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Tip-α (*hp0596* Gene Product) Is a Highly Immunogenic *Helicobacter pylori* Protein Involved in Colonization of Mouse Gastric Mucosa

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Abstract A product of the *Helicobacter pylori hp0596* gene (Tip-α) is a highly immunogenic homodimeric protein, unique for this bacterium. Cell fractionation experiments indicate that Tip-α is anchored to the inner membrane. In contrast, the three-dimensional model of the protein suggests that Tip-α is soluble or, at least, largely exposed to the solvent. *hp0596* gene knockout resulted in a significant decrease in the level of *H. pylori* colonization as measured by real-time PCR assay. In addition, the Tip-α recombinant protein was determined to stimulate macrophage to produce IL-1α and TNF-α. Both results imply that Tip-α is rather loosely connected to the inner membrane and potentially released during infection.

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Introduction

Helicobacter pylori infections are a major risk factor for gastroduodenal diseases (gastritis, duodenal ulcers, gastric carcinomas, and lymphoma). Many *H. pylori* virulence factors have been identified and characterized as either direct or indirect causes of observed pathophysiologies. A great number of these factors are highly immunogenic. Among the most intensively studied immunodominant antigens are CagA, which, upon injection into eukaryotic cells, subverts the cellular functions; proteins responsible for cell motility, urease subunits, which allow the bacterium to survive in acidic environments; vacuolating toxin VacA, which is a potent inducer of epithelial cell apoptosis; neutrophil-activating protein NapA, CagF, and other proteins encoded by the pathogenicity island PAI; and some surface-located adhesins [2, 9, 27, 42]. Initially, immunogenicity of these proteins was confirmed by classical molecular biology methods and one-dimensional (1-D) electrophoresis followed by Western blotting with antibodies raised in experimental animals or sera obtained from *H. pylori*-positive individuals with different gastroduodenal pathologies. Sequencing of the genomes of two *H. pylori* strains (26695 and J99) has enabled exploration of bacterial proteomes for seroreactive proteins, using the technology known as immunoproteomics [3, 53].

Some pioneer studies focused on the identification of *H. pylori* antigens among a whole set of cellular proteins. Atte et al. reported that among proteins of the *H. pylori* ATCC 43504 strain, 30 of them are strongly recognized by pooled human sera [34]. Most of them have been predicted or documented previously to be extracytoplasmic, and some described as immunodominant. In contrast, most immunoreactive proteins identified in the proteome of *H. pylori* G27 represented housekeeping gene products [23].

Immunoproteomes described by Attee et al. and by Kimmel et al. had only nine proteins in common. Jungblut et al. and Haas et al. screened the proteome of *H. pylori* 26695 using single sera and determined differences in the immune response of patients with various clinical manifestations [16, 19, 23, 34]. Furthermore, comparative analysis conducted by Mini et al. revealed a significant heterogeneity between selected antigens that is dependent on both the bacterial strain and the sera used for analysis [38]. Comparison of the provided data presents difficulties, due mainly to the genetic diversity of *H. pylori* as well as to methodological differences. There have been a number of significant attempts undertaken which were aimed at resolving surface or membrane proteins [5, 8]. In each assay new immunoreactive proteins were identified and some of them have been tested as potential vaccine candidates.

Despite the fact that postgenomic technology has enormously extended our knowledge on the interaction of *H. pylori* with the immune system, the list of *H. pylori* immunoreactive proteins is still incomplete. Nevertheless, comprehensive studies of *H. pylori* immunoproteomes identified several novel immunogenic proteins of unknown function. Among them, HP0410 and HP0231 have been evaluated in an animal infection model, and their protective potential was determined [45]. It should be noted that some seroreactive proteins which were not identified by immunoproteomics were proven to be protective in animal experiments.

In this report, we described the highly immunogenic, unique *H. pylori* Tip-α protein, which was missing in immunoproteomic screenings. We isolated *tip-α* gene by screening a λZAP *H. pylori* genomic DNA expression library with human and rabbit sera [31]. We showed that Tip-α is involved in stimulation of macrophages as well as in colonization of mouse gastric mucosa. Analysis conducted by others also strongly documented that Tip-α (**t**umor necrosis factor [TNF] α-**i**nducing **p**rotein) is a *H. pylori* carcinogenic factor involved in chemokine gene induction [28, 51]. Although cell fractionation methods indicated localization of Tip-α in the inner membrane, the function of this protein implies surface exposition. Additional studies are required to clarify this issue.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

H. pylori strains N6 (kindly provided by Dr. Agnes Labigne, Institut Pasteur) and SS1 were grown under microaerobic conditions as described previously [14]. *Escherichia coli* strains DH5α (Gibco BRL) and M15 (Qiagen) were grown at 37°C on LB agar or in broth. *E. coli* transformants were grown in the presence of the appropriate antibiotic (ampicillin, 100 μg/ml, or kanamycin, 25 μg/ml).

Recombinant DNA Techniques

Procedures for plasmid DNA isolation and DNA analysis (digestion with restriction enzymes, T4 ligase ligation), agarose gel electrophoresis, and transformation of *E. coli* competent cells were carried out as described by Sambrook [46]. Preparations of plasmid DNA for electroporation as well as DNA isolation from agarose gels were performed according to the manufacturer's instructions (A&A Biotechnology). DNA sequencing was performed on an ABI Prism 377 automated DNA sequencer (Perkin-Elmer) at the DNA Sequencing and Oligonucleotide Synthesis Lab, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. An *E. coli* strain that overproduces Tip-α was constructed by cloning the Tip-α-coding region into pQE31 under the control of an inducible *lacZ* gene promoter.

The $(His)_6$Tip-α recombinant protein was overproduced by IPTG-induced *E. coli* M15 (pREP4, pQE31/596) culture and purified by Ni-NTA affinity chromatography as described in the manufacturer's protocol (Qiagen). Purified $(His)_6$Tip-α was used for rabbit immunization.

Gene replacement strategy was used to construct the *H. pylori* Tip-α isogenic mutant. Two internal fragments of the gene were amplified by PCR and cloned into the *H. pylori* nonreplicable vector pBluescript II SK, with an inactivated ClaI site in the MCS. The following oligonucleotides were used: 5′-CCCGGGTTGCCCAAACACTTCACA-3′, 5′-ATCGATTGCCACTCTTATTGTCGCTCTT-3′, 5′-ATCGATCGACAATAAGAGTGGCACAAAA-3′, and 5′-TTATGCAACCCTTCGCTCAAA-3′. Primers contained SmaI and ClaI restriction site overhangs (underlined) to facilitate cloning of the amplified DNA fragments in the appropriate orientation. A ClaI-ending kanamycin-resistant cassette carrying the *Campylobacter jejuni* aph(3′)-III gene (1.4 kb) was inserted into unique ClaI sites among cloned PCR fragments. *H. pylori* N6 and SS1 strains, naturally competent for genetic transformation, were used for generation of isogenic *H. pylori* Tip-α mutants. The obtained kanamycin-resistant transformants were screened by hybridization using the cloning vector DNA as a probe to confirm that no vector DNA was integrated into the chromosome via a single crossover. Moreover, the absence of an 18-kDa protein was determined in cell extracts obtained from *H. pylori* Tip-α isogenic mutants using specific antibodies (Fig. 2A). The Km cassette lacking a transcriptional termination signal was inserted in the orientation corresponding to *hp0596* transcription, which was confirmed by

PCR. Additionally, the *hp0597* gene (located downstream) is transcribed from another DNA strand. A generated Tip-α mutant displayed normal viability, indicating that the *hp0596* gene product belongs to nonessential proteins.

Western Blot Analysis

SDS/PAGE was carried out as described by Laemmli [30]. Western blot detection of Tip-α was performed using a 1:1000 dilution of anti-Tip-α rabbit antibodies and goat anti-rabbit IgG conjugated to an alkaline phosphatase (Sigma).

Preparation of Cellular Fractions

Cellular fractions were prepared by the method described by Blaser et al. [7]. Proteins from the outer and inner membrane fractions present in the cell envelope were separated by the sodium lauroyl sarcosine procedure of Filip et al. [12]. The periplasmic fraction was prepared by the osmotic shock procedure of Ames et al. [4].

Identification of the Transcription Start Point

Total RNA samples were prepared from *H. pylori* cells using the Trizol reagents (Invitrogen). The transcription start point was mapped with the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Gibco BRL, Life Technologies), according to the manufacturer's directions. Analysis was carried out using primers provided by the manufacturer and *hp0596*-specific primers: ATCGATTGC CACTCTTATTGTCGCTCTT and TTGGATCCATCTC TTCAGTTTTCTTACCATCT. The resulting PCR product was cloned into the pGemT Easy vector (Promega) according to the manufacturer's instructions prior to sequencing.

In Silico Analysis

Secondary structure prediction and tertiary fold recognition were carried out via the GeneSilico meta-server gateway at http://genesilico.pl/meta/2 [26]. Secondary structure was predicted as a consensus of the following methods: PSI-PRED [35], PROFsec [43], PROF [41], SABLE [1], JNET [10], JUFO [36], and SAM-T02 [20]. In addition, solvent accessibility for the individual residues was predicted with SABLE [1] and JPRED [10]. The fold-recognition analysis was carried out using FFAS03 [44], SAM-T02 [20], 3DPSSM [22], BIOINBGU [13], FUGUE [48], mGENTHREADER [18], and SPARKS [57]. Fold-recognition alignments reported by these methods were compared, evaluated, and ranked by the Pcons server [33]. Fold-recognition alignments to the structures of selected templates were used as a starting point for homology modeling using the "FRankenstein's Monster" approach [24], which yielded the final model. Apart from comparative modeling, de novo modeling of the protein structure using the ROSETTA fragment insertion method was applied [49].

Infection of Mice and DNA Preparation

C57BL/6, outbred specific pathogen-free mice (6–8 weeks old) were experimentally infected with either *H. pylori* SS1 or Tip-α-mutated isogenic *H. pylori* SS1. DNA from gastric mucosa was obtained as described previously [11, 37]. Noninfected 6- to 8-week-old C57BL/6 mice were used as a control.

Real-Time PCR

Infection with *H. pylori* was quantified 3 weeks postchallenge by real-time PCR (of the gene encoding the 26-kDa species-specific antigen), as described previously [37]. Calculated amounts of *H. pylori* total DNA and the number of bacterial cells present in each sample were normalized to murine GAPDH DNA. Student's *t*-test was used to calculate the significance of the effects of the Tip-α mutant on the colonization process. $p < 0.05$ was considered significant.

Stimulation of Macrophages

Macrophages were obtained by peritoneal lavage (4- to 5-week-old C57BL/6 mice), washed twice with phosphate-buffered saline (PBS), and suspended in RPMI medium (Sigma) containing HEPES (20 mM), sodium pyruvate (1 mM), gentamicin (5 μg/ml), 2-mercaptoethanol (50 mM), and 10% autologous serum. Macrophages were incubated in PBS and the recombinant Tip-α for 8 h at 37°C in a 24-well plate. The viability of macrophages after incubation was always more than 90% as determined by trypan blue dye exclusion. After 8 h of macrophage stimulation, culture supernatants were collected. Concentrations of interleukin (IL)-1α and TNF-α released into the supernatants were determined with ELISA kits (R&D Systems, Minneapolis, MN).

Results

Our earlier studies described construction and immunological screening of *H. pylori* gene expression libraries.

Several immunopositive clones were identified using antiserum raised in rabbits exposed to formalin-killed bacterial cells and human antisera from infected patients to screen *H. pylori* genomic libraries. One of them, containing a 2.3-kb *H. pylori* DNA fragment inserted into the EcoRI site of pBK-CMV, produced an immunopositive protein with an apparent M_r of 18 000, as determined by SDS/PAGE and Western blot. Sequence analysis identified two incomplete genes: a 477-bp *hp0596* gene fragment encoding a putative protein with a calculated molecular mass of 18 kDa and a 1697-bp *hp0597* gene fragment. Both open reading frames (ORFs) are transcribed from opposite DNA strands and both lack their own promoters. The *hp0596* gene expression in *E. coli* was driven from the *lac* promoter of the pBK-CMV plasmid. Cloning of the above-mentioned fragment in the opposite orientation resulted in the absence of a product reacting with the same antiserum.

A clone containing the *hp0596* gene with its own promoter was also isolated during immunological screening of the *H. pylori* cosmid library. The whole 576-bp *hp0596* gene encodes a protein with an M_r of $\sim$21 kDa (with signal sequence). HP0596 homologues have not been detected in the genomes of related species (*Campylobacter jejuni, Helicobacter hepaticus* and *Wiolinella succinogenes*). The product of the *hp0596* gene was named TNF-α-inducing protein (Tip-α) by Suganuma's group [51].

Protein Localization

Subcellular localization of Tip-α in *H. pylori* and *E. coli* was analyzed by cell fractionation. Western blot of proteins isolated from different cell compartments probed with rabbit antiserum to recombinant $(His)_6$Tip-α protein showed that most of the Tip-α is associated with the cell envelopes and only a small fraction is soluble and present in the periplasm. Tip-α was not detected among outer membrane proteins, which signified that the protein is localized in the inner cell membrane, in both *E. coli* and *H. pylori* strains (data not shown). Tip-α forms a homodimer of an apparent M_r of 36,000, which was confirmed by Western analysis under nonreducing conditions and molecular filtration (data not shown). Data are consistent with those presented earlier by Yoshida et al. [55].

Transcription Start Point Identification

Based on genome sequence data of *H. pylori* 26695 and J99, *hp0596* is transcribed as a separate transcription unit. RACE-PCR was used to map the transcription start point (TSP) of *hp0596* to identify the potential promoter. The TSP was localized 119 bp upstream of the *hp0596* GTG translation start codon. Examination of the nucleotide sequence located upstream of the transcription start point revealed a putative −10 region and lack of nucleotide sequences recognized by RNAP with σ^{54} and σ^{28}. These data indicate that *hp0596* is transcribed by RNAP with the main sigma factor [54].

Protein Modeling

The three-dimensional model (3-D) of Tip-α, predicted based on sequence structure threading, is shown in Fig. 1. Only marginal similarity to structurally characterized proteins was detected. In addition, the Rosetta method was used to build a de novo model of Tip-α, yet it did not produce any reasonable result. Nevertheless, the majority of algorithms suggested that Tip-α may adopt a four-α-helical-bundle fold, similar to proteins such as the periplasmic ligand-binding domain of the chemotaxis aspartate receptor and proteins from the helical cytokine superfamily. Most of the hydrophobic side chains are in the structural core of the model, while hydrophilic residues are present at the surface, suggesting that Tip-α is soluble or, at least, largely exposed to the solvent.

Cytokine Production by Tip-α-Stimulated Macrophages

The level of cytokines produced by macrophages that had been exposed to the recombinant $(His)_6$Tip-α was estimated.

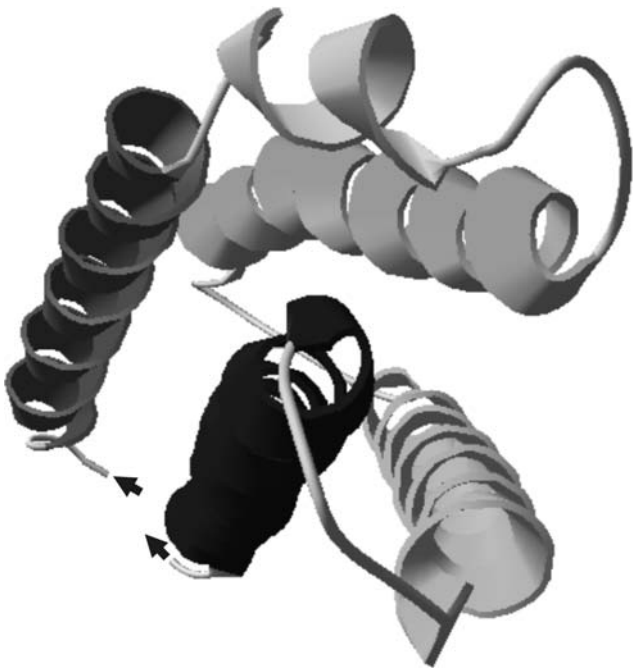

Fig. 1 A three-dimensional model of Tip-α. Arrows indicate the route of the polypeptide backbone from the N- to the C-terminus

Production of IL-1α as well as TNF-α was 15–50 times higher in macrophages stimulated by $(His)_6$Tip-α (113.3 and 1033.8 pg/ml, respectively) compared to nonstimulated cells (8.8 and 20.6 pg/ml, respectively; data not shown).

Colonization Assay

In this report we studied the involvement of the Tip-α protein in the colonization of murine mucosa by *H. pylori*. The goal was accomplished by constructing an isogenic Tip-α-mutated *H. pylori* SS1 strain by gene replacement technology and evaluating its colonization abilities using the murine infection model. The bacterial load in the mouse stomach was monitored by RT-PCR. *H. pylori* SS1 isolated by Lee et al. [32] is a mouse-adapted strain, used to elucidate some of the mechanisms involved in *H. pylori*-dependent pathologies.

Mutation in Tip-α significantly decreased the ability of *H. pylori* to colonize the stomach ($p < 0.01$). For three of six mice, real-time PCR confirmed a lower level of residual infection (6 times lower than in control mice), while the remaining animals were colonized at very low levels (100–300 times lower). The extrapolated amounts of bacterial DNA quantified by real-time PCR corresponded to 2230–18975 bacterial cells/100 ng of mouse DNA in gastric samples from seven control mice (infected by the wild-type strain SS1) and 10–606 bacterial cells/100 ng of mouse DNA in samples from six mice infected with the isogenic Tip-α mutant (Fig. 2B). *H. pylori* colonization was also examined in mucosa samples employing the qualitative rapid urease test (CLO test), which was read routinely 1 h after gastric mucosa specimen collection and, if negative, 3 and 24 h later. CLO test results were correlated with results of colonization experiments.

The in trans complementation experiment was not conducted due to the fact that the *H. pylori* SS1 strain is poorly tolerant to foreign DNA [15].

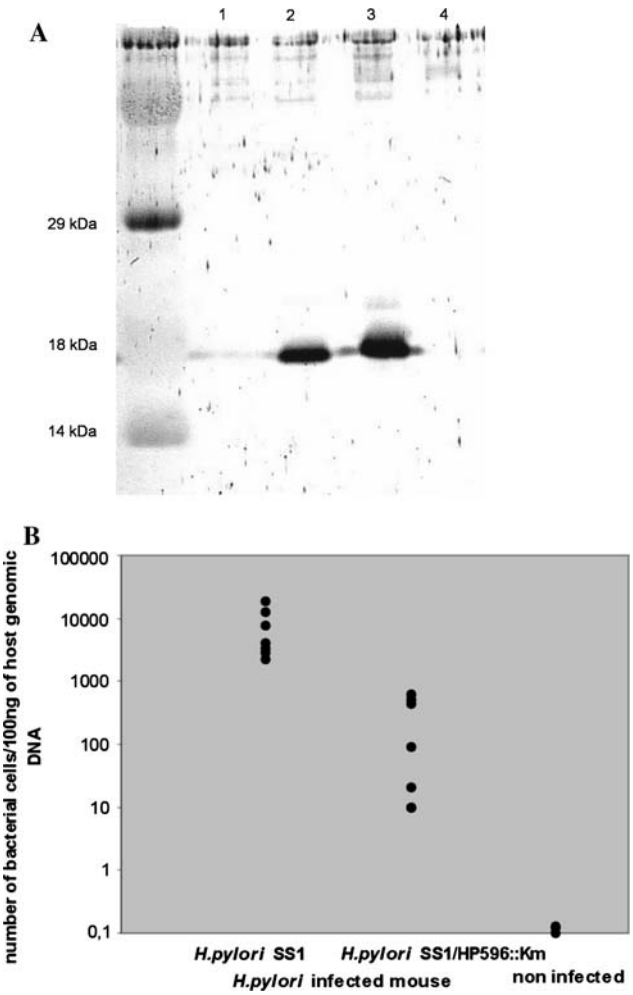

Fig. 2 **(A)** Western blot analysis of whole-cell lysates of *H. pylori* SS1 and Tip-α mutant reacted with rabbit anti-Tip-α sera. Lane 1, *H. pylori* Tip-α mutant; lanes 2 and 3, *H. pylori* SS1 strain; lane 4, *E. coli*. **(B)** Gastric colonization level of *H. pylori* in mice infected by the SS1 strain and by SS1/596::Km (Tip-α mutant)

Discussion

The high immunogenicity of Tip-α is observed not only during immunization of animals with killed bacterial cells but also during human infection. This fact was also documented by Yoshida et al. [55], who showed the presence of anti-Tip-α in the sera of *H. pylori*-positive individuals. However, Tip-α (HP0596) has not been identified as a *H. pylori* immunoreactive protein by classical immunoproteomics technology. This can be explained by several limitations of this methodological approach such as the low-abundant proteins or difficulties with solubilization of membrane-bound proteins. In addition, Tip-α was not present among immunoreactive proteins when cell surface proteins or outer membrane proteins of *H. pylori* were analyzed by two-dimensional (2-D) electrophoresis and immunoblot assays [5]. It should be pointed out that immunoproteomic experiments also failed to identify Lpp20 (HP1456) and HpaA (HP0797), which are both membrane-located lipoproteins recognized by human antibodies. On the other hand, the HpaA homologue (HP0410 gene product) was selected as seroreactive [5]. Moreover, both proteins (HP0410 and Lpp20) have an immunoprotective effect [21, 45].

Another *H. pylori* outer membrane-located lipoprotein, a product of the *hp1285* gene, which exhibits acid phosphatase activity, was recognized as immunoreactive by similar technologies. HP1285 is recognized by sera from ulcer and cancer patients but not by sera taken from gastritis patients [45].

The *hp0596* gene product, 18 kDa in size, was determined to be a homodimer located in the cell inner membrane by cell fractionation assay and Western blotting. In silico prediction of the three-dimensional structure revealed that Tip-α is most likely a soluble protein. Analysis of the Tip-α amino acid sequence showed that it contains a signal sequence with a classical LQAC motif that can be processed by a signal peptidase II specific for lipoprotein precursors. This observation strongly implies that Tip-α is a lipoprotein anchored in the inner membrane facing the periplasm. All our attempts to confirm the lipoprotein nature of Tip-α by growing *H. pylori* cells in the presence of labeled palmitic acid were unsuccessful. Similar difficulties were encountered by Kostrzynska et al. and O'Toole et al. during their work on *H. pylori* Lpp20 and HpaA [25, 40].

Another group [29] has proposed that Tip-α shows a relationship to penicillin-binding proteins (PBPs) in PSI-BLAST searches. Nonetheless, we were unable to reproduce this results. In PSI-BLAST searches carried out in the same way as described by the authors of the aforementioned article, Tip-α shows only insignificant similarity to PBPs (e-value = 1.5, which means that on the average 1.5 sequences are expected to be reported with the same or a better score due purely to chance and not to a real relationship). Therefore, we used the most sensitive tools for sequence comparisons available nowadays, including the fold-recognition approach [26] and the HHSearch method for profile-profile comparison [50]. None of them indicated any similarity between Tip-α and PBPs, at either the sequence or the structural level. In fact, structure prediction for Tip-α suggests that Tip-α is mostly α-helical, while the experimentally determined structure of PBP is α/β.

Thus, we have attempted to predict the structure of Tip-α using the currently best de novo prediction method ROSETTA [49]. We have generated 5000 decoys and clustered them using the standard procedure available in the ROSETTA package. Although we were unable to identify a single preferred topology, the largest cluster of conformations has exhibited a hairpin structure comprising two central α-helices, flanked by two additional helical regions from the N- and C-termini. Figure 1 shows the lowest-energy conformation from the largest cluster. Although this theoretical structure should be taken with a grain of salt, as it probably exhibits large deviation from the real structure, it can be used as an illustration for potential arrangement of helical secondary structures predicted for Tip-α and as an argument that it is very unlikely that Tip-α assumes a structure similar to that of PBPs.

According to the +2 rule, Tip-α, having a threonine residue next to a lipid-modified cysteine, should be outer-membrane located [47]. Lpp20 and HpaA also contained a LVGCS motif (with a serine as the +2 amino acid) at the C-terminal end of their signal sequences, which is characteristic for lipoprotein precursors. Until now, the cellular localization of HpaA and Lpp20 remains controversial. Originally, the subcellular localization of both proteins was determined by cell fractionation followed by sarcosyl solubilization of inner membrane proteins. HpaA was found to be cytoplasmic and inner membrane situated, whereas Lpp20 was isolated from both the inner and the outer membrane fractions of *H. pylori* cells [25, 40]. Further systematic examination, conducted by different methods, contradicted previous results and revealed that HpaA is a flagellar sheath protein and Lpp 20 is an outer membrane antigen released from cells inside membrane vesicles [17, 21]. Thus, it is likely that the methodology used for *E. coli* cell fractionation is not reliable when employed for *H. pylori*. Moreover, the amino acid located next to the lipid-modified cysteine, which in *E. coli* is considered to be a sorting amino acid, most likely does not exhibit this function in *H. pylori*. Additionally, in *Pseudomonas aeruginosa*, Narita and Tokuda showed that residues at positions 3 and 4 are critical for the destination of lipoproteins [39].

The role and physiological function of Tip-α in *H. pylori* pathogenesis are still not fully understood. The protein is nonessential for growth in vitro. However, this work provides experimental evidence showing that the protein is involved in colonization of mouse gastric mucosa. Inactivation of the *hp0596* gene did not eliminate colonization but greatly reduced the bacterial load in the murine stomach. It was also demonstrated that the recombinant Tip-α stimulates IL-1α and TNF-α production by macrophages, confirming data presented by Yoshida et al. [55]. Further investigations conducted by Suganuma et al. and Kuzuhara et al. demonstrated that Tip-α, being a proinflammatory mediator (TNF-α-inducing protein), can be involved in carcinogenesis [28, 52]. On the other hand, we observed a reduced ability of the mutated strain to colonize mice. One of the possible explanations for the correlation between the decrease in IL-1 secretion and the inhibition of bacterial colonization in the gastric mucosa could be the IL-1-induced proliferation of gastric epithelial cells (in in vitro experiments), which, in effect, result in cancer development and, additionally, increase the surface area for *H. pylori* colonization. It is possible that the decrease in IL-1 production results in poor colonization of mucosal surfaces by the pathogen [6].

We were not able to show the presence of Tip-α in the culture supernatant in vitro. The protein was also absent in 33 protein species which constitute the *H. pylori* 2665 secretome established by 2-D electrophoresis [8]. Obtained data do not rule out the possibility that Tip-α is unleashed by the pathogen in vivo during infection, especially that the predicted 3-D protein model suggests its soluble

character. Additionally, Suguanuma et al. demonstrated the presence of Tip-α in the culture supernatants of several *H. pylori* strains [52].

There is a great deal of evidence indicating that lipoproteins are released by Gram-negative bacteria (*Salmonella*, *Yersinia*, and *Escherichia*) and that the process is enhanced by many factors, e.g., by the presence of human serum [56]. The released lipoproteins are potential factors playing a role in bacterial pathogenesis, mainly due to the stimulation of eukaryotic cells throughout Toll-like receptors (TLR2).

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