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Targeting *Helicobacter pylori* urease activity and maturation: In-cell high-throughput approach for drug discovery

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ABSTRACT

Background: *Helicobacter pylori* is a bacterium strongly associated with gastric cancer. It thrives in the acidic environment of the gastric niche of large portions of the human population using a unique adaptive mechanism that involves the catalytic activity of the nickel-dependent enzyme urease. Targeting urease represents a key strategy for drug design and *H. pylori* eradication.

Method: Here, we describe a novel method to screen, directly in the cellular environment, urease inhibitors. A ureolytic *Escherichia coli* strain was engineered by cloning the entire urease operon in an expression plasmid and used to test in-cell urease inhibition with a high-throughput colorimetric assay. A two-plasmid system was further developed to evaluate the ability of small peptides to block the protein interactions that lead to urease maturation.

Results: The developed assay is a robust cellular model to test, directly in the cell environment, urease inhibitors. The efficacy of a co-expressed peptide to affect the interaction between UreF and UreD, two accessory proteins necessary for urease activation, was observed. This event involves a process that occurs through folding upon binding, pointing to the importance of intrinsically disordered hot spots in protein interfaces.

Conclusions: The developed system allows the concomitant screening of a large number of drug candidates that interfere with the urease activity both at the level of the enzyme catalysis and maturation.

General significance: As inhibition of urease has the potential of being a global antibacterial strategy for a large number of infections, this work paves the way for the development of new candidates for antibacterial drugs.

1. Introduction

Helicobacter pylori is a widespread Gram-negative bacterium infecting the stomach of millions of people every year, up to 50% of adults and reaching 80–90% in developing countries [1]. After colonization, untreated *H. pylori* infections persist, often asymptomatically, for the entire life-span of the host, because of the inability of the human immune response to efficiently counteract the bacterium. The infection causes chronic inflammation of the gastric mucosa, which can slowly progress to gastric adenocarcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma. Accordingly, in 1994 the WHO classified *H. pylori*, first and unique among all bacteria, as a class I carcinogen [2] and reconfirmed this classification in 2012 [3]. Several meta-analyses pointed out that eradication of *H. pylori* at the population level would have beneficial effects, such as reduction in gastric cancer incidence, peptic ulcer development, dyspepsia symptoms, and anemia occurrence [4]. In addition, *H. pylori* eradication causes complete regression in 60%–80% of already established MALT lymphomas [5]. Therefore, the Gastric Cancer Consensus Conference boldly recommended population-based screening and treatment for *H. pylori* to prevent gastric cancer [6].

Nonetheless, treatment efficacy remains a major concern. The standard first-line therapy to eradicate *H. pylori*, using a combination of three different antibiotics, fails in 20% of the cases because of antibiotic resistance, leading to an increasing number of infected individuals harboring resistant bacteria. Indeed, in 2017 WHO included *H. pylori* in the list of antibiotic-resistant bacteria for which antibiotic development is a global priority [7].

To survive inside the very acidic environment of the gastric niche, *H. pylori* has created a unique adaptive mechanism relying on the Ni(II)-dependent enzyme urease, which catalyzes the rapid hydrolysis of urea to produce ammonia and carbamate. The latter spontaneously

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hydrolyzes, generating an increase of pH that buffers the micro-environment surrounding the bacterium [8, 9]. Indeed, urease-deficient *H. pylori* strains cannot colonize the stomach niche of mice [10]. Similarly, mutants that can synthesize the apo-urease but are unable to incorporate Ni(II) into its active site are unable to colonize the gastric mucosa, thereby highlighting a link between Ni(II) activation of urease and host colonization [11]. Interestingly, standard therapy reaches significantly higher eradication rates if a nickel-free diet is maintained by infected patients, suggesting that the reduction of urease activity could increase the bacterial susceptibility to antibiotics [12]. Higher eukaryotes do not produce urease, nor other nickel enzymes, rendering urease activity an attractive target for the development of alternative and specific antibacterial strategies to overcome *H. pylori* gastric infection.

Urease proteins are generally heteropolymeric proteins with a quaternary structure $(\alpha\beta\gamma)_3$ [8]. In bacteria of the genus *Helicobacter* the trimer is of the type $(\alpha\beta)_3$, with the β subunits corresponding to the fused β and γ subunits normally found in other bacteria. The protein also presents a higher level of oligomerization, with an $[(\alpha\beta)_3]_4$ quaternary structure. The activity of urease relies on the presence of a binuclear active site containing two Ni(II) ions, bridged by the carboxylate group of a carbamylated lysine, essential to maintain the ions at the right distance for the catalysis, and by a hydroxide ion, the nucleophile of the hydrolysis reaction [8, 13]. This architecture, always present in the α subunit, is fully conserved in all known urease structures. Nickel incorporation into the active site of urease occurs post-translationally during enzyme maturation, and requires the assistance of the accessory proteins UreD, UreF, UreG and UreE, expressed from genes belonging to the urease operon [14]. UreE is a homodimeric protein responsible for Ni(II) delivery into the urease active site, while UreG is an intrinsically disordered GTPase that energizes the process of urease maturation [15]. In solution, UreE and UreG form a complex $UreE_2$-$UreG_2$, whose structure has been obtained with molecular modeling and protein docking [16], and subsequently confirmed by NMR [14]. The crystal structure of $UreD_2$-$UreF_2$-$UreG_2$ was reported [17], this larger complex being a multiprotein composite chaperone that pre-activates apo-urease before Ni(II) incorporation, a modification necessary for the enzyme to build up a correct active site.

In an attempt to provide a model system to test new antibacterial molecules that target *H. pylori*, we engineered a ureolytic *E. coli* strain to screen urease inhibitors by means of an in-cell high-throughput colorimetric assay. In addition, we set up a two-plasmid system to evaluate the ability of peptide sequences to block the protein-protein interactions (PPIs) among the accessory proteins that lead to enzyme maturation. This method was used successfully to screen the inhibition activity of a co-expressed peptide.

2. Experimental section

2.1. Gene cloning and site-directed mutagenesis

A scheme of the cloning strategy used in this work is presented in Fig. 1SI. A list of the plasmids used in this work, with a short description, and of the oligonucleotide sequences needed for the molecular biology setup is reported in Tables SI1 and SI2 respectively. Briefly, the *pGEM-ureOP* plasmid, carrying the *H. pylori* strain G27 urease operon (National Center for Biotechnology Information code NC_011333.1) [18], was used as PCR template for amplifying the *ureABIE* portion of the urease operon, containing the structural urease genes *ureA* and *ureB*, the urea membrane transporter gene *ureI* and the metallo-chaperone gene *ureE*, using ureOP_F and ureE_R. The purified PCR product was ligated (T4 DNA ligase) into the *pGEM-T* Easy vector according to the manufacturer instructions. The resulting construct (*pGEM-ureABIE*) was purified, analyzed by restriction analysis, and sequenced at the 5′ and 3′ ends to verify the correct insertion of the partial operon. Site directed mutagenesis was performed on *pGEM-ureOP* to create a urease operon containing a truncated version of *ureF* gene, lacking the C-terminal 21 residues (here called *pGEM-ureOP*ΔαF), by amplifying the entire plasmid using the mutagenic primers ureF_STOP and ureF_Blg_R. Using *pGEM- ureOP* as a template, *ureF* gene was amplified using ureF_Xho_F and ureF_Hind_R oligos. The gene was double-digested using *Xho*I and *Hin*dIII restriction enzymes and eventually cloned into the *pBAD/His-Myc* vector (ThermoFisher) digested with the same restriction enzymes. This construct was used as template for amplifying the *araC-pBAD-ureF* portion, containing the *ureF* gene downstream to the arabinose promoter, using the pBAD_XbaI_F and pBAD_BspHI_R oligonucleotides. Similarly, the *pXG-0* vector [19] was used as a template for a PCR reaction using HSC_Xba_F and HSC_BspH_R oligonucleotides, generating a DNA fragment containing the pSC101 origin of replication and the chloramphenicol resistance gene. The *pBAD-ureF* and *pXG-0* PCR products were double-digested using *Xba*I and *Bsp*HI and ligated to give the *pXG0-pBAD-ureF* construct, containing the *ureF* gene under the control of the arabinose promoter, and with the pSC101 origin of replication and the chloramphenicol acetyl transferase gene. This construct was then digested using *Xho*I and *Hin*dIII restriction enzymes and the gene coding for the 21-residues C-terminal α-helix of UreF (αF), obtained by annealing the αF_Xho_F and αF_Hind_R oligos was cloned in under the control of arabinose promoter.

Finally, the αF coding gene, deriving from the annealing of αF_Nco_F and αF_Bam_R oligos was cloned in frame with the GB1 gene in the *pETM-12* [20] previously digested with *Nco*I and *Bam*HI. Subsequently, the *GB1-αF* gene was amplified using the GB1αF-Xho_F and GB1αF-Hind_R, double digested with *Xho*I and *Hin*dIII and cloned into the *pXG0-pBAD* vector, to obtain *pXG0-pBAD*-GB1αF plasmid expressing GB1 protein fused with αF.

2.2. In-cell urease activity test

TOP10 cells harboring *pGEM-ureOP* plasmid or its derivatives, co-transformed, if needed, with *pXG0-pBAD-ureF*, *pXG0-pBAD-αF* or *pXG0-pBAD-GB1αF* were pre-cultured at 37 °C in 1 mL of lysogeny broth (LB) containing 50 μg/mL of carbenicillin (Cb), and, for the double transformant, 25 μg/mL of chloramphenicol (Cm). After 16 h, 200 μL were used to inoculate 2 mL of M9 medium (1 L contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1.25 g of $(NH_4)_2SO_4$, 0.246 g of $MgSO_4$, 4 g of glucose) containing the appropriate antibiotics and 10 μg/L of cresol red pH indicator. When OD600 reached 0.7–0.9, 100 μL of each culture were inoculated in 96-well plates with the appropriate concentration of urea, and with or without 0.5% arabinose or the appropriate inhibitor concentrations. Immediately before running the experiment, $NiSO_4$ was added to each culture. In each well, 50 μL of mineral oil were added to prevent medium evaporation during the culture. The color change of cresol red indicator was monitored over time spectrophotometrically in a multi-plate reader, measuring the absorption at 430 nm and at 580 nm.

3. Results

3.1. In-cell test of urease activity

The 6500 bp urease operon [8] (*ureOP*) coding for both the structural and accessory genes of the *Helicobacter pylori* enzyme (Fig. 1A) was cloned in an expression vector to yield *pGEM-ureOP* (Figs. 1B and 1SI) and used to express the holo-urease enzyme in recombinant BL21(DE3) *E. coli*, as previously described [18].

To evaluate the urease activity in the cytoplasm of growing bacteria, the same genetic construct was used to transform Top10 *E. coli* cells. The recombinant bacteria maintained urease expression and activity, driven by the vector-encoded *lac* promoter upstream of the *ure* operon. In addition, the *recA-* genotype of this strain ensured higher stability of the genetic constructs, preventing unwanted recombination, especially when two plasmids harboring similar sequences were co-transformed, a