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Compositional analysis of endogenous porphyrins from *Helicobacter pylori*

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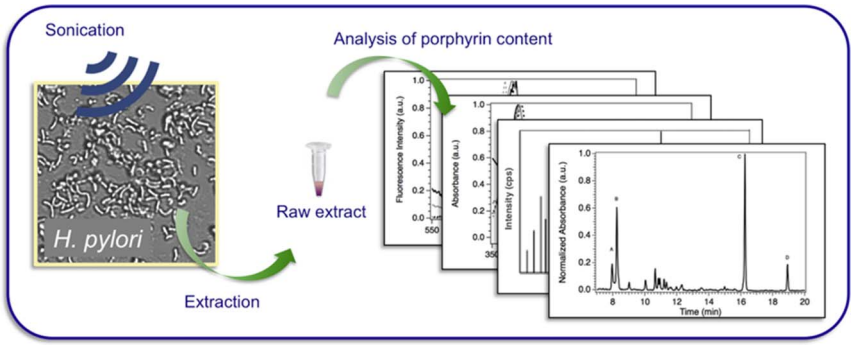
HIGHLIGHTS

- Bacteria producing photosensitizers are optimal targets for photodynamic therapy.
- *Helicobacter pylori* (Hp) produces a mixture of endogenous porphyrins.
- Porphyrins were successfully extracted from Hp.
- The main porphyrinic components of the extract were identified.
- Their relative contribution to the global red fluorescence was examined.

GRAPHICAL ABSTRACT



ABSTRACT

Bacteria able to accumulate porphyrins can be inactivated by visible light irradiation thanks to the photosensitizing properties of this class of aromatic pigments (photodynamic therapy, PDT). Since the bacterial resistance to antibiotic is growing, PDT is becoming a valid alternative. In this context, the pathogen *Helicobacter pylori* (Hp) is a suitable target for PDT since it spontaneously produces and accumulates porphyrins. It is then important to understand the spectroscopic behavior of these endogenous species to exploit them as photosensitizers, thus improving the results given by the application of PDT in the treatment of Hp infections. In this work we extracted porphyrins from both a laboratory-adapted and a virulent strain of Hp, and we performed spectroscopic and chromatographic experiments to collect information about the composition and the spectrophotometric features of the extracts. The main components of the porphyrin mixtures were identified and their relative contribution to the global red fluorescence was examined.

1. Introduction

Porphyrins are naturally occurring pigments involved in several essential biological functions like oxygen transport as well as photosynthesis. The chemical structure of porphyrinic molecules is a ring based on a substituted tetrapyrrolic aromatic macrocycle with an extensive system of delocalized πelectrons, responsible for its characteristic absorption and red fluorescence [1–3]. Porphyrins have been extensively studied as they can take part in light-induced reactions within living organisms acting as photosensitizers. Upon light absorption they can reach an excited state and then react with environmental oxygen, thus generating reactive oxygen species (ROS) able to induce irreversible photodamage to cells. This effect is involved in some human pathologies such as porphyries, a group of metabolic disorders characterized by the abnormal accumulation of porphyrins within the body. In light of the rising antibiotic resistance

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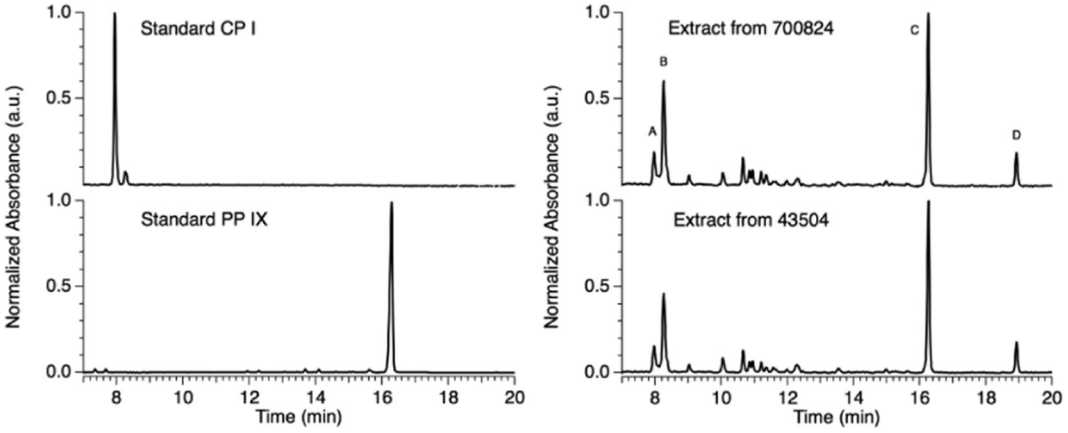

Fig. 1. Chromatographic analyses of standard CP I (top left), standard PP IX (bottom left), extract from strain 700824 (top right) and extract from strain 43504 (bottom right). Detection wavelength: 400 nm.

shown by several bacterial pathogens, efforts are being devoted to exploit the photosensitizing effect of some molecules in the development of photodynamic therapy (PDT). In detail, the therapy consists in the application of visible light to a selected district enriched with a photosensitizer molecule (endogenous or previously administered or induced); the photosensitizer reacts with triplet molecular oxygen to generate ROS, thus causing photokilling. PDT has successfully been employed against cancer, atherosclerosis and skin diseases (psoriasis, fungal infections, acne, sun damage, keratoses) [4]. Furthermore, PDT alone or in combination with antibiotics can also be used to treat bacterial infections [5,6] even when bacteria are embedded in biofilms [7], and this approach is particularly convenient when the target bacteria are able to spontaneously produce and accumulate porphyrins. Many oral bacteria responsible for periodontal diseases such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* show red fluorescence and both *A. actinomycetemcomitans* and *P. gingivalis* can be photokilled upon blue light illumination, suggesting that endogenously produced porphyrins are involved [8,9]. Several studies have also investigated the porphyrin content of the bacterium *Propionibacterium acnes*, the etiological agent of acne [10,11]. Among porphyrin-producing bacteria, it is worth citing *Helicobacter pylori* (Hp), a well-known Gram-negative, microaerophilic bacterium that lives in the stomach of half of the human population [12]. In most cases infected patients are asymptomatic or present a minimal gastric inflammation; however, in a significant percentage of patients Hp is responsible for serious manifestations such as sickness, acid reflux, gastric pain and burning. These disturbs can evolve towards gastritis, gastric or duodenal ulcers, or even provoke more severe gastric diseases such as chronic inflammation, lymphoma and other forms of cancer [13,14]. Infections by Hp have been treated so far by conventional triple or sequential antibiotic therapy; however, this approach is turning ineffective due to increasing antibiotic resistance in this pathogen [15]. It is known that Hp spontaneously produces porphyrins, making it a suitable target for PDT. In order to deepen our knowledge about the photokilling potential of porphyrins for Hp eradication, in a previous work we have characterized the spectroscopic features of Hp endogenous porphyrins in terms of absorption, steady-state and time-resolved fluorescence, both on bacterial extracts and within planktonic and biofilm growing Hp cells [16]. In this study the compositional analysis of Hp endogenous porphyrins has been performed by means of HPLC-MS combined with fluorescence techniques, with the aim of giving an estimation of the relative contribution of the main porphyrin species present in two different bacterial strains.

Table 1
Retention time (t), mass/charge ratio (m/z), fluorescence quantum yield (Φ_f), quantum yield error (Err), maximum absorption and emission wavelengths of the analyzed samples. Retention times are to be intended as intervals of ± 0.2 min. Main peaks are highlighted in bold.

Sample	t (min)	m/z [M + H]$^+$	Φ_f[a]	Err	Absorption λ_{max} (nm)	Emission λ_{max} (nm)
PP IX	16.2	563.0	0.016	± 0.0003	**410**, 556, 601[a] **401**, 504, 538, 573, 628[b]	**606**, 664[a] **633**, 699[b]
CP I	7.9	655.1	0.017	± 0.0006	**403**, 548, 592[a] **395**, 498, 531, 567, 619[b]	**595**, 616[c], 654[a] **622**, 686[b]
43504	7.9, 8.3 16.2	655.1 563.0	–	–	**405**, 552, 597[a]	**605**, 657[a] **632**, 697[b]
700824	7.9, 8.3 16.2	655.1 563.0	–	–	**404**, 551, 594[a]	**603**, 654[a] **628**, 688[b]

[a] In HCl.
[b] In MeOH.
[c] Shoulder.

2. Materials and methods

2.1. Materials

Protoporphyrin IX (PP IX), coproporphyrin I (CP I) and coumarin 153 (C153) were purchased from Sigma-Aldrich Co. and used as received. All solvents were analytical grade unless otherwise specified.

2.2. Bacterial strains and 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 extraction was carried out according to a