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Arginine 66 Residue of Fur is Required for the Regulatory Function of this Protein in the Acid Adaptation Mechanism of *Helicobacter pylori*

Héctor Toledo,* Carlos Villafaena, Manuel Valenzuela and Remigio López-Solís

*Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Avenida Independencia 1027, Casilla 70086, Santiago-7, Chile

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Reprint requests to: Héctor Toledo, Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Avenida Independencia 1027, Casilla 70086, Santiago-7, Chile.
E-mail: htoledo@med.uchile.cl

Abstract

Background: *Helicobacter pylori* colonizes the gastric mucosa and must survive the acid pH of that environment. Like other enteric bacterial pathogens, including *Salmonella enterica*, *H. pylori* develops an acid tolerance response that is dependent on the function of the transcriptional regulator protein Fur.
Objective: To explore by site-directed mutagenesis whether two particular amino acid residues in the amino acid sequence of the *H. pylori* Fur protein, arginine 66 and histidine 99, are involved in the acid response mechanism in this bacterium.
Materials and Methods: Complementation assays in *Escherichia coli* H1780 (*fur* null mutant) both with plasmids carrying the *H. pylori fur* gene bearing substitution mutations R66A or H99A or R66A/H99A and with the *H. pylori* Fur-R66A mutant were conducted. Wild-type and mutated Fur proteins from *H. pylori* were assayed by using the *fiu::lacZ* reporter gene in the *E. coli* H1780 heterologous system at various pH and iron concentrations.
Results: Both bacterial growth and repression of the reporter gene were impaired under acid conditions in *E. coli* H1780 complemented with pUC19-*fur*-R66A. Also, in the *H. pylori* Fur-R66 strain bacterial growth and *speA* gene expression were impaired under acid conditions.
Conclusions: Arginine 66 but not histidine 99 in *H. pylori* Fur is required for the regulatory function of the Fur protein in the acid adaptation mechanism of the bacterium.

To colonize the digestive tract of a mammalian host, pathogenic enterobacteria must survive the acidic stomach condition and overcome the acid stress before reaching the intestine. For instance, *Salmonella* Typhimurium survives those extreme conditions because it has evolved a mechanism to respond to the acid challenge, the so-called acid tolerance response or ATR [1]. During the logarithmic growth phase of *S.* Typhimurium, the ATR is under the control of both *rpoS* [2], the signal transduction system PhoP/Q [3], and the iron transcriptional regulator Fur [4]. Each of these regulators controls a different subset of genes. The transcriptional regulator Fur, which normally acts as a co-repressor together with iron in the mechanism of iron uptake and storage [5], also regulates the expression of several genes coding for proteins that are involved in the acid tolerance response by a mechanism that is independent from iron availability [4].

Other outstanding example of adaptation to the acid environment is that of *Helicobacter pylori,* which is a neutralophilic Gram-negative spiral-shaped bacterium that is able to persistently colonize the human stomach. *H. pylori* infection is chronic in nature causes a serious transmissible infectious disease that damages gastric structure and function and is recognized as the causative agent in gastric atrophy, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma [6]. Although urease is essential for *H. pylori* stomach colonization [7], this bacterium is able to develop both an urease independent ATR and an acid stress response [8,9]. This acid tolerance response requires de novo protein synthesis

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and is dependent on the function of the global regulatory protein Fur [9].

The transcriptional factor Fur is a 17 kDa protein that is involved in global regulation of gene expression in bacteria. This protein has been also involved in both iron and pH homeostasis [4,10–12]. The regulatory role of Fur in the acid tolerance response can be separated from its role in iron homeostasis regulation by introducing mutations in specific amino acids [4]. Thus, in *S.* Typhimurium, mutations in codons coding for glycine 51, arginine 57, and isoleucine 67 residues of Fur affect the response to acid while mutations in the codon coding for the histidine 90 residue affect the response to iron with independence of the acid stress response [4]. The present study is aimed at defining whether the amino acid residues of Fur that are important for the response to acid in *S.* Typhimurium and that are conserved in the *H. pylori* Fur protein (arginine 66 and histidine 99) may be involved in the acid response mechanism in this bacterium.

Materials and Methods

Bacterial Strains and Growth Conditions

H. pylori strains used in this study were the strain ATCC 43504, the Fur negative strain FUR504, and the substitution mutant FUR-R66A (this study), which was derived from the *H. pylori* strain FUR504. The *H. pylori* FUR504 strain was obtained by natural transformation of the *H. pylori* 43504 strain with the pUC19-*fur::cat* plasmid, as described by Valenzuela et al. [9]. Frozen stocks of *H. pylori* were recovered and routinely grown for 24 hour at 37 °C, 5.5% CO_2, and 70–80% humidity on Trypticase soy agar (Becton-Dickinson, Sparks, MD, USA) with 0.4% *H. pylori* selective supplement Dent (Oxoid, Basingstoke, Hampshire, UK), 0.3% Isovitalex (Oxoid), and 5% horse serum (HyClone, Logan, UT, USA). Chloramphenicol-resistant (Cam^R) derivatives of these strains were grown on the above-described media supplemented with Cam (10 μg/ml). For liquid growth experiments, cells were grown in Trypticase soy broth (Becton-Dickinson) with 5% fetal calf serum supplemented with Isovitalex and Dent (Oxoid). The broth was adjusted to the desired pH with HCl. Bacteria were first grown to an optical density of 0.6–1.0 at 600 nm (OD_{600}) at pH 7.0 and subsequently diluted to a starting OD_{600} of 0.05 [13]. To measure the growth of *H. pylori* in liquid medium under acidic conditions, the medium was adjusted to pH 5.5 with HCl and supplemented with chloramphenicol (Cam; 10 μg/ml), as appropriate.

Escherichia coli strain XL-10 Gold [TetrD(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lac*IqZD*M15* Tn*10* (Tet^r) Amy Cm^r] (Strategene, Santa Clara, CA, USA) and strain H1780 [*fur*, *fiu::lacZ*, Sm^r, Km^r] [14] were used as hosts for plasmid transformations and for the reporter gene assay, respectively. *E. coli* strains were grown in Luria-Bertani (LB) medium or in MacConkey lactose agar plates (Difco, Detroit, MI, USA) supplemented with ampicillin (Amp; 100 μg/ml), as appropriate.

Construction of the R66A and H99A Substitutions in the *H. pylori* Transcriptional Factor Fur by Site-directed Mutagenesis

The plasmid pUC19-fur [9], which contains the complete sequence of the *H. pylori* 43504 *fur* gene, was used to replace amino acids R66 and H99 for alanine using the PCR method. The reaction mixture (50 uL) contained 5 uL of 10 × turbo Pfu buffer, 10 ng of pUC19-fur, 125 ng of mutagenic primers (R66AF and R66AR for the R66A substitution and primers H99AF and H99AR for the H99A substitution (Table 1), 1 uL 10 mmol/L dNTPs, and 2.5 U of Pfu DNA Turbo polymerase (Stratagene). PCR conditions were as follows: one cycle of 95 °C for 8 minutes followed by 18 cycles of 95 °C for 50 seconds, 55 °C (for R66A substitution) or 58 °C (for H99A substitution) for 50 seconds, 68 °C for 5 minutes, and a final extension step at 68 °C for 7 minutes. A PTC-100 MJ Research thermocycler was used. To select the mutant plasmids, the reaction product was treated for 1 hour at 37 °C with the restriction enzyme *Dpn*I (10 U), which specifically digests methylated DNA (GMe6ATC). Recovery of mutant plasmids (pUC19-fur-R66A and pUC19-fur-H99A) was achieved by transforming the *E. coli* XL-10 Gold. The R66A/H99A double substitution was developed by using the plasmid pUC19-fur-R66A, primers H99AR and H99AF, and following the same procedure. Using this approach, we produced plasmids pUC19-fur-R66A, pUC19-fur-H99A,

Table 1 List of the primers

Partidor	Secuencia
R66AF	5′ TTTCTTCAGTCTATGCCATTTTGAATTTC 3′
R66AR	5′ AGAAATTCAAAATGGCATAGACTGAAGAAAATGC 3′
H99AF	5′ AAGAACACCATGATGCCATCATTTGTTTGC 3′
H99AR	5′ GCAAACAAATGATGGCATCATGGTGTTCTTT 3′
Hp0422- F	5′ GCGGGATCCATGCAAGAAGTCCATGATTAGG 3′
Hp0422- R	5′ GCGCTGGAGTTAGAATCGTGCGCAAATACC 3′
GST-*fur*-F*	5′ GCGCTCGAGATGAAAAGATTAGAAACTTTGGA 3′
GST-*fur*-R*	5′ GCGGATCCTAACATTCACTCTCTTGGCATT 3′
16S-F	5′ GCTAAGAGATCAGCCTATGTCC 3′
16S-R	5′ CCTACCTCTCCCACACTCTA 3′

*Used for DNA sequencing.

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and pUC19-fur-R66A/H99A, which contain substitutions R66A, H99A, and the double substitution R66A/H99A, respectively. Plasmids were recovered by transformation of the *E. coli* XL-10 Gold. The substitutions introduced in the plasmids were confirmed by DNA sequencing using the ABI PRISM method Big-DyeTM Terminator version 3.0 (Applied Biosystems, Framingham, MA, USA) in an ABI-3100 instrument.

Complementation of fur Mutations: β-galactosidase Reporter Assay

Complementation was performed by transformation of the *E. coli fur* mutant strain H1780 [15] with the constructs presented above and was assessed by two methods: phenotypic analysis on MacConkey lactose agar plates [15] and by β-galactosidase activity determination [16]. For the reporter assay on solid medium, the *E. coli* strains that had been produced by transforming the *E. coli* H1780 strain with plasmids containing one of the mutations R66A, H99A or R66A/H99A, were grown in an iron-enriched medium (60 μmol/L $FeSO_4$). Under these conditions, the Lac^+ strains are red while the Lac^- strains are colorless or white. White colonies are observed if the transcription factor Fur is functional, otherwise colonies are red.

For the reporter assay in solution, *E. coli* H1780 transformed with the mutated *fur*-plasmid was grown overnight in LB broth and an aliquot of the culture was diluted 1:50 with fresh LB, supplemented with ampicillin 100 μg/mL, and further incubated with shaking at 37 °C until approximately 0.4 to 0.5 U OD560. Subsequently, 1 mL of culture was centrifuged at 3000 × ***g*** for 5 minutes, and the pellet was resuspended in 1 mL of Z buffer, pH 7.0 (0.06 mol/L $Na_2HPO_4 \times 7H_2O$; 0.04 mol/L $NaH_2PO_4 \times H_2O$; 0.01 mol/L KCl, 0.001 mol/L $MgSO_4$; 0.05 mol/L β-mercaptoethanol). Then, 50 μL of 0.1% SDS and 100 μL of chloroform were added to the cellular suspension, and the incubation was continued for 2–3 minutes at 37 °C. Finally, 200 μL of 4 mg/mL o-nitrofenilgalactopiranosido (ONPG) in buffer Z was added, and the mix was incubated at 37 °C until the solution acquired a yellow color, after which 500 μL of 1 mol/L sodium carbonate was added to stop the reaction [16]. The mixture was centrifuged at 15,700 × ***g*** for 5 minutes, the aqueous phase was recovered, and the absorbance was measured at 420 nm. β-galactosidase activity was calculated using the following equation:

$$U.M = (OD\ 420\ nm) \times 1000/(OD\ 560\ nm \times V \times t)$$

U.M = Units Miller; OD 420 = Absorbance of o-nitrophenol; OD 560 = Absorbance of cells; V = aliquot volume (mL); t = time of color appearance.

mRNA Extraction and RT-PCR Analysis

Total mRNA from each *H. pylori* strain was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total cDNA was synthesized using cDNA CoreKit (BIOline, Randolph, MA, USA) following manufacturer's instructions. PCR was performed in a PTC-100 MJ Research thermal cycler using the Hp0422-F and Hp0422-R primers for the *speA* gene, and the 16S-F and 16S-R primers for the 16S rRNA (internal control) (Table 1). Conditions for the PCR were optimized for each primer pair. The amplification products were subsequently analyzed by electrophoresis on 1% agarose gels and staining with ethidium bromide. Signals were quantified with ImageQuant 5.0 (Amersham Biosciences, Little Chalfont, UK).

Statistical Analysis

Mean standard deviations of at least three independent experiments were processed using the INSTAT v. 3.05 program (GraphPad Software, San Diego, USA, http://www.graphpad.com). For comparisons, *p* values were determined by using the one-way analysis of variance test and the Dunnett's post-test. A *p* value of .05 or less was considered as statistically significant.

Results and Discussion

Identification of Conserved Amino Acids in *H. pylori* Fur and *S.* Typhimurium Fur

Hall and Foster (1996) had shown that in *S.* Typhimurium the amino acid residues G51, R57 (fully conserved among 10 sequenced Fur genes [4]), and I67 are related to the acid stress response, while the H90 residue is important in iron regulation but not in regulating acid tolerance. To determine whether in *H. pylori* those amino acids residues are conserved, we aligned the amino acid sequences of *H. pylori* Fur and *S.* Typhimurium Fur. We observed that the R57 and H90 residues of the amino acid sequence of *S.* Typhimurium Fur were conserved in the *H. pylori* Fur sequence (amino acid residues R66 and H99, respectively). By contrast, amino acid residues G51 and I67 of *S.* Typhimurium Fur have been replaced in the sequence of *H. pylori* Fur (amino acid residues S60 and F76, respectively) (Fig. 1). Carpenter et al. [18] have recently reported that conserved amino acid residues in *H. pylori* Fur are important for its function. In that respect, H96A, E110A, and E117A mutations were found to alter iron-bound Fur regulation. Additionally, the H96A mutation alters Fur oligomerization whereas the E110A mutation

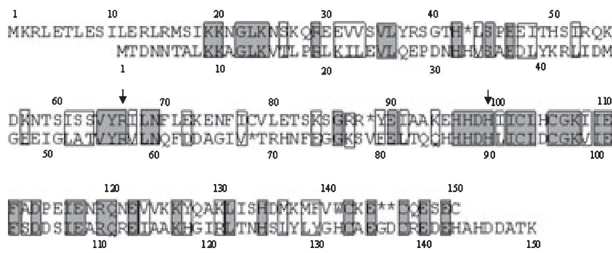

Figure 1 Amino acid sequences of the *Helicobacter pylori* and *Salmonella* Typhimurium Fur proteins. Amino acid sequence alignment of the *H. pylori* Fur protein (top) and *S.* Typhimurium Fur protein (bottom) was produced by using the Clustal X2 program [17]. Identical amino acids (filled rectangles) and similar amino acids (empty rectangles) are indicated. Arrows indicate positions of arginine 66 and histidine 99 residues in the *H. pylori* Fur protein sequence. These are conserved in the amino acid sequence of the *S.* Typhimurium Fur protein.

impacts both Fur oligomerization and its binding to DNA. Conversely, the H134A mutant exhibits altered *apo*-Fur regulation as a consequence of changes in its ability to bind DNA. Finally, the E90A mutant exhibits alterations in the *apo*-Fur regulation, with no affect on other protein functions.

Functional Analysis of the *H. pylori* Transcription Factor Fur

To characterize the role of the R66 and H99 amino acid residues in the sequence of *H. pylori* Fur, the plasmids pUC19-fur-R66A, pUC19-fur-H99A, and pUC19-fur-R66A/H99A, containing the substitution mutations R66A, H99A, and R66A/H99A, respectively, were used to transform the *E. coli* H1780 strain. This strain is a null mutant for Fur and expresses the *lacZ* gene under the control of the *fiu* (ferric ion uptake) gene promoter, which contains four Fur boxes. We used two positive controls, namely E. *coli* H1780 transformed with the plasmid pUC19-fur [9], which has a 1.5 kb fragment containing the promoter and the complete sequence of the *fur* gene derived from *H. pylori* 43504 and, secondly, the plasmid pGEM-furAf, that contains the *fur* gene of *Acidithiobacillus ferrooxidans*, which is functional in the presence of iron [14]. β-galactosidase activity was determined both in solid and in liquid media. In both conditions, the mutated transcriptional factor Fur R66A did not display repressor activity over the *lacZ* gene in the presence (60 μmol/L $FeSO_4$) or in the absence (200 μmol/L 1, 10-phenanthroline) of iron. On the other hand, with the substitution mutation H99A, the repressor function of Fur was mostly lost although to a lower extent than the repression observed with the substitution mutation R66A or with the double mutant R66A/H99A (Fig. 2A). The latter two substitution mutations displayed a similar repressive effect. Under the conditions of the assay, *H. pylori* Fur repressed the expression of the *lacZ* gene either in presence or in absence of its ferrous iron cofactor (Fe2+), while the *A. ferrooxidans* Fur repressed *lacZ* gene only in the presence of Fe2+ (Fig. 2A). These results were independent of the medium pH (Fig. 2B). These observations suggest that R66 and H99 could be involved in the repression function of the transcriptional factor Fur, and this factor represses the expression of the *lacZ* gene by the classical mechanism comprising Fur bound to Fe2+ or by the apo-Fur regulation [19].

Effect of the R66 and H99 Mutations in the Transcription Factor Fur on the Adaptive Response to Acid in *E. coli* H1780

To determine whether R66 and/or H99 are involved in acid adaptation, *E. coli* strains that were produced by

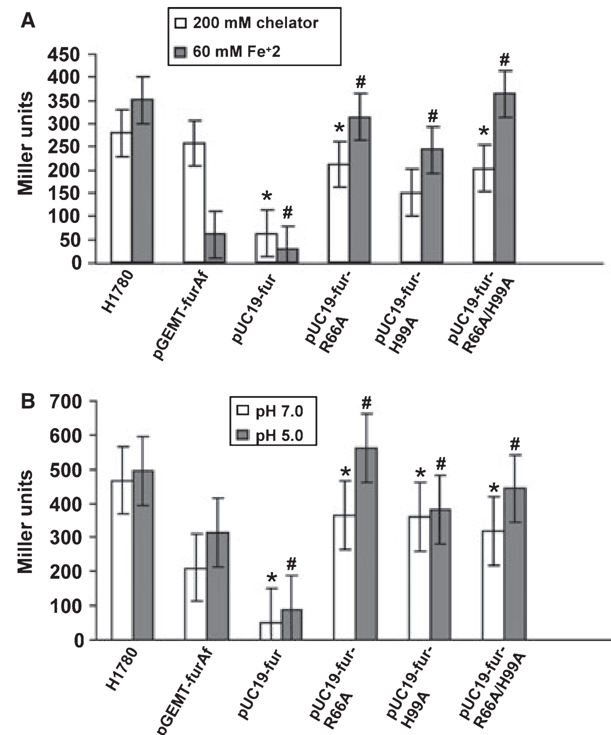

Figure 2 Reporter activity of β-galactosidase. β-galactosidase activity was measured both in the *Escherichia coli* H1780 wild-type strain and in the strains produced by transformation of *E. coli* H1780 with the plasmids containing either the R66A, H99A, or R66A/H99A substitution mutations made in the *Helicobacter pylori fur* gene. (*A*) The assay was performed under conditions of iron absence (200 μmol/L 1,10-phenanthroline) and iron presence (60 μmol/L ferrous sulfate). (*B*) Cultures were incubated for 5 hour with mechanical stirring at 37 °C in Luria-Bertani medium at either pH 7.0 or pH 5.5. Bars represent data (mean ± standard deviation) from three independent experiments (where indicated * and #, $p \leq .05$). *E. coli* strains are indicated under each bar.

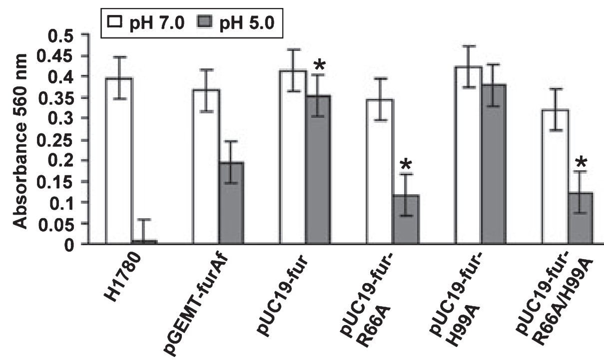

Figure 3 Growth of *Escherichia coli* H1780 transformed and untransformed under different conditions of pH. Bacteria were cultured for 5 hour at 37 °C in Luria-Bertani at either pH 7.0 or pH 5.0 just before the enzyme assay. Bars represent data (mean ± standard deviation) from three independent experiments (*$p \leq .05$). *E. coli* strains are indicated under each bar.

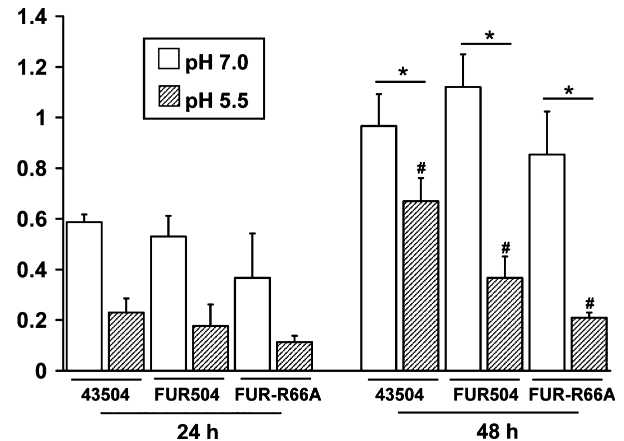

Figure 4 Growth of *Helicobacter pylori* strains ATCC 43504, FUR504, and Fur-R66A in liquid medium. Aliquots of each of the *H. pylori* strains (3×10^6 cells) were seeded in TSB medium adjusted either to pH 7.0 or to pH 5.5 and incubated for 48 hour at 37 °C, 5.5% CO_2, and 70% humidity. At 24 and 48 hour, samples of the culture medium were obtained. Bars represent data (mean ± standard deviation) from three independent experiments (where indicated * and #, $p \leq .05$).

transformation of the H1780 strain with plasmids containing any of the substitution mutations R66A, H99A or R66/H99A, were grown in LB medium adjusted to either pH 7.0 or pH 5.0 for 5 hour at 37 °C. As shown in Fig. 3, the substitution mutations R66A and R66A/H99A did affect both the growth and the ability of the two *E. coli* transformants to respond to changes in pH. On the other hand, the substitution mutation H99A did not affect the growth in the acid medium of the *E. coli* strain containing the plasmid pUC19-fur-H99A. These results suggest that R66 but not H99 is involved both in the mechanism of proton sensing by the transcriptional factor Fur and in the development of the acid adaptation mechanism, as reported in *S.* Typhimurium [4].

Delany et al. [20] had shown that substitution of the histidine 99 residue by an isoleucine residue (H99I) in the *H. pylori* Fur protein prevented the conformational change that is necessary for the binding of the protein to DNA. This observation was explained on the ground that this histidine residue acts as a ligand with the ability to bind the ferrous ion [20]. Also, Hall and Foster (1996) had shown that in *S.* Typhimurium Fur, the amino acid residue H90 (equivalent to H99 in the *H. pylori* Fur protein) is important in iron regulation but not in regulating acid tolerance. Both observations from the literature are in full agreement with the findings of our present study.

Determination of Fur-R66A Functions in the Acid Adaptation of *H. pylori*

We recently demonstrated that the *speA* gene expression is under the control of the transcription factor Fur and that its expression increases when *H. pylori* is exposed to pH 5.5 [21]. To analyze the effect of the R66A substitution mutation on the transcriptional factor Fur, the *fur::cat* gene in the *H. pylori* strain FUR504 [9] was replaced by allelic gene recombination using the plasmid pUC19-fur-R66A to produce the *H. pylori* strain FUR-R66A. The mutant strain was identified by isolating chloramphenicol sensitive colonies and the substitution R66A was confirmed by sequencing of the *fur* gene. *H. pylori* FUR-R66A displays a defective growth in liquid medium adjusted to pH 5.5 (Fig. 4), as it also occurs with *E. coli* H1780 transformed with the plasmid pUC19-fur-R66A (Fig. 3) and with the *H. pylori* strain FUR504 (Fig. 4). The parental strain of *H. pylori* 43504 grew normally after 48 hour of exposure at pH 5.5 (Fig. 4). This result lends strong support to the view that the R66 residue of the transcriptional factor Fur is involved in its regulatory role in the acid tolerance response. Further, we also observed that the *speA* gene expression in *H. pylori* 43504 increased continuously up to 2.5-fold at 15 minutes of acid exposure (pH 5.5) and after a sharp decrease showed a new significant increase at 120 minutes of growth under the same acid conditions (Fig. 5C). However, the *speA* gene regulation by Fur was lost when the mutant strain *H. pylori* FUR-R66A was exposed to pH 5.5 (Fig. 5), as it was previously observed with the *fur* null mutant strain *H. pylori* FUR504 [21]. Based on these observations, we can postulate that R66 is essential for the Fur function as a transcriptional regulator in the acid adaptation mechanism.

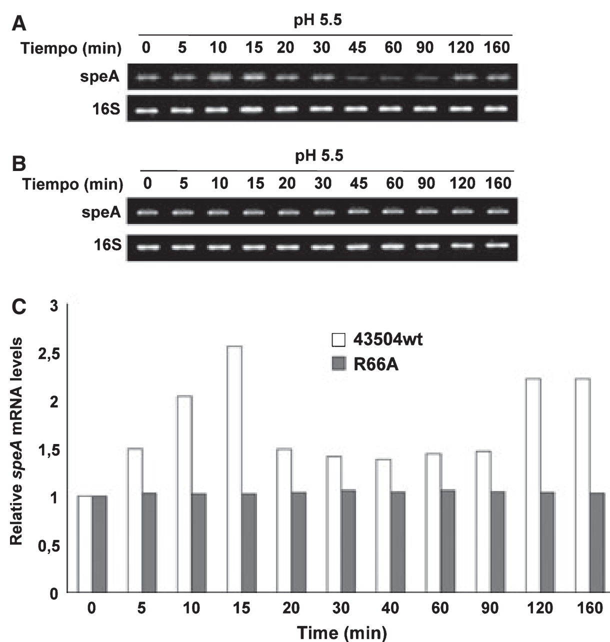

Figure 5 *speA* gene expression in *Helicobacter pylori* Fur-R66A and *H. pylori* 43504 exposed to acidic pH. A suspension of 9×10^8 cells was incubated for up to 160 minutes at pH 5.5. At various times, cell samples were collected and the incubation was stopped by centrifugation. Total RNA was extracted from each sample. *speA* gene expression was assessed by RT-PCR analysis in *H. pylori* 43504 (*A*) and *H. pylori* Fur-R66A (*B*) strains. 16S rRNA gene expression was used as a control. In (*C*), relative expressions of the *speA* gene in *H. pylori* 43504 (empty bars) and *H. pylori* Fur-R66A mutant (filled bars) strains are shown.

Recently, Rohs et al. [22] have shown that most of the amino acid residues that contact the minor grooves, particularly narrow minor grooves, are arginines. Those authors have also suggested that such interactions underlie a novel and widely used mode for protein-DNA recognition. Thus, an indirect readout mechanism based on conformational changes of DNA sequences involving the groove width would represent a connection between sequence and shape. That connection seems to be provided in part by A-tracts, that is, stretches of four or more As or Ts, which have a strong tendency to narrow the groove and to produce binding sites for arginines. According to our postulate on the interaction between Fur and the promotor of the *speA* operon [23], the transcriptional factor Fur may interact with the predicted three Fur boxes (GATAATGATAATCATTATC) [24] through arginine 66. Those Fur boxes contain short A-tracts that could provide the binding sites for arginine.

Concluding Remarks

Our results lend support to the observations of Roth et al. [22] because the Fur boxes have the potential to create arginine-binding sites and because the regulatory function of Fur on the *speA* gene expression is lost when the mutant *H. pylori* strain Fur-R66A is exposed to acid pH. This putative regulatory mechanism, together with the increasing availability of high-throughput binding data, opens the possibility of a significant progress in the understanding of how proteins can recognize specific DNA sequences and so to understand more complex genetically driven mechanisms, as it is the acid tolerance response mechanism.

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