Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements

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A defined medium has been developed for *Helicobacter pylori* that gives growth characteristics (growth rate, maximum cell number and maximum colony-forming-unit count) comparable to those in a complex medium (Isosensitest broth + 5%, v/v, foetal bovine serum). Differences found in the death rate reflected a partial (50%) conversion to a coccoid cell form of the organism in the stationary and death phase in the defined medium, versus the almost complete (> 99%) conversion seen in the complex medium. The medium was used to study the amino acids required for growth by 10 strains of *H. pylori*. All strains required arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine, and eight of the strains also required alanine; five of the strains required serine. In the absence of glucose none of the 20 amino acids tested elicited growth when added at high concentration. However, in the presence of glucose, alanine induced considerably enhanced growth over that seen in the control, consistent with its use either as a nitrogen source or possibly an additional carbon source. The medium described will facilitate investigations into the metabolism and physiology of *H. pylori*, previously only possible with sophisticated approaches such as nuclear magnetic resonance spectroscopy.

Keywords: *Helicobacter pylori*, metabolism, coccoid form, nutritional regulation

INTRODUCTION

Helicobacter pylori is now well established as a principal cause of gastritis (Marshall & Warren, 1984; Morris & Nicholson, 1987; reviewed by Robert & Weinstein, 1993) and serological evidence shows 25–34% of the UK population and 52% of the population world-wide (ranging from 87% for Poland to 15% for Australia; Megraud, 1993) to be infected. Incidence of infection shows a high correlation with duodenal ulcer and to a lesser extent gastric ulcer and it is thought that chronic infection with *H. pylori* causes these lesions (Buck *et al.*, 1986; Blaser, 1987, 1990; Hornick, 1987; Taylor & Blaser, 1991). Furthermore, there is good epidemiological evidence to support the proposal that infection over a period of decades may be linked to the development of gastric cancer (Parsonnet *et al.*, 1991; Nomura *et al.*, 1991; Forman *et al.*, 1991).

H. pylori has been widely studied since its discovery in 1982 (Warren & Marshall, 1983) but many aspects of its structure, metabolism and physiology, including its specific growth requirements, are still largely unknown. The

organism is generally grown in complex media supplemented with blood or serum, which has made the metabolic pathways utilized by the bacterium difficult to determine. For example, although early studies based on acid formation from sugars and detection of preformed enzymes found no evidence of saccharide fermentative pathways (Marshall & Warren, 1984; McNulty & Dent, 1987), more recent evidence indicates that *H. pylori* does catabolize sugars. In a series of studies by Mendz, Hazell and colleagues clear evidence has been obtained for the pentose phosphate pathway (Menz & Hazell, 1991), glucokinase activity (Menz & Hazell, 1993) and the fermentation of glucose to lactate (Menz *et al.*, 1993). Unfortunately the approach used by these workers, ^1H and ^{13}C nuclear magnetic resonance spectroscopy, is expensive to set up and use, which restricts its wider availability to researchers studying *H. pylori* metabolism.

Here we describe a simple medium in which all the constituents are defined, allowing each of them to be selectively omitted or further components added and the effect on growth studied. We have used this medium to determine the amino acid requirements of *H. pylori* and to

Table 1. Composition of the defined medium

Component	Concn (mg l ⁻¹)	Component	Concn (mg l ⁻¹)
Ca(NO ₃) ₂ ·4H ₂ O	100	Amino acids	
KCl	400	Alanine hydrochloride	44·5
MgSO ₄ ·7H ₂ O	100	Arginine	632
NaCl	6000	Asparagine	75
Na ₂ HPO ₄	800	Aspartic acid	66·5
NaHCO ₃	2000	Cystine	120
Glucose	2000	Glutamic acid	73·5
Bovine serum albumin	5000	Glutamine	300
		Glycine	37·5
Phenol red (optional)	5	Histidine	110
FeSO ₄	2	Isoleucine	262·5
		Leucine	262
Adenine	50	Lysine	362·5
Lipoic acid	3	Methionine	75·5
		Phenylalanine	165
Vitamins		Proline	57·5
D-Biotin	0·2	Serine	52·5
Choline chloride	3	Threonine	238
Folic acid	1	Tryptophan	51
<i>myo</i> -Inositol	35	Valine	234
Niacinamide	1		
<i>p</i> -Aminobenzoic acid	1		
D-Pantothenic acid	1·25		
Pyridoxine hydrochloride	1		
Riboflavin	0·2		
Thiamin hydrochloride	1		
Vitamin B12	0·005		
(cyanocobalamin)			

study the utilization of amino acids as carbon and energy sources. In addition we have compared the growth pattern of the organism in the defined medium with that in a standard complex medium and looked in particular at the extent of coccoid cell formation in the two media.

METHODS

Strain and inoculum. The *Helicobacter pylori* strains used were NCTC 11637 (type strain), Roberts (clinical isolate from Manchester, UK) and eight isolates from stomach biopsies collected during 1991–1992 in Birmingham, UK. Other strains were *Escherichia coli* K12 and a *Neisseria gonorrhoeae* strain with a requirement for the amino acids Cys, Gln and Arg (typed according to Catlin, 1973) provided by Dr N. Parsons, University of Birmingham, UK. All strains were maintained on Isosensitest agar (Unipath) containing 2% (v/v) heat-inactivated newborn calf serum (Life Technologies). Plates were incubated at 37 °C for a maximum of 3 d in a VAIN (variable atmosphere incubator set at 5% CO₂, 5% O₂, balance N₂; Don Whitley Scientific). Liquid cultures were grown in 15 ml volumes of medium in 100 ml conical flasks with gentle shaking (140 r.p.m., IKA Vibrax VXR; Janke & Kunkel) or in 1 ml volumes in the wells of a 24-well tray (NUNC) with vigorous shaking (240 r.p.m.). Inocula were grown overnight on agar plates then suspended in Hanks' balanced salts solution, washed once in the same solution and standardized to 1 × 10⁸ cells ml⁻¹.

Inoculation (150 µl suspension for flasks, 10 µl for trays) was carried out in a laminar-flow hood and the cultures immediately transferred to the VAIN incubator and shaken at 37 °C.

Chemicals. All the inorganic chemicals and glucose were obtained from BDH. The amino acids (tissue-culture grade), vitamins and other organic chemicals were from Sigma. The bovine serum albumin (BSA) was either from Sigma (Fraction V, catalogue no. A-7906) or from Advanced Protein Products, Brierley Hill, W. Midlands, UK (catalogue no. PF-201-47). Both were equally suitable, and were stored as a 14% (w/v) aqueous solution at 4 °C. Stock solutions were as follows (in water, sterilized by 0·2 µm filtration and stored at 4 °C, unless stated otherwise): amino acids, 100 × concentration in medium (Table 1) in water or water acidified with HCl, stored at -20 °C; adenine, 6·5 mg ml⁻¹ (heat needed to dissolve); NaHCO₃, 7·5%; inorganic salts were made as a single 4 × solution; lipoic acid, 10 mg ml⁻¹ in ethanol (not sterilized); glucose, 50 × solution; vitamins, a single solution containing all 11 vitamins (Table 1) at 100 ×; FeSO₄, 100 × in acidified water. The medium was made up to volume with de-ionized distilled water and the pH adjusted to 7·4.

Growth studies. Growth over time was assessed by measurement of OD₆₀₀ using 1 ml samples in semi-micro plastic cuvettes, by serial dilution viable count on Isosensitest agar plates and by phase-contrast microscopy using a Helber chamber (Weber Scientific). Proportions of coccoid and rod-shaped cells

Fig. 1. Effect of component omission on growth of *H. pylori*. Each of the components shown in the figure was selectively omitted from the medium and the resulting growth at 41 h post-inoculation (measured as OD₆₀₀) recorded as a percentage change compared to the growth seen in the complete medium (no change = 0). Where values are positive, growth is enhanced by component omission (a value of +100% corresponds to an OD₆₀₀ of 0.423); where negative, growth is suppressed by omission. The complete medium contained the components at the following concentrations: xanthine, guanine, uracil, adenine, 10 mg l⁻¹; haemoglobin 14 mg l⁻¹; BSA, FeSO₄, lipoic acid and non-essential amino acids (NE AAs: Ala, Asp, Asn, Glu, Gly, Pro, Ser) at the concentrations shown in Table 1.

were assessed by phase-contrast examination of all 16 large squares (total area 1/400 mm²) of the Helber chamber (for two or three repeat assemblies of the chamber) at a dilution of the culture to give 10–20 organisms per square. All samples taken were confirmed as *H. pylori* by microscopic examination and comparison of their protein profile, using SDS-PAGE analysis on mini-gels, with that of the inoculum. Gels were run on BioRad mini-Protean II equipment using the conditions of Laemmli (1970) according to the manufacturer's instructions.

Amino acid studies. The amino acids required for growth were determined by inoculating wells of a 24-well tray in which each well contained medium deficient in one of the amino acids listed in Table 2. Growth was assessed at 24 and 48 h; absence of growth after 48 h indicated that the missing amino acid was required for growth of that particular strain. To assess use of amino acids as a carbon and energy source, the concentrations of all 20 amino acids were reduced to a base level (20% of the concentrations in the defined medium; Table 1) giving only limited growth (see Fig. 2) and individual amino acids added at 40 times the base level. Increased growth in the presence of the test amino acid indicated that it could be used as a carbon or energy source. Growth was determined visually, then as OD₅₉₅, and confirmed as *H. pylori* by SDS-PAGE analysis. OD₅₉₅ was measured in duplicate in Falcon 96-well flexible assay plates

Fig. 2. Optimization of key media components. The concentrations of the components were individually altered from 0.125 times to twice the levels in the standard medium (Table 1) and the resulting growth measured as OD₆₀₀ for strain NCTC 11637 (a) and strain Roberts (b). The components examined were: glucose (□), lipoic acid (◇), adenine (○), FeSO₄ (△), BSA (■) and amino acids (●).

(100 µl per well) using a Titertek Multiskan MCC ELISA plate reader.

RESULTS

Development of the defined medium

Using tissue culture medium (RPMI, Life Technologies) as a basic chemically defined medium, a series of components was added until the medium would support growth. These components were chosen based on the published analysis of the Isosensitest medium used in this laboratory to grow *H. pylori*. The resulting supplemented medium was next prepared *de novo* from reagent-grade chemicals (BDH) and then simplified by sequentially removing each of the key ingredients and noting the effect on growth (Fig. 1). Reduction in growth in the absence of an ingredient indicated that it contributed towards growth in the complete medium whereas enhanced growth indicated that it had a deleterious effect. Xanthine, guanine, uracil and haemoglobin either failed to enhance or were detrimental to growth. Lipoic acid, FeSO₄, adenine, BSA and non-essential amino acids increased growth of the organism. The concentrations of these key ingredients were then adjusted to determine the optimal medium composition (Fig. 2), resulting in the defined composite medium listed in Table 1. *H. pylori*

Fig. 3. Comparison of the growth of *H. pylori* in a complex medium and the defined medium. Growth was assessed as colony-forming units (○) and total count (□) for strain Roberts grown in (a). Isosensitest broth + 5% foetal bovine serum and (b) the defined medium. The results are representative of three experiments.

strains Roberts and NCTC 11637 were both sequentially passaged in the medium five times and attained similar growth at each passage. A large inoculum was necessary to achieve growth during serial passage. Typically this was 0.75 ml of a 24 h culture (approx. 2.5×10^7 bacteria) into 15 ml medium and growth gave a 20-fold increase in numbers of bacteria at each passage of 24 h.

Growth of *H. pylori* in the defined medium

Data are only shown for strain Roberts but results for NCTC 11637 were similar. The maximum growth rate was comparable for the defined and the complex media (Fig. 3; doubling time during exponential growth phase for defined medium = 2 h 33 min, for complex medium = 2 h 37 min). The maximum total counts and c.f.u. counts were also similar, at approximately 1×10^9 bacteria ml^{-1} . There was a difference in the death rate: the mean halving time (time for the number of c.f.u. to halve) for the complex medium was lower than that for the defined medium (1 h 51 min compared to 2 h 4 min, respectively), i.e. in the defined medium the bacterial became non-cultivable less rapidly. The slower death of the culture in the defined medium was reflected in the rate of conversion to the non-cultivable coccoid form of the organism (Fig. 4). In the defined medium conversion was slower and only about 50% of the culture became coccoid even after prolonged incubation. In contrast in the complex medium

Fig. 4. Coccoid development in a complex medium (a) and the defined medium (b). Growth was assessed as colony-forming units (○) and differential microscopic counts of the rod (clear columns) and coccoid (hatched columns) forms of the organism. The results are representative of two experiments. Bars represent SD.

conversion was rapid and over 99% of the culture became coccoid.

Amino acids required for growth

The amino acid requirements of 10 strains of *H. pylori* are shown in Table 2. All these strains required Arg, His, Ile, Leu, Met, Phe and Val. In addition, most strains also needed Ala and approximately half needed Ser, with only one requiring Pro and one Trp.

As a check on the validity of the procedure and media, two entirely distinct bacterial genera, represented by *Neisseria gonorrhoeae* (known to require Cys, Gln and Arg) and *Escherichia coli* (a prototroph) were tested. Inocula were prepared as for *H. pylori* strains and the same media used. The amino acid requirements found matched the known requirements of the two strains, indicating the general applicability of the method.

Amino acids as carbon and energy sources

In the defined medium the amino acids are at relatively low levels and glucose is available as a carbon and energy source. To test whether individual amino acids could serve as alternatives to glucose, a glucose-free medium was made containing 20% of the normal medium

Table 2. Amino acid requirements of *H. pylori*

+, Growth in the absence of amino acid, i.e. amino acid not required; —, no growth in the absence of amino acid, i.e. amino acid required. The results are representative of four experiments for strains Roberts, NCTC 11637, HP79 and A654/91; two experiments for strains A681/91, Wedderburn, *N. gonorrhoeae* and *E. coli*; and single experiments for the remaining strains.

Strain	Amino acid:												Amino acid:							
	Asp	Asn	Cys	Gly	Glu	Gln	Lys	Pro	Ser	Trp	Thr	Tyr	Ala	Arg	His	Ile	Leu	Met	Phe	Val
Roberts	+	+	+	+	+	+	+	+	+	—	+	+	—	—	—	—	—	—	—	—
NCTC 11637	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
Troth	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
A654/91	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
A655/92	+	+	+	+	+	+	+	—	—	+	+	+	—	—	—	—	—	—	—	—
A681/91	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
HP2	+	+	+	+	+	+	+	+	—	+	+	+	+	—	—	—	—	—	—	—
HP79	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
HP167	+	+	+	+	+	+	+	+	—	+	+	+	—	—	—	—	—	—	—	—
Wedderburn	+	+	+	+	+	+	+	+	—	+	+	+	—	—	—	—	—	—	—	—
<i>N. gonorrhoeae</i>	+	+	—	+	+	—	+	+	+	+	+	+	+	—	+	+	+	+	+	+
<i>E. coli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

concentrations of amino acids and individual amino acids were then added at 20, 40 or 100 times this level. No growth above that in the control (in which no amino acid was at high concentration) was seen for any of the amino acids for the four strains tested (HP79, A681/91, Roberts and NCTC 11637; data not shown). The experiments were then repeated using identical media supplemented with glucose (at 2 g l⁻¹) and a distinct pattern of growth was seen. For strains A681/91 and NCTC 11637 growth in the presence of the high level of amino acid was similar to that in the control but for strains Roberts and HP79 alanine induced a considerable increase in growth over that in the control (Fig. 5).

DISCUSSION

All of the media described to date for the cultivation of *H. pylori* have been complex media based on tissue extracts (e.g. of brain or heart) or proteolytic enzyme digests of meat or casein. Here we describe a defined medium capable of supporting growth comparable to that achieved in complex media.

Initial studies during the development of the defined medium highlighted lipoic acid, FeSO₄, adenine, BSA and non-essential amino acids as key ingredients. Some of these are common additions to media used to grow other bacteria. However, the requirement for BSA, a protein known to act as a carrier for long-chain fatty acids (LCFAs), may indicate either that LCFAs are required by *H. pylori* if they are present in the BSA used in this study or that LCFAs are toxic and it is their removal by BSA that enhances growth. Alternatively, other required growth factors may be either carried by BSA or present as an impurity.

The pattern of growth in the defined and complex media was similar, with exponential growth to about 1 ×

10⁹ c.f.u. ml⁻¹ followed by a short stationary phase and a rapid death phase. This is typical for other media studied, e.g. Mueller–Hinton broth (Sorberg *et al.*, 1993) and Brucella broth (Catrenich & Makin, 1991; Cellini *et al.*, 1993). However, a major difference was that in the defined medium the rate of death in the death phase was lower and the conversion to the coccoid form reduced from 99 % to just 50 %. This is a potentially important observation as it shows that conversion to the coccoid form can be regulated by changes induced by altered nutrition. Environmental factors are known to modulate the expression of virulence determinants (Mekalanos, 1992) and similar processes might also be involved in regulating the coccoid transformation. It is possible either that a key triggering component is at reduced abundance in the defined medium or that an *H. pylori* metabolite necessary for the conversion is lowered by the lack of or reduced abundance of a nutritional factor.

Furthermore since the prevention of coccoid transition does not appear to increase the viability of the population in the death phase (c.f.u. count in both media < 10 ml⁻¹), it seems that the coccoid form is not a prerequisite for non-viability: rod forms of the organisms can also become non-viable.

The coccoid form of the organism has been proposed as a potential survival form possibly involved in transmission between individuals or reactivation of infection following apparently successful antibiotic therapy (Bode *et al.*, 1993). The ability to manipulate transition to this form would be advantageous to workers studying this aspect of the organism.

The defined medium allowed the amino acid requirements to be determined by single amino acid omission. The requirement of eight amino acids for growth of *H. pylori* was the same as for the intracellular bacterium *Legionella*

Fig. 5. Amino acids as carbon and energy sources for *H. pylori*. Individual amino acids (listed in the figure by their single-letter code) were added to the defined medium at 40× (results similar for 20× and 100×) the levels in the low amino acid medium (glucose-free, containing 20% of the normal medium concentrations of amino acids) and the resulting growth (measured as OD₅₉₅) expressed as a multiple of the growth in the control (no amino acid at high level), where growth in the control = 1. The strains tested were: (a) clinical isolate A681/91; (b) the type strain NCTC 11637; (c) clinical isolate Roberts; (d) clinical isolate HP79. The results are representative of two experiments (A681/91 and Roberts) or three experiments (NCTC 11637 and HP79).

pneumophila (George *et al.*, 1980). Indeed six of the required amino acids (Arg, Ile, Leu, Val, Met and Phe) are the same for both bacteria. This contrasts with the 16 amino acids required by the lactic acid bacterium *Leuconostoc mesenteroides* (Lehninger, 1975) and the one to five amino acids commonly required by the pathogen *Neisseria gonorrhoeae* (usually selected from Cys, Pro, Arg, Gln, Ile and Met; Catlin, 1973). The amino acid requirements of *H. pylori* may form the basis of a scheme for biotyping strains of the bacteria given that there were differences in the requirements of the 10 strains tested here.

The amino acids that were required by *H. pylori* are significant in that they generally occur at the end of biosynthetic pathways, consistent with mutations in or deletions of the genes for individual enzymes in the pathways leading to their biosynthesis (Fig. 6). The *H. pylori* genome is only 1.72 Mbp (cf. *E. coli*, which has a genome of 4.6 Mbp), suggesting that deletion of some non-essential genes has occurred. Mutations or deletions

Fig. 6. Biosynthetic pathway families for the synthesis of amino acids. Taken from Lehninger (1975).

at a point close to the root of a pathway would lead to multiple loss of biosynthetic capability and would probably have been too deleterious to permit survival of the organism in its natural habitat. One possible exception to this is the pyruvate pathway, where the ability to synthesize four amino acids has apparently been lost. However, this may simply reflect a weakness in the single amino acid omission analysis used as Leu, Ile and Val are structurally related amino acids known to be antagonistic in *E. coli* due to common active transport, biosynthesis and physiological regulation mechanisms and an imbalance in the supply of these amino acids can suppress growth (De Felice *et al.*, 1979). The finding that four of the ten *H. pylori* strains required Ser is unusual in that this amino acid occurs at a fork in the biosynthetic pathway to Gly and Cys (Fig. 6). There is no requirement for either Gly or Cys in the presence of Ser so the enzymes responsible for biosynthesis downstream of Ser clearly exist. However, in the absence of Ser, growth is arrested despite the presence of Gly and Cys, indicating that there is a defect in the biosynthetic pathway to Ser. Loss of the ability to synthesize this amino acid would only permit survival if the organism had an adequate supply of Ser in its normal environment. The fact that this occurs for less

than half of the strains tested suggests that *H. pylori* may be undergoing an evolutionary process of loss of the ability to synthesize amino acids.

Until recently it was generally thought that *H. pylori* did not use carbohydrates but was able to use amino acids as precursors for tricarboxylic acid cycle intermediates to derive energy (Dick, 1990). This is known to be the case for *Legionella pneumophila*, which uses Ser and Thr as carbon and energy sources (George *et al.*, 1980). However, there is now strong evidence that *H. pylori* can utilize glucose as a carbon and energy source (see Introduction) and the data presented here suggest that amino acids alone cannot serve as carbon and energy sources for *H. pylori*.

In the absence of glucose the addition of single amino acids at high concentration did not enhance growth. However, in the presence of glucose several amino acids induced growth above that in the control for all four strains tested. For two of the strains (Roberts and HP79) the induction of growth in the presence of Ala was considerably greater than that seen for any of the other amino acids. This correlates with earlier evidence using proton nuclear magnetic resonance spectroscopy of brain heart infusion broth (a complex medium containing both amino acids and carbohydrates) which showed that Ala was metabolized by *H. pylori* (Dick, 1990). The requirement for the presence of glucose to permit growth enhancement by Ala may indicate that the amino acid serves as a carbon source but that its assimilation is driven by energy derived from glucose metabolism. Alternatively, the primary function of Ala catabolism may be to provide nitrogen with the carbon assimilation into tricarboxylic acid cycle derivatives noted by Dick (1990) being a consequence of amino acid deamination.

The experiments described here demonstrate the potential the defined medium has for elucidating the metabolic requirements of *H. pylori* and we intend in future work to use the medium to investigate the nutritional regulation of the virulence determinants of *H. pylori*. The medium may also be generally applicable to the growth of other helicobacters and the similar campylobacters; it clearly supports the growth of *N. gonorrhoeae*. Modification of the medium to one in which all of the components are chemically defined is being pursued in this laboratory to allow us to dispense with the requirement for BSA.

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