SHORT COMMUNICATION

Evidence for the Presence of a Capsule in *Vibrio vulnificus*

By KAZUNOBU AMAKO,* KENJI OKADA AND SHUNJI MIAKE

Department of Bacteriology, School of Medicine, Kyushu University, Fukuoka 812, Japan

(Received 11 June 1984)

Vibrio vulnificus strain FCC, isolated from a patient with a wound infection, and reference strain ATCC 27562, were examined by electron microscopy for the presence of capsules. Both strains had a layer heavily stained with ruthenium red. The number of stained cells was high in strain FCC and low in strain ATCC 27562. The proportion of stained cells correlated with virulence against mice and with susceptibility to the bactericidal activity of normal human serum. Rapid freezing and substitution fixation, a mild method, revealed on the cell surface a fibrous layer of relatively low electron density, which we considered to represent a capsule.

INTRODUCTION

Vibrio vulnificus, a halophilic lactose-fermenter, causes serious wound infections or septicaemia which differ in many respects from the diseases caused by other *Vibrio* species (Blake *et al.*, 1979; Hollis *et al.*, 1976). The pathophysiology of the disease is not yet clear. To explain the pathological mechanisms of this disease several biological characteristics have been studied, e.g. resistance to the bactericidal activity of human serum (Carruthers & Kabat 1981; Tamplin *et al.*, 1983), the antiphagocytic effect on the human leucocyte (Kreger *et al.*, 1981), and the production of extracellular toxic material and some histolytic enzymes (Kreger & Lockwood, 1981; Smith & Merkel, 1982). These observations have suggested that *V. vulnificus* has surface components that resist host defence mechanisms.

In this paper we describe electron microscopical evidence for the presence of capsules by using ruthenium red staining and a newly developed rapid freezing and substitution fixation method.

METHODS

One of the strains of *V. vulnificus* was a clinical isolate from a patient with a severe wound infection (strain FCC) and the other was strain ATCC 27562, kindly donated by Dr I. Higasi of the Osaka City Institute of Public Health, Japan. These strains were cultured at 37 °C in 1% tryptone broth or on agar (Difco) supplemented with NaCl (3%, w/v).

For electron microscopy broth cultures were harvested by centrifugation (1000 g, for 30 min) and the pellet was processed for electron microscopy. For conventional thin sectioning the bacteria were fixed with 1% glutaraldehyde solution in 0.25 M-sodium cacodylate buffer (pH 7.2) and then with 1% osmium tetroxide prepared as by Ryter & Kellenberger (1958). They were then embedded in epoxy resin after dehydration with a graded series of ethanol concentrations (Spurr, 1969). Staining with ruthenium red was essentially as described by Luft (1970). Rapid freezing and substitution fixation was done as described previously (Amako *et al.*, 1983). Thin sections were made with a glass knife by a Porter-Blum type MT-2 ultramicrotome and examined by a JEOL 100C electron microscope after staining with uranyl acetate and lead citrate.

Virulence was tested by injecting serially diluted bacterial suspensions into the peritoneal cavity of ddY mice; after 5 d the LD₅₀ was calculated for each strain. Sensitivity of the strains to the bactericidal activity of human serum was tested by the procedure of Carruthers & Kabat (1981) using a pooled serum collected from healthy human volunteers. The bacterial suspension made from the broth culture (approximately 1×10^8 c.f.u.) in 0.35 ml phosphate-buffered saline and 0.65 ml of the pooled serum were mixed in small test tube and incubated at 37 °C for 30 min. At time 0 and 30 min after incubation, samples were withdrawn, diluted tenfold and plated on 3% NaCl agar plates for the viable cell count.

Fig. 1. (a) Thin-sectioned profile of *V. vulnificus* strain FCC fixed by the rapid freezing and substitution fixation method. The cell surface is covered with a fibrous layer of relatively low electron density. (b) Thin section of strain FCC stained with ruthenium red. The surface layer is heavily stained. (c) Thin section of strain ATCC 27562 stained with ruthenium red. Some cells are stained but others are not. Bars, 0.5 μ m.

RESULTS AND DISCUSSION

A typical thin-sectioned profile of strain FCC prepared by rapid freezing and substitution fixation is shown in Fig. 1(a). The surface of the cell was covered with a fibrous layer of relatively low electron density, about 60 nm thick in strain FCC. This layer stained heavily with

Table 1. Susceptibility of *V. vulnificus* to the bactericidal activity of normal human serum

See Methods for details.

Strain	10 ⁻⁷ × No. of surviving cells	
	0 min	30 min
FCC	8.2	6.8
ATCC 27562	17.2	2.3

ruthenium red (Fig. 1*b, c*), suggesting the presence of carbohydrates. Beneath the layer the typical structure of the cell envelope of Gram-negative bacteria was visible. We concluded that *V. vulnificus* has a capsule. As seen in Fig. 1(*b, c*), the layer was not stained as uniformly as might have been expected from the structure obtained by rapid freezing and substitution fixation. The stain was deposited in globular aggregates on the cell surface. This may have been a staining artefact, since condensation of capsular material during staining has been suggested (Costerton *et al.*, 1981).

Not all the cells stained with ruthenium red. The number of stained cells was high in strain FCC (60%) and low in strain ATCC 27562 (30%). The proportion of stained cells correlated with the virulence of the bacteria. For strain FCC the LD₅₀ in mice was 1.0 × 10⁶ and for strain ATCC 27562 it was 1.7 × 10⁷. The numbers of bacteria bearing the capsular layer counted under the electron microscope in sections prepared by rapid freezing and substitution fixation was higher in strain FCC than in strain ATCC 27562. We concluded that the unstained cells in preparations treated with ruthenium red were an indication of the presence of uncapsulated cells. The presence of capsules could explain some of the biological characters of this bacterium, such as serum resistance and the antiphagocytic effect. Strain ATCC 27562 was more sensitive to the bactericidal activity of normal human serum than strain FCC (Table 1). After 30 min incubation with normal serum 83% of the cells of strain ATCC 27562 were killed, compared with 17% for strain FCC.

We are grateful to Akemi Takade for his excellent technical assistance in electron microscopy.

REFERENCES

- AMAKO, K., MURATA, K. & UMEDA, A. (1983). Structure of the envelope of *Escherichia coli* observed by the rapid-freezing and substitution fixation method. *Microbiology and Immunology* **27**, 95-99.
- BLAKE, P. A., MERSON, M. H., WEAVER, R. E., HOLLIS, D. G. & HEUBLEIN, P. C. (1979). Disease caused by a marine vibrio. Clinical characteristics and epidemiology. *New England Journal of Medicine* **300**, 1-6.
- CARRUTHERS, M. M. & KABAT, W. J. (1981). *Vibrio vulnificus* (lactose-positive vibrio) and *Vibrio parahaemolyticus* differ in their susceptibility to human serum. *Infection and Immunity* **32**, 964-966.
- COSTERTON, J. W., IRVIN, R. T. & CHENG, K.-J. (1981). The glycocalyx in nature and disease. *Annual Review of Microbiology* **35**, 299-324.
- HOLLIS, D. G., WEAVER, R. E., BAKER, C. N. & THORNSBERRY, C. (1976). Halophilic vibrio species isolated from blood culture. *Journal of Clinical Microbiology* **3**, 425-431.
- KREGER, A. & LOCKWOOD, D. (1981). Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. *Infection and Immunity* **33**, 583-590.
- KREGER, A., DECHATELET, L. & SHIRLEY, P. (1981). Interaction of *Vibrio vulnificus* with human polymorphonuclear leukocytes: association of virulence with resistance to phagocytosis. *Journal of Infectious Diseases* **144**, 244-248.
- LUFT, J. H. (1970). Ruthenium red and violet. I. Chemistry, purification, method of use for electron microscopy and mechanisms of action. *Anatomical Record* **171**, 347-368.
- RYTER, A. & KELLENBERGER, E. (1958). Etude au microscope électronique de plasma contenant de l'acide deoxyribonucléique 1. Les nucléoides des bactéries en croissance active. *Zeitschrift für Naturforschung* **B13**, 597-605.
- SMITH, G. C. & MERKEL, J. R. (1982). Collagenolytic activity of *Vibrio vulnificus*: potential contribution to its invasiveness. *Infection and Immunity* **35**, 1155-1156.
- SPURR, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31-43.
- TAMPLIN, M. L., SPECTER, S., RODRICK, G. E. & FRIEDMAN, H. (1983). Differential complement activation and susceptibility to human serum bactericidal action by vibrio species. *Infection and Immunity* **42**, 1187-1197.