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Tips and Tools

Breaking the habit: isolating nicotine-degrading bacteria in undergraduate microbiology teaching labs

J. Mastenbrook,[1] E. Pathak,[1] C. Beaver,[1] F. Stull,[2] B. J. Koestler[1]

AUTHOR AFFILIATIONS See affiliation list on p. 4.

ABSTRACT Nicotine is a major alkaloid in tobacco plants and an addictive component of tobacco products. Some bacteria grow on tobacco plants and have evolved the ability to metabolize nicotine. As part of our microbiology teaching lab, we used minimal media with nicotine as the sole carbon source to isolate nicotine-degrading bacteria from tobacco leaves and commercial tobacco products. Students then identified these bacteria using 16S rRNA sequencing and biochemical assays and assessed their ability to catabolize nicotine using UV spectroscopy. Students were able to isolate and identify 14 distinct genera that can metabolize nicotine. This modification of the commonly used unknown project gave students firsthand experience using selective media, and students got the opportunity to work with largely uncharacterized microbes with a real-world connection to public health, which increased student engagement. Students had the opportunity to think critically about why nicotine-degrading microorganisms associate with tobacco plants, why there are different bacteria that use the same specialized metabolism, and how these organisms are isolated from other bacteria using selective media.

KEYWORDS nicotine, tobacco, *Arthrobacter*, *Pseudomonas putida*

Nicotine is the primary addictive component of tobacco products and poses a significant public health risk. More than 7 million people die yearly due to tobacco use, and this number is increasing (1). The biological degradation of nicotine is a potential therapeutic (2, 3). Some bacteria metabolize nicotine as a carbon or nitrogen source (Fig. 1). Bacteria use different pathways for nicotine metabolism. Some *Pseudomonas* species metabolize nicotine through the dehydrogenation of pyrrolidine to N-methylmyosmine (pyrrolidine pathway) (Fig. 1A) (4). Alternatively, *Arthrobacter* can use the pyridine pathway to hydroxylate the pyridine ring of nicotine, producing the byproduct 2,3,6-trihydroxy pyridine (nicotine blue) (Fig. 1A) (4, 5). *Arthrobacter* nicotine catabolism genes are encoded within a transposon on a large plasmid, which has driven horizontal gene transfer to other bacteria (6, 7). Other Alphaproteobacteria combine these two pathways, referred to as the VPP pathway (4). Nicotine-degrading bacteria can be isolated from tobacco leaves or commercial tobacco products (8, 9). *Pseudomonas* and *Arthrobacter* can grow on nicotine as a sole carbon source (Fig. 1B), and nicotine catabolism can be quantified using UV spectroscopy (Fig. 1C).

In microbiology teaching labs, a common curricular component is an unknown project, where students isolate and identify an unknown bacterium. We present a variation of this, where nicotine-degrading bacteria are isolated on selective media from tobacco leaves and commercial tobacco products. Students confirmed nicotine catabolism using UV spectroscopy. Our students isolated and identified 14 different genera that metabolize nicotine.

Students' feedback about this project was positive, but there were limitations to this project. Environmental isolates have variable growth rates, and some can be recalcitrant

Editor Stanley Maloy, San Diego State University, San Diego, California, USA

Address correspondence to B. J. Koestler, benjamin.koestler@wmich.edu.

J. Mastenbrook and E. Pathak contributed equally to this article. Author order was determined alphabetically by last name.

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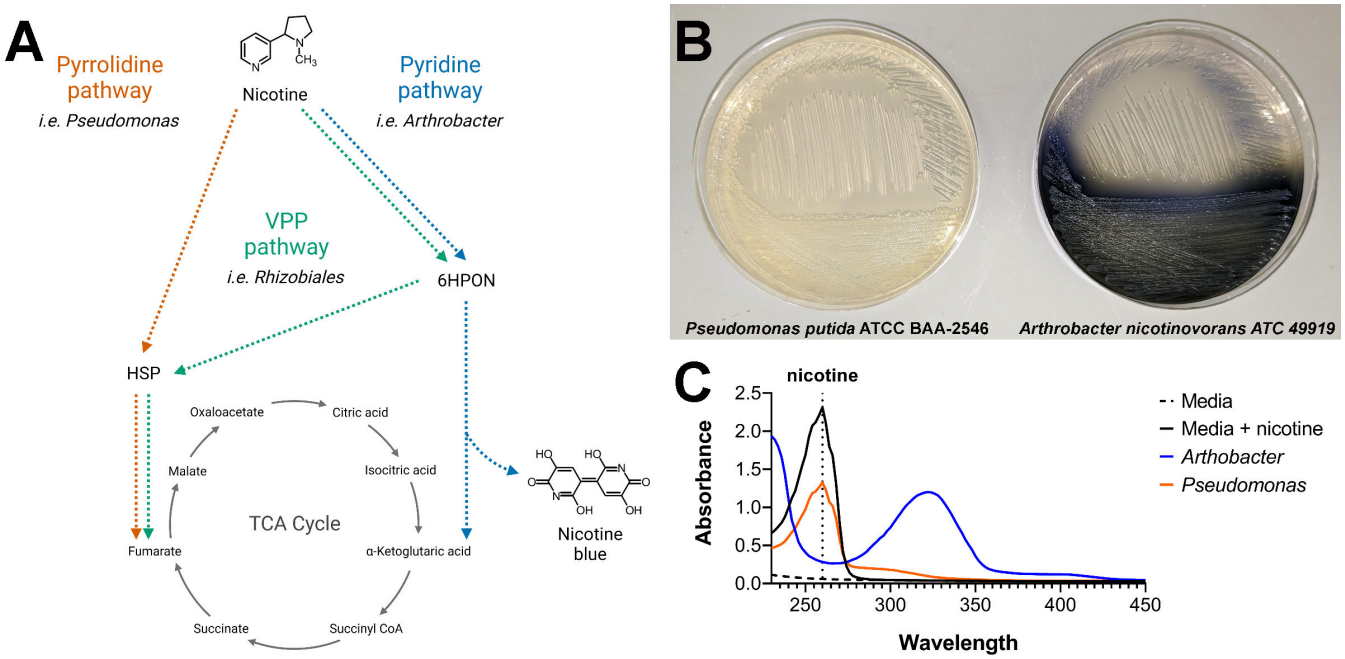

FIG 1 (A) Abbreviated map of known nicotine metabolic pathways by various bacteria, summarized from reference (4). (B) *Pseudomonas* and *Arthrobacter* strains were grown on M9 media with nicotine as the sole carbon source. *Arthrobacter* produces nicotine blue, which can be seen on the plate. (C) Wavelength scan of M9-nicotine media before and after bacterial growth. Nicotine has a maximum absorption at 260 nm. Growth of bacteria reduces absorbance at 260 nm, consistent with nicotine catabolism.

to DNA extraction. Competitive behaviors between bacteria can result in the lysis of some colonies during the initial isolation.

PROCEDURE

Safety guidelines

Purified nicotine is toxic and can cause serious health complications if inhaled, swallowed, or through skin contact, though toxicity is reduced when diluted into media. Be careful when handling pure nicotine while making nicotine media and use appropriate PPE. It is possible to grow pathogens whenever one isolates environmental bacteria; use appropriate PPE and safe handling techniques.

Isolation of nicotine-degrading bacteria from tobacco plants and products

We used media with nicotine as the sole carbon source (M9-nicotine) to isolate nicotine-degrading microorganisms. Students were provided a tobacco plant grown on campus. Tobacco plants included *Nicotiana tabacum* (Virginia Bright leaf or Burley Mammoth), *Nicotiana rustica* (Wild Rustica), and *Nicotiana sylvestris* (ornamental flowering) obtained from Victory Seed company, or *Nicotiana benthiana* (cv. Xanthi NahG or Samsun NN) obtained from Vestaron. Students were also provided a commercial tobacco product (i.e., cigarettes). For leaves, the top and bottom sides were gently pressed onto an M9-nicotine plate; bacteria differed between the top and bottom of leaves. For roots or commercial tobacco products, small clippings of the sample were placed in 10 mL of sterile water and vortexed for 30 seconds. Samples were placed upright for 10 minutes to settle debris. One hundred microliters of supernatant was added to an M9-nicotine plate and distributed using sterile spreaders. The plates were incubated at 30°C for 48 hours; colonies were visible after 48 hours of growth.

Following incubation, students re-streaked an isolated colony twice. The cultures were then maintained on tryptic soy agar (TSA) plates. All colonies initially isolated on

M9-nicotine plates were able to grow in TSA; however, some lost the ability to metabolize nicotine. This is likely due to nicotine metabolism genes being encoded on plasmids (6).

PCR amplification of 16S rRNA gene

Isolated colonies were diluted in 10 µL (small colony) or 20 µL (large colony) of H_2O. We then performed colony PCR using 8F and 1492R primers (10-minute initial denaturation, 55°C annealing). PCR reactions were confirmed using gel electrophoresis, purified using a DNA cleanup kit (Zymogen D4013), and submitted for Sanger sequencing (Genewiz).

Biochemical determination of species

Students used NCBI BLAST to determine the genus of their isolates, based on the 16S sequence. Of 61 isolates, we confirmed 14 different genera (Fig. 2). Gram stains, catalase, oxidase, and thioglycolate tests were performed; we found that 80.3% of isolates were Gram positive. 83.6% of isolates were catalase positive, 54.1% were oxidase positive, and 29.5% were facultative anaerobes. Additionally, students selected six genus-specific tests for partial species identification; these tests can be tailored to the organisms isolated or resources available.

Nicotine catabolism assay

Isolates were inoculated in M9-nicotine broth and incubated for 48 hours at 28°C with shaking. After, 1 mL of broth culture was centrifuged to a pellet. A total of 200 µL of cell-free supernatant was added to a UV-transparent 96-well plate, and the absorbance was determined between 200 and 300 nm. Nicotine has a peak absorbance at 260 nm, which can be seen in uninoculated M9-nicotine broth. Reduced 260 nm peaks indicate nicotine catabolism (Fig. 1C). Controls include *Pseudomonas* ATCC BAA2546 and the

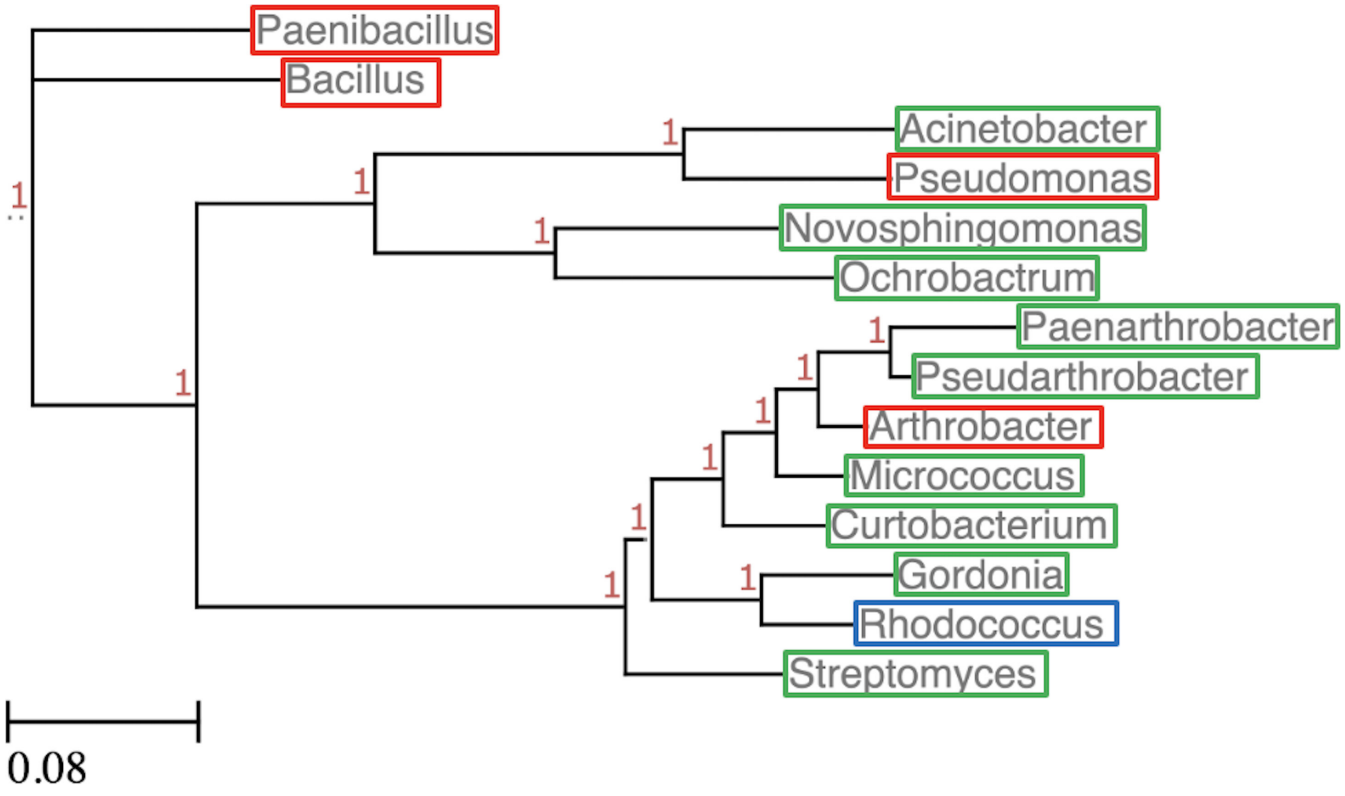

FIG 2 16S phylogenetic tree nicotine-degrading bacterial genera isolated from tobacco plants and cigarillos. Strains were isolated from tobacco plants (green), cigarette products (blue), or from both sources (red). Created using ETEToolkit using Maximum Likelihood (10).

Arthrobacter ATCC 49199 strains. Nicotine blue has a maximal absorbance at 590 nm. Sometimes a peak emerged at 325 nm, which partially overlapped with the 260 nm peak.

Conclusion

Growth on nicotine is stringently selective, making this approach easy to execute in teaching laboratories, and the straightforward connection to public health increases student interest and engagement. Tobacco plants are commonly used in teaching laboratories, and tobacco products are readily available; purified nicotine is also inexpensive, together making this approach accessible. This provides the instructor opportunities to integrate core concepts, including microbial ecology and diversity, metabolism, and horizontal gene transfer.

This project could be further modified. Alternative tobacco sources could be used, like vaping products. Students could compare environmental factors and bacterial abundance (i.e., growth conditions, different tobacco products, etc). Because tobacco products are dried, students could assess desiccation tolerance. Students could also isolate phage from tobacco plant soil using their isolates. As nicotine-degrading bacteria are understudied, this approach could be modified to be a course-based undergraduate research experience (11).

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AUTHOR AFFILIATIONS

[1]Department of Biological Sciences, Western Michigan University, Kalamazoo, Michigan, USA
[2]Department of Chemistry, Western Michigan University, Kalamazoo, Michigan, USA

AUTHOR ORCIDs

B. J. Koestler http://orcid.org/0000-0001-7213-0953

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Appendix 1 (jmbe00152-23-S0001.docx). Required materials.
Appendix 2 (jmbe00152-23-S0002.docx). Recipe for M9-nicotine media.
Appendix 3 (jmbe00152-23-S0003.docx). Lab handout.
Appendix 4 (jmbe00152-23-S0004.docx). Graphical synopsis of Nicotine degraders lab.
Appendix 5 (jmbe00152-23-S0005.docx). Notes for instructors.
Appendix 6 (jmbe00152-23-S0006.docx). Biochemical test findings from Spring 2023.

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