Histidine Residue 94 Is Involved in pH Sensing by Histidine Kinase ArsS of *Helicobacter pylori*

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Abstract

**Background:** The ArsRS two-component system is the master regulator of acid adaptation in the human gastric pathogen *Helicobacter pylori*. Low pH is supposed to trigger the autophosphorylation of the histidine kinase ArsS and the subsequent transfer of the phosphoryl group to its cognate response regulator ArsR which then acts as an activator or repressor of pH-responsive genes. Orthologs of the ArsRS two-component system are also present in *H. pylori*’s close relatives *H. hepaticus*, *Campylobacter jejuni* and *Wolinella succinogenes* which are non-gastric colonizers.

**Methodology/Principal Findings:** In order to investigate the mechanism of acid perception by ArsS, derivatives of *H. pylori* 26695 expressing ArsS proteins with substitutions of the histidine residues present in its periplasmic input domain were constructed. Analysis of pH-responsive transcription of selected ArsRS target genes in these mutants revealed that H94 is relevant for pH sensing, however, our data indicate that protonatable amino acids other than histidine contribute substantially to acid perception by ArsS. By the construction and analysis of *H. pylori* mutants carrying *arsS* alleles from the related α-proteobacteria we demonstrate that WS1818 of *W. succinogenes* efficiently responds to acidic pH.

**Conclusions/Significance:** We show that H94 in the input domain of ArsS is crucial for acid perception in *H. pylori* 26695. In addition our data suggest that ArsS is able to adopt different conformations depending on the degree of protonation of acidic amino acids in the input domain. This might result in different activation states of the histidine kinase allowing a gradual transcriptional response to low pH conditions. Although retaining considerable similarity to ArsS the orthologous proteins of *H. hepaticus* and *C. jejuni* may have evolved to sensors of a different environmental stimulus in accordance with the non gastric habitat of these bacteria.

Introduction

The human pathogen *Helicobacter pylori* thrives in the mucus layer covering the gastric epithelium. The neutralophilic bacterium has developed efficient mechanisms to cope with both the high acidity encountered during the passage of the stomach lumen in the initial phase of colonization and moderately acidic conditions expected to prevail in the mucus layer. Central to the acid adaptation of *H. pylori* is the urease system which is essential for maintaining both the cytoplasmic and periplasmic pH near neutrality when the bacteria are exposed to low pH [1]. The cytoplasmic urease enzyme is a nickel-containing dodecameric heterodimer consisting of the subunits UreA and UreB [2] which cleaves urea present in the gastric juice in millimolar concentrations to ammonia and carbon dioxide. Ammonia then acts as a buffering compound in both the cytoplasm and the periplasm. Moreover, carbon dioxide which rapidly diffuses to the periplasmic space is converted to HCO$_3^-$ by the periplasmic α-carbonic anhydrase providing an additional buffering compound [3]. The enzymatic activity of the cytoplasmic urease is controlled by the inner membrane pH-gated channel UreI, which regulates the access of the substrate urea to the bacterial cell in response to acidic pH [4,5]. Both urease and the channel protein UreI are essential for colonization in several animal infection models [6–8]. Furthermore, urease-independent mechanisms of pH-homeostasis are likely to exist [9,10]. Accordingly, global transcriptional profiling performed by several research groups revealed the differential expression of 100 to about 280 genes in response to the exposure of *H. pylori* to low pH [11–14].

The ArsRS two-component system is the master regulator of *H. pylori*’s intricate acid response. Acidic pH triggers the autophosphorylation of the histidine kinase ArsS and the subsequent phosphorylation of its cognate response regulator ArsR. Phosphorylated ArsR (ArsR$\cdot$P) then acts both as an activator and repressor of pH-responsive genes [14]. The ArsR$\cdot$P regulon comprises the urease genes, the amidase genes $amiE$ and $amiF$, hp1186 encoding a periplasmic α-carbonic anhydrase, as well as genes encoding antioxidant systems, Ni$^{2+}$-storage proteins, proteins affecting the composition of the cell envelope and *H. pylori*-specific proteins of unknown function [14–17]. Consistent with a prominent role of the ArsRS two-component system in the transcriptional control of the acid response, *arsS* null mutants of...
H. pylori were unable to colonize in a mouse infection model [18]. The metal dependent regulators Fur and NikR also contribute to pH-responsive gene regulation, since Fur- and NikR-deficient mutants showed an aberrant transcription profile upon exposure of H. pylori to low pH [13,19]. Furthermore, it was reported that in the H. pylori strain J99 the two-component system CrdRS (HP1365-HP1364) which positively regulates the expression of the copper resistance determinant CrdAB-CzcAB in response to increasing concentrations of copper ions [20] is also involved in the pH-responsive regulation of major acid-resistance determinants including the urease gene cluster [21]. This regulatory effect was not observed when CrdR-deficient mutants of the H. pylori strains 26695 and G27 were analysed [22]. Recently, the histidine kinase HP0244 which governs the expression of flagellar class II genes was also implicated in pH-responsive transcriptional control [23,24]. However, the ratios of differential expression were modest in an lp0244 negative mutant and differential expression of most target genes including several members of the ArsR~P regulon was detected only at extremely low pH [24].

In this study we investigated the mechanisms by which the sensor protein ArsS perceives acidic pH. It was assumed that protonation of specific amino acid residues in the periplasmic input domain of ArsS eliciting a conformational change of the histidine kinase is involved in pH sensing. Furthermore, we analysed the ability of ArsS orthologs from other members of the ε-proteobacteria to respond to acidic pH.

Materials and Methods

Bacterial strains and growth conditions

H. pylori 26695 and G27 are clinical isolates which have been described previously [25,26]. H. pylori strains were grown at 37°C under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood, 0.01% cycloheximide and Skirrow’s antibiotic supplement. Liquid cultures were grown in brain heart infusion (BHI) broth containing Skirrow’s antibiotic supplement and 10% fetal calf serum (FCS). When required blood agar plates or liquid broth for H. pylori culture were supplemented with kanamycin or chloramphenicol in a final concentration of 20 μg/ml. Acid exposure experiments were performed as follows: Bacteria from a liquid culture were harvested at an OD₅₆₀ of 0.7 by centrifugation and were then shifted for one hour to supplemented BHI broth whose pH had been adjusted to pH 5.0 with hydrochloric acid. In case of neutral pH controls cultivation was continued for one hour in standard BHI broth. E. coli DH5α was grown in Luria-Bertani (LB) broth which was supplemented with antibiotics in the following final concentrations when necessary: ampicillin 100 μg/ml, kanamycin 50 μg/ml, chloramphenicol 30 μg/ml.

Construction of H. pylori strains carrying mutated alleles of arsS

To construct derivatives of H. pylori 26695 expressing mutated ArsS proteins a two step allelic exchange procedure was applied. Natural transformation or electroporation of H. pylori strains was performed essentially as described previously [27,28]. First in H. pylori 26695 the wild-type arsS gene was replaced by a kanamycin resistance cassette via transformation with suicide plasmid pSL-165::km [29]. The resulting arsS null mutant 26695/arsS::km was then transformed with suicide plasmids comprising the mutated arsS allele and a cat gene from C. coli [30] flanked by DNA fragments derived from arsR and the hemB [hp0163] gene. Allelic exchange resulted in the substitution of the aphA gene by both the respective arsS allele and the cat gene. The suicide plasmids were derived from a precursor construct, pSL-arsSTD, which contains a EcoRI-SmaI fragment encoding amino acids (aa) 97–225 of ArsR (PCR amplified with primer pair arsR-5/arsR-3, Table 1), a BglII-XbaI fragment encoding aa 163–428 of ArsS (PCR amplified with primer pair arsSTD-5/arsSTD-3), a XbaI-PstI fragment containing the chloramphenicol resistance cassette, and a PstI-SacI fragment (PCR amplified with primer pair 0163-5/0163-3) encoding aa 1–100 of HP0163 (HemB, 8-aminolevulinic acid dehydratase). A SmaI-BglII fragment encoding aa 1–162 of ArsS (PCR amplified with primer pair arsSTD-5/arsSTD-3) was cloned into pSL1180 yielding the template for the generation of mutated arsS alleles via recombinant PCR [31]. To make sure that the acid response of H. pylori 26695 was not altered by the insertion of the cat gene into the hp0166-hp0162 operon or minor sequence modifications introduced for cloning purposes, a control strain (26695/arsS::H0) was constructed carrying wild-type arsS flanked by the cat gene. For the construction of double mutants a derivative of pSL-arsSTD was used whose SmaI-BglII insert encodes an ArsS input domain with a H94A substitution. Finally, the SmaI-BglII fragments containing the desired point mutations were cloned into plasmid pSL-arsSTD. All cloned PCR fragments which were amplified from chromosomal DNA of H. pylori 26695 using the primers listed in Table 1 or were obtained by recombinant PCR performed on plasmid pSL-arsSTD were subjected to automated sequencing to ensure proper PCR amplification. Transformants obtained by allelic exchange mutagenesis of H. pylori 26695/arsS::km were checked for the correct integration of the mutated arsS alleles by PCR analysis with primer pairs flanking the integration site.

Construction of H. pylori strains with substitutions of arsS by the orthologous genes of H. hepaticus, C. jejuni and W. succinogenes

The arsS orthologs HH1607 from H. hepaticus ATCC51449 [32], CJ1262 from C. jejuni 4344 [33] and WS1818 from W. succinogenes DSMZ1740 [34] were PCR-amplified from chromosomal DNA of the respective strain using primer pairs HH1607-5/HH1607-3, CJ1262-5/CJ1262-3 and WS1818-5/WS1818-3, respectively. The resulting DNA-fragments encoding HH1607, CJ1262 and WS1818, respectively, were ligated into plasmid pSL1180 containing the 393 bp EcoRI-SmaI fragment derived from arsR, the chloramphenicol resistance cassette from C. coli [30] and the 630 bp PstI-SacI fragment derived from hemB yielding suicide plasmids pSL-HH1607 cm, pSL-CJ1262 cm and pSL-WS1818 cm. These suicide plasmids were used for the transformation of H. pylori G27/HP165::km which carries a substitution of arsS by a kanamycin resistance cassette [29]. Selection for chloramphenicol resistance resulted in the replacement of the aphA gene by the respective arsS ortholog which was checked by PCR analysis of chromosomal DNA of the transformants.

RNA isolation and primer extension analysis

RNA from H. pylori was isolated from bacteria grown to the logarithmic phase in liquid broth by using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA concentration was quantified by determination of the absorbance at 260 and 280 nm, and RNA integrity was checked by visualization on a 1.5% agarose gel. Primer extension analysis was performed essentially as described previously [35] using 0.5 pMol of γ³²P-end-labelled oligonucleotides HP1432PE and HP119PE, respectively. Primer extension experiments were performed three times on two independently prepared RNAs. Quantification of the signals from the primer extension products
was performed using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences) and ImageMaster TotalLab Software (Amersham Biosciences).

Construction of plasmids expressing recombinant ArsS and ArsR orthologs from H. pylori ArsS

DNA fragments encoding the transmitter domains of histidine kinase HH1607 from H. pylori ATCC51449 (aa 181–419), CJ1262 from C. jejuni 4344 (aa 185–411) and WS1818 from W. succinogenes DSMZ1740 (aa 179–409) were PCR-amplified from chromosomal DNA of the respective strain using primer pairs HH1607-E5/H1607-E3, CJ1262-E5/CJ1262-E3 and WS1818-E5/WS1818-E3. The DNA fragments were ligated into BamHI and EcoRI-digested pQE166 encoding ArsR fused to an N-terminal His6-tag have been described earlier [29]. The His6-tagged response regulator was introduced for cloning purposes are given in lower case letters, restriction recognition sequences are underlined.

Table 1. Oligonucleotides used in this study.

| Name     | Sequence (5’ to 3’)* | Siteb | Strand | Positionc |
|----------|----------------------|-------|--------|-----------|
| arSR5    | tattgg gatcG ATTACCTCTCTAAACCCCTAG | EcoRI | —      | 174146–174167 |
| arSR3    | ctctct cccgctg ATATGATTTTCTCAATTTAA | SmaI  | +      | 173775–173796 |
| 0163-5   | aatttg TCTCAATTTTCTGAGAAGGAATAG | PstI  | —      | 172443–172464 |
| 0163-3   | agttga gatcG CATATGGGCGGTGTTGATAG | SacI  | +      | 171835–171856 |
| arsSR5   | gccctt gctgcag GTGCCACACAGCAAAGAGAATCG | PstI  | —      | 173753–173774 |
| arsSR3   | cactgg AGATCT TAACTCCTTAAAGGCAATAA | BglII | +      | 173267–173294 |
| arsSTD5  | gattga AGATCT CAAGTGAAACCTGGTCACAG | BglII | —      | 173246–173272 |
| arsSTD3  | cctct cctaga TCTACCTTCTCAGAATTTTCCG | XbaI  | +      | 172460–172480 |
| HH1607-5 | aatttt cccgctag GTGCCACAGCAAAGAGAATCG | PstI  | —      | 153356–153377 |
| HH1607-3 | aatttt cctaga CATATGTTTCTCAATTTTGG | XbaI  | +      | 1534096–1534117 |
| CJ1262-5 | aatttt cccgctag GTGCCACAGCAAAGAGAATCG | PstI  | —      | 1592449–1592469 |
| CJ1262-3 | aatttt cctaga CATATGTTTCTCAATTTTGG | XbaI  | +      | 1593685–1593705 |
| WS1818-5 | aatttt cccgctag GTGCCACAGCAAAGAGAATCG | PstI  | —      | 1712553–1712573 |
| WS1818-3 | aatttt cctaga CATATGTTTCTCAATTTTGG | XbaI  | +      | 1713781–1713801 |
| HH1607-E5 | ttgact gatcC AAACACCAAGAAATGAAATGGGAA | BamHI | —      | 1534803–1534829 |
| HH1607-E3 | tctctg gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1534110–1534133 |
| HH1608-E5 | ttgact gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1536037–1536061 |
| HH1608-E3 | tctctg gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1535384–1535408 |
| CJ1262-E5 | ttgact gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1539003–1539023 |
| CJ1262-E3 | ttgact gatcC ATTACCTCTCAGAAGTAGAT | BamHI  | +      | 1191791–1191815 |
| CJ1261-E5 | aattta cctaga TCTACCTTCTCAGAAGTAGAT | XbaI  | +      | 1192435–1192459 |
| WS1818-E5 | ttgact gatcC AAACACCAAGAAATGAAATGGGAA | BamHI  | +      | 1713094–1713115 |
| WS1818-E3 | ttgact gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1713769–1713789 |
| WS1818-E5 | ttgact gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1711878–1711899 |
| WS1818-E3 | ttgact gatcC CATATGTTTCTCAATTTTGG | XbaI  | +      | 1712591–1712611 |
| HP119PE | tctagag tctagag TCTACCTTCTCAGAAGTAGAT | BamHI  | +      | 130723–130742 |
| HP1432PE | cccgctag gatcG CATATGTTTCTCAATTTTGG | XbaI  | +      | 1502650–1502671 |

*Sequences in upper case letters are derived from the genome sequences of H. pylori 26695 [25], H. hepaticus [32], C. jejuni [33] and W. succinogenes [34]. Sequences introduced for cloning purposes are given in lower case letters, restriction recognition sequences are underlined.

**Restriction recognition sites.

cNucleotide positions refer to the genome sequence of H. pylori 26695.

In vitro phosphorylation assays

In vitro phosphorylation assays providing multiple turnover conditions were performed in a final volume of 25 μl of reaction buffer [50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.5 mM ATP, 420 mM [γ32P]ATP (3000 Ci/mmol)]. The reaction mixtures were incubated at room temperature for 10 min. The reactions were stopped by the addition of sample buffer.
oligonucleotide HP119PE was performed on equal amounts of RNA extracted from the proteins with individual histidine to glutamine mutations in the input domain. Primer extension analysis using the radiolabelled oligonucleotide HP1432PE was performed on equal amounts of RNA extracted from the presence of equal amounts of RNA in the different samples. The sequence of the -10 element of the hp0119 promoter is shown on the left. B. The gray arrow indicates the position of a cDNA band representing a non-specific primer elongation product whose unchanged intensity indicates the black arrow on the right. The sequencing ladder (lanes T, G, C, A) was obtained by annealing oligonucleotide HP119PE to plasmid pSL-119PE [39]. 8), 26695/arsS-H126Q (lane 9) and 26695/arsS::km (lane 10) which were exposed to pH 5.0 for one hour. The hp0119-specific cDNA is indicated by a black arrow on the right. The hp1432-specific cDNA is indicated by a black arrow on the right. Primer extension analysis was performed on equal amounts of RNA extracted from the acid-exposed bacteria using oligonucleotides specific for hp0119 and hp1432. As shown in Fig. 1A acid-responsive transcription of hp0119 in H. pylori 26695/arsS-H0 was indistinguishable from the wild-type strain 26695 and no significant alterations were observed in the H. pylori 26695 mutants expressing ArsS proteins with individual H35Q, H90Q, H93Q, H118Q and H126Q substitutions, respectively. However, in strain 26695/arsS-H94Q the amount of hp0119-specific transcript was reduced to about one fourth of the wild-type transcript level, while a slight increase in the amount of hp0119-specific transcript was detected in strain 26695/arsS-H44Q. The same results were obtained when pH-responsive transcription of hp1432 was analysed (Fig. 1B). Transcription of target genes at neutral pH was not affected in the mutants (data not shown). These data indicate a role of H94 in the not conserved in the N-terminal domains of the orthologous proteins from the related e-proteobacteria H. hepaticus, C. jejuni and W. succinogenes [32–34]. Derivatives of H. pylori 26695 expressing ArsS proteins with individual glutamine substitutions of these seven histidine residues were constructed and pH-responsive transcription of selected members of the ArsR−P regulon was analysed in the mutants. Target gene hp1432 encodes an Hpn-like Ni^{2+}-storage protein. In addition to being controlled by ArsR−P in response to low pH [15], hp1432 is subject to Ni^{2+}-responsive regulation by the regulator protein NikR [38]. In case of hp0119 encoding an H. pylori-specific protein of unknown function the direct binding of ArsR−P to its promoter region has been demonstrated [39]. The 26695 arsS mutants as well as the control strain 26695/arsS-H0, the 26695 wild-type and the isogenic arsS null mutant were grown to an OD$_{600}$ of 0.7 at neutral pH and were then shifted to pH 5.0 for one hour. Primer extension analysis was performed on equal amounts of RNA extracted from the acid-exposed bacteria using oligonucleotides specific for hp0119 and hp1432. As shown in Fig. 1A acid-responsive transcription of hp0119 in H. pylori 26695/arsS-H0 was indistinguishable from the wild-type strain 26695 and no significant alterations were observed in the H. pylori 26695 mutants expressing ArsS proteins with individual H35Q, H90Q, H93Q, H118Q and H126Q substitutions, respectively. However, in strain 26695/arsS-H94Q the amount of hp0119-specific transcript was reduced to about one fourth of the wild-type transcript level, while a slight increase in the amount of hp0119-specific transcript was detected in strain 26695/arsS-H44Q. The same results were obtained when pH-responsive transcription of hp1432 was analysed (Fig. 1B). Transcription of target genes at neutral pH was not affected in the mutants (data not shown). These data indicate a role of H94 in the

### Results and Discussion

H94 in the periplasmic input domain of ArsS is involved in low pH-sensing.

We hypothesized that signal perception by the ArsS sensor protein requires protonation of amino acids in the periplasmic input domain which triggers a conformational change of the protein stimulating its histidine kinase activity. Differential expression of pH-responsive genes was observed when H. pylori is shifted from neutral pH to pH 5.0 [11–14]. Histidine seemed to be best suited to participate in pH perception by ArsS, since according to its side chain $pK_a$ (6.1) free histidine residues will be protonated when the pH drops below 6, while the side chains of arginine ($pK_a$ = 12.5) and lysine ($pK_a$ = 10.0) are already protonated at neutral pH. The side chain carboxyl groups of glutamate and aspartate will change their protonation state at a pH around 4.0 ($pK_a$ = 4.3 and 3.9, respectively). Previously, protonation of histidine residues in the second periplasmic loop and the periplasmic C-terminus of the pH-gated urea channel UreC of H. pylori was shown to be required for the low pH activation of urea transport [36]. Furthermore, a histidine residue in the periplasmic input domain of histidine kinase PmrB from Salmonella enterica was reported to be involved in pH-responsive activation of the PmrAB two-component system [37].

The periplasmic domain of ArsS contains seven histidine residues (H35, H44, H90, H93, H94, H118, H126) which are

![Figure 1. Analysis of transcription of the acid-responsive genes hp0119 (A.) and hp1432 (B.) in H. pylori strains expressing ArsS proteins with individual histidine to glutamine mutations in the input domain. A. Primer extension analysis using the radiolabelled oligonucleotide HP119PE was performed on equal amounts of RNA extracted from the H. pylori strains 26695 (lane 1), 26695/arsS-H0 (lane 2), 26695/arsS-H35Q (lane 3), 26695/arsS-H44Q (lane 4), 26695/arsS-H90Q (lane 5), 26695/arsS-H93Q (lane 6), 26695/arsS-H94Q (lane 7), 26695/arsS-H118Q (lane 8), 26695/arsS-H126Q (lane 9) and 26695/arsS::km (lane 10) which were exposed to pH 5.0 for one hour. The hp0119-specific cDNA is indicated by a black arrow on the right. The sequencing ladder (lanes T, G, C, A) was obtained by annealing oligonucleotide HP119PE to plasmid pSL-119PE [39]. The gray arrow indicates the position of a cDNA band representing a non-specific primer elongation product whose unchanged intensity indicates the black arrow on the right. The hp1432-specific cDNA is indicated by a black arrow on the right. B. Primer extension analysis using the radiolabelled oligonucleotide HP1432PE was performed on equal amounts of RNA extracted from the H. pylori strains 26695 (lane 9), 26695/arsS-H0 (lane 1), 26695/arsS-H35Q (lane 2), 26695/arsS-H44Q (lane 3), 26695/arsS-H90Q (lane 4), 26695/arsS-H93Q (lane 5), 26695/arsS-H94Q (lane 6), 26695/arsS-H118Q (lane 7), 26695/arsS-H126Q (lane 8) and 26695/arsS::km (lane 10) which were exposed to pH 5.0 for one hour. The hp1432-specific cDNA is indicated by a black arrow on the right.

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Acid Sensing by H. pylori ArsS
mechanism of pH sensing by the periplasmic input domain of ArsS. To corroborate this conclusion an *H. pylori* mutant expressing a derivative of ArsS with a substitution of H94 to alanine was constructed which also exhibited reduced pH-responsive transcription of hp0119 and hp1432 (Fig. 2 and data not shown).

Since residual pH-responsive transcriptional induction of ORFs hp0119 and hp1432 was still observed in strains 26695/arsS-H94Q and 26695/arsS-H94A (Fig. 1, Fig. 2), double mutants were constructed by replacing H35, H44, H90, H93, H118 and H126, respectively, by alanine in ArsS-H94A. Compared to 26695/arsS-H94A no further decrease in the amount of hp0119- and hp1432-specific transcripts at pH 5.0 was detected in the *H. pylori* mutants expressing the proteins ArsS-H94,35AA, ArsS-H94,90AA, ArsS-H94,93AA, ArsS-H94,118AA and ArsS-H94,126AA. However, to our surprise mutant 26695/arsS-H94,44AA showed wild-type pH-responsive transcription of hp0119 and hp1432 (Fig. 2 and data not shown). When mutant 26695/arsS-H94,44AA was grown at neutral pH, transcription of hp0119 was not altered compared to the wild-type, while a marginal increase in the amount of hp1432-specific transcript was detected (data not shown). It is known that within a protein the sequence of its residues can deviate substantially from the pK\textsubscript{a} of the free amino acids. Therefore, we hypothesize that the H44A mutation in ArsS-H94,44AA might increase the side chain pK\textsubscript{a} of acidic amino acids in the input domain, whose protonation contributes to the activation of ArsS, thereby compensating for the H94A mutation.

Surprisingly, concerted substitution of all seven histidine residues in the periplasmic domain of ArsS by glutamine resulted in a *H. pylori* mutant which at neutral pH exhibited transcription of hp0119 and hp1432 at a level similar to that observed in the wild-type strain 26695 at pH 5.0, suggesting that the mutant ArsS protein adopts a locked-on conformation. However, lowering the pH from 7.0 to 5.0 still triggered an about five-fold increase in transcription of the ArsRS target genes analysed (Fig. 3 and data not shown). These data indicate that pH sensing by ArsS does not rely exclusively on the protonation of histidine residues but rather suggest a complex structural interplay of various amino acids changing charge at low pH. In other pH sensing histidine kinases protonation patterns of similar complexity have been proposed to result upon lowering of the pH. In case of PmrB from *Salmonella typhimurium*, in addition to H35, four glutamic acid residues in the periplasmic domain were shown to contribute to acid sensing [37]. Analysis of the sensor domain of the *Salmonella* histidine kinase PhoQ, which responds to low Mg\textsuperscript{2+}, acidic pH and antimicrobial peptides, by NMR spectroscopy revealed a low pH-induced conformational change involving multiple amino acid interactions [40]. The input domain of ArsS comprises five aspartic acid and nine glutamic acid residues whose contribution to pH sensing remains to be investigated in detail. Furthermore, our results suggest that depending on the protonation state of the periplasmic input domain ArsS might adopt variable conformations corresponding to different activation states of the histidine kinase. In this context it should be noted that Wen et al. observed a gradual response of the members of the low pH-stimulon of *H. pylori* when the bacteria were shifted from neutral pH to pH 6.2, 5.5 and 4.5, respectively [12,16].

The ArsS ortholog WS1818 of *W. succinogenes* responds to acidic pH

Although they have to pass through the stomach during infection of their hosts, *H. hepaticus*, *C. jejuni* and *W. succinogenes*...
and WS1818, respectively, instead of ArsS. Strain G27 was chosen for mutant construction because its restriction/modification system HpyAV [44] whose recognition sites are present in the genes hh1607, cj1262 and ws1818. RNA was prepared from liquid cultures of the wild-type strain H. pylori G27, the arsS null mutant G27/HP165::km [29] and the hybrid strains G27/HH1607, G27/cj1262 and G27/WS1818 grown at neutral pH and exposed to pH 5.0 for one hour. Primer extension analysis performed with a hp0119-specific oligonucleotide revealed similar transcript amounts in the G27 wild-type and the hybrid strain G27/WS1818 grown at pH 5.0 indicating that an efficient acid response is triggered by WS1818 upon pH downshift. In case of G27/HH1607 a slight increase in the transcription of hp0119 was observed upon exposure to pH 5.0, while similar amounts of transcript were detected in RNA extracted from G27/cj1262 at neutral and acidic pH. However, transcription of hp0119 was more pronounced in G27/ CJ1262 than in the arsS null mutant G27/HP165::km indicating that histidine kinase CJ1262 is expressed in H. pylori and causes low-level phosphorylation of ArsR (Fig. 5). These data demonstrate that histidine kinase WS1818 is indeed an acid sensor and suggest that HH1607 and CJ1262 might perceive other stimuli than low pH. At present it cannot be ruled out that the observed lack of acid responsiveness of HH1607 and CJ1262 in the genetic background of H. pylori is due to inefficient expression of the histidine kinases. So far attempts to raise an ArsS-specific polyclonal antibody suitable for proving the production of the ArsS orthologs in H. pylori have failed. However, in this context it should be noted that a response regulator protein of C. jejuni has been successfully expressed in H. pylori [45]. It is also conceivable that WS1818 or even ArsS recognize other stimuli in addition to low pH as has been demonstrated for other histidine kinases like Salmonella PhoQ [46]. Interestingly, the input domain of WS1818 harbours a histidine...
residue at position 92 corresponding to H94 in ArsS. However, substitution of H92 by glutamine did not compromise the acid-responsive transcription of hp0119 in G27/WS1818 (data not shown).

In conclusion, in this study we present evidence that H94 in the periplasmic input domain is involved in the perception of low pH by histidine kinase ArsS. However, a comprehensive mutation analysis of all the histidine residues in the input domain indicated that additional parts of the protein contribute substantially to acid sensing. Future experiments will aim at the elucidation of the role of acidic amino acids of the input domain in the mechanism of acid sensing by ArsS. Moreover we showed that the histidine kinase WS1818 of *W. succinogenes* is able to functionally complement an *ars* deficient mutant of *H. pylori*.

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Author Contributions

Conceived and designed the experiments: SM DB. Performed the experiments: SM MG. Wrote the paper: DB.
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