Microbiota of the hindgut of Periplaneta americana:
Studies of microbial diversity from culture techniques and molecular analysis

Abstract

Bacteria isolated from the hindgut of the Periplaneta americana mostly fell in the enterobacteriaceae and the Pseudomonadaceae groups. However, a limited study of the hindgut population using various molecular techniques (Checkerboard analysis, DGGE and RFLPs) revealed a hindgut containing a very species rich bacterial population. PCR amplified clones from the population hindgut yielded 16S sequence of a Cytophaga sp., and a bacterium that is related to the low GC gram positive bacteria. Checkboard analysis of the clones also hinted at the presence of a sulfate-reducer in the cockroach termite gut. The above results is partially verified by checkerboard analysis of population DNA of the Periplaneta which showed the presence of bacteria from the flavobacteria-cytophaga-bacteroides group, the sulfate-reducing bacteria and also enterics.

Introduction

The population microbiota in the gut/intestines of various vertebrates, invertebrates and insects have been studied quite extensively by various groups (1, 2, 3). Some gut/intestinal populations have been relatively well characterized while other studies have revealed only the tip of a microbial iceberg. In the latter cases, the morphological diversity of the microbiota has been observed to be very high when studied under various microscopic means. However, enrichment attempts have yielded only a fraction of those microorganisms observed invivo. One of the main reasons is the inability correctly mimic the nutrient and environmental niche of the “as yet to be cultured organisms” using various media formulations. This may partially reflect some obligate exo- as well as endosymbiotic relationships between microorganisms in the gut environment.

The scope of this investigation is to attempt to elucidate the bacterial diversity of the gut regions of the american cockroach Periplaneta americana using the traditional enrichment culture techniques as well as molecular techniques (eg. PCR using universal primers, cloning, RFLPs and DGGE). It is anticipated that enrichment culture techniques will result in the selection of a limited array of microorganisms while the molecular techniques will reveal a larger identity of the bacteria present in the gut environment.

Materials and methods

Extraction of gut contents

The gut regions (foregut, midgut, hindgut) of a cockroach Periplaneta americana were carefully dissected from the body and then the outer walls were then briefly rinsed in 95% ethanol to eliminate contaminating bacteria. The hindgut was then excised and homogenized with 100μl of sterile water.
Enrichment cultures

The following solid media was used: Medium 1- 1% glucose, 1% yeast extract and 1.5% washed bacto agar. Medium 2- 1% yeast extract, 1% casamino acids and 1.5% washed bacto agar. Medium 3- 1% yeast extract, 1% casamino acids, 1% nitrate and 1.5% washed bacto agar. The pH of above media are approximately 7.2.

Dilutions of homogenized gut contents were done with 10mM phosphate buffer (pH 6.8) and spread-plated onto petridishes with the three different media formulations. Dilutions were carried to 1x10^-9/ml.

Plates with media 1 and 2 were incubated at 37°C aerobically. Plates with medium 3 were plated under aerobic conditions and then immediately transferred into the anaerobic hood (room temperature =24°C).

Phenotypic characterizations

EMB (eosin methylene blue agar), TSI (triple sugar agar - glucose, lactose and sucrose), oxidase reagents, and SIMs citrate agar were used to characterize selected pure isolates.

Molecular techniques

PCR amplification of DNA

2 hindguts were carefully removed from cockroach bodies and rinsed with 95% ethanol. It was then added to 200µl of sterile TRIS-HCl buffer (pH 6.8) and homogenized. DNA was then extracted from 100µl of the homogenate (Appendix 1) resuspended in 20µl of sterile H2O and used as a template for amplification. Population DNA was amplified using universal primers, primers with GC clamps, digoxigenin labelled probes depending on methods used (Appendix 2). DNA of pure isolates were also obtained with methods as described in Appendix 2.

Cloning

As described in Appendix 3

DGGE (Denaturing gradient gel electrophoresis)

As described in Appendix 4

RFLPs (Restriction fragment length polymorphisms)

As described in Appendix 5

Checkerboard analysis

As described in Appendix 6 (reference)
Results

Checkerboard analysis (Figure 1)

Checkerboard analysis (Figure 1) revealed that isolate CI5 (lane 7) belongs to the alpha and enteric group. However, the probe designed for the alpha group has been shown to have high cross-reactivity to other genera of bacteria in the controls. Thus, it is most likely an enteric. This corresponds to the phenotypic characteristics of the isolate which is most probably a *Klebsiella* sp. (See below). Clones from the cockroach gut corresponding to lanes 13-16 reveals bacteria from the flavobacteria and sulfate-reducing bacterial group. This is confirmed by an earlier checkerboard (Figure 2) experiment where it was shown that the population DNA from the cockroach hindgut had very strong signals from the cytophaga-flavobacteria-bacteriodes group as well as for the sulfate-reducing bacteria. Weak signals were obtained for the spirochaetal group and the enterics.

Limited phenotypic characteristics of pure isolates (Table 1)

From a limited phenotypic characterization of pure isolates, isolates CI1 (i.e. cockroach isolate 1) and CI7 exhibited spore formation and are believed to be two *Bacillus* sp. CI3 appeared to be a *Pseudomonas* sp. and was oxidase positive. Isolate CI5 has been tentatively identified as a *Klebsiella* sp. due to its ability to ferment and grow on citrate. The other isolates could ferment sugars (TSI agar) and were presumed to be from the enterics. Sequence analysis of isolates confirm the presence of enterics and a pseudomonad (Figure 3). However, the order of the sequences were mixed up and a genus could not be assigned to any of the isolates. What is known, however, is that a majority of the pure isolates are from the family enterobacteriaceae with one isolate belonging to the pseudomonad group.

RFLPs analysis of 16s rDNA from pure isolates and clones (Figure 4)

16s rDNA from partially characterized pure isolates and clones were subjected to a limited RFLP analysis using the restriction enzyme *Hin*II. Isolate CI1 and CI7 exhibited banding patterns confirming results from phenotypic characterization and DGGE analysis (below). Isolates CI2, 5, 6, 8 and 9 show similar banding patterns suggesting that they belong to the same genus. Clones 1 - 4 exhibited different banding patterns.

DGGE analysis of 16s rDNA from some isolates and clones (Figure 5)

DGGE was run for the 16s rDNA of some of the isolates and clones used in the RFLP analysis to compare the uniformity of results between the two techniques. Again, isolates CI2 and 5 appear to be the same organisms as the bands co-migrated to the same position of the gel. Isolates CI 1, 3 and 6 have different migration patterns.
Identities of two clones obtained from sequence information

Clone c3 and c4 were characterized to be *cytophaga* sp. and a bacterium that is closely related to the low GC gram positive bacteria. (Figures 5 and 6)

Discussion

An attempt to characterize a complex microbial population from a cockroach hindgut using a limited culture based technique and three molecular tools show some good agreement between them despite limitations inherent to each technique. Some known phenotypic traits of pure isolates obtained from the cockroach hindgut were used as a reference points to compare data obtained between the three molecular techniques used. The RFLP analysis and the DGGE results, for example, confirmed the phenotypic differences (DGGE: Isolate 1 being a bacillus versus and Isolate 3 being a pseudomonad - figure 5) and similarities (RFLP: Isolate 1 being similar to Isolate 7 both of which are bacilli - figure 4) between different isolates obtained strengthening the usefulness of each techniques as a tool in a partial analysis of a complex population. However, for clones with no available phenotypic traits to study, the molecular techniques used above should only serve as a partial analysis of the clones due to their low ‘resolution’ if they are used independently. For RFLP analysis, more than one restriction enzyme needs to be chosen so that even closely related SSU rDNA clones can be differentiated (4). and for DGGE, bands at identical positions of the DGGE gel may not necessarily be derived from the same species. A more narrow gradient is needed to provide a higher resolution of a particular part of the original DGGE gel (5). The 5 clones were characterized without phenotypic information and shown to be different based on the different banding patterns RFLPs. The migrated positions of the bands of the clones in the DGGE gel were very similar and suggested that they had very low GC content. However, the likely possibility is that DGGE gel run was only run for 8 hours and not longer. The latter would allow for the 16S amplicons from the clones to migrate to their eventual positions. Controls were not done in this experiment to obtain a require run-time of the gel for these particular PCR amplicons.

The checkerboard analysis of the population DNA of the cockroach gut provided a very useful information on what genera of bacteria could be present invivo (fig 2). This would provide a useful framework with which to initiate enrichments for bacteria from a particular genus or even a particular species. In addition, after a clone has been obtained for the population DNA of the microbiota in the hindgut, a probe could be made for checkerboard analysis of the population DNA. This could help verify a clone’s presence in the gut population and not have it merely be a result of an amplification of a chance contaminant during the PCR procedure. Another verification would be to use in situ hybridisation using a constructed primer from the clonal sequence to probe the population in question. The bottleneck in the checkerboard procedure is the efficiency of the probe in its binding capacity (ie. Its binding stringency). Thus, controls would have to be run with known isolates to initially test the efficiency of the constructed probe, its ideal annealing temperature. If the efficiency of the probes have been tested and confirmed, then it may be possible for the investigator to measure species richness within a population based on the intensity of the signals.

The checkerboard confirmed the presence of the enterics in the gut population of the cockroach
when enrichment cultures were initiated in an attempt to isolate some gut microbiota. The selective nature of the medium composition was clearly inherent when mainly enterics, two bacilli and a pseudomonad was isolated. These bacteria have no fastidious growth requirements and were fast growers. Thus, they tend to be enriched more easily than the other bacteria which has more specific and narrow growth requirements (eg. *Cytophaga* sp. and the strict anaerobes - which the analysis of the clones revealed the presence of these bacteria in the hindgut).

The morphological diversity of the microbes in the hindgut of the *Periplaneta americana* was clearly evident under microscopic observation. Enrichment cultures and analysis of just 5 clones have revealed a very complex population in the termite of the hindgut. There is a need to analyze further the diversity within the hindgut of the cockroach through molecular techniques. Once the composition of the organisms within the hindgut has been elucidated, enrichment techniques can be employed to attempt to cultivate some of these organisms based on the results. This will provide a clearer picture to the already existing information regarding the contribution of these gut microbiota to the cockroach (6).

Also, there is a need to access the species richness within the population hindgut to study which bacteria actually contribute to the metabolism of the cockroach and which are merely transients. Along the same lines, perturbation experiments could be conducted to assess how the nutritional lifestyle of the cockroach can affect the population dynamics within the hindgut. These fluctuations can be followed by a combination of molecular techniques as well as the enrichment culture techniques used in this and other studies (7). The utilization of tools together will provide a more complete picture of the *in vivo* environment with regards to the population and physiological dynamics within the cockroach hindgut.
References:

7. Teske, A. et al. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments 62, 1405-1415
**PCR Protocol**

Use 0.2 ul tubes

Single reaction is as follows:

- 5 ul 10X buffer ✓
- 6 ul MgCl₂ (25 mM stock) ✓
- 4 ul dNTP mix (25-uM stock) ✓ 25 mM
- 2 ul universal forward (20 uM stock) ✓
- 1 ul Universal reverse (20 uM stock) ✓
- 1 ul template (DNA)
- 31 ul H₂O ✓

- then add 1 bead of Promega TaqBead polymerase

For multiple samples, create a master mix (minus DNA and bead) as follows:

For example for 20 reactions, use one 1.5 ml tube, add 21 times the amount of each reagent, mix, and then distribute 49 ul into each tube.

Add 1 ul of DNA template to appropriate tube, then add 1 bead to each tube.

- For every experiment, a negative control (one with no added DNA) should be run.

Run PCR (operation of EriComp tempcycler will be demonstrated).

Typical cycle for universal primers for 16S rRNA genes --

- 94°C for 5 min

then 30 cycles:

- 94°C for 45 sec denaturation
- 53°C for 45 sec annealing
- 72°C for 90 sec elongation

after the 30 cycles, 4°C hold

- After PCR, load 5 ul with 1 ul of loading buffer onto 1% agarose gel (+1 ul/ml ethidium bromide) in 1X Tris Borate EDTA buffer (TBE). 1 min under power in uncoverware.
- Run for 60 min at 100 mV. Visualize using the UV light box. Take only 1 picture (ask for directions—copies can be made on Bruce’s scanner
- Discard gels in special discard container (it will be disposed of separately).

Cautions:

- Ethidium bromide is a carcinogen—Use gloves!
- UV light can harm your eyes. Wear eye protection!
- Phenol and chloroform are harmful—use with precaution
- Do not change or add programs to Tempcycler without permission from Bruce or Dani
Using TOPO TA Cloning®

Sequence/Map of pCR®TOPO Vectors
The sequence and map of pCR®2.1-TOPO or pCR®II-TOPO are provided as separate documents with the kit you purchased. They are also available for downloading from our Website: http://www.invitrogen.com.

Materials Supplied by the User
In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.
- Taq polymerase
- Thermocycler
- DNA template for PCR product
- Primers for PCR
- 42°C water bath
- LB plates containing 50 μg/ml ampicillin or 50 μg/ml kanamycin
- 40 mg/ml X-gal in DMF (dimethylformamide)
- 100 mM IPTG in water (for use with TOP10F+)
- 37°C shaking and non-shaking incubator

Producing PCR Products
1. Set up the following 50 μl PCR reaction. Use the cycling parameters suitable for your primers and template and be sure to include a 7 to 30 minute extension at 72°C to ensure that all PCR products are full length and 3’ adenylated.
   DNA Template 10-100 ng
   10X PCR Buffer 5 μl
   50 mM dNTPs 0.5 μl
   Primers (~200 ng each) 1 μM each
   Sterile water add to a final volume of 49 μl
   Taq Polymerase 1 unit
   Total Volume 50 μl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If not, gel-purify your fragment before using TOPO TA Cloning® (see page 6). Take special care to avoid sources of nuclease contamination. Alternatively, you may elect to optimize your PCR to eliminate multiple bands and smearing (Innis et al., 1990).

Preparation
For each transformation, you will need one vial of competent cells and one selective plate.
- Equilibrate a water bath to 42°C.
- Thaw the vial of SOC medium from Box 2 and bring to room temperature.
- Warm LB plates containing 50 μg/ml ampicillin OR 50 μg/ml kanamycin at 37°C for 30 minutes.
- Spread 40 μl of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- If using TOP10F+ cells, spread 40 μl of 100 mM IPTG, in addition to X-gal, on each LB plate and incubate at 37°C until ready for use. IPTG is required for blue/white screening.
- Thaw on ice the β-mercaptoethanol and 1 vial of One Shot™ cells for each transformation.

continued on next page
Using TOPO TA Cloning®, continued

TOPO-Cloning™ Reaction

In general, 0.5 to 2 µl of a typical PCR sample (10 ng/µl) with an average insert length of 400 to 1000 bp will give the proper insert:vector ratio for TOPO-Cloning™.

1. Set up the following 5 µl TOPO-Cloning™ reaction.
   - Fresh PCR product 0.5 to 2 µl
   - Sterile Water add to a final volume of 4 µl
   - pCR®-TOPO vector 1 µl
   - Final Volume 5 µl

2. Mix gently and incubate for 5 minutes at room temperature (~25°C).
3. Place tube on ice. Proceed immediately to One Shot™ Transformation Reaction, below.

One Shot™ Transformation Reaction

1. Add 2 µl of 0.5 M β-mercaptoethanol to each vial of competent cells and mix by stirring gently with the pipette tip. DO NOT MIX BY PIPETTING UP AND DOWN.
2. Add 2 µl of the TOPO-Cloning™ reaction into a vial of One Shot™ cells and mix gently.
3. Incubate on ice for 15 minutes.
4. Heat shock the cells for 30 seconds at 42°C without shaking.
5. Add 250 µl of room temperature SOC medium and mix.
6. Cap the tube tightly and shake the tube horizontally at 37°C for 30 minutes (ampicillin selection) or 1 hour (kanamycin selection).
7. Spread 50-100 µl from each transformation on a prewarmed plate and incubate overnight at 37°C.
8. An efficient TOPO-Cloning™ reaction will produce hundreds of colonies. Pick ~10 white or light blue colonies for analysis. Do not pick dark blue colonies.

Analysis of Positive Clones

1. Take the 10 white or light blue colonies and culture them overnight in LB medium containing 50 µg/ml ampicillin or kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for sequencing, we recommend the S.N.A.P. Miniprep Kit (Catalog no. K1900-01).
3. Analyze the plasmids by restriction analysis (digest with EcoRI or refer to the vector map accompanying the manual for alternate sites) or by sequencing. M13 Forward (-20) and M13 Reverse primers are included to help you sequence your insert. If you need help with setting up restriction digests or DNA sequencing, please refer to general molecular biology texts (Ausubel et al., 1994; Sambrook et al., 1989).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and Taq polymerase. Use a 20 µl reaction volume. Multiply by the number of reactions (e.g. 10 colonies).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at +4°C.
6. Visualize by agarose gel electrophoresis.
TOPO TA Cloning® Control Reactions

Introduction

We recommend performing the following control TOPO-Cloning™ reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using it directly in a TOPO-Cloning™ reaction.

Producing Control PCR Product

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

- Control DNA Template (100 ng) 1 µl
- 10X PCR Buffer 5 µl
- 50 mM dNTP 0.5 µl
- Amplification Primer #1 1 µl
- Amplification Primer #2 1 µl
- Sterile Water 40.5 µl
- Tag Polymerase (1 unit/µl) 1 µl

Total Volume 50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.

3. Amplify using the following cycling parameters:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1 min</td>
<td>94°C</td>
<td>25X</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min</td>
<td>56°C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>1 min</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>7 min</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the Control TOPO-Cloning™ Reactions, below.

Control TOPO-Cloning™ Reactions

Using the control PCR product produced above and the TOPO TA Cloning® vector, set up two 5 µl TOPO-Cloning™ reactions as described below.

1. Set up control TOPO-Cloning™ reactions:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vector Only</th>
<th>Vector + PCR Insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PCR Product</td>
<td>--</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>4 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>TOPO TA Cloning® vector</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

2. Incubate at 25°C (room temperature) for 5 minutes and place on ice.

3. Transform 2 µl of each reaction into separate vials of TOP10 One Shot™ cells (page 3).

4. Spread 50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin (or ampicillin) and X-Gal (and IPTG, if using TOP10F® cells) (see page 8).

5. Incubate overnight at 37°C.

continued on next page
DGGE procedure as of 7/17/97, subject to change as experience increases

50x TAE-buffer (Tris-Acetate-EDTA), pH 8.3
- EDTA, Na salt 18.612 g 50 mM
- Tris, free base 242.28 g 2 M
- Glacial acetic acid 57.5 ml (17.4-N) 1 N
- adjust volume to 1000 ml with H2O.d., adjust pH to 8.3, autoclave, store at r.t.

Urea-formamide denaturing stock solutions for gel gradient
- 0% UF 6% AA
  40% Acrylamide/Bisacrylamide (37.5:1; 2.6% C; Biorad) 15 ml
  50x TAE, pH 8.3 2 ml
- adjust volume to 100 ml with H2O.d., filter through Whatman No.1, degas, store at 4°C in brown bottle. Refrigerator life 1 month.

- 100% UF 6% AA
  40% Acrylamide/Bisacrylamide (37.5:1; 2.6% C; Biorad) 15 ml
  50x TAE, pH 8.3 2 ml
  Urea 42 g
  Formamide 40 ml
- adjust to 100 ml with H2O.d., filter through Whatman No.1, degas, store at 4°C in brown bottle. Refrigerator life 1 month.

- APS 10% (w/v, (NH4)2S2O8) MW 228.20
  ammonium persulfate in 10 ml H2O.d. 1 g
  store at -20°C in 250 ul aliquots

- TEMED
  N,N,N',N'-Tetramethylenediamine

Gel loading buffer 5x, pH 8 (stored in refrigerator)
- H2O.d. 10 ml
- Bromphenol blue 5 mg 0.05% (w/v)
- Sucrose 4 g 40% (w/v)
- EDTA Na-salt 372 mg 0.1 M
- Sodium lauryl sulfate 50 mg 0.5% (w/v)

Gradient gels 30 to 50 % urea/formamide in 6% acrylamide
for 200x200x1 mm gelplates; 160x200x1 mm gels, prepare when needed
- 50 % UF (into outflow reservoir of gradient former, Vtot. 14.5 ml)
  0%UF6%AA 7.25 ml
  100%UF6%AA 7.25 ml
  TEMED 13.2 ul
  APS 66 ul
  Bromphenol blue a few crystals

- 30 % UF (into 2nd reservoir of gradient former, Vtot. 14.5 ml)
  0%UF6%AA 10.15 ml
  100%UF6%AA 4.35 ml
  TEMED 13.2 ul
  APS 66 ul

- 0 % UF (into outflow reservoir, Vtot. 5 ml)
Casting gels
- treat the spacers from time to time with a thin film of vacuum grease, put glass plates together, seal bottom part of gel plates with parafilm on the entire length of the plates (apply grease only if plates cannot be sealed leak-free otherwise)
- place plates on pad in casting device and press down firmly
- add ingredients of gelmix directly into gradient former reservoir, mix well between additions and begin casting immediately after all ingredients have been added
- make sure the connecting opening between the two gradient forming reservoirs is open and flow is not obstructed by air bubbles
- for optimal mixing when pouring gel keep stirring speed in outflow reservoir of gradient former low (larger stirring bars affects hydraulics in the mixing chambers)
- after the gradient chambers are empty, close the connecting channel between them
- fill non-denaturing acrylamide solution (0%UF+ TEMED+APS) into gradient former outflow reservoir and overlay the gradient gel with it
- fill gel volume completely before inserting well comb to a depth of only 20 mm
- clamp plates and comb together tightly during polymerization; the gel of the well divisions should adhere well to the glass plates
- let polymerize for 3 hours before conditioning gels

Conditioning gels
- fill tank with 6370 ml H2O d. and add 130 ml 50x TAE (= 6500 ml TAE 1x, pH 8.3)
- the reservoir should now be filled to “FILL” mark
- fill wells with 1x TAE, insert plates into reservoir and turn heater and pump on
- the upper reservoir will be filled automatically with buffer by the pump and the buffer level will adjust to a niveau between the marks “RUN” and “MAXIMUM”
- once the temperature has equilibrated rinse the gel wells with fresh 1xTAE from the tank before loading the samples

Loading gels
- mix 5 parts of DNA sample with 1 part of “Gel loading buffer” on a piece of parafilm
- apply 6 ul of sample per well if the sample contains DNA for which only one band is expected
- load Y*6ul per well if Y bands are expected from a community sample (large molecular weight DNA from the template which has not been specifically amplified will not enter the gel and form an ethidiumbromide stainable band at the bottom of the loading well)
do not apply sample to wells 1 and 20
- in order not to loose sample when loading proceed as follows:
  adjust the pipet volume in air to V+5ul
  take up sample volume (V) into the pipet tip
  bring pipet tip rapidly into the well to be loaded, push sample carefully out of the tip or let it fall into the well by gravity
  avoid air bubbles; otherwise the sample gets mixed and will be diluted too much with the buffer in the well. (due to rapid warming of the tip the air inside the tip expands and forces the sample out; the high density of the loading buffer assures gravity flow of the sample to the bottom of the well)

Conditions for sample migration
- 200 Volts constant, 60 °C constant, will result in a separation time of about 6 hours
- make sure that the air cushion on the lower side of the buffer reservoir is being formed after the pump has been turned on
- for overnight runs: 150 V constant, 60 °C constant, will result in a separation time of about 9 hours
  - if the run needs to be interrupted turn the power supply off before removing the lid

**Staining gels**
- remove larger glass plate and spacers and leave the gel attached to the smaller plate
- dip gel - still on glass plate - into staining solution (Ethidiumbromide 1x in TAE 1x, pH 8) and let the stain interact for 30 min; during this time the closed container is gently moved back and forth on a rocking table
- transfer gel - still on glass plate - into distilled water and rinse for 20 minutes
- remove wet gel from glass plate, transfer onto UV light box coated with Saran wrap and observe stained bands under UV, stained bands will appear pale red

**Photographing gels**
- filmtype ..........., 1/2 sec. opening 11
PROTOCOL FOR RFLP

5 µl from each 50µl PCR product
1 µl of buffer NEB 2
0.5µL of restriction enzyme (conc?)
3.5µL deionized water

Incubate at 37°C for at least one hour. Preferably for two.
After incubation, take total volume, add 1.5µl 6X loading dye and subject to gel electrograph.
"Checkerboard" DNA-DNA hybridization.

Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE

Forsyth Dental Center, Boston, MA.

A method is introduced for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane. Denatured DNA from up to 43 samples was fixed in separate lanes on a single membrane mounted in a Minislotter 45. The membrane was then rotated 90 degrees in the same device, which enabled simultaneous hybridization with 43 different DNA probes. Hybridizations were also performed on lysates of bacterial cells blotted to membranes. A MiniSlot device allowed lysates loaded in parallel channels to be aspirated through the membrane, depositing horizontal lanes on the membrane surface. Hybridizations were performed in vertical lanes with either digoxigenin-labeled whole genomic probes or 16S rRNA-based oligonucleotide probes directly conjugated to alkaline phosphatase. The method permits the simultaneous determination of the presence of multiple bacterial species in single or multiple dental plaque samples, thus suggesting its usefulness for a range of clinical or environmental samples.

MeSH Terms:
- Base Sequence
- Dental Plaque/microbiology
- DNA, Bacterial/analysis
- Human
- Molecular Sequence Data
- Nucleic Acid Hybridization
- Oligonucleotide Probes
- Support, U.S. Gov't, P.H.S.

Substances:
- Oligonucleotide Probes
- DNA, Bacterial

PMID: 7833043, MUID: 95134426
Figure 1

Checkerboard hybridization

The American cockroach, Periplaneta americana-Digestive tract microbiota Archaea
(See key)
Protocol was followed as described, except that in the prehybridization step, 2 ml of 10 mg/ml herring sperm DNA replaced the yeast RNA. No buffer was added in the last 2 lines (44-45).

Order of probes on large blot
1. Universal, 341
2. Universal 1089
3. Alpha2-T
4. Alpha1-T
5. Enterics-T
6. Deltas-T
7. SRB-T
8. Bact-T
9. Flavo-T
10. Spiro-T
11. LoGC-T
12. Cyano-T—all cyanobacteria
13. W.suc-T
14. Ntlob-T
15. Nsomon-T
16. Alc-den-T
17. Methyl-T
18. P.fluor-T
19. Nocean-T
20. Euryarch-T
21. Crenarch-T
22. Shew1-T
23. Alt1-T
24. Gaz-T

PCR dig Sample: 10µl—hybridization run at 52°C (not at 55°C)

1. E.coli (gamma proteo)
2. Cris—Dolomite PCR
3. Tom—cockroach, isolate 1
  Tom—cockroach, isolate 2
5. Tom—cockroach, isolate 3
6. Tom—cockroach, isolate 4
7. Tom—cockroach, isolate 5
8. Tom—cockroach, isolate 6
9. Tom—cockroach, isolate 7
10. Tom—cockroach, isolate 8
11. Tom—cockroach, isolate 9
12. Tom—cockroach, isolate 10
13. Tom—cockroach, clone 1
14. Tom—cockroach, clone 2
15. Tom—cockroach, clone 3
16. Tom—cockroach, clone 4
17. Tom—cockroach, mix pop. H
18. Junko—mix F +M
19. Buffer only
20. Junko—mix H
21. Junko—isolate A1
22. Junko—isolate A2
23. Junko—isolate A3
24. Junko—isolate A5
25. Junko—clone F1
26. Junko—clone F7
27. Junko—clone M6
28. Junko—clone M10
29. Junko—clone M12
30. Junko—clone M14
31. Junko—clone H4
32. Junko—clone H9
33. Junko—clone H2
34. Junko—clone M4
35. Junko—clone H14
36. Tom—termite Rf
37. Scott—thermo
38. Scott—thermo
39. Scott—fikry
40. Scott—halophyl. clone
41. Scott—Methano clone
42. Scott—Thermococcus
43. Scott—Py……dictium
Checkerboard Hybridization of Class isolates and environmental samples

Key

1-4. Toad fish isolates from the MRC
5. Debra Hughes plaque sample
6. Cockroach hindgut by Ludek
7-10. Al's chemostat (25,16,3,0 time)
11. Sewage sample from Sabine
12. Methanogen enrichment (blank lane)
13,14. Jeff's plaque samples
15. Blue sand, Sippawisset
16. Sippawisset mat
17. Scott's plaque
18. Cockroach (4X), Tom
19. Cockroach (40), Tom
20. 1/10 dilution of Vibrio gazogenes DNA from pink colonies (Udi)
21. 1X Vibrio gazogenes DNA (Udi)
22. 1/10 dilution of pink berry DNA from Sippawisset (Udi)
23. Pink berry DNA from Sippawisset (Udi)

Notes:

1. Alpha probe utilized was not good, too much cross-reactivity—A new Alpha probe was designed.
2. Beta probe did not work because Tm was too low.
3. Methanogens (lane 12) did not work since the digoxigenin labeled primer and probes were specific for bacteria not Archaea
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Table 1: Limited phenotypic characterization of pure isolates
Fig. RFLP of sequences from 10 pure isolates obtained from hindgut of cockroach as well as four clones obtained.
Fig. DGGE profiles of pure isolates, clones and hindgut population DNA of American cockroach.

Note: 1, 2, 3, 4, 5 and 6 are 16s rDNA from pure isolates. C1 – 4 are 16s rDNA from clones. 7 and 8 are population DNA from cockroach hindgut.
NCBI BLAST Search Results


Notice: this program and its default parameter settings are optimized to find nearly identical sequences rapidly. To identify weak similarities encoded in nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query= tmpseq_1
(320 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
335,268 sequences; 515,845,374 total letters.

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ORGANISM  unidentifed rumen bacterium RC5
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          Clostridiales; environmental samples.
REFERENCE  1 (bases 1 to 1444)
AUTHORS  Tajima, K., Aminov, R., Nagamine, T., Ogata, K., Sugiiura, M. and
          Benno, Y.
TITLE  Direct Submission
JOURNAL  Submitted (30-APR-1997) Rumen Microbiology Research Team,
          STAFF-Institute, 446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305,
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Score = 204 (56.6 bits), Expect = 1.5e-86, Sum P(3) = 1.5e-86
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Query: 299
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Sbjct: 432
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Sbjct: 384
Tt 385

gb|AF001699|AF001699 Unidentified rumen bacterium RC6 16S ribosomal RNA gene, partial sequence
Length = 1442

Plus Strand HSPs:

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NCBI BLAST Search Results

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**emb|Z48283| ASPS2X26** Arthrobacter sp. partial gene for ribosomal RNA (isolate S2.26)
Length = 300

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NCBI BLAST Search Results

BLASTN 1.4.9mp [26-March-1996] [Build 14:27:07 Apr 1 1996]


Notice: this program and its default parameter settings are optimized to find nearly identical sequences rapidly. To identify weak similarities encoded in nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query= tmpseq_1
(524 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
335,513 sequences; 516,533,051 total letters.

Sequences producing High-scoring Segment Pairs:

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Gelidibacter algens A296 16S ribosomal RNA gene...
Gelidibacter algens A374 16S ribosomal RNA gene...
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Cappuccinella granulosa LMG 12119 16S rRNA...
Cappuccinella algens LMG 12116 16S rRNA...
Flavobacterium sp. 16S ribosomal RNA gene...
Flavobacterium sp. 16S ribosomal RNA gene...
Flavobacterium sp. 16S ribosomal RNA gene...
Bacteroides putredinis (ATCC 29800) 16S rRNA...
Flavobacterium sp. 16S ribosomal RNA gene...
Marine psychrophile ACAM210 16S rRNA gene...
Cappuccinella algens LMG 12115 16S rRNA gene...
Cappuccinella algens FDC 7b 16S rRNA gene...
Cappuccinella algens 16S ribosomal RNA gene...
Cappuccinella algens C. ochracea gene for 16S rRNA gene...
C. ochracea gene for 16S rRNA gene...
C. salmonicolor small subunit ribosomal RNA gene...
Gelidibacter sp. IC158 16S ribosomal RNA gene...
Psychrosphera burtonensis ACAM188 16S rRNA gene...
Unidentified eubacterium SCB49 16S rRNA gene...
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Cytophaga latercula 16S ribosomal RNA gene...
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B. fragilis ribosomal RNA small subunit ribosomal RNA gene...
Bacteroides fragilis 16S rRNA gene...
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Prevotella diisens (ATCC 29426) 16S rRNA gene...
Flexibacter tructuosus 16S ribosomal RNA gene...
Flexibacter thermophilum 16S ribosomal RNA gene...
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Microscilla sericea 16S ribosomal RNA gene...
Cytophaga hutchinsonii 16S ribosomal RNA gene...
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F. marinus 16S ribosomal RNA gene...
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Flavobacterium breve 16S ribosomal RNA gene...
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Bacteroides splanchicus (NCTC 108) 16S rRNA gene...
Cappuccinella algens 16S ribosomal RNA gene...
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Ornithobacterium rhinotrichonale 16S rRNA gene...
Flexibacter polymorphus 16S ribosomal RNA gene...
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Flexibacter litoralis 16S ribosomal RNA gene...
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Psychrosphera burtonensis ACAM188 16S rRNA gene...
Cappuccinella algens LMG1602 16S rRNA gene...
Flavobacterium amnieres 16S ribosomal RNA gene...
Ornithobacterium rhinotrichonale 16S rRNA gene...
NCBI BLAST Search Results

**emb|X85210|CSRMA16S**  Cytophaga sp. partial 16S rRNA gene (type 0092)
Length = 1488

**Plus Strand HSPs:**
Score = 894 (247.0 bits), Expect = 1.8e-128, Sum P(3) = 1.8e-128
Identities = 198/222 (89%), Positives = 198/222 (89%), Strand = Plus / Plus

Query: 236 GGTTAAGGATGGGATGCCGTACCATTAGCTAGTTGAGGGTAGGGAACGCTCAACAGCGCA 295
Sbjct: 216 GGTTAAGGATGGGATGCCGTACCATTAGCTAGTTGAGGGTAGGGAACGCTCAACAGCGCA 275
Query: 296 TGATGGTTAGGTTGCTGAGGAAAGGCTGACCAGGACCTACTAGGACGACCCACAGAC 355
Sbjct: 276 CGATGGTTAGGTTGCTGAGGAAAGGCTGACCAGGACCTACTAGGACGACCCACAGAC 335
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Sbjct: 336 CTTACCGGGGAAAGCAGATCCATGTTGAGGAGACGCTCAACAGAGCGCAGCAGAC 395
Query: 416 CCGGCGATGGGAGGAAACGACGACCTTATGCCATTTCCTTTTT 457
Sbjct: 396 CCGGCGATGGGAGGAAACGACGACCTTATGCCATTTCCTTTTT 437

Score = 727 (200.9 bits), Expect = 1.8e-128, Sum P(3) = 1.8e-128
Identities = 163/185 (88%), Positives = 163/185 (88%), Strand = Plus / Plus

Query: 31 GATTTGATCGGATCCAGTGATGGAAGCGCCGCTGGATGCTTAATGACATCGGACGAAC 90
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Query: 91 GTAAGGGAGGCTTGCCTTCATTGAGGATGCGGCCACAGGTGGTTAACCAGCTACACCTAC 150
Sbjct: 70 GTAAGGGAGGCTTGCCTTCATTGAGGATGCGGCCACAGGTGGTTAACCAGCTACACCTAC 129
Query: 151 CCTTACCGGGGATGCGCATTCATGATATAGCTGATTACATTACGCTTGATGATTGG 210
Sbjct: 130 CCTTACCGGGGATGCGCATTCATGATATAGCTGATTACATTACGCTTGATGATTGG 189
Query: 211 CATCA 215
Sbjct: 190 CATCA 194

Score = 109 (30.1 bits), Expect = 1.8e-128, Sum P(3) = 1.8e-128
Identities = 29/38 (76%), Positives = 29/38 (76%), Strand = Plus / Plus

Query: 487 GAGTTGCGGAGTCTACGGAATAAGGATCAGGTCTAAGT 524
Sbjct: 466 GAGCTTGCGGAGTCTACGGAATAAGGATCAGGTCTAAGT 503

**gb|U85889|FSU85889**  Flavobacterium sp. IC001 16S ribosomal RNA gene,
partial sequence
Length = 1474

**Plus Strand HSPs:**
Score = 856 (236.5 bits), Expect = 1.0e-126, Sum P(3) = 1.0e-126
Identities = 196/227 (86%), Positives = 196/227 (86%), Strand = Plus / Plus

Query: 232 TTTTGTTAAGGATGGGATGCGGATACCATTAGCTAGTTGAGGGTAGGTAACGGCTACCAAG 291
Sbjct: 204 TATCGGTTGAAGGATGCGGATACCATTAGCTAGTTGAGGGTAGGTAACGGCTACCAAG 263