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Transcriptional responses of *Haemophilus parasuis* to iron-restriction stress in vitro

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Abstract *Haemophilus parasuis* is the causative agent of Glässer's disease, which is responsible for the increasing economic losses in the pig industry worldwide. In this study, selective capture of transcribed sequences approach was used to investigate the transcriptional responses of *H. parasuis* to iron-restriction stress. Thirty-six genes were identified to be up-regulated under iron-restricted conditions. Knowledge of the genes involved in adaptation to environments encountered during disease will help understand the mechanisms of pathogenesis for this economically significant bacterium.

Keywords *Haemophilus parasuis* · Transcriptional responses · Iron restriction · Selective capture of transcribed sequences

Introduction

Haemophilus parasuis, a member of the family *Pasteurellaceae*, is the etiological agent of Glässer's disease responsible for the increasing economic losses in the swine industry worldwide. *H. parasuis* is a common commensal bacterium of the upper respiratory tract of conventional pigs. However, it can also invade and cause severe systemic disease characterized by fibrinous polyserositis, polyarthritis and meningitis (Hoefling 1991; Oliveira and Pijoan 2004). The clinical course of this disease ranges from acute to chronic, depending on host immune status and serotype of the infecting strain (Oliveira and Pijoan 2004). To date, 15 serotypes have been identified in the world, with serotypes 4 and 5 being the most prevalent in most countries (Kielstein and Rapp-Gabrielson 1992; Oliveira and Pijoan 2004; Cai et al. 2005).

Despite many years of research, the bacterial components that are involved in *H. parasuis* infection were largely unknown. As in other Gram-negative microorganisms, lipopolysaccharide, capsule, certain outer membrane proteins, fimbriae and neuraminidase have also been linked to the virulence of this bacterium (del Río et al. 2005). Approximately 40 differentially expressed genes of this pathogen have been identified from infected pig lungs in our previous study (Jin et al. 2008). However, the overall contribution of each component to the infection process remains unclear.

Iron is essential element for nearly all living organisms and is required for processes ranging from energy metabolism to DNA, protein and sugar metabolism (Mey et al. 2005). However, the concentration of

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free iron is not enough to support the growth of bacteria in the mammal host, due to the presence of specialized protein carriers such as transferrin, lactoferrin, haptoglobin and hemoglobin in body fluids (Wandersman and Stojiljkovic 2000; Braun 2001). So, low availability of free iron in the host represents a major stress for bacterial pathogens. To understand the responses of *H. parasuis* to the iron restriction, the Selective Capture of Transcribed Sequences (SCOTS) technique (Graham and Clark-Curtiss 1999) was used in this study to evaluate the transcriptional profiles of *H. parasuis* grown under in vitro iron-restricted conditions mimicking the in vivo environment.

Materials and methods

Bacterial strains, plasmids, primers and culture conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. The *H. parasuis* 0165 used in this study is a highly virulent strain of serovar 5 isolated from the lung of a commercial pig with hemorrhagic pneumonia, fibrinous polyserositis, arthritis and meningitis. *H. parasuis* was cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) supplemented with 10 μg/ml nicotinamide adenine dinucleotide (NAD) and 5% bovine serum at 37°C. *Escherichia coli* strains were grown in/on Luria-Bertani (LB) broth/agar.

The growth conditions of iron restriction were performed as follows: Exponential growing cultures ($OD_{600} \sim 0.5$) were split into separate culture tubes containing 5 ml medium in each. One culture was grown under normal conditions (37°C, 250 r/mim; $OD_{600} \sim 0.5$) whilst the other was restricted by adding 2, 2′-dipyridyl (Sigma) to the growth medium to give a final concentration of 200 μM.

RNA isolation, cDNA synthesis and amplification

Total RNA was extracted from cells of *H. parasuis* 0165 at an optical density of 0.5 using TRIzol reagent (Invitrogen) according to the manufacture's instructions. RNA samples of bacteria cultured in normal conditions and iron-limitation were treated with RNase-free DNase I (Fermentas) and RNA concentrations and integrity were evaluated by A260/A280 spectrophotometer readings and agarose gel electrophoresis, respectively. Total RNA from three parallel samples was pooled together and converted to first-strand cDNAs by random priming with Superscript II reverse transcriptase (Invitrogen). Random priming was performed as described (Froussard 1992) using oligonucleotides containing terminal sequences at the 5′-end (5′-GTGGTACCGCTCTCCGTCCG-3′ and 5′-CTACGCATGCTCGAGGTACC-3′ for cDNAs from the iron-restriction and normal broth culture, respectively) and a random hexamer at the 3′-end (PCR primer-dN6). cDNAs were made double-stranded with Klenow fragment (Fermentas) as described (Froussard 1992). cDNAs were amplified by PCR with 30 cycles of amplification (95°C for 30 s, 66°C for 60 s and 72°C for 60 s).

Selective capture of transcribed sequences

Genomic DNA from *H. parasuis* 0165 was biotinylated as described previously (Hou et al. 2002). The biotinylated genomic DNA (12 μg) was mixed with 100 μg of plasmids pSKHP16S, pSKHP23SN and pSKHP23SC carrying *H. parasuis* 16S and 23S *rrnA* DNAs, respectively (Jin et al. 2008). The mixture was sonicated using a microprobe for 5 s at an output power of 25 W (Fisher Scientific 100, USA). After sonication, the mixture was precipitated with 1/10 volume of 3 M sodium acetate and equal volume of isopropanol, and resuspended in 160 μl of 10 mM *N*-(2-hydroxyethyl) piperazine-*N*-(3-propanesulfonic acid; EPPS)/1 mM EDTA. The mixture was divided into 20 samples of 8 μl each. For each round of SCOTS, a sample of the mixture (containing 0.6 μg of *H. parasuis* genomic DNA and 5 μg of *rrnA* DNAs) was denatured by incubation at 98°C for 3 min. Two microliters of 1 M NaCl was added to the mixture and incubated at 67°C for 30 min. This step allowed the plasmid *rrnA* DNAs to hybridize to the *rrnA* sites on the *H. parasuis* genomic DNA, thereby rendering these sites unavailable for hybridization with ribosomal DNA present in the cDNA mixtures. At the same time, total amplified cDNA from either from iron-limitation or broth-grown bacteria in 8 μl of 10 mM EPPS/1 mM EDTA was also denatured at 98°C for 3 min, followed by the addition of 2 μl of 1 M NaCl. The denatured cDNA mixture was added

Table 1 Characteristics of bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Characteristics and/or sequences	Sources/references
Strains		
H. parasuis 0165	Serovar 5, clinical isolate	Jin et al. (2008)
E. coli DH5α	*supE44* △*lacU169* (*φ80lacZ*△*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* △*luxS*	TaKaRa
Plasmid		
pMD18-T	Vector for cloning Taq polymerase-amplified PCR products	TaKaRa
pBluescript II SK(+)	Cloning vector, ampicillin resistant	Stratagene
pSKHP16S	pBluescript II SK(+) containing the 16S rRNA sequence (1,472 bp) of *H. parasuis* 0165	This work
pSKHP23SN	pBluescript II SK(+) containing the 5′-end 1,494 bp fragment of 23S rRNA of *H. parasuis* 0165	This work
pSKHP23SC	pBluescript II SK(+) containing the 3′-end 1,131 bp fragment of 23S rRNA of *H. parasuis* 0165	This work
Primers		
SCOTS-N6-01	5′-GTGGTACCGCTCTCCGTCCG-N6-3′	This work
SCOTS-N6-02	5′-CTACGCATGCTCGAGGTACC-N6-3′	Jin et al. (2008)
SCOTS-01	5′-GTGGTACCGCTCTCCGTCCG-3′	This work
SCOTS-02	5′-CTACGCATGCTCGAGGTACC-3′	Jin et al. (2008)
16S01	5′-CGTACAGGTACCAGAGTTTGATCATGGCTCAG-3′	Jin et al. (2008)
16S02	5′-CGTATCGAGCTCAATGAATCATACCGTGGTAAAC-3′; amplifies the 16S rRNA sequence (1,472 bp)	
23SN01	5′-GACTTAGGTACCACCGGGAGAACTGAAACAT-3′	Jin et al. (2008)
23SN02	5′-CACTACGAGCTCCTAGTTCCTTCACCCGAG-3′; amplifies the 5′-end of 23S rRNA sequence (1,494 bp)	
23SC01	5′-GACTTAGGTACCCCGACACAGGTGGTCAG-3′	Jin et al. (2008)
23SC02	5′-CACTACGAGCTCCTTATCTCTTCCGCATTTAG-3′; amplifies the 3′-end of 23S rRNA sequence (1,131 bp)	
fbpA01	5′-TTGGTTGCCACTGAGATA-3′	This work
fbpA02	5′-AACACTACGGTGAAGAAAA-3′; amplifies the sequence of *H. parasuis* (317 bp)	
rplM01	5′-AGTTGGCTTTGCTCACG-3′	This work
rplM02	5′-AACGGGCATAATCCACA-3′; amplifies the sequence of *H. parasuis* (293 bp)	
luxS01	5′-TTTTGTCCGTCCAAACAT-3′	This work
luxS02	5′-TTTCATCAGGCACTTTCT-3′; amplifies the sequence of *H. parasuis* (239 bp)	
tonB01	5′-ATAGTGGCTCAGCAAGTGG-3′	This work
tonB02	5′-TTAGCGATGTAGGGAAACC-3′; amplifies the sequence of *H. parasuis* (279 bp)	
exbB01	5′-AAACAAGCGGTCTGAAATT -3′	This work
exbB02	5′-ATGGCGACAAATAATCCC-3′; amplifies the sequence of *H. parasuis* (279 bp)	
exbD01	5′-GCTTGAACTACCAACCG-3′	This work
exbD02	5′-GCCAATTTTAGTTAGCC-3′; amplifies the sequence of *H. parasuis* (253 bp)	
tbpA01	5′-GTAGAGCAAGGACGAGGTG-3′	This work

Table 1 continued

Strain, plasmid or primer	Characteristics and/or sequences	Sources/references
tbpA02	5′-TGCTGCTGTAGGCACTTT-3′; amplifies the sequence of *H. parasuis* (313 bp)	
tbpB01	5′-AATGTTGACACCGTTCGT-3′	This work
tbpB02	5′-TTGGCTGTTTCACCCTCT-3′; amplifies the sequence of *H. parasuis* (254 bp)	
yfeCD01	5′-GCATTAGATGTTGATCGGTTTC-3′	This work
yfeCD02	5′-TTGCCATAATCTGTGCTGTG-3′; amplifies the sequence of *H. parasuis* 291(bp)	
recN01	5′-TATTAGCGGAAATGGCAACG-3′	This work
recN02	5′-CCGAATAGCACGATAGAGCA-3′; amplifies the sequence of *H. parasuis* (287 bp)	
hrpA01	5′-AAACGCTAAACAAGCCATCA-3′	This work
hrpA02	5′-CCCGATTAGTCCTTTCACCC-3′; amplifies the sequence of *H. parasuis* (262 bp)	
ccmA01	5′-GAGTGGTCAATGGTGGCA-3′	This work
ccmA02	5′-TTCTCGCCCAATCAACG-3′; amplifies the sequence of *H. parasuis* (298 bp)	
acrB01	5′-AGTTGCCTGATACTACGGG-3′	This work
arcB02	5′-ATCGGTTCTGACAAGACTACAT-3′; amplifies the sequence of *H. parasuis* (259 bp)	
ptnC01	5′-GCGATTGAAACAGGAGACT-3′	This work
ptnC02	5′-GTAGAAGAATGGCATTAAGTGA-3′; amplifies the sequence of *H. parasuis* (255 bp)	
gmhA01	5′-AAGTGATCGTTTGTGGCTAT-3′	This work
gmhA02	5′-ATTTAAGACCGCTTGTTCG-3′; amplifies the sequence of *H. parasuis* (254 bp)	

to the biotinylated chromosomal DNA-*rrnA* prehybridized mixture, and hybridization continued at 67°C for 24 h. cDNA was captured by streptavidin-coated magnetic beads (Dynal M280, USA) according the manufacturer's instructions. The process of elution was performed as described previously (Hou et al. 2002). For each growth condition, in the first round of SCOTS, 10 separate samples of the cDNA mixtures were captured by hybridization to rDNA-blocked genomic DNA in parallel reactions.

Enrichment for cDNA molecules under iron restriction

To identify cDNA molecules that represent transcripts from genes that specific or up-regulated for expression during growth of *H. parasuis* under iron limitation, an additional step was included in the experiments. Preparations of cDNA mixtures from *H. parasuis* grown under iron-limitation that had been obtained by three rounds of SCOTS were added to biotinylated genomic DNA that had been prehybridized with both rDNA and cDNA preparations from broth-grown *H. parasuis* (also obtained by three rounds of SCOTS). Hybridization proceeded for 24 h at 67°C, and the hybridized molecules were recovered by binding to streptavidin-coated beads as described above.

Analysis of individual cDNA clones

The up-regulated cDNAs under iron-limitation were ligated with cloning vector pMD18-T (TaKaRa). For verification, cloned inserts were amplified by PCR, spotted onto nylon membranes, and subjected to Southern dot blot analysis with digoxigenin (DIG)-labeled *H. parasuis* genomic DNA, iron-restricted and normal culture cDNA pools obtained after three rounds of normalization, respectively. *H. parasuis*

genomic DNA (1 or 0.1 μg) spotted on nylon membranes were used as positive controls exposed alongside their corresponding SCOTS clone dot blots. Hybridization signals stronger than 1 μg of genomic DNA or weaker than 0.1 μg of genomic DNA were deemed as strong or weak signals, respectively. The positive sequences of these clones should satisfy the requirement that signals were weaker or absent when hybridized to the normalized culture-specific cDNA and strong on the blot hybridizing to normalized cDNA from iron restriction, indicating that these sequences are either absent or present in much less abundance in cDNA prepared from *H. parasuis* grown under standard culture conditions (Fig. 1).

The inserts of positive cDNA clones from the pMD18-T library were sequenced using the standard Sanger method. Database searches, DNA and protein similarity comparisons were carried out by using the BLAST algorithm from the National Center for Biotechnology Information at the National Library of Medicine (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

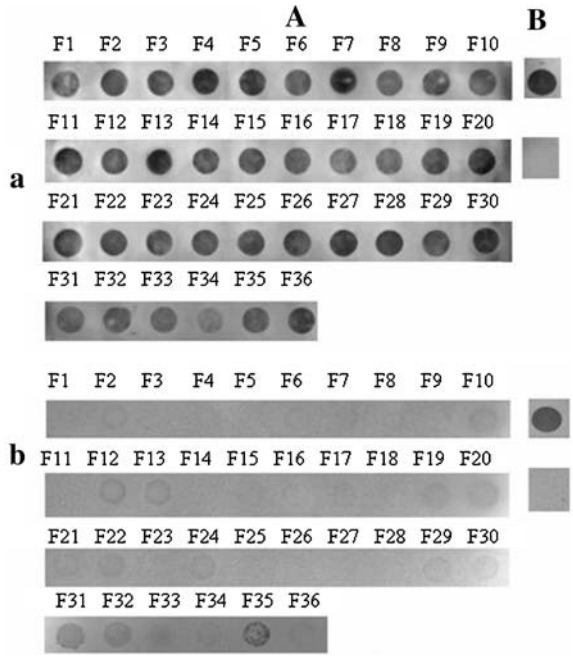

Fig. 1 Southern dot blot showing (*A*) SCOTS clones and (*B*) 0.1 μg (*top*) or 1 μg (*bottom*) of genomic *H. parasuis* DNA as positive controls, hybridized to a digoxigenin (*DIG*)-labeled probe generated from iron-limitation (**a**) or normal culture-derived cDNA (**b**) after three rounds of normalization

Dot blot hybridization

PCR amplicons of positive SCOTS clones were transferred to a positively charged membrane (Roche, Germany). Samples of *H. parasuis* genomic DNA and cDNA mixtures generated from iron restriction and normal growth conditions were labeled with DIG-dUTP (Roche) and used as probes. Dot blot hybridization using DIG Easy Hyb (Roche) was in accordance with the manufacturer's instructions.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was carried out in an Applied Biosystems 9700 instrument. Two samples were iron-limitation and normal culture cDNA pools obtained after three rounds of normalization. All reactions were run in triplicate with a cDNA template from normal culture cDNA as negative control. The fluorescent dye SYBR Green I (TaKaRa) was used in the PCRs. The sequences of primers for real-time PCR are shown in Table 1. The following cycles were performed: 3 min at 95°C followed by 40 amplification cycles of 95°C 30 s, 60°C 30 s, and 72°C 40 s. The C(t) value in the 40 cycles was recorded and a passive reference dye (ROX; TaKaRa) was included in the PCR buffer providing an internal reference to which the fluorescent green dsDNA complex signal was normalized. The 16S rRNA of *H. parasuis* from the same sample was used as internal control and the relative contents of the copy numbers of the target gene's mRNA were calculated, through which we could determine the gene expression level and its trend of change.

Results and discussion

Identification of *H. parasuis* genes preferentially expressed under iron-restricted conditions via SCOTS

To investigate the transcriptional responses of *H. parasuis* to iron restriction, the highly virulent strain 0165 of serotype 5 was grown in TSB broth containing 200 μM of 2, 2′-dipyridyl, a concentration sufficient to cause iron restriction (Melnikow et al. 2005). Using SCOTS approach, a total of 36 up-regulated genes were identified under iron limitation compared to normal

culture condition (Fig. 1; Table 2). The 36 genes could be divided into six functional groups: cell surface protein, DNA metabolism, energy metabolism, sugar metabolism, amino acid and protein synthesis, and transport and binding proteins (Table 2). To validate the results of SCOTS, six genes were selected for transcript level analysis using qRT-PCR: *recN* and *hrpA* implicated in DNA replication and repair, *ccmA* and *acrB* related to respiration, and *ptnC* and *gmhA* involved in sugar metabolism. The resulted showed that all the six genes were up-regulated more than 33-fold under the iron-limitation compared with normal cultural condition (Fig. 2).

Regarding gene expression of *H. parasuis* under iron restriction, two previous studies have been reported (Melnikow et al. 2005; Metcalf and MacInnes 2007). Using differential-display RT-PCR, Metcalf and MacInnes identified nine *H. parasuis* genes up-regulated in response to iron restriction, including homologues of genes encoding fructose bisphosphate aldolase (*fba*), adenylosuccinate synthetase (*purA*), 29, 39-cyclic nucleotide phosphodiesterase (*cpdB*), lipoprotein signal peptidase (*lspA*), pyrophosphate reductase (*lytB*), superoxide dismutase (*sodC*), tyrosyl t-RNA synthetase (*tyrS*), cysteine synthetase (*cysK*), an unknown protein, and a homologue of a hydrolase of the haloacid dehydrogenase superfamily (Metcalf and MacInnes 2007), but none of the nine genes falls in the list of Table 2 of this study. With the help of a microarray, Melnikow et al. (2005) identified 16 genes up-regulated under iron restriction. According to the functional annotation, only 2 genes identified in this study were associated with the genes identified by DNA microarray from *H. parasuis* under iron restriction (Melnikow et al. 2005). The products of the 2 genes included chaperone protein F36 or P45_B05 (the clone ID given by Melnikow et al. 2005; the same hereinafter), the phosphotransferase system (PTS) enzyme IIC F32 or P11_A05 homologous with DQ127925 (23% identity) identified in this study (Melnikow et al. 2005).

Genes expressed differentially under iron restriction

The major response of *H. parasuis* to iron restriction was the induction of genes involved in iron transport, probably to counter-balance effects of 2, 2′-dipyridyl. To obtain host iron, successful bacteria would employ one of the following strategies: (1) direct uptake of iron from host iron-containing molecules via specific receptors including receptors for hemin, hemoglobin, transferrin and lactoferrin; (2) production of siderophores that chelates iron from host iron-binding proteins, followed by uptake of the ferric siderophore via specific cell surface receptors, the iron-regulated membrane/envelope proteins (Sritharan 2006). In *H. parasuis*, like *Actinobacillus pleuropneumoniae*, the *tonB-exbBD-tbpBA* organization was simultaneous to carry out efficiently the uptake of iron bound to transferrin, indicating that the expression of these genes is likely to be under the transcriptional control of the same promoter (del Río et al. 2005). The *tonB* gene showed the highest level of up-regulation, and genes *exbB*, *exbD, tbpA* and tbpB which are transcriptionally linked to *tonB* were shown by Southern dot blot analysis to be also up-regulated (Fig. 1).

Our identification of a putative Yfe system was also of seminal interest. The Yfe system was first identified in *Yersinia pestis* and shown to allow chelated iron utilization in an *E. coli* mutant lacking enterobactin (Bearden et al. 1998). Later studies showed that the *yfeABCD* genes code for a periplasmic binding protein-dependent transport system belonging to the superfamily of ABC transporters, implicated in iron and manganese acquisition, and independent on TonB. The homologue of YfeCD was found, showing 65% nucleotide homology with their counterparts in *A. pleuropneumoniae*, were up-regulation during iron restriction.

Successful pathogens must therefore possess an effective response to regulate gene expression under the iron-limited conditions encountered upon entry into a host (Paustian et al. 2001). Several stress response genes of *H. parasuis* were up-regulated under iron-restricted growth conditions. A homologue to the gene *recN*, encoding a DNA repair protein involved in the SOS response was identified. Although the exact role of RecN in DNA repair and recombination is unclear in any organism, it appears to function in the repair of DNA double-strand breaks (Stohl and Seifert 2006). In *Neisseria gonorrhoeae recN* is the only DNA repair and recombination gene up-regulated in response to hydrogen peroxide (H_2O_2) by microarray analysis and subsequently shown to be important for oxidative damage protection (Stohl and Seifert 2006). In addition, the expression of *recN* is down regulated

Table 2 Genes of *H. parasuis* identified via SCOTS

Clone	Insert (bp)	Gene	Predicted function or property of products	GenBank acc. no.
Cell surface protein				
F1	291	*pilA*	Tfp pilus sssembly protein, major pilin	ZP_02478003.1
DNA metabolism				
F2	457	–	Predicted phage tail protein	YP_001053233.1[a]
F19	614	*sbcB*	Exonuclease I	ZP_02478433.1
F14	540	–	Replicative DNA helicase	ZP_02477758.1
F24	370	*recN*	DNA repair protein	ZP_02478756.1
F26	682	–	Exodeoxyribonuclease V beta chain	ZP_02479309.1
F29	470	*hrpA*	ATP-dependent RNA helicase	ZP_02478503.1
F5	616	–	tRNA (uracil-5-)-methyltransferase	ZP_02479177.1
F22	293	*trpD*	Anthranilate phosphoribosyltransferase	ZP_02477987.1
F30	397	–	Transposase IS4 family protein	ZP_02479496.1
F9	531	–	DNA-dependent helicase II	ZP_02478118.1
F11	508	*comJ*	Protein involved in catabolism of external DNA	ZP_02479136.1
F20	397	–	Transposase IS4 family protein	ZP_02478109.1
Energy metabolism				
F3	483	–	Homoserine kinase	ZP_02477617.1
F18	546	*aroK*	Shikimate kinase	ZP_02478349.1
F4	296	–	Dihydroxy-acid dehydratase	ZP_02478596.1
F28	424	–	3-Hydroxydecanoyl-ACP dehydratase	ZP_02477394.1
F16	399	*ccmA*	Cytochrome C biogenesis ATP-binding export protein	ZP_02477283.1
F7	382	*arcB*	Polynucleotide phosphorylase	ZP_02477898.1
F15	508	–	2-Isopropylmalate synthase	ZP_02478043.1
F25	401	*putA*	Bifunctional protein	ZP_02478770.1
Protein and amino acid synthesis				
F8	480	*clpB*	Chaperone	ZP_02478978.1
F10	414	*sui1*	Translation initiation factor	ZP_02477873.1
F27	414	*aspS*	Aspartyl-tRNA synthetase	ZP_02478507.1
F6	469	*gltB*	Glutamate synthase subunit alpha	ZP_02477869.1
F21	428	–	Putative cytosol aminopeptidase	ZP_02478931.1
Sugar metabolism				
F17	497	*ptnC*	Phosphotransferase system (PTS) enzyme IIC	ZP_02478695.1
F13	459	*gmhA*	Phosphoheptose isomerase	ZP_02477467.1
Transport and binding proteins				
F36	279	*tonB*	Energy transducing protein	ZP_02479012.1
F31	279	*exbB*	Biopolymer transport ExbB protein	ZP_02477372.1
F32	253	*exbD*	Biopolymer transport ExbD protein	ZP_02477373.1
F33	313	*tbpA*	Transferrin binding protein A	AAV68905.1
F34	254	*tbpB*	Transferrin binding protein B	AAV68904.1
F35	291	*yfeCD*	Chelated iron ABC transporter permease	HPS_05815[b]
F23	404	–	Predicted symporter, sodium:solute symporter family	ZP_02478724.1
F12	330	–	Permeases	ZP_02478255.1

[a] *Actinobacillus actinomycetemcomitans*

[b] Nucleotide, locus_tag

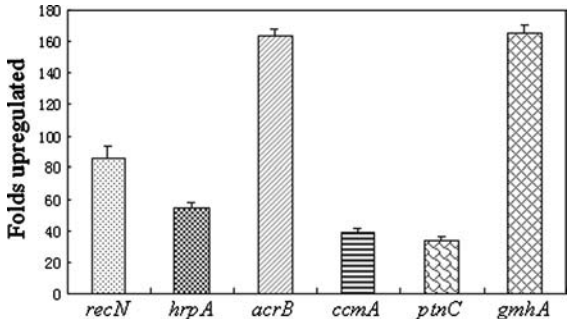

Fig. 2 qRT-PCR analysis of the expression of *recN*, *hrpA*, *arcB*, *ccmA*, *ptnC*, *gmhA*, in iron restriction and culture-specific cDNA pools obtained after three rounds of normalization. Real-time RT-PCR data for each gene relative to that obtained for 16s rRNA gene control. Data points represent the means ± SE of three independent experiments

during growth with a high iron concentration in *N. gonorrhoeae* (Sebastian et al. 2002). It was demonstrated that purified gonococcal Fur protein could bind to the operator regions of *recN* (Sebastian et al. 2002). In this study, the Fur protein of *H. parasuis* was also predicted to bind with the promoter/operator region of *recN* gene in *H. parasuis* using the Virtual Footprint promoter analysis tool (Münch et al. 2005) available at http://prodoric.tu-bs.de/vfp/, suggesting that the upregulated expression of *recN* in *H. parasuis* is related with the regulation of Fur protein.

One of the two subunits of the Clp protease (ClpB) showed up-regulation during iron restriction in this study belongs to the Clp/HSP100 family of molecular chaperons and contributes to rescuing denatured proteins from the aggregated state (Lee et al. 2003). This stress protein was also identified to be up-regulated in *H. parasuis* under heat, acidic, oxygen and iron-limiting growth conditions (Melnikow et al. 2005), and in *A. pleuropneumoniae* under iron restriction (Deslandes et al. 2007) via microarrays. Under the iron limitation the aggregation of proteins which require iron for proper folding develops and the induction of clpB expression under iron-limitation could reflect the participation of ClpB in the disaggregation of proteins (Melnikow et al. 2005).

Additionally, a homologue of ArcB sensor kinase, the component of the Arc (anoxic redox control) two-component signal transduction system, was identified in this study. This system modulates the expression of numerous operons in response to redox conditions of growth (Georgellis et al. 2001). Recent research began to investigate the relationship between ArcB and AI-2 signal transduction pathway and suggest that LuxS and ArcB may act in concert to control the adaptation of *Aggregatibacter actinomycetemcomitans* to iron-limiting conditions and its growth (Fong et al. 2003). In *Vibrio harveyi* AI-2 signal transduction pathway was well characterized and requires the LuxS, LuxP, LuxQ, LuxU, and LuxO polypeptides (Bassler 1999; Xavier and Bassler 2003). The *H. parasuis* protein exhibiting the similarity to LuxQ (32%) and LuxO (27%) was the ArcB sensor/kinase. The LuxP protein was found to be most similar to the *H. parasuis* periplasmic ribose binding protein RbsB (26%). Finally, no homolog of LuxU was identified in *H. parasuis*. All of these are consistent with the conditions of similarity in *A. actinomycetemcomitans* by Fong et al. (2003) They also found ArcB may contribute to the signal transduction cascade that directs the response of *A. actinomycetemcomitans* to AI-2. Inactivation of *arcB* decreased the expression of iron acquisition genes that are regulated by LuxS, such as *afuA*, encoding periplasmic ferric transport protein (Fong et al. 2003). *H. parasuis* 0165 *rplM* was identified with 50% of amino acid identity with *afuA* of *A. pleuropneumoniae.* So when the expression of *arcB* up-regulated, we speculated the expression of *luxS* and *rplM* would be increased as well. To address this hypothesis, the relative transcription levels of these genes were analyzed by qRT-PCR. The results showed that *luxS* and *rplM*, like *arcB*, were significantly up-regulated under iron restriction (Fig. 3). In a word, the induction of ArcB expression under iron-limitation maybe contributes to the over-expression of LuxS, which would regulate some ferric transport protein at the same time.

Virulence genes were also up-regulated under iron restriction. A homologue of the PilA was found to be up-regulated under iron-limitation. PilA, a pleiotropic transcriptional regulator in *N. gonorrhoeae*, encoded by an essential gene *pilA*, regulates pilin gene expression and stress response. It is implicated in protein export, the modulation of cell growth rate in response to different environmental conditions and the resistance to the bactericidal effect of normal human serum (Taha et al. 1991, 1992). The upstream of *pilA*, the iron regulation of pilin can bind the pleotrophic iron-responsive transcriptional regulator Fur in *Moraxella catarrhalis* identified with The FURTA (Fur titration assays; Luke et al. 2004). The

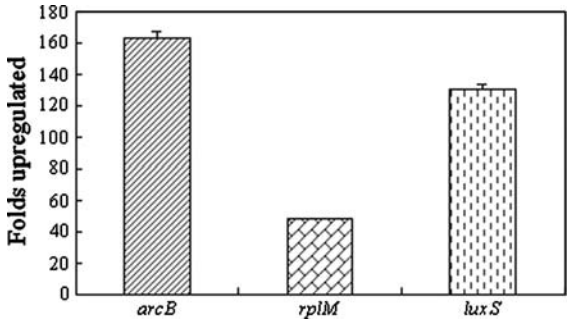

Fig. 3 qRT-PCR analysis of the expression of *arcB*, *rplM*, *luxS* in iron restriction and culture-specific cDNA pools obtained after three rounds of normalization. Real-time RT-PCR data for each gene relative to that obtained for 16s rRNA gene control. Data points represent the means ± SE of three independent experiments

fact that iron limitation has an inducing effect on pilus expression suggest that bacteria are primed to colonize host surfaces and initiate infection by responding to low-iron conditions within the host by upregulating pilus expression (Luke et al. 2004). The identification of pilA in this study by SCOTS supported that iron regulation of pilus expression was a common occurrence in other type IV pilus-expressing mammalian pathogens. Inactivation of pilA reduces the ability of *Vibrio vulnificus* to form biofilms and significantly decreases adherence to HEp-2 cells and virulence in iron dextran-treated mice (Paranjpye and Strom 2005). The PilA protein of non-typable *Haemophilus influenzae* plays an important role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract (Jurcisek and Bakaletz 2007).

A homologue of the GmhA was found to be up-regulated under iron-limitation in this study. GmhA, a phosphoheptose isomerase catalyzing the first step of the biosynthesis of ADP-L-glycero-D-mannoheptose, was involved in lipooligosaccharide expression and virulence in *Haemophilus ducreyi* (Bauer et al. 1998). Mutations in *H. influenzae* were associated with a heptoseless core lipopolysaccharide which determines increased outer membrane permeability to hydrophobic compounds (Brooke and Valvano 1996). A homologue of *gmhA* was also identified by microarray in *Pasteurella multocida* grown in iron-free medium supplemented with hemoglobin (Paustian et al. 2001). Preferential expression of *gmhA* under iron limitation was thought to consistent with the participate in cell surface biosynthesis and presence of a general sensor-effector system in bacteria that responds to stimuli, indicative of in vivo growth, and suggested that iron restriction resulted in modifications and alterations in the bacterial cell surface (Paustian et al. 2001).

In summary, 36 *H. parasuis* genes were identified that were up-regulated in response to iron restriction. Knowledge of the genes involved in adaptation to environments encountered during disease will help elucidate the mechanisms of pathogenesis for this economically significant bacterium. We should focus on the view of a major role of cell surface biosynthesis and presence of a general sensor-effector system in bacteria that responds to iron limitation. Some genes identified were virulence factors in other organism, showing its potential role as vaccine candidates.

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