CURRENT MICROBIOLOGY Vol. 27 (1993), pp. 109–113

Current Microbiology

A Study of the Nutritional Requirements of a Selected *Haemophilus ducreyi* Strain by Impedance and Conventional Methods

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Abstract. The growth and nutritional requirements of *Haemophilus ducreyi* (CIP 542^T) were determined in liquid media by impedance technology. We found that *H. ducreyi* grew in a liquid mixture of Proteose Peptone No. 3, hemin, L-serine, glucose, L-glutamine, and inorganic salts. The peptone in the medium was essential for *H. ducreyi* growth, and the growth factor (or factors) in the peptone was stable to autoclaving (15 min, 121°C) and could not be extracted with organic solvents (2 : 1 chloroform/methanol) from either acidic (1 *N* CH_3COOH), neutral (pH 7.1), or basic (1 *N* NaOH) solutions. Hemin was also an essential component for the growth of *H. ducreyi*, but a much lower concentration of hemin (5.0 μg/ml) was required than has been previously reported in the literature (25 μg/ml). Using impedance technology to measure *H. ducreyi* doubling times, we showed that bovine albumin enhances *H. ducreyi* growth.

Haemophilus ducreyi is a poorly understood pathogen that causes the sexually transmitted disease chancroid [2, 13]. One of the difficulties in studying this organism in the laboratory is that *H. ducreyi* requires fastidious growth conditions and a complex medium. The growth conditions and nutritional requirements for isolating this organism from clinical samples with solid, semi-defined media have been determined in a number of studies. These studies show that *H. ducreyi* grows best at 33°C [10, 19] in high humidity [4, 14, 19] and in an atmosphere of 3–9% CO_2 [14, 19]. The nutritional requirements for growth of *H. ducreyi* on a solid medium are: L-glutamine [17, 21], hemin [11], albumin [1, 3, 21], and an undefined ingredient found in peptone [1]. In addition to these nutritional requirements, sodium selenite has been reported to enhance the growth of *H. ducreyi* on a solid medium [21].

There is a paucity of information dealing with the growth of *H. ducreyi* in liquid media. Some studies have shown that *H. ducreyi* grows well in a complex liquid medium containing serum or whole blood [4, 6, 14, 17, 20]; however, little progress has been made towards developing a chemically defined medium that would allow a systematic examination of this organism's metabolism and nutritional needs.

The conventional method for determining the nutritional requirements of an organism is to vary singly the concentration of all components of the medium and to monitor how the variate affects bacterial growth [9]. Automated impedance technology offers an efficient method for screening nutritional requirements, since change in impedance values can be correlated with bacterial growth. The principle of this technology is based on the fact that subtle changes in the ionic composition of the medium affect its electrical conductance and capacitance [16]. As bacterial metabolism proceeds, uncharged or weakly charged substrates are transformed into charged end products. Accumulation of these products increases the conductance of the medium and the capacitance at the electrode–medium interface. The time required to cause a series of significant deviations from baseline capacitance or conductance values is referred to as the detection time (DT), and this corresponds to a bacterial concentration of 10^6–10^7/ml [8]. Since DT is inversely proportional to the bacterial concentration at the time of inoculation, this measure can be used as an index of bacterial growth.

The focus of this study was to investigate the nutritional requirements of *H. ducreyi* in a liquid

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medium. Using impedance and conventional methods, we examined the effects of amino acids, hemin, peptone, glucose, and a number of other defined and undefined ingredients on *H. ducreyi* growth. Information from these experiments was used to develop a semi-defined liquid medium for the propagation of *H. ducreyi*.

Materials and Methods

Inoculum. *Haemophilus ducreyi* CIP 542^T (Pasteur Institute, Paris) was maintained and transferred on a solid medium, EPL (Edmonton Provincial Laboratory). Bacterial growth (24 h) was scraped off the EPL medium with a sterile cotton-tipped swab and suspended in pH 7.0 phosphate-buffered saline (PBS) which consisted of 0.8% (w/v) NaCl and 0.009 M K_2HPO_4/KH_2PO_4. Fifty microliters of 10^{-2} dilution was used to inoculate the media. For experiments dealing with the development of a liquid medium, the inocula were standardized with No. 0.5 and No. 2 McFarland standards.

EPL medium. In 791.0 ml distilled and deionized water (ddH_2O), 30.1 g of Tinsdale base (Difco) and 0.8 g of Bacto agar were dissolved, adjusted to pH 7.5, and autoclaved at 121°C for 30 min. Sheep blood (7.1 ml) was mixed with 80.0 ml of ddH_2O, hemolyzed for 2 h at 60°C, cooled to 50°C in a water bath, and then added to the autoclaved Tinsdale base. To this was added: 86.3 ml of bovine serum, 10.0 ml of chemically defined enrichment [15] IsoVitaleX (BBL, Cockeysville, Maryland) or Suplex (Pro-Lab, Richmond Hill, Ontario), and 25.5 ml of sterilized 15% (w/v) Na_2HPO_4.

Culture conditions. Solid media were incubated at 33°C in an environment of 5–10% CO_2 and 90–100% relative humidity. Liquid cultures were also incubated at the same temperature, but no special atmospheric conditions were used.

Impedance equipment. Impedance was measured with a Vitek Bactometer (Vitek, Hazelwood, Missouri). This system consists of a computer terminal, printer, and two temperature-controlled incubators. Modules, which are designed to clip into an incubator, contained 16 wells; each well had two electrodes. The ability to detect significant deviations in the impedance signal was controlled by the algorithm or test type selected. Different test types permitted the user to select the desired impedance signal: capacitance or conductance.

Nutritional requirements. Minimum Essential Medium (MEM) obtained from ICN FLOW (Mississagua, Ontario, Canada) was used as a base to which selected ingredients were added in different combinations and concentrations. The following ingredients were added: L-glutamate, L-cysteine, L-asparagine, L-ornithine, L-proline, L-citruline, L-alanine, L-serine, fraction v albumins (bovine, human, dog, chicken, horse, egg), sodium selenite, sodium pyruvate, Tween 60, EDTA, glycerol, citric acid, Suplex (Pro-lab), Bacto gelatin (Difco), type A gelatin, type B gelatin, 30,000–70,000 MW polyvinyl alcohol (PVA), Proteose peptone No. 3 (Difco), yeast extract (Difco), spermidine, and hemin. Unless otherwise indicated, the ingredients were obtained from Sigma. In each medium mix, a concentrated solution of MEM (2×) was diluted 1 in 4 by the addition of the selected ingredients. Unit value concentrations were attained by diluting each medium with PBS and water. Module wells containing these media (550 μl/well) were inoculated and screened for *H. ducreyi* growth with the Bactometer.

Evaluation. Medium that supported the growth of *H. ducreyi* with minimal undefined ingredients was evaluated by conventional and impedance methods.

Cultures grown in the medium were serially transferred ten times to demonstrate that the medium supported the growth of *H. ducreyi*. For each transfer, a suspension of 24-h culture-growth medium was vortexed, and 50 μl of this suspension was added to 550 μl of fresh medium. As a check for the possibility of contamination, the last serial transfer was subcultured onto EPL medium. Colony forming units (CFU)/ml were estimated by diluting *H. ducreyi* from 24-h-old cultures and plating onto EPL medium by the spread plate method [12].

Impedance methods were used to determine *H. ducreyi* doubling times in the medium with and without selected ingredients. Doubling times of bacterial cultures can be calculated from the slope of the linear relationship between logarithm of bacterial concentration (log CFU/ml) and DT (h) by the following formula: doubling time = $(\text{slope})^{-1} \times \log 2$ [7]. *H. ducreyi* cells were suspended to a concentration of 0.5 McFarland units, serially diluted (tenfold), and plated on EPL medium (in triplicate) to estimate CFU/ml. DTs were determined by using the suspensions as inocula for module wells containing the liquid medium (in triplicate). Each doubling time was calculated from an average slope of two independent experiments.

Results and Discussion

Impedance signal for measuring bacterial growth. We found that the capacitance signal produced a more pronounced deflection of impedance values than did the conductance signal, and for this reason capacitance was used as the signal of choice for the remainder of the study. Capacitance gave positive deflections only when granular growth was visible in the module wells; negative or no deflections occurred only when there was poor or no growth.

Semi-defined liquid media for *H. ducreyi* (HDLM). HDLM components were individually prepared in ddH_2O as follows: 50.0 ml of pH 7.0 PBS, 25.0 ml of 2× MEM, 15.0 ml of 50 g/l PVA (MW 30,000–70,000), 2.0 ml of 100 g/l Proteose Peptone No. 3 (Difco), and 1.0 ml of 10 g/L-serine. These solutions and 6.5 ml of ddH_2O were autoclaved at 121°C for 10 min and mixed together. To this mixture were added filter-sterilized (0.44 μm pore-size filter) 1.0 ml of 10 g/l L-glutamine (dissolved in ddH_2O) and 0.5 ml of 1.0 g/l hemin (stock solution of 20 g/l hemin was prepared in 1 *N* NaOH, filter-sterilized, and diluted 1 in 20). Ten consecutive transfers of *H. ducreyi* in this medium showed typical *H. ducreyi* growth with no apparent contamination.

Quantitative evaluation of *H. ducreyi* growth in the medium showed an average of 3×10^7 CFU/ml ($n = 3$) after 24 h incubation.

Growth requirements of *H. ducreyi* were further investigated by amending the concentration of individual components in the HDLM. Through these experiments, we developed a simpler medium, HDLM2. HDLM2 contained the following (in g/l): 10.0 D-glucose, 6.67 NaCl, 2.0 Proteose Peptone No. 3, 0.69 $NaH_2PO_4 \cdot H_2O$, 0.20 KCl, 0.10 $MgSO_4 \cdot 7H_2O$, 0.10 $CaCl_2$, 0.10 L-glutamine, 0.10 L-serine, and 0.0050 hemin. Although Proteose Peptone No. 3 in the medium was essential for the growth of *H. ducreyi*, it could be replaced with either 0.2% (w/v) Bacto yeast extract or 1.7% (w/v) Bacto gelatin. The growth-promoting activity of this peptone was not affected by autoclaving (15 min, 121°C), nor could the growth factor(s) be extracted from the aqueous phase with organic solvents (2 : 1 chloroform/methanol) even when acidic (1 *N* CH_3COOH), basic (1 *N* NaOH), or neutral (pH 7.1) solutions of the peptone were used.

Differences between *H. ducreyi* growth in liquid and on solid media. Hemin requirement for *H. ducreyi* was dependent on whether growth occurred on a solid or in a liquid medium. According to previous studies, *H. ducreyi* required between 200 and 500 μg/ml [11] and 25–50 μg/ml [3] of hemin for growth on a solid medium. This study showed that *H. ducreyi* needed 5.0 μg/ml hemin to grow in HDLM2.

We compared *H. ducreyi* hemin requirement on solid and in liquid media. In the experiment, both media contained (in g/l): 15.0 Proteose Peptone No. 3, 5.0 NaCl, 1.0 D-glucose, 1.0 soluble potato starch, 0.259 L-cysteine, 0.100 L-glutamine, 0.100 L-serine, 0.120 K_2HPO_4, 0.030 KH_2PO_4. The solid medium also contained 1% (w/v) Bacto agar; pH of both media was 7.1 ± 0.1. Stock 20 mg/ml hemin (Bovine type I, Sigma) in 1 *N* NaOH was filter sterilized and added to the media in different amounts, giving final concentrations of 50, 25, 5, 1, and 0 μg/ml. All inoculum dilutions, as shown in Table 1, were made in 0.8% (w/v) NaCl; the first (10^0) was adjusted to No. 2 McFarland standard; these dilutions were added to the media in 10-μl drops. The solid medium plates with intact drops on the medium surface were incubated in a sealed jar with wetted paper towels inside (with careful handling of the medium plates, the drops did not run on the surface).

The results shown in Table 1 demonstrated that *H. ducreyi* requires 50 μg/ml hemin on the solid medium and 5.0 μg/ml in the liquid medium to grow.

Table 1. *Haemophilus ducreyi* growth[a] on solid and in liquid media with different hemin concentrations

Hemin (μg/ml)	Inoculum dilution[b]	48-h growth on solid medium	48-h growth in liquid medium
50	10^0	+	+
	10^{-1}	+	+
	10^{-2}	+	+
	10^{-3}	±	±
25	10^0	+	+
	10^{-1}	±	+
	10^{-2}	−	+
	10^{-3}	−	±
5	10^0	±	+
	10^{-1}	−	+
	10^{-2}	−	+
	10^{-3}	−	±
1	10^0	−	+
	10^{-1}	−	+
	10^{-2}	−	±
	10^{-3}	−	−
0	10^0	−	−
	10^{-1}	−	−
	10^{-2}	−	−
	10^{-3}	−	−

[a] Visible growth in all experiments (+); visible growth present or absent depending on experiment (±); no visible growth in all experiments (−); the results represent four experiments.
[b] The first dilution (10^0) was adjusted to No. 2 McFarland standard.

The solid medium described above supported *H. ducreyi* growth only inside a sealed jar containing wetted paper towels but not in an atmosphere of 90–100% humidity and 5–10% CO_2. We suspected that *H. ducreyi* did not grow under the latter circumstance because CO_2 affected the buffering capacity of the medium. We increased the phosphate buffer concentration of the solid medium 33-fold, and on this medium *H. ducreyi* grew in 5–10% CO_2 and required 25 μg/ml hemin.

Besides hemin requirement, morphological differences between *H. ducreyi* growth in liquid and on solid media (media ingredients listed above) were observed. In the liquid medium *H. ducreyi* formed mostly small, planktonic colonies; on the solid medium it formed large, tightly packed colonies.

It is possible that the *H. ducreyi* hemin requirement, its colonial morphology, and its growth conditions in liquid and on solid media are interrelated. The solid medium containing low concentration of hemin may have limited the growth of dense *H.*

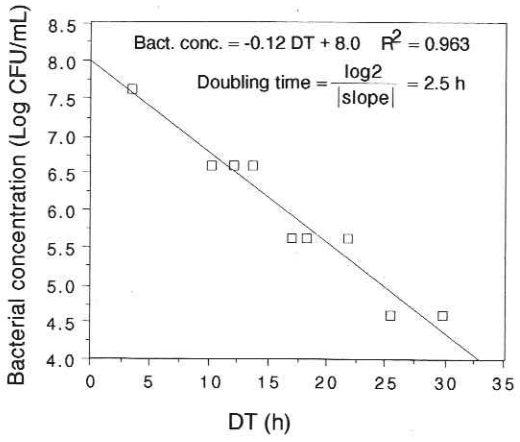

Fig. 1. Calculation of the doubling time for *Haemophilus ducreyi* grown at 33°C. Doubling time was calculated from the slope of the bacterial count and DT of 12 samples. Concentrated cells were serially diluted (tenfold) and inoculated (in triplicate) in module wells containing HDLM2 broth.

ducreyi colonies because metabolic acids produced by the bacteria decreased the pH of the medium in the immediate area of each growing colony, making hemin less soluble. Higher concentrations of hemin were required for the solid medium to compensate for the insoluble hemin. Probably, localized pH changes did not occur in the liquid medium because bacterial growth was less concentrated and diffusion of the metabolites was better facilitated.

Other growth requirements of *H. ducreyi* grown in HDLM2. Contrary to an earlier study [17], we found that *H. ducreyi* strain CIP 542^T utilized glucose as an energy source. Methods used for determining carbohydrate utilization may account for our disagreement. In our laboratory the impedance instrument could not detect growth, nor was growth visible in any of the module wells, when this strain was inoculated in HDLM2 prepared without glucose.

Salts and L-glutamine were also required for the growth of *H. ducreyi*.

Doubling time of *H. ducreyi* in HDLM2. Our initial experiments showed that the relationship between the inoculum concentration (inocula prepared in HDLM2 minus glucose, hemin, and L-serine) and the DT was linear only when heavy inocula (4×10^7–4×10^4 CFU/ml) were used. *H. ducreyi* doubling times were calculated by use of these parameters (Fig. 1).

We determined the effects of varying the concentrations of hemin, L-serine, and bovine albumin on *H. ducreyi* doubling time in HDLM2. The doubling time in HDLM2 remained the same (2.4–2.5 h) when L-serine was deleted or when the hemin concentration was increased to 25 μg/ml. However, when 0.1% (w/v) bovine albumin was added to the medium, *H ducreyi* doubling time decreased to 1.8 h. Albumin adsorbs metabolic products [5], and this may be the reason that it enhanced *H. ducreyi* growth.

Although the doubling time for *H. ducreyi* grown in HDLM2 (1.8–2.5 h) was considerably slower than doubling times of other bacteria, such as *Escherichia coli* (0.35 h), *Pseudomonas putida* (0.75 h), and *Vibrio marinus* (1.35 h) [18], the calculated doubling time was consistent with observations that *H. ducreyi* often requires 48–72 h of incubation to produce visible colonies on solid media [9, 13, 17], whereas *E. coli* requires only 12–20 h.

Growth of other *H. ducreyi* strains in HDLM2. *H. ducreyi* isolates from various sources were subcultured on EPL medium for 48 h, suspended in 0.8% (w/v) NaCl, and inoculated into the module wells containing HDLM2 medium. Thirty-six of the 48 *H. ducreyi* isolates produced capacitance profiles comparable to that of the CIP 542^T strain. These results suggest that the nutritional requirements of *H. ducreyi* may vary among strains, with some strains requiring constituents in addition to those found in HDLM2 medium.

Significance of results. This study used impedance methods to develop a semidefined liquid medium and to measure the rate of bacterial multiplication. Since the rate of bacterial multiplication is a direct and objective measure of quantifying bacterial growth in a medium, this method can be used to optimize existing media for the growth and isolation of nutritionally fastidious bacteria.

In developing a semi-defined liquid medium for *H. ducreyi*, this study has shown that CIP 542^T strain of *H. ducreyi* requires a much lower concentration of hemin in a liquid medium than on an equivalent solid medium and that albumin enhances growth of this *H. ducreyi* strain. Additional studies are required to isolate and characterize the essential growth factor (or factors) in peptone required for the growth of *H. ducreyi*.

ACKNOWLEDGMENTS

We thank the Media Preparation and Bacteriology Divisions at the Provincial Laboratory of Public Health for their assistance

in preparing media and maintaining bacterial stocks, respectively. We also acknowledge the work of E. Kadis and H. Porton, who originally developed the EPL medium in our laboratory, and the help of Erika M. Pfeiffer in improving the manuscript.

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