RNA-seq transcriptional profiling of *Herbaspirillum seropedicae* colonizing wheat (*Triticum aestivum*) roots

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Abstract *Herbaspirillum seropedicae* is a diazotrophic and endophytic bacterium that associates with economically important grasses promoting plant growth and increasing productivity. To identify genes related to bacterial ability to colonize plants, wheat seedlings growing hydroponically in Hoagland's medium were inoculated with *H. seropedicae* and incubated for 3 days. Total mRNA from the bacteria present in the root surface and in the plant medium were purified, depleted from rRNA and used for RNA-seq profiling. RT-qPCR analyses were conducted to confirm regulation of selected genes. Comparison of RNA profile of root attached and planktonic bacteria revealed extensive metabolic adaptations to the epiphytic life style. These adaptations include expression of specific adhesins and cell wall re-modeling to attach to the root. Additionally, the metabolism was adapted to the microoxic environment and nitrogen-fixation genes were

expressed. Polyhydroxybutyrate (PHB) synthesis was activated, and PHB granules were stored as observed by microscopy. Genes related to plant growth promotion, such as auxin production were expressed. Many ABC transporter genes were regulated in the bacteria attached to the roots. The results provide new insights into the adaptation of *H. seropedicae* to the interaction with the plant.

Keywords Biological nitrogen fixation · *H. seropedicae* · RNA-seq profiling · Rhizosphere · PGPB-plant growth promoting bacteria

Introduction

Endophytic bacteria are able to colonize plant internal tissues without injuring the host. Among these bacteria, the nitrogen fixers are considered promising biotechnological tools to improve agricultural productivity through biological nitrogen fixation (BNF) and plant growth promotion (Beatty and Good 2011; Geddes et al. 2015; Santi et al. 2013). *Herbaspirillum seropedicae* is a useful model to study such a group of bacteria, since this organism is easily cultivated, has its genome sequenced (Pedrosa et al. 2011) and genetic manipulation techniques were developed. *H. seropedicae* is a diazotrophic and endophytic *beta-proteobacterium* found colonizing the rhizoplane and internal tissues of crops such as wheat, sugarcane, rice and maize [for a review see (Monteiro et al. 2012)]. *H. seropedicae* was also found colonizing diverse plants such as banana and pineapple (Magalhães Cruz et al. 2001). Under laboratory conditions *H. seropedicae* was able to colonize *P. vulgaris* seedlings (Schmidt et al. 2011) and micropropagated sugarcane (Muthukumarasamy et al. 2006). The inoculation of micropropagated sugarcane with *Herbaspirillum spp.*

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resulted in 17 % increase of the rhizome presumably due to BNF (Oliveira et al. 2002). Other studies demonstrated that inoculation of aluminum tolerant rice varieties with *H. seropedicae* Z67 increased root and shoot dry weights by 38–54 % and 22–50 %, respectively. Moreover, these increases were supported by significant accumulation in total C (35–50 % for roots and 13–35 % for shoots) and total N (29–61 % for roots, 37–85 % for shoots) (Gyaneshwar et al. 2002). Convincing data of *H. seropedicae* colonizing rice plants showed that inoculated plants had an increase in total dry weight and accumulated N content by approximately 30 % (James et al. 2002). Moreover, inoculated plants exposed to an atmosphere enriched with $^{15}\text{N}_2$ for 3 days incorporated significantly more ^{15}N than control plants (James et al. 2002).

Wheat is one of the main sources of calories and proteins of the human diet. Increase in wheat productivity and protein content depends on improved absorption of inorganic nitrogen, thus this crop may benefit from endophytic nitrogen-fixing bacteria (Saubidet and Barneix 1998). Assimilation of nitrogen derived from BNF was demonstrated in wheat inoculated with *Azospirillum brasilense* Sp7 (Rennie et al. 1983), and an increase in grain yield of *T. aestivum* inoculated with *Azospirillum lipoferum* Sp108 was observed under field and greenhouse conditions (Mertens and Hess 1984). *H. seropedicae* was also found to colonize endophytically and epiphytically wheat (el-Komy et al. 2003), and inoculation with a *nifH::gusA* strain showed that *nif* genes are expressed in both bacterial cell populations (Roncato-Maccari et al. 2003). Recently, the performance of different wheat genotypes inoculated with *H. seropedicae* SmR1 were tested in greenhouse experiments; positive, genotype-specific responses were observed, such as increased biomass in the early stages of the culture and in grain yield (Neiverth et al. 2014).

The process of colonizing a plant host by *H. seropedicae* begins with the attachment of the bacteria to the root surface, followed by entry at the sites of emergence of lateral roots and injuries, and spread through intercellular spaces with later occupation of the xylem (revised by Monteiro et al. 2012). Although many authors have shown that *H. seropedicae* colonizes graminaceous plants (Elbeltagy et al. 2001; James et al. 2002; Roncato-Maccari et al. 2003), much of these observations were made at a microscopic level and little is known concerning the molecular factors that affect such interaction. Genome analyses indicated that *H. seropedicae* has several genes related to bacterial ability to colonize and promote plant growth, including: (a) *nif* genes; (b) type IV *pili* (T4P); (c) auxin biosynthesis (IAA production); (d) ACC deaminase gene; (e) genes for attachment to surfaces (hemagglutinins/

hemolysin) and biofilm formation (*eps* genes) (Pedrosa et al. 2011). In addition, other studies showed that lipopolysaccharides (LPS) are required for *H. seropedicae* attachment to maize root and internal colonization of plant tissues (Balsanelli et al. 2010) and that the flavonoid naringenin regulates the expression of genes involved in cell wall synthesis of *H. seropedicae* (Tadra-Sfeir et al. 2011). On the other hand, mutagenesis of an EPS biosynthesis cluster indicated that exopolysaccharide is necessary for biofilm formation on abiotic surface but not for plant colonization (Balsanelli et al. 2014).

In this study we aimed to determine the global gene expression of *H. seropedicae* associated to wheat roots through transcriptional profiling of the bacteria in the planktonic state compared to the cells attached to the wheat roots (Online Resource 1). RNA-seq libraries were sequenced using next generation sequencing technology and the sequences obtained were mapped on the *H. seropedicae* genome. Analyses of the up-regulated mRNA in attached cells indicated activation of nitrogen fixation, polyhydroxybutyrate metabolism, cell wall re-modeling and adhesion molecules such as adhesin, suggesting specific metabolic adaptations of the bacteria to the rhizospheric environment. Our results provide a panorama of gene expression of *H. seropedicae* during colonization of wheat roots, and reveal insights into pathways involved in plant–bacteria interaction.

Materials and methods

H. seropedicae growth conditions

The *H. seropedicae* strain SmR1 (Souza et al. 2000) was grown routinely at 30 °C with shaking at 120 rotations per minute (rpm) in NFbHP-malate medium (Klassen et al. 1997) with 20 mM of NH_4Cl added (NFbHPN-malate). Streptomycin was added as needed at the concentrations of 80 $\mu\text{g/mL}$. The bacterial strains for plant inoculation were pre-cultured overnight in 5 mL of NFbHPN-malate medium and antibiotic was added as needed. An overnight culture was used to inoculate 10 mL NFbHPN-malate medium, which was grown as previously described to an $\text{OD}_{600} = 1.0$. Bacterial cells were collected by quick centrifugation (13,100 $\times g$ for 20 s at room temperature) to ensure that the bacteria remained viable. Cell pellets were re-suspended in the same volume of Hoagland's medium (Hoagland and Arnon 1950) to a cell density of 10^7 cells/mL. 250 μL of this bacterial suspension was added to glass tube containing 25 mL of Hoagland's medium and two wheat seedlings to give 10^5 bacteria per mL. No nitrogen source was added to the Hoagland's solution.

Germination, inoculation and growth of seedlings

Seeds of *Triticum aestivum* (cv. CD104) were disinfected as described previously (Camilios-Neto et al. 2014; Döbereiner et al. 1995). In vitro plant cultivation was carried out under hydroponic and axenic conditions. Surface sterilized seeds were pre-germinated in water-agar petri dishes in the dark for 24 h at 30 °C. The seedlings were then transferred to glass tubes (two seedlings in each tube) containing 25 mL of Hoagland's medium. Polypropylene spheres (about 10 mL in volume) were added to each tube to serve as support for the seedlings (Online Resource 1). On the second day after germination, seedlings were inoculated with *H. seropedicae* to a final density of 10^5 *H. seropedicae* cells per mL of Hoagland's medium. The inoculated wheat seedlings were incubated at 26 °C with a light cycle of 14/10 h (light/dark) for 72 h.

Bacterial counts

To evaluate bacterial colonization, roots were sampled 6 to 126 h post inoculation (h.p.i.). For total bacterial counting the roots were washed and crushed in 0.9 % NaCl (m/v) using mortar and pestle. Homogenates were serially diluted and plated on NFbHPN-malate medium containing streptomycin (80 µg/mL). Bacterial growth in the hydroponic medium was determined by plating serial dilutions as described above. Colonies were counted after 2 days of incubation at 30 °C and expressed as colony forming units (CFU) per mL of medium or per gram of fresh root tissue.

RNA purification, RNA-seq library construction and sequencing

Root-attached bacteria were recovered 3 days after inoculation by vigorous vortexing (1 min) of roots from 80 seedlings in 5 mL of RNeasy lysis solution (ThermoFisher Scientific®), followed by centrifugation (2254×g; 40 min; 4 °C). Planktonic cells were collected by centrifugation (18,900×g; 1 min; 4 °C) of the hydroponic medium at the same time. The cell pellet was resuspended in RNeasy lysis solution and stored at −20 °C. The total RNA of *H. seropedicae* cells (approximately 10^6 CFU/g of fresh root tissue) was extracted using Trizol (ThermoFisher Scientific®) and treated with DNaseI (ThermoFisher Scientific®) following the manufacturer instructions. The rRNA was depleted using the Microbe Express kit (ThermoFisher Scientific®). Libraries for sequencing were prepared using the Whole Transcriptome Analysis kit (ThermoFisher Scientific®) and sequenced in next generation sequencing platform SOLiD 4 (ThermoFisher Scientific®) using the ToP Sequencing Kit (1 × 50 bp). Two independent samples were processed in

parallel for each type of bacterial cell, resulting in 4 sequencing libraries, and each library was sequenced at least twice. The sequence data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3646).

Transmission electron microscopy and scanning electron microscopy

Wheat roots colonized with *H. seropedicae* 3 days after inoculation were fixed with modified Karnovsky's fixative (Karnovsky 1965), post-fixed with 2 % OsO₄ in 0.1 M cacodylic acid buffer (pH 7.2) for 1 h and embedded in Epon 812 (Luft 1961). Subsequently, the colonized wheat roots were contrasted with 2 % uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and examined with a JEOL-JEM 1200 EX II transmission electron microscope. For scanning electron microscopy, the colonized roots were fixed with modified Karnovsky's fixative (Karnovsky 1965), washed in 0.1 M cacodylic acid buffer (pH 7.2) and dehydrated in ethanol. Critical-point dryness was obtained with a Bal-Tec CPD-030, the samples were coated with gold using a Balzers SCD-030 sputter coater and examined with a JEOL-JSM 6360 LV scanning electron microscope.

Data analyses

Obtained sequences were mapped on the *H. seropedicae* genome using the software CLC Genomics Workbench (v. 6.5.1). The following parameters were used: reads were trimmed to minimum of 40 bp, 90 % alignment to the reference sequence and 80 % identity required for inclusion as a mapped read, number of hits equal to 1, number of additional bases down and upstream of the CDS equal to 50 bp. Genes were considered as expressed if presented more than 3 times of coverage. Differential gene expression analyses was performed using DESeq (DESeq using R-package from Bioconductor's project) and were considered regulated those genes with a fold-change ≥ 2 and p -value ≤ 0.05 . RPKM values (Mortazavi et al. 2008) were calculated using CLC Genomics Workbench (v. 6.5.1). In order to evaluate the impact of up and down-regulated genes in *H. seropedicae* metabolic pathways, a gene set enrichment analysis (GSEA) was performed using the Bioconductor Project (version 2.14) (Reimers and Carey 2006) package Model-based Gene Set Analysis (MGSA) (version 1.13) (Bauer et al. 2010) in R statistical environment (version 3.1.0) (R Core Team, 2014). MGSA calculate the posterior probability of a pathway being affected by up or down-regulated genes. Pathways from KEGG database (Kanehisa et al. 2014) were employed for this analysis and only differentially expressed genes with FDR ≤ 0.05 were used.

Reverse transcription-quantitative PCR

Total RNA from planktonic and attached bacteria was obtained in independent experiments under the same conditions used for RNA-Seq. Total RNA (300 ng) was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific®). The 16S rRNA of *H. seropedicae* was used as reference. The primers used in the analyses were for the genes *nifH*, *ntnC*, *amtB*, *fixP*, *fnr2*, *ttuC*, *Hsero_2999*, *Hsero_0639*, *pilS*, *pilT* (*Hsero_0816*), *adhA*, *narG*, *nirD*, *narU*, *nasF* and *narX*, and their sequences are shown in online resource 3. Primer efficiency was calculated through cDNA dilution curve over at least 5 orders of magnitude. The RT-qPCR experiments were performed using the Power SYBR green master mix (ThermoFisher Scientific®) and the reaction run on a Step One Plus real-time PCR system (ThermoFisher Scientific®). Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ (threshold cycle) method (Livak and Schmittgen 2001).

Results and discussion

Colonization of wheat seedlings

A hydroponic model to study the interaction between wheat and diazotrophic bacteria was setup under axenic conditions. Bacterial counts showed that the number of *H. seropedicae* cells colonizing wheat roots and free-living (hereafter called planktonic bacteria) increased up to 10^8 cells/g of fresh root and 10^7 cells per mL of cultivation media, respectively, 24 h post inoculation (h.p.i), and then remained constant until 168 h.p.i (Fig. 1).

Fig. 1 Time course colonization of wheat roots by *H. seropedicae*. (Filled square) Number of bacteria colonizing the wheat roots. (Filled circle) Number of bacteria in Hoagland's medium. Each point is the average of three independent experiments and each experiment was made of 3 biological replicates. Error bars are standard deviation between the biological replicates. CFU colony forming units

To check whether wheat exudates could support bacterial growth, *H. seropedicae* was inoculated in filter-sterilized conditioned Hoagland's medium (obtained from 3-days-old wheat hydroponic culture in the presence or absence of *H. seropedicae*). The results showed that wheat exudates indeed support *H. seropedicae* growth, as seen by the increase in bacterial population in conditioned Hoagland's medium (Online Resource 2). Root exudation includes the secretion of ions, enzymes, mucilage, and many different carbon-containing primary and secondary metabolites, and these nutrients attract microbes to the plant root as well as sustain their proliferation (Bais et al. 2006). In addition to nutrients, molecules exuded by the plant could influence gene expression and the ability of the microbe to colonize the plant root.

RNA-seq profiling of *H. seropedicae* interacting with wheat seedlings

For the RNA-seq transcriptional analysis, wheat seedlings inoculated with *H. seropedicae* were incubated at 26 °C with a light cycle of 14 h for 72 h. Free-living or planktonic (PLANK) and wheat root-attached *H. seropedicae* cells (WRA) were collected and used for RNA extraction. Two independent samples (biological replicates, containing 80 seedlings [WRA] or from 1 L of Hoagland's cultivation medium [PLANK]) of each condition were used to construct 4 sequencing libraries; each PLANK library was sequenced twice, whereas WRA libraries were sequenced three times. Pearson's correlation coefficient of the transcriptome data (RPKM values) for PLANK and WRA libraries were $r = 0.98$ and $r = 0.99$, respectively, indicating the biological and technical replicates had high reproducibility. On the other hand, the correlation coefficient when the two conditions were compared was $r = 0.32$ (Table 1).

A total of 99,842,318 reads were obtained for the PLANK libraries and 46,343,321 reads for the WRA libraries; after quality trimming to a minimum of 40 bp, 90 % of alignment and 80 % of identity to reference, about 97,800,104 (98.0 %) and 45,712,110 (98.6 %) reads remained, respectively. The latter set was mapped against the *H. seropedicae* genome and wheat sequence database (Table 1).

The planktonic libraries had the highest percentage of sequences mapped to the *H. seropedicae* sequences (55–65 %) and the lowest percentage mapping to wheat sequences (7.2–7.8 %), while 23 % of the reads from the root-attached cells mapped to wheat sequences and only 12.6–15.2 % to *H. seropedicae* sequences. The different proportion of reads mapped to wheat sequences between the treatments may be due to detachment of epidermal cells from the roots during harvesting procedure, which required vigorous vortexing to recover the attached bacteria.

Table 1 Number of reads mapping to different reference databases

Biological replicate samples	Total # of reads	Reads remaining after trimming	r*	r**	Reads mapped to Hs*** tRNA genes	Reads mapped to Hs*** rRNA genes	Unique reads mapped to Hs CDS	% reads mapped to Hs sequences	Reads mapped to wheat chloroplast genome	Reads mapped to wheat mitochondrial genome	Reads mapped to wheat EST	% reads mapped to wheat sequences
WRA1	25,836,672	25,491,158	0.99	0.32	15,106	2,992,011	224,627	12.7	2,244,649	114,332	3,607,494	23.41
WRA2	20,506,649	20,220,952	(1)	0.29	14,509	2,864,544	189,357	15.2	1,444,488	111,663	3,139,598	23.22
Plank 1	46,532,584	45,581,780	0.98	(2)	654,192	22,881,973	1,532,127	55.0	461,309	213,704	2,636,029	7.26
Plank 2	53,309,734	52,218,324			484,975	31,969,105	1,474,565	65.0	615,928	140,866	3,319,709	7.81

* Pearson correlation factor between biological replicates

** Pearson correlation factor between biological replicates from different conditions

*** *Herbaspirillum seropedicae***Table 2** Number of expressed, regulated or differentially expressed genes

	PLANK	WRA
Technical replicates	2	3
Biological replicates	2	2
Total expressed genes	2167	
Regulated genes	403	
Up-regulated genes	152	
Down-regulated genes	251	

For gene expression analyses, reads were first mapped to the *H. seropedicae* genome using the CLC genomics workbench and followed by DESeq analyses with the reads mapped to the reference genome. For the PLANK libraries 3,004,584 reads were obtained and unambiguously mapped, and for the WRA libraries 413,969 reads were obtained. Our analyses revealed 985 expressed genes (with 3 times or higher coverage) in the WRA sample and 2,130 genes in the PLANK sample; out of these 403 genes were differentially expressed (fold-change higher than 2 and p -value ≤ 0.05), 152 genes up-regulated and 251 down-regulated (Table 2). Differentially expressed genes represent about 8.3 % of the *H. seropedicae* genome, indicating that ample changes in *H. seropedicae* gene expression are required to switch from free-living to attached life style.

In order to extract more information from the transcriptomic data, a gene set enrichment analysis (GSEA) was performed. Analysis of the up-regulated gene set in WRA samples allowed the identification of three over-represented KEGG pathways, as follows: oxidative phosphorylation (10 genes); chloroalkene and chloroalkene degradation (5 genes); and pyruvate metabolism (5 genes) (Table 3). A total of five KEGG pathways were over-represented among the down-regulated gene set. Ribosome pathway had the highest posterior probability of being over-represented (43 genes were identified associated with this pathway) among down-regulated genes in root-attached cells. RNA degradation (6 genes); RNA synthesis (3 genes); plant-pathogen interaction (3 genes) and protein export (5 genes) had their posterior probability also higher than 0.5 of being over-represented in the sub-set of down-regulated genes (Table 4). Down regulation of processes involved in proteins biosynthesis pathway (RNA metabolism) indicates that the attached bacteria have a lower duplication rate compared with those in the planktonic state. Whereas, repression of genes involved in plant-pathogen interaction may diminish the plant defense response. Previous work demonstrated that *H. seropedicae* cultivated in the presence of sugar cane extract also regulated proteins related with plant-pathogen interaction (Cordeiro et al. 2013). Nitrogen fixation genes were also

Table 3 Gene set enrichment analysis of up-regulated genes with $FDR \leq 0.05$

Pathway description	KEGG id	Identified genes	Probability	SE
Oxidative phosphorylation	ko00190	10	1	1.72E–003
Chloroalkene and chloroalkene degradation	ko00625	5	0.91	3.98E–003
Pyruvate metabolism	ko00620	5	0.58	6.24E–003

Table 4 Gene set enrichment analysis of down-regulated genes with $FDR \leq 0.05$

Pathway description	KEGG id	Identified genes	Probability	SE
Ribosome	ko03010	43	1	2.02E–004
RNA degradation	ko03018	6	0.99	6.93E–004
RNA polymerase	ko03020	3	0.97	2.11E–003
Plant–pathogen interaction	ko04626	3	0.66	7.34E–003
Protein export	ko03060	5	0.57	1.34E–002

over-represented (posterior probability of 1) in the up-regulated gene dataset of root-attached cells.

Nitrogen fixation and bacterial adaptation to microoxic environment

The overall expression of genes from bacteria attached to the plant roots compared to the planktonic cells suggested that the root surface environment requires specific adaptation for bacterial survival. In this environment, bacteria seem to face deprivation of some nutrients, including oxygen and ammonium. Oxygen sensing is very important for bacterial survival and successful colonization of the plant. The bacterial respiratory shift from oxic to microoxic condition relies on a strategy that ensures that cells can sense and respond to changes. *H. seropedicae* can sense oxygen through Fnr-like proteins, which act as intracellular redox sensors and regulate gene expression according to changes in oxygen levels (Batista et al. 2013). We found that expression of *fnr1* (Hsero_3197) increased 16.8-fold in root-attached cells, and *fnr2* (Hsero_2381) 38.9-fold (Online Resource 4). Up-regulation was confirmed by RT-qPCR assay (Fig. 2b). However *fnr3* (Hsero_2538) expression had only a minor change. Batista et al. (2013) observed that *fnr1* expression was apparently regulated by Fnr3 and oxygen, while *fnr3* expression was only marginally affected. They also showed that Fnr1 and Fnr3, not only regulate expression of the *cbb3*-type terminal oxidase, but also control the cytochrome content and other components required for the cytochrome *c*-based electron transport. *fixNOPQ* coding for *cbb3*-type oxidase were up-regulated in attached cells, suggesting that Fnr proteins are activated. The role of Fnr2 could not be identified yet. The finding that *fnr2* is not regulated by oxygen (Batista et al. 2013) and that is up-regulated in root-attached cells may indicate involvement of Fnr2 protein in plant-bacteria interaction.

Regulation of nitrogen fixation and N assimilation in *H. seropedicae* is well characterized (Chubatsu et al. 2012). On the other hand, little is known about nitrogen fixation during grass–bacterial interactions. BNF was well demonstrated in several varieties of rice inoculated with *H. seropedicae* (Gyaneshwar et al. 2002) and *H. seropedicae* *nif* genes expression was shown in roots, stems and leaves of rice, sorghum and maize (James et al. 2002; Roncato-Maccari et al. 2003). Here, the expression of the whole nitrogen fixation cluster was observed during the plant-bacterial association, strongly indicating active nitrogen fixation by the bacteria (Fig. 2a). NifH was among the most abundant protein expressed during interaction of *H. seropedicae* and rice seedlings (Alberton et al. 2013).

nif genes were expressed by both *H. seropedicae* colonizing wheat roots and planktonic cells. However, the expression of the *nifHDKENXHsero_2847Hsero_2846fdxB* operon was more prominent in attached bacteria (Fig. 2a). In this condition, nitrogenase structural genes *nifHDK* (Hsero_2853; Hsero_2852; Hsero_2851) were up-regulated 34- to 67-fold compared to planktonic cells, and similar levels of up-regulation occurred for the other genes in the operon. Up-regulation of the *nifH* in attached bacteria was confirmed by reverse transcription-quantitative real-time PCR (RT-qPCR) with a fold-change comparable to that of RNA-seq (Fig. 2b).

Expression of the *nif* transcription activator gene, *nifA* (Hsero_2871), *ntrC* (Hsero_3125) and *glnA* (Hsero_3127) were 60.8-, 7.4- and 2.7-fold up-regulated, respectively, in the WRA libraries. The NtrC-activated operon *nlnAgln-KamtB* (Hsero_0083; Hsero_0084; Hsero_0085) (Huergo et al. 2010; Noindorf et al. 2011) was also up-regulated in the WRA libraries with fold-changes of 14.8, 38.9 and 23.8, respectively. Up-regulation of *ntrC* and *amtB* was confirmed by RT-qPCR with a fold-change of 5.3 and 9.3-fold, respectively (Fig. 2b). Taken together the results show that cells attached to wheat roots were nitrogen deprived, since the Ntr system was up-regulated. Higher

Fig. 2 **a** Structural and regulatory *nif* genes activated in the bacteria attached to the wheat seedlings roots. Expression is represented as RPKM and the conditions are epiphytic bacteria (cells attached to wheat root) or planktonic bacteria. **b** Quantification of expression of selected genes of *H. seropedicae* colonizing wheat roots by reverse transcription-quantitative PCR. Relative gene expression of 16

candidate genes of *H. seropedicae* attached to the wheat root surface compared with cells in planktonic state. Regulation in RT-qPCR data is in agreement with RNA-seq data although the fold change varied. Data represents the mean of 3 technical replicates and 2 biological replicates. Error bars are standard deviation between the biological replicates

expression of *nif* may also be partially explained by lower oxygen levels since *H. seropedicae* NifA protein is inactivated by O_2 (Souza et al. 1999).

H. seropedicae also contains genes encoding an assimilatory nitrate reductase (NAS) (*nasAHsro_2899*), a nitrite reductase (*nirBD*) and a respiratory nitrate reductase (NAR) (*narGHJI*) (Pedrosa et al. 2011). Interestingly, genes related to nitrate assimilation (*nasAHsro_2899* and *nirBD* genes) were up-regulated in the attached cells, whereas genes related to the respiratory nitrate reductase were down-regulated. The up-regulation and down-regulation in the attached cells of *nirD* and *narG* genes, respectively, was confirmed by RT-qPCR (Fig. 2b). Additionally, RT-qPCR also confirmed the up-regulation in attached cells of *nasF* gene, which encodes to a nitrate transporter related to the NAS, and the down-regulation of *narU* gene, encoding to a nitrate transporter related to NAR (Fig. 2b).

The regulation of genes related to nitrate assimilation are well studied in *Klebsiella pneumoniae*, in which *nasA* gene was found to be induced in response to nitrate (Lin and Stewart 1998). Expression analyses of *nasF* promoter in *H. seropedicae* showed that its expression is highly induced in response to nitrate (Bonato, unpublished data).

Since no source of nitrogen was added during cultivation of wheat seedlings, the data suggest that wheat roots can exude nitrate. This result is in agreement with previous work, which shows the up-regulation of *H. seropedicae* genes encoding NAS in maize roots in response to nitrate exuded from maize (Balsanelli et al. 2015). Therefore, nitrate may be a source of nitrogen in *Herbaspirillum*-plant interaction, although the bacteria rely mainly on nitrogen fixation (Pankievicz et al. 2015).

Adhesion

The first step of the colonization process is the attachment of bacteria to the plant root, a process mediated by bacterial and host surface constituents, such as lipopolysaccharides (LPS), adhesins and lectins (Balsanelli et al. 2010, 2013). Two genes coding for filamentous hemagglutinins proteins were up-regulated in cells attached to the plant roots. *Hsro_1294* and *fhaB* (*Hsro_3251*) had a fold-change of 2.8 and 2.3-fold, respectively (p -value = 0.01 and 0.04, respectively). Filamentous hemagglutinin proteins (FHA) are important adhesins involved in mediating the attachment of the bacteria to the host. In the phytopathogen *Xanthomonas axonopodis* pv. *citri* mutation in *fhaB* gene

impaired virulence and adhesion of the bacteria *in planta* assays (Gottig et al. 2009).

Genes coding for exopolysaccharide biosynthesis (*eps* genes) were not expressed by *H. seropedicae* in any of the conditions. The transcriptomic analyses of epiphytic cells of *H. seropedicae* colonizing the maize rhizosphere also demonstrated the absence of the *eps* genes expression (Balsanelli et al. 2015), corroborating the finding that EPS production by *H. seropedicae* is not necessary for colonization of maize roots (Balsanelli et al. 2014). However, LPS seems to be involved in maize colonization by *H. seropedicae* (Balsanelli et al. 2010). The cluster for LPS synthesis, comprising CDS Hsero_4197 to Hsero_4222, was expressed in both planktonic and attached bacterial cells (Online Resource 4). On the other hand, *lpxB* (Hsero_2191) encoding a lipid-A-disaccharide synthase was 10.9-fold up-regulated in attached cells. Hsero_2761, encoding a Wza-like outer membrane polysaccharide export protein, and Hsero_4222, encoding a CapD polysaccharide biosynthesis protein, were also up-regulated in attached cells, although the difference in expression was not statistically significant. These genes (Hsero_2191, Hsero_2761 and Hsero_4222) were also up-regulated in *H. seropedicae* attached to maize root surface (Balsanelli et al. 2015). Four genes coding for membrane porins were up-regulated in attached cells: Hsero_0185, Hsero_1344, Hsero_4260 and Hsero_4295 with 6.1- 34.4- 13.1- and 2.8-fold change, respectively (Online Resource 4). Balsanelli et al. (2015) observed that the porin genes Hsero_0677, Hsero_1043 and Hsero_2393 were up-regulated in *H. seropedicae* attached to maize root and suggested that the expression of specific membrane porin genes might be involved in colonization. Interestingly, Hsero_4260 and Hsero_4295 were repressed in maize root attached cells further supporting that the set of porin expressed genes depends on the host plant and possibly on the stage of colonization.

Cell motility

Flagellar motility guides bacteria movement in response to diverse stimuli towards a desired environment. The main flagella gene cluster of *H. seropedicae* attached to the plant cells were down-regulated, however with a fold-change lower than 2 (Online Resource 4). Some flagellar related genes were significantly repressed: the flagellins *fliC* (Hsero_2070) and *flaG* (Hsero_2069) had expression decreased by 1.9 and 3.0 -fold in the attached cells, respectively. Also, *fliP* (Hsero_2049), *flgK* (Hsero_2042) and *motB* (Hsero_2985) that encode structural proteins of the flagella were 6.7, 3.4 and 2.5 fold down-regulated. In addition, two genes coding for transcription activator proteins, *fliH* (Hsero_2988) and *fliD* (Hsero_2989), were

repressed in attached cells with fold-changes of 2.4 and 2.7, respectively (Online Resource 4). *H. seropedicae* cells cultivated in presence of the flavonoid naringenin showed a marked repression of flagellar genes (Tadra-Sfeir et al. 2015). Flavonoids are signaling plant molecules that can influence the bacterial colonization process, and may modulate motility of bacteria in the rhizosphere.

Repression of flagellar genes suggests a shift from free-swimming to sessile lifestyle upon attaching to the root surface. In this new state *H. seropedicae* cells could be using twitching motility guided by type IV *pili*, since the expression of the system ATPase, encoded by *pilT* (Hsero_0816) increased 1.6-fold in the attached cells, and *pilI* encoding the twitching motility protein, increased 2.5-fold. Although, with low significance level in the RNA-seq (p -value = 0.29 and 0.25, respectively) the RT-qPCR experiments showed a slight up-regulation of *pilT* gene (1.3-fold). In addition, the gene *pilS* (Hsero_0812), coding for a putative type IV *pilus* assembly protein, was repressed in the RNA-seq (24.5-fold) (Online Resource 4) and also down-regulated in the RT-qPCR analyses (Fig. 2b). The exact function of PilS in various organisms is not clear yet; in *P. aeruginosa* PilS2 is not required for twitching motility, but may be involved with the negative transcriptional regulation controlling the expression or activity of the type IV *pili* (Mattick 2002).

Electron microscopy of wheat roots colonized by *H. seropedicae* (Fig. 3a–c) revealed structures in attached bacterial cells similar to type IV *pili* (Fig. 3b, c). These structures were 5 times longer than the bacterial cell as measured by the software ImageJ. Type 4 *pili* genes are widely distributed in both Gram-negative and Gram-positive pathogenic bacteria and play essential roles in host colonization and virulence in susceptible host targets. Comparisons between various endophytic strains suggest that twitching motility mediated by PilT retraction is likely an important feature for bacterial colonization of root surfaces (Reinhold-Hurek and Hurek 2011). The results suggest that the type IV *pili* of *H. seropedicae* might be related to the bacterial colonization of root seedlings.

Chemotaxis

The bacterial ability of sensing the environment is another important trait involved in plant colonization by the bacteria. Methyl-accepting chemotaxis proteins or MCPs seems to be important elements in this process (Grebe and Stock 1998). The RNA-seq analyses revealed that several genes coding for methyl-accepting chemotaxis proteins (MCPs) were highly up-regulated in the WRA libraries (Hsero_2914, Hsero_2915 and Hsero_2723 increased 17.6- 18.1- and 5.9- fold, respectively). Balsanelli and collaborators (2015) also found Hsero_2723 up-regulated when *H. seropedicae* were attached

Fig. 3 Electronic microscopy of *H. seropedicae* attached to wheat roots 3 days after inoculation. **a** Scanning electronic microscopy showing bacteria colonizing wheat root. **b, c** Show formation of a microcolony. White arrows indicate structures similar to type 4 pili.

In **d** transmission electron micrograph of *H. seropedicae* attached to the wheat roots 3 days after inoculation. Black arrows indicate PHB granules

to maize roots. Indeed, these authors suggested that another *H. seropedicae* gene, Hsero_3720, is a key MCP regulator responsible for sensing the rhizosphere environment. The Hsero_3720 mutant strain was a less efficient colonizer and this gene was expressed in the planktonic cells in contact with maize roots (Balsanelli et al. 2015). Hsero_3720 was also highly expressed in planktonic cells in contact with wheat roots and its expression compared to attached cells was 55-fold decreased (RPKM (WRA) = 106; RPKM (PLANK) = 4856), suggesting that the protein coded by this gene is important for cells in the planktonic state.

Transporters and stress related genes

ATP-binding cassette (ABC) transporters catalyze the translocation of various substrates across bacterial membrane. They enable the uptake of nutrients and important molecules (importers) or facilitate the extrusion of toxins (exporters) (Dawson and Locher 2006). The RNA-seq analyses showed an expressive regulation of ABC transporters in *H. seropedicae*, with 10 ABC transporters genes up-regulated and 19 down-regulated in the wheat root attached bacteria compared to planktonic cells. Among the

down-regulated genes, 4 were predicted branched-chain amino acid transporters, one proline/glycine transporter, two aspartate/glutamate transporters, two nitrate/sulfate transporters, two sugar transporters, with sorbitol or maltose as possible substrates, one ion transporter, one metal transporter and three transporters of unknown substrates. Interestingly, two toluene tolerance ABC transporter genes (Hsero_4079 and -4077) related to bacterial defense and a multidrug ABC transporter gene (Hsero_4073) were down-regulated by 2.4 (p -value = 0.03), 1.4 (p -value = 0.48) and 6.5-fold (p -value = 0.003) in wheat root attached cells, respectively (Online Resource 4). These transporters may be related to bacterial defense.

Among the up-regulated transporters were found genes for three ABC transporters of glutamate/aspartate and one dipeptide/oligopeptide transporter. An oligopeptide transporter operon (Hsero_1130-1131-1132-1133) had expression increased by 25-fold to 43-fold. In many Gram-negative bacteria this kind of transporters has a role in nutrition, recycling cell-wall peptides or adhesion to host cells (Higgins and Linton 2004). Also were up-regulated ABC transporters for sugars, branched-chain amino acids and nitrate/sulfate molecules (Online Resource 4).

The expression of one ABC exporter operon increased by 12–97 fold (Hsero_2386-2387-2388-2389; p -value ≤ 0.05). This transporter is probably related to efflux of antimicrobial compounds. The ABC transport for putrescine was also up-regulated (*potCHDA* Hsero_1078-1079-1080-1081; p -value = 0.55; 0.80; 0.02; 0.0004, respectively) (Online Resource 4). Putrescine or 1,4-diaminobutane is a biogenic polyamine present in nearly all living cells and is involved with the bacterial response to osmotic stress (Miller and Wood 1996). It is expected that the osmolarity of the rhizoplane is higher than the regions located away from the roots. The rapid bacterial adaptation to the osmolarity increase would facilitate the colonization of the rhizosphere (Miller and Wood 1996). Also, the amount of putrescine in plant cells increases in response to the osmotic stress (Flores and Galston 1982). The genomic analyses made by (Mitter et al. 2013) showed that all studied endophytes had putrescine carrier genes (e.g., *Azospirillum* sp. B510, *Burkholderia phytofirmans* PsJN, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stutzeri* A1501 and *Enterobacter* sp. 638). However, it is still not clear if these transporters are involved in protection from the putrescine produced by the plant or to the adaptation of bacteria to hyperosmolarity in the colonized environment.

Polyhydroxybutyrate metabolism

The *H. seropedicae* genome contains several genes that are likely involved in polyhydroxybutyrate (PHB) metabolism. The genes encoding enzymes related to PHB biosynthesis were up-regulated in the *H. seropedicae* attached cells. First, *phaA2* (Hsero_0239) encoding a 3-ketothiolase, which condenses two acetyl-CoA to form acetoacetyl-CoA, had a 2.7-fold increase in expression (p -value = 0.0001). Second, the expression of *phaB* (Hsero_2998) encoding a NADPH-dependent acetoacetyl-CoA reductase (PhaB), which reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, increased 1.6 times (p -value = 0.40). Third, mRNA of the *phaC1* (Hsero_2999) encoding the PHB synthase had a 1.5-fold increase (p -value = 0.33) (Fig. 4). RT-qPCR experiments also showed *phaC1* activation (1.9-fold increase) in attached cells compared to planktonic cells (Fig. 2b).

In other bacteria poly-hydroxyacids production is stimulated under low concentrations of nitrogen and oxygen (Anderson and Dawes 1990; Jendrossek 2009; Madison and Huisman 1999), which prevail in the environment of both root-attached and planktonic *H. seropedicae* cells. Furthermore, carbon excess or high carbon/nitrogen ratio also induces PHAs (polyhydroxyalkanoates) synthesis (Hervás et al. 2008). Since cells attached to roots show higher

Fig. 4 PHB metabolic pathway adaptations of *H. seropedicae* attached to wheat root. Schematic representation of a *H. seropedicae* cell attached to the wheat root cell, with bacterial PHB biosynthesis

and degradation pathways represented. Fold-change values of up-regulated genes are shown

expression of PHB biosynthesis genes, it is likely that these cells have more access to carbon sources supplied by the root exudates compared to the planktonic bacteria.

H. seropedicae genome encodes three phasin proteins associated to the PHB granule (Tirapelle et al. 2013). The phasin PhaP1 (Hsero_1639) with a 2.7-fold increase in WRA library (p -value = 0.0001), and two phasins related proteins: PhaP2 (Hsero_4759) whose expression were also increased by 2.4-fold in the WRA library (p -value = 0.04); and PhaP3 (Hsero_2401) which was not expressed in the PLANK nor WRA libraries (Fig. 4). In contrast, expression of *phbF* (Hsero_2997) gene encoding the regulatory protein associated to the PHB granule (PhbF), which may act as a transcriptional repressor of genes of PHB metabolism in *H. seropedicae* (Kadowaki et al. 2011), decreased by 1.8-fold in the attached bacteria (p -value = 0.09). The down-regulation of *phbF* in the bacteria attached to the wheat roots is consistent to increased expression of PHB biosynthesis genes and large amount of PHB granules observed in the transmission electron micrograph of *H. seropedicae* colonizing wheat roots 3 days post inoculation (Fig. 3d). Similar granules can be seen in *H. seropedicae* expressing *nifH* on rice roots (Gyaneshwar et al. 2002).

PHB production appears to be an important feature for root colonization and plant growth promotion by other rhizobacteria. For example, field experiments showed that *A. brasilense* strains that produce PHAs are more efficient as inoculants (Fibach-Paldi et al. 2012). In the rhizobacterium *A. brasilense*, accumulation of polyhydroxyalkanoate was shown to support chemotaxis, motility, and cell multiplication (Kadouri et al. 2005). *A. brasilense* attached to wheat root seedlings cultivated in Hoagland's medium also expressed genes related to the PHA biosynthesis (Camilios-Neto et al. 2014). To further support the role of PHB metabolism in root colonization, it was shown recently that knockout of *phaC1* impaired maize colonization by *H. seropedicae* (Balsanelli et al. 2015).

The genome of *H. seropedicae* contains two additional genes, *phaC2* and *phaC3*, encoding putative PHB synthases, which are located distant from *phaC1*. The *phaC2* is located on the operon with *pta-ackA* genes encoding phosphotransacetylase and acetate kinase enzymes. The mRNA levels of *phaC2* (Hsero_2405) and *phaC3* (Hsero_0265) were 36.7- (p -value = 0.01) and 12.4- fold (p -value = 0.05) increased, respectively, in the bacteria attached to the roots while *phaC1* was expressed but not regulated (Online Resource 4). The average fold change increase for the operon *phaC2-fabI-pta-ackA* (Hsero_2405-Hsero_2406-Hsero_2407-Hsero_2408) was greater than 6-fold, but the significance was higher than 0.05 only for Hsero_2408 (p -value = 0.43). This result suggests the activation of the phosphate acetyltransferase-acetate kinase pathway, which converts acetyl-CoA to acetate and produces ATP. In cyanobacteria, acetyl-phosphate seems to

play a role controlling PHA synthase. The organization of the operon and co-expression of the *pta-ackA* genes with *phaC2* in *H. seropedicae* suggests a role for acetyl-phosphate in PHB metabolism during plant colonization. Mutation of *phaC1* of *H. seropedicae* abolished PHB synthesis in vitro, suggesting that PhaC1 is the main gene responsible for PHB synthesis (Tirapelle et al. 2013). The induction of PhaC2 in the rhizoplane suggests specific role for this putative PHB synthase. The results suggest that colonization of the root surface require active PHB metabolism in *H. seropedicae*.

The conversion of acetyl-CoA to acetate may be activated to supply energy in an oxygen-deprived environment. Under this condition, PHB biosynthesis may function to recycle NAD⁺. Interestingly, a gene for an alcohol dehydrogenase, *adhA* (Hsero_0964), was also found 10-fold up-regulated (p -value $\leq$ 0.05), which may suggest oxidation of ethanol to acetate to recycle reduced co-enzymes. The induction of the *adhA* was confirmed by RT-qPCR (2.5-fold increase in the cells attached to the root) (Fig. 2b). Krause et al. (2011) showed that *Azoarcus* sp. strain BH72 expressed different alcohol dehydrogenases during the interaction with rice. Proteomic analyses of *H. seropedicae* SmR1 colonizing rice also found the expression of the AdhA enzyme in root-colonized samples (Alberton et al. 2013), suggesting an important role of this enzyme in the rhizosphere colonization by bacteria.

Phytohormones related genes

Auxins are the major class of phytohormones involved in regulation of plant growth and development, being indole-3-acetic acid (IAA) the most studied hormone of this class (Spaepen et al. 2007; Went and Thimann 1937). *H. seropedicae* possesses genes to produce IAA from four different possible pathways (Bastián et al. 1998; Pedrosa et al. 2011). Among these possible pathways there are two with up-regulated genes found in the WRA libraries: (1) conversion of indolepyruvate into indole-acetic acid through the indolepyruvate ferredoxin oxidoreductase activity (enzyme encoded by Hsero_4278) and (2) conversion of indole-3-acetonitrile to indolacetate through the activity of the nitrilase (enzyme encoded by Hsero_1422). The increase in expression of Hsero_4278 and Hsero_1422 by 3.1- (p -value = 0.04) and 2.7- fold (p -value = 0.12) in attached cells may suggest that IAA synthesis by *H. seropedicae* is regulated by the plant.

Conclusions

Beatty and Good (2011) suggested three main approaches to enhance plant nitrogen nutrition, and one of them is the utilization of diazotrophic endophytic bacteria as

biofertilizers. More recently, Gutiérrez (2012) emphasized the importance to increase the understanding of N-use efficiency by using systems biology approaches concerning N-regulatory networks and molecular mechanisms to control growth and development of plants. Indeed, the use of ^{13}N labeled N_2 showed that *Setaria viridis* incorporated N-fixed by the diazotrophic bacteria *H. seropedicae* and *A. brasilense* (Pankievicz et al. 2015), opening new avenues for utilization of plant growth promoting bacteria.

H. seropedicae is the best-characterized species of *Herbaspirillum* genus and is a model of grass-associative endophytic nitrogen fixing bacteria. This bacterium is capable of interacting with many cereals of economical importance, increasing their productivity. Such effect seems to be due, at least in part, to fixed nitrogen transferred to the plant (Monteiro et al. 2012; Pankievicz et al. 2015), thus it can potentially be used as biofertilizer. However, molecular cues and signals that trigger plant colonization are largely not understood.

Global transcriptional analysis of *H. seropedicae* during plant-bacterial interaction using two different types of bacterial cells, i.e., cells attached to wheat roots and planktonic, uncovered new facets of the endophyte-grass interaction. Expressed genes and comparative analysis between the two conditions revealed a consistent global profile, and a high correlation was observed for the two biological replicates in each condition. The expression data obtained by RNA-seq was supported by RT-qPCR of selected genes.

Many studies showed that bacteria change their gene expression to adapt to specific environment or host interaction. Under the used conditions *nif* gene expression was high, mainly in *H. seropedicae* attached to plant roots. Activation of *fixNOP* in attached cells indicated that the root surface environment is microaerophilic, and this may be more appropriate for nitrogen fixation. In addition, attached cells potentially have facilitated access to carbon sources. We also observed that the shift from free-swimming to attached lifestyle seems to lead to a reduction in flagella gene expression and up-regulation of *pilT* type 4 *pili* gene, which probably enables adhesion to wheat roots and twitching motility. Also, the up-regulation of specific genes coding for methyl-accepting chemotaxis proteins suggests that the bacteria are sensing the change in the environment. Indeed, two specific genes coding for MCPs, found up-regulated during the wheat interaction, were also found up-regulated in *H. seropedicae* colonizing maize (Hsero_2723 and Hsero_3720) (Balsanelli et al. 2015).

Among the metabolic changes undergone by *H. seropedicae*, up-regulation in attached cells of polyhydroxybutyrate synthesis coding genes was prominent. An array of transporters was also induced suggesting exudation of several carbon sources by the plant. Activation of *nif* genes

and other Ntr-regulated genes indicated N-limitation. PHB production appears to be an important feature for root colonization and plant growth promotion by *azospirilla*, and field experiments showed that strains producing PHAs are more likely to be more effective as inoculant (Fibach-Paldi et al. 2012).

In summary, this study presents a panorama of the wheat–*H. seropedicae* interaction, and allowed identification of candidate genes and pathways potentially involved with attachment and metabolic adaptations for interaction with plants.

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Author's contribution Pankievicz, V. C. S.; Camilios-Neto, D. and Souza, E.M. Conceived and designed the experiments. Pankievicz, V. C. S. and Camilios-Neto, D. Plant assays, RNA purification and constructed the RNA-Seq libraries. Tadra-Sfeir, M.Z. and Faoro, H. RNA-Seq libraries, NGS sequencing and data acquisition. Pankievicz, V. C. S., Bonato, P. and Souza, E.M. RNA-Seq analysis and interpretation of the data. Donatti, L. Electronic microscopy. Wajnberg, G. and Passetti, F. GSEA and critical revisions on bioinformatics analysis. Pankievicz, V. C. S. and Souza, E.M. drafted the manuscript. Pankievicz, V. C. S.; Balsanelli, E.; Chubatsu, L.; Monteiro R. A.; Pedrosa F. O. and Souza, E. M. Critical revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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