Niche Differentiation of *Shewanella putrefaciens* Populations from the Baltic as Revealed by Molecular and Metabolic Fingerprinting

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ABSTRACT

Shewanella putrefaciens is an important denitrifier of stratified aquatic ecosystems. 134 isolates of this species were obtained from various depth profiles of two stations in the Baltic using five different isolation procedures. These isolates were analysed for their clonal genotypes by Randomly Amplified Polymorphic DNA (RAPD) fingerprints, and for their metabolic profiles using the BIOLOG system. The results indicated clonal structure to differ more on a spatial than temporal scale. Metabolic profiles correlated, in terms of utilization of 24 carbon sources of marine relevance, very well with the 22 clonal genotypes detected by RAPD. Calculation of niche-overlap-indices (NOI) revealed differences in niche size (number of substrates) of almost 50% among the different genotypes. These findings indicate that for *S. putrefaciens*, different niches in an estuarine environment are reflected on a clonal level and not on a species level.

Introduction

Shewanella putrefaciens is of special importance to stratified aquatic ecosystems due to its capability of coupling the turnover of organic matter to anaerobic respiration with a large variety of electron acceptors, e.g. nitrate (NO_3^-), manganese (Mn^{4+}), and iron (Fe^{3+}) [10,1]. During a genotypic screening study of heterotrophic bacteria from the Baltic Sea, a large set of isolates was obtained from various depth profiles of two stations in the main basin [1]. Using Low Molecular Weight (LMW)-RNA profiling and Amplified Ribosomal DNA Restriction Analysis (ARDRA) [13], 134 isolates could be identified as *S putrefaciens* [5]. More detailed taxonomic analysis using 16S rDNA sequence analysis and determination of DNA-DNA homology of selected strains, resulted in the reclassification of *S putrefaciens* Owen's genomic group II as *S. baltica* [17]. All 134 Baltic isolates belong to this new species, and the name *S putrefaciens* used in this report is synonymous with *S. baltica*.

Recent studies indicate that pelagic bacterial communities are rather stable over time [3, 6] and mainly consist of a few abundant species dominating the community [4, 9]. Most of these studies on community structure of bacterioplankton are based on the molecular analysis of environmental ribosomal RNA and have a taxonomic resolution of about the species level or higher [14].

Here, we describe the genetic structure of a pelagic bacterium, *S. putrefaciens*, at a much finer scale, using the RAPD DNA fingerprinting technique [15]. Despite the disadvantage that this type of population structure analysis is only possible with cultured

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bacteria, the criteria observed for the structure of communities, i. e., stability over time and a limited number of abundant taxonomic units, were also fulfilled for the investigated *S. putrefaciens* population [17]. The population was observed to be dominated by five to six frequent RAPD genotypes that were present in both years, indicating a stability of the population over time.

In the present study, we extended RAPD typing [15] to the analysis of the genetic structure of all 134 *S. putrefaciens* strains isolated from the water column of two stations over two consecutive summers, thus allowing for a comparison of the *S. putrefaciens* populations over time and space. In addition to the genetic structure, the phenotypic structure of this *S. putrefaciens* population was assessed using the BIOLOG system for metabolic profiling. These physiological measurements aim to understand the correlation between genotype and phenotype, i. e. the relationship between the evolution and the ecology of an organism. More specifically, the following questions are examined: i) which niches are occupied by a pelagic bacterium; ii) does intraspecies competition occur, and; iii) at which taxonomic level are niches defined?

Material and Methods

Seawater samples were taken in the Gotland Deep and at station T aboard RV "Poseidon" from 15 to 19 August 1986, and aboard RV "Aranda" from 29 to 31 July 1987. The Gotland Deep station BY 15 (57°20.0'N, 20°03.0'E), and station T (59°25.5'N, 21°30.0'E) are located in the central part of the Baltic Sea. The Gotland Deep has a maximum depth of 240 m, with anoxic, H_2S-containing water below 140 m in 1986 and below 130 m in 1987. Station T, sampled only in 1986, displayed low oxygen but sufide-free water below 100 m. The overall hydrographic and chemical conditions in the water column of the Gotland deep for the sampling periods in 1986 and 1987 were very similar. For detailed descriptions of the station's hydrographic, chemical and microbiological parameters, see [2, 11].

Water samples were collected aseptically from the whole water column [10-235 m) using sterile champagne bottles mounted on modified ZoBell samplers. Samples were processed directly on board, i. e., initial inoculations and incubation steps for the five different isolation procedures were carried out immediately after sampling. Five different isolation procedures were used to obtain a broad range of different heterotrophic bacteria from the seawater samples, four procedures in 1986 (a, b, c, d) and two procedures in 1987 (c, e). At Station T procedure c was used only. Details for procedures a-e have been described previously [5, 16], the letters used for the isolation procedures here are directly comparable. For procedures a and b ZoBell agar plates were directly inoculated with 0.1 ml seawater, and incubated aerobically (a) or anaerobically (b) at 20°C for 14 days. For procedures c-e 0.001-10 ml seawater were inoculated in the following media: (c) nutrient broth + nitrate (Difco); (d) 1/10 strength nutrient broth + nitrate; and (e) thiosulfate-nitrate medium. Inoculated media were incubated at 20°C for five weeks.

All isolates obtained by the five isolation procedures were subjected to LMW RNA profiling for a first genotypic analysis [5]. All isolates that were identical in terms of their LMW RNA profile with *S. putrefaciens* were subjected to a more detailed taxonomic analysis using ARDRA and 16S rDNA sequence analysis to confirm their identity and establish their phylogenetic position [17]. The detailed genetic structure of all *S. putrefaciens* isolates from the Baltic was determined using RAPD fingerprints [15]. Three decamer random primers were used to generate a specific banding pattern for each primer

in agarose electrophoresis gels for each of the 134 strains. These three RAPD patterns were combined and normalized for each strain in an electronic way to enable statistical analysis and the definition of RAPD genotypes. Further details on the molecular and statistical analysis are given elsewhere [16].

Metabolic profiles of *S. putrefaciens* strains comprising five frequent RAPD genotypes were determined with BIOLOG-GN plates (BIOLOG Inc., Hayward, Calif., USA). Four strains per genotype were grown on TSA agar medium at 25°C for 2 days. Bacterial biomass was scraped off the agar plates and resuspended in 0.85 % NaCl as an inoculum with a defined optical density (OD 595nm about 0.35). Inoculated microtiter plates were incubated at 25°C for 7 days based on recommendations by Rüger and Krambeck [12]. Plates were checked for positive reactions by measuring the oxidation of substrates via the production of purple formazan from a tetrazolium redox-dye with a microplate reader (Emax precision microplate reader, Molecular devices) at 595 nm.

Results and Discussion

RAPD Genotypes from Two Consecutive Years in the Gotland Deep

S. putrefaciens exhibited substantial intraspecies diversity, as evident from the 22 RAPD genotypes shown in Table 1. The RAPD genotypes were obtained by clustering the 134 strains using two different algorithms. The frequencies of all 22 RAPD genotypes are summarized in Table 1. Frequency is defined as the percentage of a given RAPD genotype, in relation to the total number of isolated *S. putrefaciens* strains per station and year, in order to distinguish this term from the abundance of a RAPD genotype *in situ*, i. e., in the ecosystem. Six frequent RAPD genotypes (frequency of, at least, 5 %) were isolated in 1986. 83 % of all strains belonged to the frequent RAPD genotypes A, B, C, F, G and K. The remaining 17 % are distributed among RAPD genotypes D, E, H, I, L, M and N, and each of these rare genotypes was represented by one or two strains only.

The striking observation was that five of the six frequent RAPD genotypes from 1986 were still frequent in 1987, although different isolation procedures were used. The frequent RAPD genotypes A, B, C, F and K comprised 98 % of the total number of strains isolated in 1987 in the Gotland Deep. The proportion of RAPD genotype A, which was also isolated at high frequency in 1986 (30 %) almost doubled in 1987 (59 %). RAPD genotype G was the only frequent RAPD genotype not recovered in 1987. All other RAPD genotypes, which were missing in 1987 (D, E, I, L, M and N comprising 15 % of the total number of strains in 1986), were rare genotypes.

RAPD Genotypes from Two Different Stations

The *S. putrefaciens* population from station T was far different from the population at the Gotland Deep. Only two of 13 RAPD genotypes found at the Gotland deep were isolated from station T as well. The frequent RAPD genotypes A, B, C and K were missing at station T. Note the absence of the RAPD genotype A, reaching frequencies of 30 and 59%, respectively, in the Gotland Deep. Nine RAPD genotypes were isolated at station T only, but all of them had a low frequency. RAPD genotypes F and G, both with a frequency >5%, were also frequent at the Gotland Deep, indicating a wider distribution of these RAPD genotypes in the Baltic Sea. The absence of four frequent RAPD genotypes out of six from the Gotland deep and the presence of nine different RAPD genotypes

shows the spatial variation of the *S. putrefaciens* population across the Baltic Sea. Differences over space were greater than at a single station over time.

Genotype and Phenotype of S. putrefaciens at a Clonal Level

The genetic diversity of the *S. putrefaciens* populations observed was to a certain extent reflected at he phenotypic level. We chose 24 carbon sources of marine relevance out of 95 BIOLOG substrates to check for physiological differences between the RAPD genotypes [8]. The different usage of these 24 substrates is summarized in Table 2 for the frequent RAPD genotypes A, B, F, G and K from the Gotland Deep and station T. Ten substrates were used by all RAPD genotypes, e.g. N-acetyl-D-glucosamine, α-D-glucose, formic acid, leucine, serine, etc. These substrates can be considered as general substrates for *S. putrefaciens*. Other substrates could serve as marker substrates, because they were utilized by only selected RAPD genotypes. L-arabinose, for example, was used by RAPD genotype B exclusively, and N-acetyl-D-galactosamine was utilized by the RAPD genotypes G and K only. RAPD genotype F exhibited the most limited metabolic profile because it could use only ten out of the 24 substrates. RAPD genotype F was the only *S. putrefaciens* genotype that was not able to use seven substrates used by all other genotypes, e.g. acetic acid, L-asparagine or threonine. By contrast, RAPD genotype K, found only at the Gotland Deep, was the most versatile genotype and was capable of using 21 substrates out of 24. It is noteworthy that genotype F, despite its low substrate versatility, was the only genotype to be observed consistently over time and space, i.e. in the two consecutive years at the two stations.

The 24 selected substrates were used to calculate the niche overlap index (NOI) to obtain a quantitative measure of competition within a population of bacteria [7] (see Table 3). For the calculation of the NOI we determined the niche size, i.e. the number of substrates used, for each strain. The 24 carbon sources with relevance to marine ecosystems were chosen as substrates (Table 2). We then compared pairs of strains and the set of substrates used by both strains in relation to the niche size of each strain. In this way we obtained two results (in each direction) with values ranging from 0 to 1, as a measure for the capacity of the strains to coexist by using different niches. RAPD genotype F exhibited the smallest niche size (Table 3). Calculated NOIs ranged from 0.57 for genotype K versus F to 1 within one genotype indicating a 100 % overlap, i.e., identity within most of the frequent genotypes in comparison to only 57% overlap across different genotypes.

Conclusion

In conclusion, this first comparison of genotypic and phenotypic structure from a set of marine isolates, all of which belong to the same species as determined by detailed molecular analysis, showed that different physiological features can occur within genotypes of the same species derived from the same ecosystem. It can be assumed that these different features offer different niches to the genotypes in the environment. Competitiveness and filling of niches in the environment can therefore be assumed to occur in nature not only among different species, but also on the subspecies level. We suggest a need to consider the relevance of intraspecies filling of niches in the environment for the turnover of organic carbon in natural ecosystems.

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