SHORT COMMUNICATION

An Extra Layer in the Surface Layers of an Atypical *Aeromonas salmonicida* Isolated from Australian Goldfish

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The surface layers of *Aeromonas salmonicida* strain 1107/1B, isolated from Australian goldfish, had an extra layer beyond the normal outer membrane of Gram-negative bacteria. When the bacteria were grown in the presence of lithium this extra layer was not observed. The extra layer may be an important factor in both adherence and virulence.

INTRODUCTION

Recently goldfish ulcer disease has become a problem in goldfish (*Carassius auratus*) culture (Elliott & Shotts, 1980). Typically, the disease develops from a superficial inflammatory skin lesion which progresses to an ulcer involving not only the skin but also the underlying muscles. Eventually, in severe cases, death results. If valuable breeding stock are affected the losses can be financially crippling. The causative agent of goldfish ulcer disease appears to be an atypical strain of *Aeromonas salmonicida* (Shotts *et al.*, 1978). An atypical strain of *A. salmonicida* was isolated from goldfish suffering from cutaneous ulcerative lesions from five locations in the United States of America, one location in England and one location in Japan (Elliott & Shotts, 1978). Since 1974, goldfish at a goldfish farm at Boolarra in Gippsland, Victoria, Australia, have been suffering from an ulcerative disease. When the lesions were sampled at an early stage of the disease atypical strains of *A. salmonicida* were isolated (Trust & Ashburner, 1980).

Normally, the surface layers of Gram-negative bacteria, as revealed by thin-section electron microscopy, consist of an inner trilaminar membrane (the plasma membrane), an outer trilaminar membrane, and some form of intermediate layer between these two unit membranes. This intermediate layer, the peptidoglycan, is mainly responsible for the mechanical strength of the cell wall. Occasionally, extra layers beyond the normal outer membrane have been reported (Glauert & Thornley, 1969). Virulent *A. salmonicida* isolated from furunculosis in salmon have an additional layer external to the normal cell wall, whereas avirulent strains lack this layer (Udey & Fryer, 1978). Duff (1937) reported that a bacterial strain identified as *Bacillus salmonicida*, which was isolated from furunculosis in salmonids, dissociated from R-type colonies to S-type colonies when grown in broth containing 0.25% (w/v) lithium chloride. The aim of the present work was to determine whether *A. salmonicida* isolated from ulcers in goldfish had an additional layer and whether an observed alteration in colony type with lithium treatment was associated with any alterations in the surface layers of the organism.

METHODS

Aeromonas salmonicida strain 1107/1B was isolated from goldfish from the fish hatchery at Boolarra (Trust & Ashburner, 1980). This strain has been demonstrated to be virulent for goldfish (H. Kalnins, unpublished observations). The bacteria were maintained on Trypticase Soy Agar (TSA, BBL) plates. The colonies on the TSA plates were large and friable. When touched they moved like ice hockey pucks across the plates. Lithium-treated bacteria were obtained by subculturing the *A. salmonicida* on TSA plates containing 0.25% (w/v) lithium chloride (TSA/Li), for up to five subcultures until aberrant colonies (one or two per plate) appeared. The aberrant colonies were smaller, butyrous and non-friable. They lacked the brown pigment typical of strain 1107/1B and no longer slid over the plates when touched. Bacteria from these aberrant colonies were again grown on TSA/Li plates. The bacteria were harvested from both types of plates and suspended in either isotonic saline (0.9%, w/v, NaCl), or a semi-synthetic complete liquid growth medium. The bacteria were pelleted by centrifugation at 14 000 g for 40 min at 4 °C.

Pellets of bacteria were fixed in a mixture of 2% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.2) containing 2.5 mM-CaCl₂ for 24 h at room temperature. The pellets were then cut into small blocks, washed overnight in 0.1 M-cacodylate buffer (pH 7.2) and post-fixed in 2% (w/v) osmium tetroxide in distilled water at 0 °C for 2 h. The blocks were dehydrated in an acetone series and embedded in TAAB embedding resin mixture C (TAAB Laboratories, Reading). Sections were cut on an LKB Ultratome III, stained with lead citrate for 5 min at 20 °C and examined with a Philips EM301 electron microscope, which had been calibrated with a line grating replica having spacings of 2160 lines mm⁻¹ (Polaron Equipment, London).

RESULTS

The surface layers of *A. salmonicida* strain 1107/1B when grown on TSA medium and suspended in growth medium consisted of a plasma membrane, an intermediate electron-dense layer, a corrugated outer membrane and an extra layer beyond the outer membrane and separated from it by an electron-lucent layer which may or may not contain material (Fig. 1a). The extra layer was electron-dense but not as dense as the intermediate layer, and it was not a unit membrane. It was approximately 10 nm thick and separated from the outer membrane by 10 to 40 nm. The extra layer was relatively smooth in appearance and in thin sections no substructure could be discerned.

When the bacteria were suspended in isotonic saline they became aggregated. In this state they appeared to be joined by contact of the outer visible limits of the extra layers (Fig. 1b). In zones of aggregation the extra layers appeared to merge, as no electron-lucent area was visible between them. Although in thin section the extra layers appeared continuous and no substructure could be observed, they are probably somewhat porous and capable of enmeshing with each other.

When the bacteria were grown in the presence of lithium the extra layer was no longer observable by thin-section electron microscopy (Fig. 1c). The surface layers consisted only of a plasma membrane, an intermediate layer and an outer membrane which still maintained a corrugated appearance. Blebs and vesicles of a trilaminar membrane were, however, observed. This membrane appeared to be due to the production of an excess of outer membrane by the bacteria, as some of the blebs were attached to the outer membrane. Other blebs and vesicles were detached and floating free in the culture medium.

DISCUSSION

Udey & Fryer (1978) demonstrated that virulent *A. salmonicida* isolated from furunculosis in salmon possessed an extra layer and that these virulent strains aggregated and were able to adhere to cultured fish tissue cells. They speculated that it was the extra layer which enabled the bacteria to agglutinate and to adhere to fish tissue cells. We have demonstrated that virulent *A. salmonicida* isolated from goldfish ulcer disease also have this extra layer. We have shown that when the bacteria agglutinate the extra layers adhere and appear to merge. We have not investigated the adherence of these bacteria to cultured cells or to fish skins, and so cannot comment on the importance of the extra layer in adherence. If goldfish ulcer disease

Fig. 1. Electron micrographs of *A. salmonicida*. The bar markers represent $0.5\ \mu\text{m}$.

(a) Strain 1107/1B grown on TSA and suspended in growth medium. Note the extra surface layer (arrowed).

(b) Strain 1107/1B grown on TSA and suspended in isotonic saline. Note the zones of aggregation.

(c) Cells from aberrant colonies grown on TSA with 0.25% (w/v) LiCl and suspended in growth medium. Note the absence of the extra layer and the blebs of outer membrane. Cells suspended in isotonic saline were of similar appearance.

results from an invasion of the skin from the external environment the ability of the bacteria to adhere either to the slime layer or the skin of the fish could be important.

Duff (1937) showed that *Bacillus salmonicida* when grown in the presence of lithium chloride changed colony type. We have shown that the aberrant colony type contains organisms that no longer have an extra layer in the surface layers of the bacteria. The loss of the extra layer was associated with an increased production of the outer membrane. We do not know how the presence of lithium inhibits the synthesis of an extra layer or why the outer membrane should be produced in excess when this happens. Naturally occurring aberrant colony types of this organism have been found not to cause experimental ulcer disease in goldfish (L. D. Ashburner, unpublished observations), but we have not had the opportunity to study such strains by electron microscopy.

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