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Nutritional Requirements for Growth of *Helicobacter pylori*

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A chemically defined medium consisting of a buffered mineral base supplemented with amino acids, a purine, and thiamine supported growth of 23 clinical isolates and the type strain of *Helicobacter pylori*. The growth of four strains was inhibited by the presence of certain amino acids. All but one strain required alanine for growth. The amino acids leucine, valine, phenylalanine, methionine, arginine, and histidine were generally required. Isoleucine either was required or stimulated growth. Strains could be differentiated into groups on the basis of a requirement for one or more of the amino acids cysteine, serine, and proline. Only one strain however, demonstrated a requirement for all three of these amino acids.

Helicobacter pylori is a gram-negative, microaerophilic, curved rod which colonizes the human gastric mucosa. The presence of *H. pylori* on the gastric mucosa is strongly correlated to active chronic gastritis (type B gastritis) and ulcer disease (for reviews, see references 1, 5, 6, and 9). Recently, an association with development of gastric lymphomas has been proposed (4, 13). Although a large number of studies addressing *H. pylori* have been published, there is a severe lack of information regarding basal nutrition of this bacterium (3, 5, 7, 9). It is not known what nutrients *H. pylori* makes use of as sources of carbon and energy, nor is it known what growth factors are required or if strains differ in their requirements. It has, however, been reported that growth in chemostat culture is associated with depletion of glutamate, serine, and aspartate (9). Carbohydrates do not stimulate growth, and consequently *H. pylori* is considered asaccharolytic (1, 5). In contrast to the results of growth studies, a recent report, in which radioactively labeled glucose was used for tracer analysis, demonstrated incorporation of label in resting cells with lactate as the main product (7).

The present work was undertaken to disclose the nutritional requirements among 23 clinical isolates and the type strain of *H. pylori*. The results presented here demonstrate, for the first time, that *H. pylori* can grow on a chemically defined medium. The defined medium contains amino acids, a purine, and thiamine. Under these conditions, the strains demonstrated a general requirement for certain amino acids. The use of the defined medium made it possible to allocate the strains to groups according to requirements for a few amino acids.

Bacterial strains. The type strain of *H. pylori* (NCTC 11637) and the clinical isolates examined are listed in Table 1. The clinical strains were isolated from gastric biopsy specimens taken from patients referred for gastroscopy from the whole of Norway for various reasons during 1986. All of the patients were of Norwegian origin and underwent medical examination at Rikshospitalet (The National Hospital), Oslo, Norway. The biopsy samples were inoculated onto complex medium (brain heart infusion agar) enriched with 5% horse blood and incubated under microaerobic conditions. The isolates have been preserved at −70°C in brain heart infusion broth with 10% glycerol. Relatedness of the strains to the type strain has been established by demonstration of transfer of DNA and expression of streptomycin resistance by natural genetic transformation (8) and by DNA-DNA hybridization (2). Each of the strains listed in Table 1 originated from a different patient.

Composition of defined medium. The chemically defined medium developed during the present study was prepared according to the following recipe. ACES buffer [*N*-(2-acetamido)-2-aminoethanesulfonic acid] and salts were dissolved in approximately 90 ml of water in the following order: ACES, 0.5 g; KOH (as pellets), 0.11 g; NaCl, 0.45 g; KCl, 0.40 g; $MgSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 1 mg. The following amino acids were added to the buffer and salt solution (in milligrams): L-alanine, 15; L-arginine, 15; L-aspartic acid, 15; L-cystine, 7; L-glutamic acid, 15; glycine, 7; L-histidine $HCl \cdot H_2O$, 7; L-isoleucine, 15; L-leucine, 15; L-lysine, 15; L-methionine, 7; L-phenylalanine, 15; L-proline, 15; L-serine, 15; L-threonine, 15; L-tryptophan, 7; L-tyrosine, 15; L-valine, 15. A further 1 mg of hypoxanthine and 200 mg of activated charcoal were added, and the pH was adjusted to 6.85 to 6.90 at 20°C with 1 N KOH or 1 N HCl. Finally, 0.18 to 2.0 g of Noble agar (Difco Laboratories, Detroit, Mich.) was added to the medium, which was then autoclaved at 115°C for 15 min. A phosphate solution (dipotassium phosphate; 100 mg/ml) and a calcium dichloride dihydrate solution (20 mg/ml) were also sterilized by autoclaving and then placed at 50°C together with the solutions sterilized by membrane filtration: cysteine (10 mg/ml, adjusted to pH 6.85 to 6.90 with 1 N KOH), thiamine (5 mg/ml), and ferric pyrophosphate (10 mg/ml). In the following order and amounts, the solutions were added to the amino acid-containing medium: phosphate, 0.5 ml; ferric pyrophosphate, 0.5 ml; thiamine, 50 μl; calcium, 0.1 ml; and cysteine, 0.7 ml. Finally, 80 μl of trace metal solution ($ZnSO_4 \cdot 7H_2O$, 100 mg; H_3BO_3, 300 mg; $CoCl_2 \cdot 6H_2O$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $NiCl_2 \cdot 6H_2O$, 20 mg; and $Na_2MoO_4 \cdot 2H_2O$, 30 mg [water to 1,000 ml]) was added and the volume was adjusted to 100 ml. The trace metal solution was a slightly modified version of that of Schlegel et al. (10). All of the chemicals used were of analytical quality. All water used was double distilled and finally purified in a Millipore Ultra Pure water system.

Experimental media. The medium developed, or one of the variants of it, was able to support growth of all the strains. However, diversity in the requirements for some amino acids existed, and therefore, a series of media, each a modification of this basal medium, was prepared. The medium variants and the results of the experiments are summarized in Table 1 and in the text. A liquid defined medium did not contain agar, charcoal, or the amino acids cystine, glycine, lysine, and

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threonine. The liquid medium was prepared by dissolving all of the components in a final volume of 100 ml of water in the same order and amounts as for the solid medium, except that the amount of cysteine was increased to 15 mg. Liquid medium was sterilized by membrane filtration after the pH was adjusted as described.

Preparation of inoculum and incubation of solid medium. The strains were recovered on complex medium (Oxoid tryptose agar blood base supplemented with 5% human blood). Blood agar plates were incubated at 35°C in anaerobic jars with a GasPak for campylobacters (BBL Campy Pak Plus; Becton Dickinson Microbiology Systems, Cockeysville, Md.). Bacterial cells from 3- or 4-day-old growth were inoculated onto defined media by streaking with a platinum loop. To eliminate a growth effect caused by carryover of nutrients in the inoculum, the growth response was recorded after two or three subcultures on identical medium. All experiments were repeated several times with different batches of defined medium. A positive result was noted when a strain formed single colonies on a particular medium (Table 1). To estimate the effect of addition or deletion of compounds from the medium, the sizes of colonies formed by each particular strain on different variants of the medium were compared. Some strains consistently formed large colonies (diameter larger than 2 mm) on both defined and complex media, while other strains formed only small colonies (diameter less than 1 to 2 mm). Inoculated defined medium was incubated at 34 to 35°C in anaerobic jars (see below) for 2 or 3 weeks. Generally, colonies appeared within the first week of incubation but often continued to enlarge during the second week. Medium was stored in anaerobic jars with anaerobic GasPaks, which remove oxygen by a palladium-catalyzed reaction combining oxygen and hydrogen (BBL Gas Pak Plus). In studies on a possible growth stimulating effect of hydrogen, the media were stored in an anaerobic hydrogen-free atmosphere. Stored medium was kept at 4°C for 3 to 4 weeks.

Preparation of inoculum and incubation of liquid medium. Bacterial cells harvested from blood agar plates were washed twice in liquid defined medium, sedimented by centrifugation, and resuspended in the same medium to a density of 1×10^6 to 2×10^6 CFU. Inoculated medium was subsequently distributed in amounts of 1.2 ml in 50-ml glass bottles having an internal diameter of approximately 38 mm (Pyrex; Bibby Sterilin Ltd., Stone, Staffordshire, United Kingdom). The bottles were sealed with a 0.2-μm vented filter cap (Costar, Cambridge, Mass.) and placed in a jar under an atmosphere containing hydrogen, as described below. Each day one bottle was removed and examined for growth by serial dilution in liquid medium and plating onto blood agar plates for counting of CFU, and in addition a measurement of the A_{600} was performed. Three randomly selected strains (42A, 48A, and 53A) were tested for growth in liquid medium; a typical growth curve (strain 48A) is shown in Fig. 1.

Atmosphere. Two types of pressurized gas mixtures of the following composition were purchased from AGA Norgas, Oslo, Norway: 80% N_2–10% H_2–10% CO_2 or 90% N_2–10% CO_2. To these gas mixtures, oxygen was added to a final concentration of 2% (controlled by use of an industrial oxygen analyzer (Servomex, type 1100A; Servomex Ltd., Crowborough, United Kingdom). Experiments with different oxygen concentrations indicated that most strains of *H. pylori* grew best at oxygen levels of about 2%, but further studies are necessary to investigate which atmosphere is optimal for this bacterium and if strains vary in their sensitivity to oxygen. The gas mixture used in most of the experiments was a typical anaerobic gas containing 10% hydrogen. In control experi-

TABLE 1. Growth of strains of *H. pylori* on a defined medium lacking one or two components of the medium

Strain(s)	Growth[a] on medium lacking the following component(s):																			
	Ala-nine	Leu-cine	Va-line	Tyro-sine	Phenyl-alanine	Aspartic acid	Methi-onine	Threo-nine	Iso-leucine	Lysine	Serine	Glycine	Cysteine	Cystine	Cysteine and cystine	Trypto-phan	Argi-nine	Pro-line	Glutamic acid	Histi-dine
35A	−	−	−	−	−	−	−	−	−	+[b]	−	−	−	−	−	−	−	+	−	−
38A	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−
39A	−	−	−	−	−	−	−	−	−	−	−	+[b]	−	−	−	−	−	−	−	−
49A	−	−	−	−	−	−	−	−	−	+[b]	−	+[b]	−	−	−	−	−	−	−	−
36A	+	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	−
4A, 17A, 26A, 42A, 48A	−	−	−	+	−	+	−	+	−[c]	+	+	+	+	+	+	+	−	+	+	−
2A, 53A, 55A	−	−	−	+	−	+	−	+	−[c]	+	+	+	+	+	+	−	−	+	+	−
NCTC 11637, 9A, 43A, 44A, 59A	−	−	−	+	−	+	−	+	−[c]	+	+	+	+	+	+	−	+	−	+	−
5A, 11A, 32A, 33A, 54A	−	−	−	+	−	+	−	+	−[c]	+	−	+	+	+	+	−	+	−	+	−
29A	−	−	−	+	−	+	−	+	−	+	−	+	+	+	+	+	+	−	+	−

[a] +, Good growth of isolated colonies; −, no growth.
[b] Growth of small colonies.
[c] Some strains show growth of small colonies after prolonged incubation.

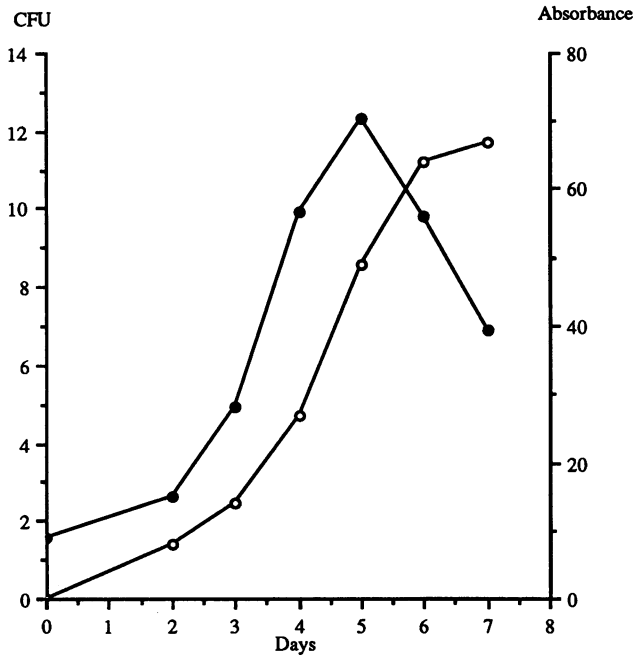

FIG. 1. Growth of *H. pylori* 48A in a chemically defined liquid medium. ●, CFU (10^6); ○, A_{600}.

ments, however, six randomly selected strains grew for several passages under an atmosphere without hydrogen, excluding the possibility that hydrogen was necessary as a source of energy. The gas in the jars containing solid medium was renewed two or three times per week. The gas in the jar containing liquid medium was replaced daily. No growth took place under anaerobic conditions.

Amino acids essential for growth. Only one strain (strain 36A) did not require alanine. All strains had an absolute requirement for leucine, valine, methionine, arginine, histidine, and phenylalanine. Isoleucine was either required for growth or stimulatory; the strains able to grow for several passages on a medium not containing isoleucine formed only small colonies after prolonged incubation. Addition of isoleucine at concentrations of up to approximately 5 to 10 mg/100 ml stimulated growth of correspondingly larger colonies. Diversity was found in the requirements for cysteine (or cystine, which could substitute for cysteine), serine, and proline. All of the serine- or proline-requiring strains also had a requirement for cysteine. Only strain 29A required serine and proline in addition to cysteine. As cysteine and cystine could substitute for each other, cysteine was used in a liquid medium because of its high solubility at room temperature.

Amino acids stimulating growth. Although glutamic acid was not essential for growth, some strains formed smaller colonies in its absence. Doubling the concentration of glutamic acid, however, did not result in the formation of larger colonies (data not shown).

Amino acids not required for growth. None of the strains demonstrated a requirement for aspartic acid, glutamic acid, glycine, lysine, threonine, tryptophan, or tyrosine.

Amino acids that may decrease or inhibit growth. Some strains seem to be particularly sensitive to growth inhibition by certain amino acids (Table 1). Strain 35A was able to grow with relatively large colonies (up to a diameter of 2 mm) on a medium not containing proline. Strain 35A also grew, though with smaller colonies, if lysine was omitted from the medium. Strain 38A grew only on a medium deficient in glycine, lysine, or both. As the lack of growth of strains 35A, 38A, 39A, and 49A on most of the media tested probably was caused by an inhibitory effect of certain amino acids, these strains are listed separately in Table 1. Strain 9A showed better growth on media not supplemented with glycine or lysine. It was also noted that many of the strains formed larger colonies when glycine was omitted from the medium.

Miscellaneous requirements. All of the strains examined had an absolute requirement for thiamine and a purine; hypoxanthine was generally adequate, although a few strains, such as 43A and 49A, seemed to form larger colonies on a medium containing adenine and guanine (data not shown). In some experiments the medium was supplemented with ammonium chloride (0.5 mg/ml), but no growth stimulatory effect was found (data not shown). Preliminary data indicate that addition to the medium of other vitamins and other putative growth factors (biotin, folic acid, vitamin B_6 complex, vitamin B_{12}, *para*-aminobenzoic acid, thymine, cytosine, uracil, and spermidine) does not influence growth significantly.

Conclusions and discussion. At present it is not known how amino acids are synthesized in *H. pylori*, nor is it known how amino acids are assimilated and metabolized, but under the conditions described one or more amino acid(s) must provide energy and act as a substrate for biosynthetic reactions. The defined medium should be useful for further detailed physiological and biochemical investigations. Compared to a solid medium, a liquid medium offers important advantages such as ease of detection and purification of bacterial products in response to changes in the composition of the medium. A liquid medium can be used to optimize growth conditions for each strain by analysis of the cell mass produced from each component. For these reasons a liquid variant of the defined medium was tested. Although only a few strains were tested for growth in liquid medium, Fig. 1 show that such a medium also supports growth.

The present study indicates that strains of *H. pylori* are auxotrophic for several amino acids and consequently are dependent on a steady supply of these growth factors from the environment. The requirement for several growth factors together with a dependency on a low oxygen tension supports the view that *H. pylori* is a highly adapted parasite. Most strains of *H. pylori* can incorporate closely related DNA from the environment into the chromosome by natural genetic transformation and thereby acquire new inheritable traits (8), a complicated mechanism which may add to the chances of success in the adaptation to environmental changes. Other studies have shown genetic diversity among strains (11, 12), and the results of the present study expand these findings by allowing for a differentiation of strains on the basis of requirements for amino acids. It seems that a requirement for one or more of the amino acids cysteine (or cystine), serine, and proline differentiates the strains into major groups. Although the biological explanation for the observed clustering must await further investigations, such a grouping system could be useful in epidemiological as well as taxonomic studies. *H. pylori* has very few phenotypic markers such as positive reactions for oxidase, catalase, and urease. The demonstration here that diversity exists in the requirements for amino acids greatly expands the number of phenotypic markers.

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