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# Quantification of Conserved Antigens in *Helicobacter pylori* during Different Culture Conditions

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**In this study, we raised monoclonal antibodies (MAbs) against three conserved *Helicobacter pylori* antigens, i.e., the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin, HpaA; the flagellin subunits, FlaA and FlaB; and a species-specific 26-kDa protein. The MAbs were used for the development of sensitive inhibition enzyme-linked immunosorbent assays for quantification of these antigens in *H. pylori* during various culture conditions. The quantities of these antigens varied considerably (up to 8-fold) during different culture procedures and between strains (up to 10-fold).**

*Helicobacter pylori* causes chronic antral gastritis and peptic ulcers and is associated with stomach cancer (2, 21, 25). Despite the considerable genetic diversity of *H. pylori*, several phenotypic characteristics are relatively well conserved (3, 4, 18). Among the conserved *H. pylori* antigens is the *N*-acetylneuraminyllactose-binding hemagglutinin, HpaA, which mediates binding to sialic acid in vitro (8). However, whether HpaA also mediates adhesion in vivo is still controversial (19, 24). The flagella of *H. pylori* consist of two subunits, FlaA and FlaB, and have been suggested to play a role in bacterial virulence by providing motility and promoting colonization. Another preserved *H. pylori* antigen is the species-specific 26-kDa protein, of unknown function. Conserved *H. pylori* antigens may be useful for bacteriological as well as immunological studies and may be important protective antigens. The influence of different culture conditions on the growth of *H. pylori* has been evaluated in several studies (16, 17), but there are only few reports describing the quantitative variability of *H. pylori* antigens during various culture conditions or stages of growth (15, 23). In this study, we raised monoclonal antibodies (MAbs) against three conserved *H. pylori* antigens, i.e., the HpaA, the flagellins, and the 26-kDa protein, and developed inhibition enzyme-linked immunosorbent assays (ELISAs) for the quantification of these antigens in different strains of *H. pylori* during various culture conditions.

The strains analyzed were the stock strain CCUG 17874 (Culture Collection, Göteborg University) and the clinical isolates Hel 73 and Hel 305, isolated from two Swedish patients with gastritis and duodenal ulcer disease, respectively. Also, stock strains E32 and E50 (kindly provided by J.-P. Butzler) were used for antigen production. After one subculture from frozen stocks, bacteria were grown on horse blood agar plates and in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% (2.6-di-*O-methyl*-β)-cyclodextrin (kindly provided by Teijin Ltd., Tokyo, Japan) (17). Cultures were incubated at 37°C in a microaerobic incubator; some liquid cultures were incubated as described by Deshpande et al. (6) by injection of a gas mixture (6% $O_2$, 10% $CO_2$ and 84% $N_2$) into the culture flasks. Broth cultures were supplemented with vancomycin (10 μg/ml), trimethoprim (5 μg/ml), amphotericin B (5 μg/ml), and polymyxin B (20 U/ml) and incubated with or without agitation at 150 rpm. Bacterial samples taken at different time intervals were washed with phosphate-buffered saline (PBS), and the bacterial concentration was adjusted to $5 \times 10^9$ bacteria/ml, corresponding to an optical density at $A_{600}$ of 1.5. Some broth culture supernatants were concentrated 20 times by filtration (Filter type YM100; Amicon Inc., Beverly, Mass.). All antigens were produced from plate-grown bacteria, and the protein content was estimated by spectrophotometric readings ($A_{280}$ minus $A_{310}$ values). A membrane preparation (MP) was produced from strain CCUG 17874 as described previously (1). The 26-kDa protein was purified from a water extract of strain E32 essentially as described previously (7). In short, the extract was precipitated with 70% ammonium sulfate and centrifuged at 16,000 × *g* for 60 min. The pellet was resuspended in 20 mM phosphate buffer, pH 7.0 (PBS), and dialyzed overnight against PBS prior to size exclusion chromatography on a Sepharose CL 6B column (Pharmacia LKB, Uppsala, Sweden). The early fractions after elution with PBS were pooled and dialyzed as described prior to anion-exchange chromatography by fast protein liquid chromatography (Pharmacia) on a MonoQ column. The first peak after elution with a linear gradient from 0 to 500 mM NaCl contained the 26-kDa protein as verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting essentially as described by Laemmli (11). *H. pylori* flagellins were purified from strains E32 and E50 by CsCl equilibrium density gradient centrifugation of flagellum-containing pellets pretreated with trypsin (Boehringer-Mannheim, Scandinavia AB, Stockholm, Sweden) as previously described (10). The flagellin-subunit-containing band was dialyzed against PBS. SDS-PAGE and immunoblotting with *H. pylori*-specific rabbit serum and two flagellin-specific MAbs, HP50F-48:13 and HP32F-2:1, raised against purified *H. pylori* flagellins were used to confirm the flagellin content. The HpaA was prepared from strain CCUG 17874. Whole-cell lysates were separated by SDS-PAGE, and the HpaA band was identified by immunoblotting with rabbit serum raised against whole *H. pylori* cells and an HpaA-specific MAb (HP30-1:1:6) after the transfer of vertical gel strips to nitrocellulose membranes. Subsequently, several 1-mm-thick horizontal strips were cut from the 1.5-mm-thick 17% polyacrylamide gel and were electroeluted for 17 h at 3 mA with an Electro-Eluter (model 422; Bio-Rad Laboratories AB, Sundbyberg, Sweden). The HpaA-containing fractions were identified by immunoblotting against *H. pylori*-specific

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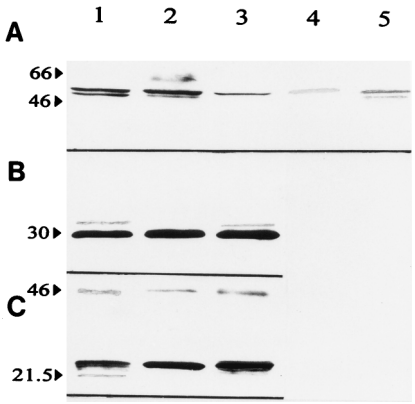


FIG. 1. Immunoblot identifying different *H. pylori* antigens in bacterial samples with MAbs. (A) HP50F-48:13, specific for FlaA and FlaB diluted 1:5; (B) HP30-1:1:6, specific for the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin, HpaA, diluted 1:25; (C) HP26-18:1, specific for the 26-kDa protein diluted 1:3. Lane 1, strain Hel 73 cultured in shaken broth; lane 2, strain Hel 305 cultured in shaken broth; lane 3, strain CCUG 17874 cultured in shaken broth; lane 4, strain CCUG 17874 cultured in static broth; and lane 5, strain CCUG 17874 cultured on agar plates. The samples were taken after 96 h of culturing, and $5 \times 10^7$ bacteria were added to each well.

rabbit serum and the HpaA-specific MAb HP30-1:1:6. MAbs against the flagellins, the HpaA, and the 26-kDa protein were produced as described previously (5) by immunizing BALB/c mice with flagellin, MP, or the 26-kDa protein, respectively. The hybridomas showing the highest titers against the antigens in ELISAs were subcloned and expanded. The MAb HP30-1:1:6 (4) reacting with a 30-kDa surface-exposed antigen reacts strongly with the HpaA (7a). The 26-kDa protein-specific MAb, HP26-18:1, reacts with both monomeric and dimeric forms of the protein (Fig. 1C) (18). The flagellin-specific MAbs, MAb HP50F-48:13 and MAb HP32F-2:1 (not shown), raised against flagellins from strain E50 and E32, respectively, identified two distinct bands corresponding to molecular weights of approximately 56,000 and 57,000, suggesting that they react with both the FlaA and the FlaB subunits (Fig. 1A). The MAbs were isotyped by double diffusion in gel (20) with specific antisera (Sigma). Single radial immunodiffusion (14) with the specific antisera and mouse sera of known concentration as standards was used to determine the immunoglobulin (Ig) concentration. All MAbs were found to be of the IgG1 subclass. The MAbs were titrated in ELISA as described previously (12). Plates were coated at room temperature (RT) overnight with 25 μg of MP or 5 μg of flagellins or 26-kDa protein diluted in PBS per ml. The bacterial antigens were quantified by inhibition ELISA with plates coated as described and the specific MAbs against each antigen. Samples were threefold serially diluted in 0.1% bovine serum albumin-PBS in noncoated plates. Thereafter the specific MAb, diluted in 0.1% bovine serum albumin-PBS to a concentration of approximately 10 times the ELISA titer against the corresponding antigen, was added. After incubation with agitation at RT for 1 h, the mixtures were transferred to antigen-coated plates and incubated at RT for 90 min. After three washes in PBS-0.05% Tween, the plates were developed with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories) and *o*-phenylenediamine–$H_2O_2$. Plates were read at 450 nm in a Multiscan PLUS reader (Labsystems), and the sample concentration causing 50% inhibition of binding of the respective MAb to solid-phase antigen was determined. All samples were analyzed in duplicate. Purified flagellins, HpaA, and 26-kDa protein were used as positive controls, and *Escherichia coli* K12C600 was used as a negative control. The total protein contents in lysed bacterial samples and in culture supernatants were determined by Peterson's modification (22) of the method of Lowry et al. (13) with a kit obtained from Sigma (Sigma-Aldrich Sweden AB, Göteborg, Sweden) according to the manufacturer's instructions. The specific MAbs were used to determine the surface localization of the antigens by immunoelectron microscopy as previously described (9). The inhibitory titers were given as geometric means of three experiments plus standard errors of the means. A Mann-Whitney test was used for the statistical evaluation of the differences in titers between different strains.

**Bacterial growth during different growth conditions.** The best growth, as studied by optical density measurements at $A_{600}$, of strains Hel 73, Hel 305, and CCUG 17874 was obtained in shaken (150 rpm) brucella broth cultures compared to that obtained in static cultures. The maximal exponential growth in shaken broth was reached within 24 to 48 h, whereas the bacterial number increased slowly in static broth throughout the 168 h of incubation. Furthermore, the injection of a gas mixture consisting of 6% $O_2$, 10% $CO_2$, and 84% $N_2$ (6) directly into the culture flasks before they were sealed resulted in better bacterial growth than that obtained by incubation in a microaerobic incubator. This finding supports the importance of adequate dispersion of the appropriate gases throughout the culture medium. Phase-contrast microscopy of the liquid cultures showed clumping of the bacteria, which was more pronounced in shaken cultures.

**Inhibition ELISAs.** We have previously shown that inhibition ELISA is a very sensitive method for the quantification of bacterial antigens in purified or secreted form or on whole bacteria (12). In this study, inhibition ELISAs were modified to allow quantification of the HpaA, the 26-kDa protein, and the flagellins in different *H. pylori* strains during various culture conditions. The lower limits for detection of the antigens studied were between 0.2 and 2 μg of protein/ml, and the minimal number of bacteria required for the detection of the antigens was $2 \times 10^8$ per ml. Even though the inhibition ELISA could be used for the quantification of bacterial antigens, e.g., the HpaA and the 26-kDa protein, on the surface of nontreated bacteria, we froze the bacterial samples prior to testing, since this allowed the analysis of samples obtained at different time points in the same assay. By comparing frozen and fresh bacterial samples, we found 1.7- to 2.8-fold-higher titers for the HpaA and the 26-kDa protein after freezing; for detection of the flagellin subunits by our MAbs in the inhibition ELISA, freezing was necessary. The reactivity of the MAbs HP50F-48:13 and HP32F-2:1 for flagellin subunits but not for assembled flagella was confirmed by immunoelectron microscopy; immunogold staining was obtained with fragmented (frozen) flagella but not with intact flagella, regardless of whether the flagellar sheet was present (not shown).

**Antigen release into culture supernatants.** Broth culture samples were separated into supernatants and bacterial pellets by centrifugation, and the bacteria were washed and then resuspended to the original sample volume. Thereafter, the supernatants and the corresponding bacterial suspensions were analyzed by inhibition ELISA. These analyses revealed that only the 26-kDa protein was found in significant amounts in the supernatants; this protein could be detected in unconcentrated supernatants of shaken cultures in approximately 2 times and 4 to 10 times the concentrations of the corresponding bacterial samples for strains Hel 305 and CCUG 17874, respectively. However, the protein was not found in 20-fold-concentrated supernatants of static cultures grown for up to 96 h; thereafter, the inhibitory titers were comparable to the

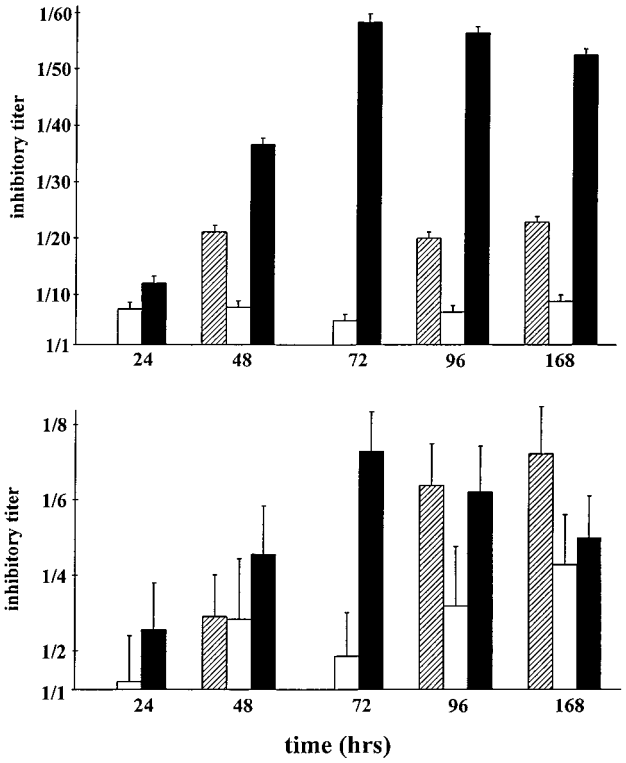


FIG. 2. Quantification of the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin, HpaA, as measured by inhibition ELISA in strain CCUG 17874 (top) and strain Hel 305 (bottom). Black bars, liquid shaken cultures; white bars, liquid static cultures; hatched bars, solid media culturing. Bacteria were tested at an initial concentration of $5 \times 10^9$ cells/ml, and geometric means of inhibitory titers in three experiments plus standard errors of the means are shown.

titers of the corresponding bacterial samples. The HpaA and the flagellins were not detected by inhibition ELISA in unconcentrated supernatants. Analysis of supernatants concentrated 20 times showed that the HpaA was present in low concentrations, i.e., less than 25% of the corresponding bacterial sample titers in supernatants from late-stationary-phase shaken cultures only.

**Quantification of the *N*-acetylneuraminyllactose-binding hemagglutinin, HpaA.** The HpaA was found to be present in all strains and during all the culture conditions and at all time points tested, the HpaA concentration being 2- to 10-fold higher in strain CCUG 17874 than in strains Hel 305 (Fig. 2) and Hel 73 (not shown). The titers of strain CCUG 17874 varied up to eightfold between the different culture conditions. The maximal titers of all strains were seen after culturing with shaking for 72 h, the HpaA expression being significantly higher in strain CCUG 17874 than in Hel 305 ($P < 0.01$). In some experiments very low concentrations of HpaA were detected in strains Hel 73 and Hel 305, whereas the expression by strain CCUG 17874 was highly reproducible from one experiment to another, possibly depending on the laboratory adaptation of the stock strain. Phase-contrast microscopy showed that most organisms were coccoids after 72 to 96 h of culturing. The high HpaA titers at these time points indicated that the HpaA was preserved in the coccoid form of *H. pylori*, a finding that was confirmed by immunoelectron microscopy; MAb HP 30-1:1:6 showed immunogold staining of the surface on both coccoid and bacillary organisms (not shown). This finding may have implications for protective immunity against *H. pylori*, i.e., that specific immune responses against the HpaA may also be effective against chronic *H. pylori* infection.

**Quantification of the 26-kDa protein.** The highest titers of the 26-kDa protein were seen in the shaken broth cultures (Fig. 3). The 26-kDa protein content was comparable in the different strains when they were cultured in the same way. The kinetics of the 26-kDa protein expression in broth cultures was similar to that of the HpaA expression in all strains. The 26-kDa protein has been reported by O'Toole et al. (18) to be localized subcellularly, since no membrane-spanning segments are present, as judged by computer analysis of the amino acid sequences, and a signal sequence in the chromosomally located gene is absent, as confirmed by DNA sequence determination. However, we could detect this protein on the surfaces of intact, repeatedly washed bacteria by inhibition ELISA and immunoelectron microscopy with MAb HP26-18:1, which reacted with the bacterial surface and shedded membrane material (not shown). However, we cannot exclude the possibility that the membrane association of the 26-kDa protein can be explained by the suggested property of *H. pylori* of absorbing cytoplasmic proteins, e.g., urease and HspB, to the surface during spontaneous autolysis (23).

**Quantification of flagellins.** Also, the flagellins showed the highest concentrations in shaken broth cultures, the expression being significantly higher ($P < 0.05$) in strain Hel 305 than in CCUG 17874 (Fig. 4). The flagellin concentration was 3 to 10 times higher in strains Hel 305 and Hel 73 (not shown), respectively, than in CCUG 17874. The culture procedure seemed to be particularly important for the production of

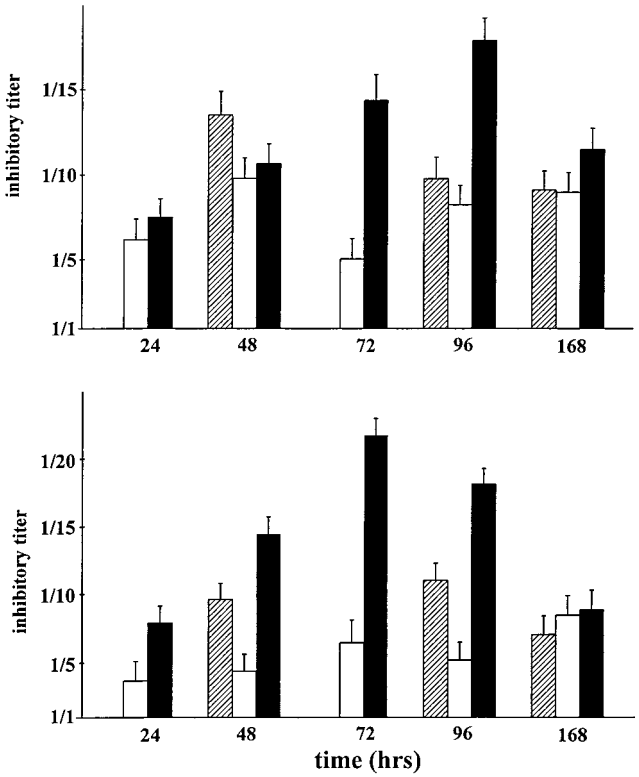


FIG. 3. Quantification of the 26-kDa protein as measured by inhibition ELISA in strain CCUG 17874 (top) and strain Hel 305 (bottom). Black bars, liquid shaken cultures; white bars, liquid static cultures; hatched bars, solid media culturing. Bacteria were tested at an initial concentration of $5 \times 10^9$ cells/ml, and geometric means of inhibitory titers in three experiments plus standard errors of the means are shown.

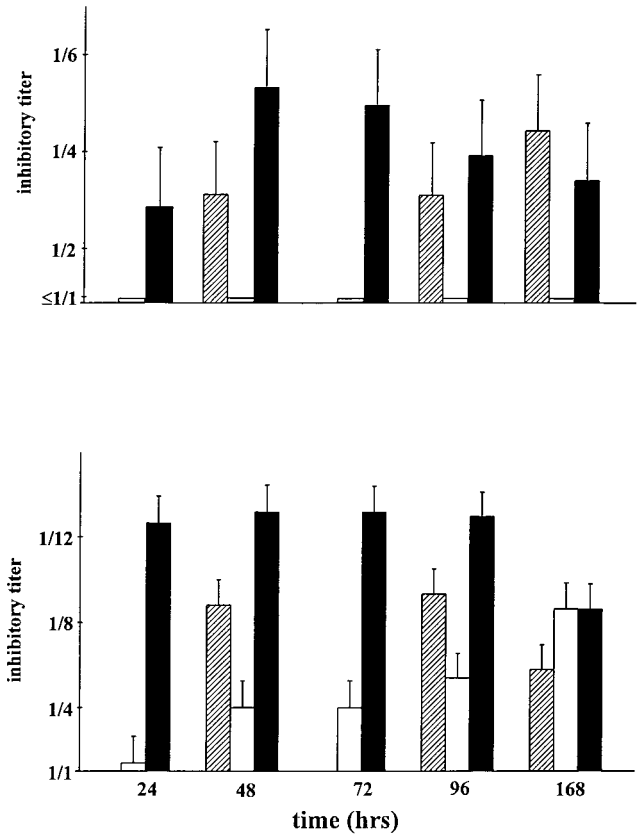


FIG. 4. Quantification of flagellin subunits as measured by inhibition ELISA in strain CCUG 17874 (top) and strain Hel 305 (bottom). Black bars, liquid shaken cultures; white bars, liquid static cultures; hatched bars, solid media culturing. Bacteria were tested at an initial concentration of $5 \times 10^9$ cells/ml, and geometric means of inhibitory titers in three experiments plus standard errors of the means are shown.

flagellins in strain CCUG 17874; i.e., no FlaA or FlaB could be detected in samples from static broth cultures as analyzed by inhibition ELISA, whereas both subunits seemed to be present in samples of plate-grown organisms (and the larger subunit was detected after culturing in shaken broth) as shown by immunoblotting (Fig. 1A). This result may be due to lower production of the flagellins during static broth culturing compared to production under the other culture conditions tested. This conclusion is supported by the weak reaction seen in the immunoblotting and by immunoelectron microscopy showing that the organisms from such cultures had flagella only occasionally (not shown).

**Total protein concentration during different culture conditions.** The total protein concentration in bacterial samples (optical density at $A_{600}$ of 1.5) taken at various time intervals varied between 200 to 300 and 400 to 700 μg/ml for static and shaken broth cultures, respectively (Table 1). Thus, the up-to-eightfold-higher antigen concentrations in shaken broth cultures (compared with those in static broth cultures) could be explained only partly by the approximately twofold-higher protein concentrations in shaken cultures. The higher specific antigen titers could be due to the higher growth rate in the agitated cultures, suggesting that the regulation of the studied antigens might be growth rate dependent as described for the production of K1 capsule antigen and the fimbrial adhesins K99 and F41 of *E. coli* (26). Furthermore, whereas the total protein concentrations in samples obtained at different time points from the same culture were rather constant throughout the study period, the antigen-specific inhibitory titers varied considerably during different phases of bacterial growth (Table 1).

TABLE 1. Comparison of total protein concentrations and HpaA titers in static and shaken cultures of CCUG 17874

| Time (h) | Shaken culture (150 rpm) | | Static culture | |
|---|---|---|---|---|
| | Protein concn (μg/ml)[a] | HpaA titer[b] | Protein concn (μg/ml) | HpaA titer |
| 24 | 445 ± 45 | 12.0 ± 1.2 | 220 ± 40 | 7.5 ± 1.2 |
| 72 | 605 ± 105 | 42.3 ± 1.4 | 295 ± 105 | 5.4 ± 1.1 |
| 168 | 520 ± 20 | 52.3 ± 1.1 | 165 ± 35 | 8.8 ± 1.1 |

[a] Total protein concentration as determined by Peterson's modification (22) of the method of Lowry et al. (13) after adjustment of the bacterial concentration to $A_{600}$ of 1.5. Data are given as arithmetic means ± standard errors of the means.

[b] Data given are geometric means of three experiments ± standard errors of the means.

In conclusion, we have shown that inhibition ELISA is a sensitive method for the quantification of different *H. pylori* antigens and that the specific antigen titers vary considerably among different *H. pylori* strains and during various culture conditions. Thus, the best bacterial growth and the largest amounts of the three conserved *H. pylori* antigens, i.e., the HpaA, the flagellin subunits, and the 26-kDa membrane-associated protein, were obtained in shaken liquid cultures of *H. pylori*. These differences in antigen levels among different *H. pylori* strains and after different culture procedures may be important, e.g., for antigen production and diagnostic purposes.

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