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Spectroscopic characterization and fluorescence imaging of *Helicobacter pylori* endogenous porphyrins

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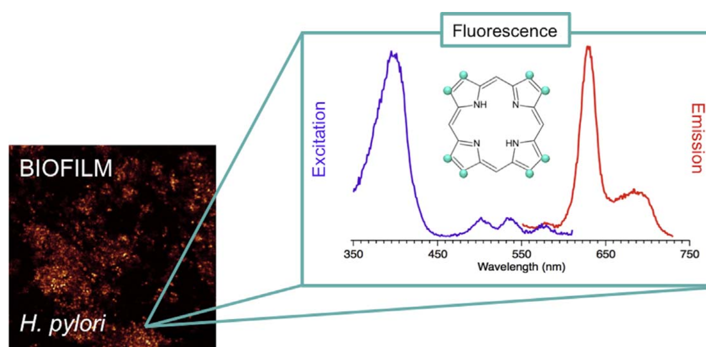
HIGHLIGHTS

- *Helicobacter pylori* (Hp) produces porphyrins acting as endogenous photosensitizers.
- PhotoDynamic Therapy can be a promising treatment for Hp eradication.
- Hp endogenous porphyrins have been spectroscopically characterized.
- The fluorescence properties of Hp biofilm have been studied for the first time.

GRAPHICAL ABSTRACT



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ABSTRACT

Conventional antimicrobial strategies have become increasingly ineffective due to the rapid emergence of antibiotic resistance among pathogenic bacteria. In order to overcome this problem, antimicrobial PhotoDynamic Therapy (PDT) is considered a promising alternative therapy. PDT has a broad spectrum of action and low mutagenic potential. It is particularly effective when microorganisms present endogenous photosensitizing pigments. *Helicobacter pylori* (Hp), a pathogen notoriously responsible of severe gastric infections (chronic gastritis, peptic ulcer, MALT lymphoma and gastric adenocarcinoma), produces and accumulates the photosensitizers protoporphyrin IX and coproporphyrin, thus it might be a suitable target of antimicrobial PDT. With the aim to design and develop an ingestible LED-based robotic pill for intragastric phototherapy, so that irradiation can be performed *in situ* without the use of invasive endoscopic light, photophysical studies on the Hp endogenous photosensitizers were carried out. These studies represent an important prerequisite in order to select the most effective irradiation conditions for Hp eradication. The photophysical characterization of Hp porphyrins, including their spectroscopic features in terms of absorption, steady-state and time-resolved fluorescence, was performed on bacterial extracts as well as within planktonic and biofilm growing Hp cells.

1. Introduction

Helicobacter pylori (Hp) is a microaerophilic, Gram-negative bacterium which colonizes the stomach of humans where it induces severe mucosal inflammation and local and systemic immune response. Thanks to the presence of critical factors for colonization and persistence of infection such as urease, flagella and adhesins, Hp is highly adapted to the gastric mucosa, with a unique array of features that

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permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response [1,2]. After reaching the gastric mucus layer and the epithelium, the bacterium delivers its virulence factors, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), the two major toxins that damage the gastric mucosa and cause disease. Chronic infections are most often associated with the formation of biofilms highly resistant to detergents, antiseptics and antibiotics. Bacterial biofilm is defined as a complex microbial ecosystem where bacterial cells, adhered to abiotic and biotic surfaces, are embedded in an extracellular polysaccharide matrix. It plays a crucial role in promoting the survival and the spread of pathogenic microorganisms in the host [3].

The association between chronic Hp infection and development of gastric cancer is well established. The International Agency for Research on Cancer has categorized Hp as a group I carcinogen [4]. Today, Hp is accepted as the causative agent of acute and chronic gastritis, and a major predisposing factor for peptic ulcer disease, gastric carcinoma, and gastric lymphoma. Hp infection occurs worldwide and the clinical treatment requires the use of several antimicrobials such as a combined intake of a proton pump inhibitor with amoxicillin and one of two antibiotics, clarithromycin or metronidazole (or tinidazole). The efficacy of antibiotic therapy is decreasing worldwide (< 70–80%) mainly due to increased antibiotic resistance rates, demanding new therapeutic strategies [5]. In the last years, a non-pharmacological approach has been considered for Hp eradication: Photo-Dynamic Therapy (PDT), a light-based antimicrobial treatment able to efficiently eradicate a wide range of bacteria, viruses, fungal and protozoan parasites. PDT concerns the use of harmless visible light combined with a light sensitive dye, a photosensitizer (PS), and with the oxygen present in and around cells. When light of the appropriate visible wavelength is applied, the non-toxic PS goes to an excited state that can react with oxygen, resulting in the formation of cytotoxic reactive oxygen species (ROS) and thus triggering a destructive action in biological systems, leading to cell death. PDT is a highly selective and useful treatment because cell death is spatially limited to regions where light of the appropriate wavelength is applied.

It has long been known that some bacteria accumulate porphyrins and are susceptible to photokilling. Indeed, PDT treatments of bacterial infections like acne, gastric infection by Hp and brain abscesses in selected animal models and in clinical trials were reported [6].

Hamblin et al. in 2005 showed that Hp produces and accumulates a mixture of protoporphyrin IX (PP IX) and coproporphyrin (CP) that can sensitize and kill the bacteria upon illumination by visible light, particularly blue light. This finding suggested a novel phototherapy approach to eliminate Hp infection in the human stomach [7].

The main absorption band of these porphyrins peaks at about 415 nm but an interesting longer wavelength band is present at about 630 nm [8]. The main limitation of PDT relies on the poor light penetration in the tissues: commonly violet-blue light is used for porphyrins sensitization, but tissues and blood vessels absorb this spectral range. Red light seems to be a promising alternative as it is considerably less attenuated by blood [9].

Phototherapy is generally administered by voluminous light sources through mirrors or fiber optics. The sources are mainly lasers or discharge lamps; more recently LED arrays have been considered. Light treatment for Hp infection was experimented in 2009 by Lembo et al. using an intra-gastric fiber optic system tethered with an external violet light source. Bacterial load, measured by biopsy just before and after the single treatment, showed the greatest reduction in the stomach antrum (> 97%), followed by the body (> 95%) and fundus (> 86%) [10]. This type of treatment is clearly not well accepted by patients who will unlikely tolerate a subsequent session.

In the framework of the project "CapsuLight - Design of an ingestible robotic pill based on LED sources for the treatment of gastrointestinal disorders" devoted to the realization of an ingestible LED-based robotic pill for intragastric PDT able to perform *in situ* irradiation without the use of invasive endoscopic devices, the effectiveness of Hp photokilling in models has to be determined. In order to select the best irradiation conditions for Hp eradication, it is crucial to spectroscopically characterize Hp endogenous porphyrins extracted from bacteria as well as on viable bacteria and their biofilms.

2. Materials and methods

2.1. Materials

Protoporphyrin IX (PP IX) and coproporphyrin I (CP I) were purchased from Sigma-Aldrich Co. and used as received. All solvents were analytical grade.

2.2. Bacterial strains culture conditions

A laboratory-adapted strain, Hp ATCC 43504, and a virulent strain (*cag*A + and *vac*A +), Hp ATCC 700824 (J99), purchased from LGC Standards S.r.l. (Milan, IT), were used in this study. Both strains were stored at − 80 °C in Brucella Broth (BB, Thermo Fisher Scientific Remel Products, Lenexa, KS) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) and 20% (v/v) glycerol.

From frozen stocks, bacteria were grown in BB supplemented with 10% (v/v) FBS and incubated at 37 °C for two days in a microaerophilic atmosphere (CampyGen Compact, Oxoid Hampshire, UK) with shaking at 170 rpm in the dark.

2.3. Extraction of porphyrins from Hp strains

Bacterial porphyrins were extracted from Hp cultures (100 ml) as described by Mancini and Imlay with minor modifications [11]. Briefly, Hp cells were harvested by centrifugation (7000 × *g* at 4 °C for 10 min), washed in 20 ml pre-chilled buffer (0.05 M Tris pH 8.2–2 mM EDTA) and suspended in 10 ml of the same buffer. Following centrifugation, an aliquot (1,5 ml) of a mixture of ethyl acetate and acetic acid (3:1, v/v) was added and bacterial cells were lysed by sonication on ice (6 cycles: 30 s, stop, 60 s). Cell debris was removed from the ethyl acetate-acetic acid mixture by centrifugation (7000 × *g* at 4 °C for 10 min), and the non-aqueous layer was washed twice with distilled water. Then, porphyrins in ethyl acetate were solubilized by adding 100 μl of HCl 3 M. After vigorous vortexing, this solution was centrifuged (7000 × *g* for 5 min) and then the bottom layer, containing porphyrins, was collected for spectroscopic analysis.

2.4. Biofilm formation assay and cell viability

Hp biofilm was grown according to a protocol by Yang et al. [12], applying minor modifications. Hp strains were cultured on Brucella agar plates supplemented with 7% laked horse blood (Oxoid, Hampshire, UK) and incubated at 37 °C for three days in microaerophilic conditions. After incubation, bacteria were harvested with a sterile cotton swab and suspended in brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) supplemented with 0.5% β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) and 0.4% yeast extract (Oxoid, Hampshire, UK). This suspension was adjusted to a concentration of approximately 5×10^6 CFU/ml. An aliquot (2 ml) of this suspension was inoculated into each well of the 12-well culture plates (BD Falcon, Franklin Lakes, NJ), containing sterilized round glass coverslips placed vertically to allow adherence of Hp cells at the air-liquid interface. As negative control, BHI broth without Hp was used. Plates were incubated at 37 °C under microaerophilic conditions for 4 and 7 days without shaking. After incubation, coverslips were washed twice with phosphate-buffered saline (PBS, Gibco, Life Technologies, Carlsbad, CA) to remove non-adhered bacterial cells and imaged immediately. Hp biofilms were examined also for cell viability by confocal microscopy using the Live/

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