A Selective Interaction Between Ferrous Ions and Lipopolysaccharide in Desulfovibrio vulgaris

By G. BRADLEY, C. C. GAYLARDE* AND J. M. JOHNSTON
Department of Biological Sciences, City of London Polytechnic, Old Castle Street, London E1 7NT, UK

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Lipopolysaccharide and protein material were released from cells of Desulfovibrio vulgaris on treatment with 10 mM-EDTA. Preincubation with up to 15 mM-ferrous ions caused increased removal of lipopolysaccharide from bacteria grown in iron-limited medium, but preincubation with calcium, magnesium, or zinc ions was unable to induce this response. Ferrous ions appear to have an important role in the stabilization in vivo of the outer membrane of D. vulgaris. The selective interaction between ferrous ions and D. vulgaris lipopolysaccharide may play a part in iron uptake.

INTRODUCTION

Desulfovibrio vulgaris is a Gram-negative, anaerobic sulphate-reducing bacterium, which has a high requirement for inorganic iron (Postgate, 1981). The cells associate with exposed iron surfaces and may cause costly corrosion problems in metal structures wherever anaerobic conditions arise. Much work has been performed on the chemical and electrochemical processes of anaerobic iron corrosion and several theories have been proposed to explain this action of Desulfovibrio (for a review, see Miller, 1971; Iverson, 1974; Iverson & Olson, 1983), but little or no work has been carried out on the interactions between iron and the Desulfovibrio surface at the molecular level.

It is well established that the lipopolysaccharide (LPS) located in the external leaflet of the outer membrane (OM) of Gram-negative bacteria can interact with various metal cations. This is important for membrane assembly (Galanos et al., 1977) and for the barrier function of the OM (Leive, 1974). Leive et al. (1968) have demonstrated that the metal cation chelator EDTA releases LPS and protein material from the surface of some Gram-negative bacteria. The divalent cation availability in the growth medium, together with the affinity of the LPS for these cations, determines in part the amount of LPS held in place in the OM by cationic binding. This, in turn, influences the fraction of LPS that can be released by EDTA treatment of whole cells. Lipopolysaccharides of enteric bacteria contain a number of potential cation-binding sites (Schindler & Osborn, 1979) having a high affinity for calcium and magnesium ions. The replacing of these metal cations is the proposed mode of action for various cationic antibiotics (Hancock, 1981) and for the development of resistance to these drugs by the synthesis of cation-replacing proteins (Nicas & Hancock, 1983).

In the studies reported here, we have compared the effects of preincubation with different divalent metal cations on the release of LPS by EDTA treatment, using cultures of D. vulgaris (Woolwich) grown in media of varying iron availability.

METHODS

Growth media and conditions. Desulfovibrio vulgaris Woolwich (NCIB 8457) was grown as 500 ml batch cultures at 30 °C. Media used were: Medium C (Postgate, 1981) modified by the omission of sodium citrate (C); Medium C

Abbreviation: OM, outer membrane.

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Fig. 1. Change in release of LPS by EDTA treatment of *D. vulgaris* after preincubation in metal cation solutions. Points represent mean values of at least three separate experiments. Cells grown in (a) iron-limited (C) medium, (b) iron-free (C-Fe) medium, and (c) iron-rich (C+Fe) medium. □, Iron; ○, calcium; Δ, magnesium; ▽, zinc.

including 0.25% (w/v) sodium citrate with a mild steel coupon (40 × 15 mm) added to provide a relatively iron-rich medium together with an exposed iron surface (C + Fe); Medium C modified by the omission of ferrous sulphate and sodium citrate providing a medium free of added iron (C-Fe). All media were adjusted to pH 7.5.

**Release of LPS.** Bacteria were harvested in late-exponential phase by centrifuging (3000 g, 30 min), washed in PBS (0.15 M-NaCl; 0.05 M-PO₄; pH 7.4) and resuspended in PBS to a density of approximately 10¹⁰ cells ml⁻¹. This suspension (2 ml) was added to tubes containing 0.5 ml 10 mM-ascorbate, the required volume of 200 mM-metal cation salt solution and distilled water to a final volume of 4 ml. Final concentrations of cations varied between 0.01 mM and 17 mM. Distilled water replaced cations in the control treatments. Salts used were: FeSO₄.7H₂O, CaCl₂.6H₂O, MgSO₄.7H₂O and ZnSO₄.7H₂O. Tubes were incubated at 0 °C for 15 min. Cells were then pelleted and resuspended in 2 ml PBS containing 10 mM-EDTA. After incubation at 45 °C for 15 min, the suspension was centrifuged and the supernatant was assayed for protein by the Lowry method using bovine serum albumin as a standard, and for LPS using the carbocyanine dye assay of Zey & Jackson (1973). LPS released was calibrated against standards of LPS extracted from *D. vulgaris* cultures. The change in LPS release was calculated by subtracting the control values from those obtained after ion treatment.

**LPS extraction.** Envelopes of *D. vulgaris* were isolated by resuspending washed cells in 20 ml ice-cold PBS and lysing by sonication (MSE Soniprep 150). Unbroken bacteria and debris were removed by centrifuging (5000 g, 10 min) and the supernatant was recentrifuged (30000 g, 20 min) to pellet the envelopes. LPS was extracted from this fresh preparation by the hot aqueous phenol procedure of Westphal & Jann (1965).

**Analytical PAGE.** SDS-PAGE was performed on slab gels containing 15% (w/v) acrylamide pH 8.6. Gels were stained using Page blue 83 (BDH, Coomassie blue equivalent) or the silver method of Morrissey (1981), modified by the use of 15% (w/v) acetic acid plus 10% (w/v) methanol as the fixative.

**RESULTS**

**Release of LPS by EDTA treatment**

Preincubation of cells in ferrous salt solutions caused a marked increase in the EDTA-induced release of LPS above that of the control (Fig. 1). This occurred for cells grown either in iron-limited (C) or in added iron-free (C - Fe) medium. Other divalent cations used (Zn²⁺, Ca²⁺ and Mg²⁺) were unable to mimic this effect, although there was a slight increase in LPS release from medium C-grown cells preincubated with Ca²⁺ at levels above 1.8 mM (Fig. 1a). Cells grown in iron-rich (C + Fe) medium (Fig. 1c) and all cells preincubated with Fe²⁺.
concentrations greater than 10 mM showed a gradual decrease in this response. Although up to 150 µg protein ml\(^{-1}\) was removed from the cells by EDTA treatment, no correlation was observed between this and the cation concentration (data not shown).

**SDS-PAGE analysis**

LPS extracted from cells by the aqueous phenol method could not be visualized by Page blue 83. However, the silver stain allowed the detection of three broad bands, the fastest being a dense black colour (Fig. 2). Identical bands were found in EDTA-released material, where again the fast dense black band was the most heavily stained. Numerous polypeptide bands were also revealed, both by silver and Page blue 83 staining, the major protein having an approximate molecular weight of 54000. No changes in the number of bands were noted with different ion concentrations, nor was there any difference between LPS extracted from cells grown in the various media (results not shown).

**DISCUSSION**

This study demonstrates an important interaction between ferrous ions and the LPS of *D. vulgaris in vivo*. A short incubation with ferrous ions increases the amount of EDTA-releasable LPS in *D. vulgaris* grown in iron-limited media. The inability of other cations, in particular Zn\(^{2+}\) which has a higher affinity for EDTA than ferrous ions (log\(_{10}\) stability constants 16·7 and 14·3, respectively), to mimic this effect indicates that the interaction is specific. The decrease in LPS release shown in iron-rich cultures and with ferrous ion preincubation concentrations above 10 mM in other cultures could be due to the binding of ferrous ions to molecules other than LPS, or to the precipitation of ions once LPS saturation is reached during preincubation. This would effectively reduce the amount of EDTA available for reaction with cation-bound LPS.
SDS-PAGE analysis using the silver stain shows that some of the material released by EDTA treatment is identical to LPS extracted from cell envelopes by the aqueous phenol method. The nature of the bands seen in the latter extract is unknown, but the slower running cluster (position 35 kDa, Fig. 2) bears some resemblance to that reported in Pseudomonas aeruginosa by Kropinski et al. (1982), who suggested that these bands correspond to the O antigen polysaccharide side chains. In addition to these LPS-associated bands, EDTA causes the release of other material which may also be visualized by Page blue 83. These bands correspond to some of those found in the purified OM of D. vulgaris (authors’ unpublished observations) and are, presumably, components released on partial breakdown of the membrane.

The possession of ferrous ion-selective sites in the LPS of D. vulgaris may aid the bacteria in the uptake of iron from their anaerobic environment. Although iron is more soluble in its reduced state (expected maximum concentrations from solubility products at pH 7 are $10^{-1}$ M for ferrous, $10^{-18}$ M for ferric ions), its precipitation as ferrous sulphides by the hydrogen sulphide produced during bacterial metabolism will lower its availability to the cells. Since Desulfovibrio has a high requirement for iron, the ability to bind ferrous ions selectively is obviously desirable. Alternative methods of iron uptake have been reported in other bacterial genera. Siderophores (Neilands, 1974) or other low-affinity uptake systems (Konisky, 1979) have been found in a number of cells and the possibility of their presence in Desulfovibrio should not be excluded. Induction, as well as repression, of certain Gram-negative OM proteins in response to iron limitation has been reported (McIntosh & Earhart, 1976; Meyer et al., 1979). Such iron-specific compounds have, as yet, not been detected in Desulfovibrio and this report is the first evidence of any molecular interaction between these bacteria and ferrous ions. Further work will be required to elucidate more fully the nature of the iron binding and its significance in Desulfovibrio.

REFERENCES


