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Environmental persistence of *Pseudomonas aeruginosa* and *Burkholderia multivorans* in sea water: preliminary evidence of a viable but non-culturable state

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Cystic fibrosis (CF) is the most common inherited fatal disease in persons of a white and European background, and currently affects approximately 30,000 adults and children in the USA.[1] The defective gene carrying the mutation responsible is carried by one in every 31 Americans (one in 28 Caucasians), which equates to more than 10 million symptomless carriers of the defective gene.[1] It is an autosomal recessive condition whereby two alleles carrying a polymorphism in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene phenotypically manifest the disease state through a variety of multi-organ problems associated with a pharmacological dysfunction to regulate anion (chloride) secretion across cell membranes.

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The most common complication of CF is the recurrence of chronic chest infection usually caused by bacterial pathogens.[2] Cystic fibrosis patients continue to suffer from recurrent and chronic respiratory tract infection and most of their morbidity and mortality is due to such infections throughout their life.[3] These infections are usually dominated by Gram-negative organisms, especially pseudomonads such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. However, modern antibiotic management using improved antimicrobial agents, such as the aminoglycosides and carbapenems, means that CF patients have an improved survival, resulting in more adults in employment.

Water has been documented as an important environmental source of *P. aeruginosa*[4] and has been associated with various clinical episodes of infection, including mainly dermatological infections[4] and otitis externa.[5] Acquisition of *P. aeruginosa* is particularly important for patients with respiratory disorders such as CF and bronchiectasis, as chronic colonisation with this organism has been shown to lead to a poor prognosis.[6] Hence, it is important to establish environmental and clinical reservoirs of this organism, as well as the survival dynamics of Gram-negative pathogens in such environments.

As many CF patients question their healthcare professionals about where they might acquire *P. aeruginosa* and respiratory pathogens in the *Burkholderia cepacia* complex, and how such pathogens survive in these environments, it is the aim of this study to examine the survival of three important Gram-negative bacterial pathogens (*P. aeruginosa*, *B. multivorans* and *B. cenocepacia*) in sea water over a one-year period.

Three Gram-negative organisms were employed in this study, namely *P. aeruginosa* (NCTC 10662), *B. multivorans* (formerly *B. cepacia* genomovar II) and *B. cenocepacia* (formerly *B. cepacia* genomovar III). The *Pseudomonas* isolate was a reference strain (NCTC 10662) and was obtained from the National Collection of Type Cultures, Health Protection Agency, Colindale, London, and the *Burkholderia* isolates were obtained from the sputum of adult CF patients. The identity of all isolates was confirmed initially using the phenotypic API 20NE identification scheme (bioMérieux, France), as well as by molecular techniques including 16S ribosomal DNA (rDNA) polymerase chain reaction (PCR) and automated sequencing, as described previously.[7] All isolates were subcultured on Columbia agar base (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood (Oxoid SR0048), and incubated at 37°C for 48 h.

Fresh natural sea water was obtained from Strangford Lough, Co. Down, Northern Ireland (54.591° N 5.68° W), courtesy of Dr. Niall McDonough, Marine Biology Research Institute, Queen's University of Belfast, Portaferry, Co. Down. The sea water was sterilised by filtration through a 0.2 µm cellulose nitrate membrane filter and aliquoted aseptically into 3 x 25 mL volumes in plastic sterile universal containers (Sterilin, UK), for individual inoculation with the three organisms. Each sea water microcosm was inoculated with approximately 10^4 colony-forming units of each organism and was incubated at approximately 18°C in natural sunlight for 12 months. After this period, 20 µL of

each sea water/organism microcosm was plated on Columbia agar base (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood (Oxoid SR0048) and incubated for up to five days at 37°C.

In addition, each sea water sample was centrifuged at 13,000 x*g* for 5 min and the supernatant was discarded. Assessment of bacterial viability was also performed on each sea water pellet using the BacLight bacterial viability assay (Invitrogen, UK), following the manufacturer's instructions. Each sea water pellet was enriched in non-selective nutrient broth medium (Oxoid CM0001) and incubated at 37°C for up to five days. The broth was them plated, as detailed above. The identity of resulting colonies from any source was confirmed by the API 20NE scheme, following the manufacturer's instructions.

Culture results yielded viable culturable growth of *P. aeruginosa*, but not *B. multivorans* or *B. cenocepacia*. Use of the BacLight bacterial viability assay demonstrated the presence of viable cells of *P. aeruginosa* and *B. multivorans*, but not *B. cenocepacia*. However, it was not possible to culture the *B. multivorans* isolate in non-selective enrichment broth, even though the differential uptake of the viability dyes indicated that a significant proportion of the *B. multivorans* organisms remained viable.

P. aeruginosa is a Gram-negative organism that is highly adapted to survival in the environment. Although there is no reported data on the tolerance of CF-associated strains of *P. aeruginosa* to NaCl, this organism is capable of survival and proliferation in elevated salt concentrations, as observed in the airway surface fluid; hence, it may have the ability to tolerate the marine environment, which on average has a salinity of 3.5% (0.6 mol/L NaCl) and a mean alkaline pH range of 7.5–8.4. If nitrates are available instead of oxygen, or if the amino acid arginine is available, *P. aeruginosa* can also grow anaerobically. Utilisation of nitrates could also explain the ability of *P. aeruginosa* to survive in many environments. The production of proteases and the anaerobic exploitation of their end-products, particularly arginine, might enable *P. aeruginosa* to initiate infections in parts of the body where little or no molecular oxygen is available.[8]

Another reason for the organism's ability to survive in the environment such as water amenity sites is because it is an excellent scavenger of essential nutrients. Basic properties of *P. aeruginosa*, including those that distinguish it from other *Pseudomonas* spp., probably contribute to its environmental persistence and ultimately to its role as an opportunistic pathogen.[8] Furthermore, the recently sequenced large genome of *P. aeruginosa* (approximately 6,264,404 bp), combined with the other available genome sequence (PA14, Harvard Medical School) and the six forthcoming additional genome sequences (Liverpool epidemic strain, 2192, pacs416, pacs5296, PA7 and C3719) allows for multiple open reading frames intrinsically associated with its environmental survival and ability to catabolise complex organic molecules.

Cystic fibrosis patients derive therapeutic benefit from swimming, as this promotes clearance of sputum from colonised/infected lungs. To date, there has been no evidence linking recreational/social or therapeutic activities in contaminated sea water with colonisation of the respiratory tract in CF patients. This may be due to a lack of archived isolates from water and CF patients that would allow typing studies to examine their genetic relatedness.

Hence, further work is now required to examine the genotypic relatedness of *P. aeruginosa* from sea water sources and those infecting patients with CF, to discover if marine-related environmental sources are important potential reservoirs of infection with this organism. However, it is more likely that CF patients would be exposed to, and infected with, *P. aeruginosa* via freshwater aerosols than through sea water contact, given the frequency of exposure to such freshwater aerosols during daily living.

Demonstration of cells of *B. multivorans*, which appeared viable by viability staining combined with microscopy, but which were unable to be cultured successfully, is of interest to the dynamics of survival of this species in the environment. This suggests the existence of a dormant or resting form of *B. multivorans*, possibly due to elevated NaCl concentration or to nutrient deprivation. Dormant forms have been described for many food- and waterborne pathogens, including *Listeria monocytogenes*,[9] *Vibrio chlorera*,[10] *V. vulnificus*,[11] *Mycobacterium tuberculosis*,[12] *Francisella tularensis*,[13] *Helicobacter pylori*,[14] *Salmonella typhi*,[15] *Escherichia coli*,[16] *Legionella pneumophila*,[17] as well as in *Campylobacter* spp.[18]

The concept of bacterial dormancy is not new to bacteriology. As early as 1952, Bissett wrote that "...nearly all forms of bacteria have resting cells or specialised distributive stages", which he termed "microcysts".[19] Various genera (including *Pseudomonas* and *Proteus*) have been described as producing microcysts in response to nutrient limitations.[20] Roszak *et al*.[21] described a viable but non-recoverable stage for *Salmonella enteritidis* in river water microcosms, and Turpin *et al*.[22] described a viable but non-culturable *Salmonella* sp. in soil.

Sussman and Halvorson[23] defined dormancy as "any rest period or reversible interruption of the phenotypic development of an organism", and further characterised dormancy into two types (i.e., constitutive and exogenous). Constitutive dormancy as typified by endospore formation in *Clostridium* spp. and *Bacillus* spp. may be triggered by the environment, but it is an innate property of the cell and is under strict genetic regulation. In contrast to this, exogenous dormancy, which is not as clearly understood as constitutive dormancy, may be seen in the enteropathogenic campylobacters.

Jannasch[24] proposed that, as a survival mechanism, organisms had the ability to become temporarily inactive (non-culturable) below a threshold substrate concentration. A study of a nutrient-starved strain of *Klebsiella aerogenes* demonstrated that only 20% of the population was viable after 24 h by culture techniques, although the remaining 80% of the population was still intact and responsive to mild changes in the medium composition.[25] As a result of such work, the 'pseudosenescent' state was proposed in which bacteria lose the ability to multiply as a result of certain stresses but remain completely functional as individual units – the so-called viable but non-culturable state.

The possible existence of such a resting stage for *Burkholderia multivorans* would therefore be not unusual. This organism would suffer from nutrient deprivation as well as environmental stress, and therefore the ability to produce a physiological resting stage would be advantageous for survival until more favourable conditions prevailed. In the clinical context, this condition could be brought about by antibiotic use, in that organisms might be lethally or sub-lethally stressed due to the presence of an antibiotic agent to which the organism was sensitive.

This can be a major problem in clinical microbiology, as significant pathogens can go undetected in non-selective culture media because the clinical specimen had been taken after the initiation of antibiotic therapy. In such cases, it can be difficult to isolate bacterial pathogens because they are non-culturable but may remain viable. In these cases, organisms are not thought to develop any elaborate resting stages, but may actively promote mechanisms of antibiotic resistance, such as point mutations (*gyr*A) and efflux pumps.

Thus, additional studies are required with larger numbers of isolates within these species to examine different aqueous environments, including tap water and fresh (river/lake) water, in order to fully elucidate the survival dynamics of these pathogens in such environments.

In conclusion, this short study demonstrates that *Pseudomonas aeruginosa* is capable of survival in sea water for prolonged periods of up to a year, whereas isolates of *B. multivorans* and *B. cenocepacia* could not be cultured after this period. Use of the bacterial viability assay indicates that, although non-culturable, cells of *B. multivorans* remained viable, as determined by the uptake of viability dyes, possibly indicating the presence of a viable but non-culturable (VNC) state for *B. multivorans*. □

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Enhancement of diaminobenzidine staining of chorioretinal specimens by cobaltous ions

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Chorioretinal specimens normally contain melanin, which is dark brown in colour. Diaminobenzidine (DAB) is a widely used substrate for immunohistochemistry and lectin histochemistry, as it is insoluble in alcohol and other organic

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