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Gastric Cancer

Helicobacter pylori urease and flagellin alter mucin gene expression in human gastric cancer cells --Manuscript Draft--

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Corresponding Author:	Isabelle VAN SEUNINGEN, Ph.D. Inserm UMR837/JPARC/Team 5 LILLE cedex, FRANCE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Inserm UMR837/JPARC/Team 5
Corresponding Author's Secondary Institution:	
First Author:	Michael PERRAIS, Ph.D.
First Author Secondary Information:	
Order of Authors:	Michael PERRAIS, Ph.D. Christel ROUSSEAU, Ph.D. Marie-Paule DUCOUROUBLE René COURCOL, M.D, Ph.D. Pascal VINCENT, Ph.D. Nicolas JONCKHEERE, Ph.D. Isabelle VAN SEUNINGEN, Ph.D.
Order of Authors Secondary Information:	
Abstract:	<p>Helicobacter pylori (Hp) is one of the etiological agents in human gastric adenocarcinoma and is known to interact with mucus gel and alter mucin gene expression. The aim of this work was to study, using an in vitro model of cell infection, the effects of urease, flagellin and CagA virulence factors on the regulation of the four 11p15 mucin genes (MUC2, MUC5AC, MUC5B and MUC6). KATO-III and AGS gastric cancer cells were infected for 1h, 3h or 6h with Hp wild-type strains (ATCC 43504, N6 and SS1) or corresponding isogenic mutants deficient for urease subunit B, flagellin subunit A and CagA. mRNA levels of MUC2, MUC5B, MUC5AC and MUC6 were assessed by RT-PCR and functional activity of their promoters was measured by transient transfection assays. Infection of KATO-III cells with Hp wild-type strains resulted in an early (at 1h) transient expression of MUC2, MUC5AC and MUC6 mRNA concomitant with those of IL-1beta, IL-8 and TNF-alpha cytokines. In these cells, UreB-isogenic mutant induced a strong activation of MUC5AC expression and UreB-responsive elements were located in the -486/-1 region of the promoter. FlaA- and CagA- mutants had no effect on mucin gene mRNA levels in KATO-III cells. In AGS cells, Hp-responsive elements were identified in all promoters and overexpression of NF-kappaB induced up-regulation of MUC5AC promoter activity when infected with UreB- isogenic mutant. In conclusion, these results indicate that Hp infection of gastric cancer cells alters 11p15 mucin gene transcription and that MUC5AC down-regulation is mediated by urease virulence factor.</p>

***Helicobacter pylori* urease and flagellin**

alter mucin gene expression in human gastric cancer cells

Michaël Perrais^{a*}, Christel Rousseaux^{b*}, Marie-Paule Ducourouble^a, René Courcol^b, Pascal Vincent^b, Nicolas Jonckheere^a, and Isabelle Van Seuningen^a

^aInserm, UMR837, JPARC, Team « Mucins, epithelial differentiation and Carcinogenesis », Rue Polonovski, F-59045 Lille cedex, France

^bInserm, E9919, Université JE 2225, Institut de Biologie de Lille, F-59021 Lille, France

* Equal participation of the two authors

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To whom correspondence should be sent:

Isabelle Van Seuningen, PhD

Inserm UMR837, JPARC, Team “Mucins, epithelial differentiation and carcinogenesis”

Bâtiment G. Biserte,

Rue Polonovski,

59045 Lille cedex, France

Phone: +33.320.29.88.67, FAX: +33.320.53.85.62

E-mail: isabelle.vanseuningen@inserm.fr

Abbreviations

bp: basepair

Fla: Flagellin

Hp: *Helicobacter pylori*

IL: Interleukin

NF- κ B: Nuclear factor- κ B

PCR: Polymerase chain reaction

RT: Reverse transcriptase

TNF- α : Tumor necrosis factor- α

Ure: Urease

CagA: Cytotoxin Associated Antigen

Abstract

Background: *Helicobacter pylori* (Hp) is one of the etiological agents in human gastric adenocarcinoma and is known to interact with mucus gel and alter mucin gene expression. The aim of this work was to study, using an *in vitro* model of cell infection, the effects of urease, flagellin and CagA virulence factors on the regulation of the four 11p15 mucin genes (*MUC2*, *MUC5AC*, *MUC5B* and *MUC6*). **Methods:** KATO-III and AGS gastric cancer cells were infected for 1h, 3h or 6h with Hp wild-type strains (ATCC 43504, N6 and SS1) or corresponding isogenic mutants deficient for urease subunit B, flagellin subunit A and CagA. mRNA levels of *MUC2*, *MUC5B*, *MUC5AC* and *MUC6* were assessed by RT-PCR and functional activity of their promoters was measured by transient transfection assays. **Results:** Infection of KATO-III cells with Hp wild-type strains resulted in an early (at 1h) transient expression of *MUC2*, *MUC5AC* and *MUC6* mRNA concomitant with those of IL-1 β , IL-8 and TNF- α cytokines. In these cells, UreB⁻ isogenic mutant induced a strong activation of *MUC5AC* expression and UreB-responsive elements were located in the -486/-1 region of the promoter. FlaA⁻ and CagA⁻ mutants had no effect on mucin gene mRNA levels in KATO-III cells. In AGS cells, Hp-responsive elements were identified in all promoters and overexpression of NF- κ B induced up-regulation of *MUC5AC* promoter activity when infected with UreB⁻ isogenic mutant. **Conclusion:** these results indicate that Hp infection of gastric cancer cells alters 11p15 mucin gene transcription and that *MUC5AC* down-regulation is mediated by urease virulence factor.

Keywords: MUC5AC, H pylori, mucin, urease, stomach

Introduction

Gastric cancer is the second common cancer in humans worldwide. In 1994, the International Agency for Research on Cancer recognized *Helicobacter pylori* (Hp), a Gram negative bacterium, which natural habitat is restricted to the stomach, as one of the etiological agents in human gastric adenocarcinoma and as a type I carcinogenetic agent [1-3]. Indeed, epidemiological studies performed in different countries have shown a correlation between gastric cancer rate and Hp seroprevalence in populations [4].

As the major glycoprotein component of mucus, mucins play a major role in maintaining mucosae integrity [5, 6]. The genes encoding mucins are now classified in two families, the large secreted gel-forming mucins and the membrane-bound mucins [7, 8]. In normal stomach, *MUC1* and *MUC5AC* are the main mucin genes expressed in surface/foveolar epithelial cells whereas *MUC6* is expressed in the antral glands [9, 10]. During infection, Hp associates with the mucus layer [11], interacts with *MUC5AC* [12-14] and binds gastric epithelial cells [15]. In Hp-induced gastritis, the pattern of mucin gene expression is altered [16] and is characterized by an aberrant expression of the gland-type gastric mucin *MUC6* and a decreased expression of the normal surface-type mucin *MUC5AC* in the surface epithelium [9, 10, 16-19]. However, until now nothing is known about the intracellular molecular mechanisms responsible for *MUC5AC* altered profile of expression. *MUC5AC* being the major secreted mucin in stomach with an altered profile of expression during Hp infection, we undertook to decipher the molecular mechanisms responsible for *MUC5AC* transcriptional regulation by Hp.

Since the discovery of Hp and its involvement in gastric pathologies, many studies have been conducted to identify determinants of bacterial pathogenicity. Among Hp attributes, urease and flagellin have been recognized as essential for bacterial colonization of the gastric mucosa [20, 21]. Urease degrades urea into ammonia and carbon dioxide and provides an

acid-neutralizing cloud of ammonia that could protect the bacterium from gastric acidity. Flagella (5 to 7 per cell), which confer motility, are made of polymers of two subunits, the major flagellin FlaA and the minor flagellin FlaB. The *cagA* gene is one of the 31 genes of the pathogenicity island *cag* (*cag* PAI), which encodes a type IV secretion system allowing the translocation of CagA into the gastric epithelial cells. CagA modifies the phosphorylation pattern of intracellular proteins leading to the cytoskeleton rearrangement of infected cells [22].

To better understand Hp-induced molecular mechanisms associated with altered expression of secreted mucins and especially MUC5AC in the gastric mucosa, we undertook to study the transcriptional regulation of these genes using an *in vitro* model of epithelial gastric cell infection by Hp in which the impact of urease, flagellin and CagA Hp virulence factors on the regulation of the mucin genes was studied at the transcriptional level. In this report, we establish a direct link between Hp infection of gastric cancer cells and down-regulation of *MUC5AC* gene expression by the urease virulence factor.

Materials and methods

Cell lines

KATO-III and AGS human gastric adenocarcinomatous cell lines were cultured as previously described [23].

Bacterial strains

The *Helicobacter pylori* wild type strain ATCC 43504, the parental strain N6 [24], N6 flaA Ω Km (flagellin (FlaA)-deficient [25]) and N6 ureB :: Tn3Km (urease (UreB)-deficient [24]) mutants, the parental strain SS1 and the deficient mutant SS1 CagA⁻ (SS1 cag Ω Km) [26, 27] were a kind gift of Dr. A. Labigne (Institut Pasteur, Pathogénie bactérienne des muqueuses, Paris). Bacteria were grown on Columbia agar (Becton Dickinson) supplemented with 0.5% glucose, 0.05% cystein chlorhydrate and 10% horse blood. Plates were incubated for 48h in 5% O₂ and 11% CO₂.

Infection of cell monolayers

Forty-eight hour cultures of Hp on agar plates were swabbed and bacteria were immediately suspended in 0.85% NaCl at 6.2 Mc Farland unit density (10⁹ viable bacteria per ml). One ml of this bacterial suspension was added to cell cultures and infected cells were placed at 37°C in a 5% to 10% CO₂ and 80-90% air atmosphere incubator. Cells were incubated with bacteria up to 24h. No antibiotics were added to the co-culture. Cellular integrity, determined by measuring the amount of lactate dehydrogenase in the culture medium using Olympus System Reagent Kit, was performed after 24h cell infection.

RT-PCR

Cells were harvested at confluence and total RNA (1.5 μ g), extracted with the Qiang RNA blood mini-kit (Qiagen), was used to prepare cDNA using the Advantage™ RT-for-PCR kit (Clontech). PCRs on 5 μ l of cDNA using specific pairs of primers for the mucin genes and analyses of PCR products were performed as described before [23, 28, 29]. Primers

used to study the expression of IL-1 β , IL-6, IL-8, TNF- α and NF- κ B p65 subunit are indicated in table I. 18S rRNA subunit was used as an internal control. The gene of interest/18S ratio was calculated by densitometric analysis of the DNA bands using the Gel Analyst Gel Smart software (ClaraVision, Paris, France). All PCR studies were performed in triplicate on samples from three different sets of experiments.

Plasmid constructions

MUC2 (-2627/-1 and -2096/+27), *MUC5AC* (-1366/-1, -486/-1 and -202/-1), and *MUC5B* (-223/-1, -1329/-1117 and -2044/-1117) promoter deletion mutants were previously described [23, 28, 29]. Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Transient transfection assays

Cell transfections with pGL3 deletion mutants of *MUC2*, *MUC5AC*, *MUC5B* and *mMuc5ac* promoters were performed using Effectene[®] reagent (Qiagen) as previously described [28]. Briefly, cells (0.5 x 10⁶ cells/well in a 6-well plate) were passed the day before the transfection. Transfected cells were then left for another 24h incubation at 37°C. Infection with Hp strains (10⁹ cfu/ml) was carried out 1h, 3h, or 6h before cell harvesting. Total cell extract preparation, luciferase activity and protein content measurement (BCA kit, Pierce) were carried out as previously described [28]. Each plasmid was assayed in triplicate in at least three separate experiments. Co-transfection studies were performed in the same conditions in the presence of 1 μ g of the pGL3-deletion mutant and 0.25 μ g of the pCMV4-NF- κ B p65 expression vector. Empty pCMV4 vector was used as the reference.

Statistics

The statistical analyses were carried out using both Excel and GraphPad 4.0 softwares (GraphPad softwares Inc., La Jolla, USA). The differences in the means of samples were analyzed using student t test or one way anova test with selected comparison using tukey post

hoc test with differences less than 0.05 considered as statistically significant (*). ** indicates $p < 0.01$ and *** $p < 0.001$.

Results

Influence of Hp virulence factors on mucin gene mRNA levels in gastric cancer cells

When KATO-III cells were infected with the wild-type strains, no effect was observed on *MUC5B* mRNA levels whereas *MUC2*, *MUC5AC* and *MUC6* expression noticeably increased at 1h infection (Fig.1). This effect was transient as it was not maintained at 3h or 6h infection. More interestingly, infection with the UreB⁻ mutant resulted in a strong increase of *MUC5AC* expression at 1h (7-fold) when compared with the cells infected with N6 wild-type strain. *MUC2*, *MUC5B* and *MUC6* mRNA levels were not affected by the UreB⁻ mutant. Infection with FlaA⁻ or CagA⁻ mutants had no additional impact on the expression of the four 11p15 mucin genes when compared with the respective wild-type strains. In AGS cells, no effect was seen on mucin gene expression (not shown). In conclusion, a transient up-regulation of *MUC2*, *MUC5AC* and *MUC6* mucin genes was observed in KATO-III cells at 1h infection with the three Hp wild-type strains. This effect was dramatically enhanced for *MUC5AC* after infection with UreB⁻ isogenic mutant.

Influence of Hp and UreB, FlaA and CagA virulence factors on *MUC2* promoter activity

Having shown that mucin gene mRNA levels were altered after Hp infection, we undertook to study the effect of Hp at the promoter level and identify Hp-responsive regions. In KATO-III cells, wild-type strain ATCC 43504 increased luciferase activity of the -2096/+27 region of *MUC2* promoter (2 fold) (Fig. 2). N6 wild-type strain and UreB⁻ and FlaA⁻ isogenic mutants had little effect on *MUC2* promoter activity. Absence of CagA increased activity (2 fold) of the -2627/-1 fragment whereas it decreased that of the -2627/-1 promoter region. In AGS cells, activation of the -2627/-1 *MUC2* promoter region was observed with the three wild-type strains at 3h infection followed by a 50% inhibition at 6h (Fig. 3). This activation was lost with fragment -2096/+27. Isogenic mutant UreB⁻ had no

effect whereas FlaA⁻ enhanced activity of the fragment -2627/-1 at 1h (2 fold). Infection with CagA⁻ mutant resulted in a two-fold activation of the two fragments. Altogether these results indicate that *MUC2* promoter contains positive and negative regulatory elements to Hp virulence factors that show cell-specific activity.

Influence of Hp and UreB, FlaA and CagA virulence factors on *MUC5AC* promoter activity

In KATO-III cells, Hp wild-type strains ATCC 43504 and N6 induced a time-dependent inhibition of the -202/-1 *MUC5AC* promoter region resulting in at least 50% loss at 6h infection (Fig. 3). This inhibition was lost with the -1366/-1 region of the promoter. Infection with wild-type strain SS1 induced the activity of the fragment -486/-1 in a time-dependent manner. Activation of the -486/-1 deletion mutant was found after cell infection with UreB⁻ at 3h (2.2 fold), with FlaA⁻ at 6h (2 fold) and with CagA⁻ at 6h (1.6 fold). The activation by UreB⁻ was delayed at 6h post-infection with promoter fragment -202/-1. In AGS cells, the three wild-type strains induced activation of the -202/-1 and of the -1366/-1 promoter regions at 1h. Altogether, this indicates that cell-specific urease, flagellin, and CagA positive and negative elements are present within the *MUC5AC* promoter.

UreB⁻ showed a transactivating effect of the -1366/-1 fragment at 3h (2 fold) that was inverted (inhibition of 75%) when using the -486/-1 promoter region. These results indicate that UreB responsive elements are present within the -1366/-203 region of *MUC5AC* promoter. FlaA⁻ had a strong transactivating effect on the -486/-1 *MUC5AC* promoter region that was lost on the -202/-1 region. This indicates that FlaA responsive elements are present within the -486/-203 region of *MUC5AC* promoter. Infection with CagA⁻ deficient mutant resulted in transactivation of the -202/-1 region, indicating that CagA responsive elements are located in that part of the promoter.

Influence of Hp and UreB, FlaA and CagA virulence factors on *MUC5B* promoter activity

Infection of KATO-III cells with ATCC 43504, N6 and SS1 wild-type strains decreased *MUC5B* promoter activity in a time-dependent manner and resulted in 60-80% inhibition of both promoters at 6h infection (Fig. 4). The effect was different with -2044/-1117 construct with which activation was first seen at 3h followed by an inhibition at 6h. UreB⁻ and FlaA⁻ isogenic mutants had no effect. Infection with CagA⁻ resulted in 50% inhibition of the -223/-1 construct at 1h infection. In AGS cells, a transient inhibitory effect of ATCC 43504 strain was found on the proximal promoter (-223/-1) at 1h infection (75% inhibition) and on the distal promoter (-2044/-1117) at 3h (50% inhibition). FlaA⁻ and CagA⁻ both induced activation of the distal promoter (-1329/-1117) at 1h and 3h infection, respectively. These effects were lost when using the -2044/-1117 deletion mutant indicating that FlaA and CagA responsive elements are present within the -1329/-1117 of *MUC5B* distal promoter. UreB⁻ induced a 3-fold transactivating effect of the -2044/-1117 deletion mutant at 6h infection that was lost with the -1329/-1117 mutant indicating that UreB responsive elements are present within the -2044/-1330 region of *MUC5B* distal promoter.

Effects of Hp infection on cytokines and NF- κ B expression in KATO-III and AGS cells

In order to correlate mucin gene altered expression after gastric cell infection by Hp and virulence factors to known pro-inflammatory cytokines and signaling pathway regulated by Hp, expression level of *IL-8*, *IL-1 β* , *IL-6*, *TNF- α* and *NF- κ B* mRNA were measured. Our results indicate that *IL-8* and *TNF- α* mRNA levels were increased in infected KATO-III cells as soon as 1h (Fig. 5A). *IL-6* mRNA was neither expressed nor up-regulated in these cells (data not shown). *IL-8* induction by N6 wild-type strain seemed to be mediated by the urease factor since the activation was decreased when cells were infected with UreB⁻ mutant. *TNF- α* mRNA was strongly induced by wild-type strains as early as 1h infection. Early activation of

TNF- α by Hp seems also to be mediated by the urease factor since *TNF- α* mRNA activation was postponed to 6h infection when using UreB⁻ mutant. *IL-1 β* mRNA activation occurred at 3h infection with ATCC 43503 strain and at 1h with N6 wild-type strain. *IL-1 β* activation is also mediated by urease since the activation was strongly decreased as early as 1h when using UreB⁻ isogenic mutant. *NF- κ B* mRNA level was increased as soon as 1h infection with N6 wild type strain and this effect was lost with virulence factors UreB. In AGS cells, Hp infection with both wild-type strains induced an early (1h) transient expression of *IL-8* mRNA that was lost thereafter (Fig. 5B). The activation at 1h was maintained with FlaA⁻ mutant whereas it was lost with UreB⁻ confirming the role of urease in mediating *IL-8* activation by Hp. *NF- κ B* mRNA expression increased as of 3h infection with both wild type strains and was maintained at 6h. This effect was mediated by UreB since infection with this deficient mutant did not show any induction of *NF- κ B* expression. All the other cytokines studied (*IL-1 β* , *IL-6* and *TNF- α*) were neither expressed nor induced in these cells (data not shown). Altogether these results indicate that Hp infection of KATO-III cells activates *IL-8*, *IL-1 β* and *TNF- α* and *NF- κ B* expression and that this process is mediated by the UreB virulence factor. In AGS cells, the same mechanism involving UreB was found for *IL-8* and *NF- κ B*.

Role of NF- κ B in the urease- and flagellin-mediated transcription of *MUC5AC*

Since we showed in this report that, (i) urease and flagellin alter mucin gene transcription, (ii) urease- and flagellin-response elements are present within *MUC5AC* promoter, and (iii) UreB mediates activation of cytokines and NF- κ B, we undertook to establish a link between NF- κ B and *MUC5AC* regulation by urease and flagellin. To this aim, co-transfection experiments were performed in the presence of the promoter of *MUC5AC* and a NF- κ B expression vector encoding the p65 transactivating subunit of NF- κ B. In KATO-III cells, no effect was observed with UreB⁻ whereas activation of -202/-1 *MUC5AC* promoter

region (4.2 fold increase) was found at 6h with FlaA⁻ (Fig. 6, upper panel). In UreB⁻-infected AGS cells (Fig. 6, lower panel), overexpression of NF- κ B p65 subunit led to the activation of the -1366/-1 region of *MUC5AC* promoter (2 fold, black bars). This effect was lost when using the -202/-1 fragment (white bars). This result indicates that the effects of UreB⁻ on *MUC5AC* promoter activity in AGS cells may be mediated by NF- κ B.

Discussion

Helicobacter pylori, a gram-negative bacterium, has been implicated as a causative agent for gastritis, peptic ulcer and gastric carcinoma. Byrd *et al.* recently showed that Hp-induced gastritis is characterized by a decrease of MUC5AC production and an aberrant expression of MUC6 by the surface epithelial cells (MUC6 is normally expressed in gastric glands) [16, 18]. Moreover, Hp-infected preneoplastic and neoplastic gastric mucosae also show altered expression of MUC2, MUC5AC and MUC6 mucins [17].

However, while extensive characterization of Hp adhesins and MUC5AC oligosaccharidic structures involved in Hp-MUC5AC interaction has been described in the literature [12-15, 19], nothing is known about the molecular mechanisms driven by Hp that regulate mucin gene expression in the stomach. We therefore undertook to study the effects of Hp on mucin gene expression at the transcriptional level in KATO-III and AGS gastric cancer cells. We focused our work on two virulence factors involved in Hp colonization of the stomach, that is urease and flagellin and a virulence factor involved in injury genesis, CagA. Urease produces abundant amounts of ammonia and flagellin promotes Hp motility. Kawano *et al.* showed that ammonia chronically administered orally in rats leads to changes in gastric mucosal structures and functions and induces a dose- and time-dependent decrease of intracellular mucin [30]. No relation between Hp flagella and mucin expression has been studied so far. Interestingly, some data obtained in the lung are in favor of a role for flagellin as a mucin regulator since it was shown that *Pseudomonas aeruginosa* flagellin induces transcription and expression of the mucin MUC2 after binding to lung epithelial cells *via* the membrane glycolipid ASGM1 [31]. The presence of *cagA* is statistically associated with duodenal ulceration, gastric mucosal atrophy and gastric cancer. The precise functions of the *cag* PAI, that confers inflammatory power to the strain, are still unknown. CagA is able to modulate the activity of MAP kinases and consequently the cellular pathways which regulate

cell proliferation, cell differentiation, apoptosis, reaction to stress and inflammatory responses. Activation of MAP kinases by Hp *cag* PAI positive strains [32] may be crucial in developing gastroduodenal inflammation and alteration of mucin gene expression especially since MAP kinases are also known to regulate mucin gene expression [33, 34].

In this report, we show for the first time that urease mediates down-regulation of *MUC5AC* transcription in gastric cancer cells in a cell-specific manner. We also have identified UreB-responsive elements in the -486/-1 and -1366/-202 regions of *MUC5AC* promoter in KATO-III and AGS cells, respectively. These results suggest that *MUC5AC* promoter contain UreB-responsive elements that are cell-specific. FlaA, another virulence factor involved in Hp motility and colonization, did not alter mucin gene mRNA levels in either cell line. However, FlaA-responsive elements were found in *MUC2* and *MUC5AC* promoters, which suggests that in another cellular context FlaA may alter mucin gene expression. In AGS cells, mucin gene mRNA levels were not altered after Hp infection. This is most likely the consequence of methylation as we had previously shown that 11p15 mucin genes are methylated in that cell line [35]. Despite that, AGS cells proved to be a great model to study the regulation of mucin genes at the promoter level and identify Hp-responsive elements. Indeed in these cells, we have identified UreB- and FlaA-responsive elements in the promoters of *MUC2*, *MUC5AC* and *MUC5B* and CagA-responsive elements in the promoters of *MUC2* and *MUC5B*. Since, these two genes are expressed in the stomach during the precancerous step of intestinal metaplasia [17], it is an argument in favor of an association between CagA presence in the stomach and severity of the disease. These results also suggest that the different virulence factors of Hp act chronologically during infection and development of the disease. Moreover, urease- and flagellin-mediated inhibition of *MUC5AC* gene expression (this report) is well correlated with the decrease of MUC5AC apomucin observed in the stomach [16-18], which may favor mucus penetration by Hp during stomach colonization. After anchoring of the bacteria on gastric mucosa, which persists without

adapted antibiotic treatment, the other virulence factors then become effective and create mucosal injuries. CagA, could be one of the numerous factors participating to the gastric cancer development by altering *MUC2* and *MUC5B* gene expression.

Previous data have shown that urease is a proinflammatory molecule that up-regulates production of potent proinflammatory cytokines [36] and most likely that effect is mediated by NF- κ B transcription factor [37, 38]. Moreover, NF- κ B has already been shown to up-regulate *MUC2* and *MUC5AC* transcription in cultured airway epithelial cells and bronchial explants after cell infection by the gram-negative bacterium *Pseudomonas aeruginosa* [33, 39]. In that case, the bacteria adheres to the cell and then activates NF- κ B pathway *via* lipopolysaccharide binding to a receptor yet to be characterized [40]. Two κ B *cis*-elements are present in *MUC5AC* promoter at -958/-949 and -227/-215 [41] that could be involved in the response to urease *via* NF- κ B in AGS cells and lead to down regulation of *MUC5AC* promoter activity. In KATO-III cells, this phenomenon is NF- κ B-independent. Thus, it seems that Hp uses different cell-specific signaling pathways to regulate *MUC5AC* expression in gastric epithelial cells.

The three wild type strains of Hp used in this work induced a transient activation of *MUC2*, *MUC5AC* and *MUC6* mucin gene expression at 1h infection in KATO-III cells. There was no effect on *MUC5B* expression. The reversal after 3h is most likely due to the fact that enough mRNA was produced following infection and that the cell has enough then to express and secrete mucins. Moreover, it has already been shown in our laboratory and it is known that mRNA encoding secreted mucins have quite a long half-life [42] and thus as mentioned above, a cell has enough mRNA to express a secreted mucin and does not need transcription to go on for long periods.

This early response leading to the up-regulation of mucin gene expression is to be correlated with early response of the cells to the infection by up-regulating the expression of IL-8 chemokine, IL-1 β and TNF- α pro-inflammatory cytokines. This indicates that mucins

can be considered as an early response of the cell to protect itself against bacterial injury. Hp infection of the stomach is associated with the development of inflammation [2]. Virulence factors of Hp participate in maintaining that inflammatory state [36, 43]. We thus hypothesize that gastric cells may, in an autocrine system, respond to Hp infection by up-regulating a panel of chemokines and pro-inflammatory cytokines in order to attract inflammatory cells but also to express mucins to protect themselves against injury. Indeed the mucus secretion is the first protective line of mucosal surfaces against pathogens and irritants by providing an efficient mucus layer with specific rheological properties [44]. Mucin secretion in the stomach, by modifying the gastric environment for colonization, may thus play a role of antibiotic against the bacteria [45-47], increase immunoreactivity in the mucosa [48], but also inhibit H. pylori proliferation [49]. In a healthy adult, this autocrine system would suffice to trap and eradicate the bacteria, whereas in other situations such as chronic or more severe inflammation and more vulnerable patients mucin secretion would be either not sufficient or worse used by the bacteria to adhere, develop and in some cases cause gastric neoplasia leading to cancer.

In conclusion, we have shown that *Helicobacter pylori* regulates mucin gene expression at the transcriptional level in gastric cancer cells. Hp-, urease-, flagellin- and CagA-responsive elements were found in *MUC2*, *MUC5AC* and *MUC5B* promoters and urease-mediated inhibition of *MUC5AC* in KATO-III cells is independent of NF- κ B activation. Finally, concomitant early up-regulation of mucin genes and pro-inflammatory cytokines is in favor of autocrine systems activated by gastric cells in response to Hp infection.

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Figure Legends

Figure 1: Effect of Hp on 11p15 mucin gene expression in KATO-III cells. Expression of *MUC2*, *MUC5AC*, *MUC5B* and *MUC6* mRNAs was evaluated by the means of RT-PCR after KATO-III cell infection with Hp wild-type strains (ATCC 43504, N6 and SS1) and N6 isogenic mutants (N6 UreB⁻, N6 FlaA⁻ and SS1 CagA⁻). 10 µl of PCR products were loaded on a 1.5% agarose gel and electrophoresed in 1X TBE buffer containing ethidium bromide.

Figure 2: Regulation of *MUC2* promoter by Hp wild-type strains and UreB⁻, FlaA⁻ and CagA⁻ isogenic mutants in KATO-III and AGS cells. Results are expressed as fold induction of luciferase activity of the promoter constructs in infected cells with deficient mutants over activity in infected cells with wild-type strain (UreB⁻/N6, FlaA⁻/N6, CagA⁻/SS1). The results are means +/- S.D. and represent three different experiments in triplicate for each fragment.

Figure 3: Regulation of *MUC5AC* promoter by Hp wild-type strains and UreB⁻, FlaA⁻ and CagA⁻ isogenic mutants in KATO-III and AGS cells. Results are expressed as fold induction of luciferase activity of the promoter constructs in infected cells with deficient mutants over activity in infected cells with wild-type strain (UreB⁻/N6, FlaA⁻/N6, CagA⁻/SS1). The results are means +/- S.D. and represent three different experiments in triplicate for each fragment.

Figure 4: Regulation of *MUC5B* promoters by Hp wild-type strains and UreB⁻, FlaA⁻ and CagA⁻ isogenic mutants in KATO-III and AGS cells. Results are expressed as fold induction of luciferase activity of the promoter constructs in infected cells with deficient mutants over activity in infected cells with wild-type strain (UreB⁻/N6, FlaA⁻/N6, CagA⁻/SS1).

The results are means \pm S.D. and represent three different experiments in triplicate for each fragment.

Figure 5: Expression of cytokines and NF- κ B p65 subunit in Hp-infected gastric cancer cells by RT-PCR. Cells were treated for 1h, 3h or 6h with Hp wild-type strains (ATCC 43504 or N6) or FlaA⁻ and UreB⁻ isogenic mutants. **(A):** KATO-III cells and **(B):** AGS cells. 10 μ l of the PCR products were loaded on a 1.5% agarose gel and electrophoresed in 1X TBE buffer containing ethidium bromide.

Figure 6: Role of NF- κ B on *MUC5AC* promoter activity after Hp infection. Co-transfections studies were performed in KATO-III and AGS cells in the presence of pCMV4-NF- κ B p65 expression vector encoding the transactivating subunit of NF- κ B and the pGL3-*MUC5AC* deletion mutants covering either the -1366/-1 (black bars) or the -202/-1 (white bars) regions of the promoter. Co-transfections with empty vector pCMV4 was performed and luciferase value considered as 1 (Ref, gray bar). Cells were infected for 1h, 3h or 6h with Hp wild-type strain N6 or its isogenic deficient mutants for urease B and flagellin A. Results are expressed as fold induction of luciferase activity in infected cells with deficient mutants over activity in infected cells with wild-type strain (UreB⁻/N6 or FlaA⁻/N6). The results are means \pm S.D. and represent three different experiments in triplicate for each fragment.

References

1. Correa P. *Helicobacter pylori* as a pathogen and carcinogen. *J Physiol Pharmacol*. 1997 Sep;48 Suppl 4:19-24.
2. Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev*. 1997 Oct;10(4):720-41.
3. Peek RM, Jr., Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer*. 2002 Jan;2(1):28-37.
4. Barreto-Zuniga R, Maruyama M, Kato Y, Aizu K, Ohta H, Takekoshi T, et al. Significance of *Helicobacter pylori* infection as a risk factor in gastric cancer: serological and histological studies. *J Gastroenterol*. 1997 Jun;32(3):289-94.
5. Corfield AP, Carroll D, Myerscough N, Probert CS. Mucins in the gastrointestinal tract in health and disease. *Front Biosci*. 2001 Oct 1;6:D1321-57.
6. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer*. 2004 Jan;4(1):45-60.
7. Dekker J, Rossen JW, Buller HA, Einerhand AW. The MUC family: an obituary. *Trends Biochem Sci*. 2002 Mar;27(3):126-31.
8. Jonckheere N, Skrypek N, Frenois F, Van Seuning I. Membrane-bound mucin modular domains: from structure to function. *Biochimie*. 2012 Nov 20.
9. Buisine MP, Devisme L, Maunoury V, Deschodt E, Gosselin B, Copin MC, et al. Developmental mucin gene expression in the gastroduodenal tract and accessory digestive glands. I. Stomach. A relationship to gastric carcinoma. *J Histochem Cytochem*. 2000 Dec;48(12):1657-66.
10. Teixeira A, David L, Reis CA, Costa J, Sobrinho-Simoes M. Expression of mucins (MUC1, MUC2, MUC5AC, and MUC6) and type 1 Lewis antigens in cases with and without *Helicobacter pylori* colonization in metaplastic glands of the human stomach. *J Pathol*. 2002 May;197(1):37-43.
11. Allen A, Newton J, Oliver L, Jordan N, Strugala V, Pearson JP, et al. Mucus and *H. pylori*. *J Physiol Pharmacol*. 1997 Sep;48(3):297-305.
12. Kocer B, Ulas M, Ustundag Y, Erdogan S, Karabeyoglu M, Yldrm O, et al. A confirmatory report for the close interaction of *Helicobacter pylori* with gastric epithelial MUC5AC expression. *J Clin Gastroenterol*. 2004 Jul;38(6):496-502.
13. Linden SK, Wickstrom C, Lindell G, Gilshenan K, Carlstedt I. Four modes of adhesion are used during *Helicobacter pylori* binding to human mucins in the oral and gastric niches. *Helicobacter*. 2008 Apr;13(2):81-93.
14. Van de Bovenkamp JH, Mahdavi J, Korteland-Van Male AM, Buller HA, Einerhand AW, Boren T, et al. The MUC5AC glycoprotein is the primary receptor for *Helicobacter pylori* in the human stomach. *Helicobacter*. 2003;8(5):521-32.
15. Magalhaes A, Reis CA. *Helicobacter pylori* adhesion to gastric epithelial cells is mediated by glycan receptors. *Braz J Med Biol Res*. 2010 Jul;43(7):611-8.
16. Byrd JC, Yan P, Sternberg L, Yunker CK, Scheiman JM, Bresalier RS. Aberrant expression of gland-type gastric mucin in the surface epithelium of *Helicobacter pylori*-infected patients. *Gastroenterology*. 1997 Aug;113(2):455-64.
17. Babu SD, Jayanthi V, Devaraj N, Reis CA, Devaraj H. Expression profile of mucins (MUC2, MUC5AC and MUC6) in *Helicobacter pylori* infected pre-neoplastic and neoplastic human gastric epithelium. *Mol Cancer*. 2006;5:10.
18. Byrd JC, Yunker CK, Xu QS, Sternberg LR, Bresalier RS. Inhibition of gastric mucin synthesis by *Helicobacter pylori*. *Gastroenterology*. 2000 Jun;118(6):1072-9.

19. Morgenstern S, Koren R, Moss SF, Fraser G, Okon E, Niv Y. Does *Helicobacter pylori* affect gastric mucin expression? Relationship between gastric antral mucin expression and *H. pylori* colonization. *Eur J Gastroenterol Hepatol*. 2001 Jan;13(1):19-23.
20. Dunn BE, Phadnis SH. Structure, function and localization of *Helicobacter pylori* urease. *Yale J Biol Med*. 1998 Mar-Apr;71(2):63-73.
21. O'Toole PW, Lane MC, Porwollik S. *Helicobacter pylori* motility. *Microbes Infect*. 2000 Aug;2(10):1207-14.
22. Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, et al. Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol*. 2000 Apr;2(2):155-64.
23. Perrais M, Pigny P, Buisine MP, Porchet N, Aubert JP, Van Seuningen-Lempire I. Aberrant expression of human mucin gene MUC5B in gastric carcinoma and cancer cells. Identification and regulation of a distal promoter. *J Biol Chem*. 2001 May 4;276(18):15386-96.
24. Ferrero RL, Cussac V, Courcoux P, Labigne A. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. *J Bacteriol*. 1992 Jul;174(13):4212-7.
25. Suerbaum S, Josenhans C, Labigne A. Cloning and genetic characterization of the *Helicobacter pylori* and *Helicobacter mustelae* flaB flagellin genes and construction of *H. pylori* flaA- and flaB-negative mutants by electroporation-mediated allelic exchange. *J Bacteriol*. 1993 Jun;175(11):3278-88.
26. Chevalier C, Thiberge JM, Ferrero RL, Labigne A. Essential role of *Helicobacter pylori* gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Mol Microbiol*. 1999 Mar;31(5):1359-72.
27. Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology*. 1997 Apr;112(4):1386-97.
28. Perrais M, Pigny P, Copin MC, Aubert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem*. 2002 Aug 30;277(35):32258-67.
29. Van Seuningen I, Perrais M, Pigny P, Porchet N, Aubert JP. Sequence of the 5'-flanking region and promoter activity of the human mucin gene MUC5B in different phenotypes of colon cancer cells. *Biochem J*. 2000 Jun 15;348 Pt 3:675-86.
30. Kawano S, Tsujii M, Fusamoto H, Sato N, Kamada T. Chronic effect of intragastric ammonia on gastric mucosal structures in rats. *Dig Dis Sci*. 1991 Jan;36(1):33-8.
31. McNamara N, Khong A, McKemy D, Caterina M, Boyer J, Julius D, et al. ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc Natl Acad Sci U S A*. 2001 Jul 31;98(16):9086-91.
32. Meyer-ter-Vehn T, Covacci A, Kist M, Pahl HL. *Helicobacter pylori* activates mitogen-activated protein kinase cascades and induces expression of the proto-oncogenes c-fos and c-jun. *J Biol Chem*. 2000 May 26;275(21):16064-72.
33. Basbaum C, Lemjabbar H, Longphre M, Li D, Gensch E, McNamara N. Control of mucin transcription by diverse injury-induced signaling pathways. *Am J Respir Crit Care Med*. 1999 Nov;160(5 Pt 2):S44-8.
34. Van Seuningen I, Pigny P, Perrais M, Porchet N, Aubert JP. Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? *Front Biosci*. 2001 Oct 1;6:D1216-34.
35. Vincent A, Perrais M, Desseyn JL, Aubert JP, Pigny P, Van Seuningen I. Epigenetic regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (MUC2, MUC5AC, MUC5B, MUC6) in epithelial cancer cells. *Oncogene*. 2007 Oct 4;26(45):6566-76.

36. Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology*. 1996 Aug;111(2):419-25.
37. Keates S, Hitti YS, Upton M, Kelly CP. Helicobacter pylori infection activates NF-kappa B in gastric epithelial cells. *Gastroenterology*. 1997 Oct;113(4):1099-109.
38. van Den Brink GR, ten Kate FJ, Ponsioen CY, Rive MM, Tytgat GN, van Deventer SJ, et al. Expression and activation of NF-kappa B in the antrum of the human stomach. *J Immunol*. 2000 Mar 15;164(6):3353-9.
39. Dohrman A, Miyata S, Gallup M, Li JD, Chapelin C, Coste A, et al. Mucin gene (MUC 2 and MUC 5AC) upregulation by Gram-positive and Gram-negative bacteria. *Biochim Biophys Acta*. 1998 Apr 28;1406(3):251-9.
40. Li JD, Feng W, Gallup M, Kim JH, Gum J, Kim Y, et al. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells. *Proc Natl Acad Sci U S A*. 1998 May 12;95(10):5718-23.
41. Li D, Gallup M, Fan N, Szymkowski DE, Basbaum CB. Cloning of the amino-terminal and 5'-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. *J Biol Chem*. 1998 Mar 20;273(12):6812-20.
42. Debailleul V, Laine A, Huet G, Mathon P, d'Hooghe MC, Aubert JP, et al. Human mucin genes MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 express stable and extremely large mRNAs and exhibit a variable length polymorphism. An improved method to analyze large mRNAs. *J Biol Chem*. 1998 Jan 9;273(2):881-90.
43. Harris PR, Weber HC, Wilcox CM, Jensen RT, Smith PD. Cytokine gene profile in gastric mucosa in Helicobacter pylori infection and Zollinger-Ellison syndrome. *Am J Gastroenterol*. 2002 Feb;97(2):312-8.
44. Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. *Nat Rev Gastroenterol Hepatol*. 2013 Mar 12. doi: 10.1038/nrgastro.2013.35
45. Fukuda M, Kawakubo M, Ito Y, Kobayashi M, Lee H, Nakayama J. Assay of human gastric mucin as a natural antibiotic against Helicobacter pylori. *Methods Enzymol*. 2006;415:164-79.
46. Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, et al. Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. *Science*. 2004 Aug 13;305(5686):1003-6.
47. Kobayashi M, Lee H, Nakayama J, Fukuda M. Roles of gastric mucin-type O-glycans in the pathogenesis of Helicobacter pylori infection. *Glycobiology*. 2009 May;19(5):453-61.
48. Matsuzwa M, Ota H, Hayama M, Zhang MX, Sano K, Honda T, et al. Helicobacter pylori infection up-regulates gland mucous cell-type mucins in gastric pyloric mucosa. *Helicobacter*. 2003 Dec;8(6):594-600.
49. Skoog EC, Sjoling A, Navabi N, Holgersson J, Lundin SB, Linden SK. Human gastric mucins differently regulate Helicobacter pylori proliferation, gene expression and interactions with host cells. *PLoS One*. 2012;7(5):e36378.

Target gene	Nucleotide sequence (5'→3')	Size of PCR product (bp)	Annealing temperature (°C)
18S RNA	S: GGA CCA GAG GCA AAG CAT TTG CC AS: TCA ATC TCG GGT GGC TGA ACG C	480	64
IL-1 β	S : ATT CTG ATG AGC AAC CGC TT AS: GCA CAC CAG TCC AAA TTG AA	156	55
IL-6	S: GGG AAG CTT GCT ATG AAC TCC TCC TCC AGA AS : GGG GAA TTC ATG CTA CAT TTG CCG AAG AGC	639	68
IL-8	S: AAG GAA CCA TCT CAC TG AS: GAT TCT TGG ATA CCA CAG AG	352	50
NF- κ B	S: CTG CCG AGC TCA AGA TCT GCC GAG TAA AC AS : GGA GGA GTC CGG AAC ACA ATG GCC ACT TGC C	196	69
TNF- α	S: CAG AGG GAA GAG TTC CCC AG AS: CCT TGG TCT GGT AGG AGA CG	325	61

Table I: Sequences of the pairs of primers used in RT-PCR experiments to study the expression of the proinflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) and transcription factor NF- κ B. S: sense orientation, AS: antisense orientation.

Figure 1

Figure 1 : Perrais *et al.*

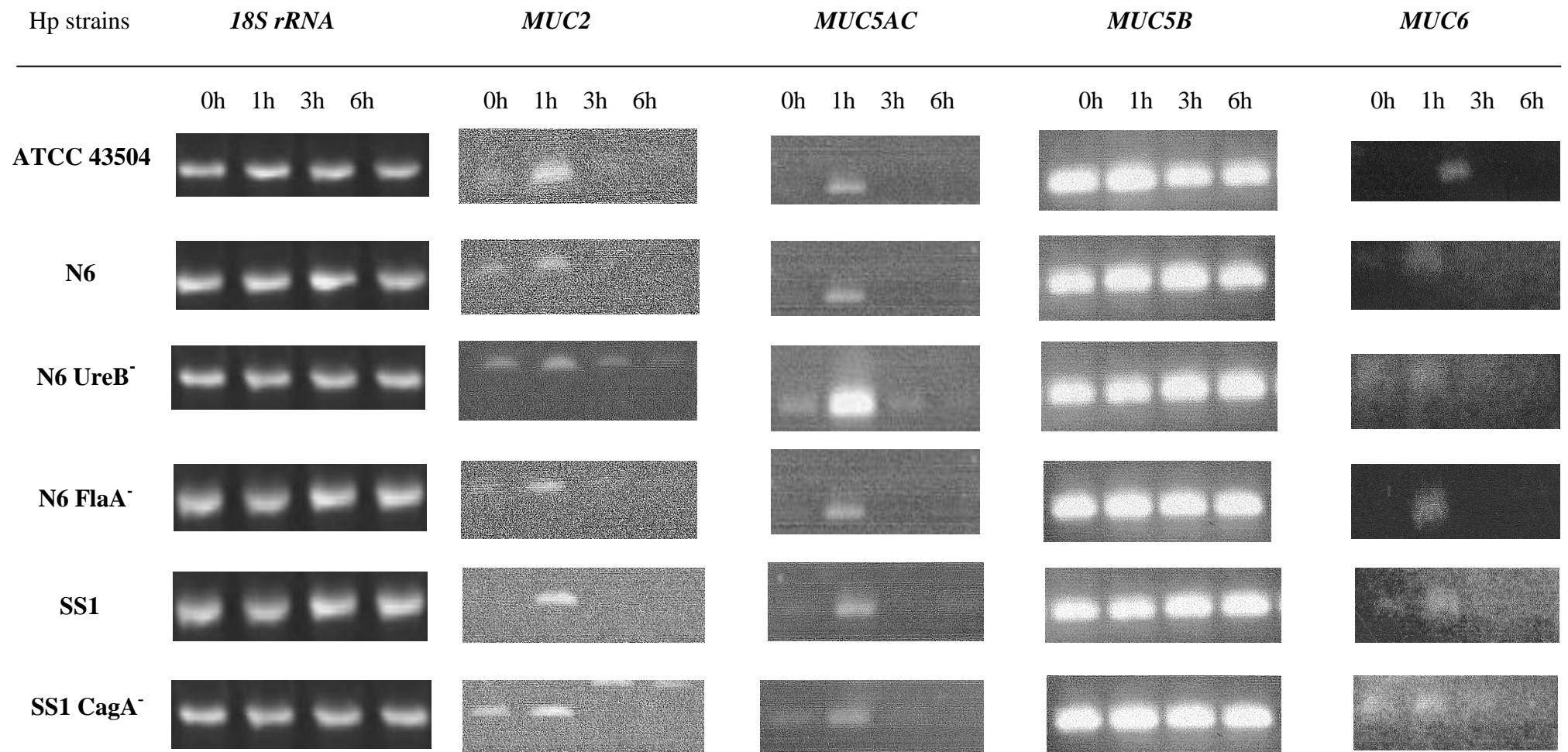


Figure 2 : Perrais *et al.*

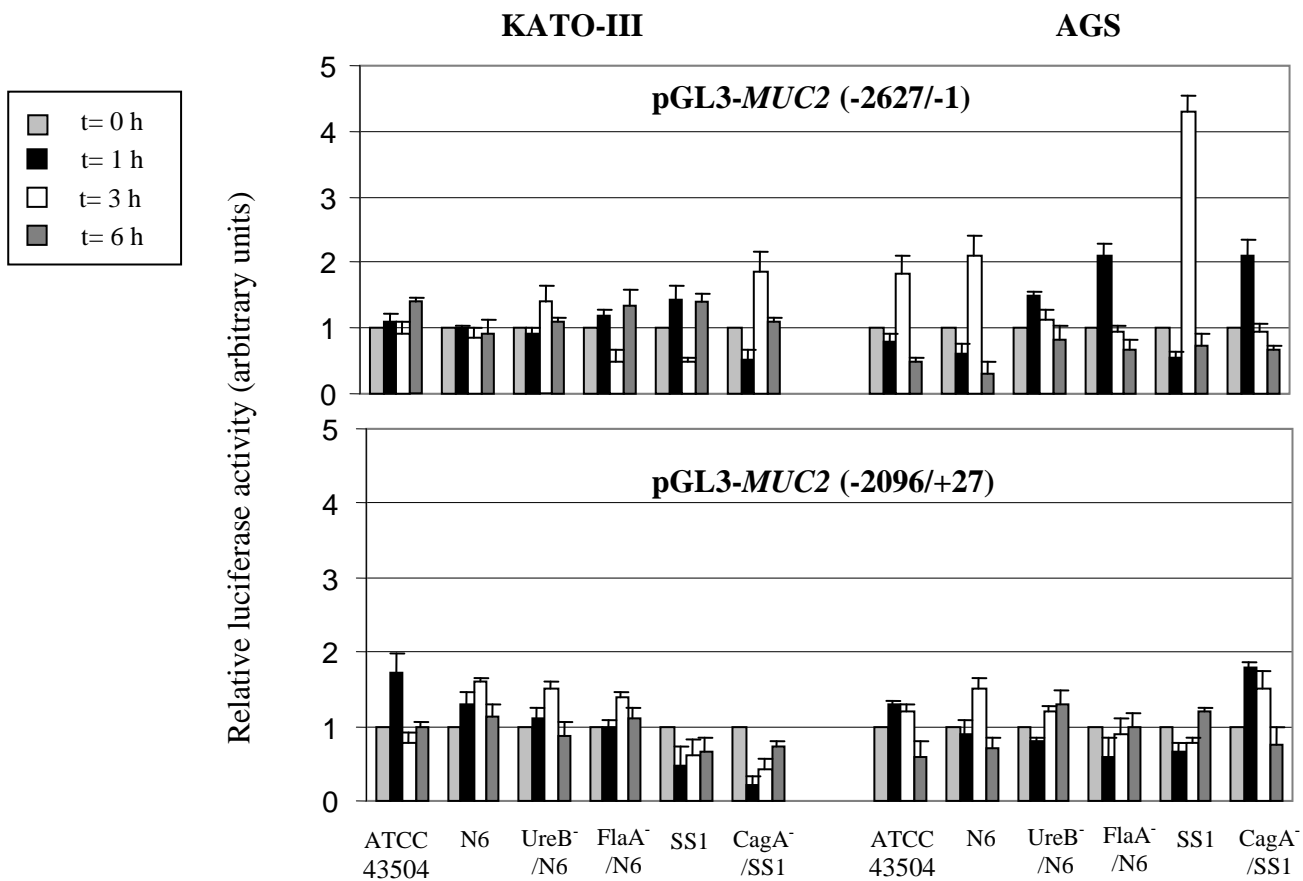


Figure 3 : Perrais *et al.*

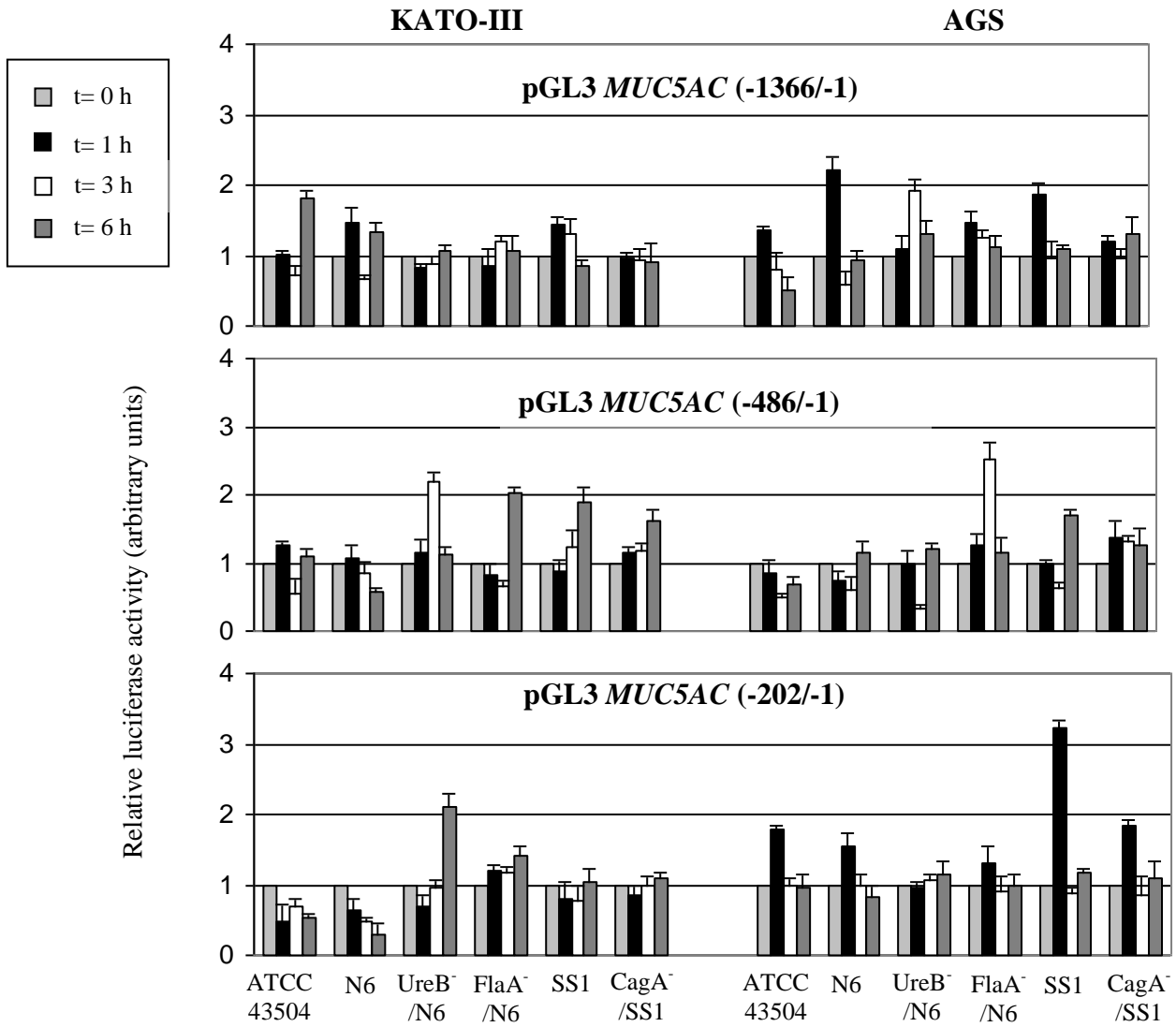
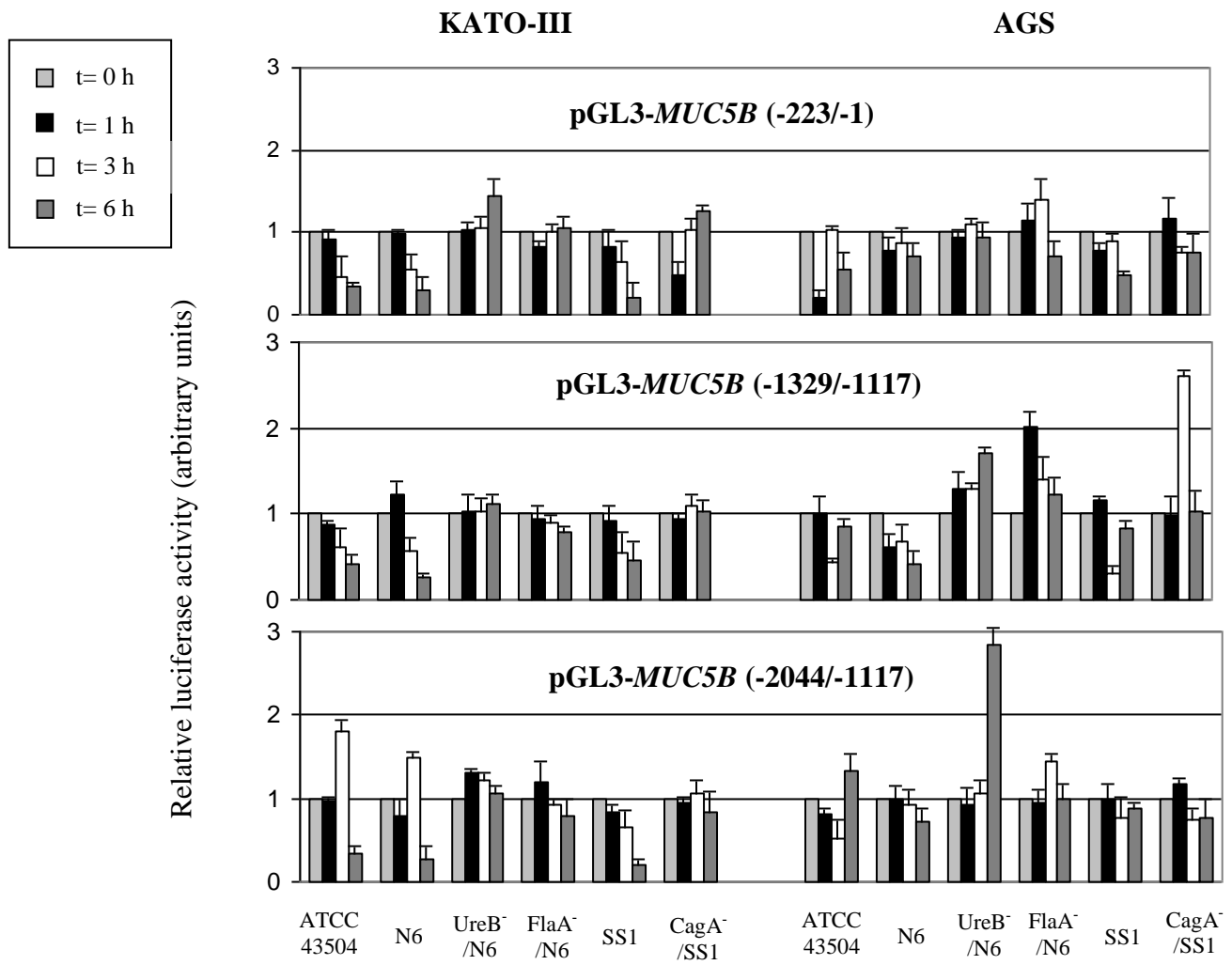
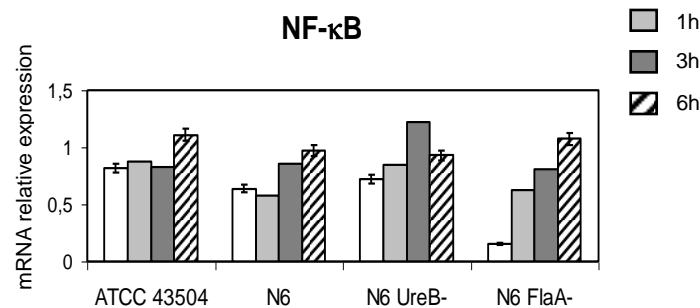
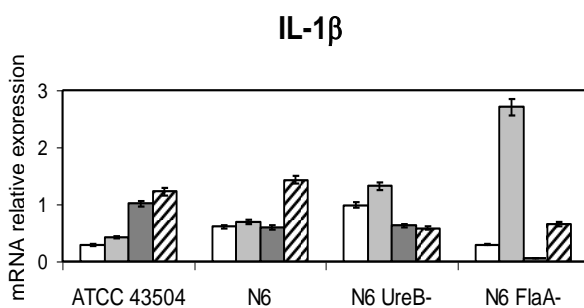
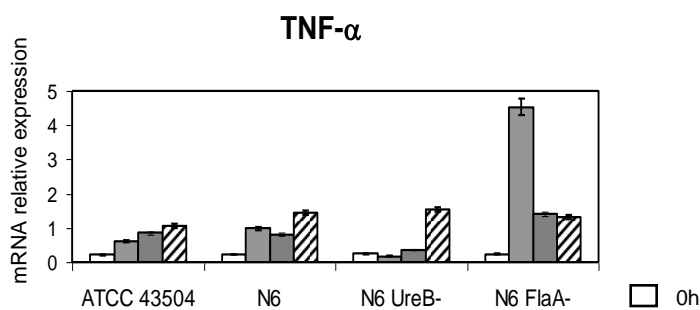
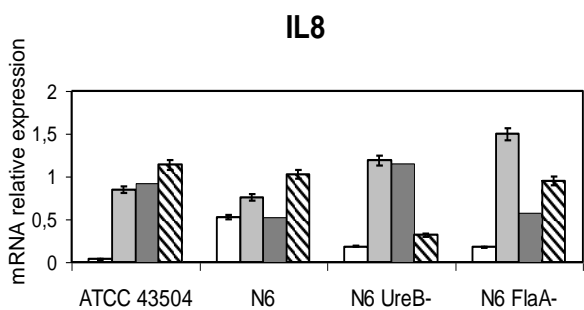
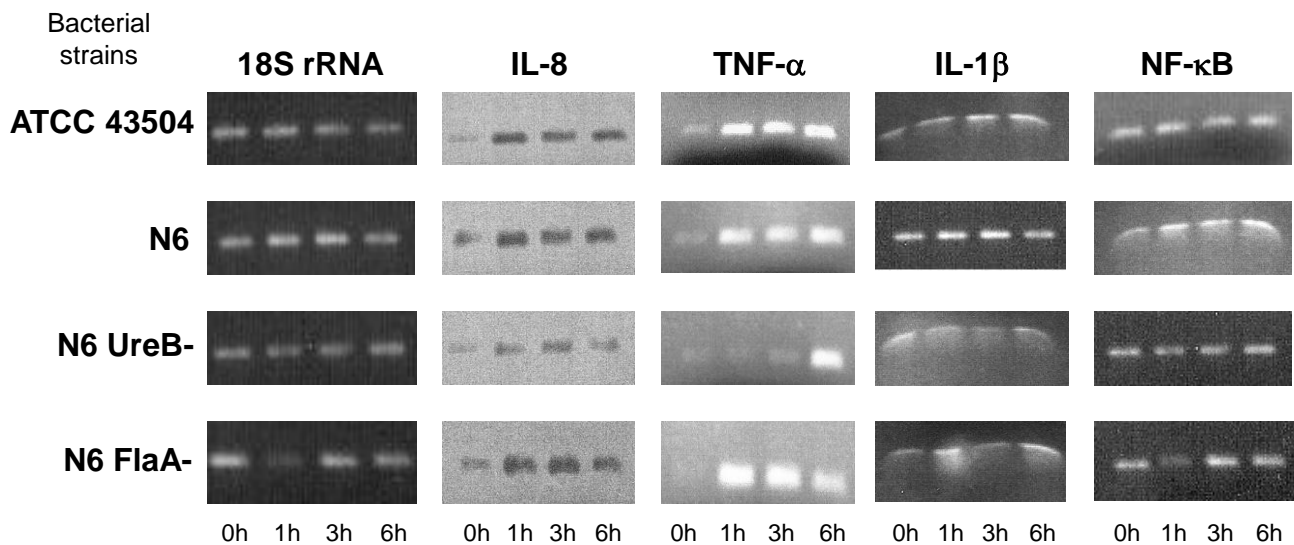
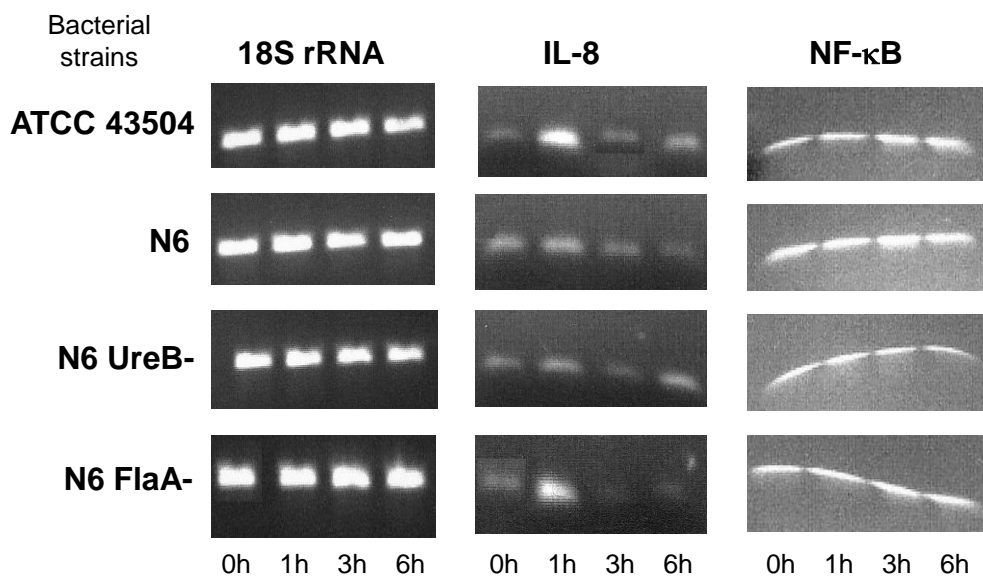


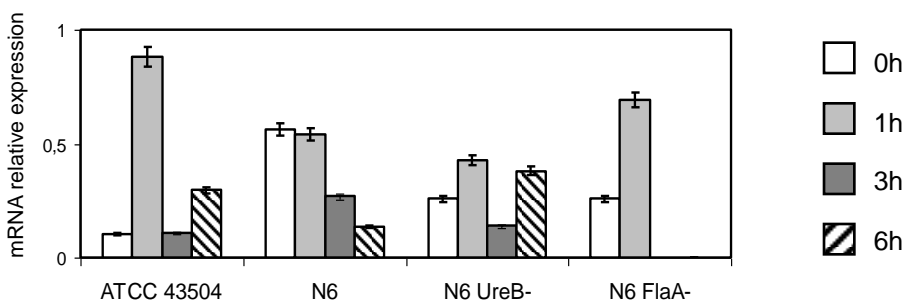
Figure 4 : Perrais *et al.*







IL8



NF-κB p65

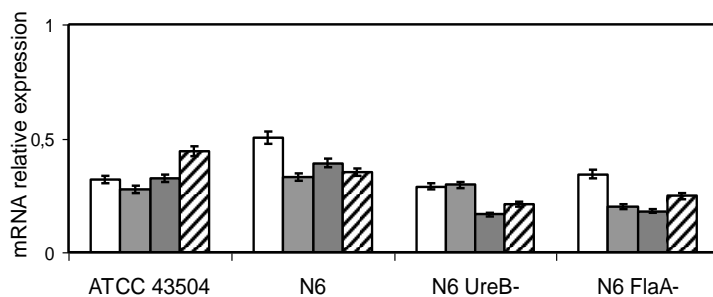
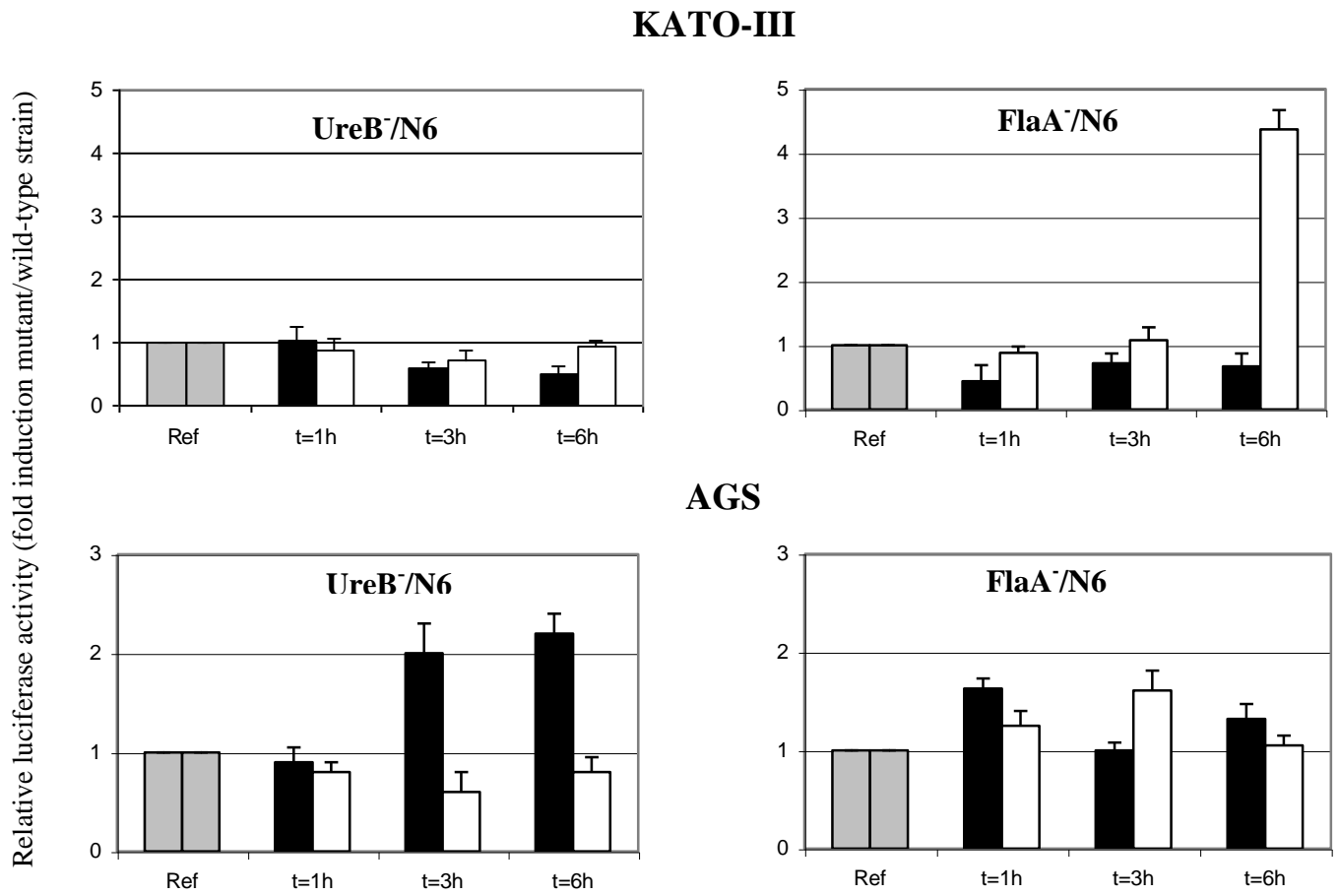


Figure 6 : Perrais *et al.*

Helicobacter pylori (Hp) interacts with gastric mucus gel and alter mucin gene expression. We show that Hp alters 11p15 mucin gene transcription and that *MUC5AC* down-regulation is mediated by urease.

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