Genotyping of Heterotrophic Bacteria from the Central Baltic Sea by Use of Low-Molecular-Weight RNA Profiles

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The Gotland Deep, an anoxic basin, was investigated for its heterotrophic microflora as a station representative of the central Baltic Sea and as an example of a brackish water environment. One hundred twenty-three bacterial strains were isolated along the water column by use of four different cultivation procedures. High-resolution electrophoresis of the low-molecular-weight (LMW) RNA (5S rRNA and tRNA) was used for analysis of the taxonomic position of the strains. The banding pattern of the LMW RNA generated by the electrophoresis allowed a taxonomic grouping at the species level of the 123 strains into 24 different genotypes. This grouping was confirmed by use of long-range gels with a substantially better resolution than that of standard gels; i.e., about 60% more tRNA bands were obtained on the long-range gels, and the distance between the bands was increased by about two-thirds. The majority of the strains (76%) could be identified to the species level by comparison with LMW RNA profiles from reference strains stored in an electronic database. Eighty-seven percent of the strains could be assigned to the families *Vibrionaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* (rRNA group I). The most abundant species among the isolates were *Shewanella putrefaciens* (48%) and a new *Pseudomonas* species (24%). The remaining fraction of 28% of the isolates was split into 22 other genotypes. Thirteen of these genotypes were represented by single isolates. This study demonstrates the utility of LMW RNA profiling for a rapid assessment of genotypic diversity of heterotrophic isolates from natural environments.

The Baltic Sea is the world's largest brackish water environment and can be described as a large estuary. Our sampling site (Gotland Deep, station BY 15) is a stratified basin of 240-m depth with anoxic deep water in the center of the Baltic Sea that is considered to be representative of the central Baltic (15, 47). We have chosen to investigate the heterotrophic microflora of this station for the following two reasons: (i) it serves as a good example for estuarine and brackish water environments, and (ii) the strong and stable physical and chemical gradients provide different conditions along the water column that are expected to support rather different heterotrophic bacteria. Additionally, a comprehensive set of data on chemical and physical parameters and microbiological activities for this station during our sampling program is available, and it allows the bacterial genotypes obtained to be related to the conditions and activities observed in the ecosystem (7, 10, 11, 17, 36).

There have been a few studies on heterotrophic bacterial isolates from the Baltic Sea (6, 32). Some of them give interesting insight into the changes in and relatedness among the heterotrophic bacteria at different times and locations (5, 6). However, like most older studies, all of these studies lack a solid taxonomic classification based on molecular data. Reliable determination of the taxonomic position of bacterial isolates from the environment is a crucial point for the value of an isolate in many respects. The features of the isolate can be related to the knowledge available for the respective species or

genus only if the taxonomic position is known. Results obtained by other groups working on bacteria from the same or a different ecosystem can be compared only on the basis of a solid taxonomic classification. The discovery of any new species basically relies on a reliable taxonomy.

Despite the fact that isolates cannot be assumed to represent the true taxonomic composition of the microbial community of an ecosystem, isolated strains are still needed as a basic link between research in the laboratory and the understanding of the functioning of the ecosystem (1). Physiological features of strains and thus their biogeochemical potential for the ecosystem can be described in the laboratory only via work on isolates (42, 43). Furthermore, isolates provide a basis for the direct analysis of natural bacterial communities in the following two ways: (i) antibodies and nucleic acid probes can be produced against isolates and used for direct single-cell detection in the ecosystem (2, 9), and (ii) 16S or 5S rRNA sequences of strains from the environment under consideration can be used as a reference basis for later analysis of the environmental rRNA by direct sequencing techniques (25, 26, 44).

On the other hand, for the description of the heterotrophic bacterial community and its diversity, a large number of strains must be isolated and identified (1, 12). Molecular identification methods, such as rRNA sequencing or DNA-DNA hybridization, describe the taxonomic position of a strain in a way unbiased by its physiological responses, which are often variable (4). However, for the analysis of a large set of environmental strains, these techniques are far too laborious. To follow Tiedje's recommendation for a reduction of the taxonomic redundancy, i.e., to identify all strains of the same species derived from one habitat and choose one representative strain for further studies (43), a rapid screening method is needed. Therefore, we used the more rapid and recently developed approach of high-resolution electrophoresis of low-molecular-weight (LMW) RNA (5S rRNA and tRNA) to analyze a large

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set of strains isolated from the central Baltic Sea (20, 23). High-resolution electrophoresis of the LMW RNA generates a pattern of 5S rRNA and tRNA bands based on their number of nucleotides with the 5S rRNA and the classes 1 and 2 of the tRNA forming three distinct groups of bands (22). This complex pattern of bands, called a profile or fingerprint, is unique for most species and allows identification of isolates by use of references in an electronic data bank (23).

LMW RNA profiling has been applied to several sets of reference strains from the domain *Bacteria* but not to larger sets of environmental isolates (14, 20, 21, 22, 24, 30, 37, 40). In the present study, we report the application of LMW RNA analysis by standard and long-range gels to 123 strains isolated along the water column of the Gotland Deep. Four different cultivation approaches were used to counteract to some extent the selectivity of a single isolation approach. LMW RNA profiles were obtained from all strains and allowed a genotypic grouping of all isolates. Comparison of the LMW RNA profiles with the reference data bank enabled identification of the majority of the isolates to the species level. It was demonstrated that LMW RNA profiling reduces the taxonomic redundancy of a set of environmental strains at the species level by about 80%, i.e., the single genotypes are subtracted from the total number of strains.

MATERIALS AND METHODS

Sampling and field measurements. Seawater samples were taken from the Gotland Deep aboard R.V. *Poseidon* from 15 to 17 August 1986. The position of station BY 15 in the Gotland Deep is 57°20.0'N, 20°03.0'E, located in the central part of the Baltic Sea. The Gotland Deep at station BY 15 has a maximum depth of 240 m, with anoxic water below 140 m. For a detailed description of the station's hydrographical, chemical, and microbiological parameters, see references 7, 11, 17, and 36. Water samples were collected aseptically from the whole water column (10 to 235 m) with sterile champagne bottles mounted on modified ZoBell samplers. Samples were processed directly on board, i.e., with inoculation and incubation for the four different isolation procedures.

Isolation procedures. Four different isolation procedures were used to recover as many different heterotrophic bacteria from the Baltic seawater samples as was possible. For procedure A, a yeast extract-peptone agar medium with a final salinity of 8‰ (i.e., ZoBell agar, modified from that described before [33], composed of 5.0 g of Bacto Peptone [Difco Laboratories, Detroit, Mich.], 1.0 g of yeast extract, 15.0 g of Bacto Agar [Difco], 250 ml of aged North Sea seawater, and 750 ml of deionized water [pH 7.2]) was inoculated with 0.1 and 0.01 ml of seawater and incubated in the dark at 20°C for 2 weeks. For isolation procedure B, ZoBell agar was inoculated with 0.2 ml of the water sample and incubated anaerobically in a desiccator in the dark for 3 weeks at 20°C. Oxygen was removed from the desiccator by use of the GasPak system (Becton Dickinson Co., Frankfurt, Germany). For procedure C, 5 ml of a nutrient broth plus nitrate medium modified from that described before (41) (composed of 3.0 g of meat extract [E. Merck AG, Darmstadt, Germany], 5.0 g of Bacto Peptone [Difco], 2.0 g of KNO₃, 250 ml of aged seawater, and 750 ml of deionized water [pH 7.4]) added to Hungate tubes was used. The inoculum from the Hungate tubes ranged from 10 to 0.001 ml of the original seawater sample, applied in three replicates. Procedure D was identical to procedure C except that the medium contained only 1/10 the organic material, i.e., 0.3 g of meat extract per liter and 0.5 g of Bacto Peptone per liter. For procedures C and D, inoculated cultures were incubated for 5 weeks in the dark at 20°C. Since all handling of the liquid culture medium was done aerobically, inoculated cultures started to grow aerobically and oxygen was reduced during growth as determined by gas chromatography (8).

From these four different sets of isolation procedures, bacterial isolates were obtained in the following way. For isolation from solid media (procedures A and B), 50 colonies were picked at random from the plates of samples for each depth and transferred to ZoBell agar plates with sterile toothpicks. After 2 weeks of aerobic incubation all typical colony types were isolated from these master plates. During this transfer process, an average of 12% of the colonies were lost as a result of insufficient growth on the next agar plates. This loss was less pronounced (6%) in water samples from the oxic part of the water column than it was from the anoxic part (18%). Master plates generated from anaerobically incubated plates were processed in the same manner, i.e., they were incubated aerobically after it was demonstrated that there was no significant loss of isolates in comparison with that during anaerobic incubation. This indicated that all colonies that were obtained from anaerobically incubated agar plates were only facultative anaerobes. Isolates from the liquid media (procedures C and D) were generated by streaking on ZoBell agar. We used for this isolation the growth-

TABLE 1. Number of isolates per genotype and taxonomic identification of the different genotypes and their relative size of the major 5S rRNA band in number of nucleotides

RNA profile type (genotype) ^a	Taxonomic identification	No. of isolates ^b	Size of 5S rRNA band(s) (nt)
S	<i>Shewanella putrefaciens</i>	59	120
V3	<i>Listonella anguillara</i>	1	120
V4	<i>Vibrio</i> sp.	2	120
P1	<i>Pseudomonas</i> sp.	29	120
P2	<i>Pseudomonas aeruginosa</i>	2	120
P3	<i>Pseudomonas</i> sp.	2	120
P4	<i>Pseudomonas fluorescens</i>	1	120
P5	<i>Pseudomonas pseudoalcaligenes</i>	1	120
E1	<i>Enterobacteriaceae</i> ^c	2	120
E2	<i>Enterobacteriaceae</i>	1	120
E3	<i>Enterobacteriaceae</i>	2	120
E4	<i>Enterobacteriaceae</i>	5	120
E5	<i>Enterobacteriaceae</i>	1	120
R1	NI ^d	1	126, 120
R2	NI	2	126, 120
G	NI	1	126, 116
D	NI	3	120
K ^e	NI	1	120
B	NI	3	119
C	NI	1	119
H	NI	1	119
F	NI	1	118
M	NI	1	117
L	NI	1	116

^a Total number of profile types, 24.

^b Total number of isolates, 123.

^c Similar to *Enterobacter aerogenes* and *Escherichia coli*.

^d NI, not identified.

^e Profile not shown in Fig. 5.

positive liquid cultures with the lowest and the two highest dilutions of the inoculum. By this procedure, only 33% of the growth-positive tubes showed growth on agar plates irrespective of the sampling depth. All different colony types were isolated on ZoBell agar plates and purified at least three times by serial transfer to new agar plates. The purified strains were kept as stock cultures for all further studies at -70°C in a solution of marine broth (Difco) with reduced salinity (8‰) and 20% glycerol.

Extraction of total RNA and electrophoretic analysis of LMW RNA. Bacterial strains were grown on ZoBell agar, and their biomass was scraped off the agar plate with a rubber spatula and harvested by centrifugation. Total RNA was extracted from some 10 mg of bacterial biomass (dry weight) with hot sodium dodecyl sulfate-phenol, followed by several steps of reextraction and chloroform treatments by the procedure given earlier (20). Finally, the total RNA was precipitated with ethanol. Total RNA was purified and enriched in the LMW RNA fraction by use of the Tip-100, recently renamed the total RNA Midi kit, as indicated by the manufacturer (Qiagen Co., Hilden, Germany). The Qiagen purification procedure for the total RNA extracts purifies the LMW RNA fraction by retaining the high-molecular-weight RNA and impurities, like polysaccharides, on an ion-exchange column and washing the LMW RNA off the column with low-salt buffer. The LMW RNA was then precipitated by the addition of 1 volume of ice-cold isopropanol. Further technical details on the extraction and purification of LMW RNA are given elsewhere (20, 22, 23, 25a).

Separation of LMW RNA was done on denaturing polyacrylamide gels (10% acrylamide, 7 M urea, in Tris-borate-EDTA buffer [100 mM Tris, 83 mM boric acid, 1 mM EDTA {pH 8.5}]) of two different sizes and different commercially available systems, namely, (i) standard gels (550 by 170 by 0.4 mm; Pharmacia LKB Co., Freiburg, Germany) and (ii) long-range gels (800 by 340 by 0.4 mm; Bio-Rad Co., Hercules, Calif.). Standard gels were run starting with a temperature of 60°C and at a constant power that was increased stepwise from 40 to 160 W (i.e., 1 h at 40 W, about 1.5 h at 80 W, and the last 15 to 30 min at 160 W) during the 3-h run. Long-range gels were warmed up to around 55°C during the run by increasing the power from 100 to 200 W during the 5-h run, starting with 100 W at 25°C for 1.5 h and then continuing with 200 W. RNA bands were visualized after electrophoresis by a modified ammoniacal silver staining procedure (20). Silver-stained gels were scanned by a computerized scanner (Hirschmann Co., Munich, Germany). These gel scans were normalized and stored in an electronic database. LMW RNA profile data were evaluated in two ways: (i) profiles of all isolates were compared and matched electronically, and (ii) the

FIG. 1. Comparison of LMW RNA profiles of *S. putrefaciens* ATCC 8071^T with Baltic Sea isolates of the most abundant RNA profile type S on a standard gel. Baltic Sea isolates were isolated from different depths and by all four isolation procedures (A to D). Lanes: a, *S. putrefaciens* ATCC 8071^T; b, strain OS 169, from 180 m, isolation procedure A; c, strain OS 186, 120 m, B; d, molecular weight standards containing 5S rRNA hydrolysate, tRNA^{Tyr}, and tRNA^{Phe} from *E. coli*; e, strain OS 204, 110 m, C; f, strain OS 107, 120 m, C; g, strain OS 161, 120 m, A; h, strain OS 202, 110 m, C; i, strain OS 223, 120 m, D; j, strain OS 106, 90 m, C; k, strain OS 230, 130 m, D; l, strain OS 177, 235 m, A; m, strain OS 155, 90 m, A.

different RNA profile types (i.e., different genotypes) were compared with a data bank of reference strains for identification. More details on the high-resolution electrophoresis of LMW RNA and the evaluation of the resulting LMW RNA profiles are given elsewhere (20, 23, 24, 25a).

RESULTS

Grouping of isolates by LMW RNA profiles by use of standard and long-range gels. Total RNA was extracted from all 123 strains and analyzed on standard gels. This first LMW RNA screening allowed an immediate grouping of the strains based on identical and different RNA profile types. The 123 strains could be grouped into 24 genotypes (Table 1). A major fraction of the strains could be assigned to two genotypes: 48% of the strains belonged to the genotype S, and 24% belonged to genotype P1. The remaining 28% of the strains showed a variety of 22 different RNA profiles that were often represented by single strains.

The most abundant RNA profile, designated S (identical with RNA profile type V1 from reference 23), was tested for homogeneity by use of two types of gels after the first screening by standard gels. For this purpose, we used a set of S-genotyped strains derived from different depths and isolation series. Total RNA extracts of these strains were purified and enriched in LMW RNA and run on a standard and a long-range gel (Fig. 1 and 2). Neither gel type shows differences between strains from different isolation series or depths, and both gel types demonstrate the homogeneity of the genotype S at the strain level. Bands were separated better on the long-range gel, and additional bands were observed for both tRNA classes. The resolution on the long-range gel is improved by two-thirds with respect to the distance between single bands differing by one nucleotide (an average of 2.8 mm per nucleotide on standard

FIG. 2. Comparison of LMW RNA profiles of *S. putrefaciens* reference strains with Baltic Sea isolates of RNA profile type S on a high-resolution gel. Baltic Sea isolates were isolated from different depths by all four isolation procedures (A to D). Strains labelled with an asterisk below are also shown in Fig. 1. Lanes: a, molecular weight standards containing 5S rRNA hydrolysate, tRNA^{Tyr}, and tRNA^{Phe} from *E. coli*; b, *S. putrefaciens* DSM 1818; c, *S. putrefaciens* ATCC 8071^T; d, strain OS 215, from 110 m, isolation procedure D; e, OS 169*, 180 m, A; f, strain OS 186*, 120 m, B; g, strain OS 204*, 110 m, C; h, strain OS 161*, 120 m, A; i, strain OS 223*, 120 m, D; j, strain OS 230*, 130 m, D; k, strain OS 155*, 90 m, A; l, strain OS 202*, 110 m, C; m, strain OS 106*, 90 m, C; n, strain OS 210, 110 m, D; o, strain OS 273, 110 m, C.

gels versus 4.6 mm per nucleotide on long-range gels). By comparing the gel scans from both types of gels, two new bands in the class 2 tRNA and three new bands in the class 1 tRNA were detected (Fig. 3, arrows). This means that the total number of tRNA bands which are used for identification was increased significantly (from 8 to 13 bands).

Also, purified RNA of the second most abundant RNA profile type, designated P1, was run on a long-range gel to detect differences among isolates from different depths (Fig. 4). There were no differences observed among isolates from different depths. Six additional tRNA bands could be detected by gel scanning if long-range runs were compared with standard runs (for direct comparison, compare Fig. 4, lanes d to k, with Fig. 5, lane m).

Next to the two major RNA profile types S and P1 (more than 25 strains each), all of the other RNA profile types were of minor abundance, i.e., one to five strains of each. Examples of all of these minor abundant RNA profiles are shown in Fig. 5, sorted by size of the 5S rRNA and the similarity of the tRNA

FIG. 3. Comparison of baseline-corrected gel scans from representative S-type RNA profiles (strain OS 161 [Fig. 1, lane g, and Fig. 2, lane h]) on a standard (a) and a high-resolution (b) gel. Arrows indicate additional bands on the high-resolution gel. Scan c shows the matching type strain *S. putrefaciens* ATCC 8071^T from the high-resolution gel (Fig. 2, lane c).

patterns. RNA profile type P1 is given in this figure only as an example for the five *Pseudomonas* species isolated because these profiles have been shown elsewhere (23). All 19 RNA profile types shown in Fig. 5 have unique patterns of tRNA bands, but some show strong similarities to each other, e.g., R1 and R2, E2 and E4, and V3 and V4 (Fig. 5, compare lanes b and c, e and f, and j and k). Differing bands in those similarity groups are labelled by arrows in Fig. 5. The size of the 5S rRNA bands, ranging from 126 to 116 nucleotides (nt) in the profiles of the single genotypes, varies a lot less than the tRNA band pattern, with most (i.e., 15) of the 5S rRNA bands sized around 120 nt (Table 1). Only three profiles have double major 5S rRNA bands (Fig. 5, lanes a to c). All of the other profiles show only a single major 5S rRNA sometimes with minor bands (<10% of the total 5S rRNA measured by gel scanning in the range from 105 to 130 nt).

Identification of isolated strains by LMW RNA profiles.

Taxonomic identification of environmental bacterial isolates by analysis of LMW RNA was performed by use of the following criteria that were established earlier (22, 23): (i) strains with identical 5S rRNA and tRNA patterns in terms of number and position of bands belong to the same species, and (ii) strains can be grouped together at the genus level if the majority of the tRNA bands are identical and only minor changes occur (22). For analysis on a standard gel, this usually means one to two varying bands in each class of tRNA in a group of strains belonging to the same genus with an overall identical pattern of tRNA. These identification criteria are on a relative basis if only unidentified isolates are compared with each other. For an absolute identification of new isolates, their LMW RNA profiles have to be compared with those of known reference strains from culture collections.

The comparison with reference strains was done with a set of electronically stored gel scans, comparable to the one shown in Fig. 3, of representative strains from five major eubacterial groups (gram positives, *Cyanobacteria*, *Planctomyces*, *Flavobacteria*, and *Proteobacteria*) and more specifically with representatives of marine genera (*Aeromonas*, *Alteromonas*, *Deleya*, *Pseudomonas*, and *Vibrio*). For the most abundant RNA profile, type S, the type strain of *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens*) and a second *S. putrefaciens* reference strain showed identical RNA profiles (Fig. 1, lane a, and

Fig. 2, lanes b and c). Long-range RNA profiles confirmed the results obtained with the standard gel by having five additional matching bands, which was especially useful because the S-type RNA profiles had no exceptionally specific bands like the P1 profiles did (Fig. 3).

The P1 profiles had a specific, very small, class 1 tRNA band at the bottom of the gel below the bulk of the class 1 tRNA (Fig. 5, lane m, position around 71 nt). It had been shown that this band is indicative for all true pseudomonads of rRNA group I as described by Palleroni (34; see also references 22 and 24). Therefore, all P-type RNA profiles could be easily identified as belonging to the genus *Pseudomonas* (Fig. 4, compare lane a with lanes d to k). On the other hand, species designation was only possible for three of the five P-type isolates (23). The RNA profile types P1 and P3 could not be matched with any of the twelve *Pseudomonas* species of rRNA group I described in *Bergey's Manual* (34) when compared on a long-range gel (data not shown). This indicates that they could represent two new species within the genus *Pseudomonas*.

From all other genotypes given in Fig. 5, only profile type V3 could be identified at the species level as *Listonella anguillara* (formerly *Vibrio anguillarum*). This identification was confirmed on long-range gels by use of *L. anguillara*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio natriegens*, and *Vibrio fischeri* as reference strains (gel not shown). The second *Vibrio* RNA profile V4 clearly belonged to the family *Vibrionaceae*, as evident by the results on the standard gel (Fig. 5, compare lanes j and k), but in comparison with the above-mentioned reference strains, no perfect match, i.e., species identification, could be obtained.

The E type of the RNA profiles was characterized by a fairly significant double band of class 2 tRNAs around 94 nt, followed by a set of three to six other class 2 tRNA bands (Fig. 5, lanes d to h). The pair with closest similarity was E2 and E4, differing by only a single additional band for E4 as indicated by the arrow (Fig. 5, lane e). RNA profile type E4 most closely resembled *Escherichia coli* and *Enterobacter aerogenes*. The number of bands for E4 was identical to that for *E. coli*, but slight differences in the migration distances of two class 2 tRNA bands were noticed. Since the only other member of the family *Enterobacteriaceae* available in the reference library of

FIG. 4. Analysis of the LMW RNAs from Baltic Sea isolates of the second most abundant RNA profile type P1 along the depth profile by use of a high-resolution gel. Lanes: a, *Pseudomonas aeruginosa* DSM 50163; b, *Pseudomonas fluorescens* DSM 50108 (very small amount of purified RNA); c, molecular weight standards containing 5S rRNA hydrolysate, tRNA^{Tyr}, and tRNA^{Phe} from *E. coli*; d, strain OS 148, from 30 m; e, strain OS 151, 50 m; f, strain OS 154, 90 m; g, strain OS 158, 120 m; h, strain OS 162, 130 m; i, strain OS 166, 140 m; j, strain OS 171, 180 m; k, strain OS 174, 235 m. All isolates were obtained from Baltic Sea ZoBell agar plates by procedure A.

standard gel scans was *Enterobacter cloacae*, which has only one band position different from that of *Enterobacter aerogenes*, a more defined taxonomic identification of this similarity group than of the family *Enterobacteriaceae* was not possible.

None of the other genotypes given in Fig. 5 could be identified because of the lack of conformity with any of the gel scans in the reference library. These 11 genotypes (R1, R2, G, D, C, H, B, K, F, M, and L) are all different at least at the genus level, except for R1 and R2. R1 and R2 have identical bands except for one (Fig. 5, lane c, arrow), indicating that they belong to the same genus. Despite a certain similarity in the class 2 tRNA bands, genotypes B and F were not similar because of major differences in the class 1 tRNA as revealed by

their gel scans (data not shown). Nine of these 11 unidentified genotypes were represented by only single isolates (Table 1).

Influence of isolation procedure on isolated genotypes. The influence of the different isolation procedures on the isolated genotypes is apparent in the number of isolates derived from the different procedures for genotypes P1 and S (Fig. 6). While *S. putrefaciens* was obtained rather evenly by all four isolation procedures, the *Pseudomonas* sp. genotype P1 was primarily (about 83% of all strains) isolated from ZoBell agar plates incubated aerobically. Not a single isolate of P1 was obtained from anaerobically incubated ZoBell agar plates. All other genotypes have abundances too low to show an effect of preference of the different isolation procedures and are therefore presented as a group in Fig. 6 for comparison.

DISCUSSION

Genotypic grouping of strains by use of LMW RNA profiles. High-resolution electrophoresis of the LMW RNA of 123 bacterial isolates from the Gotland Deep allowed us to group the strains within 24 genotypes. Furthermore, the majority of the strains could be assigned to the families *Vibrionaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae*. The two dominant genotypes S and P1 could be identified as *S. putrefaciens* and a presumably new marine *Pseudomonas* species.

The level of taxonomic information that can be obtained by grouping strains in genotypes by means of their LMW RNA profiles has been established earlier and can be summarized in some observations on the taxonomic relevance of the different groups of LMW RNA (22, 23). The 5S rRNA band is in an identical position for bacteria of the same genus. The tRNA pattern enables differentiation between species of the same genus, with the overall pattern of the tRNA being similar for members of the same genus; differences between species of the same genus are revealed by single species-specific bands in the tRNA pattern (22). Grouping on the species level by LMW RNA profiles has been confirmed for marine isolates by DNA-DNA homology determinations (37). Strains with a DNA-DNA homology higher than 56% showed the same LMW RNA profile. In general, grouping is done by use of standard gels (55cm). For bacteria where distinction is more critical, long-range gels (80cm) can be used. As exemplified for strains of *S. putrefaciens* and *Pseudomonas* sp. genotype P1, long-range gels enable a more precise comparison of the isolates because about five additional tRNA bands in class 2 and class 1 tRNA that confirm the identity of isolates from different isolation procedures and different depths are obtained. The use of long-range gels has already been demonstrated for the *Pseudomonadaceae* (24) and several genera of other families such as *Bacillus*, *Xanthobacter*, and *Hydrogenobacter* (14). Recently, a possible route to automation of LMW RNA profiling for bacteria was demonstrated by use of capillary electrophoresis with entangled polymers (27, 28).

Identification of strains and detection of new species. Identification of strains grouped into genotypes was done by comparing their LMW RNA profiles with those of reference strains from culture collections. Our reference data bank consists of about 200 species with emphasis on aerobic heterotrophic bacteria from aquatic environments. While all isolates of the families *Pseudomonadaceae* and *Vibrionaceae* could be identified to the species or genus level, isolates of the family *Enterobacteriaceae* could not be identified because of a lack of adequate references. Among the five *Pseudomonas* genotypes, two genotypes (P1 and P3) could represent new species. Like all other isolated *Pseudomonas* strains, the P1 and P3 genotypes belong to the rRNA group I (34) on the basis of their overall

FIG. 5. Comparison of all different RNA profiles found in the Baltic Sea isolates on a standard gel (except type S and types P2 to 5 that are shown in Fig. 1 or elsewhere [23]). Capital letters at the bottoms of the lane indicate the genus-specific genotypes; a number following the capital letter indicates the species subdivision (see also Table 1). Arrows indicate tRNA bands that differ among species of the same genus-specific RNA profile, except the long arrow in lane f that indicates a double band characteristic of members of the family *Enterobacteriaceae*. Lanes: a, strain OS 153, RNA profile type G; b, strain OS 323, type R1; c, strain OS 111, type R2; d, strain OS 136, type E1; e, strain OS 196, type E4; f, strain OS 159, type E2; g, strain OS 206, type E5; h, strain OS 146, type E3; i, molecular weight standards containing 5S rRNA hydrolysate, tRNA^{Tyr}, and tRNA^{Phe} from *E. coli*; j, strain OS 216, type V3; k, strain OS 188, type V4; l, strain OS 217, type D; m, strain OS 148, type P1; n, strain OS 137, type C; o, strain OS 123, type H; p, strain OS 140, type B; q, strain OS 147, type F; r, strain OS 233, type M; s, strain OS 139, type L.

tRNA pattern (24). On the other hand, their LMW RNA profiles were different from those of all 12 other *Pseudomonas* species of rRNA group I listed in *Bergey's Manual of Systematic Bacteriology* (34). To this end, LMW RNA profiling allows the detection of new species if a complete reference database bank for a genus is available, i.e., all type strains of the relevant species have been profiled.

The utility of identification and grouping of environmental strains by LMW RNA profiling is the rapid overview of their diversity at the species level that renders the strains accessible for further studies on various levels, namely, (i) determination of the 16S rRNA sequence of the unidentified genotypes to find their closest phylogenetic relatives, (ii) determination of DNA-DNA homology among related genotypes or within the same genotype, or (iii) analysis of the clonal structure of single genotypes by DNA fingerprinting techniques (35). In this way, LMW RNA profiling functions as a selection mechanism for the strains in two directions, i.e., (i) universal identification within the domains *Bacteria* and *Archaea* and (ii) studies with a fine level of resolution of the genotype beyond the species level. For progress in either direction, reliable taxonomic information is essential. For the first case, reduction of taxonomic redundancy is important because full-size sequencing of the 16S rRNA is time-consuming and costly (43). To this end, LMW RNA profiling reduced substantially the taxonomic redundancy of the whole set of strains, i.e., 87% of the strains were identical and identified at the species level, leaving 16 genotypes or strains for further molecular identification. For the second case, DNA fingerprints cannot be used to compare strains of different genera, and for most DNA fingerprinting

techniques, strains of the same species have to be compared (35).

Selectivity of the isolation procedure. It is always difficult to estimate to what extent bacterial isolates are representative of the ecosystem under consideration. Similar to results of other studies of aquatic environments, the plate counts in the central Baltic Sea reached only a maximum of 0.7% of the microscopic

FIG. 6. Number of isolates of the two main genotypic groups (S, S-type RNA profile, i.e., *S. putrefaciens*; P, P1-type RNA profile, i.e., *Pseudomonas* sp. genotype P1; other, all other genotypes) obtained by the four different isolation procedures, aerobic modified ZoBell seawater agar (■), anaerobic modified ZoBell seawater agar (▨), liquid nutrient broth plus nitrate (▤), and 1/10 concentrated nutrient broth plus nitrate (▥). All media had a final salinity of 8‰.

direct counts of the bacteria after acridine orange staining (8, 48). To compensate somewhat for the selectivity of a single medium, four different isolation procedures were used. As exemplified for the most abundant genotypes of *S. putrefaciens* S and *Pseudomonas* sp. genotype P1, isolation procedures may support one species and suppress another (Fig. 6). *Pseudomonas* sp. genotype P1 was suppressed in all liquid media and absent on the anaerobically incubated plates, which might be easily explained by the lack of oxygen in these media and a strictly aerobic metabolism of this species. *S. putrefaciens* genotype S is able to use a large array of electron acceptors that enables it to grow under aerobic and anaerobic conditions (8, 31). This might explain that it was isolated quite equally by all four isolation procedures. On the other hand, many factors other than simply oxygen supply (e.g., quality and concentration of carbon source, nutritional status of cells, physical conditions, etc.) might influence the selectivity of isolation procedures for a certain species, and far more sophisticated approaches are now being developed (39). However, one of the main reasons we used rather traditional isolation procedures was to compare our findings with earlier studies.

Comparison with other studies of marine bacteria. Goodfellow and Dickinson (19) gave an overview of the taxonomic results obtained by numerical analysis of bacterial populations from different ecosystems. For marine and estuarine ecosystems, 26 genera were listed, with only two genera agreeing with our study, i.e., *Pseudomonas* and *Vibrio*. It was striking that the genera classified most by numerical taxonomy were *Vibrio*, *Pseudomonas*, *Flavobacterium*, and *Achromobacter*. Unfortunately, these genera served at the time these studies were done as a heterogeneous collection of species from different genera (13, 16) and were split up later. For marine environments, Baumann et al. (3) described a large number of marine species, mainly *Alteromonas*, marine *Pseudomonas*, and *Alcaligenes* species (the last now called *Deleya*). These species were not found among our isolates of the central Baltic Sea as revealed by comparison with the appropriate reference strains from our database of LMW RNA profiles.

Extensive studies of heterotrophic isolates from the Baltic Sea were done by Bölter and Rheinheimer (5, 6). They used the same modified ZoBell agar medium as we did and grouped the strains by numerical analysis on the basis of 40 morphological and physiological features especially adapted to bacteria from the Baltic Sea. These studies provided interesting insights into the spatial and temporal relatedness of heterotrophic bacteria in the Baltic Sea. In the central Baltic, *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Enterobacter* spp. and coryneforms were reported to be the most important taxa. However, these taxa are difficult to relate to any species or genus defined by molecular taxonomy because the names used for these taxa can be considered to have represented in those days a large collection of rather heterogeneous bacteria that are now split up in different genera and even families (16, 18, 29, 45, 46). We tried to relate the taxonomy obtained by this numerical taxonomy to our genotyping. However, we failed because the majority of the strains under consideration in this study were analyzed as and termed *Pseudomonas* spp. while only a minor fraction of strains were classified by LMW RNA as *Pseudomonas* spp. and the major fraction were classified as *S. putrefaciens* (25b). On the other hand, it is useful to combine both techniques, i.e., genotypic screening of the strains with LMW RNA profiles and then subgrouping of larger genotypic groups like *S. putrefaciens* genotype S and *Pseudomonas* sp. genotype P1 by numerical analysis of the physiological characters. This would allow the

combination of an independent genotypic screening with the interesting physiological features of every genotypic group.

For the denitrifying fraction of the Baltic isolates, physiological features were provided elsewhere (8). Seventy-seven strains of the present study were able to denitrify. These strains consisted of 13 different genotypes, with the major fraction (59 strains) being *S. putrefaciens*. On the basis of abundance and physiological features, a major role of *S. putrefaciens* in the biogeochemical cycling of carbon and nitrogen in the suboxic part of the water column of the Gotland Deep was hypothesized (8, 11, 38).

Our taxonomic results fitted best with the findings of Neilson (32). He investigated coastal regions of the Baltic Sea with different impacts of pollution. He combined biochemical tests with determination of the G+C content of a few selected strains. He classified the isolates as members of the family *Enterobacteriaceae*, of the genus *Pseudomonas*, and the species *Alteromonas haloplanktis* and *S. putrefaciens*. In his study, a rather small number of Baltic Sea isolates (19 strains) was used, and five different phenotypes were detected at the species level. About a quarter of these strains were identified as *S. putrefaciens*.

In conclusion, we think that investigations of microbial diversity in specific ecosystems need approaches that rapidly genotype and identify environmental isolates and that are independent of the physiological reaction of the strains as is the case for phenotyping by the biochemical tests. For marine isolates, these physiological reactions can be highly variable or even undetectable. Molecular methods which provide a reproducible set of data that can be stored in electronic databases and compared whenever needed with any other data set are required. This would make the determination of microbial diversity less susceptible to changes in the actual systematics of microorganisms and conserve the observations for future studies. The problem of the changing bacterial systematics is very obvious when the classification of marine bacteria during the last two decades is examined (19). However, with the advent of a generally accepted bacterial systematics based on the phylogenetic analysis of rRNA sequences, a more stable bacterial taxonomy can be expected in the future (1, 45). Molecular methods using stable RNA for genotyping of environmental isolates, such as LMW RNA profiling, will allow a genotypic screening of a large array of strains that enables detection of new genotypes and species, even those of low abundance. Finally, this genotypic characterization of environmental isolates has to be compared with the direct analysis of natural bacterial communities by use of molecular methods to meet the challenge of exploring and inventorying microbial diversity of natural ecosystems (1).

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