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Characterization of the Arginine Decarboxylase Gene (ORF HP0422, *speA*) Involved in Acid Tolerance in *Helicobacter pylori*

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speA, *adiA*, arginine decarboxylase, acidic pH, *Helicobacter pylori*, agmatine.

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Abstract

Background: *Helicobacter pylori* is a motile microaerophilic bacterium that colonizes the human stomach. *H. pylori* infection triggers gastric diseases, such as gastritis, peptic ulcer and gastric cancer. Stomach represents a barrier for microorganism colonization, particularly because of its high hydrochloric acid concentration. The main mechanism developed by *H. pylori* to maintain intracellular pH homeostasis in this environment is the urease activity. However, urease negative strains can be also isolated from clinical samples, suggesting that *H. pylori* presents other components involved in acid resistance.
Objective: Here, we present some evidence that the arginine decarboxylase gene (*speA*) in *H. pylori* could be involved in an acid adaptation mechanism similar to the one in Enterobacteriaceae, which is dependent on the presence of arginine.
Methods: Indeed, *speA* mRNA and protein expression are acutely induced by acid stress.
Results: Moreover, we showed that *H. pylori* uses arginine in an acid response mechanism required for its growth in acid conditions.
Conclusion: Altogether, these results provide novel information regarding the *H. pylori* physiology and acid response mechanism.

Bacterial survival in adverse environments depends on the presence of adaptive systems that sense and coordinate molecular and cellular responses upon the harmful conditions. *Helicobacter pylori* is a motile Gram-negative human pathogen that causes gastritis, duodenal and gastric ulcers and increases the risk of gastric cancer. This pathogen survives intragastric acidity long enough to colonize the stomach and to live within the gastric mucous layer [1,2]. The pH in the gastric mucous layer varies between 4.0 and 6.5, with occasional decreases to less than pH 2.0 [3]. Thus, *H. pylori* is subjected to changes in the internal milieu that include transient pH fluctuations [4]. Specialized to live in a single environment, *H. pylori* has a small genome (1.67 megabases) that contains a minimal set of metabolic genes, including specialized factors required for survival and colonization [5,6]. The bacterium must then overcome acidic conditions using acid-protective mechanisms [7]. One striking feature of *H. pylori* is the constitutive high-level expression of a urease with an optimum activity at neutral pH [8–10]. Urease increases the pH of the bacterial microenvironment by converting urea into ammonia and carbon dioxide [8]. Both products protect the bacterium by buffering the gastric acid [4, 11–14]. Moreover, *H. pylori* also displays an acid resistance response to maintain the internal pH after an acid challenge [15]. Such response is characterized by a robust change in transcription and protein translation, whereby many *H. pylori* proteins are either downregulated or upregulated [6,14,15]. Interestingly, studies with a *H. pylori ureI* mutant, which is unable to incorporate urea, have shown that *H. pylori* develops an effective urease-independent acid stress response mechanism [14]. For instance, specific activities of several enzymes in *H. pylori* are altered depending on the pH of the growth media [4,8]. Conversely, acidic pH causes changes in the lipopolysaccharide composition [16], increases the expression of chaperone-like proteins [17], and induces ammonia-producing pathways [18]. Furthermore, some recent experiments in *H. pylori* have explored the relationship between this genome-wide

response mechanism and some regulatory systems, such as NikR, Fur, and the ArsRS two-component system [19]. Thus, *H. pylori* has evolved multiple mechanisms to resist acid conditions. In spite of the relevance of these adaptive mechanisms, the effects of pH on gene expression and the analysis of protein expression in response to pH stress still remain largely uncharacterized. Clarification of these response mechanisms may be critical to the understanding of *H. pylori* physiology and pathogenesis.

Interestingly, *Escherichia coli* survives extremely acidic conditions by displaying three adaptive acid resistance strategies defined as acid resistance (AR) systems 1, 2, and 3 (AR1, AR2, and AR3), which are thought to maintain the internal pH in the bacterium [20]. AR1 is displayed by Luria–Bertani-grown stationary-phase cells and protects *E. coli* at pH 2.5 in defined minimal medium [20,21]. AR2 requires glutamic acid to protect cells at pH 2.5. Two isoforms of a glutamate decarboxylase convert glutamic acid to γ-amino butyric acid in a process involving the consumption of an intracellular proton [21]. The third acid resistance system, AR3, requires extracellular arginine to protect cells at pH 2.5 and appears to function much like AR2. In this system, it is critical the *adiA* gene that encodes an inducible arginine decarboxylase induced by arginine, anaerobiosis, and low pH [20]. This enzyme decarboxylates arginine to agmatine in a mechanism that is similar to that of glutamate decarboxylase [20]. In addition, a putative antiporter is required to exchange the extracellular arginine for the intracellular agmatine [22]. Bioinformatics analyses of the *H. pylori* genome addressed to the identification of AR components of *E. coli* have shown that all the *H. pylori* sequenced genomes contain an arginine decarboxylase gene, *speA* (*hp0422* locus in the *H. pylori* 26695 chromosome). Therefore, we postulate that *H. pylori* presents a type AR3 system, similar to that observed in *E. coli*. In this study, we investigate how pH influences *speA* gene transcription and its regulation. We found that the *speA* induction in acid conditions is a Fur-regulated process. Moreover, our results suggest that SpeA plays an important role in the acid adaptation mechanism of *H. pylori* in presence of arginine. The study of these response mechanisms may be critical to the understanding of *H. pylori* physiology, colonization, and pathogenesis processes.

Material and Methods

Bacterial Strains and Culture Conditions

H. pylori strains (listed in Table 1) were cultured at 37 °C in an Shel Lab incubator with 5.5% CO_2 and 80% humidity on trypticase soy agar plates (TSA plates) containing 5% v/v horse serum, Vitox, and antibiotic Dent supplement (Oxoid; Basingstoke, Hampshire, UK).

Table 1 Bacterial strains used in this study

Strains	Source
Hp 26695	American Type Culture Collection (ATCC700392)
Hp 43504	American Type Culture Collection (ATCC43504)
Hp FUR504	*fur*::cat, cam^r. This study
Hp 60190	American Type Culture Collection (ATCC49503)
Hp U2.1	Kindly gifted by Dr. Guillermo Pérez-Pérez. Urease-negative strain derived from *H. pylori* 60190
E. coli BL21	*hsdS*, *gal*, *lcIts*857, *ind*1,*Sam*7, *Nin*5, *lac*UV5-T7gene1
E. coli BL21 Δ*adiA*	*adiA*::*aph*, Km^r, This study

E. coli strains (listed in Table 1) were cultured in Luria–Bertani (LB) media containing 100 μg/mL ampicillin, 20 μg/mL chloramphenicol, or 30 μg/mL kanamycin.

RNA Extraction, RT-PCR, and qRT-PCR

Total mRNA was isolated from *H. pylori* strains grown on TSA plates adjusted to pH 5.5 or pH 7.2 for 18, 24, 36, and 48 hours or incubated in RPMI 1640 media adjusted to pH 5.5 or pH 7.2 for 0–160 minutes to a final concentration of 3×10^8 CFU/mL using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total cDNA was synthesized using the cDNA CoreKit (BIOline, Randolph, MA, USA) following manufacturer's instructions. PCRs were performed in a PTC-100 MJ Research Thermal Cycler using forward (F) and reverse (R) primers to amplify *speA*, 16S *rrna,* and intergenic transcripts (listed in Table 2 and indicated in the operon diagram in Fig. 1). PCR conditions were optimized for each primer pair. All reaction products were analyzed after 30 amplification cycles, each of which involved consecutive 1-minute steps at 94, 55, and 72 °C. Amplification products were subsequently analyzed by electrophoresis on 1% w/v agarose gels stained with ethidium bromide. UV-revealed amplicons images were acquired and quantified with the ImageQuant 5.0 program (Amersham Biosciences, Little Chalfont, UK). For qRT-PCR, complementary DNA synthesis was performed by the cDNA Synthesis AffinityScript™ kit (Stratagene, La Jolla, CA, USA). The reactions were performed in an ABI7300 equipment (Applied Biosystems, Foster City, CA, USA) using the SYBR Sensimix Kit reagents (Bioline, Tauton, MA, USA). Complementary DNAs were amplified by PCR using the primer pairs Sp*e*A-Fq and SpeA-Rq or 16S-F and 16S-R for *speA*

Table 2 Primers used in this study

Primer	Sequence
SpeA-Fq	5′AGAGGAGTGCCTTTGTTGCGA3′
SpeA-Rq	5′ACTTGCCCTCTACAGTCCATC3′
SpeA-F	5′ATGAAATGCTAGACTTGCTC3′
SpeA-R	5′TCGCTATCGCAACTAATATC3′
16S-F	5′GCTAAGAGATCAGCCTAT3′
16S-R	5′CCTACCTCTCCCACACTCTA3′
Hp 0416F (1)	5′AAGGCAAGACTAACGCATGG3′
Hp 0417R (2)	5′TTGATGTTCGTCTGTGG3′
Hp 0417F (3)	5′AGTAGAGATCAAAGTGGGGC3′
Hp 0418R (4)	5′AGCTTGATCCATGCCACATC3′
Hp 0418F (5)	5′ACAACGGCAAGGGTGATTGT3′
Hp 0419F (6)	5′TTAGCGCGTTAAAAGGGTGG3′
Hp 0420R (7)	5′ACGCAATAAGCACAAACCCG3′
Hp 0421R (8)	5′AAGGCACTTGCATTTCTCGC3′
Hp 0421F (9)	5′AATAGCCCTTTAAGCGCGAC3′
Hp 0422R (10)	5′GGGAAAACCGCCTTAAAAGC3′
Hp 0422F (11)	5′TTTATTCACGCACCCTACGG3′
Hp 0423R (12)	5′AAGCCCTATCCTTGACACTC3′
HP0422BamHI	5′GCGGGATCCATGCAAGAAGTCCATGATTATG3′
HP0422XhoI	5′GCGCTCGAGTTAAGAAATCGTGCGCAAATAC3′
WAdiAF	5′ATGAAAGTATTAATTGTTGAAAGCGAG TTTCATATGAATATCCTCCTTAG3′
WAdiAR	5′TTACGCTTTCACGCACATAACGTGGTAAATGT GTAGGCTGGAGCTGCTTCG3′
AdiAF	5′AAGACGATATCAGTATCAGC3′
AdiAR	5′CGGCGTAATGTTATTTAAAC3′
SpeA-NdeIF	5′ATACATATGCAAGAAGTCCATGATTA3′
SpeA-XhoIR	5′TCCTCGAGTTAAGAAATCGTGCGCAAAT3′
adiA-NdeIF	5′CCGCATATGATGAAAGTATTAATTGT3′
adiA-XhoIR	5′CATCTCGAGTTACGCTTTCACGCACATAA3′

and *16S rrna*, respectively (Table 2). PCR conditions were optimized for each primer pair, under the same amplification conditions, namely initial step one cycle for 10 minutes at 95 °C; second step of 40 cycles of 15 seconds at 95 °C, 15 seconds at 58 °C, and 30 seconds at 72 °C; third step performing a dissociation curve as follows: 15 seconds at 95 °C, 1 minute at 60 °C, 15 seconds at 95 °C, and finally, 15 seconds at 60 °C. The qRT-PCR assays data were analyzed in the 7300 System SDS software, version 1.4.0.27 software (Foster City, CA, USA) provided by the real-time thermal cycler.

GST Fusion Protein Production and Anti-SpeA Antibody Generation

The *speA* gene was PCR-amplified from *H. pylori* 26695 genomic DNA using the HP0422BamHI and HP0422XhoI primers (listed in Table 2). The amplicon was digested and cloned into *BamHI*/*Xho*I sites of pGEX-KG plasmid to generate a pGEX-SpeA plasmid. The GST-SpeA fusion protein was purified from the *E. coli* BL21/DE3 strain transformed with pGEX-SpeA. To this end, these cells were grown overnight at 37 °C in LB broth with mechanical shaking. Next, GST-SpeA protein expression was induced by the addition of IPTG 0.2 mmol/L at 16 °C for 2 hours. Bacterial cells were harvested by centrifugation, resuspended in wash buffer (150 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4), and lysed by sonication in lysis buffer (150 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% v/v Triton X-100, pH 7.4) containing protease inhibitors (1 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin). The lysate was centrifuged for 45 minutes at 20,200 × *g* at 4 °C, and the supernatant was subjected to affinity purification on Glutathione Sepharose 4B (GE Amersham Pharmacia, Champaign, IL, USA) at 4 °C for 2 hours. The matrix was washed five times with wash buffer, and the GST-SpeA protein was eluted with 10 mmol/L glutathione in 150 mmol/L Tris-HCl (pH 8.0). The purified protein (2.0 mg) was used for anti-SpeA rabbit polyclonal antibody generation (Bios-Chile, Santiago, Chile).

Western Blot Analysis

Bacteria were disrupted by sonication in lysis buffer (20 mmol/L HEPES pH 7.4, 12.5 μg/mL leupeptin, 10 μg/mL antipain, 100 μg/mL benzamidine, 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate, 0.1% w/v SDS, 0.05% v/v NP-40) (IGEPAL CA-630). Protein concentration was determined using the BCA protein assay reagent following the manufacturer's instructions (Pierce, Rockford, IL, USA). Total protein extracts (30 μg per lane) were size fractionated by 10% SDS-PAGE. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then blocked for 1 hour with a blocking solution (5% w/v nonfat milk, 0.1% v/v Tween-20 in PBS) followed by overnight incubation with the rabbit polyclonal anti-*H. pylori* SpeA antibody (1 : 15,000 dilution). After three washes with 0.1% v/v Tween-20/PBS, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1 : 2,500 dilution) for 1 hour. After three washes with 0.1% v/v Tween-20/PBS, the immunoblots were visualized by EZ-ECL Chemiluminescence reagent (Biological Industries, Kibbutz Beit Haemek, Israel) and exposure to BioMax MR film (Kodak, Rochester, NY, USA) [23].

Mutagenesis of *Escherichia coli* adiA and *Helicobacter pylori* fur Genes by Homologous Recombination

Mutagenesis in *E. coli* was performed by the Red/Swap method of Datsenko and Wanner [24] to produce a

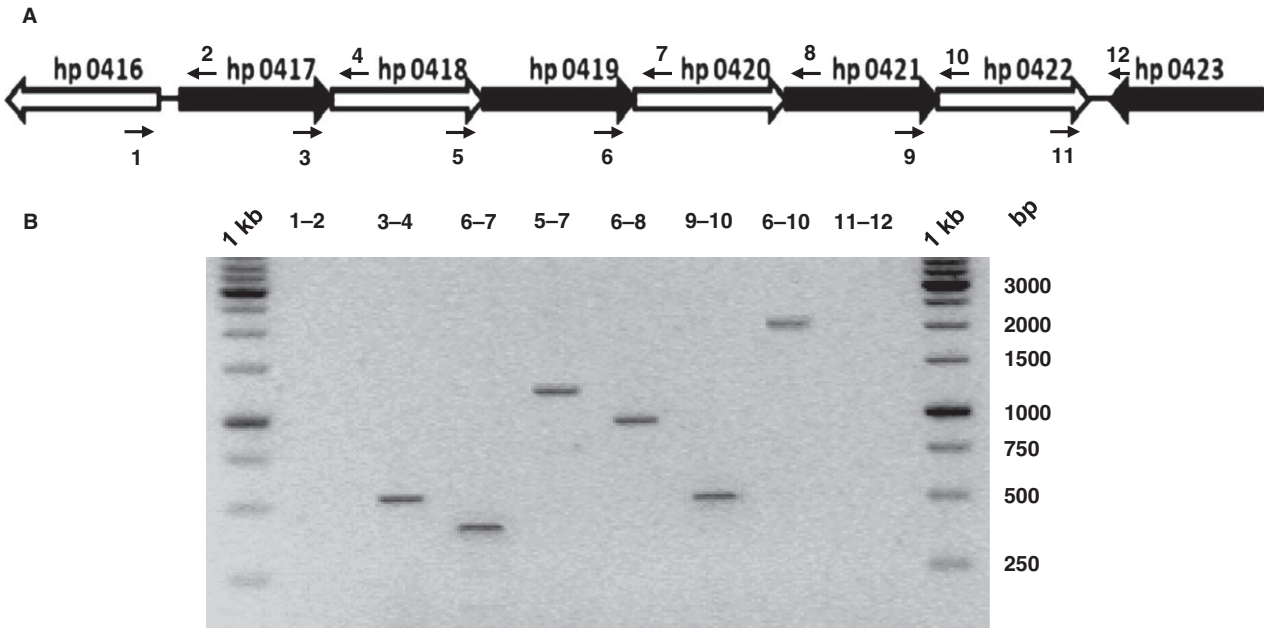

Figure 1 Mapping the *speA* operon in *H. pylori* 26695. (*A*) Schematic representation of the chromosome region surrounding the *hp0422* gene and the oligonucleotide annealing sites. (*B*) *H. pylori* cDNA was generated by reverse transcription of total RNA (Materials and Methods) and amplified by PCR using combinations of forward and reverse oligonucleotides (listed in Table 1). The amplicons were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and further exposure to UV light. A representative picture is shown. Molecular sizes of DNA standard fragments (1 kb DNA ladder) are indicated on the right.

homologous recombination using PCR products. Briefly, *E. coli* BL21 cells containing the pKD46 plasmid, which contains the λ-Red recombinase system genes, were transformed with PCR products that were generated using the pKD46 plasmid as template, which contains the FRT-flanked kanamycin resistance gene (*aph*). Each primer pair also carried 25 base segments that were homologous to the edges of the gene targeted for disruption, WAdiAF and WAdiAR (Table 2). Kanamycin-resistant colonies were replica plated in the absence of antibiotic selection at 37 °C and assayed for ampicillin sensitivity to confirm the loss of pKD46. Correct insertional gene replacement was confirmed by PCR using the AdiAF and AdiAR primers. The mutant was named as *E. coli* BL21 Δ*adiA*. The *fur* mutant was obtained by allelic exchange in the *H. pylori* 43504 strain using the pUC19 *fur::cat* plasmid described in [7].

Expression Vectors and Bacterial Transformation

H. pylori speA and *E. coli adiA* genes were PCR-amplified using the SpeA-*Nde*IF plus SpeA-*Xho*IR or the adiA-*Nde*IF plus adiA-*Xho*IR primer pairs, respectively (listed in Table 2), and subsequently cloned into the *Nde*I and *Xho*I restriction sites of the pET21a+ plasmid (Novagen, Darmstadt, Germany), generating the pET-*speA* and pET-*adiA* plasmids, which were used to transform the chemocompetent *E. coli BL21* Δ*adia* strain, as described in [25]. Protein expression was induced by adding 1 mmol/L IPTG to the culture media.

Extraction and Detection of Agmatine and Putrescine

Differential extraction and detection of agmatine and putrescine was performed according to the protocol described by Goldschmidt and Lockhart [26]. Briefly, 2 mL of synthetic media at pH 7.2 (3 mmol/L L-arginine, 1.5% w/v glucose and 1 mmol/L IPTG) contained in a screw-cap test tube was inoculated with a *E. coli* suspension from a 16-hour liquid culture to reach a final OD_{560} = 0.4. For *H. pylori* cultures, extraction was performed from tubes with 2 mL of TSB broth supplemented with 0.35% w/v L-arginine and 5% horse serum with a suspension of bacteria equal to OD_{560} = 0.8. After 12–48 hours incubation at 37 °C without agitation, cell suspensions were mixed with 2 mL of NaCl-saturated KOH solution (10% w/v) and 2 mL of n-butanol, then the tubes were agitated for 2 minutes and allowed to settle. The upper butanol layer (extract) was removed, and a 20 μL aliquot was separated by ascending one-dimensional thin-layer

chromatography on silica gel plates (TLC Silica gel 60 F254; Merck, Darmstadt, Germany). The solvent system consisted of a phenol/acetic acid/water (6 : 1 : 6, v/v/v) mixture. The chromatographic plates were dried and developed with a ninhydrin spray reagent (0.1 g of ninhydrin in 100 mL of chloroform). Alternatively, 20 μL of the extract was directly developed on Whatman paper, when indicated. Also, putrescine (3 μg) and agmatine (5 μg) standards (both from Sigma-Aldrich, St Luis, MO, USA) were included.

Escherichia coli Acid Resistance Assay

E. coli acid resistance assay was performed as described in [20]. Briefly, bacteria were grown in LBG broth (LB supplemented with 0.4% w/v glucose) at pH 5.0 until stationary phase (18–20 hours at 37 °C). Acid shock resistance was assayed by diluting a 10-μL aliquot of the cell suspension with 1 mL of prewarmed acid shock medium (40 mmol/L KCl, 80 mmol/L KH_2PO_4, 33 mmol/L H_3PO_4, 1.7 mmol/L sodium citrate, and 20 mmol/L glucose, pH 2.5) supplemented with or without 3 mmol/L L-arginine at 37 °C. Survival was determined after 2–3 hours of acid exposure by plating serial dilutions of bacterial suspensions on LB agar plates. The cell survival rate (percentage) was obtained by comparing the number of colony-forming units (CFU) observed after acid exposure with those observed at the start of the acid challenge.

Bacterial Growth under Acidic Conditions

For experiments in liquid medium, a suspension of *H. pylori* (OD_{560} = 0.05) obtained from fresh cultures on TSA plates were used to inoculate a 100-mL flask containing 30 mL of trypticase soy broth (Becton-Dickinson, Sparks, MD, USA), supplemented with 5% v/v fetal horse serum, Dent, and Vitox. The broth was adjusted to the desired pH with 1 N HCl and subsequently sterilized by filtration. When indicated, 5 mmol/L L-arginine was added to the culture media. Bacteria were grown under the conditions described before with agitation. Bacterial viability was determined by counting CFU after seeding serial dilutions on TSA plates.

E. coli strains were cultured in the synthetic minimum medium described by Goldschmidt and Lockhart [26], containing 1.5% w/v glucose and 5 mmol/L L-arginine (when indicated) adjusted to pH 4.0 with 1 N HCl [26]. A flask with 15 mL of this medium was inoculated with a bacterial suspension (OD_{560} = 0.025) and cultured for 0–5 hours at 37 °C with agitation. Bacterial viability was determined by CFU counting.

Results and Discussion

Mapping of a *speA* Operon

Three acid resistance systems have been identified in *E. coli*. AR1 relies directly on σ^S, the stationary-phase sigma factor, and protects cells at pH 2.5 in minimal media [27,28]. The other two systems involve specific amino acid decarboxylases and rely on extracellular supplied glutamate (AR2) or arginine (AR3) for protection during acidic shock. These two systems are thought to provide acid resistance by consuming intracellular protons via the amino acid decarboxylation reaction [8,29]. These amino acid-dependent acid resistance systems involve dedicated pairs of amino acid decarboxylases and antiporters [20,29]. Bioinformatics analyses revealed that a unique arginine decarboxylase gene, equivalent to the *E. coli adiA* gene, is present in the *H. pylori* 26695 genome (*hp0422*). These data suggest that *H. pylori* have an arginine decarboxylase, which could play the role of the *E. coli* AR3.

Genome sequencing of six different *H. pylori* strains showed that all of them contain the *speA* gene. Interestingly, the *speA* gene (*hp0422*) appears to be part of a short array of genes sharing the same DNA strand, which is highly suggestive of an operon structure. According to the *H. pylori* 26695 DNA sequence [5], such putative operon would contain six open reading frames (ORF): *hp0417*, methionyl-tRNA synthetase; *hp0418*, hypothetical protein; *hp0419*, hypothetical methyl-transferase protein; *hp0420*, hypothetical thioesterase protein; *hp0421*, type 1 capsular polysaccharide biosynthesis protein J; and *hp0422*, arginine decarboxylase. To confirm whether these genes are actually transcribed as a poly-cistronic mRNA, we examined the presence of these cotranscripts by RT-PCR. As shown in Fig. 1, the amplicons produced (Fig. 1B) using different combinations of oligonucleotides (Fig. 1A) corroborated that the *speA* gene in *H. pylori* is transcribed into a polycistronic mRNA that includes from the *hp0417* to the *hp0422* ORFs (Fig. 1B). These results confirmed the postulate of Sharma et al. [30], who predicted the operon structure including those ORFs by complementing their transcriptional start site maps with DOOR [31].

Effect of Arginine on the Growth Curve of *Helicobacter pylori*

Ammonia production by the enzymatic activity of urease is one of the most important mechanisms involved in *H. pylori* protection against acid challenge. However, urea concentration in gastric juice is around 1 mmol/L,

which may be insufficient to ensure *H. pylori* survival [6]. On the other hand, the occurrence of urease-negative strains [32] with the ability to colonize and induce gastric ulcers in Mongolian gerbils [33] also suggests the existence of alternative mechanisms for acid resistance. One of those mechanisms could involve the decarboxylation reaction of amino acids and the consequent production of basic molecules that buffer the enhanced concentration of protons in the intracellular environment during exposure to acid pH in a similar manner to *E. coli* [20–22]. Therefore, we studied whether decarboxylation of arginine by the enzymatic activity of SpeA could be also an important mechanism in *H. pylori* acid resistance.

First, we compared the rates of growth of *H. pylori* strains in presence or absence of arginine at different pH. In these experiments, we observed that supplementation of the medium with arginine 5 mmol/L improved the growth of the *H. pylori* 43504 (Fig. 2A) and the urease-negative U2.1 (Fig. 2B) strains at pH 5.5, suggesting that arginine supports the bacterial growth in acid conditions by serving as substrate for agmatine but not urea production. Conversely, the addition of arginine did not result in a faster growth of the 43504 and U2.1 strains at pH 7.0 (Fig. 2A,B, respectively), suggesting that the arginine overload was used mainly to support bacterial growth in acid conditions as a resistance mechanism but not as an essential factor for bacterial growth.

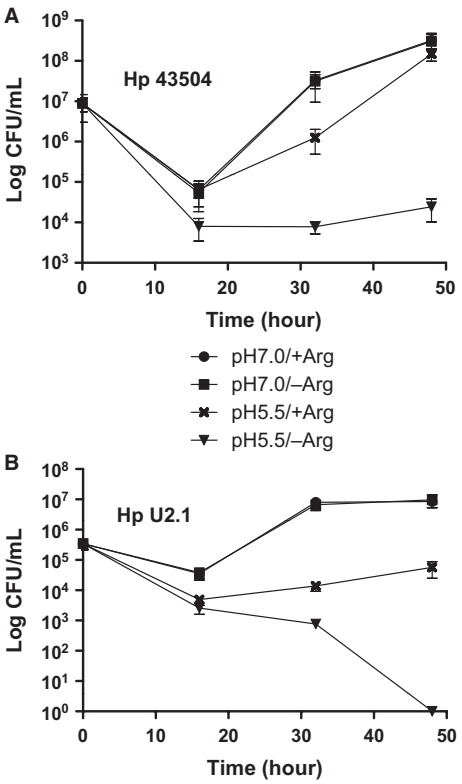

Figure 2 Effect of arginine supplementation on the growth of *H. pylori* in acidic conditions. *H. pylori* strains 43504 (*A*) and U2.1 (*B*) grown on TSA plates were used to inoculate nutritive broths at pH 7.0 and pH 5.5. Cultures were supplemented with or without 5 mmol/L L-arginine (+/−Arg). Bacteria were grown at 37 °C for 48 hours. At the indicated times, aliquots from each culture were taken, serially diluted, and seeded on TSA plates. After 3 days of growth, CFU were counted. Values from three independent experiments are shown (mean ± SD, $p \leq .05$).

Effect of Acid pH on the *speA* Gene Transcription

Having identified that arginine enhances bacterial growth in acid conditions, we next analyzed the effect of acid exposure on the *H. pylori speA* gene expression. *H. pylori* 26695, 43504, and U2.1 strains were incubated in acid conditions over various time periods between 0 and 160 minutes, and the *speA* mRNA levels were analyzed using RT-PCR. In these experiments, we determined that when *H. pylori* 26695 bacteria were incubated at pH 5.5, the *speA* mRNA levels experienced a biphasic increase with a first peak at 15–20 minutes and a second peak between 90 and 160 minutes (Fig. 3A). Similar changes were observed with the 43504 and U2.1 strains (Fig. 3B,C, respectively).

We confirmed these results by measuring the *speA* mRNA levels in the 26695 strain using qRT-PCR (Fig. 3D). In these experiments, we observed a similar progression of the mRNA expression, with an increase at 10 minutes. Then, we concluded that *speA* is acutely induced after acid exposure.

To determine whether the *speA* gene is overexpressed during a long-term acid exposure, we cultured *H. pylori* on TSA plates at pH 5.5 for 12, 24, 36, and 48 hours. Here, we observed an approximately 4.0-fold increase in *speA* mRNA expression at 12 hours of acid exposure (pH 5.5) (Fig. 3E). This increase was persistent over the various times of incubation (Fig. 3E). No differences in *speA* mRNA expression were observed at neutral conditions (Fig. 3E). Altogether these results suggest that *speA* expression is induced by acid external pH. Loss of arginine-dependent acid resistance in *E. coli* was not completely complemented by the *H. pylori speA* gene.

E. coli can survive in extreme pH conditions by at least three different mechanisms, one of which, AR3, involves arginine decarboxylation. To determine whether SpeA participates in a similar mechanism in *H. pylori*, we designed a complementation test using an *adiA*-deficient *E. coli* strain, because we have failed to produce an *H. pylori speA* knockout mutant probably

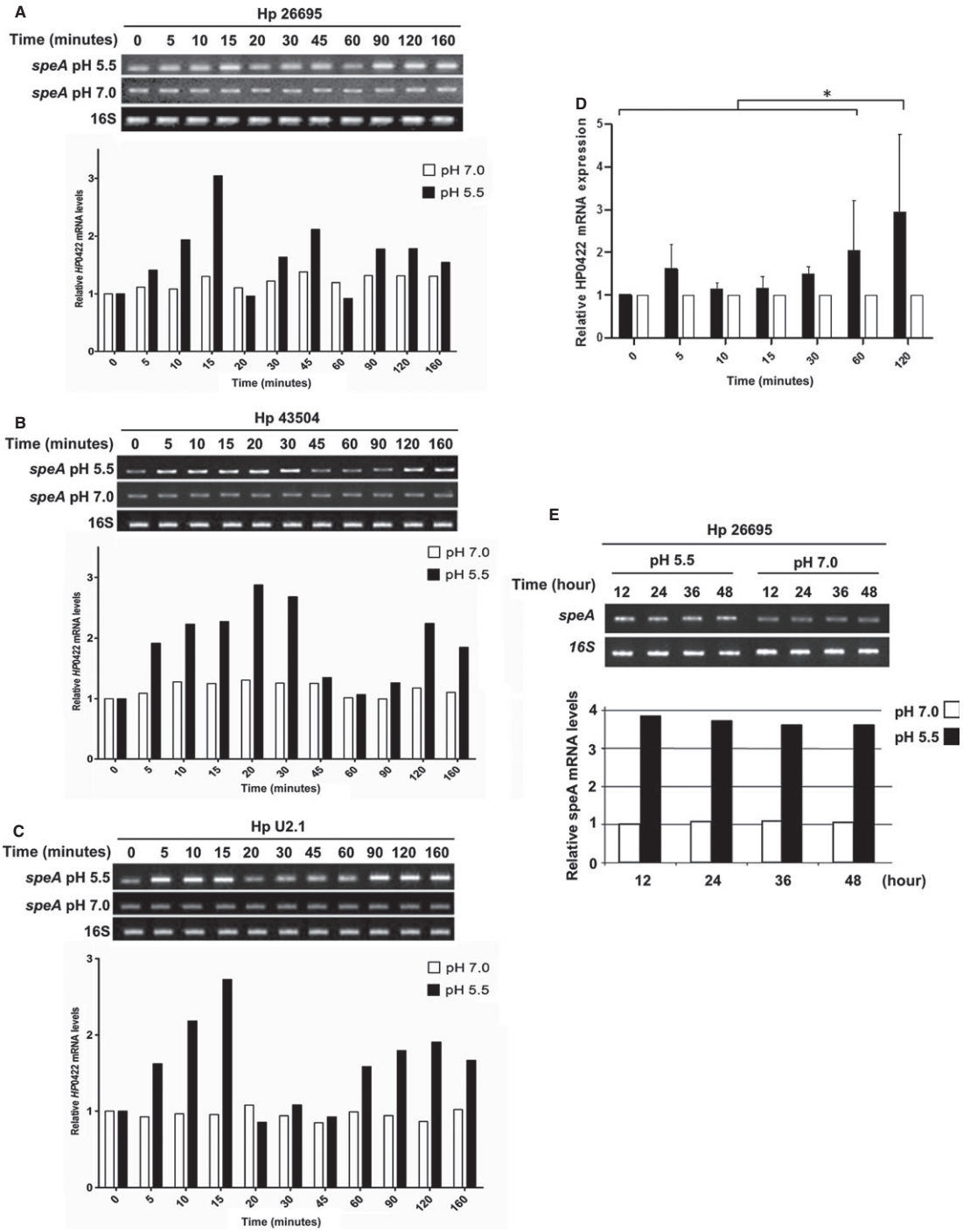

Figure 3 Effect of acid pH on *speA* gene expression. *Helicobacter pylori* strains 26695 (*A*, *D,* and *E*), 43504 (*B*), and U2.1 (*C*) were incubated in RPMI 1640 media adjusted to pH 7.0 or pH 5.5 for 0–160 minutes. Also, strain 26695 (*E*) was incubated at pH 5.5 or pH 7.0 for 12–48 hours. Semi-quantitative reverse-transcription polymerase chain reaction analysis of *speA* mRNA levels after short-term exposure to acid pH is shown (*A*, *B*, *C*). The amplicons resolved by agarose gel electrophoresis and visualized by UV light exposition were quantified by scanning densitometry. *speA* mRNA levels were standardized to *16S* rrna of the corresponding samples and normalized to the level observed at time zero (value 1). White bars represent the relative expression of *speA* obtained from bacteria exposed to pH 7.0, and black bars represent the relative expression of *speA* in samples obtained from bacteria exposed to pH 5.5. (*D*) qRT-PCR analysis of *speA* mRNA levels in *H. pylori* exposed to acid conditions (pH 5.5). With asterisk (*), ($p < .05$) values are designated among one pH condition, which are significantly different as analyzed by one-way ANOVA Tukey's post-test. (*E*) Representative semiquantitative RT-PCR of *speA* mRNA levels after long-term acid exposure. Analysis was performed as in *A*–*C*.

due to an essential character. As shown above, arginine supplementation improves *H. pylori* growth under acid conditions (Fig. 2). Thus, we tested whether or not *H. pylori* SpeA could improve growth of *E. coli* BL21 Δ*adiA* under acid conditions in the presence of arginine. As shown in Fig. 4, that was the case when growth of the parental, Δ*adiA* mutant and complemented (pET21a+ or pET-speA) *E. coli* strains were compared in the presence or absence of 3 mmol/L arginine at pH 4.0. Interestingly, *speA* expression both restored and improved the deficient growth of the BL21 Δ*adiA* in presence of arginine at pH 4.0 over a 5-hour culture period. Given this result, we wondered whether the *speA* expression could also protect against a more lethal exposure to acid in a similar manner to that observed in the *E. coli* AR3 system. As expected, deletion of the *adiA* gene by allelic exchange resulted in the complete loss of the arginine-dependent acid resistance in the *E. coli* BL21 strain at pH 2.5 (Fig. 5A). Phenotypic reversion in this strain was obtained by recombinant expression of the autologous AdiA protein after transforming the mutant bacteria with the pET-*adiA* plasmid (Fig. 5A). However, recombinant expression of *H. pylori speA* gene in the *E. coli* BL21 strain did not completely rescue the resistant phenotype when compared with the positive control (pET-*adiA*). This observation was particularly clear after a 3-hour acid challenge.

Then, we conducted an assay to extract and identify agmatine and putrescine from the *E. coli* and *H. pylori* cultures. As shown in Fig. 5B, TLC analysis of butanol extracts from *E. coli* cultures in synthetic medium revealed the increasing presence of agmatine from 12 hours of culture onwards and, as expected, it was accompanied by putrescine production. Note that agmatine and putrescine were the only polyamine compounds detected on TCL plates by the ninhydrin–chloroform reagent. As anticipated by the acid resis-

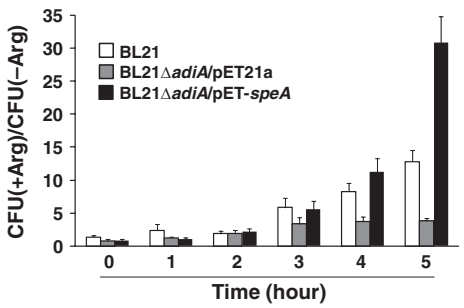

Figure 4 Effect of the expression of *H. pylori speA* on the growth of *E. coli* BL21Δ*adiA* mutant in acid conditions. BL21 and BL21Δ*adiA*/pET21a and BL21Δ*adiA*/pET-*speA* strains were grown overnight in LBG medium at pH 5.0. Volumes of the three cell suspensions corresponding to OD_{560} = 0.025 (approximately 1.8×0^7 CFU/mL) were used to inoculate 15 mL of synthetic medium adjusted to pH 4.0 and supplemented with or without 3 mmol/L arginine. Cells were grown at 37 °C for 0–5 hours, and samples were taken at 1-hour intervals for CFU counting. Bars represent the CFU (+Arg)/CFU(−Arg) ratios from three independent experiments ± SD (*$p \leq .05$).

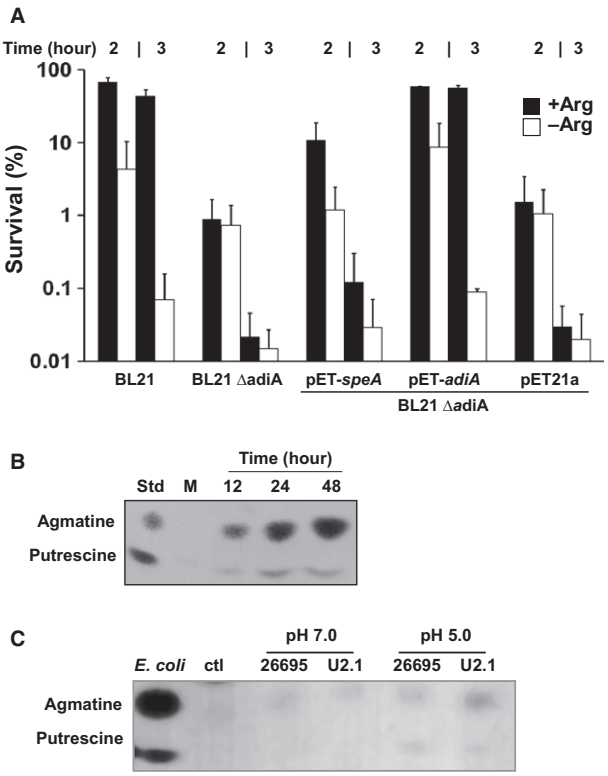

Figure 5 Heterologous expression of *H. pylori* SpeA protein in the *E. coli* BL21 Δ*adiA* mutant. (*A*) Determination of arginine-dependent acid resistance in *E. coli*. Bacteria were grown overnight in LBG medium adjusted to pH 5.0. A 10-μL aliquot was taken from the culture, added to 1 mL of acid shock medium pH 2.5, and incubated for 2 and 3 hours in the presence or absence of 3 mmol/L arginine. Survival under each experimental condition is represented by the percentage of cell viability (CFU) compared with bacteria maintained in synthetic medium (pH 7.0). Data represent mean values and standard deviations from three independent experiments ($p < .05$). Arginine-dependent acid resistance was assayed in *E. coli* BL21, BL21 Δ*adiA*, and in the BL21 Δ*adiA* strain complemented with either the pET-*speA* or pET-*adiA* plasmids, in presence of 1 mmol/L IPTG. (*B*, *C*) Detection of agmatine and putrescine production in *E. coli* and *H. pylori strains*. (*B*) *E. coli* was incubated at pH 7.0 for 12, 24, and 48 hours in synthetic medium supplemented with 3 mmol/L arginine; (*C*) *H. pylori* was incubated at pH 7.0 or 5.0 for 24 hours in TSA broth supplemented with 3 mmol/L arginine. For comparison, a butanol extract from *E. coli* culture (24 hours) is shown. Aliquots of butanol extracts from the cultures were chromatographed on TLC plates and revealed with ninhydrin. Agmatine and putrescine standards (3 and 5 μg, respectively) and a noninoculated sample were routinely included as controls.

tance assay, polyamine production by the Δ*adiA* mutant strain was undetectable, and *speA* complementation was not suffice to fully restore polyamine production, and agmatine and putrescine were poorly detected (data not shown). Based on these results, we tested agmatine and putrescine production directly in *H. pylori* 26695 and U2.1 cultures. Interestingly, both polyamines were present in butanol extracts from *H. pylori* grown at pH 5.0 (Fig. 5C), thus suggesting a role for an arginine decarboxylase in an acid adaptation mechanism, similar to the *E. coli* AR3. This result suggests that under acidic conditions, *H. pylori* expels agmatine to control pH homeostasis and is concordant with a previous report showing that agmatine levels are higher in the gastric juice of *H. pylori*-infected patients than in uninfected controls [34]. However, the amounts of agmatine and putrescine produced by *H. pylori* U2.1 were significantly higher than those produced by the *H. pylori* 26695 strain and notably less pronounced when compared with *E. coli*. It is still not clearly understood why the plasmid-borne *speA* gene (pET-*speA*) failed to fully complement the acid resistance defective phenotype of the *E. coli adiA* mutant. It is possible that the pET-*speA* is not as active as the *H. pylori* chromosomal gene or that the cloned fragment lacks a downstream DNA element of the *speA* gen that participates in the acid resistance mechanism. According to our results, the *speA* gene is the last ORF of that operon, and our construction (pET-*speA*) does not contain the DNA promoter elements that could be regulating its expression.

Transcriptional Factor Fur Regulates the Expression of the *speA* Gene in the Acid Response

Three *H. pylori* regulators have been implicated in acid adaptation: NikR, Fur, and the ArsRS two-component system [19]. The transcriptional factor Fur has been characterized as a regulator of gene expression during acid exposure in *H. pylori* [7]. Furthermore, *fur* knockout *H. pylori* strains present a sensitized phenotype to acid challenge in association with downregulation of several genes [7]. In agreement with this observation, Clustal analysis (http://www.ebi.ac.uk/Tools/es/egi-bin/jobresults.cgi/clustalw2/clustalw2-20090723-19004517 30.aln) revealed that putative binding sites for Fur described previously by Ernst et al. [35], namely Fur, Pfr I, and Pfr II boxes, actually are present in the *speA* operon promoter (data not shown). Therefore, we wondered whether Fur is involved in the observed increase in *speA* mRNA levels following acid exposure. To that purpose, we isolated mRNA from *H. pylori fur* mutants after long-term cultures in acid condition (pH 5.5, 12–36 hours), and the *speA* mRNA levels were analyzed by RT-PCR. Interestingly, we observed that the levels of the *speA* mRNA remained unaltered in *H. pylori* FUR504 strain (Fig. 6A,B). This lack of effect was in open contrast with the responsiveness of the corresponding parental strain (Fig. 6A,B). Interestingly, *speA* gene transcription changes were related to changes in protein levels. As shown in Fig. 6D, an in-house rabbit polyclonal antibody raised against *H. pylori* SpeA protein (Fig. 6C) revealed a strong increase in the levels of the SpeA protein after 24 hours of growth in acid conditions. Also, the SpeA protein levels remained basically unaltered when the Fur-negative strain was grown in acid conditions in contrast to the control condition at pH 7.0 (Fig. 6D, FUR504).

Adaptation of *H. pylori* to the acidic pH environment involves a plethora of biochemical processes. *H. pylori* responds to acid stress through multimechanisms including many proteins and depending on the acidity level [36]. Urease has been shown to play an important role in this process [4]. A urease-independent acid resistance mechanism in *H. pylori* has been also reported [14,37]. This mechanism is regulated by the ferric uptake regulator Fur [7]. Our results are in agreement with those reported by Bijlsma et al. [38] as to the growth defect of a *fur* mutant at low pH is consistent with findings of acid-sensitive *fur* mutants. This might be explained by failure in the increase in transcription of genes that are involved in the control of pH homeostasis under Fur regulation. In this study, the *speA* gene downregulation observed in the *H. pylori fur* knockout mutant supports that postulate. Such finding strongly supports our hypothesis that Fur is involved in the urease-dependent and urease-independent acid resistance-associated arginine decarboxylase activity in *H. pylori* [7].

Recently, we demonstrated that the arginine-66 residue of the transcriptional factor Fur is necessary for its regulatory function in the acid adaptation mechanisms of *H. pylori* [39]. However, the mechanism by which Fur influences arginine decarboxylase and acid resistance in *H. pylori* remains unknown and requires further investigation on Fur in association with gastric acidity.

Concluding Remarks

Our results showed that the *speA* gene (*adiA* in *E. coli*) is induced by acid conditions and that growth of *H. pylori* is enhanced in the presence of arginine, the amino acid substrate of the arginine decarboxylase, SpeA. Moreover, upregulation of the *speA* mRNA and protein levels was observed early after *H. pylori* exposure to pH 5.5 and also

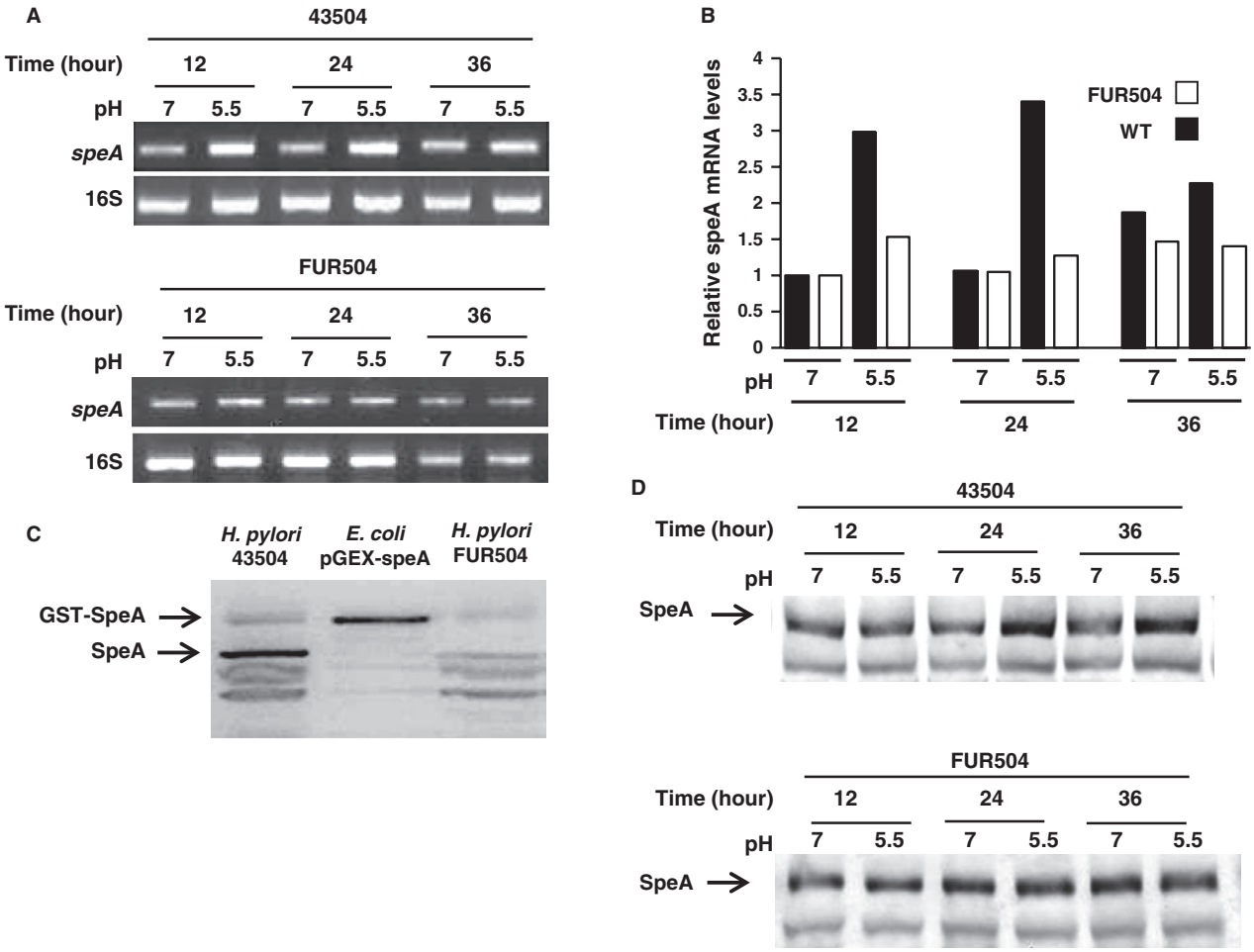

Figure 6 Effect of Fur deletion on the acid pH response of *speA* gene. *H. pylori* 43504 and the isogenic *fur::cat* mutant strains were incubated at pH 5.5 or pH 7.0 for 0–36 hours. (*A*) Representative RT-PCR analyses of *speA* mRNA levels at various times of incubation are shown. (*B*) Experimental data were analyzed and represented as described in the legend to Fig. 3C. (*C*) Anti-SpeA antibody validation. Immunoblot analysis of the SpeA protein levels in *H. pylori* 43504, the recombinant SpeA-GST protein expressed in *E. coli* (pGEX-*speA*) induced with IPTG and in *H. pylori* FUR504. (*D*) Immunoblot analyses of SpeA in the *H. pylori* 43504 and FUR504 strains incubated at pH 5.5 or pH 7.0 for 0–36 hours. A representative blot is shown.

when *H. pylori* was cultured under acidic conditions, thus suggesting that the arginine decarboxylase activity could play a role in protecting the bacterium from the acid pH and in contributing to maintain pH homeostasis through the exchange of intracellular protons while expelling agmatine to the medium. Interestingly, enhanced *speA* mRNA levels following acid stress were totally dependent on Fur presence in either short- or long-term acid exposures. Agmatine production by *H. pylori* was detectable when bacteria were grown at pH 5.0, although the amounts detected were notably lower than those of *E. coli* cultures. This observation would suggest that *speA* generates agmatine and contributes to the habituation of *H. pylori* to the acid environment rather than being part of an acute response to the acidic pH stress, as anticipated in the complementation acid stress assay at pH 2.5 (Fig. 5). Conversely, our results suggest that the SpeA activity constitutes a urease-independent acid resistance system in *H. pylori*.

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