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# Activation of Human THP-1 Cells and Rat Bone Marrow-Derived Macrophages by *Helicobacter pylori* Lipopolysaccharide

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**The mechanism by which *Helicobacter pylori*, which has little or no invasive activity, induces gastric-tissue inflammation and injury has not been well characterized. We have previously demonstrated that water-extracted proteins of *H. pylori* are capable of activating human monocytes by a lipopolysaccharide (LPS)-independent mechanism. We have now compared activation of macrophages by purified LPS from *H. pylori* and from *Escherichia coli*. LPS was prepared by phenol-water extraction from *H. pylori* 88-23 and from *E. coli* O55. THP-1, a human promyelomonocytic cell line, and macrophages derived from rat bone marrow each were incubated with the LPS preparations, and cell culture supernatants were assayed for production of tumor necrosis factor alpha (TNF-α), prostaglandin $E_2$ ($PGE_2$), and nitric oxide. THP-1 cells showed maximal activation by the LPS molecules after cell differentiation was induced by phorbol 12-myristate 13-acetate. Maximal TNF-α and $PGE_2$ production occurred by 6 and 18 h, respectively, in both types of cells. In contrast, NO was produced by rat bone marrow-derived macrophages only and was maximal at 18 h. The minimum concentration of purified LPS required to induce TNF-α, $PGE_2$, and NO responses in both types of cells was 2,000- to 30,000-fold higher for *H. pylori* than for *E. coli*. Purified LPS from three other *H. pylori* strains with different polysaccharide side chain lengths showed a similarly low level of activity, and polymyxin B treatment markedly reduced activity as well, suggesting that activation was a lipid A phenomenon. These results indicate the low biological activity of *H. pylori* LPS in mediating macrophage activation.**

*Helicobacter pylori* causes persistent infection of the human stomach and is now recognized as the most common cause of chronic superficial gastritis (1). Although usually asymptomatic, *H. pylori*-induced chronic gastritis is an important risk factor for the development of peptic ulcer disease and adenocarcinoma of the stomach (12, 24), and consequently this lesion is clinically important. However, intense inflammation may lead to loss of gastric glandular structure and function, and with the development of atrophic gastritis, the ecological niche for *H. pylori* is progressively lost (13). Thus, there exists selective pressure for *H. pylori* to modulate induction of tissue injury (3).

At present, much about the pathogenesis of *H. pylori*-induced gastric inflammation and injury is not well understood. Although the organism does not invade the lamina propria, it induces an infiltrate with T-lymphocytes, plasma cells, mononuclear phagocytes, and neutrophils (26, 34), and expression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukins-1, -6, and -8, also is enhanced (6, 27). We have previously demonstrated that water-extracted proteins of *H. pylori* are chemotactic for human polymorphonuclear leukocytes and monocytes (17) and also activate these cells by a lipopolysaccharide (LPS)-independent mechanism (18). For many other bacterial species, LPS is a powerful activator of mononuclear phagocytes (31, 42).

LPS is a major component of the outer membranes of gram-negative bacteria (15), with a lipid core and polysaccharide side chains of variable length. The carbohydrate portion contains a core region which usually is genus or species specific and an outermost chain that is strain specific and forms the basis of the O-antigenic classification (16). The lipid portion (lipid A) represents the endotoxic principle of biologically active LPS (9). LPS from members of the family *Enterobacteriaceae* has been demonstrated to be highly proinflammatory (31), and LPS from *Escherichia coli* and *Salmonella* spp. is representative of the group. Compared with the lipid A of members of the *Enterobacteriaceae*, the lipid A of *H. pylori* has an unusual composition of fatty acids (10) and also a different phosphorylation pattern, with 1′- but not 4′-phosphate present in the backbone of lipid A D-glucosamine disaccharide (21). *H. pylori* LPS has been reported to be biologically less active than LPS from members of the *Enterobacteriaceae* when mitogenicity, pyrogenicity, and lethal toxicity were compared in in vivo assays (23).

The purpose of the present study was to compare the ability of purified LPS from *H. pylori* and *E. coli* to activate monocytic cells in vitro. We hypothesized that evolutionary pressure for *H. pylori* persistence in the stomach (3) would select for LPS molecules with relatively low activity.

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## MATERIALS AND METHODS

**Cell line.** THP-1 (ATCC TIB202), originally isolated from a child with acute leukemia, are mature cells in the monocyte/macrophage lineage with a normal

TABLE 1. Chemical composition of whole-cell and LPS preparations from *H. pylori* and *E. coli* strains

| Strain | Whole cell[a] | | | LPS preparation[a] | | | Ratio B/A |
|---|---|---|---|---|---|---|---|
| | Protein concn (mg/ml) | KDO concn (mg/ml)[b] | Ratio A[c] | Protein concn (mg/ml) | KDO concn (mg/ml) | Ratio B[c] | |
| *H. pylori* 88-22 | 7.4 | 0.013 | 0.002 | 0.23 | 0.009 | 0.04 | 20 |
| *H. pylori* 88-23 | 4.8 | 0.013 | 0.003 | 0.33 | 0.011 | 0.03 | 10 |
| *H. pylori* 84-182 | 2.4 | 0.007 | 0.003 | 0.17 | 0.005 | 0.03 | 10 |
| *H. pylori* 84-183 | 5.5 | 0.009 | 0.002 | 0.18 | 0.011 | 0.06 | 30 |
| *E. coli* O55 | ND[d] | ND | ND | 0.05 | 0.027 | 0.54 | ND |

[a] All values shown are the means of two separate determinations.
[b] KDO, 2-keto-3-deoxyoctonate.
[c] Ratios A and B were calculated as the proportions of KDO to protein in whole cells (ratio A) and in purified LPS (ratio B).
[d] ND, not determined.

diploid karyotype (38), and they produce TNF-α and other cytokines in response to purified endotoxin (20). These nonadherent cells were maintained in continuous culture with RPMI 1640 (GIBCO/BRL, Grand Island, N.Y.), 10% fetal bovine serum (GIBCO/BRL), and 0.05 mM 2-mercaptoethanol (GIBCO/BRL) in an atmosphere of 5% $CO_2$ at 37°C. The doubling time for these cells under these conditions is approximately 48 h. THP-1 cells were treated with phorbol 12-myristate 13-acetate (Calbiochem Co., La Jolla, Calif.) to induce maturation of the monocytes and became macrophage-like; differentiated macrophages were identified by morphological features and their ability to adhere to plastic, as described elsewhere (37). Before experimentation or treatment with phorbol 12-myristate 13-acetate, THP-1 cells were washed three times with culture medium without fetal bovine serum and resuspended to a concentration of $10^6$ cells per ml. Cell viability was determined to be >95% by the trypan blue dye exclusion method (35).

**Rat bone marrow-derived macrophage culture.** Rat bone marrow macrophages were obtained from precursor bone marrow cells as described previously (30). Femoral bone marrow cells were grown in a 150-mm tissue culture dish at $5 \times 10^7$ cells per ml for 6 days in 50 ml of culture medium (Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and penicillin-streptomycin) plus 10% L-cell-conditioned medium as previously described (38). The mature rat bone marrow macrophages were removed from the dishes with cold 5 mM EDTA in phosphate-buffered saline (PBS) and replated into 24-well culture plates (Sarstedt, Inc. Newton, N.C.) at $5 \times 10^5$ cells per ml. They were incubated at 37°C in 5% $CO_2$ in the presence of different concentrations of the bacterial products and controls. Aliquots were obtained at 0, 6, and 18 h of incubation, and the supernatants were collected and either tested immediately or stored at −70°C.

**Bacterial strains and culture conditions.** *H. pylori* 84-183, 84-182, 88-22, and 88-23, clinical isolates in the Vanderbilt *Campylobacter*/*Helicobacter* culture collection (5, 28), were stored at −70°C until use. Bacteria were inoculated onto Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 48 to 72 h in ambient atmosphere plus 5% $CO_2$. Bacteria were suspended in sterile pyrogen-free saline and centrifuged at $3{,}000 \times g$ for 20 min, and the pellet was resuspended in distilled water.

**LPS preparations.** The LPS from the four *H. pylori* strains was prepared by the hot-phenol–water method of Westphal and Jann (41), and subsequent purification steps were performed essentially as described by Daniels et al. (7). In brief, bacterial cells from blood agar plates were scraped into saline, centrifuged ($5{,}000 \times g$ for 15 min), and resuspended in water with an equal volume of 90% phenol at 60°C for 15 min. After the mixture was cooled to 10°C and centrifuged ($10{,}000 \times g$ for 20 min), the aqueous layer was removed. This extraction procedure was repeated twice, and the pooled water-extracted layers were dialyzed for 48 h against several changes of water and lyophilized. As a control, LPS purified by the hot-phenol–water method from *E. coli* O55:B5 (List Biological Laboratories, Inc., Campbell, Calif.) was used in each experiment. In several experiments, the LPS from *E. coli* and *H. pylori* strains were treated with polymyxin B (Sigma Chemical Co., Saint Louis, Mo.) to determine the effect of binding to lipid A on the biological activities of these molecules (22). Preparations of LPS were preincubated for 1 h at 37°C with twofold concentrations of polymyxin B, and the activity was assayed in the systems used for the untreated LPS.

**Analytical methods.** Protein concentrations were measured by the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). For determination of 2-keto-3-deoxyoctonate concentrations, the thiobarbituric acid method (39) with the modifications described by Keleti and Lederer (14) was used with 3-deoxyoctulosonic acid ammonium salt (Sigma) as the standard.

**Monokine release.** Aliquots of purified *H. pylori* or *E. coli* LPS were added in duplicate to THP-1 cells ($10^6$ cells per ml) in 24-well tissue culture plates or to rat bone marrow macrophages ($5 \times 10^5$ cells per ml) and then incubated at 37°C in a 5% $CO_2$ atmosphere. Cell suspensions obtained at 0, 6, and 18 h after incubation were transferred to 1.5-ml polypropylene tubes and centrifuged at $12{,}000 \times g$ for 3 min at room temperature, and the supernatants were stored at −70°C until the monocyte-derived factors could be assayed.

**Measurement of TNF-α.** Biological activity of TNF-α was measured by an assay of L929 fibroblast lysis, as previously described (4). Briefly, L929 cells ($5 \times 10^5$ cells per ml) in alpha minimal essential medium (GIBCO BRL) with 10% fetal bovine serum were incubated overnight at 37°C with 5% $CO_2$ in 96-well flat-bottom plates (Sarstedt). An aliquot of 100 μl of fresh alpha minimal essential medium plus 15 μg of actinomycin D (Sigma) per ml and 100 μl of the cell culture supernatant were added, and the plates were again incubated overnight at 37°C with 5% $CO_2$. Subsequently, plates were washed with Dulbecco's PBS and cells were stained with 0.1% crystal violet in 100% methanol. The number of remaining cells as assessed by $A_{540}$ was used to determine cell lysis; purified TNF-α was used as a positive control and for development of a standard curve.

**Nitric oxide synthase assay.** Nitric oxide activity was measured as nitrite production (19) in stimulated rat bone marrow-derived macrophages and THP-1 monocytes; each assay was performed in triplicate. Nitrite was measured by mixing 100 μl of the cell-free culture supernatant with an equal volume of Griess reagent (1 part of 0.1% naphthylethylenediamine dihydrochloride to 1 part of 1% sulfanilamide in 5% phosphoric acid) (11). The $A_{550}$ was determined, and the nitrite concentration was calculated from a standard curve with sodium nitrite as the reference.

**$PGE_2$ assay.** Prostaglandin $E_2$ ($PGE_2$) production in cell supernatants was determined by a gas chromatography-mass spectrometry method involving stable isotope dilution techniques with $[^2H_4]PGE_2$ as an internal standard, as previously described (25).

## RESULTS

**Chemical analysis of purified LPS.** The four strains were selected because the LPS profile for each was different as observed by polyacrylamide gel electrophoresis (PAGE) analysis (28). However, the chemical analysis of the four purified LPS preparations from these *H. pylori* strains showed similar protein and 2-keto-3-deoxyoctonate contents (Table 1). In total, protein contamination was less than 5%. In most of the subsequent studies, the preparation from strain 88-23 was used.

**Activation response to LPS in undifferentiated and differentiated THP-1 cells.** We first evaluated the ability of LPS to activate either differentiated or undifferentiated THP-1 cells. LPS preparations from both *H. pylori* and *E. coli* were able to activate both types of cells to induce the production of TNF-α and $PGE_2$ (Table 2). However, undifferentiated THP-1 cells were $1.5 \times 10^5$-fold less responsive to *E. coli* LPS than were differentiated THP-1 cells, as assessed by production of TNF-α. The effects of *E. coli* LPS on macrophages are markedly enhanced by an LPS-binding protein present in serum (43). The poor response obtained with *E. coli* LPS in undifferentiated THP-1 cells is at least in part because no serum source (and thus no LPS-binding protein) was added to the medium. When $PGE_2$ was used as the indicator of activation, undifferentiated cells were $>8 \times 10^3$-fold less sensitive than differentiated cells to activation by *E. coli* LPS (Table 2). The undifferentiated cells also were poorly activated by *H. pylori* LPS. In contrast, the differentiated THP-1 cells were markedly responsive to LPS from *E. coli* but poorly activated by *H. pylori* LPS.

TABLE 2. Minimal concentrations of LPS from *H. pylori* or *E. coli* inducing responses in undifferentiated and differentiated THP-1 cells

| LPS source | Concn of LPS (μg/ml) | | | |
|---|---|---|---|---|
| | Undifferentiated | | Differentiated | |
| | TNF-α[a] | $PGE_2$[b] | TNF-α | $PGE_2$ |
| *H. pylori* | 10 | 2.5 | 0.15 | 2.5 |
| *E. coli* | >50 | 2.5 | 0.0003 | 0.0003 |

[a] Culture supernatants were obtained after a 6-h incubation of the THP-1 cells with differing concentrations of LPS. A positive response was defined as TNF-α induction of ≥0.07 μg/ml. This value represents twice the concentration of TNF-α obtained at baseline. Results shown are means of two replicate determinations.

[b] Culture supernatants were obtained after 24 h of incubation of the THP-1 cells with different concentrations of LPS. Response was defined as $PGE_2$ induction of ≥100 pg/ml; this value represents the concentration of $PGE_2$ obtained as baseline. Results shown are means of two replicate determinations.

On the basis of these preliminary results, the remainder of the experiment focused only on differentiated THP-1 cells.

**Minimal LPS concentration to induce activation in differentiated THP-1 cells.** In a second series of experiments, the differentiated THP-1 cells again were much more responsive to LPS from *E. coli* than from *H. pylori* (Table 3). Results for the commercial preparation from an *E. coli* O55 strain and a preparation from an O157 strain made in this laboratory showed nearly identical results in the range from 1 ng to 1 μg (data not shown). The minimal concentration required to activate the THP-1 cells was between $2 \times 10^3$- and $30 \times 10^3$-fold lower for *E. coli* than *H. pylori* LPS. However, as expected (36), both LPS preparations failed to induce nitric oxide production in this system.

**Minimal LPS concentrations to induce activation in rat bone marrow macrophages.** We then compared the ability of the *E. coli* and *H. pylori* LPS preparations to induce responses in rat bone marrow macrophages. Induction of nitric oxide, TNF-α, and $PGE_2$ responses were used as markers of activation. In all instances, *E. coli* LPS was a substantially better activator than was *H. pylori* LPS. Depending on the assay, $2 \times 10^3$- to $20 \times 10^3$-fold less *E. coli* LPS was required to induce the same level of responses than was *H. pylori* LPS (Table 4).

**Comparison of *H. pylori* LPS preparations inducing activation.** We then compared the relative ability of purified LPS preparations from four *H. pylori* strains to induce activation in rat bone marrow macrophages and differentiated THP-1 cells (Table 5). The results indicated that the minimal concentrations required to induce responses in macrophages were high

TABLE 3. Minimum concentrations of LPS from *H. pylori* or *E. coli* inducing responses in differentiated human THP-1 cells

| Indicator of activation | Concn of LPS (μg/ml) | | Fold difference |
|---|---|---|---|
| | *E. coli* | *H. pylori* | |
| TNF-α[a] | 0.00007 | 2.5 | $3.6 \times 10^4$ |
| NO[b] | >10 | >10 | NA[c] |
| $PGE_2$[d] | 0.0003 | 0.6 | $2 \times 10^3$ |

[a] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results shown are means of two replicate experiments.

[b] After 24 h of incubation of the THP-1 cells with differing concentrations of LPS, culture supernatant was used for determinations. Response was defined as NO induction of ≥ 0.001 nM $NO_2$ per ml. Results shown are means of two replicate experiments.

[c] NA, not applicable.

[d] Response is defined as $PGE_2$ induction of ≥100 pg/ml, as indicated in Table 2. Results shown are means of two replicate experiments.

TABLE 4. Minimum concentrations of LPS from *H. pylori* or *E. coli* that induce responses in rat bone marrow macrophages

| Indicator of activation | Concn of LPS (μg/ml) | | Fold difference |
|---|---|---|---|
| | *E. coli* | *H. pylori* | |
| TNF-α[a] | 0.3 | 600 | $2 \times 10^3$ |
| NO[b] | 0.015 | 300 | $2 \times 10^4$ |
| $PGE_2$[c] | 0.3 | 2,500 | $8 \times 10^3$ |

[a] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results are means of two replicate experiments.

[b] Response is defined as NO induction of ≥0.001 nM $NO_2$ per ml, as indicated in Table 3. Results are means of two replicate experiments.

[c] Response is defined as $PGE_2$ induction of ≥100 pg/ml, as indicated in Table 2. Results are means of two replicate experiments.

for all four strains, with only small (≤fourfold) differences. These data indicate that the macrophage-activating constituents of *H. pylori* LPS are conserved and thus suggest that these structures may be present in the lipid A moiety.

**Polymyxin B blocks LPS-induced TNF-α production.** By binding to lipid A, polymyxin B is a well-known inhibitor of activation properties of LPS from the members of the *Enterobacteriaceae* (22). To determine whether the effect of polymyxin B on *H. pylori* LPS is similar, we preincubated different concentrations of *H. pylori* LPS, with or without polymyxin B, before adding these preparations to THP-1 cells. Preincubation with polymyxin B markedly inhibited the ability of *H. pylori* LPS to induce TNF-α release (Fig. 1). These results indicate that the basis for activation of macrophages by *H. pylori* LPS is lipid A mediated and that polymyxin neutralizes this activity, despite the differences in lipid A structure in comparison with members of the *Enterobacteriaceae* (10, 21).

## DISCUSSION

The cardinal lesion of *H. pylori* colonization of the stomach is gastric inflammation (1), but since these organisms do not invade tissue, the proinflammatory effects of superficial or released bacterial products are of interest. Bacterial LPSs are classic mediators of inflammation because of their activation of phagocytic cells, endothelial and epithelial cells, and lymphocytes (32). However, despite a general conservation of LPS structure, large differences in their proinflammatory activity have been noted (33).

Thus, it is reasonable to explore whether the LPS of *H. pylori* is involved in induction of the characteristic inflammatory in-

TABLE 5. Minimum concentrations of LPS from four *H. pylori* strains that induce responses in macrophages

| *H. pylori* strain | Concn of LPS (μg/ml) in: | | | | | |
|---|---|---|---|---|---|---|
| | Rat bone marrow macrophages | | | Differentiated THP-1 cells | | |
| | NO[a] | TNF-α[b] | $PGE_2$[c] | NO | TNF-α | $PGE_2$ |
| 88-23 | 2.5 | 0.6 | 2.5 | >10 | 2.5 | 2.5 |
| 88-22 | 2.5 | 2.5 | 2.5 | >10 | 0.6 | 0.6 |
| 84-182 | 0.6 | 2.5 | 2.5 | >10 | 2.5 | 0.6 |
| 84-183 | 2.5 | 2.5 | 2.5 | >10 | 0.6 | 2.5 |

[a] Response is defined as NO induction of ≤0.001 nM $NO_2$ per ml, as indicated in Table 3. Results are means of two replicate experiments.

[b] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results are means of two replicate experiments.

[c] Response is defined as $PGE_2$ induction of ≥ 100 pg/ml, as indicated in Table 2. Results are means of two replicate experiments.

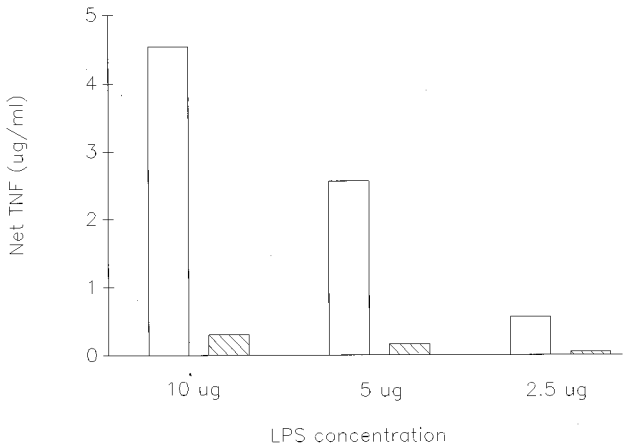


FIG. 1. Effect of polymyxin B on *H. pylori* LPS-induced TNF-α production by THP-1 cells. Empty bars, LPS without polymyxin B treatment; hatched bars, LPS treated with a twofold concentration of polymyxin B. The different LPS concentrations were incubated with or without polymyxin B at 37°C for 1 h, and then the preparations were incubated with THP-1 cells for 6 h. Culture supernatants were harvested and TNF-α concentrations were determined as described in Materials and Methods. Results shown are the means of two replicate determinations.

filtrate. Previous studies have shown that *H. pylori* LPS is substantially less active than LPS from members of the *Enterobacteriaceae* in a variety of biologic activities including rabbit pyrogenicity, B-cell mitogenicity, and ability to gel *Limulus* lysates (10, 21, 23). However, interactions with phagocytic cells were not addressed.

In our studies, *H. pylori* LPS was substantially less stimulatory to macrophages than was *E. coli* LPS. In studies of two different types of cells and assays of three different products of activation, the results are highly consistent. This multiplicity of approaches increases our confidence that the observation is correct. Furthermore, we have also performed experiments with *E. coli* O157 LPS that was prepared in our laboratory by the Westphal technique, and the results were similar to those observed for the commercially obtained *E. coli* O55 LPS. Although *H. pylori* strains may show marked differences in LPS profiles by PAGE (28), their abilities to activate macrophages were highly similar. This observation suggests that as with other gram-negative organisms, the ability to activate macrophages is a function of conserved core structures. The studies with polymyxin B confirm the important role of lipid A in this phenomenon. Our observations are consistent with results of previous studies indicating its low level of biological activity (9, 15, 16) and suggest that *H. pylori* lipid A features, including long-chain fatty acids and the lack of a phosphate group (21), may be responsible. *Bacteroides fragilis* is another gram-negative organism that is a persistant colonizer of the human gastrointestinal tract (8), and similar to *H. pylori*, its LPS is a poor activator of macrophages (40).

Among individual persons infected with *H. pylori*, there are differences in both degree of inflammation and clinical outcome of infection (29). The LPS structure is one of the few phenotypes of *H. pylori* that shows diversity. However, we found no substantial differences in proinflammatory activity despite this diversity, suggesting that LPS differences do not explain divergent outcomes of infection.

One question raised by our findings is why the LPS of *H. pylori* shows such low proinflammatory activity. Our experimental data are consistent with the general observation that toxicity is a function of the core lipid A moiety, which is likely to be highly conserved. One explanation is that there is selective pressure on *H. pylori* cells to minimize proinflammatory activities to permit long-term colonization (3), since enhanced inflammation, leading to atrophic gastritis, would lead to loss of niche (13). *H. pylori* and *B. fragilis* may be analogous in their requirement for maintaining a low profile at baseline to ensure persistence. Despite the low-level LPS activity, *H. pylori* possesses proteins that are highly efficient in recruiting and activating inflammatory cells (17, 18). Since *H. pylori* may require inflammation to provide a source of nutrients (2), the combination of a constitutively expressed LPS with low-level activity and inducible proteins with high-level activity may be beneficial. Tight regulation of proinflammatory activities could be a mechanism selected by *H. pylori* to maximize the duration of colonization.

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