The Dilution Rate Affects the Outer Membrane Protein and Lipopolysaccharide Composition of *Haemophilus influenzae* Type b Grown under Iron Limitation

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When *Haemophilus influenzae* type b was grown under iron limitation in continuous culture, the dilution rate affected the outer membrane protein and lipopolysaccharide composition. Investigations of the effect of the reduced availability of iron or other environmental parameters on these surface components should be controlled for growth rate.

Our laboratory is investigating the role of the outer membrane proteins (OMPs) and lipopolysaccharide (LPS) of *Haemophilus influenzae* type b (Hib) in virulence and their potential as vaccine candidates. There is a paucity of knowledge concerning the effects of environment and growth rate on the expression of these cell surface components. In vivo, only a small amount of iron is available for bacterial growth because of sequestration by host iron-binding proteins such as lactoferrin and transferrin (19). Comparison of Hib cells harvested directly from the intraperitoneal cavity of infected rats and cells grown under iron-restriction in vitro has led to the suggestion that Hib may produce iron-regulated OMPs (17), including transferrin-binding proteins (TBPs) (4), in vivo. OMPs of Hib with various apparent molecular masses have been reported to be expressed during conditions of iron restriction (3, 9, 10, 13, 20). Invariably, these studies were done with complex media in batch culture, with different iron chelators and with no control of growth rate. Such conditions make comparison between these studies difficult (5, 16). Therefore, we have investigated the effect of a limited iron supply on the regulation of Hib OMPs and LPS in continuous culture. The use of a chemostat allows the growth rate of the organism to be fixed while the environment can be altered, or conversely the growth rate can be varied with no change in the environment other than the concentration of growth-limiting substrate (11). A comparison with iron-replete (cystine-limited) cells was performed.

Hib 760705 (RM7004) (18) was grown under cystine limitation in continuous culture in a modification of the defined medium of Catlin (2) at pH 7.4 with constant aeration at 37°C as previously described (7). Iron limitation was obtained by raising the cystine concentration from 1 to 80 µg/ml and adding a 15 µM concentration of the iron chelator ethylenediamine-di-(*o*-hydroxy)-phenylacetic acid free from contaminating iron (12). All glassware was washed with concentrated hydrochloric acid and then with deionized water to remove adsorbed contaminating iron. Bacteria were harvested at steady state, after a minimum of seven volume changes, as determined by A_{500} . With iron limitation, use of dilution rates (D) of $\geq 0.15 \text{ h}^{-1}$, comparable to those used in a previous study with cystine limitation (7), resulted in washout. Dilution rates of 0.065 and 0.095 h^{-1} correspond to

culture doubling times of 10.7 and 7.3 h, respectively. Outer membrane preparations were obtained by sarcosyl extraction and OMP profiles determined on a sodium dodecyl sulfate (SDS)-polyacrylamide gel after silver staining (1). Protein concentration was measured by the method of Stoscheck (15) with bovine serum albumin as the standard. The OMP profiles of Hib grown under iron limitation at dilution rates of 0.065 and 0.095 h^{-1} and under cystine

FIG. 1. Effect of dilution rate on OMP profiles of Hib. Lanes: A, 0.065 h^{-1} (iron limitation); B, 0.095 h^{-1} (iron limitation); C, 0.095 h^{-1} (cystine limitation). The OMP profiles of cystine-limited cells were found to be the same for dilution rates between 0.065 and 0.28 h^{-1} (7). Each lane contains 20 µg of protein. Molecular mass markers in kilodaltons are shown on the left. The molecular masses of the indicated proteins are 12 kDa (■), 22 kDa (△), 26 kDa (□), 27 kDa (●), and 86 kDa (○). The 27-kDa protein in lane B is indicated by an arrow.

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FIG. 2. Effect of dilution rate on electrophoretic mobility of Hib LPS in SDS-PAGE. Lanes: A, 0.065 h⁻¹ (iron limitation); B, 0.095 h⁻¹ (iron limitation); C, 0.065 h⁻¹ (cystine limitation); D, 0.095 h⁻¹ (cystine limitation); E, 0.15 h⁻¹ (cystine limitation). Each lane contains 20 μ l of lysate. Only the lower part of the silver-stained gel is shown. Cystine limitation data are from reference 7.

limitation at 0.095 h⁻¹ are shown in Fig. 1. OMPs of 12 and 27 kDa were present at 0.095 h⁻¹ with both nutrient limitations but not at 0.065 h⁻¹ with iron limitation. A 22-kDa OMP was expressed strongly with cystine-limited cells but weakly with iron-limited cells. In a previous study, the OMP profiles of RM7004 grown under cystine limitation were found to be the same between 0.065 and 0.28 h⁻¹ (7). An OMP of 26 kDa was present only at 0.065 h⁻¹, and an 86-kDa OMP was present at 0.065 and 0.095 h⁻¹, under iron limitation. Thus, some OMPs appear to be regulated by the dilution rate under iron but not cystine limitation. Only the 26- and 86-kDa OMPs were unique to iron-limited cells. Mid-logarithmic-phase cells grown in iron- and cystine-replete defined medium expressed the 12-kDa OMP but not the 22-, 26-, 27-, or 86-kDa OMPs (8). *H. influenzae* RM7004 produces two TBPs with molecular masses of 90 kDa (TBP2) and 105 kDa (TBP1) (14). It is possible that the 86-kDa OMP found under iron limitation is the same as TBP2, although this possibility was not pursued further.

For LPS analysis, bacteria were resuspended to an A_{600} of 0.8 in 10 ml of phosphate-buffered saline (pH 7.4), and LPS was obtained by the proteinase K digestion method (6). LPS was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and silver stained (21) or electroblotted (7). The relative reactivity of electroblotted LPS to monoclonal antibodies (MAbs) was assessed visually as 3+ (presence of strongly reactive bands or diffuse regions), 1+ (presence of weakly reactive bands or diffuse regions), or - (no reactivity). Mouse MAbs directed against Hib LPS were kindly provided by E. J. Hansen (Department of Microbiology, University of Texas, Dallas). The effect of the dilution rate on the LPS composition of RM7004, as judged by silver staining of proteinase K whole-cell digests, is shown in Fig. 2. In a previous study with cystine-limited RM7004, the same LPS profile was obtained at dilution rates of 0.065 and 0.095 h⁻¹, although a shift to an LPS structure with a greater electrophoretic mobility was observed at ≥ 0.15 h⁻¹ (7). With iron limitation, the predominant band at 0.065 h⁻¹ had a greater electrophoretic mobility than the predominant band at 0.095 h⁻¹. At the latter dilution rate, profiles similar to those obtained with cystine limitation at 0.065 and 0.095 h⁻¹ were found. At 0.065 h⁻¹ with iron limitation, a slight reduction in staining intensity in comparison with the intensities at the same dilution rate (cystine limitation) and higher dilution rates (both nutrient limitations) was found, suggesting that less LPS was produced per cell under this growth condition. There were clear differences in MAb reactivity between the two nutrient limitations at comparable dilution rates (Table 1). In contrast to cystine-limited cells, no

TABLE 1. Effect of dilution rate on relative reactivity of Hib LPS^a with MAbs

Nutrient limitation	<i>D</i> (h ⁻¹)	Reactivity with MAb ^b :				
		4C4	12D9	5G8	7D7	9D8
Cystine ^c	0.065	3+	3+	3+	3+	1+
	0.095	3+	3+	3+	3+	1+
	≥ 0.15	3+	-	-	3+	-
Iron	0.065	3+	-	-	1+	-
	0.095	3+	-	-	3+	-

^a 20 μ l of lysate.

^b 3+, strong reaction; 1+, weak reaction; and -, no reaction (see text for details).

^c Data from reference 7.

reactivity occurred with MAbs 12D9, 5G8, and 9D8 with iron-limited cells at dilution rates of 0.065 and 0.095 h⁻¹. It remains to be determined whether the changes in LPS and OMP composition observed in this study have any effect on the ability of Hib to cause invasive disease. *Neisseria gonorrhoeae* grown under iron limitation ($D = 0.1$ h⁻¹) survived in subcutaneous chambers in guinea pigs, unlike cells grown under cystine limitation ($D = 0.1$ h⁻¹) (5). Kimura and Hansen (6) showed that antigenic variants of Hib strains with the 4C4⁺ 5G8⁺ phenotype had enhanced virulence in the infant rat model in comparison with the 4C4⁻ 5G8⁻ phenotype. However, the 4C4⁺ 5G8⁻ phenotype was associated with full virulence with one strain and low virulence with another.

In vivo, restricting the availability of free iron forms part of the host's defense strategy against infection. This study has shown that the OMP and LPS composition of Hib can change with the dilution rate under iron limitation. Further studies investigating the effect of the reduced availability of iron or other environmental parameters on these surface components should be controlled for growth rate.

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