

Phenotypic and genetic diversity within a colony morphotype

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Abstract

Isolates showing different and similar colony morphologies were selected from spread plates of bacteria from seawater samples taken in the northern Adriatic Sea. All isolates were characterised by restriction fragment length polymorphism (RFLP) patterns of their PCR-amplified 16S rRNA gene and by 95 physiological tests (Biolog system). Cluster analysis of both genetic and phenotypic patterns showed that different colony morphotypes were related to different species or biotypes. However, isolates belonging to the more well-defined, conspicuous colony types had a high similarity, whereas those from the less conspicuous colony morphotypes showed high genetic diversity. Although colony morphotypes clearly underestimate taxonomic diversity, they can be used combined with PCR-RFLP analysis and as a preliminary approach for ecological studies aimed at the isolation of different species. Furthermore, for some species forming very conspicuous pigmented colonies, such as some photosynthetic aerobic bacteria, colony morphology may be useful for a rapid and low-cost screening of their distribution in the natural environment, especially when combined with other molecular techniques. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

The recent application of molecular biological techniques to investigate the diversity of marine bacterial communities has revealed 16S rRNA sequences of previously unsequenced and possibly uncultured 'new' bacteria [1,2]. At the same time, data demonstrating the dominant occurrence of culturable bacteria in the sea have been reported [3]. It was shown that marine bacteria with the ability to form colonies

on solid media occupy a considerably larger fraction of the marine bacterial community than the number of CFU would suggest [3]. Although molecular techniques are of increasing use in microbial ecology, isolation of bacteria remains an essential task to relate taxonomic and metabolic diversity of organisms.

Use of colony morphotypes is a common procedure in microbial ecology to select clones from complex environments for diversity measurements and/or isolation of the dominant species within culturable communities [4–7]. Colony morphotypes are also used to isolate different species for physiological and genetic purposes [8–10]. Recently, it has been

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shown by fatty acid methyl ester analysis of different isolates that colony morphotype can provide an accurate basis on which to define recoverable diversity [11]. However, the extent of the variability within a colony morphotype is unknown and may be underestimated when working with the most common morphotypes. Thus, a rapid and low-cost method to discriminate more species within a given colony morphotype should be very useful. Furthermore, the most typed colony morphotypes, such as those of pigmented strains, may be used in microbial ecology to obtain rapid and low-cost data on the spatial and temporal distribution of some relevant species.

The purpose of this study was to investigate (i) the genetic and phenotypic diversity within and between colony morphotypes and (ii) the validity of using typed morphotypes to obtain rapid information on the presence and distribution of a given species in natural samples.

2. Materials and methods

2.1. Sampling strategy

Bacteria were isolated on marine agar plates from samples taken at two different fixed stations from the shore (station A located in the Trieste Bay, Italy) to the open sea (station B) in the northern Adriatic sea in June 1995. Samples at stations A and B were taken at different times during 12 days.

2.2. Isolation of bacterial strains

All the strains were isolated on marine agar plates (Difco, Detroit, MI). Bacterial colonies were isolated from each agar plate under aerobic conditions at 20°C after 7–14 days of incubation. Aerobic conditions were chosen because of our interest to isolate and to analyse the temporal distribution of aerobic heterotrophic bacteria. Preliminary tests have shown that marine agar yields more colonies than a low-nutrient medium such as R2A. All isolates representing morphologically different colonies and/or similar morphotypes were picked and purified for further analysis. Total bacterial counts were determined by DAPI staining.

2.3. Phenotypic characterisation

All isolates were tested for their cellular and colonial morphologies, Gram reaction, for their ability to produce catalase and oxidase, to oxidise or ferment glucose and to grow at different salinities (8 and 35‰). Moreover, each isolate was characterised using Biolog GN microplates (Biolog, Hayward, CA). The Biolog identification system has been previously described and evaluated [12]. Cells were grown at 20°C for 24–48 h (depending on their growth) on saline trypticase soya agar (bioMérieux, Lyon, France) plates and then were resuspended in artificial seawater (ASW, 35‰, pH 7.5; Seasalts, Sigma, St. Louis, MO). Strains were rinsed three times by centrifugation to remove organic matter and resuspended in 20 ml of ASW. Inocula were adjusted to the same cell densities and plates were inoculated with 125 µl of the cell suspension per well. The microplates were incubated at 20°C and the results recorded after 1, 2, 3 and 5 days of incubation [13]. Cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) [14] was performed with profiles obtained after a 24-h stabilised reading (corresponding to 72 h or 5 days).

2.4. Genomic DNA extraction and purification

DNA templates were prepared from 3–5-day cultures on marine agar plates. A colony of each isolate was resuspended in 20 ml of marine broth (Difco, Detroit, MI) and incubated at room temperature overnight. A 10-ml sample of the bacterial culture was centrifuged at 10,000×g for 2 min. The pellet was resuspended in 2.5 ml of cold (4°C) sucrose lysis buffer (sucrose 25% w/v, Tris (50 mM), EDTA (50 mM), pH 8.8) supplemented with lysozyme (2 mg ml⁻¹) and the preparation incubated at 37°C for 15 min. After addition of 500 µl sodium dodecyl sulfate (SDS) 10% (w/v) and 30 µl of proteinase K (20 mg ml⁻¹), the mixture was incubated at 55°C for 30 min. To ensure bacterial lysis, the tubes were placed in a boiling water bath for 2 min and then frozen in dry ice for 10 min.

High-molecular-mass bacterial genomic DNA was purified from the cell lysates by two sequential phenol:chloroform:isoamyl alcohol (50:49:1) extractions. The residual phenol was removed by extract-

ing the aqueous phase with an equal volume of chloroform:isoamyl alcohol (24:1).

The genomic DNA was precipitated with the addition of 0.6 vol. isopropanol. The pellet was resuspended in 1 ml isopropanol, centrifuged at $10\,000 \times g$ for 30 min and washed twice in 2 vol. of cold (4°C) ethanol 70%. The resulting pellet was vacuum-dried and resuspended in 100 µl distilled water with 1 µl RNase (10 mg ml⁻¹). The DNA concentration was adjusted to 100 µg ml⁻¹, as determined at OD₂₆₀.

2.5. Amplification of 16S rRNA genes

Oligonucleotides used for PCR priming were designed from the 16S rDNA and 23S rDNA genes. The primers were FGPS6 (5'-GGAGAGTTA-GATCTTGGCTCAG-3') corresponding to positions 6–27 of *Escherichia coli* 16S rRNA [15], and the reverse primer FGPS1509' (5'-AAGGAGGT-GATCCAGCCGCA-3') corresponding to the complement of positions 1455–1474 [15]. When needed, the FGPL132' (5'-CCGGGTTTCCCCATTCGG-3') and FGPS6 primers were used to amplified the 16S rDNA, the intergenic spacer and the 5' part of the 23S rDNA [15]. PCR reactions were performed as previously described [15].

Amplification reactions were done in a thermal cycler (Mini Cycler, MJ Research) using a hot-start technique (3 min at 85°C) prior to the addition of the polymerase to prevent non-specific amplification; it was performed after an initial denaturation for 3 min at 95°C. Parameters for the amplification cycles consisted of 1 min at 94°C (denaturation), 1 min at 60°C (primer annealing), and 2 min at 72°C (chain extension), with an additional extension time of 3 min at the final cycle, for a total of 35 cycles. After the reaction, samples were stored at -20°C. Efficiency of the reactions was estimated by agarose gel electrophoresis of 5 µl of PCR-amplified gene products (0.8% (w/v) agarose (Bioprobe, Paris, France) in TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) supplemented with ethidium bromide (1.0 µg ml⁻¹).

2.6. Restriction fragment analysis

Approximately 1–2-µg aliquots of each PCR product were digested separately with *Cfo*I (BRL,

Eragny, France), *Hinf*I, *Hae*III and *Msp*I (Eurogentec, Seraing, Belgium). The suppliers' instructions were followed and samples were incubated for at least 2 h at 37°C to ensure complete digestion. The resulting fragments were separated by horizontal electrophoresis in 3% agarose (Sigma) with TBE buffer supplemented with ethidium bromide (1 µg ml⁻¹). A 1-kb DNA ladder (BRL) was used as a molecular mass marker. DNA fragments were visualised and photographed with a Polaroid 667 film under UV illumination (fragments shorter than 90 bp were not considered for analysis because they were usually below the detection limit).

2.7. Statistical analysis

The results for all phenotypic characters were scored as positive (1) or negative (0). A similarity matrix for all pairwise combinations of strains was constructed using the simple matching coefficient [14] as a measure of proximity. A dendrogram was constructed from the distance matrix by using UPGMA [14]. Computations were carried out with the 'R package for numerical taxonomic analysis' distributed by Pierre Legendre (University of Montréal, Québec, Canada). For PCR-RFLP analysis, pairwise comparisons of the band patterns were manually performed considering each band as a character. The sequence distances between 16S rDNA amplicons using RFLP patterns were estimated by using the mathematical model of Nei and Li [16]. Computations were done using a Macintosh hypercard pile developed by Marc Neyra (ORSTOM, Dakar, Sénégal). Graphic representation of the resulting dendrograms was done using the NJPlot software of M. Gouy (Laboratoire de Biométrie, UMR CNRS 5558, Université Lyon I) available at <http://biomaster.uio.no/phylogeny.html>.

3. Results and discussion

3.1. Phenotypic and genomic comparison between 30 isolates having different colony morphotypes

A total of 30 morphotypes were found from the different seawater samples taken at different times. One isolate of each morphotype was purified and

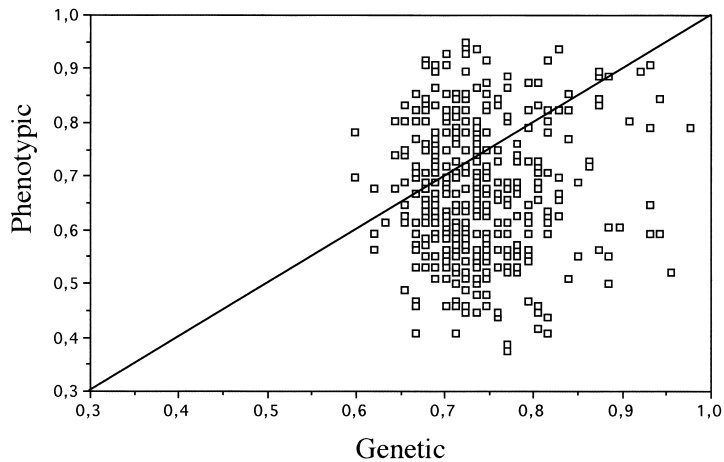


Fig. 1. Correlation between similarity values recorded by phenotypic (Biolog system) and genetic (PCR-RFLP) characterisation for 435 pairs of isolates ($y = 0.146x + 0.559$, $r^2 = 0.006$). The line corresponds to a 1:1 relationship.

characterised. All isolates were Gram-negative, rod-shaped bacteria except one isolate which was Gram-positive. PCR-RFLP analysis using four restriction enzymes resulted in the observation of specific patterns for each isolate. A similarity level of 96% was used to group the different isolates. They were also characterised phenotypically using Biolog-GN microplates. To determine the level of reproducibility of the Biolog identification system, six strains corresponding to six morphotypes were used and for each strain, eight replicates were analysed (including four replicates from the same suspension and four replicates from four different suspensions of the same strain). It was found (data not shown) that two to six descriptors varied between subcultures and three to 10 descriptors varied between replicates of the same subculture. The variability was observed within carbohydrates, carboxylic acids and amino acids. This suggests that within the same clonal strain, variability is at least 11% and a similarity level of 89% is the highest that can be used reliably.

The correlation between phenotypic and genetic patterns was studied by comparing the similarities recorded by both methods between each pair of isolates, each one corresponding to one of the 30 different morphotypes (435 similarity values) (Fig. 1). This shows that the two methods cannot be correlated ($r^2 = 0.006$) due to the presence of a large fraction of pairs of isolates which have very close patterns by one method and very different patterns by the

other. All 30 isolates showed different RFLP patterns and phenotypic traits since the similarity values were less than 1, suggesting that each morphotype belongs to a different bacterial species or group. However, a large number of genetically closely related isolates were not closely related by phenotypic analysis and inversely.

3.2. Phenotypic and genomic comparison between isolates showing the same morphotype

Among the 30 different morphotypes, four morphotypes of colonies were selected based on pigmentation or form and 63 colonies showing one of these four morphotypes were picked from plates inoculated with seawater samples taken at different stations and times. The morphotypes were selected because they were dominant in most samples.

Colonies showing morphotype A (MA) were 3–4 mm, not pigmented, cream and circular, flat with a smooth texture, opaque with entire margins and a translucent area around the colony. Morphotype B (MB) was characterised by 4–6-mm colonies, not pigmented, cream and circular, flat with a smooth texture, opaque with entire margins and could be distinguished from the MA type by the absence of a translucent area around the colony. All of the isolates from morphotype MC produced 3–4-mm orange colonies, flat and smooth, circular, bright and translucent with an opaque area in the centre. MD

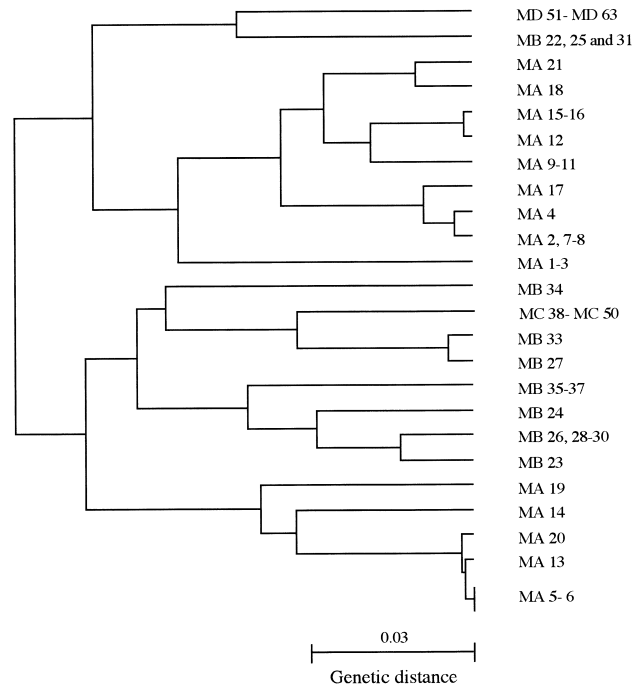


Fig. 2. Dendrogram of strains representing the four different colony morphotypes (MA, MB, MC and MD). Genetic distances were calculated by transformation of the PCR-RFLP patterns in terms of nucleotide substitutions (see text).

isolates were characterised by 3–5-mm bright colonies showing a pink-brown pigmentation, circular, convex with an entire margin. Among the 63 colonies isolated from different samples, the morphotypes MA, MB, MC and MD were represented by 21, 16, 13, and 13 colonies, respectively.

Clustering analysis based on nucleotide substitutions (after transformation of PCR-RFLP data) of

all isolates representing the four morphotypes shows that only two of them (the two pigmented ones, MC and MD) are coherent (Fig. 2). All the isolates within each of these two morphotypes show identical substitutions. Moreover, amplification of the spacer region between the 16S and 23S rDNA genetic loci gave similar products and RFLP patterns (data not reported). On the other hand, the two morphotypes

Table 1

Comparison of clustering results at different similarity levels between phenotypic (Biolog system) and genetic (nucleotide substitution) analysis of isolates within each of the four different colony morphotypes (MA, MB, MC and MD)

Morphotype	Analysis	Similarity cutting level				
		1.00	0.97	0.95	0.90	0.85
MA	Phenotypic	14	11	10	7	1
	Genetic	14	6	3	1	1
MB	Phenotypic	21	19	17	9	8
	Genetic	8	5	4	1	1
MC	Phenotypic	13	6	2	1	1
	Genetic	1	1	1	1	1
MD	Phenotypic	13	12	11	7	5
	Genetic	1	1	1	1	1

characterised by fewer distinguishing features (MA and MB) are interspersed with one another and thus cannot be used for distribution studies. One isolate of both pigmented strains was identified by 16S rDNA full sequences (data not shown). The strain showing the MC morphotype was identical (99% similarity) to *Erythromicrobium longum* and the MD morphotype was identified as a member of the genus *Roseobacter*.

The number of clusters recorded by both methods at different levels of similarity differs (in terms of number of clustered isolates and in term of isolates) for the four morphotypes (Table 1). The isolates showing morphotypes MA and MB were distributed in different clusters by both methods at similarity levels higher than 0.95. All isolates within morphotype MC showed genetic homologies and were clustered by phenotypic analysis at a similarity level lower than 0.95 (corresponding to the reproducibility limit of the Biolog system). In contrast, isolates within the MD morphotype, showing 100% homology with regard to PCR-RFLP patterns from the both the 16S rRNA gene and the intergenic spacer, were phenotypically clustered at a similarity level of only 78%. This suggests that the variability of phenotypic traits within different clones of a given species or group may be greater than that recorded between replicates of the same clone (11%). Such variability can explain the large discrepancies recorded between both methods when we characterised 30 isolates showing different morphotypes. An important part of the phenotypic variability may be due to the low reproducibility of the method which can also explain the problems of bacterial identification already reported with the Biolog system [13]. This system works relatively well with the most common bacteria but not with marine isolates which sometimes require longer incubation times and show lower rates of substrate oxidation (unpublished data). Similar disagreement between genetic and phenotypic results has already been reported [7,17]. Jimenez [7] reported that marine deep-subsurface isolates with the same phenotype had different DNA structures in terms of mol% G+C and DNA similarity. Partekh et al. [17] showed that phenotypically similar isolates had different RFLP patterns.

From a genetic point of view, our results suggest the existence of different species or groups within the

same morphotype since organisms that have less than 97.0% 16S rDNA sequence similarity may be considered as belonging to different species corresponding to less than 60–70% genomic DNA similarity [18]. Although this 97% similarity level should be confirmed by DNA-DNA hybridisation, isolates within the MA and MB morphotypes were clustered at less than 92% sequence similarity and thus may represent different species or groups. This may be explained by the fact that these morphotypes are very common on marine agar plates and can be shared by a wide variety of species. In contrast, isolates within one of the two pigmented morphotypes (MC and MD) show 100% 16S rDNA sequence similarity suggesting that all isolates within each pigmented morphotype probably represent the same species. This result was confirmed by the presence of similar RFLP patterns in the 16S-23S spacer region since it was shown that length and sequence polymorphism in the spacers within the *rrn* loci can be used to discriminate between different species of prokaryotes [19].

3.3. The ecological interest and limitation of colony morphotypes for dynamic studies

Although applicable to a small number of bacterial species, the use of morphotypes having very marked characteristics may be of ecological relevance to obtain information on the spatial and temporal distribution of some species in the environment (Table 2). The importance of the *Roseobacter* genus in marine pelagic ecosystems has recently been reported from phylogenetic studies [20]. However, the ecological role of this genus as well as the metabolic

Table 2

Percentages of culturable cells showing the MD colony morphotype in seawater samples taken at different times at stations A and B in the northern Adriatic in June 1995 (standard deviation, $n=3$)

Day	Station A	Station B
1	18.4 ± 5.2	16.7 ± 6.4
2	2.9 ± 1.8	5.8 ± 3.6
4	0 ± 0	50 ± 2.8
5	0 ± 0	10 ± 2.8
7	0 ± 0	12.5 ± 4.9
10	0 ± 0	10 ± 3.5
12	0 ± 0	6.8 ± 3.9

characteristics and the distribution of its species remain unknown. In this study, the dynamics of a pigmented strain identified as a *Roseobacter* species (MD morphotype) was analysed. To our knowledge, this is the first report on the temporal and spatial dynamics of a *Roseobacter* strain in the marine environment. This culturable species, which was dominant at station B, represented more than 50% of the culturable community and less than 1% of total bacterial counts.

In conclusion, our results suggest that the diversity of colony morphotypes underestimates the diversity of species present on a solid agar medium. However, colony morphotypes can be combined with PCR-RFLP analyses of different isolates within the most common morphotypes to improve the discrimination of species. In microbial ecology, this approach can be applied to obtain rapid data on the distribution of species forming very conspicuous pigmented colonies, such as some photosynthetic aerobic bacteria like *Roseobacter* species.

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