# Acquisition of iron and production of siderophores in *Helicobacter pylori* (b)

**Résumé**

*Helicobacter pylori* est un facteur étiologique important de la gastrite chronique, de l'ulcère ainsi que du cancer gastrique. L'acquisition du fer chez H.pylori joue un rôle important dans la virulence et la pathogénicité de H.pylori. Dans notre étude, les souches de H.pylori ont été cultivées dans un milieu carencé en fer en utilisant le chélateur de fer 2-2'dipyridyl pour induire la synthèse possible des siderophores et leurs récepteurs membranaires spécifiques. La production des siderophores a été mise en évidence par les tests chimiques. Toutes les souches de H.pylori testées sont capables de produire les siderophores de type hydroxamates, révélés par le test de Csaky. Parallèlement, sous ces conditions de stress ferrique, le profil électrophorétique des souches de H.pylori a détecté la présence d'une protéine de 74 KDa chez toutes les souches de H.pylori étudiées comme étant un récepteur membranaire spécifique, exprimé uniquement quand le milieu est carencé en fer. Par ailleurs, les expériences des bioessais (feed-crossing) ont montré l'interchangeabilité intersouches des siderophores de H.pylori d'une part, et l'inaptitude de ces souches d'utiliser des siderophores exogènes d'autre part.

**Mots clés** : Helicobacter pylori, siderophore, acquisition du fer, lactoferrine, hème.

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**Abstract**

*Helicobacter pylori* is know to be an important etiological agent of chronic gastritis and peptic ulcer in human and also associated with gastric cancer. Iron acquisition plays an important role in bacterial virulence. In this study, H.pylori was cultured under iron restricted medium by using the iron chelating compound, 2.2'- dipyridyl to induce synthesis of possible siderophores and iron-regulated outer membrane proteins. Siderophores production was detected by chemical assays and all H.pylori strains tested were able to produce hydroxamate type siderophores, as revealed by Csaky test, for growth in low iron medium. Parallely, under these conditions, electrophoretic analysis by S.D.S. page detected the presence of protein with molecular weight of 74 KDa in all strains of H.pylori examined. This appeared to be iron-repressible outer membrane proteins , and might be specific receptor expressed only when the medium is deprived of iron. Also, the experiments of bioassays (feed Crossing) showed that there was an interchangeability of siderophores between H.pylori strains tested, but these strains were unable to use exogenic siderophores.

**Key words**:  Helicobacter pylori, siderophore, iron uptake, lactoferrin, Haem

**ملخص**

*H.pylori* .

. *H.pylori*

2.2'–dipyridyl .

. *H.pylori*

Csaky .

(S.D.S. page ) 74 KDa *H.pylori*

bioessai .

*H.pylori*

.

: *Helicobacter pylori*.

*Helicobacter pylori* (*H.pylori*) is one of the most common pathogen in the world [1], and is now one of the best studied bacteria regarding various aspects of genetics, physiology and virulence factors [2]. *H.pylori* is a fastidious Gram negative, spiral shaped bacterium that colonizes the human gastric and duodenal mucosa [3,4,5]. *H.pylori* causes active chronic gastriris, plays an etiologic role in the development of peptic ulcer disease, and is considered a risk factor in the development of gastric adenocarcinoma and lymphoid tissue lymphoma [6,7,8,9].

As for all bacteria, iron is an essential nutrient for the growth and multiplication of *H. pylori* and is an essential cofactor of various metabolic and enzymatic processes [10,11,12,13], but the availability of iron for microbial assimilation in human is limited by the high affinity iron binding proteins such as transferrin in serum and lactoferrin on mucosal surface, makes this element difficult to obtain [12,14,15,16,17,18].The ability of a microbial pathogen to efficiently obtain iron from the host environment is critical to its ability to cause disease and considered as important determinants of pathogenicity [14,19,20] Therefore, for their survival, pathogenic bacteria have evolved highly efficient iron acquisition systems [16,18,21].

The most common method of acquisition is the secretion of low molecular weight compounds, high affinity iron chelators called siderophores, which are able to remove ferric iron ($Fe+3$) from the environment and then transported back into the cell by means of iron regulated receptor proteins [13,22,23,24,25]. Siderophores production is only one of several strategies that bacteria use to acquire iron from humans, which include the direct uptake of iron from host proteins such as haeme, transferrin or lactoferrin via specific uptake systems [23,26,27]. Few reports have appeared in the literature concerning the iron uptake system of *H. pylori* [10].

Therefore, the mechanism by which *Helicobacter pylori* acquires iron in the stomach remains unclear [23,28]. We had previously demonstrated that heme or human lactoferrin (HLf) supported full growth of the bacteria in media lacking other iron sources [10,13,20,29]. Since lactoferrin (HLf) has been found in significant amounts in human stomach, which can bind iron with high affinity even at low pH values, thus decreasing the availability of iron to *H.pylori* [10,23,30]. the iron uptake of *H. pylori* via a specific HLf receptor may play a major role in the virulence of *H. pylori* infection [10].This mechanism is common to several pathogens such as the genus *Neisseria, Haemophilus influenzae* [10].

The aim of this study was to examine the potential of *H.pylori* to produce siderophores and iron-regulated outer membrane proteins in laboratory culture media under iron stress conditions.

## MATERIAL AND METHODS

### Isolation and identification of *h.pylori*

Strains of *H.pylori* were isolated from antral mucosal biopsies of patients with chronic gastritis or duodenal ulcer in the Gastroenterology service military hospital Oran Algeria. Strains were cultured on blood agar plates (10% vol/vol human blood) and incubated under microaerophilic conditions at 37°C for 5 days. The strains were identified on the basis of the colony morphology, Gram negatif staining and positive reaction in biochemical tests (catalase, urease and oxidase) [31,32,33], All strains were stored for long term maintenance at – 70°C in brain heart infusion (BHI) broth containing 10% (vol/vol) glycerol [20]. 4 strains belonging to *H.pylori* (*H.pylori*1, *H.pylori*2, *H.pylori*3, *and H.pylori*4) were selected to study siderophore production. *H.pylori* BM 46 (*H.pylori* 5) was obtained from National Collection of cultures type of the experimental institute of medicine of Chieti. Italy, and used as standard control strain.

### Media and growth condition

*H.pylori* strains were precultured for 48h on blood agar plates at 37°C in a microaerophilic environment. The bacterial suspensions were prepared in nutrient broth plus 0.2% (w/v) bovine serum albumine (BSA), and adjusted to a McFarland standard No 5 (approximately $5 \times 10^9$ CFU.ml$^{-1}$). Cultures were incubated in a microaerophilic environment on a rototary orbitol incubator 200 rpm for 72 h at 37°C [23].

### Effect of 2.2'- dipyridyl on the growth of *H.pylori*

To remove traces of iron, all glassware were *j*soaked overnight in 0.5% EDTA and rinsed extensively with distilled deionized water [20]. Iron chelated media were prepared using an standard chelator 2.2'-dipyridyl supplemented to the liquid medium (BN+SAB) with different concentrations of the following : (0, 0.02, 0.05, 0.1, 0.15, 0.20 and 0.30 mg/ml). These media were inoculated in 2 days blood agar cultures of *H.pylori*. Cultures (25 ml in 1OO ml conical flasks) were shaken at 2OO rpm under microaerobic conditions in an orbital incubator at 37°C [23]. Bacterial growth was estimated turbidimetrically at 650 nm on a spectophotomter type « Spectronic » with uninoculated broth serving as a black.

### Detection of siderophore

To determine wether strains of *H.pylori* possessed siderophore-mediated iron acquisition systems, a simple screening chemical assay for siderophore production was performed. 5 strains of *H.pylori* were grown in iron limited broth medium and supernatants were collected. Csaky test was used to detect the presence of hydroxamate type siderophore [34]. Arnow test was used for detection of catechols type siderophore [35].

## Bioassays (siderophore utilization assay)

An assay similar of that described by Miles and Khimji (36) was used. In this assay, the iron chelator EDDA was added to the agar media to bind the iron and make it less accessible for growth bacterial. The medium was remitted, inoculated with $10^3$ bacteria per ml, and poured into sterile Petri dishes. Volume of 10 ul of the siderophore solution was placed on sterile disk on the surface of the agar. Plates were examined after 48h of incubation at 37°C for zones of growth around the disks.

## SDS page

Outer membrane proteins of *H.pylori* strains were prepared using a modification of the method of Carlone *et al*. [37] by Illingworth *et al*. [23]. Proteins were separated by S.D.S. page as described by Laemmli [38]. Apparent molecular weights were determined using standard proteins as reference markers. After electrophoresis, the gels were stained by using the silver staining method [39].

## RESULTS

### Effect of 2.2'-dipyridyl on the growth of *H.pylori*

Strains of *H.pylori* were tested for their ability to grown under iron restrictive conditions. All strains prove a sensitivity to chelating the 2.2'- dipyridyl , expressed by a reduction of the bacterial growth until total inhibition (fig.1). The growth decreases considerably with the concentration 0.15 mg/ml at the strains *H.pylori*1, *H.pylori2, H.pylori*3, *H.pylori4* and *H.pylori5*. The iron chelating agent was inhibitory at concentration 0.2 mg/ml and above in all strains of *H.pylori* tested. Growth of *H.pylori* strains in low iron medium suggested that *H.pylori* possess high affinity uptake systems by producing siderophore to solubilise and transport iron into the cell to response to iron limitation.
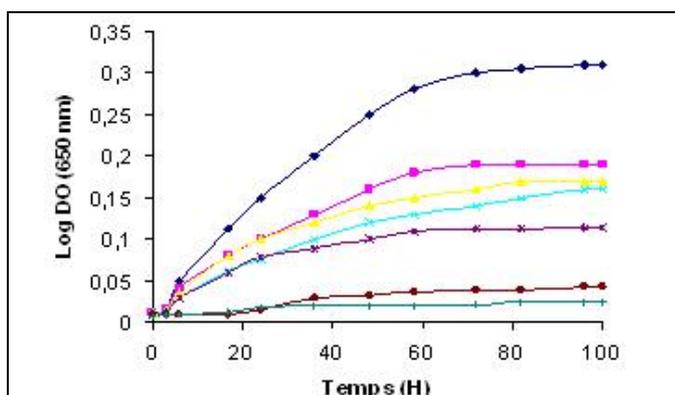


**Figure 1:** Kinetics of growth of *H. pylori* in medium (BN+ SAB) container different concentrations of 2.2'- dipyridyl expressed in mg/ml (0 (♦),0.02 (■),0.05 (▲),0.1(×),0.15 (✳), 0.2 (●) et 0.3 (I) ).

### Kinetics of growth of Hp1 (A) and Hp2 (b) in

### Detection of siderophore

To determine the type of siderophore produced by *H. pylori*, all strains were tested by Csaky test for hydroxamate and the Arnow test for the catechols. Culture supernatants were found to contain a compound with reacted with iron to give a red color, and a test for hydroxamate group, the Csaky reaction, gave a positive result indicating the presence of hydroxamic acids. Supernatants fluids from cultures of all strains of *H. pylori* gave consistently negative results when assayed from catechol-type siderophores by the method of Arnow (tab.1). Theses results concluded that all strains of *H.pylori* tested produced hydroxamate type siderophore.

**Table 1:** chemical detection of siderophores in *H. pylori strains*

| Strains | Hydroxamates | Catecholates |
|---|---|---|
| *H. pylori 1* | + | - |
| *H. pylori 2* | + | - |
| *H. pylori 3* | + | - |
| *H. pylori 4* | + | - |
| *H. pylori 5* | + | - |

**+:** Production of siderophores ; **-:** No Production of siderophores

### Bioassays

Siderophore utilization was detected by a cross-feending method (bioassays). Media containing EDDA to bind iron inhibited the growth from inocula of all bacterial strains tested of *H.pylori*. Only usable siderophore relived this inhibition. The results obtained after 48 h of incubation showed that growth of *H.pylori* strains in the EDDA medium was stimulated by its own siderophore, and siderophores secreted by other *H.pylori* strains tested (tab.2).

**Table 2:** Interchangeability of the siderophores between the different strains of *H.pylori* tested

| Strains | siderophores of *H. pylori* | | | | |
|---|---|---|---|---|---|
| | HP1 | HP2 | HP3 | HP4 | HP5 |
| HP1 | + | + | + | + | + |
| HP2 | + | + | + | + | + |
| HP3 | + | + | + | + | + |
| HP4 | + | + | + | + | + |
| HP5 | + | + | + | + | + |

Growth zones were observed around disks containing this hydroxamate type siderophore. Also, theses results showed that some bacteria such as *E.coli*, *Salmonella spp* and *Klebsiella spp*. were stimulated by siderophore secreted by *H.pylori* strains (tab.3).

However, all strains of *H.pylori* tested were apparently unable to utilize the supernatants the bacteria test for iron

transport explained by zones of inhibition of *H.pylori* strains around disks containing the supernatants (tab.4).

**Table 3:** Interchangeability of the siderophores of *H.pylori* with other bacterial species

| Bacteria tested | Siderophores of *H.pylori* | | | | |
|---|---|---|---|---|---|
| | HP1 | HP2 | HP3 | HP4 | HP5 |
| *E.coli* | + | + | + | + | + |
| *Ps. aeruginosa* | - | - | - | - | - |
| *Bifidobacterium spp.* | - | - | - | - | - |
| *Salmonella spp.* | + | + | + | + | + |
| *Shigella spp.* | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - |
| *Proteus vulgaris* | - | - | + | - | - |
| *Klebsiella spp.* | + | + | + | + | + |
| *S.aureus* | - | - | - | - | - |
| *Bacillus cereus* | - | - | - | - | - |

**Table 4 :** Interchangeability between the supernatants of bacteria tests and the strains of *H.pylori*

| Bacteria tested | Strains of *H.pylori* | | | | |
|---|---|---|---|---|---|
| | HP1 | HP2 | HP3 | HP4 | HP5 |
| *E.coli* | - | - | - | - | - |
| *Ps. aeruginosa* | - | - | - | - | - |
| *Bifidobacterium spp.* | - | - | - | - | - |
| *Salmonella spp.* | - | - | - | - | - |
| *Shigella spp.* | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - |
| *Proteus vulgaris* | - | - | - | - | - |
| *Klebsiella spp.* | - | - | - | - | - |
| *S.aureus* | - | - | - | - | - |
| *Bacillus cereus* | - | - | - | - | - |

### Electrophoresis

The outer membrane proteins profiles of strains of H.pylori (the reference strain and medical strains) grown under high and low iron conditions were examined. All strains gave a typical and identical response to iron limitation by 2.2'-dipyridyl, expressing a polypeptide observed in outer membrane preparation with apparent molecular of 74 KDa (fig.2). This protein was not produced under iron sufficient condition and the presence of the iron-chelating compound in the medium induced their production to become major constituents of the outer membrane preparation**.**
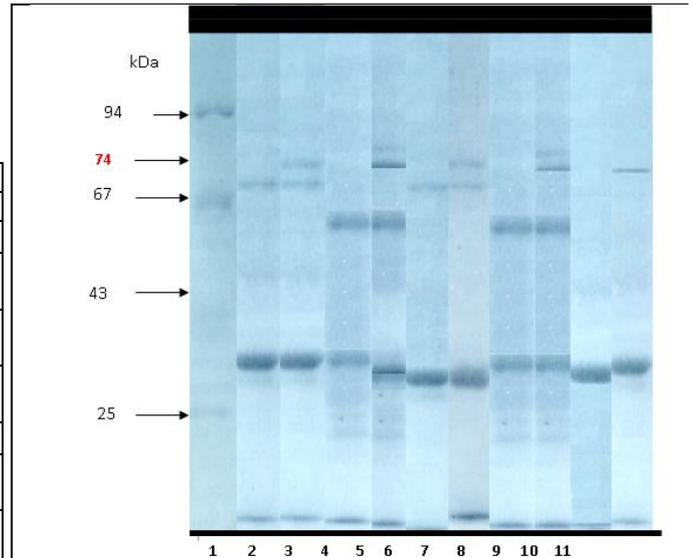


**Figure 2**: Electrophoretic profiles of *H.pylori* cultivated in presence and absence of 2.2'-dipyridyl. 1: marker protein (Sigma); 2: Hp1 (control); 3: Hp1 (test); 4: Hp2 (control); 5: Hp2 (test); 6: Hp3 (control); 7: Hp3 (test); 8: Hp4 (control); 9: Hp4 (test); 10: Hp5 (control); 11: Hp5 (test).

### DISCUSSION

Under iron restricted conditions, growing bacteria usually express iron uptake systems which are comprised of iron chelating compounds (siderophores) and outer membrane proteins that act as receptors for the ferric siderophores [16,24,25).These metabolites were detected by different assays [34,35,40].

In the present study, we attempt to detect the siderophore production in *H.pylori* strains by chemical assays (Csaky and Arnow tests). Our ability to limit iron was therefore to the addition of the chelators 2.2'-dipyridyl. However, we have shown that all strains of *H.pylori* grown under iron limiting conditions have the potential to express siderophore *in vitro*. *H.pylori* strains produced one type of siderophore which was detected by the Csaky test for hydroxamates. Theses results correlated with the findings of Illingworth *et al*. [23] who had evidence that all 23 strains of the *H.pylori* strains tested and cultured under iron limited conditions produced extracellular siderophore on the modified universal detection medium in response to low iron availability by chrome azurol S Assays. *H.pylori* strains were usually sensitive to 2.2'- dipyridyl, with inhibition of growth at concentrations 0.2 mg/ml and above.

The work undertaken by Illingworth *et al*. [23] bring back the inhibiting effect of the 2.2 dipyridyl on the growth of *H.pylori* strains tested at concentration above 25 ug/ml. Same investigators showed that the addition of the iron-chelating agent deferoxamine mesylate inhibited the growth of all 10 strains *H.pylori* tested at low concentrations (10 ug/ml) . Some bacterial species which fail to produce their own siderophores can use compounds secreted by other microbes [20].

Therefore, interstrain utilization of siderophore among *H.pylori* strains was showed the identical nature of the siderophores secreted by *H.pylori* facilitating their interchangeability. However, all strains of *H.pylori* studied were unable to use siderophores secreted by other microbes such as *E.coli*, *Salmonella spp*, *Klebsiella* spp.for growth in low iron medium. The similar results shown that the presence of either enterochelin or pyochelin in the low iron medium did not affect the growth of *H.pylori* [20]. Indeed, *H.pylori* sets up an ecologically isolated microbial group, whose exchanges with other micro-organisms miss practically [41, 42]. This is in connection with its particular localization in the acid environment of the stomach, generally hostile site to bacteria [43, 44].

The effect of iron starvation on the outer membrane proteins composition of strains of *H.pylori* tested (the reference and a medical strains) was determined by S.D.S. page. The presence of an iron regulated protein with an apparent molecular mass of 74 KDa was showed. This protein was synthesized in iron deficient conditions, but its absence in bacteria grows in iron sufficient conditions. These results suggested that the specific membrane protein acts as the receptor of ferric siderophore. Similar results were found with strains of *H.pylori* 11637 and 85035 that showed expression of a protein of molecular weight 78 KDa in the presence of 2.2'-dipyridyl. This effect was shown to be dependent on the concentration of 2.2'-dipyridyl [23]. Likewise, Husson *et al*. [20] observed the same effect at strains of *H.pylori* cultivated in the presence of EDDA.

The presence of the iron chelating compound in the medium induced the production of proteins to become major constituents of the outer membrane preparation [23]. *H.pylori* possesses iron assimilation well adapted to the host and ecological niche [45].

This study demonstrated that *H.pylori* strains studied produce siderophores *In vitro* but *in vivo H.pylori* can use human lactoferrin, present in significant amounts in stomach and heme as an iron source though a direct interaction between the receptors and these molecules [10,13,20,29,45]. This mechanism is common to several pathogens that are able to sequester iron directly from heme, hemoglobin, transferrin, or lactoferrin such as the genus of Neisseria and *Haemophilus influenza* [10,26,27,46]. The possession of a specific human heme or lactoferrin uptake systems in *H.pylori* would contribute greatly to the virulence of this microorganism [10,13,20].

*H.pylori* has traditionally been difficult to work with because of its complex nutritional growth requirements [13,20,23], Some other techniques for the study of iron limitation on bacteria were found to be unsuitable for *H.pylori* [23].

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