

Allelic Exchange Mutagenesis of *nixA* in *Helicobacter pylori* Results in Reduced Nickel Transport and Urease Activity

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Received 16 February 1996/Returned for modification 26 March 1996/Accepted 18 April 1996

***Helicobacter pylori*, an etiologic agent of gastritis and peptic ulceration in humans, synthesizes urease, a nickel metalloenzyme, as its most abundant protein. NixA, a high-affinity nickel transport protein, allows synthesis of catalytically active urease when coexpressed with *H. pylori* urease in an *Escherichia coli* host. To determine whether NixA is essential for the production of active urease in *H. pylori*, *nixA* was insertionally inactivated with a kanamycin resistance cassette (*aphA*) and this construct was electroporated into *H. pylori* ATCC 43504; allelic exchange mutants were selected on kanamycin-containing medium. The *nixA* mutation, confirmed by PCR, reduced urease activity by 42% ($140 \pm 70 \mu\text{mol of NH}_3/\text{min}/\text{mg}$ of protein in the mutant versus $240 \pm 100 \mu\text{mol of NH}_3/\text{min}/\text{mg}$ of protein in the parent ($P = 0.037$). Rates of nickel transport were dramatically reduced ($P = 0.0002$) in the *nixA* mutant ($9.3 \pm 3.7 \text{ pmol of Ni}^{2+}/\text{min}/10^8$ bacteria) of *H. pylori* as compared with the parent strain ($30.2 \pm 8.1 \text{ pmol of Ni}^{2+}/\text{min}/10^8$ bacteria). We conclude that NixA is an important mediator of nickel transport in *H. pylori*. That residual nickel transport and urease activity remain in the *nixA* mutant, however, provides evidence for the presence of a redundant transport system in this species.**

Helicobacter pylori, a gram-negative, microaerophilic, spiral-shaped bacterium, is the most frequently cited etiologic agent of human gastritis and peptic ulceration (11). This species, whose niche is highly restricted to the gastric mucosa of humans, has adopted a strategy of survival that includes synthesis of urease as its most abundant cellular protein (3, 15). This enzyme hydrolyzes urea, releasing ammonia, which allows colonization by this acid-sensitive organism at low gastric pH (4, 20, 22, 28).

The *H. pylori* nickel transport gene *nixA* allows synthesis of catalytically active urease in *Escherichia coli*, independent of growth conditions (21). Recombinant *H. pylori* urease expressed in *E. coli* is only weakly active unless urease structural subunits are overexpressed, exogenous NiCl_2 is added to the medium, and the host strain is grown in medium that does not chelate free Ni^{2+} (16). Since wild-type *H. pylori* does not require such conditions for very high levels of urease expression, we reasoned that an additional factor is required to accumulate the metal ion. NixA, a polypeptide of 34,317 Da with characteristics of an integral membrane protein, was shown to be that factor when expressed in *E. coli*.

A number of observations support the role of NixA as a mediator of Ni^{2+} transport including (i) homology of NixA with HoxN, an Ni^{2+} transport gene found in the hydrogenase gene cluster of *Alcaligenes eutrophus* (5, 6, 30), and other nickel transporters, HupN (9) and UreH (17); (ii) the increased sensitivity to NiCl_2 of clones expressing NixA; and (iii) the synthesis of catalytically active urease by clones expressing both urease genes and *nixA* (21). The transporter displays a very high affinity for Ni^{2+} ions ($K_T = 11.4 \text{ nm}$) and transports the divalent cation when present at low concentrations ($<1 \mu\text{M}$) (21). If serum levels, which range from 140 to 650 ng/liter (i.e., 2 to 11 nM) (27), approximate the concentration encountered

by *H. pylori* in the gastric mucosa, the affinity of the transporter would be well suited to these low physiological levels of metal ion. The purpose of this study was to determine whether NixA is functional in *H. pylori* and necessary for synthesis of active urease.

H. pylori ATCC 43504, obtained from the American Type Culture Collection (Rockville, Md.), and UMAB41, isolated from a gastric biopsy taken by endoscopy from a patient with complaints of abdominal pain and a history of peptic ulcer disease (22), were used in this study. Biopsies were cultured on brucella agar containing 10% sheep blood and Skirrow's selective supplement (26). Cultures were passaged on blood agar at 37°C in an anaerobic jar with palladium catalyst and activated Campypak (Becton Dickinson, Baltimore, Md.). Isolates were stored at -70°C in Trypticase soy broth (BBL) supplemented with 15% (vol/vol) glycerol.

For liquid culture, *H. pylori* strains were inoculated from fresh blood agar plates into 250 ml of Mueller-Hinton broth supplemented with 4% (vol/vol) fetal calf serum in a 500-ml flask. Cultures were incubated for 48 h with shaking (200 rpm) at 37°C in an anaerobic jar containing an activated Campypak.

E. coli DH5 α [*supE44* Δ *lacU169* ($\phi 80$ *lacZ* Δ *M15*) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used as the recipient for electroporation (18). *E. coli* SE5000 [*F*⁻ *araD193* Δ (*argF* *lac*)*U169* *rpsL150* *relA1* *flbB5301* *deoC1* *ptsF25* *rbsR* *recA56*] (10), transformed with plasmid pUEF202, which carries *nixA* (21), was used for transport assays.

Plasmid DNA was isolated by alkaline sodium dodecyl sulfate (SDS) extraction (1) and purified on plasmid purification columns (Qiagen tip-100) as directed by the manufacturer. Chromosomal DNA was isolated from bacterial cells lysed with SDS, treated with proteinase K, and extracted with phenol, chloroform, and ether by the method of Marmur (19).

To determine whether the NixA nickel transport protein is essential for the production of catalytically active urease in *H. pylori*, we constructed a mutation within *nixA* that would result in synthesis of an inactive truncated transport protein. All but the first nucleotide of the *nixA* open reading frame (bp 2 to 923) plus 144 bp of downstream sequence was subcloned from plasmid pUEF202 (21) on a 1,067-bp *MscI*-*HindIII* fragment

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into the *Sma*I-*Hind*III site of pBluescript SK- to make plasmid pUEF203. A 1.4-kb kanamycin resistance cassette (*aph*A) (provided by H. Kleanthous, Oravax, Boston, Mass.) was inserted into the *Ssp*I site within the *nixA* open reading frame of plasmid pUEF203 to make plasmid pUEF401 (Fig. 1A). The mutation is such that it is predicted that only 112 of the total 308 amino acids of NixA would be synthesized. Constructs that produced truncated NixA polypeptides of 100 and 114 amino acids (pUEF304 and pUEF303, respectively) (21) have previously been shown to carry out no appreciable nickel transport beyond the background level of the *E. coli* SE5000 host strain.

Construct pUEF401, which contains 337 bp of homologous DNA upstream of the mutation and 729 bp downstream, was electroporated into *H. pylori* ATCC 43504 and UMAB41 and plated onto blood agar containing kanamycin (50 μ g/ml). Kan^r colonies were isolated and passaged on the same medium. Isolated colonies were selected and screened for the presence of a double-crossover mutation in the chromosome of the Kan^r mutants (Fig. 1A).

PCR analysis, using chromosomal DNA from parent and mutant *H. pylori* strains as templates and primer pairs for *nixA* alone (NIXA1 and NIXA2) or *nixA* and *aphA* (NIXA1 and KAN) (Fig. 1A), demonstrate conclusively that the Kan^r cassette is inserted into the chromosomal copy of *nixA* (Fig. 1B). The primer pair NIXA1-NIXA2 amplified the expected 0.84-kb fragment in the parent strain but resulted in a 2.24-kb fragment in the mutant strain, consistent with insertion of the 1.4-kb *aphA* cassette. To demonstrate that the *aphA* cassette was indeed contiguous with the 5' end of *nixA* in the chromosome, the NIXA1-KAN primer pair was used for amplification. No amplification was seen in the parent strain since it lacks the *aphA* sequences. However, the predicted-size 1.24-kb fragment was amplified from the mutant, demonstrating that the chromosomal insertion mutation in the mutant strain is identical to the pUEF401 construct used for allelic exchange. Plasmid vector sequences were not detected by PCR of *H. pylori* genomic DNA (data not shown), verifying that the insertion mutation was the result of a double-crossover event. Interestingly, no single-crossover cointegrates were observed. An identical mutant was isolated for strain UMAB41 but was not further characterized.

The parental strain ATCC 43504 and its *nixA* Δ *aphA* derivative were tested for urease activity. We predicted that the mutant would lack urease activity because of lack of sufficient intracellular Ni²⁺. The urease activity of cells grown for 2.5 days in Mueller-Hinton broth plus 4% fetal calf serum was quantitated for *H. pylori* ATCC 43504 and its *nixA* mutant. Seven independent determinations revealed mean values (\pm standard deviations) of 240 \pm 100 μ mol of NH₃/min/mg of protein for the parent strain and 140 \pm 70 μ mol of NH₃/min/mg of protein for the *nixA* mutant (Fig. 2). While this 42% drop in urease activity was statistically significant ($P = 0.037$), more than half (58%) of the urease activity remained. This finding suggested that while *nixA* is required to synthesize fully active urease, other systems in *H. pylori* are apparently also capable of Ni²⁺ acquisition.

We had previously characterized NixA-mediated nickel transport in *E. coli* (21) but not in *H. pylori*. To compare the nickel transport of *H. pylori* with that of *E. coli* carrying *nixA* on plasmid pUEF202, uptake studies were done using cell suspensions of these strains (data not shown). *H. pylori* accumulated more than five times the amount of ⁶⁵Ni²⁺ that *E. coli*(pUEF202) did. We had previously shown that *E. coli*(pUEF202) transported nickel ions at nearly 10 times the rate of *E. coli* transformed with a vector control (21). We conclude

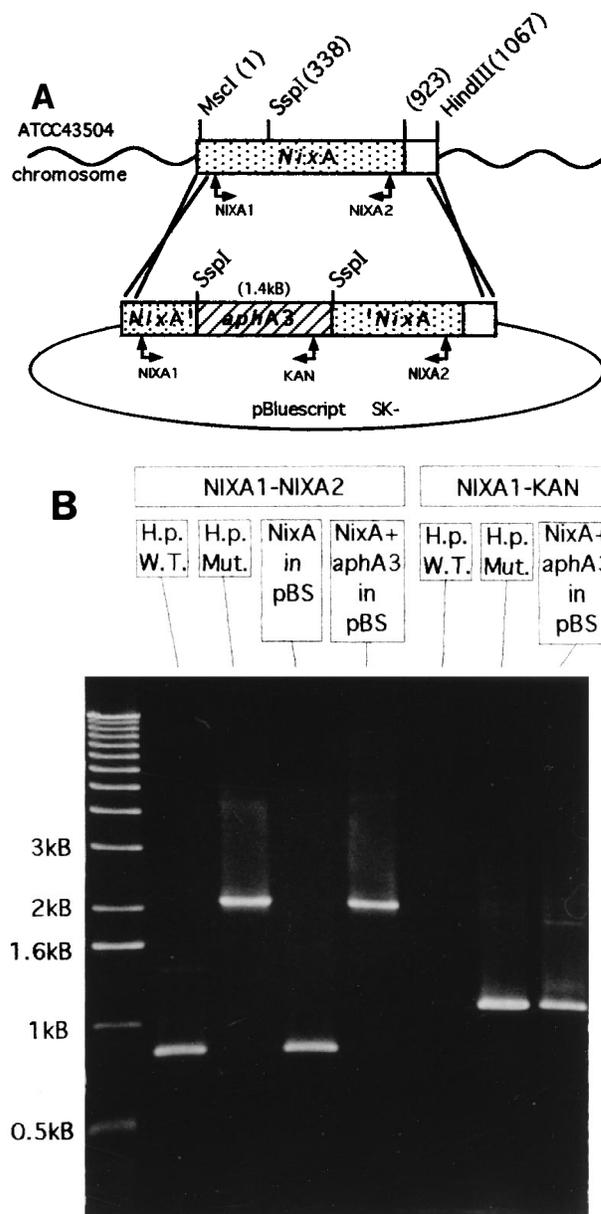


FIG. 1. Construction of *nixA* mutant. (A) Allelic exchange mutagenesis of *nixA*. A kanamycin resistance cassette (*aph*A) was inserted into the *Ssp*I site within the *nixA* open reading frame of plasmid pUEF203 to make plasmid pUEF401 (bottom). This construct was electroporated into *H. pylori* ATCC 43504 by using a Gene Pulser electroporator (Bio-Rad) in 15% glycerol-9% sucrose at 12.5 kV/cm, 25- μ F capacitance, and 200 Ω according to the method of Ferrero et al. (7). Kan^r colonies were isolated, passaged, and used for confirmation of a double-crossover mutation in the chromosome of the parental strains. Numbers in parentheses (top) refer to nucleotide numbers beginning with the first nucleotide of *nixA*. (B) Documentation of the *nixA* mutation. PCR products, amplified from chromosomal DNA of *H. pylori* ATCC 43504 and its *nixA* mutant, using primer pairs to *nixA* alone and *nixA-aphA*, were electrophoresed on 0.7% agarose gels. The presence of the pBluescript vector was not detected by PCR using the chromosomal DNA as template (data not shown). Far left lane: 1-kb ladder. Abbreviations: H.p., *H. pylori*; W.T., wild-type (parental) strain; Mut., mutant; pBS, pBluescript. Boxes above the lanes refer to the source of DNA used for PCR amplification. PCR was performed as previously described for *H. pylori* (8, 29) using primers designed from known *nixA* (NIXA1: 5'-GGCTTACATGCTA GGGGCAAAG-3'; NIXA2: 5'-CCTAGAAACGCTATTACAAATAA-3' [21], *aphA* (KAN: 5'-GAGCTGTATGCGGAGTGCATCAGG-3'), and vector pBluescript SK- (GenBank accession no. X52330) sequences. Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems automated DNA synthesizer model 380B.

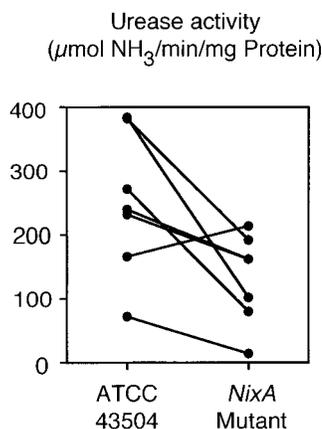


FIG. 2. Urease activities of *H. pylori* parental strain ATCC 43504 and its *nixA*-deficient isogenic mutant. Urease activities of bacterial cells grown for 2.5 days in Mueller-Hinton broth containing 4% fetal calf serum were quantitated for *H. pylori* ATCC 43504 and its *nixA* mutant. Seven independent determinations were made under identical growth conditions for the wild-type and mutant strains. Paired samples evaluated on the same day are connected by lines. Mean urease activities were 240 ± 100 μmol of NH_3 /min/mg of protein for the parent strain and 140 ± 70 μmol of NH_3 /min/mg of protein for the *nixA* mutant ($P = 0.037$ using the paired *t* test). For preparation of cell lysates, *H. pylori* was harvested from liquid culture by centrifugation ($10,000 \times g$, 10 min, 4°C), washed twice with 20 mM sodium phosphate (pH 6.8), suspended in 5 ml of 20 mM sodium phosphate (pH 6.8)–5 mM dithiothreitol–1 mM EDTA, and ruptured in a precooled French pressure cell at 20,000 lb/in^2 . Lysates were centrifuged ($27,000 \times g$, 30 min, 4°C), and supernatants were removed with a Pasteur pipette and used directly for assay. Protein concentration was determined by the BCA method according to the instructions of the manufacturer (Pierce), using bovine serum albumin as a standard. Rates of urea hydrolysis were measured by the spectrophotometric assay of Hamilton-Miller and Gargan (12) calibrated for the measurement of ammonia as described previously (23).

that *H. pylori* strains display significantly higher nickel uptake or binding than *E. coli* expressing NixA.

Since urease activity is completely dependent on nickel acquisition and because *nixA* is known to mediate nickel transport when expressed in *E. coli*, Ni^{2+} transport was measured using bacterial suspensions of *H. pylori* ATCC 43504 and the *nixA* mutant. Uptake of Ni^{2+} was determined by addition of carrier-free $^{63}\text{NiCl}_2$ to the suspension at a concentration of 50 nM followed by vacuum filtration for from 0.25 to 5 min (Fig. 3). The parent strain transported significantly more nickel ions than the isogenic *nixA* mutant. Over the first 2 min, values at each time point were 3.9- to 4.5-fold higher for the parent than for the mutant ($P < 0.001$). The mean initial rates of transport calculated from the first minute were 30.2 ± 8.1 pmol of Ni^{2+} /min/ 10^8 bacteria for the parent strain compared with 9.3 ± 3.7 pmol of Ni^{2+} /min/ 10^8 bacteria for the mutant ($P = 0.0002$); that is, the parent strain transported nickel ions an average of 3.2-fold faster than the *nixA* mutant. These data demonstrate that mutation of *nixA* results in a dramatic reduction in nickel transport in *H. pylori*, consistent with the drop in urease activity.

The detection of significant differences in rates of transport between the parent strain and the *nixA* mutant required that transport assays be concluded by washing the vacuum-filtered suspensions with buffer containing 1 mM NiCl_2 . In the absence of such treatment, nonspecific binding of isotope obscured differences in nickel ion accumulation (data not shown).

To demonstrate that uptake in *H. pylori* was a property of intact cells and not simply a function of binding to cell constituents, nickel transport was measured using cell suspensions of *H. pylori* ATCC 43504; half of the suspension was subjected

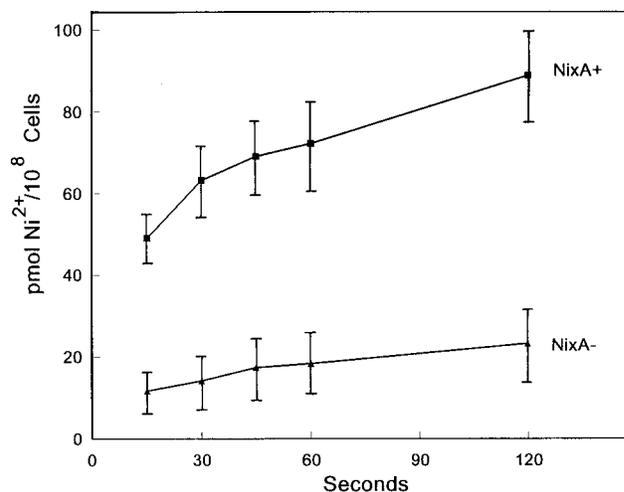


FIG. 3. Nickel transport by *H. pylori* ATCC 43504 and its *nixA* mutant. Nickel uptake was measured as described below for strain ATCC 43504 and its *nixA* mutant at a $^{63}\text{NiCl}_2$ concentration of 50 nM. Values represent the means of three experiments each conducted in duplicate. Values for parent and mutant strains were found to be statistically significantly different ($P \leq 0.001$) at every time point as compared by the *t* test. For transport, *H. pylori* strains were grown on brucella agar containing 10% sheep blood agar supplemented with vancomycin (10 $\mu\text{g}/\text{ml}$), polymyxin (2.5 IU/ml), and trimethoprim lactate (5 $\mu\text{g}/\text{ml}$). Cultures were incubated at 37°C in a microaerobic environment generated by an activated Campybak (Becton-Dickinson, Baltimore, Md.). *H. pylori* was cultured for 48 h prior to use in experiments, harvested from agar plates with a sterile cotton swab, suspended, and washed twice in 50 mM Tris-HCl (pH 7.0) at 23°C . Before each experiment, the cell morphologies of the mutant and wild-type strains were assessed by microscopy after crystal violet staining and found to be identical. After being washed, the cells were resuspended in 50 mM Tris-HCl buffer to an optical density at 600 nm of 0.5. Uptake was initiated by addition of $^{63}\text{NiCl}_2$ (specific activity, 6.35 mCi/ml; 0.46 mg of Ni/ml; Amersham) to a final concentration of 50 nM. Samples of 200 μl were taken at 0.25, 0.5, 0.75, 1, and 2 min by vacuum filtration (20 lb/in^2) through 0.45- μm -pore-size filters and washed with 10 ml of ice-cold 50 mM Tris-HCl (pH 7.0) containing 1 mM unlabeled NiCl_2 . Filters were added to scintillation vials along with 5 ml of Biosafe 11 biodegradable counting cocktail (RPI), and radioactivity was quantitated by liquid scintillation counting using a window of 5 to 650.

to lysis by passage through a French pressure cell at 20,000 lb/in^2 . Intact cells accumulated $^{63}\text{Ni}^{2+}$ (experiments conducted at 50 nM NiCl_2) over time, whereas lysed cells showed no accumulation (data not shown).

Urease is an unusual protein in that it requires nickel ions in the active site to successfully hydrolyze its substrate (2, 13, 14). *H. pylori*, however, lives in an environment in which free divalent cations such as Ni^{2+} are most likely bound by cellular components including any histidine- or cysteine-rich proteins, negatively charged glycoproteins, or nucleic acids (25). In a situation analogous to iron sequestration, the successful pathogen must possess a high-affinity system to scavenge Ni^{2+} ions from these human macromolecules. Although there is no evidence of a siderophore-like molecule, *H. pylori* is apparently able to scavenge these ions when the bacteria are cultured in complex medium such as Mueller-Hinton broth containing 4% fetal calf serum. Bacteria produce a highly active urease under these conditions, whereas *E. coli* harboring the cloned urease gene cluster, but not *nixA*, produces only the inactive apoenzyme.

While studies of cloned *H. pylori* urease genes expressed in *E. coli* in *trans* to *nixA* are clearly useful, it is also important to directly examine the phenotype in strains of *H. pylori* lacking this factor. If we were to rely solely on the observations made with *E. coli*, we would have certainly come to the conclusion that *nixA* is the only important element outside the urease gene

cluster that is necessary for activation of this enzyme. However, when the *nixA* mutant of *H. pylori* was constructed, we observed that while the mutant had significantly reduced urease activity, more than half of the activity remained (Fig. 2). This suggested that other nickel binding, nickel transport, or nickel processing factors were also required for full enzymatic activity in the wild-type organism. While *nixA* is necessary for the synthesis of fully active urease, other ion transport systems can apparently also be used by *H. pylori* for Ni²⁺ acquisition. As in *E. coli*, at least two systems can transport Ni²⁺ (24) although apparently not at rates sufficient to activate cloned *H. pylori* apourease. Surface proteins capable of binding Ni²⁺ may also be required as well as outer membrane proteins that selectively admit Ni²⁺ to the periplasmic space.

A more dramatic effect of a *nixA* mutation on urease activity was expected but not observed. This raises a number of questions. First, are there multiple copies of *nixA* present in the chromosome? Southern blots of chromosomal DNAs from several strains always reveal only single bands that hybridize with a *nixA* probe. In addition, PCR amplifications would have also revealed normal copies of *nixA* in the allelic exchange mutant. Second, does the growth medium (Mueller-Hinton broth plus 4% fetal calf serum) contain sufficient Ni²⁺ to overcome the effect of the *nixA* mutation? This is unlikely since *E. coli* containing a *nixA* clone and the urease gene cluster is urease positive while a similar plasmid combination that contains a mutation in *nixA* is not urease positive. Third, is *nixA* completely inactivated by the Kan^r cassette insertion? The *nixA* gene appears to be very sensitive to mutation. Every insertion or deletion (both in frame and out of frame) mutation of the *nixA* open reading frame examined thus far has resulted in inactivation, including a mutation located within two codons of the position described in this report (21). Since the chromosomal mutation was carefully verified by PCR and a reduction in both urease activity and nickel transport was observed, we are confident that the gene has been inactivated in the allelic exchange mutants. It would be desirable, however, to demonstrate in *trans* complementation of the mutation in *H. pylori* using a cloned *nixA* determinant. Unfortunately there has been no report of a shuttle vector capable of replicating in *H. pylori*, and thus complementation cannot be demonstrated because of this technical limitation.

The data reported in this study finally led us to conclude that like many bacterial pathogens, *H. pylori* possesses redundancy with respect to critical virulence determinants. Since the urease is apparently so crucial for colonization and virulence of *H. pylori*, backup systems for Ni²⁺ accumulation must exist that partially complement the *nixA* mutation.

This work was supported by Public Health Service grants AI23328 and AI25567 from the National Institutes of Health. P.B. was supported by Schweizerische Stiftung für med. biol. Stipendien.

We thank Harry Kleantous for generously providing the Kan^r cassette and Irene Corthesy-Theulaz for helpful suggestions.

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