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Growth of *Helicobacter pylori* in Media Containing Cyclodextrins

R. OLIVIERI,[1] M. BUGNOLI,[1] D. ARMELLINI,[1] S. BIANCIARDI,[1] R. RAPPUOLI,[1] P. F. BAYELI,[2] L. ABATE,[2] E. ESPOSITO,[2] L. DE GREGORIO,[2] J. AZIZ,[2] C. BASAGNI,[2] AND N. FIGURA[2]*

Immunobiological Research Institute Siena[1] and Istituto di Patologia Speciale Medica, Università di Siena, P.zza Duomo 2,[2] 53100 Siena, Italy

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We show that solid and liquid media, supplemented only with cyclodextrins and free of blood and its derivatives, support the growth of *Helicobacter pylori*. These media can be used for primary isolation of the bacteria from biopsy samples, routine laboratory growth, and large-scale industrial fermentation.

Helicobacter pylori is a gram-negative microaerobic bacterium often isolated from gastroduodenal biopsy samples which has been proposed to be the agent of type B gastritis and also to be a cause of peptic ulcers (2). Laboratory growth of *H. pylori* is difficult and can be achieved by using complex media containing serum, blood, or blood derivatives. Good growth of *H. pylori* may be obtained in the presence of starch, charcoal, and catalase; however, flourishing growth cannot be obtained under these conditions. The development of simplified media, possibly devoid of serum or blood derivatives, which can support flourishing growth would be useful to make cultivation easier and to increase our knowledge of the physiology of this microorganism. The purification of *H. pylori* antigens would also be facilitated by the development of serum-free media. In fact, we have observed that serum-derived proteins, which are the major components of culture supernatants and are associated with bacterial cells, can make the purification of *H. pylori* products very difficult. Washing of the microorganism, on the other hand, may lead to loss of superficial proteins. Large-scale growth of *H. pylori* that would be useful for antigen purification has not yet been described, and this may also be due to the difficulties of growing the bacteria in the media currently available. For instance, bacteria have never been grown in volumes larger than 600 ml (5), and in all instances the media contained serum in amounts ranging from 5 to 15%. In this study, we demonstrate that chemically defined compounds belonging to the cyclodextrin group can substitute for serum in solid and liquid media for the growth of *H. pylori*. These media can be used for the primary isolation of *H. pylori* strains from gastroduodenal biopsy samples (3), routine laboratory growth, and large-scale fermentation (up to 20 liters).

Organisms and growth conditions. The following *H. pylori* strains were used in this work: CCUG 17874 (type strain; Culture Collection, University of Göteborg) and G39, G47, G25, G56, and G17-91 (clinical isolates). Stock cultures were stored at −80°C in Wilkins-Chalgren broth with 20% glycerol.

All tests were performed in a microaerobic environment obtained with the gas mixture generated by the BBL Campy Pak envelopes (Becton Dickinson) inside an anaerobic jar.

The following solid and liquid media were used: solid media, Columbia blood agar base (CBA) (Difco) and Mueller-Hinton agar (MHA) (Difco); liquid medium, brucella broth (BB) (Difco). Supplements were as follows: fetal calf serum (FCS) (HyClone, Logan, Utah), (2,6-di-*O*-*methyl*)-β-cyclodextrin (CD) (Teijin Lim, Tokyo, Japan), Skirrow selective supplement (Oxoid), and horse blood (HB) (Sclavo, Siena, Italy).

The suitability of *H. pylori* strains for growth on media containing CD was assayed in the following manner. Bacteria were thawed and seeded onto CBA with 5% HB. After 3 days of incubation in a microaerobic environment at 37°C, strains were subcultured onto CBA and onto MHA plates with 1 g of CD per liter or with 50 ml of HB per liter. As a control, strains were cultivated onto CBA and MHA without any supplement. In preliminary tests we had seen that selected strains grew on media containing a CD concentration higher than 0.125 g/liter. The size of the colonies increased until the CD concentration reached 1 g/liter; beyond this concentration no increase in colony size was observed. Thus, a CD concentration of 1 g/liter was chosen for all tests. Strains were subcultured 10 times, at 3-day intervals, onto CBA-CD to see whether consecutive subcultures caused loss of viability of the microorganisms.

All strains developed well on either CBA-CD or MHA-CD. After 4 days of incubation on CBA-CD, *H. pylori* colonies were about 1 mm in diameter, yellowish, and raised and had entire edges. Colonies on CBA were bigger than those on MHA. Thus, CBA was used for further tests. All microorganisms retained their viability after 10 subcultures.

The culture of *H. pylori* strains in broth was carried out with BB with 1 g of CD per liter or 50 ml of FCS per liter. As a negative control, strains were grown in BB without CD or FCS. Flasks of 700 ml, each containing 100 ml of broth, were each inoculated in duplicate with 10 ml of plain BB containing 10^7 microorganisms per ml from a 3-day CBA-CD culture and incubated in a microaerobic environment at 37°C for 72 h (150 oscillations per min). In preliminary tests we had seen that growth was not enhanced by prolonging the incubation. After 3 days of incubation in BB with 1 g of CD per liter, the yield was 8.3×10^8 to 1.5×10^9 CFU/ml, depending on the strain tested, whereas the yield on BB-serum was 1.5×10^8 to 3×10^8 CFU/ml. The appearance of coccoidal forms in the broth with CD occurred later than in the broth with serum (4 versus 2 days, as seen in additional tests). The strains tested did not grow in media without sera or CD.

Batch fermentations were carried out with *H. pylori*

* Corresponding author.

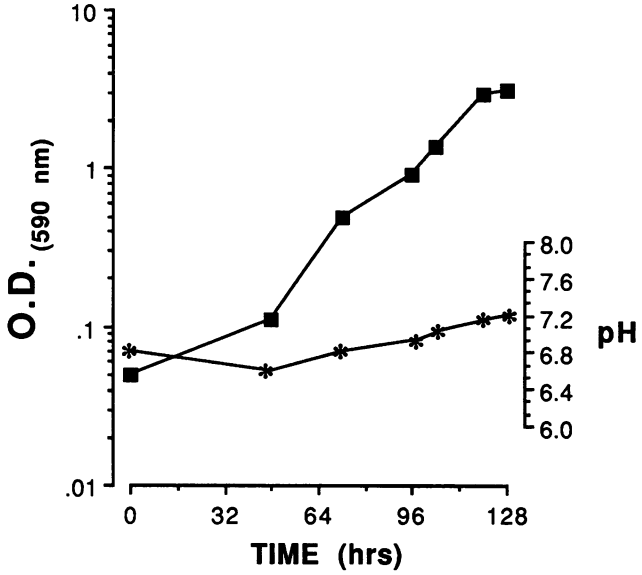

FIG. 1. Kinetics of growth of *H. pylori* CCUG 17874 in BB with 1 g of CD per liter in a 30-liter bioreactor. O.D.$_{(590\ nm)}$, optical density at 590 nm. ■, growth; *, pH.

CCUG 17874 in a 30-liter bioreactor (MBR Bioreactors, Wetzikon, Switzerland) containing 20 liters of BB with 1 g of CD per liter and the Skirrow selective supplement for 128 h. The broth was inoculated with 2 liters of a 3-day flask culture in BB-CD. Cultures were grown at 37°C. The initial pH was 7.0 and was not controlled. The inlet atmosphere was composed of a mixture of 3 liters of nitrogen and 0.3 liters of carbon dioxide per min. Oxygen was used to maintain the dissolved oxygen tension at 5% saturation. The calibration of the probes was performed in the medium, before inoculation, at 37°C with 4 liters of air per min and with an agitation of 200 rpm. Growth was monitored by measuring the optical density of broth samples at 590 nm (Varian Spectrophotometer DMS 80; light path of 1 cm). Purity checks of the samples were made by Gram staining and by subculturing samples on CBA-HB plates which were incubated in a normal atmosphere at 37°C for 24 h.

The fermentation parameters were not optimized. However, many different batch cultures were made under the above-mentioned conditions in order to obtain large amounts of biomass for purification of the interesting proteins. During the fermentation, the pH was not controlled by addition of acid or alkali. The CO_2 content of the sparging gas was sufficient to maintain the pH close to neutrality (Fig. 1).

Biochemical characterization and protein profile. After five subcultures on CBA-CD, the biochemical characteristics and the protein profiles of strains were determined together with those of strains cultured on CBA-HB.

We assayed oxidase, catalase, urease, alkaline phosphatase, DNase, leucine and arginine arylamidase, nitrate, and hippurate (API campy; Anident system, API). The protein content was assayed as follows. Strains were suspended in phosphate-buffered saline at a density of approximately McFarland no. 6 and lysed with 1% sodium dodecyl sulfate (SDS). Proteins were denatured with Laemmli's solution at 95°C for 5 min and run electrophoretically in an SDS–12% polyacrylamide gel in duplicate. Proteins were then either stained with Coomassie blue or transferred on to nitrocellulose sheets and then reacted with a pooled serum

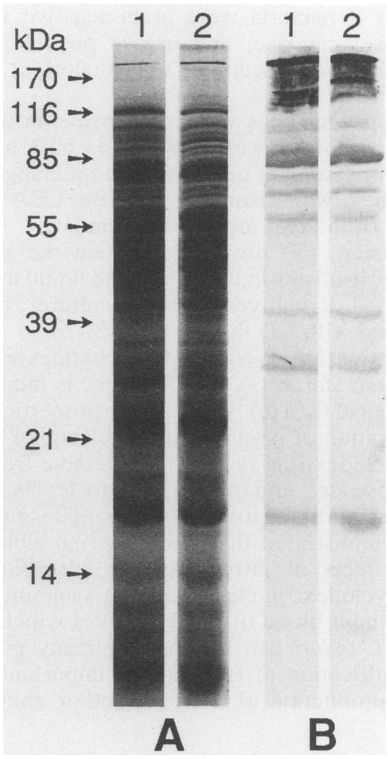

FIG. 2. SDS–12% polyacrylamide gel electrophoresis (A) and immunoblotting (B) of *H. pylori* CCUG 17874 cultured on CBA with CD (lane 1) and on CBA with HB (lane 2). Molecular size markers are shown to the left.

sample from patients infected by cytotoxic *H. pylori* strains diluted 1:1,000 (Fig. 2). Patients (n = 10) underwent diagnostic digestive endoscopy for dyspepsia. All patients were adults, five had duodenal ulcers, and all had severe chronic gastritis, determined histologically. Sera from all patients reacted with a cytotoxin-associated protein of 130 kDa by Western immunoblotting. After overnight incubation at room temperature, anti-human immunoglobulin G serum conjugated with alkaline phosphatase was added. After 90 min of incubation at room temperature, the positive reaction of the antibodies was revealed by adding the substrate.

The biochemical characteristics of *H. pylori* strains cultured on the media containing CD were fully retained. Strains were oxidase, catalase, urease, alkaline phosphatase, DNase, and leucine and arginine arylamidase positive and nitrate and hippurate negative. The protein profiles and the antigenicities of the proteins of strains cultured on media with CD or with blood were similar. In particular, the 130-kDa protein and urease subunits occurred in 6 M guanidine extracts of organisms grown with either CD or FCS.

Primary isolation of *H. pylori*. The suitability of the CD-containing agar for supporting growth of *H. pylori* from gastric biopsy samples was assayed as follows. Sixty biopsy samples from the gastric antrum and 60 from the bodies of 45 patients with dyspepsia were streaked on selective CBA plates containing 5 mg of cefsulodin per liter, 5 mg of teicoplanin per liter, 10 mg of trimethoprim per liter, 5 mg of amphotericin B per liter, and 1 g of CD per liter or 50 ml of HB per liter. Plates were incubated in a humidified microaerobic environment at 37°C. They were inspected for colonies after 48 h and then daily for 5 days. Colonies were identified

as *H. pylori* if bacteria were gram-negative spiral-shaped rods; oxidase, catalase, and urease positive; nitrate and hippurate negative; susceptible to cephalothin; and resistant to nalidixic acid.

Thirty biopsy samples yielded *H. pylori* in culture. In 28 cases microorganisms were isolated on both media, in one case they were isolated only on CD-containing agar, and in one case they were isolated only on CBA plates. The selectivities of the two media were similar.

In conclusion, CD proved to sustain the growth of *H. pylori* microorganisms in both solid and liquid media without blood or blood derivatives. In broth cultures, better results were obtained with CD than with FCS.

Cyclodextrins are cyclic oligosaccharides enzymatically produced from starch (1), and their use is increasing in the biotechnological field (6). One of their properties is based on the complexation of organic substrates and/or products. In this way cyclodextrins reduce the effective free concentrations of substrates and/or products to levels below those which are inhibitory or toxic to microorganisms. Recently it has been demonstrated that bovine serum albumin reduces the toxic effects of fatty acids by adsorbing them (4). Similarly, cyclodextrin can act by the same mechanism.

Media without blood or its derivatives which support the growth of *H. pylori* can be used for many purposes. The industrial cultivation of *H. pylori* is important to obtain a large-scale production of antigens and/or enzymes, which can be employed in the serological diagnosis of gastric infection, in the study of virulence characteristics of this microorganism, in the differentiation of isolates for epidemiological purposes, and in the preparation of vaccines. On the basis of our results, agar plates containing cyclodextrins and selective supplements can also be used for primary isolation of *H. pylori* from gastric biopsy samples.

The suitability of the medium we propose is finally demonstrated by the fact that the strains tested fully maintained their biochemical characteristics, protein profiles, and antigenic properties.

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