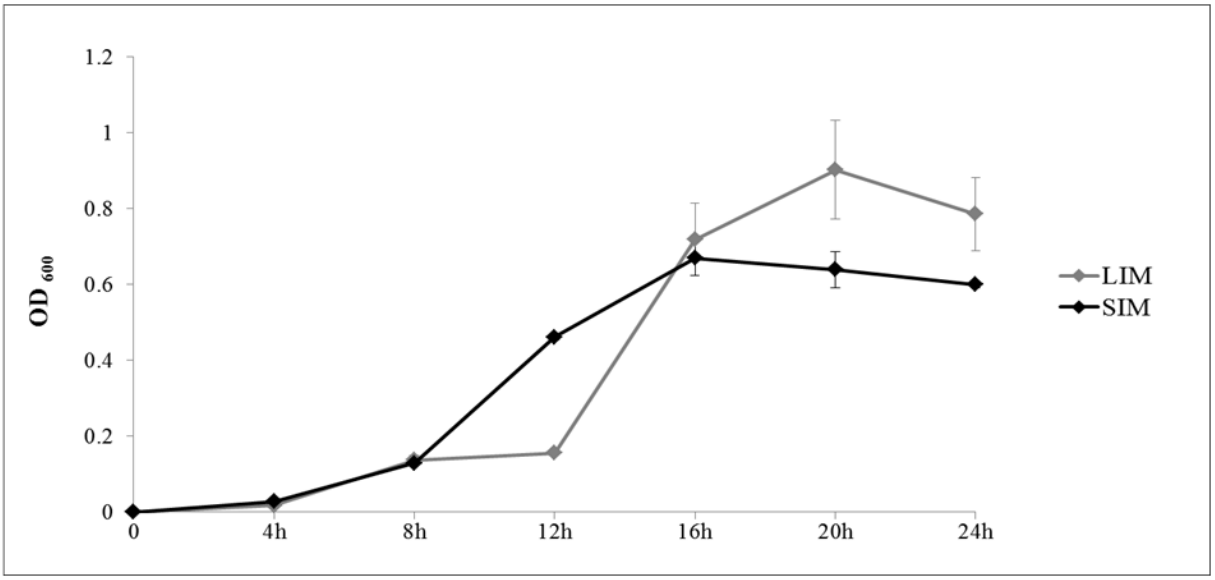

Fig. 1

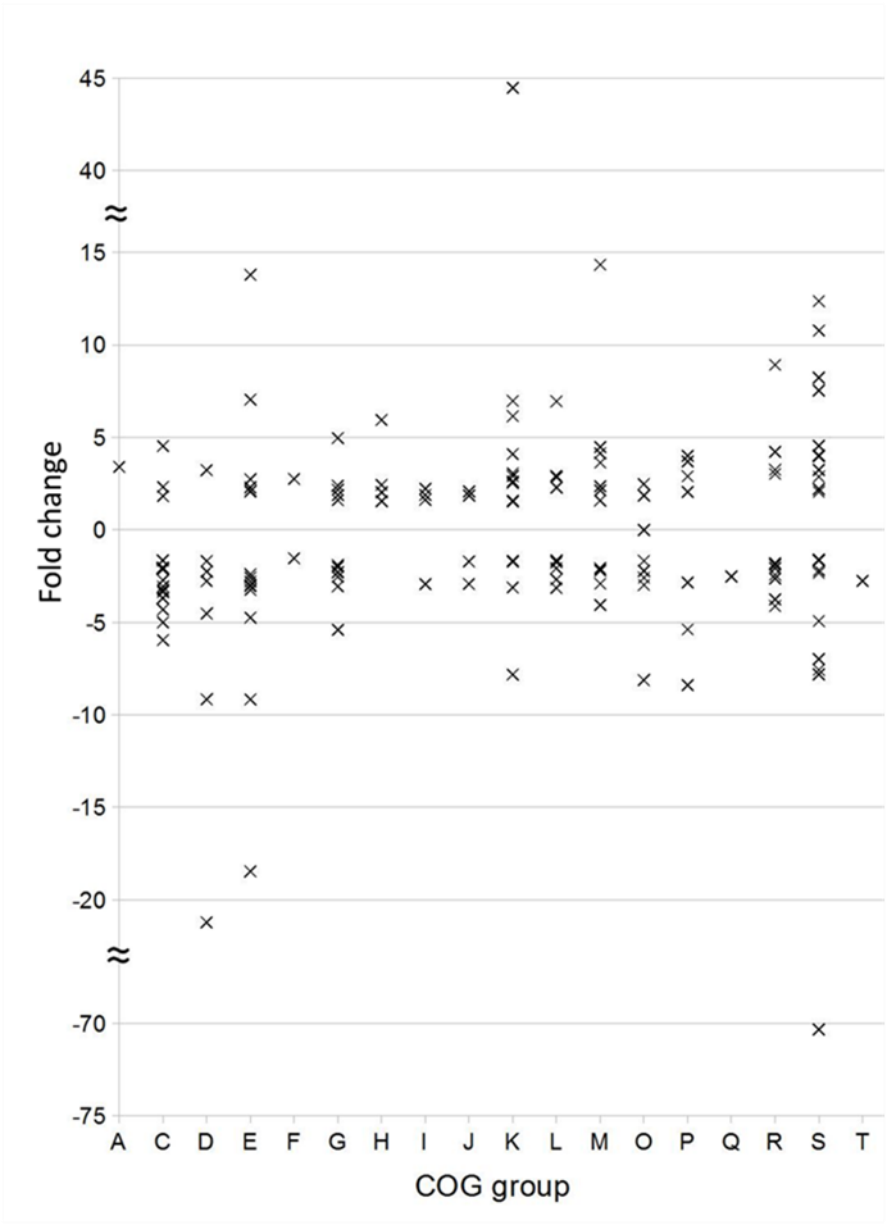
45
40
≈
15
10
5
0
-5
-10
-15
-20
≈
-70
-75
Fold change
A C D E F G H I J K L M O P Q R S T
COG group

Fig. 2

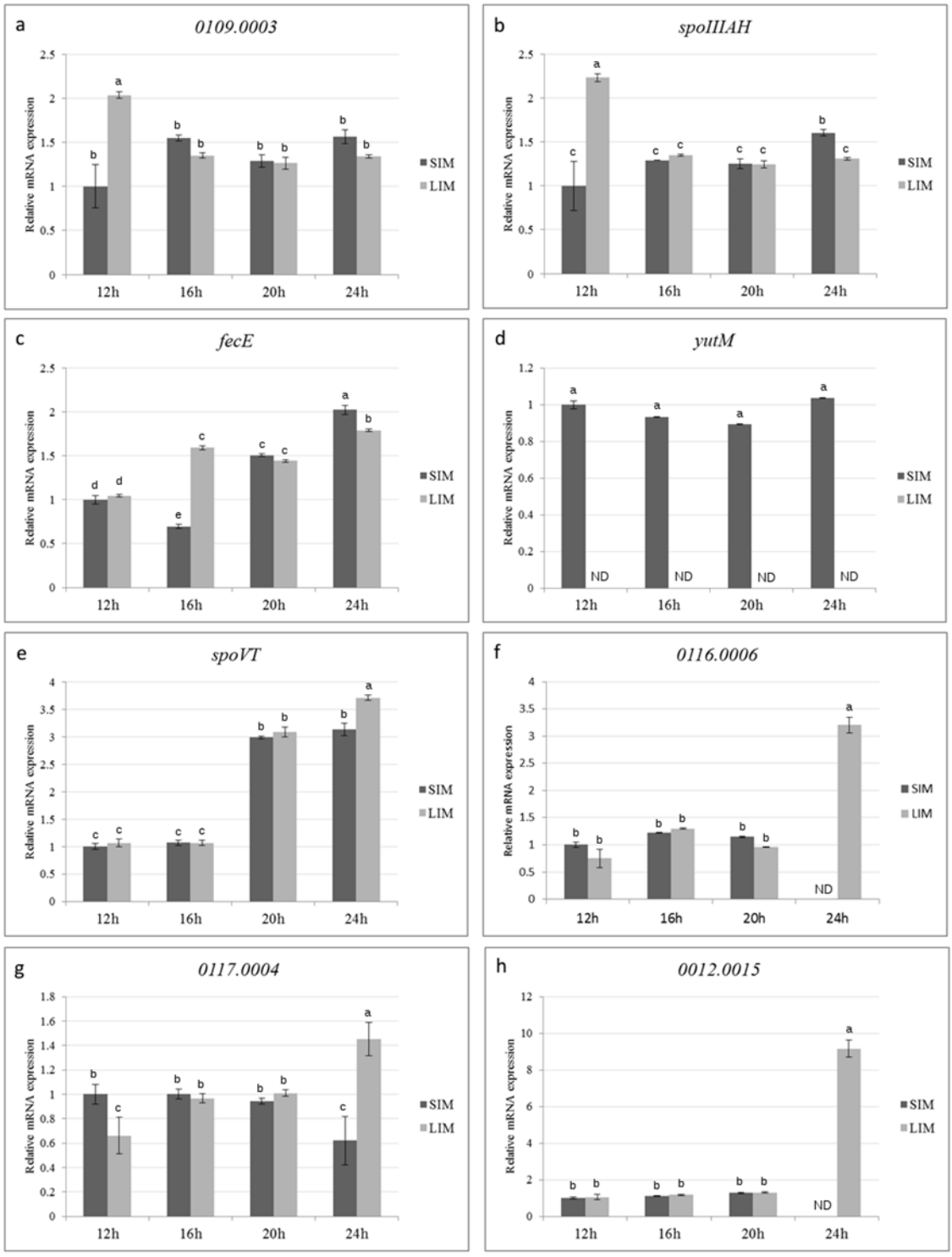
a
0109.0003
b
spoIIIAH
c
fecE
d
yutM
e
spoVT
f
0116.0006
g
0117.0004
h
0012.0015
Relative mRNA expression
SIM
LIM
ND
12h
16h
20h
24h

Fig. 3

Supplementary material for on-line submission

Table S1: PFAM number, BLAST accession and COG group (A: RNA processing and modification; C: Energy production and conversion; D: Cell cycle control and mitosis; E: Amino acid metabolism and transport; F: Nucleotide metabolism and transport; G: Carbohydrate metabolism and transport; H: Coenzyme metabolism; I: Lipid metabolism; J: Translation; K: Transcription; L: Replication and repair; M: Cell wall/membrane/envelop biogenesis; O: Post-translational modification, protein turnover, chaperone functions; P: Inorganic ion transport and metabolism; Q: Secondary structure; R: General function prediction only; S: Function unknown; T: Signal transduction) of down-expressed genes grown in limited iron condition related to sufficient iron condition. *: No matches were found in PFAM database. **: No matches were found in NCBI database.

Identity	PFAM number	BLAST accession	COG group
perR	PF01475	WP020426262.1	K
0100.0005	PF13345	CQR58242.1	S
sigY	PF04542	WP039785613.1	K
0100.0018	PF14595	WP046505765.1	R
0101.0011	*	CQR58260.1	S
0103.0005	PF01145	WP020426291.1	E
0103.0007	*	LN831776.1	S
amyC	PF00528	WP020425880.1	G
0105.0005	PF13416	WP020426315.1	G
0105.0009	PF01370	WP020426318.1	G
0106.0002	*	WP020426322.1	S
fecE	PF00005	WP020426326.1	P
0109.0003	PF07690	WP046505657.1	G
mrdA	PF03717	WP020426339.1	M
yutJ	PF07992	WP039785694.1	C
thiH	PF04055	WP020426354.1	E
yutM	PF01521	WP020426355.1	O
0109.0024	PF12847	WP039785697.1	R
yumC	PF07992	CQR58028.1	O
yqfD	PF06898	WP039836979.1	D
phoH	PF02562	WP020426374.1	I
dnaG	PF08275	WP039785724.1	L
sigA	PF03979	WP020426386.1	K
0112.0029	PF00082	WP039836993.1	O
0115.0006	PF00884	WP046505616.1	M
0117.0012	PF13416	WP020426427.1	G
0117.0014	PF00528	WP020426429.1	D
0119.0006	PF00583	WP020425578.1	E
0011.0005	PF00795	WP020425579.1	M
0124.0003	PF00005	WP020426445.1	G
0124.0022	PF07730	WP020426445.1	Q
0126.0005	PF12681	WP020426472.1	E
0126.0012	PF00528	WP020426479.1	D
0126.0021	PF00072	CQR51525.1	T
0126.0022	PF00072	CQR51526.1	D

0128.0001	PF00933	WP020426493.1	D
0128.0003	PF02518	WP020426496.1	D
ald	PF05222	WP020426501.1	E
0128.0012	PF12733	**	R
0128.0018	*	WP020426508.1	S
0128.0019	PF00535	CQR51601.1	M
0128.0002	PF01385	WP020426495.1	L
0128.0023	PF04434	WP020426513.1	R
0128.0028	PF00528	WP020426518.1	E
argE	PF01546	CQR51589.1	E
0012.0019	PF01814	WP020425598.1	S
0135.0003	PF02687	WP020426534.1	R
ygjM	PF00724	CQR57155.1	C
spoIIIAA	*	WP025705503.1	L
spoIIIAD	*	WP025703761.1	L
spoIIIAH	PF12685	WP020426554.1	L
0143.0002	PF13243	CQR52979.1	S
0145.0001	PF04392	CQR58828.1	R
mrsF	CL0023	LN831776.1	O
0147.0029	*	CQR518873.1	S
queA	*	WP 0240426616	J
0153.0015	CL0301	CQR53120.1	C
0153.0029	PF007691	WP0421185189	C
0153.0030	CL0058	**	C
0153.0031	CL0110	**	F
0153.0033	CL0063	**	C
0153.0037	CL0110	WP020426693.1	C
0153.0038	CL0222	**	G
0153.0043	CL0222	**	C
gtaB	CL0222	**	C
0015.3005	CL0158	WP039833961.1	C
0153.0051	CL0546	**	C
0156.0003	CL0404	**	P
0156.0004	CL0404	**	P
0157.0015	CL0105	WP020426735.1	E
licT	CL0166	WP020426736.1	K
uppP	*	WP020426532.1	C
0157.0034	CL0023	WP025691279.1	E
0162.0006	DUF4269	**	S
0162.0009	*	WP_020426779.1	O
0164.0005	*	CL0063	R
0164.0013	CL0023	WP_025704026.1	J
0164.0015	CL0063	WP_046507702.1	O
0164.0017	CL0142	**	D

Table S2: PFAM number, BLAST accession and COG group (A: RNA processing and modification; C: Energy production and conversion; D: Cell cycle control and mitosis; E: Amino acid metabolism and transport; F: Nucleotide metabolism and transport; G: Carbohydrate metabolism and transport; H: Coenzyme metabolism; I: Lipid metabolism; J: Translation; K: Transcription; L: Replication and repair; M: Cell wall/membrane/envelop biogenesis; O: Post-translational modification, protein turnover, chaperone functions; P: Inorganic ion transport and metabolism; Q: Secondary structure; R: General function prediction only; S: Function unknown; T: Signal transduction) of over-expressed genes grown in limited iron condition related to sufficient iron condition. *: No matches were found in PFAM database. **: No matches were found in NCBI database.

Identity	PFAM number	BLAST accession	COG group
0105.0001	*	WP020426312.1	S
yqxD	PF02639	WP020426384.1	S
0137.0018	*	WP020426560.1	S
0112.0004	PF09424	WP020426560.1	S
yqfG	PF02130	WP020426376.1	A
0115.0014	*	WP039785764.1	G
0011.0002	PF09148	WP020425576.1	S
0012.0015	PF10298	WP020425594.1	S
0124.0006	PF05336	WP020426448.1	S
0112.0027	PF00392	WP046505272.1	K
0116.0006	PF02575	WP039832492.1	K
0117.0004	PF01022	WP020426420.1	K
0135.0006	PF12802	WP039785930.1	K
spoIIIAB	PF09548	**	L
spoVT	*	WP020425614.1	K
0101.0004	PF00528	WP039785631.1	G
0115.0008	*	CQR57974.1	K
asnB	PF00733	WP039838269.1	E
rpmE	PF01197	WP020425948.1	J
rph	PF03725	WP020426432.1	H
pgpA	PF04608	WP039838101.1	I
tpiA	PF00121	WP020425585.1	G
ycsK	PF13472	WP020425590.1	E
0012.0017	PF03668	WP020425596.1	C
0012.0023	PF14559	WP020425602.1	R
0012.0025	PF02811	WP039833567.1	E
0128.0032	PF13561	WP020426522.1	I
prs	PF13793	WP020425603.1	O
hisF	PF00977	WP020425606.1	E
hisA	PF00977	WP020425608.1	E
lysC	PF00696	WP020426544.1	E
0147.0011	CL0186	CD01005	P
0147.0012	CL0186	WP_039838957.1	C
0147.0013	CL0186	**	C
0147.0014	*	WP_038585188.1	M
yhaQ	CCK17507.1	**	M

bofC	*	WP_039835903.1	D
0149.0017	*	WP_039835903.1	S
0149.0036	*	WP_020426647.1	S
0149.0037	*	WP_046505430.1	K
0151.0001	*	WP_039834959.1	M
0151.0002	CL0292	WP_039834959.1	P
hesA	*	WP_039834965.1	H
0153.0004	*	WP_042266168.1	F
0153.0011	PF00528	WP_039833949.1	P
0153.0019		WP_020426679.1	S
0153.0023	PF00210		P
0153.0034	PF00534	WP_020426690.1	M
0153.0039		WP_020426695.1	S
tagA	PF03808	WP_020426696.1	M
0153.0042	PF00535	CQR53100.1	M
0153.0046	PF02706	WP_039838875.1	M
0154.0001	PF13358	CQR55436.1	L
0154.0002	PF06056	WP_044648838.1	L
0155.0003	PF00664	WP_039786189.1	V
0156.0001	PF00356	WP_020426717.1	K
0156.0005	PF01547	WP_020426721.1	G
0156.0007	PF00251	**	R
0157.0005	PF04055	WP_046502291.1	R
0157.0014	PF00232	WP_039833798.1	G
yknV	PF00664	CQR54609.1	R
0157.0035	PF00072	WP_020426753.1	K
sufB	*	WP_020426758.1	O
sufS	*	WP_020426760.1	E
0162.0005	PF00106	WP_020426775.1	E
sdaAB	PF03315	WP_039834356.1	L
0164.0007	PF00440	**	K
0164.0008	PF13396	WP_020426788.1	I
0164.0016	PF00873	WP_020426786.1	V
0166.0002	PF00809	**	H
0166.0004	PF01288	**	H
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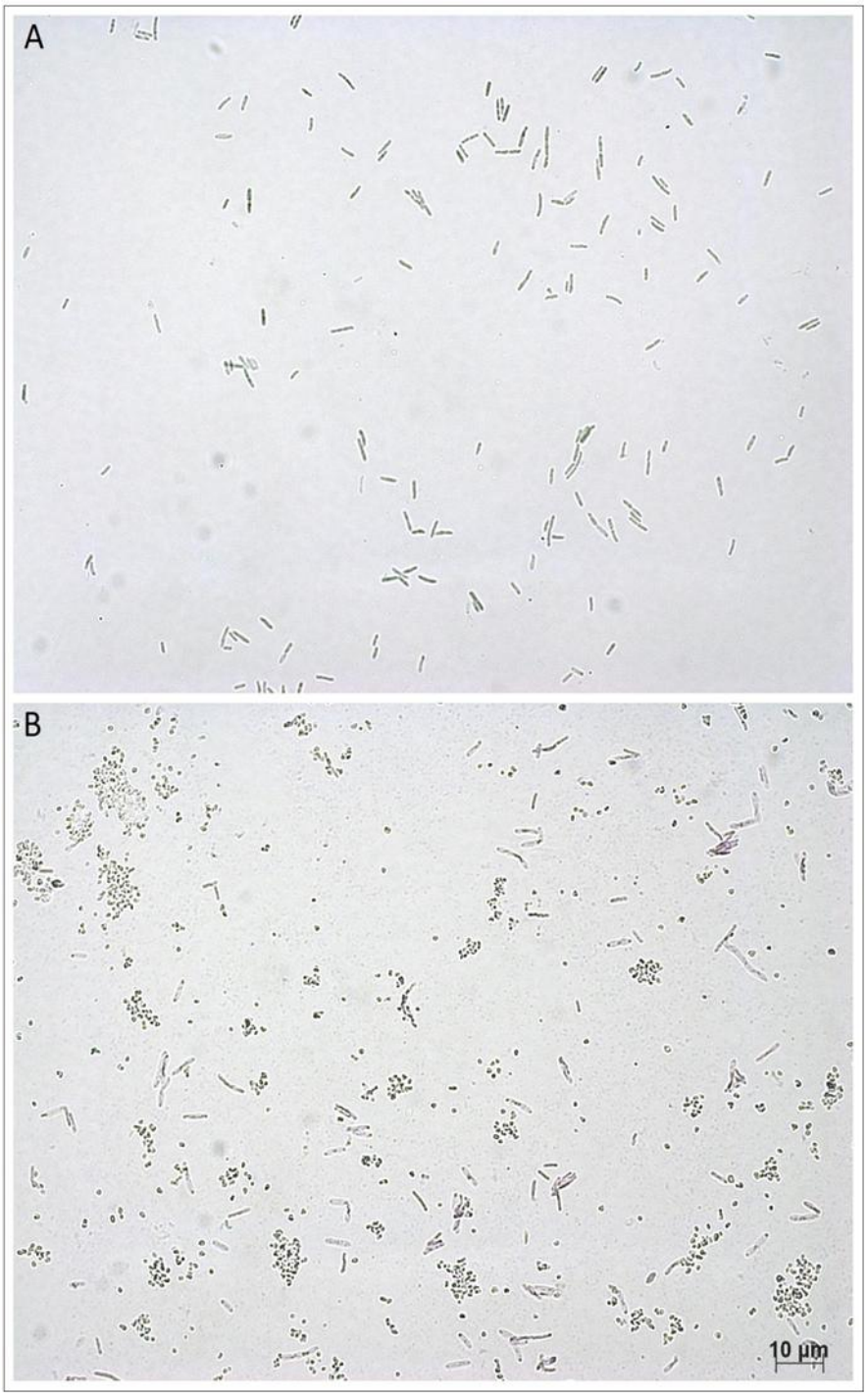

Fig. S1. Micrographic picture of SBR5 grown in sufficient iron condition (A) and limited iron condition (B) during 24 hours.

5. Considerações finais

A técnica de sequenciamento de RNA tem sido amplamente utilizada nos dias de hoje para avaliar a expressão diferencial de genes em determinadas condições. Porém, há pouquíssimos trabalhos empregando PCR quantitativa em tempo real (RT-qPCR) para validação de dados de RNA-seq. A grande maioria dos trabalhos já publicados utiliza a técnica de RNA-seq para validar dados obtidos com microarranjos. Neste trabalho nós demonstramos que RT-qPCR, além de ser adequada para a validação dos dados obtidos com RNA-seq, possibilita a expansão da análise de genes de interesse, conforme demonstramos na análise da expressão de determinados genes ao longo de intervalos de tempo. RNA-seq e RT-qPCR podem, juntas, avaliar a expressão de genes em organismos para os quais não há microarranjos disponíveis, como é o caso de *P. riograndensis*, uma espécie recentemente descrita.

O presente trabalho também demonstrou, pela primeira vez, uma associação entre o estresse causado por deficiência de ferro e a expressão de genes ligados à esporulação bacteriana. Para verificar se *P. riograndensis* realmente está esporulando em maior quantidade quando submetido à deficiência de ferro, serão empregadas técnicas de microscopia óptica e eletrônica, com o objetivo de contabilizar os esporos presentes em cada condição.

Vários genes relacionados à captação e ao metabolismo de ferro, cujas funções ainda não foram descritas, foram identificados nesse trabalho, abrindo novas possibilidades de estudo desses sistemas em *Paenibacillus*.

As sequências obtidas nesse trabalho já estão disponíveis arquivo SRA (Sequence Read Achive) do banco de dados do NCBI (National Center for Biotechnology Information) com o acesso GSE66816.

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