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ULTRASTRUCTURE AND CHARACTERISTICS OF A DEEP-SEA BACTERIUM

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The ultrastructure of a bacterium, isolated from rusticles found on the wreck of the *Royal Mail Steamship (R.M.S.) Titanic*, was studied. The bacterium was rod-shaped, gram-negative and produced circular, off-white, opaque colonies on marine agar. Transmission electron microscopy revealed that the bacterium had a typical gram-negative cell wall structure; the nucleoid region was scattered throughout the cytoplasm and darkly stained inclusions were found in the cytoplasm. Negative staining illustrated the presence of 2-6 peritrichous flagella on the bacterium. This bacterial isolate may be part of transient consortia involved in the formation of the rusticles.

On a étudié l'ultrastructure d'une bactérie, isolée de concrétions de rouille en stalactite provenant de l'épave du *Royal Mail Steamship (R.M.S.) Titanic*. La bactérie, en forme de bâtonnet, était Gram négatif et produisait des colonies circulaires, blanc cassé et opaques sur de l'agar marin. La microscopie électronique à transmission a révélé que la structure de la paroi cellulaire de la bactérie était typiquement Gram négatif; la région du nucléoïde était dispersée dans le cytoplasme, caractérisé par des inclusions foncées. La coloration négative a révélé la présence de 2 à 6 flagelles péritriches. Cet isolat bactérien pourrait faire partie des consortiums transitoires qui contribuent à la formation des concrétions de rouille.

Introduction

In 1985, a group of research scientists led by Dr. Robert Ballard aboard the *Akademik Keldysh* succeeded in locating the site of the *R.M.S. Titanic* wreck. The ship was found in the North Atlantic Ocean at a depth of 3.8 km; it had undergone extensive corrosion and was covered by rusticles (Ballard 1987). The surrounding waters were well-oxygenated at a temperature of about 4°C.

During the *Akademik Keldysh* expedition to the *Titanic* wreck site, in 1991, rusticle samples were retrieved. Preliminary analysis of their structure and rate of formation indicated that these rusticles might be of biogenic origin (Ballard 1987, Stoffyn & Buckley 1992). Later work showed that they are highly complex, porous structures that support a wide range of microbial and higher life forms (Stoffyn-Egli & Buckley 1995, Wells & Mann 1997). Cullimore et al. (2002) reported that rusticular microbes may exist in the form of consortia: associations of microbial species that function synergistically. These consortia may be vital or transient depending upon whether the strains involved in their formation can function independently when removed from the consortium. Attempts have been made to isolate and identify organisms that might be involved in the formation of rusticles. Pure cultures of some of the bacteria from the *Titanic* rusticles have been studied and identified as members of *Leptothrix/Sphaerotilus* genera (Wells 1996).

In an extension of these studies we have used the transmission electron microscope to record the ultrastructure of a bacterium isolated and cultured from a *Titanic* rusticle sample.

Key words: deep-sea bacterium, gram negative, ultrastructure, *R.M.S. Titanic*, rusticles

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Materials and Methods

Rusticle collection and storage

Rusticle samples were collected during the Akademik Keldysh *expedition, in 1991, to the* R.M.S. Titanic *wreck site by using the articulating arm of the* Mir-2 *submersible. They were transferred to plastic collection bags, along with sea water and then transported aseptically to the surface (Low 1991, Stoffyn-Egli & Buckley 1995). Until 1993, they were stored under dark, vacuum-sealed conditions at 4°C at the Bedford Institute of Oceanography, Dartmouth, NS.*

Isolation of bacteria

The rusticle sample was aseptically removed from the collection bags and rinsed three times in sterile seawater. The rusticle was then dissected aseptically and a portion removed from the interior used to streak plates of #2216 marine agar medium (DIFCO Laboratories, Detroit) for isolation and enumeration of the heterotrophic, halophilic bacteria found in conjunction with the rusticle. These plates were incubated at 4°C±2°C and the bacterial isolates obtained were cultured and stored in 50% glycerol at -20°C. These stock cultures were sub-cultured periodically to ascertain whether contamination had occurred. In 2000, one of the bacterial stocks was re-cultured on #2216 marine agar and broth for this study. Colonial morphology was described after 24-48 h growth on marine agar. Cultures from the marine broth and agar were examined for general morphology and gram staining reactions (Benson 1998) using a light microscope.

Acidity, temperature and salt-tolerance growth ranges

To establish the range of the bacterium's growth at different pH values, marine broth solutions of pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were prepared and growth was monitored at 27 °C. The temperature range was determined with marine broth samples kept at 4 °C, 15 °C, 27 °C, and 37 °C. Salt concentrations of 0.26%, 2.2%, 8.0%, 14.5%, 20.0% and 32.0 % (wt/vol) were prepared by adding NaCl to the 0.01 M glucose plus basal salts medium of Vreeland & Martin (1980) to ascertain the salt tolerance limit of the bacterium at 27°C. Bacterial growth in all trials was monitored by recording the optical density at 660 nm using a 4040 Novaspec II spectrophotometer.

Electron microscopy protocol

Bacterial cells were harvested from the late stationary phase by centrifugation at 15000 g for 5 minutes. Primary fixation with 2.5% glutaraldehyde in sodium cacodylate buffer for 2h at 4°C was followed by washing in buffer, then postfixation in 1% osmium tetroxide for 2h, followed by staining in 0.25 - 0.5% uranyl acetate for 24h. The cell preparation was then dehydrated with acetone and embedded in Epon Araldite resin (4.6g Araldite, 6.1g TAAB 812, 11.9g DDSA, 0.5g DMP30). Thin sections were cut using a Reichert Jung Ultracut E ultramicrotome. Silver and gold sections (60-90 nm) were picked up on uncoated copper grids. Double staining was performed using lead citrate for 10 minutes and uranyl acetate for 4 minutes. Sections were observed using a Phillips 300 Transmission Electron Microscope at 60 eV; micrographs were obtained with fine grain positive film and printed on resin coated Kodak paper.

Negative staining

Unfixed bacterial cells were placed on formvar coated copper grids and allowed to settle. The grids were then covered with uranyl acetate stain for 30 secs and excess stain was removed. The grids were air-dried for 10-15 minutes and observed using the transmission electron microscope.

Results

Colony characteristics, morphology and gram staining

On marine agar, the cells produced circular, smooth surfaced, raised, off-white, opaque and glistening colonies. Under the light microscope, the bacteria appeared as long and short rod shaped single cells and also formed chains of 2-6 cells. The bacteria were gram-negative.

Acidity, temperature and salt-tolerance growth ranges

The bacterium showed growth in the pH range from 6.0 to 9.0 and no growth at pH values of 4.0 and 5.0. Maximum growth was observed at pH 8.0 and 9.0. The bacterium grew over the temperature range 4 °C to 37 °C; the maximum occurred at 27 °C. The bacteria showed growth at NaCl concentrations of 0.26%, 2.2%, 8.0%, 14.5% and 20.0% (wt/vol) with no growth at 32% NaCl; at 20% NaCl, the growth was very slow. Maximum growth occurred at NaCl concentrations between 0.26 and 8.0% NaCl.

Ultrastructure

Well-preserved bacterial cells were obtained by the double fixation procedure. At a low magnification (14,000X), transverse, oblique and longitudinal cross sections of the bacterial cells were observed. The bacterium ranged in size from 2-4 μm in length and 0.3-0.5 μm in width.

Three distinct layers were visible in the cell envelope (Fig. 1). The outermost layer, the outer membrane (om) was electron dense and double layered, varying between 8 and 10 nm in thickness. A thick (16-20 nm) lightly stained layer, the periplasmic space (ps), occurred on the inner side of the outer membrane. The innermost layer, the cell membrane (cm) was also electron dense and varied in size from 13-15 nm. These 3 layers are typical of gram-negative cell wall structures.

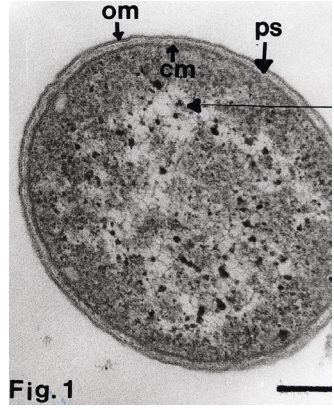

Fig 1 Ultrathin section of the rusticle bacterium showing three distinct layers of the cell envelope: dense outer membrane (om), middle light zone periplasmic space (ps) and inner dense cell membrane (cm). The lightly stained nucleoid region designated nr showing the nucleoplasm containing what may be dense thin DNA fibrils and the cytoplasm containing numerous ribosomes. Bar = 0.2 μm.

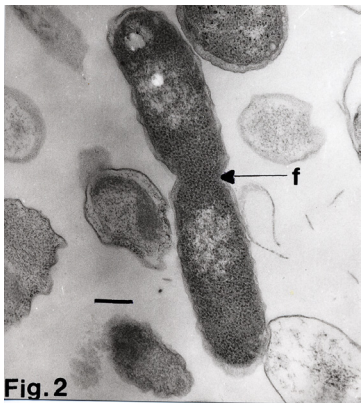

Fig 2 Thin-section transmission electron micrographs showing cells of the rusticle bacterium undergoing division; note the furrowing (f) of cell envelope. Bar = 0.3 µm

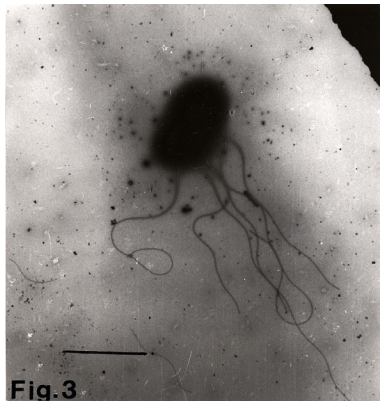

Fig 3 Negative stained (uranyl acetate) cells of the rusticle bacterium showing flagellar arrangements. The lengths of the flagella varied between 9-12 µm and their diameters ranged between 12-22 nm. Bar = 1.6 µm

The nucleoid region (nr) appeared lightly stained (Fig. 1). It was scattered in the cytoplasm and was not restricted to the center of the cell. Dense and thin fibrils were seen in this region and may represent the deoxyribonucleic acid (DNA). Ribosomes were observed as dark almost circular spots spread throughout the cytoplasm. The size of these ribosomes ranged from 14-18 nm.

Fig. 2 shows a cell which is in the process of cell division. The cell undergoing division was elongated along the longitudinal axis and showed two distinct nucleoid regions. Large numbers of ribosomes were visible in the cytoplasm. Cell division seemed to occur via transverse fission; invagination of the cell envelope appeared to start near the center of the cell and proceed inwards.

Apart from the ribosomes, a large number of darkly stained inclusions of varying sizes were observed in the cytoplasm. Others had a more regular circular or ellipsoid contour; these may be a kind of volutin granule composed of polyphosphate or poly-_-hydroxy butyrate. Histochemical and immunocytochemical tests are needed to determine their molecular composition.

Negative staining

Negative staining illustrated the presence of flagella on the bacterium (Fig. 3); varying numbers of flagella (2-6) were found. These unsheathed flagella were 9-12 µm in length and 12-22 nm in diameter. Some of the flagella had detached and were lying free in the stain. At higher magnification, the points of origin of the flagella were observed and it was found that these were not restricted to any particular region of the bacterial cell. They appeared to arise from lateral and polar ends of the bacterium, i.e. they were peritrichous.

Discussion

The complex structures of the rusticles, found on the *Titanic*, with their goethite-dominated mesh-like matrix, iron-plate like structures, water channels, ducts and thread-like spans, provide various sites with a range of physicochemical conditions suitable for the growth of diverse and site-focused microbes (Stoffyn-Egli & Buckley 1995, Mann 1997, Pellegrino & Cullimore 1997, Wells & Mann 1997). Stoffyn-Egli & Buckley (1995) identified a wide array of mineral precipitates within the rusticles that are stable at very different oxidation-reduction potentials and pH conditions (6.0-8.2). In addition, the restricted flow of water within some of the structures creates higher salt concentrations than occur in the surrounding seawater (Brown 1997). Since the isolate studied has a wide pH and salt tolerance range, it would be able to survive in a number of different formations within the rusticles. The rod-shaped formations covered by lepidocrocite found in the outer surface of the iron-oxyhydroxide shell (Stoffyn-Egli & Buckley 1995, Wells & Mann 1997) are very similar in size and shape to the isolate. They look like solid chains of bacteria with iron deposits around their cells. Furthermore, the *RMS Titanic* rests in highly oxygenated waters that would support this aerobic bacterial isolate.

According to Cullimore et al. (2002), different species within the rusticular consortium perform different activities which support the survival of the consortium and in turn, the growth of the rusticles. Since the isolate studied was successfully cultured in the laboratory, it lends support to the consideration that the rusticles are made by transient consortia containing species that may have arrived via the water column (Simon et al. 2002) or surrounding waters (Takami et al. 1997) and attached to the *Titanic* or the rusticles and helped in their growth.

The halotolerant nature of the isolate studied suggests its suitability for inclusion in a couple of bacterial families: *Halobacteriaceae* and *Halomonadaceae*. Its typical gram-negative cell wall structure and its ability to grow even at low salt concentrations (0.26%) however, precludes its inclusion in the family *Halobacteriaceae*. The family *Halomonadaceae* appears to have more characteristics that are closely related to those of the isolate studied. The type genera for this family, *Halomonas* (Holt 1984), is rod shaped, gram-negative, motile with 4-7 unsheathed lateral or polar flagella, halotolerant, forms white to yellow colonies and is facultatively anaerobic. Our isolate shows most of these characteristics. Thus, while it is possible that this bacterium belongs to the family *Halomonadaceae*, our results are not adequate to show its exact identity.

Conclusion

The isolate studied has characteristics suitable for its survival within the rusticles of the *Titanic*. It may play a role in the formation of these rusticles or it may be a species simply occupying a niche within the structural formations of the rusticles. Further investigation into its characteristics and nutritional capabilities are needed to provide a clearer understanding of the role of this isolate.

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References

Ballard RD (1987) The discovery of the *Titanic*. Warner Books, New York

Benson HJ (1998) Microbiological applications: Laboratory Manual of General Microbiology, 7th ed. The McGraw-Hill Companies Inc, Dubuque, Iowa

Brown M (1997) The identification of a bacteria pure cultured from and analysis of the elemental and structural composition of a rusticle taken from the *R.M.S. Titanic*. BSc Thesis, Dalhousie University, Halifax

Cullimore DR, Pellegrino C, Johnston L (2002) *RMS Titanic* and the emergence of new concepts on consortial nature of microbial events. Rev Environ Contam Toxicol 173:117-141

Holt JG (ed) (1984) Bergey's Manual of Systematic Bacteriology, vol 1, 1st ed. Lippincott Williams and Wilkins, Baltimore

Low S (1991) Titanica. IMAX Film, Imax Corporation/TMP (1991) I Limited Partnership

Mann H (1997) A close-up look at the *Titanic's* rusticles. Voyage 25:47-48

Pellegrino C, Cullimore DR (1997) A study of the bioarcheology of a physically disrupted sunken vessel. Voyage 25:39-46

Simon M, Grossart HP, Schweitzer B, Ploug H (2002) Microbial ecology of organic aggregates in aquatic ecosystems. Aqua Microb Ecol 28:175-211

Stoffyn P, Buckley DE (1992) The *Titanic* 80 years later: Initial observations on the microstructure and biogeochemistry of corrosion products. In: Bailey GW, Bentley J, Small JA (eds) Proc 50th Ann Mtg Electron Microsc Soc Amer. San Francisco Press Inc, San Francisco, p 1330-1331

Stoffyn-Egli P, Buckley DE (1995) The micro-world of the *Titanic*. Chem Br 31:551-553

Takami H, Inoue A, Fuji F, Haricot K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279-285

Vreeland RH, Martin EL (1980) Growth characteristics, effects of temperature, and ion specificity of the halotolerant bacterium *Halomonas elongata*. Can J Microbiol 26:746-752

Wells W (1996) Microbiology and formation of microbially assisted rusticle from the *R.M.S. Titanic*. BSc Thesis, Saint Mary's University, Halifax

Wells W, Mann H (1997) Microbiology and formation of rusticles from the *R.M.S. Titanic*. Resour Environ Biotechnol 1:271-281

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