Characterization of Bacterial Populations using Molecular Techniques

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INTRODUCTION

Historically, the study of bacteria has relied upon our ability to culture organisms in the laboratory. Organisms unable to be cultured have often been left uncharacterized. The role that uncultivable organisms play in a population is typically defined using only speculative evidence. This evidence is often based upon observations of the population as a whole rather than the specific metabolic activities of the individual organism. The recent application of molecular techniques to the study of bacterial populations has allowed for the roles of various genera and even species to be determined within complex communities (Bond et al., 1995; Fredrickson et al., 1995; Liu et al., 1997; Muyzer et al. 1993).

These various molecular techniques have been extremely valuable in the study of bacterial populations. When deciding to use such methods in the study of bacterial populations, one must realize that each method has strengths and weaknesses. While there have been numerous publications demonstrating the utility of these techniques (Braun-Howland et al., 1992; Moyer et al., 1994), there have been few studies where the limitations of these methods have been examined (Suzuki and Giovannoni, 1996).

Defined populations can be used as tools to test the sensitivity of a method to detect a particular species in a population. Also, each method can be tested for its ability to distinguish the differences between various populations and to determine the minimum amount of change required in a population for an effect to be detected. Defined populations consisting of five bacterial species at different relative concentrations were characterized in the present study using polymerase chain reaction (PCR) amplified 16S rRNA genes with restriction fragment length polymorphism characterization (RFLP), density gradient gel electrophoresis (DGGE), and reverse-capture checkerboard hybridization as well as in situ hybridization of whole cells. The use of defined populations allowed for the limitations of each method to be defined and for a direct comparison of each method to be made.

METHODS

Strain isolation

A water sample from the Great Harbor (Woods Hole, Mass.) was serially diluted to $10^6$ in 2% NaCl then plated on Marine agar 2216 (Difco) and incubated at 23°C. Colonies were
picked and restreaked on 2216 agar. This was repeated three times to ensure culture purity. Microscopic examination was also used to monitor purity.

**Population construction**
Each strain was grown in 40 mL of 2216 broth at 23°C with agitation (125rpm). Once a culture reached late logarithmic growth it was refrigerated. After all cultures reached late log, defined mixtures (Table 1) were prepared in sterile test tubes. These populations were then sampled for further characterization.

**Dilution plating**
Each population was serially diluted in 2% NaCl and plated (100 μL) on 2216 agar. Plates were counted after 3 days of incubation at 23°C.

**DNA extraction**
Pure cultures and population mixtures (1 mL) were centrifuged and resuspend in 100 μL of 0.01 mM Tris buffer, pH 7.6. Phenol (100 μL) and sterile glass beads(1-2 mg) were added. This mixture was shaken using a Beadbeater for 1 minute on homogenize setting. Following centrifugation (1 minute) the aqueous layer was transferred to a sterile, 0.5 mL tube. An equal volume of chloroform was added followed by vortexing and centrifugation. The aqueous layer was transferred to a sterile, 0.5 mL tube and mixed with 0.1 volume of 3M sodium acetate and an equal volume of 2-propanol. The DNA was precipitated on ice for 10 minutes. After centrifugation for 5 minutes, the supernatant was removed and 200 μL cold 70% ethanol was added. The sample was centrifuged for 5 minutes, the ethanol was removed and the sample was allowed to air dry. The DNA was resuspended in 20 μL of distilled water and stored at -20°C.

**Amplification of the 16s rRNA gene using PCR**
A standard reaction mixture for PCR amplification consisted of 5 μL of 10X buffer, 6 μL 25 mM MgCl$_2$, 4 μL dNTP mixture (6.25 μM each dNTP), 1 μl DNA template, 2 - 4 μL primers, 30 - 32 μL dH$_2$O and 1 bead of Promega TaqBead polymerase. Primer amount depended upon the type of primer being used. PCR product generated for characterization using restriction enzymes and sequencing was produced in reactions using 2 μL universal forward primer (Ufw: 5’ A(8)GA GTT TGA TYM TGG C(23) 3’; 20 μM), 1 μL universal reverse primer (Urev: 5’ GYT ACC TTG TTA CGA CTT (1492) 3’; 20 μM) and 31 μL dH$_2$O. The PCR method for this amplification was 94.5°C for 5 minutes, followed by 30 cycles of 94.5°C for 45 seconds, 53.5°C for 45 seconds and 72.5°C for 100 seconds after
which time the block was cooled to 4°C. Products from all PCR were confirmed on 1.0% agarose gels run at 100V.

Product generated for characterization using denaturing gradient gel electrophoresis (DGGE) was produced in reactions using 4 μL of a mixture containing DGGE forward (DGGE fw: 5’ CGC CCG CCG CCC GCG CCC GTC CCG CCG CCG CCG CCC T(341)AC GGG AGG CAG CAG 3’; 20 μM) and DGGE rev (5’ CCG TCA ATT CMT TTG AGT TT 3’20 μM) primers and 30 μL dH2O. The PCR method for this amplification was one cycle of 94°C for 5 minutes, 65°C for 1 minute, 72°C for 1 minute followed by 18 cycles of 94°C for 60 seconds, 64°C for 60 seconds and 72°C for 60 seconds. After each cycle the annealing temperature was lowered by 0.5°C. Cycles 20 to 30 were as follows: 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 60 seconds, after which time the block was cooled to 4°C.

PCR product used for characterizing samples by reverse-capture checkerboard hybridization was produced in reactions using 2 μL of digoxigenin labeled forward primer (5’ L(8)AG AGT TTG ATY MTG GC(23) 3’; 20 μM) with , 2 μL Urev (20 μM) and 30 μL dH2O. The PCR method for this amplification was 94°C for 5 minutes followed by 29 cycles of 94°C for 45 seconds, 53°C for 45 seconds and 72°C for 90 seconds. After each cycle the elongation time was increased by 5 seconds. The block was cooled to 4°C after the last cycle.

**Restriction enzyme digestion of 16s rRNA PCR product**

PCR product from each population and pure culture was digested with three separate restriction enzymes: HinP I, Hae III and Msp I. The reaction mixture for each enzyme was the same: 0.77 μL of NE buffer 2 (New England Biolabs), 0.38μL enzyme, 2.68 μL dH2O and 5 μL PCR product. Samples were digested at 37°C for 18 hours. Reaction products were run on 2.5 or 3.0% agarose gels at 100V. Bands were detected using ethidium bromide staining.

**Density gradient gel electrophoresis**

Gradient gels (1.0 mm) utilizing urea/formamide as denaturants in 6% acrylamide were used. Gradient composition was either 30 to 50% or 35 to 45% denaturants. The upper 5 cm of each gel was non-denaturing. Both gels were run at 200V and 60°C for 6 to 7 hours. Gels were stained with ethidium bromide in 1X TAE, pH8. Samples were mixed with loading buffer (5:1) prior to loading.
Reverse capture-checkerboard hybridization

This procedure was performed using a minislot/blot apparatus using a nylon membrane. One milliliter of each probe was applied to the membrane (50 pmole/mL of 10 mM Tris HCl, 1 mM EDTA), crosslinked using UV light and washed in 5X SSPE, 0.5% SDS for 30 minutes at 55°C with gentle shaking. The membrane was then treated with prehybridization solution in a sealed container overnight at 55°C with gentle shaking. Prior to loading the DNA, 9.6 µL of each sample was mixed with an equal volume of denaturing solution and 108.1 µL of 1.25X hybridization buffer that was warmed to 55°C. This mixture was heated to 55°C then 7.7 µL of neutralizing solution was added. This entire mixture (135 µL) was loaded. After all samples were loaded, the membrane was sealed and incubated at 52°C for 2 hours. The membrane was then washed with 1X SSPE, 0.1% SDS followed by conjugate buffer (1 minute) then blocked with blocking buffer/buffer 1 (1:5) for 1 hour with gentle shaking. The membrane was then washed in buffer 1 containing antibody (1000:1) for 30 minutes with gentle shaking. This was followed by two 10 minute washings with buffer 1. Substrate buffer (#3) was then added to the membrane which was incubated for 2 minutes, drip dried, then overlayed with 5 mL of DTTphos. The membrane was then exposed for 2 hours in the dark and visualized using a Storm system. The following probes were used:

1) universal 341: 5’ 20T’s-CTGCTGCCTCCCGTAGG 3’
2) universal 1089:5’ 20T’s-CTCGTTGCGGACCTAACT 3’
3) flavobacteria:5’ 20T’s-T(308)CAGTRCCAGTGTGCGGGG 3’
4) alpha proteobacteria:5’ 20T’s-C(500)GRAGTTAGCCGCGGC 3’
5) enterics:5’ 20T’s-C(1416)CTTTGCAARCCACTCC 3’

In situ hybridization

One milliliter of each population was centrifuged and resuspended in 4% formaldehyde. These fixed samples were then stored at 4°C for 2 weeks prior to hybridization. One hundred microliters of fixed pure cultures and populations were centrifuged and resuspended in 600 µL of 0.1% gelatin. Fifteen microliters of each sample were loaded and dried on a slide at 37°C. The slides (6 total each containing the five pure cultures and five populations) were treated with ethanol/formaldehyde (90/10; v/v) for 60 minutes at room temperature. These were washed twice for 2 minutes in distilled water then dried at 37°C. All spots (10) on a single slide were treated with a single probe by applying 40 µL of probe to each spot. The six probes, all at 340ng/mL hybridization solution, used were:

1) a mixture of three universal probes
primer A: 5’G(AT)ATTACCGCGGC(GT)GCTG3’
primer C: 5’ACGGGCAGGTGT(AG)C3’
primer 342: 5’CTGCTGC(CG)(CT)CCCGTAG3’
2) high G+C (5’CC(CG)GATATCTCGCGA3’)
3) low G+C (5’TGTAGCCCA(AG)GTCATA3’)
4) flavobacteria (5’TCA(TG)CCAGTGGGGG3’)
5) alpha proteobacteria (5’CG(AG)AGTATAGCCGGGGC3’)
6) enterics (5’CTTTTGGCA(AG)CCACCT3’)

The probes were allowed to hybridize overnight at 37°C in a seal container to prevent evaporation. Slides were then washed three times for 20 minutes in 1X SET at 37°C. The slides were then dried at 37°C and stored in the dark until observed.

RESULTS AND DISCUSSION

Strain identification and population composition

A portion of the 16S rRNA gene from each of the five isolates was sequenced and compared to pre-existing sequences in a publicly available database (Genbank). The genus name of each isolate, based upon high BLAST score results and manual sequence alignment, are as follows: isolate 12: Alteromonas; isolate 13: Pseudoalteromonas; isolate 15: Alteromonas; isolate 18: Shewanella; isolate 20A: Vibrio. All strains are aerobic, heterotrophic gamma proteobacteria and are highly related to one another. Recently, Gauthier et al. (1995) proposed placing many species of the genus Alteromonas into the genus Pseudoalteromonas. Given that only a fraction of the 16S rRNA genes for these strains was sequenced and that no biochemical tests were conducted, the only conclusion that can be made about isolates 12, 13 and 15 are that they are highly related to species present in the Alteromonas and Pseudoalteromonas genera. Further sequencing and biochemical data are also required in order to conclude that strains 18 and 20A are actually species of Shewanella and Vibrio, respectively.

Each population (Table 1) was serially diluted on 2216 agar to determine its true composition (Fig. 1). In general, the relative concentrations of strains 12, 13 and 20A remained equal while the concentration of strain 15 was typically 2 to 3 orders of magnitude lower. The variable isolate, number 18, was more than three orders of magnitude lower than isolates 12, 13 and 20A in population A and increased to being two
orders of magnitude greater than these same isolates in population E. Populations B, C and D represent a continuum between these extremes.

The effect of DNA concentration on the polymerase chain reaction
All of the methods used, except for in situ hybridization, require the amplification of the 16S rRNA gene from all populations and isolates. Figure 2 demonstrates the results of PCR run on the five isolates and five populations using undiluted DNA. No bands were visualized for isolates 12 (lane 1) and 20A (lane 5), indicating their 16S rRNA genes were not amplified. Upon dilution of the DNA (Fig. 3, lanes 1, 2, 6 and 7), amplification did occur. A reasonable explanation for this result is that the high concentration of DNA in the original reactions inhibited amplification. An alternative explanation could be that isolates 12 and 20A contain some inhibitor that is ineffective at higher dilution. This explanation is not supported by the results of PCR from the mixed populations since an inhibitor should have prevented amplification in these samples also. However, the presence of an inhibitor could not be ruled out. Also, the relative and total amounts of DNA may result in biased amplification (Suzuki and Giovannoni, 1996). For these reasons, undiluted and DNA diluted 1:10 was used with select methods (DGGE and checkerboard) to determine if any PCR bias could be observed.

Characterization of populations using RFLP
Ribosomal RNA genes amplified using PCR with undiluted DNA from each population and isolate were treated with three different restriction enzymes: HinP I, Hae III and Msp I (Fig. 4). The results for HinP I illustrate a technical challenge of this method. High density agarose gels are required in order to resolve all of the bands yet these gels are extremely difficult to pour. This can be seen the apparent downward stretching of lanes 2, 3, 4 and 5 in Fig. 4A, preventing a comparison of bands from these lanes to others on the gel. Inconsistencies in the gel can be avoided by using polyacrylamide gels, which also have much higher resolving power. The drawback to this is the toxicity of acrylamide. More importantly, in order to follow a specific member of a bacterial population using RFLP, it must have a unique band that can be used to distinguish it from all the other members of the population. Such a band (approximately 450bp) is noticeable in the HinP I digest for isolate 18 (Fig. 4A, lane 4). However, the poor resolution of the gel and the stretching prevents a comparison of the populations based upon this band. No such unique band exists in the Hae III digest (Fig.4B). A strong band of approximately 750bp exists in the Msp I digest (Fig.4C, lane 5) that allows strain 18 to be monitored in the various populations. Lanes 8 (population E) through 12 (population A) illustrate that as strain 18
becomes a smaller component of the population, the intensity of the band decreases (lane 10 to lane 11). Unfortunately, the band disappears in lane 11 despite the fact that strain 18 is still present in the population. This sensitivity problem can also be illustrated using the Hae III digest and isolate 15 (Fig. 4B, lane 4). A band of approximately 1000bp is evident in the lane for the isolate yet is undetectable in all of the population digests. This can be explained in two ways, the 16S rRNA gene for isolate 15 was never amplified or there is not enough DNA present for a band to be detected. The latter explanation seems more likely since isolate 15 is a minor component of all the populations (Fig. 1).

**Characterization of populations using DGGE**

Ribosomal RNA genes amplified using PCR with undiluted and diluted DNA from each population and isolate were analyzed using DGGE (Figs. 5 and 6). Figure 5, lanes 1 to 5 and 16 to 12 demonstrate that differences between populations can be visualized. This is best illustrated by the loss of the lowest band in lane 1 (representing isolate 18, see lanes 6 and 11) as the composition of the bacterial population changes (population E to A). The changes illustrated on the gel for the sequential populations represents what is actually happening between the samples: isolate 18 changes from the most abundant species to the least (Fig. 1). Two significant limitations are evident from this experiment. Similar to the RFLP result, the apparent loss of the band for isolate 18 would suggest that isolate 18 was no longer present in the population (Fig. 5, lanes 5 and 12) when it actually is present. Also, the density gradient used in this first experiment, 30 to 50%, was insufficient to resolve isolates 12, 13 and 15 (Fig. 5, lanes 7, 8 and 9). Again, without the benefit of knowing the true composition of populations A through E, the results for the populations in Fig. 5 would suggest that there are only two, perhaps three species present in the population. This greatly underestimates the true composition of the population.

A second DGGE experiment was conducted using a narrower density gradient (35-45%) in order to effect a better separation between isolates 12, 13 and 15. The results (Fig. 6) indicate that isolate 13 could be resolved from isolates 12 and 15 but these latter two isolates were still unresolved. This greater resolution of isolates 12, 13 and 15 is apparent in the lanes for the various populations with undiluted (lanes 10 to 15) and diluted (lanes 1 to 5) DNA, allowing for a better, yet still conservative, estimation of the number of bacterial species within each population. Also, while the resolution of isolates 12, 13 and 15 was greater, the band resolution for isolates 18 and 20A deteriorated (Fig. 5, lanes 6, 10 and 11 compared to Fig. 6, lanes 6 and 10). It is clear from these results that DGGE does allow for the visualization of changes in the composition of a bacterial population.
However, the technique can lead to underestimating the complexity of a population if a variety of gradient conditions are not employed. The results from PCR product generated using undiluted DNA (Fig. 5, lanes 12 to 16 and Fig. 6, lanes 11 to 15) do not differ significantly from those generated with diluted DNA (Figs. 5 and 6, lanes 1 to 5). It appears, at a qualitative level, that no bias in amplification occurred.

Characterization of populations using reverse-capture checkerboard hybridization
Ribosomal RNA genes amplified using PCR with undiluted and diluted DNA from each population and isolate were analyzed using reverse-capture checkerboard hybridization (Fig. 7). Five probes demonstrated the ability to hybridize to DNA present in any sample. Two of these probes, Univ341 and Un1089, are universal eubacterial probes that act as positive controls. The probes specific for bacteria in the alpha subdivision of the proteobacteria and for the flavobacteria also demonstrated the ability to hybridize to all of the isolates and the populations. These results are in conflict with one another since the proteobacteria are divergent from the flavobacteria. DNA from an isolate should not hybridize to both probes. These results are also questionable since most, if not all, of the other samples tested in this experiment appear positive for hybridization to both of these probes, demonstrating non-specific hybridization. Lastly, preliminary identification based upon 16S rRNA gene sequence analysis indicates that none of the strains fall into either of these groups. The last probe to show positive hybridization was that for enteric bacteria. Lanes 24 to 28 indicate hybridization, suggesting that an enteric organism is a component of each population. If no PCR bias occurred, the same result should be seen in lanes 34 to 38. Given the high level of background, it is difficult to conclude that there is a true positive signal in all of those lanes. The results for the isolates suggest that strains 13 (lane 30) and 20A (lane 33) are enteric bacteria. This result was confirmed for isolate 20A using in situ hybridization (data not shown). Isolate 13 did not show a positive result for enteric bacteria when tested using in situ hybridization. Identification by partial 16S rRNA gene sequencing places strain 20A in the family Vibrionaceae. The results from the checkerboard and in situ experiments suggests that the probe used to detect only enteric bacteria is not specific enough to distinguish members of the Vibrionaceae from those of the Enterobacteriaceae. This conclusion is dependent upon the a more definitive classification of isolate 20A as a Vibrio sp.

Restriction fragment length polymorphism analysis and density gradient gel electrophoresis analysis both detected predicted changes in defined bacterial populations (Figs. 4C, 5 and 6). However, both techniques indicated the loss of a member of the population when in fact
that member was present at low concentrations relative to other community members (Fig. 4C, lane 12; Fig. 5, lane 5 and 12; Fig. 6, lanes 5 and 13). DGGE allowed for a conservative yet incorrect estimation of the diversity of the populations studied. RFLP did not allow for any such estimation. Checkerboard hybridization analysis failed to detect any changes in the populations. Checkerboard results for individual strains were also in direct conflict with one another, with in situ hybridization data and with 16S rRNA sequence data. These results indicate that many of these molecular techniques can be useful in monitoring gross changes in bacterial populations. In order to fully characterize a population (i.e. define all of the bacterial components), each method must be operated under a variety of conditions to encourage the detection of all of the members of a population. This implies that no one set of conditions, be it a DNA extraction protocol, PCR method, gel density gradient or hybridization scheme, is sufficient to characterize a bacterial population. Conclusions about the nature and composition of a bacterial population can only be made after a number of molecular methods are employed repeatedly under a variety of conditions.
LITERATURE CITED


Table 1. Construction of bacterial populations using aerobic, marine heterotrophs. Values represent milliliters of each culture used to construct the populations.

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Fig. 1. Composition of various population mixtures determined by direct plate counting.
Fig. 2. PCR product using undiluted DNA. Lanes: 1, isolate 12; 2, isolate 13; 3, isolate 15; 4, isolate 18; 5, isolate 20A; 6, population A; 7, population B; 8, population C; 9, population D; 10, population E; 11, negative control.

Fig. 3. PCR product using diluted DNA. Lanes 1, isolate 12, 1:10; 2, isolate 12, 1:100; 3, isolate 13, 1:10; 4, isolate 15, 1:10; 5, isolate 18, 1:10; 6, isolate 20A, 1:10; 7, isolate 20A, 1:100; 8, positive control; 9, negative control; 10, blank lane; 11, 100bp ladder.
Fig. 4A. Restriction fragment length polymorphism of 16S rRNA gene using HinP I. Lanes: 1, 12, 13, 100kb ladder; 2, isolate 12; 3, isolate 13; 4, isolate 18; 5, isolate 15; 6, isolate 20A; 7, population E; 8, population D; 9, population C; 10, population B; 11, population A. Gel: 5.0% agarose.

Fig. 4B. Restriction fragment length polymorphism of 16S rRNA gene using Hae III. Lanes: 1, 7, 13, 100kb ladder; 2, isolate 12; 3, isolate 13; 4, isolate 15; 5, isolate 18; 6, isolate 20A; 8, population E; 9, population D; 10, population C; 11, population B; 12, population A. Gel: 2.5% agarose.

Fig. 4C. Restriction fragment length polymorphism of 16S rRNA gene using Msp I. Lanes: 1, 7, 13, 100kb ladder; 2, isolate 12; 3, isolate 13; 4, isolate 15; 5, isolate 18; 6, isolate 20A; 8, population E; 9, population D; 10, population C; 11, population B; 12, population A. Gel: 2.5% agarose.
Fig. 5. Density gradient gel electrophoresis (DGGE) of 16S rRNA genes after PCR. Gel gradient 30 to 50%. Lanes: 1, population E, DNA diluted 1:10 before PCR (0.1X); 2, population D, 0.1X; 3, population C, 0.1X; 4, population B, 0.1X; 5, population A, 0.1X; 6, isolate 18, 0.1X; 7, isolate 12, 0.1X; 8, isolate 13, 0.1X; 9, isolate 15, 0.1X; 10, isolate 20A, 0.1X; 11, isolate 18, 0.1X; 12, population A, DNA undiluted before PCR (1X); 13, population B, 1X; 14, population C, 1X; 15, population D, 1X; 16, population E, 1X.

Fig. 6. Density gradient gel electrophoresis (DGGE) of 16S rRNA genes after PCR. Gel gradient 35 to 45%. Lanes: 1, population E, DNA diluted 1:10 before PCR (0.1X); 2, population D, 0.1X; 3, population C, 0.1X; 4, population B, 0.1X; 5, population A, 0.1X; 6, isolate 18, 0.1X; 7, isolate 12, 0.1X; 8, isolate 13, 0.1X; 9, isolate 15, 0.1X; 10, isolate 20A, 0.1X; 11, population A, DNA undiluted before PCR (1X); 12, population B, 1X; 13, population C, 1X; 14, population D, 1X; 15, population E, 1X.
Fig. 7. Reverse-capture checkerboard hybridization of 16S rRNA genes after PCR. Lanes: 24, population E, DNA undiluted before PCR (1X); 25, population D, 1X; 26, population C, 1X; 27, population B, 1X; 28, population A, 1X; 29, isolate 12, DNA diluted 1:10 before PCR (0.1X); 30, isolate 13, 0.1X; 31, isolate 15, 0.1X; 32, isolate 18, 0.1X; 33, isolate 20A, 0.1X; 34, population E, 0.1X; 35, population D, 0.1X; 36, population C, 0.1X; 37, population B, 0.1X; 38, population A, 0.1X.