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# Sphingomonads from marine environments

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***Sphingomonas*** **species play an important role in the ecology of a range of marine habitats. Isolates and 16S-rRNA clones have been obtained from corals, natural and artificial sources of marine hydrocarbons and eutrophic and oligotrophic waters, and have been isolated as hosts for marine phages. In addition they are found in oceans spanning temperature ranges from polar to temperate waters. While less is known about marine sphingomonads in comparison to their terrestrial counterparts, their importance in microbial ecology is evident. This is illustrated by, for example, the numerical dominance of strain RB2256 in oligotrophic waters. Furthermore, the known marine sphingomonads represent a phylogenetic cross-section of the** ***Sphingomonas*** **genus. This review focuses on our present knowledge of cultured isolates and 16S-rDNA clones from marine environments.**

**Keywords:** marine; *Sphingomonas*; phylogeny; oligotroph

## Introduction

Prior to 1990, sphingomonads were typically described as *Pseudomonas* and *Flavobacterium* spp. In 1990, the genus *Sphingomonas* was first described by Yabuuchi *et al* who defined the chemotaxonomic and phylogenetic characteristics of the genus and four species [38]. Since then, numerous new *Sphingomonas* spp have been described (reviewed in Ref. [39]). Many of the *Sphingomonas* spp or related genera have been isolated from soils and deep subsurfaces, and much attention has been focused on their ability to degrade xenobiotic compounds, particularly aromatic hydrocarbons. In comparison to terrestrial environments, little is known about the presence or significance of sphingomonads in marine environments.

This paper describes the ecology and physiology of marine sphingomonads. For these purposes, 'marine' has been interpreted as any environment associated with the sea including the water column and sediment. The description of marine as stated by ZoBell [40] and reviewed by Larsen [16], which describes a marine microorganism as requiring salt, will not be used. This is due to the limitations it places on microorganisms that have been isolated from the ocean that do not have an obligate requirement for NaCl (eg *Sphingomonas* sp strain RB2256 [6]).

Most marine sphingomonads have been described in only one or two research papers and only one isolate, strain RB2256, has been studied in any detail. As a result, the coverage in this review reflects the available information. However, while the extent of knowledge is limited, the potential importance of sphingomonads in marine environments should not be underestimated. Their importance is highlighted by, for example, reports of their numerical dominance in oligotrophic regions, their role in coral diseases and their involvement in hydrocarbon degradation at polluted sites.

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## Coral pathogens

Based on its metabolic properties using Biolog GN plates and 16S-rDNA sequencing, a new species of *Sphingomonas* has recently been identified as the pathogen causing disease in corals [23]. As a coral pathogen the *Sphingomonas* strain was unusual in that it caused tissue damage to at least 16 varieties of corals and hydrocorals, in addition to *Dichocoenia stokesi*, from which it was originally isolated from in the Florida Reef Tract in 1995. In addition, the pathogen caused a high rate of tissue damage (up to 2 cm per day) and the progression of the disease initiated from the base of the coral. The virulence of the strain was exemplified by its spread over 200 km of the Florida Keys within 4 months of its discovery. In *D. stokesi* populations the incidence of disease was highest at 14 m depth. The concentration of bacteria collected from the surface of the diseased tissue from three coral spp was $10^5$ cells $ml^{-1}$. The isolated strain was shown to be the cause of the disease by producing complete tissue loss to healthy *D. stokesi* colonies that had been inoculated with the strain and then placed on marine agar.

## Hosts for temperate phage

Using API-NFT bacterial identification kits, strain D0 was shown to have a very good match with *Sphingomonas paucimobilis* [18]. Strain D0 harbored a lysogenic *Siphoviridae* phage (T-$\phi$D0) and was isolated from Ke'ehi Lagoon, Mamala Bay, Oahu, Hawaii, in 1994 [18]. Single colonies of strain D0 always contained phage particles indicating that the strain was a lysogen. In addition, plaques formed from T-$\phi$D0 were turbid indicating that the phage was temperate.

*S. paucimobilis* was previously known as *Pseudomonas paucimobilis*, and *Pseudomonas* phages have been isolated frequently from marine environments (see Ref. [18] and references within). If further taxonomic and genetic analyses confirm D0 to be a strain of *S. paucimobilis*, it would suggest that *Pseudomonas*-like *Sphingomonas* spp and associated phages may be common in marine environments.

## Hydrocarbon degraders

A *Sphingomonas* sp (2MPII) that can utilise 2-methylphenanthrene as the sole carbon and energy source was isolated from the top 2 cm of sediment from a polluted site near a petroleum refinery located at Lavera in the Gulf of Fos on the French Mediterranean coast [13]. The strain was also able to oxidise phenanthrene, napthalene, salicylaldehyde and catechol. Oxidation of these compounds was stimulated by growth on 2-methylphenanthrene in comparison to growth on acetate, indicating the presence of inducible metabolic pathways. The ability of the strain to utilise methyl-substituted aromatic compounds is of environmental interest for bioremediation of polluted marine sites, such as those associated with oil spills and the petroleum industry.

The strain was yellow pigmented, grew over a range of 15–38°C, pH 6–8 and had an obligate requirement for NaCl (0.4 M optimum). The visible absorption spectra of acetone extracts of the yellow pigment were identical to peaks seen for extracts from *S. paucimobilis*, characteristic of the carotenoid nostoxanthin. The fatty acid profiles were typical of the deep subsurface *Sphingomonas aromaticvorans*, strain F199 [11], and the 16S-rDNA sequence was >95% similar to *S. capsulata* [13].

Six *Sphingomonas* strains were isolated from deep (180–407 m) saturated Atlantic coastal plain sediment [1]. The boreholes at the Savannah River Plant near Aiken, South Carolina where the strains were isolated are drilled into fluvial, deltaic and marine sediments that consist of unconsolidated and clayey sands deposited since the late Cretaceous period (66–100 million years ago) [21,24]. While the isolation sites are terrestrially based, the geological history of the region and the geographical proximity close to the ocean (see map in Ref. [21]) indicates that the isolates may have a marine origin. *S. aromaticvorans* F199, which was isolated at a depth of 407 m, is an obligate aerobe that can utilise toluene, napthalene, dibenzothiphene, salicylate, benzoate, *p*-cresol and all isomers of xylene as sole carbon and energy sources [10,11]. Recently the complete sequence of a 184-kb plasmid from this strain was sequenced, providing unique insight into the genes involved in the aromatic degradative pathways [32].

A number of *Sphingomonas* isolates were obtained from sediment samples taken from natural hydrocarbon reserves at Mermaid Reef, Scott Reef and Pee Shoal on the northwest Australian shelf [19]. Concentrations of light aromatic and *n*-alkane oils in some samples were comparable to those in sediments surrounding an oil platform in the local area. The presence of significant concentrations of hydrocarbons and associated hydrocarbon-degrading bacteria from seabed mounds, suspected to be modern carbonate bioherms associated with hydrocarbon seepage, indicates that indigenous marine microbiota have evolved the capacity to degrade xenobiotic pollutants.

The *Sphingomonas* strains from the natural hydrocarbon-containing sites were isolated according to their ability to oxidise a mixture of phenanthrene, anthracene, fluorene and dibenzothiophene as sole carbon and energy source. The polyaromatic hydrocarbon degraders were characterised by 16S-rDNA sequencing. Strain JJ043 was phylogenetically closely related to the marine ultramicrobacterium, *Sphingomonas* sp, strain RB2256 (see below).

## Antarctic sphingomonads

A *Sphingomonas* strain (SW154) was isolated from seawater at a depth of 2 m, below 1 m thick ice in Taynaya Bay in the Vestfold Hills, Eastern Antarctica [2,3]. The water temperature was −2°C and the strain appeared to be numerically significant as it was isolated from the highest dilution of an extinction dilution series at ~$10^4$ cells $ml^{-1}$. It requires NaCl for growth and is psychrotolerant with fastest rates of growth between 20 and 25°C and a growth temperature maximum below 37°C.

## Culturable *vs* non-culturable sphingomonads

In a recent study examining the types of microorganisms identified from the same seawater samples using 16S-rRNA methods in comparison to isolates grown on solid media, numerous *Sphingomonas* isolates were cultured. Seawater from a depth of 10 m was sampled from the sea, 8 km west of the mouth of Yaquina Bay, Oregon [31]. Colonies were cultured on a complex marine R2A medium which contains a carbon content orders of magnitude higher than natural seawater. At least 15 *Sphingomonas* isolates representing a single restriction fragment length polymorphism (RFLP) type were isolated (represented by strain SYK6), while no *Sphingomonas* spp were detected from sequencing the 16S-rDNA clones. Due to the overall lack of correlation between the types of strains isolated on plates and those identified using 16S-rDNA clones, the authors concluded that many of the most abundant bacterioplankton species are not readily culturable by standard methods. It is important to note, however, that the complex medium used is not likely to support the growth of many marine species, particularly those adapted to nutrient-limited (oligotrophic) growth.

## Sphingomonads from oligotrophic seawater

The most well-characterised marine sphingomonad is the oligotrophic ultramicrobacterium, *Sphingomonas* sp strain RB2256 (LMG 18877) [7–9,25–29]. Strain RB2256 was isolated early in 1990 from Resurrection Bay, Alaska, where it was the numerically most dominant bacterium [4,26], as part of the total bacterioplankton community of approximately $10^6$ cells per ml. The isolation procedure used to obtain these dominant bacteria in culture was based on dilution to extinction in filtered-autoclaved seawater (FAS). In most dilution mixtures, very small and quite large rods, spirilla and cocci were found. However, most (> 50%) of the subcultures from the highest dilution series consisted predominantly of single, very small bacteria with a volume of approximately 0.05–0.06 $\mu m^3$ (length 0.9 $\mu$m and diameter 0.3 $\mu$m). All dilution cultures, with the exception of those containing only the very small rod-shaped bacteria (based on flow cytometric determinations), could be subcultured on full strength nutrient marine broth and agar plates. The latter small bacteria could only be subcultured in synthetic seawater amended with no more than 2 mg cas-

amino acids per litre (ie oligotrophic medium). Based on these observations these bacteria are considered obligately oligotrophic.

Repeated attempts to isolate these bacteria on the same medium solidified with agar failed when plates were inoculated from the various subcultures in liquid oligotrophic medium. However, if these same subcultures were kept at 5–8°C for at least 6 months after they had reached stationary phase, colonies could be obtained on agar plates made from ZoBell 2216E agar or oligotrophic agar. These colonies were very small, barely visible with the naked eye, but they could clearly be distinguished using a binocular microscope at a magnification of 25×. The same results were obtained after storage of liquid subcultures for up to at least 1 year at 5°C prior to streaking on agar. Most significantly, flow cytometric analysis of these pure cultures revealed that both the cell volume and the apparent DNA content of the cells were the same as those of the cells in the original dilution cultures.

The single colony type of these Resurrection Bay isolates was smooth, translucent and yellow-pigmented. To demonstrate that success with this procedure to obtain numerically dominant, oligotrophic bacteria from the sea was not an isolated event, the same procedure was used with different dilution series, again from Resurrection Bay, and from North Sea samples. In all cases growth of small colonies was obtained after prolonged incubation of stationary phase cultures and subsequent streaking on agar plates. Although for the North Sea samples this did not yield similarly yellow-pigmented colonies, the repeated dilutions from Resurrection Bay did yield this type of colony again. Based on morphological characteristics and on the colony appearance it thus seemed that the same or very similar species were obtained.

To investigate the phylogeny of these isolates in more detail, strain RB2256 and six additional representative strains obtained from Resurrection Bay were studied to determine their taxonomic positions. It was established that all seven strains (including strains RB2515, RB2510 and RB255), with RB2256 as the type strain, belong to the same species with a similarity, based on 16S-rDNA sequences, of >98%. This high degree of similarity was further substantiated on the basis of DNA-DNA hybridizations between all members of this group (80–100% identity). Based on these phylogenetic data and on the results of fatty acid methyl-ester (FAME) analysis, all strains belong to the same new, and as yet undescribed species of the genus *Sphingomonas* [37]. The most closely related species is *S. macrogoltabidus*, which on the basis of DNA-DNA hybridization is clearly distinguished from the new species (ie <30% relatedness) [37].

Characteristics that denote RB2256 as a 'typical' or 'model' oligotrophic ultramicrobacterium [12,14,15,29,30] include: constant ultramicro-size ($<0.1\ \mu m^3$) irrespective of whether it is growing or starved, and a mechanism for avoiding predation (ultramicro-size); relatively slow maximum specific growth rate ($<0.2\ h^{-1}$); ability to utilise low concentrations of nutrients; high affinity, broad specificity uptake systems; ability to simultaneously take up mixed substrates [7,28]. The strain can utilise a range of substrates as sole carbon sources including glucose, mixed amino acids, acetate and alanine, and can assimilate trehalose, maltose and malate [29,37]. Based on Michaelis–Menten constants for substrate transport ($K_t$) and the available concentrations of mixed amino acids in the ocean, strain RB2256 is predicted to have an *in situ* doubling time of 12 h to 3 days [4,26]. As the average doubling time for microorganisms in oligotrophic waters is estimated to be 5–15 days [8], RB2256 is likely to be a significant contributor to biomass turnover in oligotrophic ocean waters where it is a numerically dominant species.

Recently, it has been shown that strain RB2256 requires vitamin B12 as a growth factor [5]. Vitamin B12 is one of the largest organic molecules known (excluding polymers) [17] and consists of a highly decorated corrinoid ring attached to D-1-amino-2-propanol esterified to the nucleotide dimethyl-benzimedazole. At present, the exact sequence of biosynthetic reactions is unclear; however, it is estimated that about 30 enzymes may be uniquely required for synthesis of vitamin B12 [17]. Since the genome size of strain RB2256 is abbreviated (~1/3 that of *E. coli* [4,25]), it may not encode all the genes necessary for the biosynthesis of vitamin B12 and instead may rely on its acquisition from the environment. The requirement of vitamin B12 as a growth factor does not seem to be specific to strain RB2256. About 10% of total viable marine bacteria in oligotrophic seawater have been shown to require vitamin B12 as a growth factor [5]. Hence, a small amount of vitamin B12 added to oligotrophic media may be of general use for isolating marine bacteria representative of oligotrophic environments.

Over a number of years of study it has been clearly established that the oligotrophic physiotype of strain RB2256 is distinctly different from its eutrophic counterparts. Marine eutrophic bacteria (eg *Vibrio angustum* S14) tend to grow in bursts, exhibit pronounced lag phases when glucose-limited chemostat cultures are challenged with excess glucose, undergo reductive cell division when starved resulting in resting-stage cells, exhibit pronounced starvation-induced cross-protection, and at any stage of growth are markedly less stress-resistant than strain RB2256 [7,20,22]. In contrast, strain RB2256 retains essentially the same ultramicro-size irrespective of carbon concentrations or growth phase (log/starved), and does not undergo reductive cell division [7]. Little variation in maximum specific growth rate ($0.13–0.16\ h^{-1}$) results in response to a 1000-fold difference in the concentration of complex growth medium (VNSS). Strain RB2256 is extremely resistant to high temperature (56°C), ethanol (20%) and hydrogen peroxide (25 mM) in comparison to *V. angustum* S14. Growing and starved cells are equally resistant to stresses such as high temperature and hydrogen peroxide; ie no starvation cross-protection.

Consistent with a genome ~1/3 the size of the *E. coli* genome [4,25], these morphological, growth response and stress resistance characteristics indicate that strain RB2256 may have evolved a genotype that produces very few phenotypic adjustments to changes in the environment and that it may have largely constitutive gene expression.

The regulation of ribosome content, the number of ribosomes per cell and growth rate responses in strain RB2256 are also fundamentally different from those in fast-growing

heterotrophs like *E. coli* and *V. angustum* S14. For example, there is only one copy of the rRNA operon in strain RB2256, in comparison with 8–11 copies in *Vibrio* spp [8]. The number of ribosomes per cell is highly regulated throughout the growth phase, reaching maximum levels during mid-log phase growth but decreasing rapidly to 10% of maximum during late log phase through to 7 days of starvation. While the maximum number of ribosomes per cell (2000) is low compared to *E. coli* or *V. angustum* S14 (40 000), on the basis of cell volume, strain RB2256 has a concentration of ribosomes at least as high as for the two faster growing eutrophs. When cells are abruptly confronted with excess glucose, the maximal specific growth rate occurs immediately (as high as a 7-fold increase in growth rate). This occurs for cells grown in glucose-limited chemostats, acetate or alanine batch cultures, or glucose batch cultures throughout the growth phase from log through to 7 days of starvation. Therefore, while strain RB2256 has a single copy of the rRNA operon and is not genetically geared to high growth rates, a ribosome content 10% of maximum is sufficient to allow cells to respond immediately to nutrient upshift and achieve maximum rates of growth.

## Characteristics of a typical marine sphingomonad?

Performing a BLAST search with the 16S-rRNA sequence from strain RB2256 (GenBank Z73631) revealed numerous related species. Most of these belonged to the genus *Sphingomonas*; however, sequences of *Blastomonas*, *Rhizomonas*, *Erythromonas*, *Erythromicrobium* and *Porphyrobacter* species were also clearly related. This heterogeneity has been documented [33–36] and indicates that the classification of members of the genus *Sphingomonas* and related species may need revision.

A phylogenetic tree was constructed using 16S-rDNA sequences from the available marine isolates (Figure 1). Strains mentioned in this review that did not have 16S-rDNA sequences available (the phage host strain D0 and the hydrocarbon degrader JJ043) or those with less than 500 bp of contiguous sequence (coral pathogen), were excluded. Although the number of sphingomonad isolates from marine environments is limited, it is apparent from Figure 1 that they represent a broad phylogenetic range within the *Sphingomonas* genus.

The diversity of the marine strains is also reflected in the ecological niches they inhabit. It is perhaps not surprising that marine hydrocarbon degraders have been isolated as terrestrial and subterranean strains; however, the presence of coral pathogens and numerically dominant species in oligotrophic waters broadens the range of sphingomonad habitats. Less is known about their physiology; however, they are likely to possess unique metabolic pathways that are characterisitic of the natural substrates they encounter and as a consequence of their particular life-styles, eg hydrocarbon degradation *vs* oligotrophic growth *vs* pathogenesis of corals.

The Antarctic *Sphingomonas* sp is not especially phylogenetically related to the Alaskan ultramicrobacterium, *Sphingomonas* sp, strain RB2256. During isolation, the

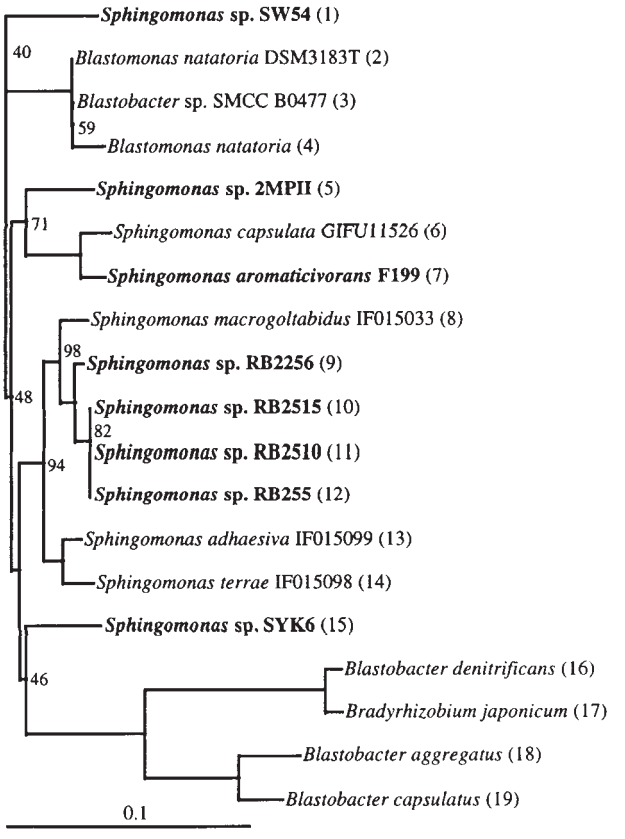


**Figure 1** Distance-matrix tree of marine sphingomonads. The 16S-rRNA sequences were aligned using the programs PILEUP and CLUSTALW (GCG-software package). Gaps and ambiguous positions were deleted manually and distances were calculated using Jukes–Cantor matrices in the program DNADIST. Distance matrix trees were constructed using the neighbourhood joining method in the program NEIGHBOR. Bootstrap analysis was performed using ESEQBOOT. Scale bar = 1 substitution for every 10 base pairs. Numbers on branches indicate boot-strap values for 100 repeats (unless otherwise stated, all values are 100). Database accession numbers are as follows: 1, U85838; 2, AB024288; 3, U20772; 4, X73043; 5, U90216; 6, D16147; 7, U20756; 8, D13723 (D12640); 9, Z73631; 10, AF145753; 11, AF145754; 12, AF145752; 13, D17322; 14, D13727; 15, D16149; 16, X66025; 17, X66024; 18, X73041; 19, X73042.

temperatures of the Alaskan and Antarctic waters were 4–8°C and −2°C, respectively. Interestingly, while both strains were isolated from cold water, they are able to grow at temperatures significantly above ambient: 45°C [17] and 35°C [2,3] for the Alaskan and Antarctic strains respectively. This indicates that while they are different species, their physiological responses to temperature may be similar.

An interesting feature of the oligotrophic sphingomonad, RB2256, is its ultramicro-size. With the exception of strain RB2256 [7], accurate cell volumes of the marine sphingomonads determined throughout growth and starvation have not been reported; however, the size estimates that have been reported indicate that only strain RB2256 is an ultramicrobacterium ($<0.1\ \mu m^3$). The ultramicro-size of strain RB2256 is consistent with the characteristics expected for a microorganism living under nutrient-limited conditions in the ocean. It will be interesting to determine how common the ultramicro-size is in marine sphingomonads in general.

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