Novel function assignment to a member of the essential HP1043 response regulator family of epsilon-proteobacteria

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HP1043 of Helicobacter pylori is an orphan response regulator (RR) with a highly degenerate receiver sequence incapable of phosphorylation, which is essential for cell viability. In contrast, the orthologous RR protein of Helicobacter pullorum, an enterohepatic Helicobacter species mainly isolated from poultry, harbours a consensus receiver sequence and is associated with a cognate histidine kinase (HK). Here, we show that this two-component system of H. pullorum, denoted HPMG439/HPMG440, is involved in the control of nitrogen metabolism by regulating the expression of glutamate dehydrogenase, an AmtB ammonium transporter and a P II protein. However, the role of the RR HPMG439 is not restricted to nitrogen regulation since, in contrast with the HK HPMG440, HPMG439 is essential for growth of H. pullorum under nutrient-rich conditions.

INTRODUCTION

The genome of the gastric pathogen Helicobacter pylori encodes only a small number of proteins that are involved in signal transduction and transcriptional regulation (Tomb et al., 1997; Alm et al., 1999). This fact is commonly interpreted to be a consequence of the strict adaptation of the bacterium to its exclusive niche, the human stomach, which is thought to provide a rather constant environment. However, a recent genome-wide analysis demonstrated the presence of quite a large number of small regulatory RNAs and antisense transcripts to annotated genes, suggesting that gene expression control in H. pylori might rely largely on regulatory mechanisms acting at the post-transcriptional level (Sharma et al., 2010). Among the few transcriptional regulators of H. pylori are three two-component systems (TCS), which are involved in the control of processes with fundamental importance for successful colonization, i.e. motility, acid resistance and heavy-metal resistance (Panthe1 et al., 2003; Beier & Frank, 2000; Spohn & Scarlato, 1999; Pflock et al., 2005, 2006; Waidner et al., 2005), as well as two orphan response regulators (RRs) whose functions are less well defined. The orphan RR HP1043 belongs to the OmpR family and contains a strongly degenerate receiver domain lacking the phosphate-accepting aspartic acid residue as well as other conserved amino acids of the consensus sequence. The hp1043 gene could not be deleted unless a second gene copy was integrated into the H. pylori chromosome, demonstrating that HP1043 is essential for cell viability (McDaniel et al., 2001; Schar et al., 2005). From NMR spectroscopy and X-ray crystallography of the receiver it was concluded that HP1043 forms a symmetrical dimer with two functional domains, which are separated by a short linker domain (Hong et al., 2004, 2007; Lee et al., 2006). The interface of the HP1043 dimer, which is formed by the secondary structure elements $\alpha$4-$\beta$5-$\alpha$5 of the receiver domain, strongly resembles that of the active phosphorylated forms of other OmpR-type RRs such as ArcA or PhoB, suggesting that HP1043 function does not require covalent receiver modification (Hong et al., 2007). HP1043 has been shown to bind in vitro to the promoter regions of its own gene and to the tlpB gene encoding a methyl-accepting chemotaxis protein (Delany et al., 2002), yet other target genes accounting for the essentiality of HP1043 have not been identified so far.

Helicobacter pullorum is an enterohepatic species which was originally isolated from the caeca and livers of broiler and laying hens, and from faeces of humans with diarrhoeal disease (Stanley et al., 1994). Infection with H. pullorum is prevalent among farm-raised birds like chickens, turkeys and guinea fowls (Nebbia et al., 2007; Zanoni et al., 2007, ...
2011). Recently, natural infection of laboratory mice and rats from a commercial rodent breeding facility in the USA has also been reported (Boutin et al., 2010). In both chickens and rodents the preferred colonization sites of H. pullorum are the caecum and the colon (Ceelen et al., 2006; Cacioppo et al., 2012; Turk et al., 2012). Although H. pullorum infection in chickens has been linked to vibriotic hepatitis and enteritis (Stanley et al., 1994), experimental infection of broiler chickens did not result in overt clinical signs and resulted in only mild lesions in the caeca, with no detectable histological changes in the liver (Ceelen et al., 2007). Similarly, rodents which were experimentally infected with H. pullorum did not develop histological changes of the liver and intestinal tract (Cacioppo et al., 2012; Turk et al., 2012). However, H. pullorum has been isolated from patients with diarrhoeal illness (Burnens et al., 1994; Stanley et al., 1994; Steinbrueckner et al., 1997) and bacteraemia (Schwarzew-Zander et al., 2010; Tee et al., 2001), and has been associated with inflammatory bowel and hepatobiliary disease in humans (Hansen et al., 2011; Castéra et al., 2006; Fox et al., 1998; Veijola et al., 2007). Although H. pullorum is now considered an emerging human pathogen, apart from an analysis of its N-linked protein glycosylation system (Jervis et al., 2011), there have been other studies showing that H. pullorum is a human pathogen, apart from an analysis of its protein glycosylation system (Jervis et al., 2011) and has been associated with inflammatory bowel and hepatobiliary disease in humans (Hansen et al., 2011; Castéra et al., 2006; Fox et al., 1998; Veijola et al., 2007).

### METHODS

**Bacterial strains and growth conditions.** H. pylori G27 and H. pullorum NCTC12827 are clinical isolates and have been described previously (Xiang et al., 1995; Stanley et al., 1994). H. pylori and H. pullorum strains were grown at 37 °C under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood and Dent’s antibiotic supplement. Liquid cultures were grown in brain heart infusion broth containing Dent’s antibiotic supplement and 10% FCS. When required, blood agar plates or liquid broth for Helicobacter culture were supplemented with kanamycin or chloramphenicol at a final concentration of 20 µg ml⁻¹. Escherichia coli strain DH5α was grown in lysogeny broth which was supplemented with antibiotics at the following final concentrations if required: 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, and 30 µg chloramphenicol ml⁻¹.

**Construction of H. pylori and H. pullorum mutants.** Helicobacter mutants were obtained via natural transformation (H. pylori G27) or electroporation (H. pullorum) with appropriate suicide plasmids, as described previously (Ferrero et al., 1992; Beier et al., 1997). The PCR fragments generated with the primers listed in Table S1 (available with the online version of this paper) were cloned into pSL1180 vector DNA and were checked by sequence analysis for correct amplification. The Helicobacter mutants were analysed by PCR with appropriate primer combinations. PCR was performed using ReproFast DNA polymerase (Genaxxon Bioscience) with the following conditions: an initial denaturation of 95 °C for 3 min; followed by 30 cycles each of 95 °C for 30 sec, 50 °C for 30 s and 72 °C for 30 s–1.5 min (30 s per 500 bp to be amplified); and a final polymerization of 72 °C for 10 min.

**H. pylori G27/439Δ.** An 833 bp BamHI–KpnI fragment comprising ORF HPMG439 and its upstream region was amplified with primer pair S439-5/S439-3 and was cloned between DNA fragments derived from the cagCD and cagA genes from H. pylori [830 bp, EcoRI–BamHI, amplified with primer pair cagD-5/cagC-3; 862 bp, KpnI–SacI, amplified with primer pair cagA-5(KpnI)/cagA-3]. Finally, a chloramphenicol resistance cassette from Campylobacter coli (Wang & Taylor, 1990) was inserted downstream of hpmg439. The resulting plasmid was transformed into H. pylori G27. A chloramphenicol-resistant clone with the desired insertion of hpmg439 into the cag locus (designated G27/439) was then transformed with knockout plasmid pSL-R35, which contained the aphA gene of Campylobacter coli (Labigne-Roussel et al., 1988) flank by a 793 bp PstI–SacI fragment and a 510 bp EcoRI–BamHI fragment derived from the upstream and downstream regions, respectively, of hpi1043 (amplified with primers pairs Δ5490-12/Δ1043-2 and Δ1043-3/Δ1043-4).

**H. pullorum Δ440.** In the suicide plasmid pSLΔ440, used for the construction of H. pullorum Δ440, the aphA gene of Campylobacter coli is flanked by a 507 bp EcoRI–BamHI fragment (amplified with primer pair Δ440-1/Δ440-2) and a 610 bp PstI–SacI fragment (amplified with primer pair Δ440-3/Δ440-4) derived from the upstream and downstream regions of hpmg439, respectively.

**Knockout of HPMG439.** In order to delete hpmg439 the suicide plasmids pSLΔ439-1 and pSLΔ439-2 were constructed. The plasmid pSLΔ439-1 contained PCR fragments derived from the coding and downstream sequences of hpmg439, which were amplified with primer pairs Δ439-12/Δ439-22 (494 bp, EcoRI–BamHI) and Δ439-3/Δ439-4 (506 bp, PstI–SacI) flanking the aphA gene from Campylobacter coli. Plasmid pSLΔ439-2 was obtained by replacing the 494 bp EcoRI–BamHI fragment in pSLΔ439-1 by a 601 bp EcoRI–BamHI fragment (amplified with primer pair Δ437-2/Δ439-23) from the upstream region of hpmg439. In both plasmids the non-polar kanamycin resistance cassette had the same transcriptional orientation as hpmg439 and hpmg440.

**H. pullorum S440Δ.** In order to reintegrate hpmg440 into H. pullorum Δ440 the upstream region of hpmg439 was fused to ORF HPMG440 via recombinant PCR providing a Shine–Dalgarno (SD) sequence for efficient translation of the hpmg440 mRNA. Overlapping PCR fragments were amplified with primer pairs S439-5/440-com1 (170 bp) and 440-com2/S440-3 (1361 bp). The amplified fragments were used as templates in a PCR with primer pair S439-5/S440-3, yielding a DNA fragment of 1501 bp which was cloned between DNA fragments derived from the H. pullorum flaA gene (hpmg4128) and its 3’ flanking region (fragment 1–422 bp, EcoRI–BamHI, amplified with primer pair fla-12/fla-22; fragment 2–537 bp, PstI–SacI, amplified with fla-3/fla-4). Finally, a chloramphenicol resistance cassette was inserted downstream of hpmg440. The resulting plasmid, pSLΔ440C, was used for the transformation of H. pullorum Δ440.

**H. pullorum S439 and H. pullorum S439Δ.** An 833 bp BamHI–KpnI fragment comprising ORF HPMG439 and its upstream region was amplified with primer pair S439-5/S439-3 and was cloned between DNA fragments derived from the flaA gene of H. pullorum and its 3’ flanking region. A chloramphenicol resistance cassette was inserted downstream of hpmg439 and the resulting plasmid pSLΔ439 was transformed into H. pullorum, yielding the strain H. pullorum S439, which was subsequently transformed with plasmid pSLΔ439-2 to delete the natural copy of hpmg439.
H. pullorum \text{S439DS1N} and H. pullorum \text{S439DS1N}\Delta. A mutated hmpg439 allele, containing a substitution of the phosphate-accepting aspartic acid residue D51 with asparagine, was constructed via recombinant PCR. Overlapping PCR fragments with the desired point mutations were amplified with primer pairs S439-5/S439-N2 (308 bp) and 439-N1/S439-3 (558 bp). The annealed PCR products were used as templates in a PCR with primer pair S439-5/S439-3, resulting in an 833 bp \text{BanHI–KpnI} fragment comprising the mutated hmpg439 allele as well as the promoter region of hmpg439. The wild-type allele in plasmid pSL439 was replaced by the hmpg439 D51N allele, and the resulting plasmid pSL439D51N was used to transform H. pullorum, yielding strain S439D51N. This strain was subsequently transformed with suicide plasmid pSLA439-3 which was constructed by \text{PstI–SacI} digestion of pSLA439-2 and insertion of a 507 bp fragment obtained via PCR with primer pair A439-32/A439-4, using pSL440C as template DNA. Thereby, hmpg440 which presumably is translationally coupled to hmpg439 in the H. pullorum wild-type is provided with a 5D sequence in the H. pullorum mutant S439D51N. Chloramphenicol- and kanamycin-resistant clones from the transformation of H. pullorum S439D51N with pSLA439-3 were checked for the presence of the D51N mutation by sequence analysis of an appropriate PCR fragment.

Separation of proteins by 2D gel electrophoresis and protein identification. H. pullorum cells grown on Columbia agar plates were harvested, washed once in 1 M Tris/HCl, pH 7.0, and resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 2 % CHAPS, 40 mM Tris, 1 % \text{Zwittergent 3–10} (Calbiochem), 0.8 % carrier ampholyte 3–10 (Serva) and 20 mM DTT. The cells were disrupted by glass bead homogenization in a FastPrep machine (MP). The supernatant obtained after centrifugation (5 min, 12000 g, 4 °C) was stored at −20 °C. Three biological replicates were generated. Immobiline DryStrips (18 cm; non-linear pH 3–11 gradient) were rehydrated overnight with 340 µl DeStreak Rehydration Solution (GE Healthcare) supplemented with 1 % IPG buffer (pH 3–11 non-linear; GE Healthcare) using an Immobiline DryStrip reswelling tray (GE Healthcare). Total protein (250 µg) was loaded onto the Immobiline DryStrips via cup loading. Isoelectric focusing was performed using an IPGPhor isoelectric focusing unit (GE Healthcare) by sequentially applying 300 V for 4 h, 600 V for 6 h, 1000 V for 4 h, 8000 V for 5 h and finally 8000 V until 24000 Vh was reached. The strips were equilibrated for 15 min in equilibration buffer (6 M urea, 35 % glycine, 2 % SDS, 75 mM Tris/HCl, pH 8.8, 0.1 % bromophenol blue) containing 1 % DTT and then for 15 min in equilibration buffer containing 2.5 % iodoacetamide. The strips were sealed onto 12 % SDS polyacrylamide gels and gel electrophoresis was run overnight at 18 °C in a Hoefer DALT vertical system (Pharmacia) by applying 80 V. The gels were stained with the colloidal Coomassie stain and documented using ImageScanner III (GE Healthcare). At least three technical replicates were performed per biological replicate. Images were analysed with software Progenesis SameSpots (Non-linear Dynamics).

For MS, gel bands derived from 2D PAGE were washed, reduced, alkylated and dried equilibrated, and proteins were proteolytically digested with trypsin as described previously (Winkler et al., 2007). Generated peptides were extracted twice with 20 µl 0.1 % trifluoroacetic acid and once with 20 µl 0.1 % trifluoroacetic acid with 60 % acetonitrile for 15 min at 37 °C. The acetonitrile content of the pooled extracts was subsequently reduced by evaporation in a SpeedVac (Thermo Fisher Scientific) prior to MS analysis. Peptide mixtures were analysed by nano-LC-MS/MS on an Orbitrap XL (Thermo). An UltiMate 3000 RSLCnano-LC system (Thermo Scientific) was used in a preconcentration set-up comprising a 100 µm x 2 cm pre-column (Acclaim PepMap 100, pre-column, C18, 5 µm, 10 mm; Thermo Scientific) and a 75 µm x 15 cm Acclaim PepMap RSLC separation column (C18, 2 µm, 10 mm; Thermo Scientific). Peptides were separated using a binary gradient (solvent A, 0.1 % formic acid; solvent B, 0.1 % formic acid, 84 % acetonitrile) from 5 to 50 % B in 52 min, at a flow rate of 300 nl min⁻¹ and tempered to 35 °C. For detection of analytes, a survey scan (300–2000 m/z) was followed by tandem MS scans of the five precursor ions with the highest intensities. Data interpretation was carried out using Proteome Discoverer 1.2 (Thermo Scientific). Thus, all data were searched against the Uniprot_HPullorum database (Feb 2012; 2035 sequences) using Mascot 2.3.2 (MatrixScience) with the following settings: trypsin was chosen as a protease with a maximum of two missed cleavage sites. Acetylation of the N-terminus, carboxymethylation of cysteine, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as dynamic modifications. MS tolerance was set to 10 p.p.m. and MS/MS tolerances to 0.5 Da (# 13C=1). Only peptides with Mascot scores above 30 (P<0.05) were considered for further validation.

**Immunoblot analysis.** Western blot analysis was performed as described previously (Müller et al., 2007).

Preparation of RNA and quantitative real-time (qRT) PCR. mRNA was isolated from H. pullorum using TRI-Reagent LS (Sigma) and was further purified using the RNasy Mini kit (Qiagen). cDNA was prepared from 1 µg RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas). The reaction mixture was diluted 25-fold. qRT-PCR samples contained 5 µl diluted cDNA, 10 µl PerfeCTa SYBR Green FastMix, Rox (Quanta Biosciences) and gene-specific primers (Table S1) in a concentration of 250 nM in a final volume of 20 µl. qRT-PCR was run on a StepOne Plus Real-time PCR System (Applied Biosciences). The relative expression levels of the genes studied was normalized to the housekeeping gene \text{gpoB}. Data were analysed using the ΔΔC\text{t} method as described by Livak & Schmittgen (2001). Three qRT-PCR experiments were performed in triplicate with cDNA, which was reverse transcribed from independent RNA preparations.

Mapping of transcriptional start sites. Transcriptional start sites were mapped by the rapid amplification of 5′ cDNA ends (RACE) using the 5′ RACE System (Invitrogen). The oligonucleotide primers used are listed in Table S1 and are specific for \text{gdhA} (hmpg199), \text{amtB} or \text{ghk} (hmpg1845 and hmpg1846) and hmpg439.

**RESULTS**

HMPG439 can replace its essential orthologue HP1043 in H. pylori

RR HP1043 is highly conserved in the \(\varepsilon\)-proteobacteria. **BLAST** analysis (www.ncbi.nlm.nih.gov/protein) revealed an average sequence identity and similarity of 62.9 % and 76.2 %, respectively, for the bacterial species from this group. When only the DNA-binding effector domain of the RR was considered, sequence conservation was even more pronounced (84.6 % identity, 92.9 % similarity), suggesting similar DNA-binding properties of the HP1043 orthologues. While in *Wolinella succinogenes*, many *Campylobacter* species, *Arcobacter butzleri* and *Arcobacter nitrofigilis*, and the environmental \(\varepsilon\)-proteobacteria the hp1043 orthologue is flanked by a gene encoding the cognate HK, the HP1043 orthologues of the members of the genus *Helicobacter* are mostly orphan RRs with degenerate receiver sequences. A complete TCS is present only in the enterohpetic species *H. pullorum*, *Helicobacter*...
canadensis and Helicobacter whigmansensis, suggesting that in these organisms the activity of the HP1043 orthologue is still linked to an external or internal stimulus. We set out to characterize the H. pullorum TCS, consisting of the HP1043 orthologue HPMG439 and its cognate HK HPMG440, by testing whether HP1043 in H. pylori can be functionally replaced by the RR HPMG439, which shows 65% identity and 77% similarity to the H. pylori orthologue. The genome sequence of H. pullorum MIT 98-5489 (www.broadinstitute.org) suggests that the RR gene hpmg439 is co-transcribed with the cognate HK gene hpmg440 and ORF hmg441 encoding a conserved protein of unknown function from a promoter (P439) with high similarity to the hp1043-specific promoter of H. pylori (Delany et al., 2002). This operon structure was confirmed by RT-PCR (Fig. S1). Since we assumed that P439 would be recognized in H. pylori the hpmg439 gene and 146 bp of its upstream sequence as well as a chloramphenicol resistance cassette were integrated into the cag pathogenicity island of H. pylori G27 by homologous recombination, thereby replacing the genes cagC and cagB, and the 5’ part of cagA. The resulting strain G27/439 produced similar amounts of the RR proteins HP1043 and HPMG439, as demonstrated by immunoblot analysis with a HP1043-specific polyclonal antibody (Fig. 1). Unexpectedly, the two RR proteins which have the same size (223 aa, 25.5 kDa) showed remarkably different electrophoretic mobilities. However, since this result was also observed when the recombinant proteins were expressed in E. coli, a H. pylori-specific modification of HP1043 accounting for this anomaly can be ruled out (Fig. 1). Transformation of G27/439 with a suicide plasmid designed for the substitution of the essential gene hp1043 with the apha gene of Campylobacter coli yielded strain G27/439Δ. The mutant which, according to immunoblot analysis, produces only HPMG439 (Fig. 1) grew equally well as the G27 wild-type both on plate and in liquid culture (data not shown), demonstrating that the consensus RR HPMG439 of H. pullorum can functionally replace the atypical HP1043 protein in H. pylori.

**RR HPMG439 is essential, while HK HPMG440 is not**

To delete the genes encoding RR HPMG439 and HK HPMG440, respectively, appropriate suicide plasmids were constructed. Transformation of H. pullorum with plasmid pSLA440 yielded clones whose characterization proved the substitution of hpmg440 with the apha gene of Campylobacter coli. However, in several independent attempts to create hpmg439 deletion mutants via transformation of H. pullorum with two different suicide plasmids, pSLA439-1 and pSLA439-2, no transformants were obtained, suggesting that HPMG439 is essential, as is its H. pylori orthologue HP1043. To prove this assumption we intended to integrate a second copy of hpmg439 and its upstream region into the chromosome of H. pullorum and to delete the primary allele from this mutant in a subsequent step. As the swarming behaviour of H. pullorum Δ440 on soft agar plates was not altered compared with that of the wild-type (data not shown), a plasmid for insertion of the second hpmg439 allele into the flaA gene was constructed. The successful deletion of flaA had previously demonstrated that, as expected, motility was dispensable for in vitro growth of H. pullorum (data not shown). Clones from the transformation with this plasmid carried the desired insertion of hpmg439 in the flaA gene. One clone was subsequently transformed with suicide plasmid pSLA439-2, yielding mutant H. pullorum S439A which, according to PCR analysis, carries a deletion of the RR gene in the hpmg439–hpmg440 locus, thus confirming that hpmg439 is an essential gene in H. pullorum. The observation that deletion of hpmg440 did not affect the in vitro growth of H. pullorum suggested that phosphorylation is dispensable for the essential function of HPMG439. Therefore, strain H. pullorum S439D51N carrying a mutated hpmg439 allele with a substitution of the phosphate-accepting aspartic acid residue D51 with asparagine inserted into the flaA gene was constructed. By the subsequent deletion of the wild-type allele, HPMG439 was substituted by the phosphorylation-deficient HPMG439D51N protein in strain H. pullorum S439D51NΔ, arguing in favour of an essential function of the unphosphorylated RR.

**HPMG440 is involved in the regulation of nitrogen metabolism**

In order to identify genes which are controlled by the HPMG439–HPMG440 TCS, comparative proteome analysis was performed with H. pullorum Δ440 and the isogenic wild-type strain harvested from blood agar plates. As shown in Fig. 2, 13 protein spots were found to be differentially expressed in H. pullorum Δ440. Three protein spots were slightly downregulated in the mutant, while ten
H. pullorum and H. pullorum Δ440 were found to be more highly expressed in wild-type pullorum D pullorum wild-type (WT) and mutant (Δ440) are marked by arrows. Spots 1, 2 and 3 were found to be more highly expressed in wild-type H. pullorum, while the remaining spots were overexpressed in H. pullorum Δ440.

spots were found to be upregulated. Nine of the upregulated spots showed similar isoelectric points and almost identical electrophoretic mobility in the second dimension (4, 5, 6, 7, 8, 9, 10, 11 and 12 in Fig. 2) and were identified as glutamate dehydrogenase (GdhA) by MS, while the remaining spots could not be identified unambiguously. To test differential expression of gdhA at the transcriptional level qRT-PCR was performed on cDNA reverse transcribed from RNA isolated from H. pullorum Δ440 and wild-type bacteria grown on blood agar plates and in liquid culture, respectively. Irrespective of the growth conditions a strong increase in the amount of gdhA-specific mRNA was observed in H. pullorum Δ440 (Fig. 3a), suggesting repression of gdhA transcription by HPMG439–HPMG440 under nutrient-rich conditions and a role of the TCS in the regulation of nitrogen assimilation. Therefore, the transcription of other genes involved in nitrogen metabolism was analysed. H. pullorum, like H. pylori, encodes gdhA and glutamine synthetase (glnA), but no glutamine 2-oxoglutarate aminotransferase (GOGAT). In contrast with H. pylori, H. pullorum contains a glnK–amtB operon (hpmg1846–hpmg1845) encoding a small regulatory protein of the PII family and an ammonium transporter. While compared with the wild-type, glnA transcription was not significantly altered in H. pullorum Δ440, amtB transcription was strongly derepressed in the mutant (Fig. 3a). The transcription of the genes encoding aspartate aminotransferase (HPMG1773), L-asparaginase (HPMG535) and aspartase (HPMG484), which catalyse the interconversion between glutamate and aspartate, the synthesis of aspartate from asparagine and the conversion of aspartate to fumarate, respectively, was not significantly affected by the deletion of the HK gene hpmg440, nor was transcription of gltS, encoding a glutamate transporter (HPMG866) (Fig. 3b).

To verify that derepression of gdhA and glnK–amtB in H. pullorum Δ440 is due to the knockout of the HK gene, we attempted complementation via insertion of hpmg440 into the flagellin gene of the deletion mutant. For this purpose hpmg440 was fused to the promoter region of the upstream RR gene hpmg439 and an artificial SD sequence was introduced upstream of the start codon of hpmg440. It should be noted that the sequence of H. pullorum NCTC12872 differs from that of the sequenced strain MIT 98-5489 by a 14 bp deletion located 27 bp downstream of the stop codon of hpmg439 which results in an extension of the N-terminal sequence of the encoded HK protein compared with the annotation of HPMG440 and translational coupling with the upstream ORF. The gdhA- and amtB-specific transcript amounts in H. pullorum S440Δ were found to be intermediate between those of H. pullorum Δ440 and the wild-type strain, demonstrating a partial complementation of the hpmg440 knockout (Fig. 3a).

The expression profile of the Δ440 mutant suggested that in wild-type H. pullorum transcription of gdhA and glnK–amtB is repressed under nutrient-rich conditions by the phosphorylated RR HPMG439 (HPMG439-P). To address the possibility that HPMG439 acts as a transcriptional activator of gdhA and glnK–amtB is cross-phosphorylated in the absence of HPMG440 by other HKs or low-molecular-mass phosphate donors, transcription of these target genes was quantified in mutant H. pullorum S439D51NA expressing the phosphorylation-deficient HPMG439 protein. As observed for H. pullorum Δ440 gdhA and glnK–amtB were also derepressed in H. pullorum S439D51NA, suggesting that phosphorylation of HPMG439 is not a prerequisite for their transcription (Fig. 3a).

The P439, PglnK and PgdhA promoters are preceded by sequence motifs resembling the putative HP1043 binding site

In the case of H. pylori HP1043 a 29 bp consensus binding motif could be derived from footprint analyses of the PripB and Phyp1043 promoters (Delany et al., 2002). A sequence motif corresponding to 23 positions to this consensus sequence is present in the upstream region of hpmg439 which, as is the case for Phyp1043 is located in a distance of 5 bp from a putative −10 promoter element (TATAAT, Fig. 4). By 5’ RACE analysis the transcriptional start site of hpmg439 was mapped to position −32 with respect to the translational start codon corresponding to the aforementioned −10 box. Therefore, we checked for autoregulation
of the HPMG439–HPMG440 TCS by quantifying the amount of hpmg439-specific mRNA in H. pullorum Δ440 and the wild-type strain. However, transcription of the RR gene hpmg439 was not altered in the deletion mutant (Fig. 3b). The 5′ ends of the gdhA- and glnK–amtB-specific transcripts were mapped to position −228 with respect to the start codons of gdhA and glnK, respectively. These transcriptional start sites correspond to −220 boxes of the sequence TAAAAT (gdhA) and TATAAT (glnK–amtB) which are also preceded by sequence motifs resembling the proposed HP1043 binding motif. These motifs match the 29 bp HP1043 consensus sequence at 22 positions (P glnK) and 21 positions (P gdhA), respectively, and are located at a distance of 10 bp from the respective −10 boxes (Fig. 4).

DISCUSSION

The HP1043 orthologues show a remarkably high degree of homology throughout the ε-proteobacteria, suggesting a similar and possibly essential function of the RR in this group of bacteria. This prediction is based on the observation that all HP1043 orthologues studied in some detail so far, i.e. HP1043 of H. pylori, Cj0355 of Campylobacter jejuni and HPMG439 of H. pullorum, proved to be essential proteins (Raphael et al., 2005; Schär et al., 2005). The construction of mutant H. pullorum S439D51N indicated that the essential function of HPMG439 is exerted by the unphosphorylated protein. Moreover, HPMG439 could replace HP1043 in the absence of the cognate HK, suggesting that in H. pylori HPMG439 recognizes the same promoters as HP1043. However, we cannot exclude the possibilities that HPMG439D51N adopts a locked-in active conformation or that in H. pylori HPMG439 is phosphorylated by low-molecular-mass phosphate donors or a non-cognate HK. Interestingly, expression of the C. jejuni Cj0355 is reduced in the presence of paraquat (Garéniaux et al., 2008; Hwang et al., 2011) and the RR has been shown to control the transcription of oxidative-stress-defence genes such as sodB and ahpC encoding superoxide dismutase and alkyl hydroperoxide reductase, respectively.
(Hwang et al., 2011). How oxidative stress is sensed by the atypical RR Gj0355, which contains asparagine in the position of the phosphate-accepting aspartic acid residue of the receiver domain, is so far unclear. Our preliminary experiments did not reveal an effect on the protein levels of HP1043 and HPMG439 upon paraquat exposure of H. pylori and H. pullorum bacteria (data not shown).

Upregulation of gdhA and glnK–amtB transcription in the mutants H. pullorum Δ440 and S393Δ51ΔA indicated that the phosphorylated HPMG439 is involved in the regulation of nitrogen metabolism by repressing the above-mentioned target genes under nitrogen-rich conditions. GlnK belongs to the family of PII proteins found in Eubacteria, Archaea and plants, which function as signal integrators in the control of nitrogen assimilation via the binding of ATP, ADP and 2-oxoglutarate. In fact, the known key residues involved in nucleotide and 2-oxoglutarate binding (Huergo et al., 2013) are conserved in H. pullorum GlnK (Fig. S2). In addition PII proteins are frequently modified by uridylylation or adenylylation of tyrosyl residue 51 (Forchhammer, et al., 2008; Huergo et al., 2013), and in unicellular cyanobacteria phosphorylation of PII proteins at seryl residue 49 has been observed (Forchhammer & Tandeau de Marsac, 1995). Depending on effector molecule binding and covalent modification state the PII proteins adopt different conformations which affect the interaction with various signal receptor proteins involved in nitrogen metabolism and regulation (Ninfa & Atkinson, 2000; Forchhammer, 2008; Huergo et al., 2013). In most prokaryotes glnK is genetically coupled to amtB, encoding a high-affinity ammonium transporter to which the unmodified GlnK protein binds, thereby inhibiting the function of the AmtB transporter (Forchhammer, 2008; Huergo et al., 2013). In many bacterial species glnK transcription is induced under nitrogen shortage (Huergo et al., 2013; Atkinson et al., 2002; Jack et al., 1999; Amon et al., 2008; Hervás et al., 2009; Nolden et al., 2001; Drepper et al., 2003), which also favours covalent modification of GlnK (Forchhammer, 2008; Huergo et al., 2013). Presumably H. pullorum glnK–amtB is also upregulated upon nitrogen deprivation; however, covalent modification is unlikely since the putative modification sites Y51 and S49 are not conserved in H. pullorum GlnK (Fig. S2) and no homologues of GlnD uridylyl- or adenylyltransferases are encoded in the H. pullorum genome sequence. In Bacillus subtilis complex formation between GlnK, which does not seem to be covalently modified, and AmtB is ATP-dependent and the GlnK–AmtB complex has been shown to tether the nitrogen stress transcription factor TnrA to the membrane (Detsch & Stülke, 2003; Heinrich et al., 2006), possibly sequestering it under conditions of energy limitation (Forchhammer, 2008). The role of GlnK in the control of nitrogen metabolism in H. pullorum remains to be investigated.

GdhA could have a dual function in H. pullorum and might be predominantly involved in nitrogen assimilation or the catabolic degradation of glutamate. In fact, during growth in culture the preferred carbon sources of the related bacteria H. pylori and C. jejuni are the amino acids aspartate, asparagine, glutamate, glutamine, proline and serine (Mendz & Hazell, 1995; Stark et al., 1997; Guccione et al., 2008). H. pylori, in addition, uses alanine and arginine (Mendz & Hazell, 1995; Stark et al., 1997). In accordance with these nutritional preferences it was shown that in H. pylori genes involved in the uptake of aspartate (dcaU) and glutamate (glnS) and those involved in the deamination of glutamine (HP1118, γ-glutamyltranspeptidase) and asparagine (asntB) are required for efficient colonization in animal models (Chevalier et al., 1999; McGovern et al., 2001; Merrell et al., 2003; Kavermann et al., 2003; Leduc et al., 2010). Actually, H. pylori is unable to take up glutamine and asparagine directly and therefore their periplasmic deamination by γ-glutamyltranspeptidase and asparaginase is required (Leduc et al., 2010). Accordingly, glutamine synthetase is an essential enzyme in H. pylori (Garner et al., 1998). In C. jejuni, which lacks glutamate dehydrogenase, aspartate is required for growth on aspartate, asparagine, glutamate, glutamine and proline, and aspartate aminotransferase, converting glutamate and oxaloacetate to aspartate and 2-oxoglutarate, is essential for the catabolism of glutamate (Guccione et al., 2008). Both enzymes required for the catabolism of glutamate in C. jejuni are present also in H. pullorum (HPMG484 and HPMG1773), suggesting that in this Helicobacter species glutamate might be catabolized essentially as in C. jejuni, and GdhA might function predominantly in nitrogen assimilation. This assumption is in accordance with the observation that gdhA transcription is repressed during growth in rich broth and is co-regulated with the ammonium transporter amtB. Furthermore, Helicobacter GdhA belongs to the NADP+-specific enzymes (EC 1.4.1.4) which are normally involved in nitrogen assimilation. How the nitrogen status of the cell is sensed by HPMG440, a membrane-bound HK with two transmembrane domains flanking a periplasmic domain of 89 amino acids, is unclear.

Interestingly, gdhA transcription in H. pullorum is conversely regulated compared with most other bacteria, where gdhA is repressed under conditions of nitrogen deprivation, whereas expression of glnA is induced due to the considerably higher affinity of glutamine synthetase for ammonium (Leigh & Dodsworth, 2007; Tiffert et al., 2008; Hervás et al., 2010; Harper et al., 2010; Schwacha & Bender, 1993; Ueki & Lovley, 2010; Camarena et al., 1998). However, co-regulation of gdhA and glnA in response to nitrogen limitation has been reported for Streptococcus pneumoniae, Corynebacterium glutamicum and Corynebacterium diptheriae, and Ruminococcus flavefaciens (Kloosterman et al., 2006; Hänßler et al., 2009; Nolden et al., 2002; Antonopoulos et al., 2003). In S. pneumoniae transcription of glnA, gdhA and glnPQ encoding a glutamine uptake system is repressed by the MerR family regulator GlnR under nitrogen-rich conditions (Kloosterman et al., 2006). In corynebacteria, genes involved in nitrogen metabolism are controlled by the repressor protein AmtR belonging to the TetR family of transcriptional regulators (Amon et al., 2010), while the
mechanism underlying nitrogen-dependent regulation in R. flavefaciens has not been investigated. It seems probable that in H. pullorum glnA transcription is also upregulated upon nitrogen shortage; however, surprisingly glnA does not appear to be under control of the HPMG439–HPMG440 TCS. So far, nitrogen regulation could not be addressed experimentally, since we did not succeed in growing H. pullorum in a chemically defined medium.

The promoter regions of hpmg439, glnK and gdhA show strong similarities to the consensus sequence derived for HP1043 of H. pylori (Delany et al., 2002). Surprisingly, deletion of the HK gene hpmg440 had no significant effect on the transcription of hpmg439 despite the presence of a putative HPMG439 binding site in the upstream region. A similar observation has been made for the regulator GlnR of S. pneumoniae, where target genes comprising GlnR operators with a perfectly conserved inverted repeat sequence exhibited different degrees of GlnR-dependent regulation (Kloosterman et al., 2006). Furthermore, due to the different spacings of the putative HPMG439 binding site to the −10 box in the P 439 promoter and the P glnK and P gdhA promoters HPMG439 might act as a transcriptional activator on P 439 and this function could be independent of phosphorylation via HPMG440.

National Center for Biotechnology Information database analysis revealed that the HPMG439–HPMG440 regulon is conserved within the environmental bacteria of the order Campylobacterales, but is not conserved in the host-associated members of that order. All environmental Campylobacterales bacteria contain the TCS and encode GlnK, AmtB, GdhA and/or GOGAT. Among the host-associated Campylobacterales only A. butzleri, W. succinogenes, H. pullorum, H. canadensis and Campylobacter fetus encode a HPMG439–HPMG440-like TCS as well as the aforementioned genes involved in nitrogen metabolism. Campylobacter strains featuring the TCS usually encode GdhA, but lack glnK and amtB, as do the Campylobacter and Helicobacter species containing an orphan or degenerate HP1043 orthologue (Table S2). This observation might imply that sophisticated regulation of nitrogen metabolism is less important in ε-proteobacteria which are adapted closely to a host organism.

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