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Curriculum

Pseudomonas Isolation and Identification: An Introduction to the Challenges of Polyphasic Taxonomy †

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The ability to isolate an organism in pure culture from the environment is a manageable task for undergraduate students; the identification of that organism requires integration of both genotypic and phenotypic data and illustrates the challenges inherent in contemporary bacterial taxonomy. In this ten-laboratory period series of exercises, students isolate a strain of *Pseudomonas* from soil and characterize its biochemical and physiological properties, as well as determine the DNA sequence of its 16S rRNA genes. Integrating these data positions students to defend their classification of the isolate as a new species or as a member of a validly described species. Assessment data demonstrate that both knowledge of and confidence in understanding of the principles of laboratory handling of *Pseudomonas* and bacterial taxonomy increased following the exercises.

INTRODUCTION

Bacterial identification is a cornerstone of the microbiology teaching laboratory. Many classroom exercises to identify an unknown give students a short list of possible organisms that can be distinguished with a few laboratory manipulations. These demonstrations can lead to a narrow view of the challenges inherent in strain identification, as well as the microbial complexity of most environments.

Soil, in particular, has an astounding number and diversity of microbes, and constitutes a fertile, easily accessible, and generally safe resource for the isolation of bacteria. *Pseudomonas* species are ubiquitous in soil and although some have long been recognized as plant pathogens (6), others are emerging as plant-associated growth promoters with potential roles in biocontrol (1, 12). The nutritional versatility of this genus renders it a common isolate in bioremediation surveys (7, 13), and provides an enrichment strategy. Atypical carbon and nitrogen sources in a minimal enrichment medium exploit the degradative capacity of *Pseudomonas* species and increase their abundance for facile isolation, as demonstrated by Mulet et al. (10).

As discussed by Tindall et al. (14), bacterial taxonomy relies on characterization, classification, and nomenclature. The laboratory exercise described here directs students to isolate a strain of *Pseudomonas* following a nutritional enrichment and to characterize it using genetic (16S rRNA gene sequencing) and phenotypic (enzyme assays, growth conditions) methods. Classification is attempted using a polyphasic approach (15) to integrate these data and compare with validly published species. This provides students the opportunity to isolate a potentially new organism in pure culture, to describe its properties, to place it in a phylogenetic framework and evaluate its similarities to type strains, and to present a logical argument to justify their classification.

This series of exercises is demonstrated to introduce students to the principles of bacterial isolation and identification. Knowledge of the principles and confidence in understanding and applying those principles increased following completion of the activities.

Intended audience

This series of exercises is performed in an introductory microbiology laboratory course for students majoring in microbiology and other life sciences.

Learning time

This series of exercises spans ten two-hour laboratory sessions, as diagrammed in Table 1. It is adapted to a two-hour, twice-weekly lab format (e.g., Tuesday/Thursday), and is timed for two-day and five-day incubation times between class meetings. While some labs require the full two hours, others are brief and the additional time is used for short lectures to explain the techniques and to demonstrate the software for DNA sequence analysis.

Prerequisite student knowledge

Students should have experience in the routine techniques of the microbiology laboratory including generating

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†Supplemental materials available at http://jmbe.asm.org

TABLE 1.
Timeline for activities.

Class	Task(s)	Time Required (Hours)	Incubation Time (Days)
1	Formulate and inoculate broth enrichments	0.5	2
2	Microscopy; streak enrichments on solid media	1	2–5
3	Screen colonies by microscopy for likely pseudomonads; streak on selective media	2	2
4	Streak to generate a pure culture; Gram and oxidase reactions	1	2
5	Streak to generate a pure culture; Gram and oxidase reactions	1	2
6	Inoculate media for phenotypic testing; freeze pellets for later DNA extraction	1	2–7
7	Purify genomic DNA and assess on gel; interpret results of phenotypic tests; inoculate media for phenotypic testing	2	2
8	Perform PCR to amplify 16S rRNA gene; interpret results of phenotypic tests	2	none
9	Agarose gel electrophoresis of PCR; prepare samples for DNA sequencing	2	none
10	*In silico* analysis of DNA results	1	none

a pure culture, microscopy, Gram stain, oxidase test, interpretation of differential media, and the use of micropipettors and microcentrifuges. These exercises are scheduled as the final project of the semester to give students the opportunity to apply these skills. A separate lecture or recitation should cover concepts of bacterial media and their use in nutritional enrichment, microbial identification and the concept of species, use of 16S rRNA for taxonomy, and PCR.

Learning objectives

Upon completing these activities the student will be able to:

1. Describe the characteristics of the *Pseudomonas* genus and the conditions under which it may be isolated from its natural environments.
2. Define microbiological enrichment, and list the components of a minimal enrichment medium.
3. Judge whether a culture is pure based on observation of a three-phase streak and generate a pure culture from a single colony.
4. Interpret growth and reactions on selective and differential media, including *Pseudomonas* Isolation Agar, *Pseudomonas* F agar, starch agar, and nitrate broth.
5. Discuss the steps in the extraction and purification of genomic DNA and identify chromosomal and plasmid DNA bands on a gel.
6. Explain the purpose of PCR amplification of the 16S rRNA gene, enumerate the steps of PCR, and interpret the bands present on a gel following electrophoresis.
7. Analyze a current journal article describing a new species of *Pseudomonas* and state the criteria used for the creation of a new species.
8. Perform a search on the Ribosomal Database Project (RDP) website and identify the closest matches to the input sequence.
9. Demonstrate competence with *in silico* DNA sequence manipulation to align multiple sequences and interpret the program output.
10. Defend the classification of their isolate as a new or an existing species of *Pseudomonas* by a comparison of sequence data and laboratory observations with validly published species.

PROCEDURE

Materials, student instructions, faculty instructions, sample data

Because of the length of these components, they are included as appendices in the supplementary materials. Appendix 1 is a materials and equipment list for each of the ten class meetings. Students will supply their own soil samples in the first lab. Appendix 2 is the lab manual provided for students; Appendix 3 is the instructor's manual. Sample data are provided in Appendix 4.

Suggestions for determining student learning

Learning objectives 1 through 6 relate to technical competence in the laboratory and understanding the background of the exercise. The formative assessment questions listed in the faculty instructions (Appendix 3) and questions like those found in the pre/posttest (Appendix 6) will measure the student's knowledge. Technical competence is best evaluated by laboratory demonstration of results.

Learning objectives 7 through 9 are assessed by homework assignments (Appendix 5). Following a lecture or recitation on bacterial classification and taxonomy, students

are provided a recent publication from the International Journal of Systematic and Evolutionary Microbiology (IJSEM) describing a new species of *Pseudomonas* (see for example, refs. 2 and 3). The publication illustrates the standard to which new isolates are held for classification, and provides a context for organizing the data that the student will obtain from their isolate. Assignment 1 directs students to examine the primary data presented for the new species and its closest relatives from whom it is distinguished, making note of how the sequence and phenotypic data are balanced. Assignment 2 directs students to explore the Ribosomal Database Project (RDP) (4). A live demo of the website in class precedes the assignment and sample partial 16S sequences provide the opportunity to gain hands-on experience with querying the database and interpreting the output. This assignment is critical as a prelude to analyzing the sequence data derived from the student's own isolate; it builds confidence in the student's ability to handle *in silico* sequence analysis.

The final learning objective is the essence of this project: having isolated a strain of *Pseudomonas* and collecting phenotypic data and the DNA sequence of its 16S rRNA gene, the student is asked to classify the strain and to defend that classification in a written report in the format of a publication in IJSEM. This report constitutes the greatest portion of the student's grade, and the grading rubric is included in Appendix 5.

Safety issues

Most students succeed in isolating a strain in pure culture, and most strains are *Pseudomonas*; species pathogenic to humans have not been recovered. Other organisms isolated by this procedure and identified by partial 16S rRNA sequences include *Sphingomonas*, *Pseudoxanthomonas*, *Variovorax*, and *Achromobacter*; such non-*Pseudomonas* strains occur with about 5% frequency. Because some of the isolates are potential pathogens, students should work at Biosafety Level 2. Determination of fluorescent pigment production and documentation of agarose gels require the use of ultraviolet light; eye protection must be provided. Zinc dust for the evaluation of nitrate broth must be used in the hood.

DISCUSSION

Field testing

This activity has been included in the laboratory portion of the second half of the introductory microbiology course at The Ohio State University for six years. Class sizes range from 65 to 200 in sections of up to 36 students. When the exercise is timed to follow a break (winter or spring break), students are invited to bring soil samples from their travels and a wide variety of species are isolated; when it is timed to occur in the middle of a semester, soil samples obtained from campus and close environs yield less diverse isolates, primarily strains related to *P. putida* and *P. fluorescens* (9).

One common experimental difficulty (~25% of students) is in generating a pure culture. Figure 1A illustrates the diversity of colony morphologies in an enrichment, and Figures 1B and 1C demonstrate typical student results when streaking from such an enrichment. Classes 4 and 5 (Table 1) are both dedicated to achieving a pure culture and confirming that culture as *Pseudomonas*. As most isolates grow well overnight, students still struggling to achieve a pure culture after Class 5 are asked to return to the lab outside of the regular meeting times for additional attempts.

A second experimental challenge (~25% of students) is to perform a successful PCR. The reaction template, the student's genomic DNA preparation, is visualized on an agarose gel (Fig. 1D) to confirm the presence of a chromosomal DNA band. In most cases, carefully repeating the reaction assembly results in success. Students unable to achieve this result and those whose DNA sequencing reactions fail are provided with the data from another student in the class with a similar isolate.

Informal surveys of students generate positive responses, with many students noting that it felt like "a real research experiment" and "it was cool to see a clump of dirt become a specific DNA sequence." Students appreciate

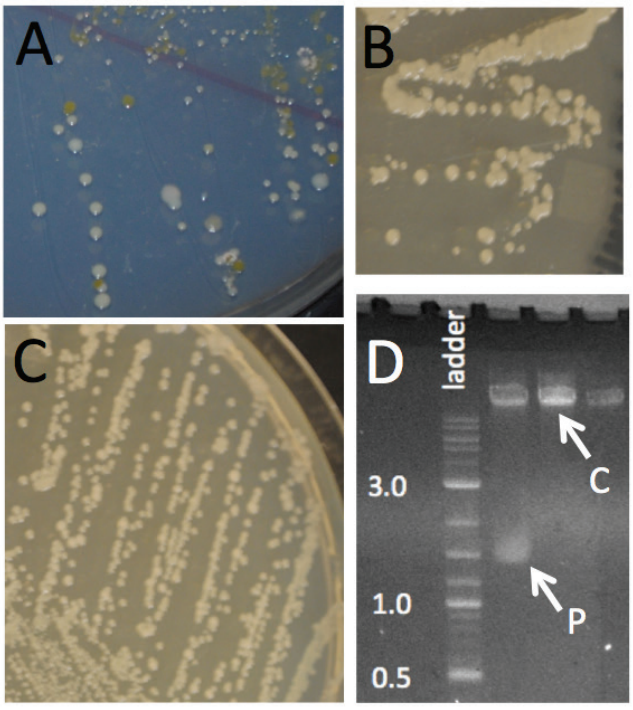

FIGURE 1. A. Streak from the liquid enrichment on a solid medium of the same composition, as observed following Lab 2. These are not pure cultures and a variety of colony morphologies are visible. B and C. Student streaks on *Pseudomonas* Isolation Agar as observed following Lab 3. B is not yet a pure culture; C is a pure culture. D. Agarose gel image of genomic DNA preparations, annotated to show the molecular weight ladder (2 log ladder with bright bands at 3.0, 1.0, 0.5 kb), chromosomal DNA fragments (C) and plasmid band (P).

the fact that the data collected are comparable to those described in the current publication that they read. In addition, many commented favorably on the synthesis of concepts and techniques learned earlier in the course.

Evidence of student learning

In two semesters, fall 2013 and spring 2014, students were instructed to read the background information in the lab manual before the first laboratory period. A no-stakes, anonymous, ten-question, multiple-choice pretest (Appendix 6) aimed at learning objectives 1 through 6 was administered. In addition to selecting the best answer for each question, students were asked to indicate if they were confident of their answer or if they were guessing. The identical test was administered during the final laboratory period, again no-stakes and anonymous.

As illustrated graphically in Figure 2, both accuracy and confidence increased following the exercise, with an average 22% increase in correct answers. The most dramatic increase was in student confidence, a testament to the power of experiential learning (5). Correct answers in the pretest were most often good guesses; only 35% of the correct answers were reported confident. The posttest, however, showed an average of 82% confidence.

The assignments relating to learning objectives 7 through 9 (Appendix 5) are essential prerequisites to adequately prepare a student for the final assignment, the written lab report. Reading the IJSEM publication provides the students with a model for their report on the isolation and identification of their strain. A dry run through the RDP website with sample sequences acquaints students with the platform before using it for their "real" data. Students typically perform well on both assignments, with ~90% of students submitting the assignment and most (~80%) earning an A or B grade.

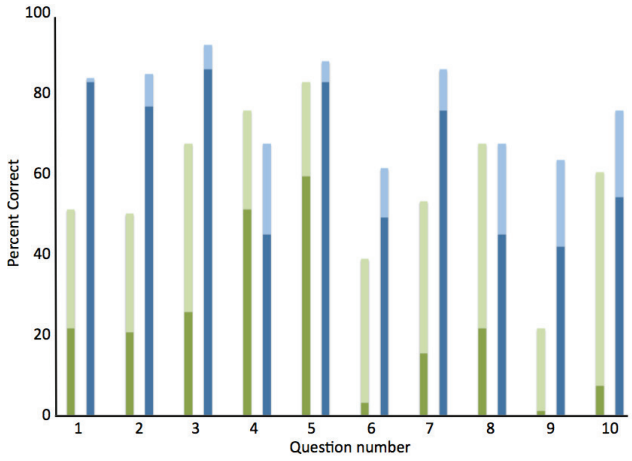

FIGURE 2. The percentage of students answering correctly in the pretest (green) vs. the posttest (blue). For each bar the dark lower portion illustrates the percentage of students who were both correct and confident; the lighter upper portion denotes the percentage who were correct but guessing. The questions are included in Appendix 6.

Preparing the final lab report in the IJSEM format provides the opportunity for the student to apply the skills acquired in the two previous assignments (analysis of the criteria for establishing species and *in silico* manipulation of 16S rDNA sequences). In the report, the student must present a narrative to describe the isolation of their *Pseudomonas* strain, propose a species-level identification and defend that decision on the basis of the data acquired. The top "hits" from their sequence in the RDP dictate which strains are investigated, and the student amasses the biochemical data for those strains from the primary literature. Although the quantity and quality of data collected by the students in this exercise is typically insufficient for classification to the species level, students' understanding of the classification process, in terms of what additional information is needed, can be assessed from their writing. As with the assignments described above, ~90% of the students submitted a report with 55 to 70% of students earning a grade of A.

Possible modifications

Given the nutritional versatility of *Pseudomonas*, many other compounds can serve as the basis for enrichment, and it is not necessary to perform the anaerobic enrichment if the equipment is not available. Fresh water samples may be substituted for or provided in addition to soil as the source of microbes. PCR amplification of the 16S rRNA gene may be accomplished using either the general bacterial primers described here or the primers described by Widmer et al. for the specific amplification from *Pseudomonas* (16).

The Ribosomal Database Project was chosen as the reference database for 16S rRNA sequences for its ease of use and interpretation for novices. Alternately, students may be instructed to use phylogenetic tree building programs like GreenGenes (8) or Silva (11).

SUPPLEMENTAL MATERIALS

Appendix 1: Materials and equipment list
Appendix 2: Student laboratory manual
Appendix 3: Instructor's guide
Appendix 4: Sample data
Appendix 5: Sample assignments and exam questions with answers and grading keys
Appendix 6: Pre-/posttest to assess student learning

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