Supplemental Materials
for
A Multi-Unit Project for Building Scientific Confidence via Authentic Research in Identification of Environmental Bacterial Isolates
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Table of Contents
(Total pages 6)

Appendix 1: Metabolic testing assignment sheet
Appendix 2: 16s PCR and sequencing primers
Appendix 3: Two methods for quantification of biofilm growth
Appendix 1: Metabolic testing assignment sheet.

Metabolic testing assignment: identification of unknown bacteria

For metabolic testing of your unknown, your group will complete a minimum of four metabolic tests from the list below. You might try to select tests that are common for the Gram reaction and arrangement/morphology of your unknown.

As a group you will have to prepare all the needed media tubes or plates. Positive controls will need to be included with each test. A list of available strains is provided below, you will have to select a positive control from that list for each test you perform.

To figure out how to prepare each test, there are numerous options:
1. Look over the protocol in the course notebook
2. Examine the Lammert “Techniques in microbiology” reference handbook
3. Check in the course protocols binder
4. Read the instructions on the side of the container of the agar media
5. Check the Difco manual entry for reagent preparation

Keep good notes on the protocol to record in your lab notebook entry. It is acceptable to note the source of the protocol in those entries; the entire protocol is not needed in the notebook entry.

Inoculate the tests and monitor them as needed (a member of your group will need to observe all 24 hour results!). For tests requiring 48-hour incubations notify the instructor, and arrangements will be made to remove them on Saturday to the refrigerator until you can examine them later.

Tests available:
1) Anaerobic replication (three different options for tests):
   a. Thioglycolate media
   b. BHI agar deeps (melt, inoculate and solidify)
   c. Anaerobe growth chamber
2) Catalase activity
3) Citrate metabolism
4) Fermentation and carbohydrate utilization tests:
   a. Phenol red broth with: Sucrose, lactose, glucose, etc. (don’t forget Durham tubes!)
   b. Triple sugar iron agar
   c. OF-media with your choice of carbohydrate (Glucose, sucrose, lactose, etc.)
5) Motility agar (prepare any complex media with only 0.5% agar, in a tube or a petri dish)
6) MR and VP tests
7) Nitrate and nitrite reduction
8) Oxidase activity
9) Starch hydrolysis
10) Urea broth/urease test

Strains available as positive controls: (others may be available by request)
B. subtilis
E. coli
E. aerogenes
K. pneumoniae
P. mirabilis
P. aeruginosa
S. aureus
S. epidermidis
Appendix 2: 16s PCR and sequencing primers.

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<th>Primer set:</th>
<th>Primer name:</th>
<th>Sequence (5' to 3'):</th>
<th>Variable regions:</th>
<th>Ref.</th>
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<td>Year 1 set:</td>
<td>E334F</td>
<td>CCAGACTCTCTACGGGAGGCAGC</td>
<td>V3 and V4</td>
<td>Rudi et al., 1997</td>
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<td></td>
<td>E939R</td>
<td>CTTGTGCGGGCCCCGGTCAATTC</td>
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Result: One band at ~600bp, good sequencing results with either primer

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<td>E9F</td>
<td>GAGTTTGATCCTGGCTCAG</td>
<td>V1 and V2</td>
<td>McInnery et al., 1995</td>
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<td></td>
<td>E338R</td>
<td>GCTGCCTCGGCCTAGGAGT</td>
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<td>Amann et al., 1990</td>
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Result: One band at ~300bp, good sequencing results with either primer

<table>
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<td>V3 to V6</td>
<td>Rudi et al., 1997</td>
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<td>E1115R</td>
<td>AGGGTTGCGCTCGTGG</td>
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<td>Reysenbach and Pace, 1995</td>
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</table>

Results: Multiple bands with major band at ~700bp. Since gel purification or PCR optimization would be needed was not sent for sequencing.


Appendix 3: Two methods for quantification of biofilm growth.

METHOD 1: Biofilm quantification by crystal violet staining

Materials:
- 0.25% crystal violet in water (not Gram’s crystal violet!)
- biofilm sample (on a plastic/borosilicate tube, glass slide or plastic well in 96 or 24 –well plate)
- pipets/pipet tips (as needed)
- pipet bulb/ pipettor (as needed)
- Sterile water

Protocol A: best when applied to samples with minimal visible biofilm accumulation

1) Add crystal violet as a 1:4 dilution (ex: for a 2 ml sample, add 0.5 ml)
2) Stain for 15 minutes
3) Remove non-adherent bacteria from the biofilm sample (dispose of as infectious waste for autoclaving, use appropriate pipet-transfer method for the volume of the sample)
4) Wash biofilms with sterile water three times (add the same volume of water as was in the tube/well before step 1, remove with sterile pipet, dispose as in #1, repeat 2 more times)
5) Add Ethanol to the tube/well at the same volume as initially started
6) De-stain for 10 minutes
7) Mix ethanol in the tube/well by moving liquid in and out of a pipette or pipette tip multiple times, transfer a portion to a glass cuvette
8) Using clean ethanol as the blank, read the OD 600 of the ethanol (revealing the quantity of ethanol retained in the initial tube/well). Multiple replicates per species tested are needed for accurate measurements.
   a. Note: if the number is outside range of the spectrophotometer, dilutions of the sample may be required: example, dilute 1 ml of crystal violet sample in 2 ml of ethanol and multiply resulting OD 600 by 3 to get initial value.

Protocol B: better for samples with abundant biofilm visible in the tube/well

1) Remove non-adherent bacteria from the biofilm sample (dispose of as infectious waste for autoclaving, use appropriate pipet-transfer method for the volume of the sample)
2) Wash biofilms with sterile water three times (add the same volume of water as was in the tube/well before step 1, remove with sterile pipet, dispose as in #1, repeat 2 more times)
3) Add water to the sample volume as initially in the tube/well
4) Add crystal violet at a 1:4 dilution
5) Stain for 15 minutes
6) Wash biofilms with sterile water three times (add the same volume of water as was in the tube/well before step 1, remove with sterile pipet, dispose as in #1, repeat 2 more times)
7) Add Ethanol to the tube/well at the same volume as initially started
8) De-stain for 10 minutes
9) Mix ethanol in the tube/well by moving liquid in and out of a pipette or pipette tip multiple times, transfer a portion to a glass cuvette

10) Using clean ethanol as the blank, read the OD 600 of the ethanol (revealing the quantity of ethanol retained in the initial tube/well). Multiple replicates per species tested are needed for accurate measurements.
   a. Note: if the number is outside range of the spectrophotometer, dilutions of the sample may be required: example, dilute 1 ml of crystal violet sample in 2 ml of ethanol and multiply resulting OD 600 by 3 to get initial value.
METHOD 2: Harvesting biofilms for quantification by serial dilution and drop plating:

1) Remove non-adherent bacteria from the biofilm sample by pipeting-off all media in the tube/well, and take care to maintain aseptic conditions
   a. dispose of the media as infectious waste for autoclaving, use appropriate pipet-transfer method for the volume of the sample
   b. perform in biosafety hood if available to help minimize contamination

2) Wash biofilms with sterile water three times (add the same volume of water as was in the tube/well before step 1, remove with sterile pipet, dispose as in #1, repeat 2 more times)

3) Add sterile water at the same volume as initially in the tube

4) Disrupt biofilms with sound waves in water-bath sonicator for 5 minutes. Place tubes in a beaker with water inside to transmit waves to sample.

5) Use 1:10, serial dilutions in sterile water to dilute sample 3-8 times, depending on visibility of biofilms

6) Use drop plating method (10ul drops, in triplicate, placed in one quadrant of an R2A plate from each serial dilution) to quantify CFU from biofilms