

Genetic analysis of housekeeping genes reveals a deep-sea ecotype of *Alteromonas macleodii* in the Mediterranean Sea

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Summary

The genetic diversity of 19 strains belonging to *Alteromonas macleodii* isolated from different geographic areas (Pacific and Indian Ocean, and different parts of the Mediterranean Sea) and at different depths (from the surface down to 3500 m) has been studied. Fragments of the 16S rRNA gene, the internal transcribed spacer (ITS) between 16S and 23S rDNA genes, the *gyrB* and the *rpoB* genes, have been sequenced for each strain. Amplified fragment length polymorphisms were used to characterize similarity at the level of the whole genome. Most of the diversity reflected the existence of a cluster of strains isolated from deep Mediterranean waters and two isolates from the Black Sea. Particularly the isolates from the deep sites were consistently different from all the others indicating the existence of a specific ecotype adapted to these conditions. Amplification of *gyrB* gene and ITS directly from DNA retrieved from deep Mediterranean waters and one Atlantic sample showed that presence of this deep-sea ecotype is widespread and is not a product of culture bias. On the other hand, strains isolated from surface tropical waters showed a remarkable level of resemblance to the first isolate of this species obtained from Hawaii in 1972. The results indicate the existence of both lineages of global distribution and ecotypes adapted to specific conditions such as deep or more diluted (the Black Sea) waters.

Introduction

The genera *Alteromonas* and *Pseudoalteromonas* (Gauthier and Breittmayer, 1992; Gauthier *et al.*, 1995) are among the most common heterotrophic bacteria living in open marine waters all around the world (Suzuki *et al.*, 1997; Acinas *et al.*, 1999; Eilers *et al.*, 2000; Hagström *et al.*, 2000; Rappé *et al.*, 2000; García-Martínez *et al.*, 2002; Pinhassi and Berman, 2003). The use of molecular approaches has helped in providing a general idea of the predominance and kind of niche that these organisms occupy. It was found that 16S rDNA molecules belonging to *Alteromonas macleodii* could be recovered abundantly from Mediterranean offshore samples both from the surface and down to 400 m (Acinas *et al.*, 1999). Although mostly associated to particles some sequences were also retrieved from the picoplankton size fraction. *Alteromonas macleodii* 16S rDNA sequences were found also to be predominant in mesocosm experiments with Mediterranean seawater enriched with organic nutrients (Pukall *et al.*, 1999; Schäfer *et al.*, 2000). The association of *Alteromonadaceae* with particulate material was also shown by fluorescence *in situ* hybridization (FISH) in North Sea samples (Eilers *et al.*, 2000). By dot blot hybridization the presence of this kind of organisms was ascertained in many other tropical and temperate seas (García-Martínez *et al.*, 2002). Using specific primers for amplification of the 16S–23S internal transcribed spacer (ITS) of *A. macleodii* polymerase chain reaction (PCR) products from different oceanic regions provided a collection of amplicons that showed a widespread distribution of this species and certain geographic associations. Particularly a cluster of sequences from the tropical areas with coral reefs seemed well established by both alignable sequence and indels affecting the spacer. However, in Antarctic waters *A. macleodii* could not be detected neither by hybridization nor by PCR-specific primers.

The information is consistent with *A. macleodii* being a *r* strategist (successful competition based on reproduction rather than survival) that can grow rapidly when organic nutrients are readily available. It has relatively large cell size and strong degradative capabilities that could be an important asset for this kind of strategy (Curds, 1982;

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Morita, 1997). That would explain its frequent association to the particulate fraction where decomposing faecal pellets and other microplanktonic remains might provide the right conditions for rapid growth. Low temperatures would be one of the factors that might preclude this strategy being successful as it imposes an absolute barrier to rapid growth rate resulting from physic-chemical limitations. This would explain the apparent absence of representatives of this species in cold latitudes (García-Martínez *et al.*, 2002). Still, some representatives were found by PCR in deep samples (down to 1000 m in the Mediterranean and 400 m in the Atlantic). These results were puzzling as deep waters are normally cold.

Contrastingly with other widespread marine prokaryotes, *A. macleodii* can be grown in pure culture easily. Isolates belonging to this species are relatively frequent and have been obtained in many studies carried out at different locations. For example, in a work carried out with North Sea samples, 10 out of 145 strains were classified by the 16S rDNA sequence as *A. macleodii* (Eilers *et al.*, 2000). Recently, some isolates belonging to this species were retrieved from 3500 m deep waters at the interphase between brines and deep Mediterranean waters of the Urania Bassin (Sass *et al.*, 2001). The Mediterranean Sea is special in maintaining a relatively warm temperature (c. 13°C) throughout the water column regardless of depth. Therefore, it is understandable that an organism that requires relatively mild temperatures, for the standards of the deep ocean, might thrive here at high depth. In a previous work carried out with biomass from offshore Western Mediterranean waters (Acinas *et al.*, 1999) by PCR amplification of 16S rDNA, two clusters of sequences belonging to *A. macleodii* were detected. Although highly similar, the sequences clustered accordingly with the depth of sampling (surface or 400 m). This was taken as an indication of the possible existence of a deep-sea ecotype of this organism. However, the 16S rRNA sequence is much too stable to allow the identification of ecotypes. In the present work, we have studied the isolates from the Urania Basin mentioned above, together with two isolates from different geographic origins and depths in the Mediterranean. Our aim was to study the variation in more variable genes to establish how different the deep-sea isolates were. The results indicate that indeed the deep-sea isolates represent a special lineage within the species that could have specific adaptations for living in deep waters.

Results

16S rRNA genes

For this study a total of 19 strains were collected, nine from surface waters and 10 from deep Mediterranean

samples (1000 or 3500 m). *Alteromonas macleodii* ATCC 27126 is the original Baumann isolate obtained from Oahu, Hawaii (Baumann *et al.*, 1972). The others were obtained from different locations (most from different parts of the Mediterranean) and isolated by different laboratories (Table 1). The sequences of the 3' end of the 16S rRNA gene (435 nucleotides) of all the isolates were obtained and used for a BLAST search against sequences in GenBank in order to assess their taxonomic affiliation. The comparisons clearly indicated that all the isolates belonged to the species *A. macleodii* (nucleotide similarity higher than 98%) except the isolates SCB56 and H4-6. SCB56 belonged to the recently described *Alteromonas stellipolaris* (99% similarity) while H4-6 was 100% identical to a *Pseudoalteromonas* sp. S511-1 and was used as outgroup. The relationships among the 19 strains sequenced are shown in the dendrogram in the inset of Fig. 1. Although 16S rRNA is a gene that usually varies too slowly to show interspecies variability, two groups could be distinguished as was found with PCR products (Acinas *et al.*, 1999). The sequences of the isolates retrieved from superficial waters were all nearly identical to *A. macleodii* ATCC 27126. In fact, the isolates MED64, H94-8 and H84-3 were identical in all the sequenced nucleotides to *A. macleodii* ATCC 27126, while isolates AND1, U8, AD 45, and the sequence gb/AACY01090398.1/, retrieved from a database of shotgun cloned sequences from the surface of the Sargasso Sea (Venter *et al.*, 2004), present only one different nucleotide. The only significantly different surface isolate was AND3 that differed in three nucleotides from the reference strain. On the other hand, the isolates from the deep samples had two to three different positions (Adriatic1, Adriatic2, UM4b, U4, U12 and U7), or seven to eight (isolates UM8, UM7 and U10).

Internal transcribed spacer (ITS)

The use of the spacer between 16S and 23S rRNA genes to characterize microdiversity in bacteria is well documented (García-Martínez *et al.*, 1999; García-Martínez and Rodríguez-Valera, 2000; Boyer *et al.*, 2001; Rocop *et al.*, 2002.). This region is located between highly conserved rRNA genes (Gürtler and Stanisich, 1996) so can be easily amplified by PCR with universal (or specific) primers, and the sequence is highly variable in both nucleotides and length (García-Martínez *et al.*, 1999; Rocop *et al.*, 2002; Ferris *et al.*, 2003). Intragenomic cistron heterogeneity can complicate comparison (Boyer *et al.*, 2001), but at the level of alignable sequence, the homogenization trend in each genome (Gürtler, 1999) is enough to allow reliable similarity estimations. Particularly when similarity is very high, e.g. identical sequences, it provides a strong argument for very similar or identical genomes.

Table 1. Origin of the strains.

| Strain | Species | Obtained from | Isolated from | Depth | Reference |
|------------|----------------------------------|--|---|-------------------------------|-------------------------------|
| ATCC 27126 | <i>Alteromonas macleodii</i> | American Type Culture Collection (ATCC) | Pacific Ocean Oahu, Hawaii | Superficial waters | Baumann <i>et al.</i> (1972) |
| H84-3 | <i>Alteromonas macleodii</i> | Olga Onyschenko, Academy of Sciences, Ukraine | Black Sea. Karadag Nature Reserve of Crimea, Ukraine | Superficial waters | Unpublished |
| H94-8 | <i>Alteromonas macleodii</i> | Olga Onyschenko, Academy of Sciences, Ukraine | Black Sea. Karadag Nature Reserve of Crimea, Ukraine | Superficial waters | Unpublished |
| H4-6 | <i>Pseudoalteromonas</i> sp. | Olga Onyschenko, Academy of Sciences, Ukraine | Black Sea. Karadag Nature Reserve of Crimea, Ukraine | Superficial waters | Unpublished |
| MED64 | <i>Alteromonas macleodii</i> | Ake Hagström, Marine Science, Kalmar University, Sweden | Aegean Sea off Lebanon, Mediterranean (32°81'N, 34°69'E) | Superficial waters (5 m deep) | Hagström <i>et al.</i> (2000) |
| AND1 | <i>Alteromonas macleodii</i> | Ake Hagström, Marine Science, Kalmar University, Sweden | Andaman Sea off Thailand (08°00'N, 98°00'E) | Superficial waters | Hagström <i>et al.</i> (2000) |
| AND3 | <i>Alteromonas macleodii</i> | Ake Hagström, Marine Science, Kalmar University, Sweden | Andaman Sea off Thailand (08°00'N, 98°00'E) | Superficial waters | Hagström <i>et al.</i> (2000) |
| SCB56 | <i>Alteromonas stellipolaris</i> | Ake Hagström, Marine Science, Kalmar University, Sweden | Pacific Ocean, Scripps Pier, California Bight (32°53'N, 117°15'W) | Superficial waters | Hagström <i>et al.</i> (2000) |
| U4 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Ionian Sea, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| U7 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| U8 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| U10 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| U12 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| UM4b | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| UM7 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| UM8 | <i>Alteromonas macleodii</i> | Henrik Sass, University of Oldenburg, Germany | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | Sass <i>et al.</i> (2001) |
| Adriatic1 | <i>Alteromonas macleodii</i> | This work | Urania Basin, West of Crete, Ionian (35°13.83'N, 21°28.29'E) | Deep waters (3500 m) | This work |
| Adriatic2 | <i>Alteromonas macleodii</i> | This work | Adriatic Sea, (41°37'N, 17°22'E) | Deep waters (1000 m) | This work |
| AD 45 | <i>Alteromonas macleodii</i> | M ^a Jesus Pujalte, Universidad de Valencia, Spain | Adriatic Sea, (41°37'N, 17°22'E) | Deep waters (1000 m) | This work |
| | | | Balearic Sea | Superficial waters | Pujalte <i>et al.</i> (2003) |

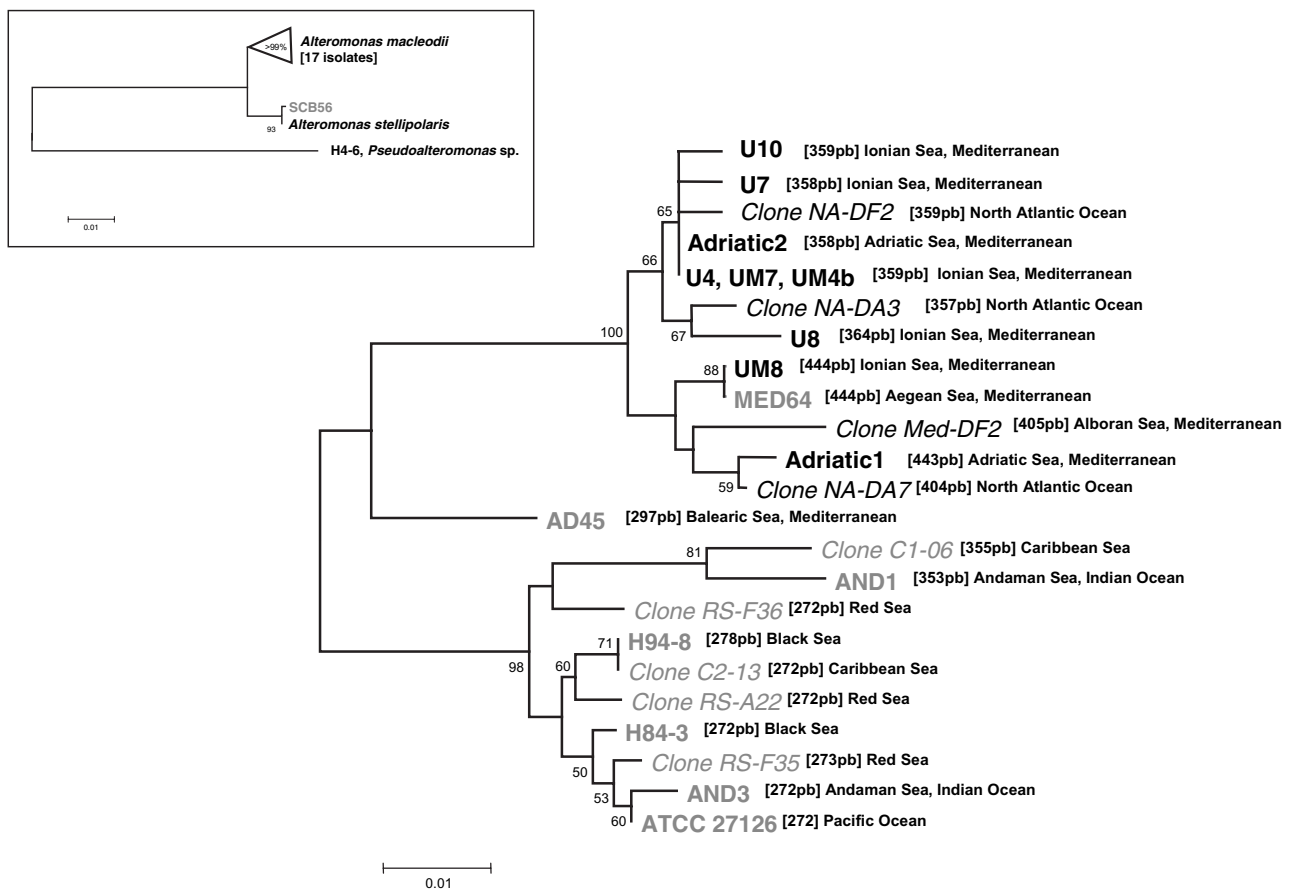


Fig. 1. Neighbour-joining tree showing relationships among the isolates (bold) and some environmental clones (italics) based on the ITS sequences. Length of the spacers is shown between brackets. Inset shows the dendrogram obtained when comparing partial sequences (435 nucleotides) of 16S rDNA gene for each isolate. The geographic origin is specified and the colours indicate the deep (black) or superficial (grey) origin of the strains/clones. Bootstrap values over 50% from the neighbour-joining analysis are shown. Scale bar, 0.01 substitutions per nucleotide position.

In a previous work specific primers for *A. macleodii* were used to amplify this same stretch from total prokaryotic DNA extracted from different marine samples. Always two bands of different size appeared showing two size types of ITS in this organism. This is the case also for *Escherichia coli* and many other *Proteobacteria*. García-Martínez and colleagues (2002) selected the smaller band aiming to amplify operons corresponding to the same loci. Here we have sequenced four clones from the smaller band for each strain to check if indeed there is only one operon with this spacer size. The similarity that we found among the four clones for each isolate was always so high (>99% similarity) that, even if there is more than one operon included in the comparison, inter-cystronic heterogeneity is not high enough to blur the inferred relationships among the isolates.

We had also already available a collection of ITS sequences for *A. macleodii* obtained by direct PCR amplification from marine samples from around the world (García-Martínez *et al.*, 2002). Figure 1 shows a dendro-

gram comparing the sequences determined here and others from that previous PCR-based study. The differences between isolates from surface and deep waters that started to appear in the comparison of the 16S rRNA sequences appear here much more obvious. The length of the sequenced ITS varied from 272 to 444 pb (see Fig. 2), and generally shorter spacers corresponded with superficial isolates. In the alignment (data not shown) it was evident a large insertion of 141 pb starting at position 79 that was present only in some of the deep-sea isolates and was responsible of the larger sizes. In the rest of the sequence there are smaller insertions/deletions events (1–30 pb) that affect in different ways each sequence. In the surface sequences, a subcluster including the ATCC 27126 strain, one isolate from the Andaman Sea and a PCR product from the Red Sea is remarkable. The existence of very similar sequences of this organism associated to tropical and temperate seas has been already described (García-Martínez *et al.*, 2002). The deep Mediterranean isolates showed a much more conserved

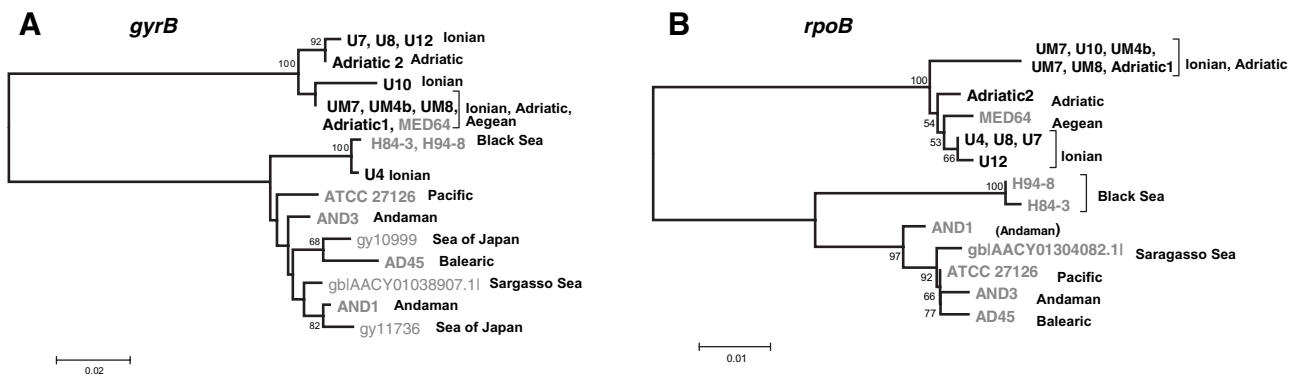


Fig. 3. Neighbour-joining trees showing the relationships of the partial sequences of genes (A) *gyrB* (508 nucleotides) and (B) *rpoB* (511 nucleotides). Colour code indicating origin of the isolates/clones as before. Scale bars, 0.01 and 0.02 substitutions per nucleotide position.

wide and the deep Mediterranean strains. In *rpoB* we found an exchange in the position 229 of a serine (alcohol polar R-group) in all surface isolates for an asparagine (amine R-group) in the deep Mediterranean strains. A similar change was found in *gyrB*, where glutamic acid located in position 139 in superficial isolates was exchanged by glutamine (amine R-group) in deep-sea isolates. Both non-synonymous changes are located in the protein regions that connect secondary structure motives (data not shown). In addition, in the two strains from the Black Sea, the amino acid sequence of the proteins showed a replacement of a threonine located in the position 216 by a serine (both polar with alcohol groups) in *rpoB* and again a serine located in position 152 was substituted by cysteine (hydrophobic) in the *gyrB* protein.

The dendrograms obtained with the sequences of these coding genes for all the isolates tested are shown in Fig. 3. Within the surface water isolates two clusters were evident for both genes. In the case of *gyrB*, one of the clusters contains both isolates from the Black Sea in addition to one deep Ionian isolate, while the other contains sequences from tropical and temperate waters, including one *gyrB* sequence retrieved from the database of environmental sequences of the Sargasso Sea (Venter *et al.*, 2004), two *gyrB* sequences from the surface of the Sea of Japan (Watanabe *et al.*, 2001), the type strain, the two Andaman isolates and the Balearic isolate. The *gyrB* from isolate U4 seems to be totally misplaced in the *gyrB* tree. This could result from recombination affecting this deep-sea isolate and a surface donor. In the case of *rpoB* both isolates from Black Sea again cluster together. For both genes sequences of isolates from deep Mediterranean waters form a different cluster, and again this cluster included MED64 that was isolated from surface waters in the Levantine Sea.

As an additional tool to evaluate the distribution of the *A. macleodii* genotypes, we designed species-specific primers of *gyrB*. We used them to analyse environmental

DNA samples (data not shown). The samples from cold waters that included 50 and 2000 m deep in the Greenland Sea and 50 and 1000 m in the Arctic produced no amplification product illustrating again the apparent absence of this species in permanently cold waters. Contrastingly, all the Mediterranean samples produced PCR products. Ten clones were obtained from the Adriatic Sea at 1000 m and ten clones from the Ionian Sea at 3000 m (at a different location from the one in which the Ionian isolates were obtained). The *gyrB* sequences retrieved were all highly related (more than 99% similarity) to some of the sequences of deep Mediterranean strains, except two that clustered with the superficial isolate AD 45, from the Balearic Sea. This result confirms the prevalence and ubiquity of this ecotype in deep Mediterranean waters as by using environmental DNA and PCR bias resulting from pure culture isolation is avoided.

Amplified fragment length polymorphism (AFLP) fingerprinting

Although the similarity of housekeeping genes might be an indication of genome similarity, there are many examples, particularly in large genomes, where dramatic differences in gene content have been found in organisms that by individual genes appear to be highly related (e.g. Rocap *et al.*, 2003). To analyse genome similarity among our collection of isolates, Amplified fragment length polymorphism (AFLP) fingerprinting was used. This method combines the advantages of PCR and restriction pattern fingerprinting methods and provides a pattern of the genome variation that, particularly for high similarity values, indicates high genome similarity and can be used to detect high level of relationships, for example, in clinical microbiology where this is required to trace epidemiological outbreaks.

The AFLP patterns shown in Fig. 4 identify clusters of closely related strains. The two isolates from the Black

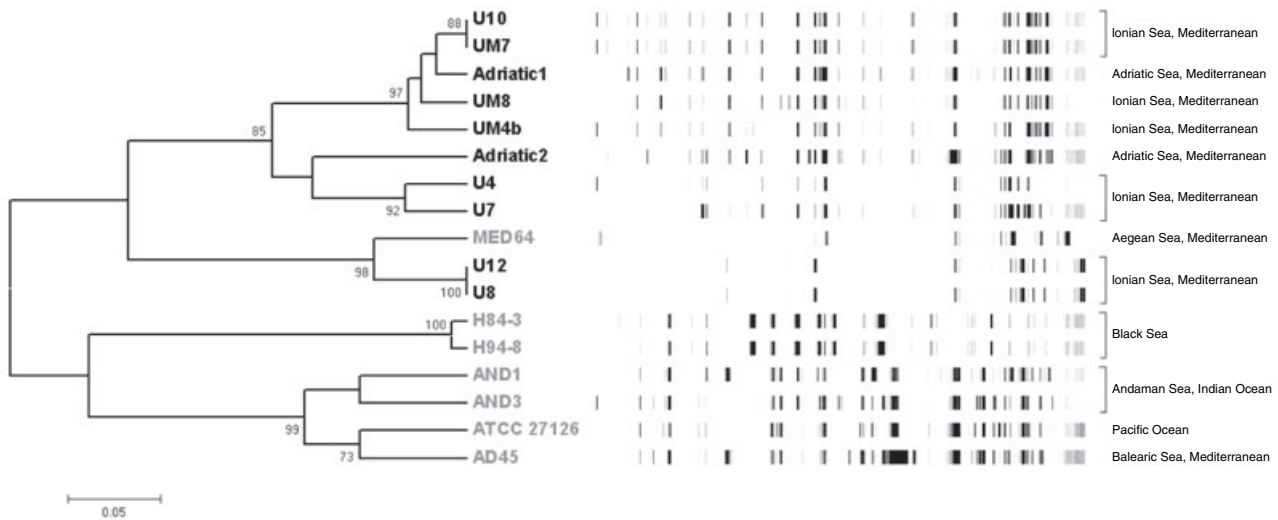


Fig. 4. Unweighted pair group method with arithmetic mean (UPGMA) analysis of the AFLP genomic fingerprints of the *Alteromonas macleodii* strains. Colour code as before. Bootstrap values over 50% are shown. Scale bar, 5% percentage of similarity.

Sea show nearly identical patterns and therefore correspond to a single clonal lineage. The similarity of the cluster from surface tropical waters (Hawaii, Andaman and Mediterranean) was also quite conserved at the level of the genome fingerprinting. Here again the main differences found were with the isolates from the deep. They were markedly different from the surface isolates and relatively similar to each other. One Adriatic isolate (from 1000 m) had a pattern nearly identical to two Ionian ones (3500 m) underscoring homogeneity of the deep-sea group regardless of the location.

Discussion

The 16S rRNA dendrogram depicted in Fig. 1 shows that all the isolates from geographic distant regions form a coherent cluster consistent with a well-delimited species, i.e. all belong to *A. macleodii*, and that they differ significantly from the closest taxon described as *A. stellipolaris* that was isolated from Antarctica (Van Trappen *et al.*, 2003). Most surface isolates belong to one genotype that is widely distributed and well conserved worldwide. The Baumann isolate ATCC 27126 is remarkably similar to two isolates from the Andaman Sea (about 4500 NM away and in a different ocean basin). The original strain isolated in Hawaii in 1972 is so similar to AND3 from the Andaman Sea that in clinical microbiology they could be considered members of a single epidemiological outbreak. It is also noteworthy that a PCR product from the Red Sea had an ITS sequence identical to ATCC 27126 (García-Martínez *et al.*, 2002). Moreover, we have found a nearly identical *rpoB* sequence in the Sargasso Sea shotgun database (99.6% similarity) (Venter *et al.*, 2004). Strain AD 45 obtained from the Balearic Sea was also

very similar. Overall, these results indicate that organisms very similar to the original Baumann isolate are found all around the world in temperate or tropical surface waters. It is remarkable that organisms that are genetically so homogeneous show such cosmopolitan distribution. Although we do not know how abundant they really are in the locations from which they were isolated, the description of PCR products directly amplified from the biomass at different locations (without culture) that could also correspond to very similar organisms indicates that their presence is not only the product of culture selection (this clone being particularly well endowed to grow in the laboratory).

The deep Mediterranean ecotype

Contrastingly, the isolates from deep waters studied here were similar to each other and markedly different from isolates from the surface waters. These isolates came from two sites in the Eastern Mediterranean that were only 350 NM apart. Nevertheless, the two sites were located in basins separated by the Italian peninsula. Besides, a group of ITS-16S rDNA PCR products from 200 m deep of the North Atlantic Ocean clustered with the deep Ionian Sea isolates and the same is also true for a PCR product from 500 m deep in the Alboran Sea, Western Mediterranean (Fig. 1). Former work indicated that *A. macleodii* is restricted to temperate waters (García-Martínez *et al.*, 2002) and there are no reports of isolates or PCR products that could be assigned to this species from waters permanently below 10°C. Here again we failed to get any product with PCR-specific primers for 16S–23S rRNA and *gyrB* genes from the Greenland Sea and the Antarctic Polar Front regardless of depth. In the Atlantic or Pacific

temperature decreases rapidly with depth even at tropical latitudes, while in the Mediterranean never varies significantly, staying *c.* 13°C regardless of depth. If there is a lower temperature limit to the distribution of this species, it would explain its absence in deep and permanently cold superficial waters. If this is so the deep-sea genotype of *A. macleodii* would be restricted to relatively warm deep waters as found also, for example, in the Red Sea. Some organisms closely related to *A. macleodii* have been isolated from deep waters but always from relatively warm environments. *A. macleodii* ssp. *fijiensis* (Ragueneas *et al.*, 1996) has been isolated from a diluted hydrothermal fluid at 2000 m at the North Fiji Basin and *A. macleodii* ssp. *fijiensis* biovar *deepsane* (Cambon-Bonavita *et al.*, 2002) was isolated from a polychaete annelid from a similar environment and depth (East Pacific Rise). We could not include these strains in our comparison because they are retained by commercial purposes. However, the 16S rDNA sequence comparisons strongly suggest that the isolates from the two sites in deep Mediterranean waters are markedly different from the other isolates, but that they are still closer to the type strain than other subspecies of *Alteromonas* isolated from the deep sea mentioned above. The protein-coding genes *gyrB* and *rpoB* supported strongly the existence of this deep-sea genotype. Furthermore, the amino acid changes located in the protein regions that connect secondary structure motives could reflect adaptations to the high pressure or other factors acting at deep waters. The amino acid changes detected do not necessarily affect the tertiary structure; however, it is well known that pressure modifies the solubility properties of solutions (Bartlett, 2002) and these connecting regions are totally exposed to the aqueous phase. Therefore the changes could affect tertiary structure of the proteins, being an adaptation for enzyme optimal functionality under high pressure. The similarity of the changes detected for two independent proteins, involving similar amino acid changes, are intriguing and deserve further investigation. In any case the results are consistent with the idea that the deep-sea isolates are representatives of a different ecotype adapted to live in deep waters. The existence of ecotypes with highly similar 16S rRNA sequences but adapted to different environmental conditions appears to be a very common motive in microbial ecology (e.g. Rocap *et al.*, 2002). Some results seem to indicate that there is no genetic isolation between the surface and the deep-sea ecotype. Strain U4 from the deep Ionian has an atypical version of the *gyrB* gene that clusters with surface sequences, especially with the Black Sea isolates (all three share the cysteine in position 152). However, the 16S rRNA gene, ITS and *rpoB* sequences, and AFLP data show that this strain fits very well within the deep-sea ecotype. This atypical *gyrB* is probably a result of a recombination event involving a surface and a

deep-water representative of the species and proves that both populations maintain some gene exchange. Also the mosaic ITS found in the isolate AD 45 could be attributed to recombination taking place between representatives from both ecotypes.

An apparent anomaly is strain MED64, a surface isolate that comes from the Levantine Sea, 25 km off the coast of Lebanon, and belongs by all markers to the deep-sea genotype. We conceive that this strain has been transported from deep waters to the surface by upwelling. This location is *c.* 1000 m deep and is at the Eastern end of the salinity-driven Mediterranean conveyor belt; it is one of the few places in the Mediterranean where the stratification of the water column is broken regularly (Malanotte-Rizzoli *et al.*, 1996). Curiously, there are several reports of animals found at atypical depths in the Levantine Sea (Sorbe and Galil, 2002). Besides, one single exception is not enough to modify our conclusions.

The comparative study of the superficial and deep-sea ecotypes at the level of physiology, molecular biology or genomics could be very relevant for the field of the pressure effect on bacteria and the microbiota of the deep ocean, one of the most important and less known aspects of marine microbiology.

The two sequences from cultures isolated from the Black Sea were also considerably different. Here the environmental conditions are also different from most oceanic provinces as salinity is about half of the typical values found in the Mediterranean and most ocean waters. We have found cases of non-synonymous amino acid substitutions in *rpoB* and *gyrB* that might reflect the adaptation to the brackish waters of this water body. Unfortunately, we had only two isolates from the same location and they show a considerable level of similarity with all the markers used.

Experimental procedures

Sampling and DNA extraction from Adriatic, Ionian, Greenland and Antarctic Seas

Variable volumes of deep-seawater samples (between 100 and 200 l) were collected in different cruises in the sampling sites, situated at coordinates: 41°36'98"N, 17°22'08"E (Adriatic); 36°30'N, 15°50'E (Ionian); 59°22'S, 55°46'W (Antarctic); and 72°54'N, 4°42'E (Greenland). The depth of the sampling sites were 1000 m in Adriatic, 3000 m in Ionian, 50 and 2000 m in Greenland, and 50 and 1000 m in Antarctica. Seawater was pre-filtered through 5 µm pore size filters (Millipore) and the remaining picoplankton was collected in 0.2 µm Sterivex filters (Millipore). Following a proteinase K-SDS lysis step, nucleic acids were extracted as previously described (Massana *et al.*, 1997). Briefly, lysates were extracted twice with phenol-chloroform-isoamyl-alcohol and once with chloroform-isoamyl-alcohol and nucleic acids concentrated after washing with sterile water using microconcentrators.

trator (Centricon 100, Amicon). DNA integrity was checked using agarose gel electrophoresis and samples were stored at -20°C until use.

Isolation and identification of *A. macleodii* strains

The isolates used in this study were kindly provided by various researchers or isolated by ourselves (Table 1). The type strain of *A. macleodii* was obtained from the American Type Culture Collection. Strains Adriatic1 and Adriatic2 were isolated from deep-water samples (1000 m) of the Adriatic Sea ($41^{\circ}36'98''\text{N}$, $17^{\circ}22'08''\text{E}$). Marine agar plates (Laboratorios Conda) were inoculated with 100 μl of seawater and incubated at 13°C in the dark. Individual colonies were picked, and resuspended in sterile water. The mix was heated (98°C for 10 min) to lyse the cells and the lysate was centrifuged for 1 min at 13,000 r.p.m. An aliquot of 1 μl of the supernatant was used for the PCR (see below). The PCR amplification was carried out by using the *gyrB*-specific primers (see below). This appeared to be the most useful primer combination to discern *A. macleodii* strains. A total of 275 colonies were screened and two de-replicated strains belonging to this species were found. Once the identification was confirmed by the 16S rRNA gene sequence (see below), positive colonies were re-grown in Marine Broth (Laboratorios Conda) and the DNA was extracted with phenol-chloroform method (Massana *et al.*, 1997). The strains provided by other researchers were grown in the same liquid media and their DNA was extracted with the same method.

The identification of the strains was carried out by amplifying and partial sequencing of the 16S rRNA gene plus the ITS region. Polymerase chain reaction primers used for amplification were B1055 (5'-AATGGCTGTCGTCAGCTCGT-3'), within the 16S rRNA gene, and 23S1R (5'-GGGTTCCCATTCGGAAATC-3'), a universal primer for rRNA eubacterial genes (García-Martínez *et al.*, 1999). Polymerase chain reactions were performed under the following standard conditions: 35 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 2 min) preceded by 2 min denaturation at 94°C and followed by 5 min extension at 72°C .

The primers amplify the last 550 nucleotides of the 16S rRNA gene plus the ITS region and a small fragment of the 23S rRNA gene. The amplicons were run in an agarose gel and different patterns were obtained; most often two bands ranging in size between 1000 and 1500 bp were found. However, in some strains up to four bands were seen (data not shown). This reflects the existence of at least four different rRNA operons in this species that probably cluster into two ITS size types (such as is the case in *E. coli*). To compare the equivalent (orthologous) operons of the different strains, the smallest band (*c.* 1200 nt), the size of which was very similar in all the strains, was sliced from the gel and cloned into pCR2.1-TOPO vector with the TOPO TA Cloning kit (Invitrogen). The selected clones (four clones of each strain) were purified using the QIAquick PCR purification system (Qiagen) and sequenced using primers B1055 and 23S1R in an ABI Prism 377 (PE Applied Biosystems) using the ABI prism Big Dye Terminator Cycle Sequencing Ready reaction Kit V2 (PE Applied Biosystems) according to the manufacturer's recommendations.

Amplification of the *gyrB* and *rpoB* genes

Two specific primers were designed to amplify a fragment of 668 bp of the *gyrB* gene: primer AGYRF (5'-TAGGTGTATCAGTAGTAAACGC-3') and primer AGYRR (5'-AAGCTTATCTTTAGTCTGTGAGC-3'). The sequences used to design the primers were taken from the ICB database (Watanabe *et al.*, 2000). Polymerase chain reactions were performed as described above and the amplified fragments were purified and fully sequenced with primer AGYRF. In subsequent experiments, it was verified that this primer pair was specific for *A. macleodii* strains, even discerning between them and very closely related species (like *A. stellipolaris*).

Primers RPOBF349 (5'-AARGARSRAGAAGTMTWYATGGG-3') and RPOBR1540 (5'-TCDGAHARYGGRTTGTTYTGRTCC-3') were designed to obtain an amplicon of around 1200 bp. The design of the primers was performed by aligning sequences of *rpoB* genes from some related γ -*Proteobacteria* representatives available in GenBank database (accession numbers are specified in brackets): *Vibrio cholerae* (AAF93501) *Vibrio vulnificus* (AAO09671), *Pseudomonas aeruginosa* (AAG07658), *Pseudomonas fluorescens* (ZP000829), *Shewanella violacea* (BAA99392), *Shewanella oneidensis* (AAN53309), *Microbulbifer degradans* (ZP000678) and *E. coli* K12 (CAA23625). The PCR conditions were the same as those used for the amplification of the 16S rRNA gene (see above). The amplified fragments were purified and fully sequenced with primer RPOBR1540.

The mean distances at synonymous sites (Ks parameter) of the sequenced coding genes were calculated by applying the formula $K_s = -3/4 \ln [1 - (4M_s/3N_s)]$, with M_s being the number of synonymous differences and N_s the number of synonymous sites (see Fig. 3). For calculations, both *rpoB* and *gyrB* sequences of isolate MED64 were included as deep-sea sequences for its homology with deep-sea isolates. In the same way, U4 sequence was considered as a superficial isolate for the analysis of *gyrB*.

Amplified fragment length polymorphism (AFLP)

DNA digestions and adaptor ligation were carried out at 37°C overnight with five units of *EcoRI*, one unit of *MseI*, one unit of T4 ligase (Invitrogen) and the recommended proportion of *EcoRI* and *MseI* adaptors in a final volume of 20 μl . Reactions of pre-selective (with amplification core mix) and selective amplifications (with fluorescently labelled primers *EcoRI*-A and *MseI*-G) were performed using the reagents supplied in the AFLP Microbial Fingerprinting Kit (PE Applied Biosystems) according to the manufacturer's recommendations. The products of the selective fluorescently amplification were denaturalized with 5 volumes of formamide, mixed with 1 volume of Genescan-500 (PE Applied Biosystems) and 1 volume of gel loading buffer. Two microlitres of this mixture were charged onto an ABI PRISM 377 to obtain the fluorescent band patterns. Fragment analysis was carried out using GENESCAN software (PE Applied Biosystems).

Nucleotide accession numbers

Nucleotide sequences presented in this work have been assigned GenBank accession numbers AY831564–AY831633.

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