Growth of *Haemophilus influenzae* type b in continuous culture: Effect of dilution rate on outer-membrane protein and lipopolysaccharide expression

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1. SUMMARY

*Haemophilus influenzae* type b (Hib) was grown in continuous culture under cystine-limitation between dilution rates (D) of 0.065–0.28 h\(^{-1}\). A similar outer-membrane protein profile, as adjudged by SDS-PAGE, was found at all dilution rates. However, a shift to a lipopolysaccharide structure with a greater electrophoretic mobility on SDS-PAGE with accompanying changes in monoclonal antibody reactivity was observed at D ≥ 0.15 h\(^{-1}\). Growth rate per se can affect the expression of outer-membrane components of Hib.

2. INTRODUCTION

Bacteria can alter their metabolism and composition in response to environmental change [1]. Invariably in vitro studies designed to elucidate the effect of environment and growth rate on expression are done in batch culture. Such organisms are in a state of continual physiological change [2]. The use of a chemostat in such studies has many advantages: (1) the growth rate of the organism can be fixed while the environment is altered; (2) the growth rate can be varied with no change in the environment other than the concentration of growth limiting substrate; and (3) substrate-limited growth with a constant growth rate can be maintained [3].

*Haemophilus influenzae* type b (Hib) is a major cause of meningitis in young children [4]. The outer membrane proteins (OMPs) and lipopolysaccharide (LPS) of this organism have been the focus of considerable research because of their
vaccine potential and the role they may have in pathogenesis [5]. However, no fundamental study of the effect of growth rate on OMP or LPS expression has been done. This may have important implications in vivo since colonising bacteria may be growing at a slower rate than those in blood or cerebrospinal fluid. The effect of dilution rate (D, h⁻¹) on OMP and LPS expression of Hib in continuous culture under cystine-limitation was investigated. Cystine-limitation was chosen since preliminary experiments in batch and, subsequently, in continuous culture, established that it was possible to limit Hib in a defined medium in a manner analogous to that reported by Keevil et al. [6] for Neisseria gonorrhoeae.

3. METHODS

3.1. Bacterium and medium
The Hib strain used was 760705 (RM7004) [7]. A modification of the medium of Catlin [8] was used to obtain cystine limitation. The complete medium was made up from stock solutions, double-distilled water (DDW) being used as the diluent. Salts (Analar grade) were obtained from BDH and all other compounds from Sigma. Cellulose acetate filters (0.22 µm, Costar) were used for sterilisation unless indicated. Stock 1: L-aspartic acid 5 g; L-glutamic acid 13 g; sodium chloride 58 g; potassium sulphate 10 g; magnesium chloride hexahydrate 2 g; calcium chloride dihydrate 0.28 g; EDTA 0.04 g and ammonium chloride 0.10 g; made up to 1 l with DDW (pH 7.4), autoclaved and stored at room temperature. Stock 2: di-potassium hydrogen orthophosphate trihydrate 22.8 g and potassium dihydrogen orthophosphate 13.6 g; made up to 1 l with DDW (pH 7.4), autoclaved and stored at room temperature. Stock 3: sodium acetate 34 g; L-arginine 1.5 g; glycine 0.25 g; L-isoleucine 0.3 g; L-leucine 0.9 g; L-valine 0.6 g; L-alanine 1 g; L-lysine monohydrochloride 0.5 g; L-tryptophan 0.8 g; L-threonine 0.5 g; L-phenylalanine 0.25 g; L-asparagine 0.25 g; L-glutamine 0.5 g; L-histidine 0.18 g; L-methionine 0.15 g; sodium hydrogen carbonate 0.42 g; choline 14 mg and myo-inositol 3.6 mg made up to 1 l with DDW (pH 7.4), filter-sterilised and stored at 4°C. Stock 4: calcium pantothenic acid 0.1 g; thiamine 0.1 g; NAD 0.1 g; made up to 20 ml with DDW, filter-sterilised and stored at −20°C. Stock 5: protoporphyrin IX 2 mg/ml; autoclaved and stored at 4°C. Stock 6: uracil 1% (w/v) in 0.1 M sodium hydroxide, filter-sterilised and stored at 4°C. Solution A consisted of 1 mg L-cystine, 7 mg L-tyrosine and 8 mg hypoxanthine dissolved in 80 ml of 1 M HCl. Complete medium consisted of 100 ml stock 1, 100 ml stock 2, 100 ml stock 3, 0.80 ml stock 4, 1 ml stock 5 and 2 ml stock 6 made up to approximately 850 ml with DDW followed by the addition of 5 g glucose and 0.2 g inosinc. When the latter two compounds had dissolved, solution A, 10 M NaOH (to adjust pH) and DDW were added to give a final volume of 1 l (pH 7.4).

3.2. Culture vessel and growth conditions
The culture vessel (Fig. 1) was made of borosilicate glass with the dimensions indicated and was
maintained at 37°C by means of a water jacket. A magnetic stirring bar (0.8 x 3.5 mm) on a magnetic stirrer (Gallenkamp, Loughborough, UK) at speed setting 8 provided aeration. Medium was pumped into the vessel from a reservoir bottle immersed in ice (to prevent decay of NAD) by a 101U peristaltic pump (Watson-Marlow, Falmouth, UK) via a Pasteur pipette mounted in an SQ13 fitting on the lid. Of the three remaining SQ13 fittings on the lid, two were sealed and the other connected to sterile air filter. With the conditions listed above a constant volume of 80 ml was maintained. Hib was grown overnight in batch culture in the culture vessel and the pump switched on at the desired flow rate. The volume of spent culture was recorded over the time course of each experiment to give an accurate measure of dilution rate. Dilution rates of 0.065, 0.095, 0.15, 0.20 and 0.28 h⁻¹ correspond to culture doubling times of 10.7, 7.3, 4.6, 3.5 and 2.5 h respectively. Cells were left for a minimum of seven volume changes and until steady state has been reached as judged by A600 readings. Steady-state cells taken directly from the culture vessel were centrifuged (8000 x g, 4°C, 20 min) and the pellet stored at -20°C until required.

3.3. Isolation of outer membranes and SDS-PAGE

Outer membranes were isolated by sarkosyl (2%) extraction of whole membranes and OMP profiles determined by SDS-PAGE on 12.5% acrylamide gels after silver staining using the Amersham (RPN.17) kit as described by Allan et al. [9]. Protein concentration was measured by the method of Stoscheck [10] using bovine serum albumin as the standard.

3.4. LPS analysis by SDS-PAGE and immunoblotting

Bacteria were resuspended to an A600 0.8 in 10 ml phosphate buffered saline (pH 7.4) and LPS obtained by the proteinase-K digestion method as described by Kimura and Hansen [11]. LPS was resolved by SDS-PAGE using a 15% (w/v) polyacrylamide-4 M urea gel and visualised by silver staining as described by Zamze and Moxon [12]. The reactivity of LPS to monoclonal antibodies (mAbs) 4C4, 12D9, 5G8, 7D7 and 9D8 (kindly provided by Dr. E.J. Hansen) was determined as described [13] except that mouse IgG-alkaline phosphatase conjugate (Sigma) was used as the secondary antibody and

Fig. 2. Effect of dilution rate on OMP profiles of Hib. Lanes A, 0.065 h⁻¹; B, 0.095 h⁻¹; C, 0.15 h⁻¹; D, 0.20 h⁻¹ and E, 0.28 h⁻¹. 20 µg of protein per lane. Nomenclature of Hib OMPs on the left. Molecular-mass markers (kDa) on the right.
development was by the method of Blake et al. [14].

4. RESULTS

4.1. Analysis of OMPs by SDS-PAGE

The effect of dilution rate on OMP profile of RM7004 grown under cystine-limitation is shown in Fig. 2. A similar OMP profile was found at all dilution rates used.

4.2. Analysis of LPS by SDS-PAGE and immunoblotting

The effect of dilution rate on LPS composition of RM7004, as adjudged by silver staining of proteinase-K whole-cell digests is shown in Fig. 3. There was a shift to a lower molecular mass form between $D = 0.095 \, \text{h}^{-1}$ and $0.15 \, \text{h}^{-1}$ with an accompanying change of reactivity to some mAbs (Table 1). There was reactivity to mAbs 4C4 and 7D7 at all dilution rates and to mAbs 12D9, 5G8 and 9D8 at $D \leq 0.095 \, \text{h}^{-1}$.

Table 1

<table>
<thead>
<tr>
<th>$D$ (h$^{-1}$)</th>
<th>mAb</th>
<th>12D9</th>
<th>5G8</th>
<th>7D7</th>
<th>9D8</th>
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<td>3+</td>
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<tr>
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3+ indicates a strong, 1+ a weak, and − no, reaction.

5. DISCUSSION

Previous information regarding the possible effect of growth rate on outer-membrane composition of Hib has come from comparison of log- and stationary-phase cells. Loeb and Smith [15] reported that the OMP profile of strain Eagan varied with the phase of growth, there being more minor proteins in stationary phase compared with log-phase grown cells. In contrast Barenkamp et al. [16] found that Eagan had the same OMP profile from early-log to early-stationary-phase grown cells. Qualitative differences in minor OMPs were observed by van Alphen et al. [17] between log- and stationary-phase grown cells of RM7004. Comparison between these studies is difficult since bacteria were grown on complex media in batch culture. Changes in OMP profile between log- and stationary-phase cells may be due to differences in nutrient composition or oxygen-limitation (stationary phase) rather than growth rate. In this study, under cystine-limitation, growth rate per se did not appear to affect the expression of the major or minor OMPs of Hib over the range of dilution rates used.

In contrast, a clear change in LPS composition and antigenicity with dilution rate was observed with a lower molecular mass form present at $D \geq 0.15 \, \text{h}^{-1}$. Kimura and Hansen [11] showed that antigenic variants of two Hib strains with the LPS phenotype 4C4 + 5G8 − had LPS with a greater electrophoretic mobility than 4C4 + 5G8 + variants upon SDS-PAGE analysis. Similarly, in this study, the LPS of greater electrophoretic mobility was 4C4 + 5G8 − and that of lesser mobility 4C4 + 5G8 +. With the exception of mAb 4C4 which recognises α-galactosyl-1,4-β-galactose [13], the epitopes that are recognised by the mAbs are unknown. However 12D9 and 5G8 are believed to recognise epitopes in the core region of LPS [18]. That LPS structure can alter with dilu-
tion rate has been documented for other species including *Pseudomonas aeruginosa* [19], *Escherichia coli* [20], with strains containing smooth LPS, and *Neisseria gonorrhoeae* [21] which has a rough form of LPS like that of *H. influenzae* [22]. Morse et al. [21] found that quantitatively less LPS was produced at low dilution rate (D = 0.12 h⁻¹) compared to a high dilution rate (D = 0.56 h⁻¹) when cells were grown under glucose limitation. The decrease in LPS was associated with an increase in sensitivity to the bactericidal activity of normal human serum, an increase in cell hydrophobicity and an increase in access to a mAb recognising an epitope in the core region of LPS. However the 4C4 + 5G8 + genotype in Hib was associated with enhanced virulence in rats, as measured by the number of animals bacteraemic after intra-nasal or intra-peritoneal infection, compared to a 4C4 + 5G8 − antigenic variant [11]. Whether the changes in LPS composition observed in this study would have any affect on the ability of Hib to cause invasive disease remains to be determined.

This study has established that changes in outer-membrane components of Hib can occur with growth rate per se. Further chemostat studies will be invaluable in determining the effect of growth rate and environment on the regulation of virulence factors and vaccine candidates as well as extending the scant knowledge that exists concerning the general physiology of Hib. Such information may lead to a greater understanding of the disease process.

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REFERENCES