Overview: Plastic-Eating Fungi & Other Wonders Discovered by Yale Undergraduates

Prepared by Dean's Fellow, Lian Hai Guang for Yale-NUS Centre for Teaching and Learning

1. Focus Articles

a. Anderson, S. (2014, December 15). The Plastic-Eating Fungi That Could Solve Our Garbage Problem. Retrieved February 26, 2016, from http://www.newsweek.com/2014/12/26/plastic-eating-fungi-could-solve-our-garbage-problem-291694.html

This quick and easy to digest paper from *Newsweek* offers an idea of the research Prof. Strobel and his students have conducted. It offers an entry way into Prof. Strobel's talk, the impact and potential of this particular research, as well as other similar efforts in the field.

b. Strobel, S. A., & Strobel, G. A. (2007). Plant endophytes as a platform for discovery-based undergraduate science education. Nature chemical biology, 3(7), 356-359.

Coming in from a more pedagogical angle, this article shares how a research project can be beneficial and motivating for learners in a particular field, the various design elements that contribute to its success, as well as the description of some features of the authors' own intensive research module.

2. Supplementary Materials

a. Russell, J. R., Huang, J., Anand, P., Kucera, K., Sandoval, A. G., Dantzler, K. W., ... & Marks, D. H. (2011). Biodegradation of polyester polyurethane by endophytic fungi. Applied and environmental microbiology, 77(17), 6076-6084.

The original paper consolidating the research that Prof. Strobel has done with his students. This paper is focused on the topic of **bioremediation**, which is the practice of using naturally occurring organisms to breakdown and treat waste or hazardous substances. The paper describes the research that was carried out, the experimental methods applied and the obtained results.

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The Plastic-Eating Fungi That Could Solve Our Garbage Problem

Stacey Anderson

Of the approximately 251 million tons of trash dropped annually into American landfills, items made of polyurethane are particularly destructive to the planet. The hardy, widely used polymer is found in foam insulation, bike seats, garden hoses, Spandex fabric and more, and its use has increased steadily since it was first developed in the 1930s. The material has long been considered nonbiodegradable because its chemical bonds are so strong. It is usually reduced by incineration, which releases harmful gases into the ecosystem, or it languishes indefinitely in landfills or the ocean, where, alternately, ultraviolet rays from the sun or waves very slowly break it into still-harmful microplastic particles that poison marine life when ingested. And it is not going away anytime soon; polyurethane production is rising both in America and overseas.

However, the pesky plastic may have a natural combatant, found in the most unlikely of places: the Amazon rain forest. In 2011, 20 undergraduates from Yale University's Department of Molecular Biophysics and Biochemistry traveled to Ecuador with professor Scott Strobel for an annual research trip and discovered a fungus that eats only polyurethane. It's the first microbe found to survive exclusively on the plastic, and, most notably, it can do so in

anaerobic (oxygen-free) environments, meaning it could potentially thrive at the bottom of landfills. Strobel and his students published a paper, "Biodegradation of Polyester Polyurethane by Endophytic Fungi," in the journal *Applied and Environmental Microbiology* in 2011.

"It's interesting research. I think this approach of bioremediation could be very useful in treating accumulated plastic waste," says David Schwartzman, professor emeritus of biology at Howard University. He has long studied the ecological properties of fungi and lichens and published several papers on the subject. "Landfills are sources of serious problems. They're leaking methane as well as other pollutants that get into the groundwater. Some bioremediation may be necessary to deal with the huge mountainous accumulation of these waste."

In their paper, the Yale researchers discussed finding the fungus, *Pestalotiopsis microspora*, in the Amazon, one of the most biologically diverse areas on the planet. The students brought a sample back to the United States, with permission from the Ecuadorean government, and isolated the enzymes in the fungus that enable its plastic-chomping properties.

Although Strobel does not believe there's enough in the study to warrant media coverage, other researchers in the field are more optimistic. If applied successfully to landfills, the fungus could mark a major advancement in conservationists' ongoing attempt to change our society's linear model of manufacturing, says Joel Cohen, head of the Laboratory of Populations at Columbia

University and Rockefeller University. “Under that model, we extract [a] resource, we process the material, we sell it, and we discard it after use,” he says. “The problem with that model is, there is no longer a way to throw things away; what we discard comes back to bite us, one way or another. This study is part of a larger effort to replace that with a circular model.”

Researching fungi for their bioremediation properties is nothing new in the scientific community; it even has its own subset of studies, called “mycoremediation.” The book *Mycelium Running: How Mushrooms Can Help Save the World* by Paul Stamets earned media attention in 2005 for arguing that certain fungi may be able to combat smallpox and anthrax, as well as stem the tide of pollution. Some fungi have been studied by scientists over the past decade for their ability to clean crude oil spills.

Along these lines, Schwartzman says the Yale results are the promising first steps of a much longer scientific examination. “This research is of value, but we should be quite cautious about the application,” he says. “I would be very leery of releasing some organism into [a new] environment. That’s fraught with a lot of potential dangers.”

Schwartzman cites the plastic trash and microplastic particles clogging the oceans. “I’m just imagining a scenario where we say, ‘Let’s get rid of the marine plastics by spraying these fungi’ [into the ocean]. To me, that’s not a viable solution.” He offers an alternate plan: “Collecting the debris and then applying bioremediation to break them down would seem to be a valuable approach.”

Beyond that are potential horror-movie implications within the

microbe's structure, says Cohen. He points to one of the fungus's properties, as outlined in the Yale paper: its potential ability to adopt other species' genes and infect other species with its own genes. As the Yale study notes briefly, "*Pestalotiopsis microspora*...have a propensity for horizontal gene transfer."

Cohen cites the Uruguayan-bred virus myxomatosis, which was introduced to Australia in the 1950s in an attempt to curb the enormous rabbit population and quickly went wild across the continent. "What is the possibility of horizontal gene transfer for the gene that produces this digesting chemical to be transferred to other organisms that weren't intended to have that gene?" asks Cohen. "Is there a risk that the process of degradation could spread out of control?" In other words, the trash-eating enzyme could possibly mutate with other organisms in the landfill and start breaking down materials besides polyurethane—great for horror-movie writers, less so for people living nearby.

The Yale students posited that they might be able to breed the fungus in laboratory settings and test them on plastic-heavy zones, though they have not shared further information on that yet. (They did not theorize on transportation or containment strategies, which would be key.) They also urged future scholars to pick up their research, writing, "The relative ease with which organisms can be isolated and screened makes this a highly accessible and environmentally relevant project for engaging undergraduate students in scientific research."

"This brings home the importance of conservation of biodiversity," says Cohen. "The bacteria and fungi are older and wiser than we are. They've been around for a very long time, maybe hundreds to

thousands of times longer than the entire existence of the human species. We have to protect the reserves that we don't understand yet."

Plant endophytes as a platform for discovery-based undergraduate science education

Scott A Strobel & Gary A Strobel

Project ownership is an essential but sometimes overlooked ingredient for a successful undergraduate research experience. We have embarked on an experiment in undergraduate education that targets isolation of microbes from rainforest plants and characterization of natural products as objectives for discovery-based undergraduate research.

We have initiated an undergraduate research program that includes a two-week expedition to the Amazonian rainforest and a ten-week summer research experience. Our objective is the discovery of (i) novel microbes that are endophytically associated with plants and (ii) the potentially biologically active molecules that these microbes produce. Although the foreign travel is a strong enticement for student participation, we have found that the opportunity to own and independently direct their research program is key to program success.

Intensive research is often used as a capstone experience to successfully enrich an undergraduate student's science education[1]. Typically, a student joins a research group and participates in an ongoing project under the supervision of a professor, or a senior member of the laboratory. This experience provides personalized training that can be inspirational and highly motivating. Undergraduate students become exposed to a group of people engaged in inquiry-based research who communicate in the 'foreign language' of that scientific discipline. Some students realize that science is not beyond their abilities and that it is much more exciting than the vocabulary-focused courses they have taken, particularly at the introductory levels. Several studies have demonstrated that an independent research experience is the best way to get undergraduates excited about a career in science. In fact, the vast majority of science faculty in US universities had an undergraduate research experience during their training.

Despite the critical role hands-on research has in scientific career development, some very good students still come away from the experience frustrated and discouraged. In our experience working with undergraduate students in a variety of different laboratories, their frustration often resulted from the nature of the project to which they had been assigned. Undergraduates were assigned work that was primarily technical support and were provided insufficient opportunity to formulate hypotheses and design experiments to test their ideas. This might reflect the need to provide projects designed to fit within the short timescale available to students, whose time is often interrupted by classes, vacations and other activities.

Given that project design is critical to a successful undergraduate research experience, what are the elements of a research project that make it well suited to the abilities and time constraints of a motivated undergraduate student? Graham Hatfull, a Howard Hughes Medical Institute professor at the University of Pittsburgh, has proposed a series of criteria based on the successful implementation of his phage hunters program[2]. He argues that undergraduate research projects should be technically feasible and should not require a large body of prior knowledge. The projects should include multiple success points, allowing confidence to be gained as the project grows to greater levels of complexity. For projects involving large numbers of students, the projects should be parallel in nature, thereby simplifying mentoring and maximizing the number of students that can be involved. This also provides an opportunity to publish the collective observations made by individual students. The projects must be scientifically 'real'—that is, capable of producing scientific results and providing motivation through scientific discovery. Most importantly, the project must provide a sense of intellectual ownership, with sufficient freedom for the student to pursue his or her own experimental questions and observations. This feeling of empowerment to control the scientific direction of an inquiry is critical to the success of any research experience, regardless of the student's academic level.

Hatfull's program, which has involved both undergraduates and high school students, has focused on the isolation, sequencing and annotation of mycobacteriophage from soil samples. The initial stages of the work are technically simple, but invoke the students' imaginations as they consider where to collect their soil samples. The abundance and diversity of phage provide a high likelihood that each student can isolate, name and characterize a new virus, thus creating a strong sense of intellectual ownership and project

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control. Though not all high school or college students who participated in the phage hunt concluded that a scientific career is in their future, every student achieved at least the first success point and several went on to scientific publication[3,4].

We have set out to incorporate the conceptual elements of Hatfull's phage hunters program in an intensive undergraduate research experience of our own. Our focus is the discovery of biologically active natural products produced by endophytic microorganisms associated with plants. Endophytes represent a diverse potential source of new products for use in medicine, agriculture and industry[5,6]. They colonize living tissues of plants without inflicting negative effects and can be either fungal or bacterial organisms. Of the nearly 300,000 plant species on earth, each is likely to be host to at least one endophyte[7]; but relatively few of these organisms have been characterized. Many endophytes make bioactive natural products to inhibit the growth of other organisms or provide a selective advantage to their plant host[5–8]. In some cases they can even acquire the ability to synthesize the same defensive natural products produced by the plant[9]. Our focus is on endophytes associated with plants of the neotropics, an ecosystem in which the hypervariability of the plants is expected to be matched by the hypervariability of their associated microbes[10]. Many plants have an extensive ethnobotanical history of use by indigenous people that provides an intriguing starting point for each student's project design. For example, students may wish to explore microbes associated with the cinchona (the "Fever tree" or Jesuits' Bark), which is responsible for quinine production. Quinine was used worldwide for the treatment malaria, based on original observations of its utility by the Malacatos Indians of present-day Ecuador in the early 1600s[11]. Alternatively, they may wish to explore plants that were indigenous to ancient Gondwanaland, given the potential for long-term and potentially beneficial association between plant and microbes. No matter how the students design their projects, bioprospecting for endophytic natural products in the rainforest presents an open-ended, inquiry-based opportunity for students to make original discoveries about the natural world.

We are halfway through the first year of a four-year bioprospecting program funded by the Howard Hughes Medical Institute and the US National Science Foundation (NSF). The program includes three major components: a semester-long seminar course designed to prepare the students for the expedition and laboratory work, a two-week expedition to one of the world's rainforests during spring break, and an intensive summer laboratory experience isolating and characterizing endophytic microbes associated with the collected plant samples (**Fig. 1**).

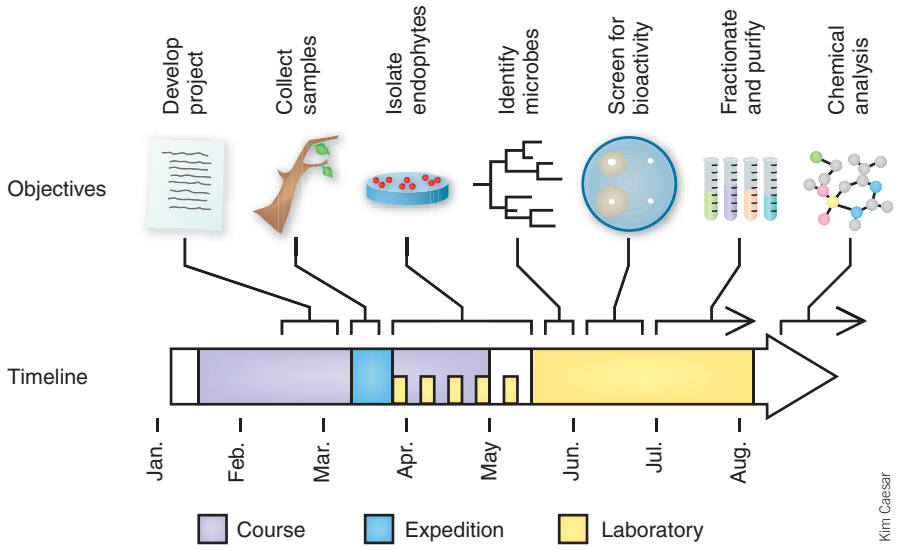

Figure 1 Approximate timeline for the progressive success points of the rainforest expedition and laboratory.

The course is designed to cover a breadth of topics relevant to both the field and the laboratory experience, including conservation, ethnobotany, plant phylogenetics, mycology, pharmacology, patenting and intellectual property, and even a lecture on ants by Mark Moffett, a National Geographic photographer. The intention is to prepare students to see more than just plants when they visit the jungle. Many of these subjects are whole courses unto themselves, so the goal is not for them to master each discipline, but instead to expose the students to key ideas that drive these fields. Students were selected for participation in the course based primarily on two questions: what can you contribute to the course, and what do you wish to learn from this experience? Some answers to the first question were particularly helpful, making it possible to include two Spanish translators and an EMT among the students on the expedition.

A key assignment in the class was for each student to define the theme for his or her plant collection based on the published ethnobotanical history of various plants and his or her own imagination and interests. This maximized each student's sense of project ownership. One student collected plants used by native people to treat tuberculosis infections; another selected plants likely to produce antioxidants. One targeted carnivorous plants, while another selected plants used in the treatment of wounds. One particularly creative student selected trees related to *Tabernanthe iboga*, a tree indigenous to Africa that produces ibogaine, an indole alkaloid that has been postulated as a treatment for drug addictions[12]. He hopes to identify a microbial source for this natural product. The extensive scientific possibilities and the intellectual license to design their own experimental plan has been highly motivating to the students.

In March, 15 students (most in their sophomore year) and one high school biology teacher traveled to the upper Amazonian basin of South America, where we collected plant samples (**Fig. 2**). With the help of Percy Nunez, a field biologist with an encyclopedic knowledge of neotropical botany, each student found most of the plants on his or her list (**Fig. 2**). "I found MY plant!" was a common cry heard along the jungle trail. Even more encouraging was the call "We found YOUR plant" over the group's walkie-talkie network. The group developed a strong sense of collegiality and cooperation during the expedition that was manifest in the willingness of students to collect samples from each other's lists. Since our return, the plant stems have been dissected and the segments placed in Petri dishes. Growth is evident on all of the plates, as almost every plant collected had several endophytic associations. We will spend the rest of the summer characterizing these microbes and screening for any bioactive compounds they may produce. The program is designed to provide students with multiple success points, beginning with plant identification and collection, and followed soon after with microbial isolation. The objectives then

Figure 2 Members of the rainforest expedition 2007 (left); plant collecting under the guidance of Percy Nunez from the University of Cusco (right).

build in complexity as they phylogenetically classify the microbes by molecular analysis, screen them for bioactivity, and undertake chemical fractionation (**Fig. 1**).

This is the first large-scale attempt to use endophyte isolation and characterization as a tool for empowering undergraduate research. Though student enthusiasm is anticipatory of success, it is still too soon to judge the value of our experiment in science education. However, work done on a more limited scale has established that undergraduates can use this platform to successfully design and implement their own scientific plan. In the three cases listed below, the students embarked on the laboratory studies after the fieldwork was completed by others, but the examples demonstrate the feasibility of using natural product isolation as a format for inquiry-based undergraduate research. These three students were undergraduates at Montana State University working in the laboratory of G.A.S. during summer break and subsequent semesters.

A novel endophytic fungus (*Muscodor albus*) was isolated from a cinnamon tree growing along the Caribbean coast of Honduras (**Fig. 3a**). Emily Dirkse focused on this organism during an NSF-sponsored summer undergraduate program. *M. albus* produces volatile compounds that inhibit and/or kill a wide spectrum of fungi and bacteria. Emily identified 28 of these natural products by GC/MS and prepared artificial chemical mixtures that mimicked the antibiotic effects of the fungus. Individual fungal compounds had some inhibitory effect against test fungi and bacteria, but none was lethal. However, when added in combination they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria, including *Escherichia coli*. A report describing the "mycofumigation" effects of *M. albus* was published in the journal *Microbiology* and highlighted in the editor's choice section of *Science*[13,14], and a patent was issued for its use in the treatment of human waste.

Plant materials isolated from the jungles of the Peruvian Amazon were given to Bryn Daisy, another undergraduate working on endophytic microorganisms. Her goal was to identify relatives of *M. albus* by virtue of their resistance to its antimicrobial volatiles when grown on the same Petri plate. She isolated and characterized a novel fungus, *Muscodor vitigenus*, that produces naphthalene as its sole volatile compound, and she demonstrated that the fungus produces naphthalene at sufficient levels to repel insects[15,16]. This provided a potential clue about the selective advantage provided to the plant by its microbial association.

Lindsey Browne became particularly adept at isolating endophytes from plants collected throughout the world. She recovered six novel bioactive streptomycetes from *Notofagus* spp. growing in Patagonian Chile. The isolates were characterized by molecular and morphological features and found to be active against such plant pathogens as *Phytophthora cinnamomi* and *Pythium ultimum*[17]. She isolated endophytes from plants collected on Socotra, an island off the coast of Yemen in the Arabic Sea[18], and had a major role in the isolation of 139 endophytic streptomycetes from an Australian snakevine plant (**Fig. 3b**). Many of these organisms were found to be novel based on their recombinant DNA sequences, unusual morphologies and biological activities[19].

Our hope is that a group of students involved in an institutionalized program will also feel intellectually empowered to become creatively engaged in science. Because the program includes both plant collection and microbial characterization, students will have the opportunity to take the project from the field to the laboratory. The program is a fishing expedition by its very design, so it is uncertain what will be found, but the diversity of microbial life in these environments is sufficiently rich that each student is all but assured to 'catch' something interesting. The program provides a holistic view of scientific training that incorporates experience in the subjects of ecology, microbiology, biochemistry, bioinformatics, pharmacology and chemistry.

Though funding for students to visit a rainforest is beyond the budget of most college courses, a less expensive variation on the program could still be implemented at other institutions. Plant materials can be collected from many locations that do not

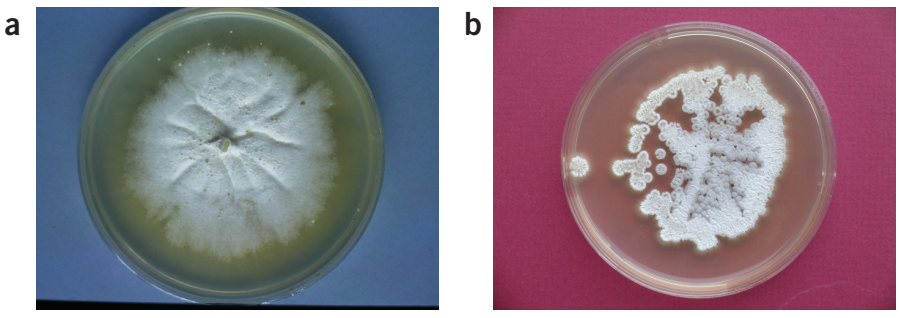

Figure 3 Endophytic microbes isolated from plants. (**a**) *M. albus* from Honduras. (**b**) One of the streptomycetes isolated from the Australian snakevine.

require extensive travel. There are many old-growth forests or other unique ecosystems within driving distance of most campuses that could provide the source of materials for endophyte-targeted programs. An enormous amount of biological and chemical diversity remains to be discovered[20]. We anticipate that the range of scientific possibilities will inspire the scientific imagination of most of the students who participate.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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Biodegradation of Polyester Polyurethane by Endophytic Fungi[▽]

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Bioremediation is an important approach to waste reduction that relies on biological processes to break down a variety of pollutants. This is made possible by the vast metabolic diversity of the microbial world. To explore this diversity for the breakdown of plastic, we screened several dozen endophytic fungi for their ability to degrade the synthetic polymer polyester polyurethane (PUR). Several organisms demonstrated the ability to efficiently degrade PUR in both solid and liquid suspensions. Particularly robust activity was observed among several isolates in the genus *Pestalotiopsis*, although it was not a universal feature of this genus. Two *Pestalotiopsis microspora* isolates were uniquely able to grow on PUR as the sole carbon source under both aerobic and anaerobic conditions. Molecular characterization of this activity suggests that a serine hydrolase is responsible for degradation of PUR. The broad distribution of activity observed and the unprecedented case of anaerobic growth using PUR as the sole carbon source suggest that endophytes are a promising source of biodiversity from which to screen for metabolic properties useful for bioremediation.

Tremendous increases in the manufacture and consumption of plastics over recent decades have led to numerous ecological and economic concerns. The persistence of synthetic polymers introduced into the environment by human industry poses a major threat to natural ecological systems. The low cost and ease of manufacture have increased global plastic demand more than 150-fold, with the production of 1.5 million tons in 1950 and 245 million tons as of 2006 (21). Despite recognition of the persistent pollution problems posed by plastic, global production is still increasing, with the largest increases expected in developing nations. The sheer volume of plastics produced each year presents a problem for waste disposal systems. The scale of this problem and the recalcitrance of some polymers to degradation necessitate investigation into effective methods for biodegradation of plastics. By gaining an understanding of the mechanisms of polymer degradation, a more efficient technique for the biodegradation of plastic waste can be achieved. To accomplish this goal, researchers need greater knowledge of how compounds are metabolized by existing organisms, an investigation of new organisms with bioremediation potential, and the characterization of novel metabolic capabilities. A basic understanding of the biological processes that lead to biochemical degradation will advance the development of new bioremediation techniques.

Polyester polyurethane (PUR) is a plastic widely used in industry and manufacturing that has been shown to be susceptible to biodegradation (6, 10). The polymer is generated by the condensation of a polyisocyanate and a polyol. This results in a carbon polymer composed of a series of urethane linkages. Variations in the spacing between urethane linkages, as well as the nature of the substitutions, can change the properties of the resulting polymer from linear and rigid to branched and flexible. In a liquid suspension PUR appears milky white and completely opaque. Like other polyurethanes, this product is synthesized commercially for the manufacture of textiles and textile coatings.

Enzymatic degradation of PUR by both fungi (4, 5, 6, 19) and bacteria (11, 12, 14, 15, 17, 18, 23) has been demonstrated. Soil fungi comprise the majority of organisms screened for PUR degradation activity (4, 5). Fungi of the genera *Alternaria*, *Aspergillus*, *Phoma*, *Pennicilium*, *Plectosphaerella*, *Geomyces*, *Nectria*, and *Neonectria* were isolated with access to mixed nutrient sources from buried PUR samples (4). *Geomyces pannorum* was the most commonly isolated PUR-degrading organism with this method (4). Few organisms have been shown to degrade PUR as a sole carbon source. *Aspergillus niger* has some reported degradation activity; however, it was observed to be quite slow, with visible signs of degradation occurring only after 30 days (7).

Putative polyurethanases have been isolated and characterized from protein extracts of several organisms, including the bacteria *Pseudomonas chlororaphis* (25) and *Comamonas acidovorans* (1, 2), as well as the fungus *Candida rugosa* (8). The active enzymes have been classified as esterases (5, 13, 16), lipases (26), and proteases and ureases (19), suggesting degradation of the PUR substrate by cleavage of the ester bond.

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In an effort to identify new organisms with novel metabolic capabilities for polymer degradation, we have embarked on an effort to explore the biological and chemical diversity of endophytes. This was achieved as part of an educational program to engage undergraduate students in discovery-based research (9, 28). Endophytes are hyperdiverse microorganisms, including bacteria and fungi, that live within the inner tissues of plants without causing overt disease symptoms (3). These organisms enter their hosts by penetrating exterior surfaces, and some play a key role in plant decomposition following host tissue death (3). Indeed, fungi such as these contribute to decomposition of lignocellulose polymers and are major contributors to the carbon cycle (3, 22, 24). The ability of these microorganisms to degrade a polymer as complex as lignocellulose would suggest that these organisms offer promise for their ability to degrade other complex polymers, such as those present in plastics. The unique biological niche of endophytes as endosymbionts of tissues rich in complex carbon polymers justifies the investigation of their wider metabolic capabilities. Each of the more than 300,000 land plant species on Earth potentially hosts multiple endophyte species. Only a small sampling of plants have been examined for their endophytic associations, yet many of these organisms can be readily cultured (3, 25, 28). Endophytes reach their greatest diversity in tropical forests. Individual trees can harbor hundreds of endophytic species, some of which are known but many of which are new to science (25).

In the current study, endophytes were isolated from plant stems collected in the Ecuadorian rainforest. A subset of these organisms was screened for their ability to degrade polyurethane. Several active organisms were identified, including two distinct isolates of *Pestalotiopsis microspora* with the ability to efficiently degrade and utilize PUR as the sole carbon source when grown anaerobically, a unique observation among reported PUR biodegradation activities.

MATERIALS AND METHODS

Plant sampling. Woody plants of various families were collected in the Yasuni National Forest in the Ecuadorian Amazonian rainforest. Some plants were targeted for their purported ethnobotanical uses, while others were sampled randomly. A 10-cm stem sample was collected and stored in an air-tight polyethylene bag at 4°C. Herbarium samples were prepared and deposited in the Peabody Herbarium at Yale University and the Herbario Nacional del Ecuador (QCNE).

Endophyte isolation. Plant stems were surface sterilized by immersion in ethanol for 10 s followed by brief flaming. Outer layers of tissue were removed from the stems, and three sections of the inner tissues of each sample were plated on potato dextrose agar (PDA) (Difco), a 1:10 dilution of potato dextrose medium in water agar (WA), a 1:10 dilution of glycerol arginine medium (GAM) in WA, and WA plates (25). All plates were sealed with Parafilm and monitored every 2 to 3 days for growth. As growth of microbes was detected, fungal organisms were isolated by transferring a hyphal tip to fresh PDA plates. The plates were again wrapped with Parafilm and stored in plastic containers. Permanent stocks of each organism were made by growing the organisms on triple-autoclaved barley seeds and storing them at −80°C.

Sequencing and phylogenetic analysis. Endophyte cultures were grown on PDA plates for 1 to 2 weeks at room temperature. DNA was extracted from approximately 100 mg of fungal material using the Qiagen DNeasy plant minikit. Approximately 10 ng of DNA was used as a template to amplify the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) by PCR as previously described (30). The primers used for ITS sequencing were ITS1 (5′-TCCGTAGGTGAACCTGCGGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), corresponding to the forward and reverse primers (30). The amplified fragments were verified for length using agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen). Sequencing was performed at the Yale University W. M. Keck Facility on Applied Biosystems 3730cL DNA Analyzer machines. Forward and reverse sequences for each organism were aligned using the programs Pregap4 and Gap4 (25). Endophyte sequences were aligned to organisms present in the GenBank database on 1 August 2008 using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/GenBank/index.html).

Initial PUR clearance screen. Endophytes were first assayed for their ability to degrade PUR by growing them in the presence of Impranil DLF an anionic aliphatic aqueous PUR dispersion with 4% *N*-methyl pyrrolidone (NMP) (Bayer MaterialScience). Fifty-nine fungal endophytes were grown on solid PUR medium (PUR-A) containing 19 mM NaH_2PO_4, 33.5 mM K_2HPO_4, 7.6 mM $(NH_4)_2SO_4$, 2.5 mM Na citrate, 250 μM $MgSO_4$, 19 μM thiamine, 0.05% Casamino Acids, 147 μM $FeCl_3 \cdot 6H_2O$, 14 μM $ZnCl_2 \cdot 4H_2O$, 12 μM $CoCl_2 \cdot 6H_2O$, 12 μM $Na_2MoO_4 \cdot 2H_2O$, 10 μM $CaCl_2 \cdot 2H_2O$, 11 μM $CuCl_2$, 12 μM $MnCl_2$, 12 μM H_3BO_3, and 1.8 mM HCl. To 1 liter of this mixture was added 10 ml Impranil DLF and 15 g of agar. The polymer was added after autoclaving the medium to prevent deformation.

The solid medium screening assay followed the general method of Crabbe et al. (5). The PUR-A solid medium was added to sterile culture tubes in 10-ml aliquots. A 0.5-cm^3 plug of fungus grown on PDA was added to each test tube using aseptic technique and allowed to grow undisturbed at 23°C. The bacterium *Pseudomonas chlororaphis* (ATCC 55729, from Gary Howard) and the fungus *Aspergillus niger* (from Gary Strobel, Montana State University) were used as positive controls for polyurethane degradation (6, 7, 13). PUR degradation was evidenced by a change in medium appearance from opaque to translucent. After 2 weeks of incubation, the depth of polyurethane clearance was measured from the top of the medium to the lowest point of visible clearance.

The liquid medium assay followed the method described by Gautam et al. (8). PUR-L liquid medium was prepared using the same recipe as for the solid PUR-A medium without agar. PUR-L medium was added to sterile culture tubes in 10-ml aliquots and inoculated with a 0.5-cm^3 plug of fungus grown on PDA. At the end of 2 weeks, the liquid cultures were homogenized by vigorous shaking. A 1.5-ml portion of each culture was transferred to an Eppendorf tube and centrifuged for 1 min at 4,200 × *g* in an Eppendorf MiniSpin centrifuge to selectively pellet the fungal matter. The absorbance of the supernatant liquid was measured on a Varian Cary 50 Bio UV-visible spectrophotometer at a wavelength of 600 nm, with sterile water as a blank. Dilutions of PUR-L medium with sterile water were measured to construct a standard curve for converting absorbance to percent clearance. Samples were measured each day for 2 weeks for optical absorbance to determine a relative rate of clearance.

Sole carbon source assay. Organisms identified as having PUR-degrading activity were tested for their ability to use PUR as the sole carbon source. For these studies, the substrate was Impranil DLN, which contains PUR suspended in only water (no *N*-methyl pyrrolidone is present). This isolates the Impranil as the sole source of carbon for metabolism and growth. The top five organisms from the initial activity screens were grown on Impranil DLN with no other carbon sources (PUR-L_{min}). The fungal samples were washed prior to inoculation to remove all residual medium. These two considerations—the wash and the DLN substrate—ensure that the polymer is the sole source of carbon for fungal metabolism and growth. PUR-L_{min} was prepared in a manner similar to that for the PUR-L medium but in the absence of sodium citrate, thiamine, Casamino Acids, or agar. The organism *Aspergillus niger* was tested as a basis for comparison.

Fungal cultures were grown for 1 week in potato dextrose broth (PDB). Stock cultures were homogenized by vigorous shaking, and 1 ml of each culture was centrifuged at 12,100 × *g* for 1 min. The supernatant was removed, and the fungal pellet was resuspended in 1 ml of the PUR-L_{min} liquid medium. Samples were centrifuged and resuspended a second time to ensure removal of all residual PDB. The 1-ml sample of washed fungal material was added to sterile culture tubes to a final volume of 10 ml PUR-L_{min}. The cultures were monitored for visual clearance of the opaque medium. Samples were measured every 2 days for 2 weeks for optical absorbance at 600 nm to determine an approximate rate of clearance. An increase in fungal mass correlating to PUR degradation was measured by lyophilizing mycelial mass from triplicate cultures containing minimal medium with and without PUR.

Anaerobic degradation of PUR. Culture tubes containing 9-ml aliquots of PUR-L_{min} medium and 1 ml of washed fungal inoculums were incubated under anaerobic conditions. The anaerobic environment was generated using a BD GasPak anaerobic chamber. Liquid cultures of each isolate were inoculated in duplicate. One set was placed inside the anaerobic chamber, and the control set was placed outside the chamber in an aerobic environment. Both sets were

incubated at 25°C for the duration of the study. After 1 and 2 weeks, samples were removed and clearance was determined by absorption at 600 nm.

IR analysis of PUR degradation. Infrared (IR) spectra of liquid PUR-L_{min} suspensions were collected every 48 h using a Nicolet 6700 infrared spectrometer. A 50-μl portion of each sample was removed for each measurement and centrifuged for 60 s at 4,200 × *g* to settle out fungal material from the samples without significant sedimentation of the polymer. Liquid sample spectra were collected using deionized water as a background spectrum. Measurements were performed until complete degradation of PUR in the 10-ml culture was observed within 2 weeks.

Enzymatic characterization of putative polyurethanase. An extracellular enzyme fraction was prepared by growing a 1-liter liquid culture of the most active organism, *Pestalotiopsis microspora* E2712A, in PUR-L_{min} and PDB media. After 10 days of incubation at 30°C, the culture was filtered through a 0.22-μm filter into a sterile container. Crude extracellular extract was stored at 4°C. To test for the activity of an extracellular enzyme, 4 ml of extract was added to 6 ml of PUR-L_{min} medium. Samples were incubated for 2 h in a rotary incubator at 30°C.

Three mechanism-based inhibitors were used to characterize the activity. Phenylmethylsulfonyl fluoride (PMSF), a serine hydrolase inhibitor, was added to an aliquot of enzyme extract and added to PUR-L_{min} medium to a final concentration of 1 mM. Iodoacetate, a cysteine hydrolase inhibitor, was added to a final concentration of 10 μM. EDTA at a concentration of 5 mM was used as a metallohydrolase inhibitor. Extract that was heat treated at 98°C for 20 min served as a negative control. Samples were incubated in a rotary incubator at 30°C, and clearance was observed macroscopically after 2 h.

Based upon initial observations that a serine hydrolase was implicated in PUR degradation, activity-based probes were used to test for a serine hydrolase protein in crude cell-free filtrates. The probe molecules contain a fluorophosphonate moiety that reacts specifically with the active-site serines of enzymes in the serine hydrolase family and a tetramethylrhodamine (TMR) tag (20). Reactions of the crude protein extract with the probe were carried out as previously described (20). The resulting SDS-polyacrylamide gel was visualized using a Fujifilm FLA-5100 fluorescent bed scanner with excitation at 532 nm and a 570-nm emission filter. The same gel was silver stained to visualize all proteins in the samples.

Crude protein from cell-free filtrate of *Pestalotiopsis microspora* E2712A grown in 1-liter cultures of PUR-L_{min} minimal medium and PDB rich medium were concentrated and purified to approximately 90% purity by gel filtration chromatography (data not shown) using a Superdex 200 column (GE Healthcare) in buffer comprised of 10 mM MES (morpholineethanesulfonic acid) (pH 5.5) and 50 mM NaCl. The ability of the purified protein to degrade PUR was assayed as described above.

RESULTS

Initial PUR clearance screen. Impranil DLN, a polyester polyurethane (PUR), is an opaque milky suspension that becomes transparent upon degradation. Organisms capable of degrading this polymer display a zone of clearance around the growing culture (5). A collection of 59 fungal endophytic organisms isolated from plant samples in the Ecuadorian Amazon were screened for their ability to grow on and degrade polyester polyurethane using the PUR halo assay as the initial screen. Of the organisms screened, 18 organisms produced a halo of clearance such as that shown in Fig. 1. Two other organisms, identified by ITS sequencing as *Guignardia mangiferae* (E2702C) and *Zopfiella karachiensis* (E2719A), could grow on but not degrade PUR-A medium. These were used as negative controls in the subsequent studies. The host plants, isolation media, and identities of the 18 active fungi and two inactive control fungi are listed in Table 1.

Several organisms of the *Pestalotiopsis* genus were represented among the active organisms in the first PUR-A clearance screen. Based upon initial activity observed from this genus, all of the *Pestalotiopsis* isolates in the collection were screened for activity. Some organisms within the genus exhibited high activity (E2712A, E3317B, and E2711A), some exhibited moderate activity (E3314A and E2708A), and others exhibited no detectable activity (E4112A). This variable expression was observed under identical growth conditions for each organism. While it is notable that some *Pestalotiopsis microspora* strains demonstrated some of the highest levels of activity, the level was quite variable among the various isolates.

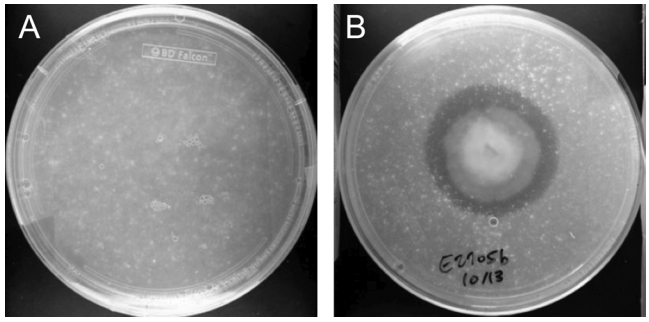

FIG. 1. Example of PUR-A plates initially used to screen for polyurethane-degrading activity after 2 weeks of fungal growth. (A) Negative control. (B) *Pleosporales* sp. strain E2705B.

The active organisms from the plate clearance test were assayed in two additional PUR clearance assays, one using a solid medium and the second using a liquid medium. In the solid PUR-A clearance assay, fungal plugs were used to inoculate test tubes of PUR-A medium and vertical clearance was measured after 2 weeks of growth (Fig. 2). All of the 18 active fungi visibly cleared the PUR-A medium. Sixteen of these fungi demonstrated activity that was at least twice that of the organism *Pseudomonas chlororaphis*, a positive control for PUR degradation (13). The top clearing organism in this solid medium assay was identified by ITS sequencing as a *Lasiodiplodia* sp. (strain E2611A). Four of the six most active organisms belonged to the *Pestalotiopsis* genus, with high relatedness at the species level to *Pestalotiopsis microspora*. These top six organisms all cleared PUR more efficiently than the positive-control fungus *Aspergillus niger*. The two negative-control organisms, *Guignardia mangiferae* (E2702C) and *Zopfiella karachiensis* (E2719A), demonstrated no visible clearance of the Impranil substrate.

The relative rate of PUR clearance was also measured in a liquid culture assay. Optical absorbance at 600 nm, due to the scattering of the suspended polymer, was measured as an indication of the extent of clearance by the fungus. The liquid cultures were cleared to the point of visual transparency at the surface of the liquid culture, which extended down the length of the tube as time progressed (Fig. 3A). The relative order of the liquid clearance assay was different from that observed for the solid clearance assay (Fig. 2A). A *Lasiodiplodia* sp. (strain E2611A) was the most active organism in the liquid medium clearance screen, along with a *Pleosporales* sp. (strain E2812A) and a *Bionectria* sp. (strain E2910B), followed by *Pestalotiopsis microspora* (E2712A) and *Pestalotiopsis microspora* (E3317B). These organisms all cleared as well or better than *Aspergillus niger*, a positive fungal control, after 2 weeks of growth. Almost all of the active organisms tested showed more activity than the positive bacterial control *Pseudomonas chlororaphis*. The two negative-control organisms did not produce detectable clearance of the PUR-L liquid medium.

TABLE 1. Endophytes studied and the host plant species from which the endophytes were isolated

Endophyte[a]	Highest-homology organism (maximum % identity)[b]	Host plant	Isolation medium[c]
E2524A	*Alternaria* sp. (100)	Annonaceae, *Annona muricata*	PDA
E2914A	*Alternaria dauci* (100)	Malvaceae, *Malva alcea*	1:10 PDA
E2910B	*Bionectria* sp. (99)	Gesneriaceae, *Drymonia semicordata*	1:10 PDA
E2705G	*Plectosphaerella* sp. (94)	Piperaceae, *Piper arboretum*	1:10 PDA
E3432O	*Edenia gomezpompae* (99)	Fabaceae, *Erythrina smithiana*	1:10 GAM
E2702C	*Guignardia mangiferae* (99)	Melastomataceae, *Axinaea sodiroi*	1:10 PDA
E2611A	*Lasiodiplodia* sp. (100)	Moraceae, *Naucleopsis oblongifolia*	WA
E2711A	*Pestalotiopsis microspora* (100)	Monimiaceae, *Siparuna aspera*	1:10 PDA
E2712A	*Pestalotiopsis microspora* (100)	Myrtaceae, *Psidium guajava*	1:10 PDA
E2708A	*Pestalotiopsis microspora* (100)	Mimosaeae, *Calliandra angustifolia*	1:10 PDA
E2911H	*Pestalotiopsis microspora* (100)	Commelinaceae, *Dicrorisandra ulei*	PDA
E3317B	*Pestalotiopsis microspora* (100)	Annonaceae, *Annona muricata*	WA
E3412F	*Pestalotiopsis microspora* (99)	Fabaceae, *Lonchocarpus glabrescens*	1:10 PDA
E3314A	*Pestalotiopsis* sp. (100)	Sterculiaceae, *Guazuma ulmifolia*	1:10 PDA
E2520A	*Pestalotiopsis microspora* (99)	Myrtaceae, *Psidium acutangulum*	PDA
E3432K	*Phaeosphaeria* sp. (95)	Fabaceae, *Erythrina smithiana*	PDA
E2104E	*Nectria* sp. (97)	Onagraceae, *Fuschia hybrida*	1:10 PDA
E2705B	*Pleosporales* sp. (99)	Piperaceae, *Piper arboretum*	1:10 GAM
E2812A	*Pleosporales* sp. (99)	Sterculiaceae, *Theobroma kakau*	WA
E2719A	*Zopfiella karachiensis* (99)	Myrtaceae, *Psidium guajava*	WA

[a] Each distinct fungal isolate was given a unique identification number.
[b] DNA sequence identity for each endophyte was determined by ITS-5.8s rDNA sequencing and comparison to sequences in the GenBank database.
[c] PDA, potato dextrose agar; 1:10 PDA, 1:10 dilution of potato dextrose medium in water agar; 1:10 GAM, 1:10 dilution of glycerol arginine medium in water agar; WA, water agar.

Sole carbon source assay. The five most active organisms were tested for their ability to degrade PUR in liquid culture using PUR as the sole carbon source (PUR-L_{min}). For these studies we used Impranil DLN, which is equivalent to the Impranil DLF (an anionic aliphatic PUR dispersion in water) used for the previous studies but does not contain *N*-methyl pyrrolidone. If an organism grows in Impranil DLN, it can use PUR as the sole carbon source for metabolism and growth. All fungal inocula were washed with minimal medium to remove residual carbon and enzymes from the stock culture. The top five organisms from the two screen assays were tested for this activity, and all five were capable of PUR degradation activity under this minimal medium condition. Each fungus showed significant mycelial growth as monitored by visual inspection. For *Pestalotiopsis microspora* E2712A, cultures grown with PUR showed increased growth of 0.110 g ± 0.031 g of fungal

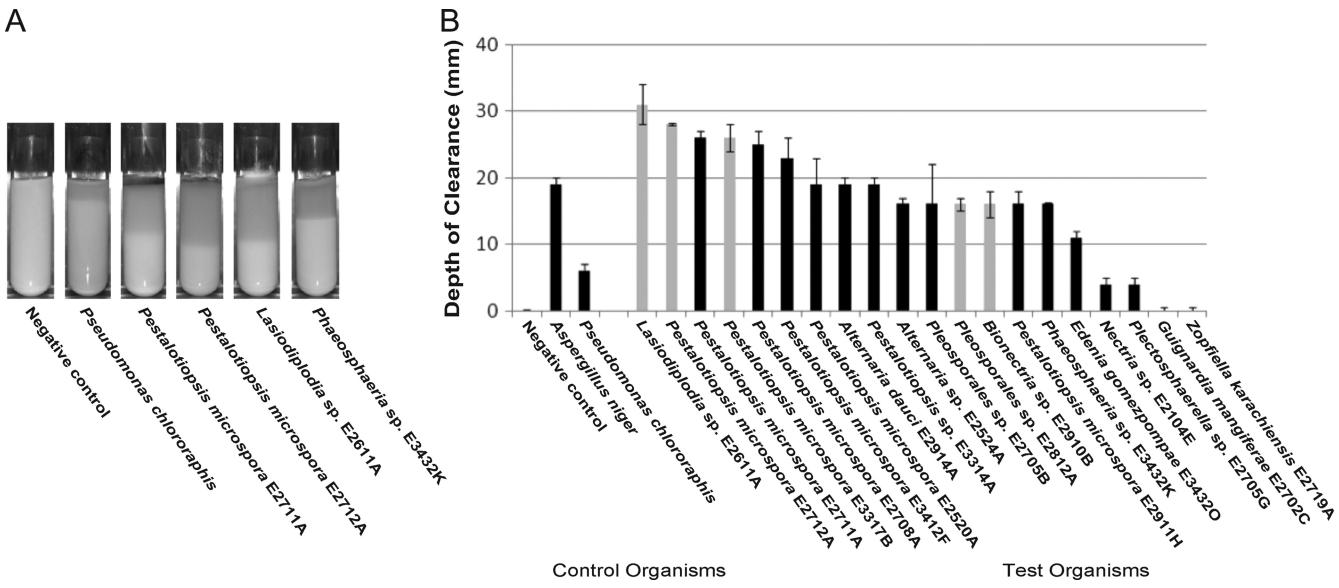

FIG. 2. (A) Examples of PUR-A solid agar cultures at 2 weeks after inoculation, including *Pseudomonas chlororaphis* as a positive control. (B) Fungal cultures were grown in triplicate on solid PUR-A medium for 2 weeks. Depth-of-clearance measurements were made after 2 weeks of growth. The positive-control test organisms, *Pseudomonas chlororaphis* and *Aspergillus niger*, are represented at the left. The five highlighted organisms (*Lasiodiplodia* sp. strain E2611A, *Pestalotiopsis microspora* E2712A, *Pestalotiopsis microspora* E3317B, *Pleosporales* sp. strain E2812A, and *Bionectria* sp. strain E2910B) were selected for further screening. Error bars represent the standard deviation for each data set.

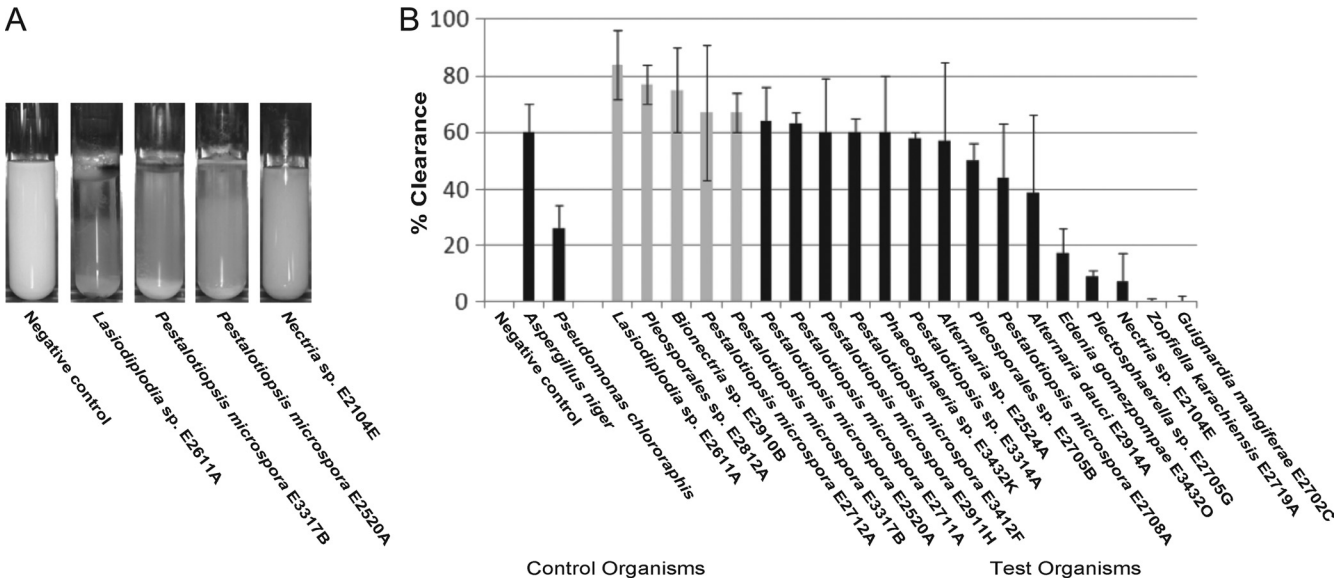

FIG. 3. (A) Examples of PUR-L liquid cultures at 2 weeks after inoculation. (B) Fungal cultures were grown in triplicate in liquid PUR-L medium containing Impranil DLF for 2 weeks. Percent clearance was determined by a decrease in light scattering at 600 nm as measured by UV-visible spectrophotometry. Percent clearance measurements were taken after 2 weeks of growth. All data were normalized to the negative control. The positive-control test organisms, *Pseudomonas chlororaphis* and *Aspergillus niger*, are represented at the left. The 5 highlighted organisms (*Lasiodiplodia* sp. strain E2611A, *Pestalotiopsis microspora* E2712A, *Pestalotiopsis microspora* E3317B, *Pleosporales* sp. strain E2812A, and *Bionectria* sp. strain E2910B) were selected for further screening. Error bars represent the standard deviation for each data set.

material over that of a control culture grown under identical conditions with no PUR added. In addition, the positive-control fungus *Aspergillus niger* was able to slowly degrade PUR as a sole carbon source, as previously reported (7).

In order to compare the rates at which PUR was degraded under this condition, the top five organisms in the solid and liquid screen assays were grown in duplicate and tracked for 16 days (Fig. 4A). Two organisms, *Pestalotiopsis microspora* E2712A and E3317B, demonstrated the highest rate of PUR clearance. The approximate half time for clearance for these two organisms was 5 days. The cultures were visually transparent by the end of the 16-day period, consistent with complete degradation of the Impranil DLN (Fig. 4B). The only nontranslucent material remaining in the flask consisted of fungal growth. In comparison to other standards, the half time for clearance by the control organism *Aspergillus niger* was 15 days, and the bacterium *Pseudomonas chlororaphis* did not reach a state of half clearance within the 16-day time course. Experiments performed using Impranil DLF as the substrate showed that the presence of *N*-methyl pyrrolidone did not affect the growth rate for the organisms tested. This established that PUR alone is sufficient for fungal growth for these organisms. As a result, Impranil DLN was used for subsequent studies to characterize anaerobic growth and the nature of the degradation reaction.

Anaerobic degradation of PUR. Some of the organisms tested during the liquid culture assays, including *Pestalotiopsis microspora* E2712A and *Pestalotiopsis microspora* E3317B, were observed growing from the bottom of the culture tube rather than from the top. This suggested that these fungi are capable of anaerobic degradation of the polyurethane. To test this possibility, the assay using PUR-L_{min} medium with Impranil DLN substrate was repeated under anaerobic conditions. The top five organisms from the previous assay were inoculated in triplicate and grown for 1 and 2 weeks in a BD GasPak anaerobic chamber. A corresponding set of cultures was grown outside the chamber, and the extents of clearance under the two conditions were compared (Fig. 5). The *Pestalotiopsis microspora* E2712A fungal isolate used in this assay showed equivalent rates of degradation under both anaerobic and aerobic conditions (Fig. 5B). *Lasiodiplodia* sp. strain E2611A and *Pleosporales* sp. strain E2812A showed diminished rates of clearance, while *Bionectria* sp. strain E2910B and the control *Aspergillus niger* showed negligible substrate degradation under anaerobic conditions. All organisms showed mycelial growth under anaerobic conditions as evinced by visual inspection.

IR analysis of PUR degradation. The mechanism of PUR degradation was initially investigated by infrared spectroscopy (16, 24). PUR samples of Impranil DLN display a large absorption peak at 1,735 cm^{-1} corresponding to the C(O)-O ester linkage in the polyurethane polymer (Fig. 6A). Fungal material was added to the PUR substrate and spectra collected throughout the course of the degradation experiment. A progressive reduction in the relative intensity of the peak at 1,735 cm^{-1} was observed and was accompanied by more subtle changes at other wave numbers. By the time the culture had became visually transparent, there was a complete loss of the absorbance peak at 1,735 cm^{-1} (Fig. 6B). Loss of this peak is consistent with hydrolysis of the ester bond in the urethane linkage.

Enzymatic characterization of putative polyurethanase. Clearance zones in both the solid and liquid media occurred at a significant distance from the site of fungal growth, which is suggestive of an enzymatic activity that was extracellularly excreted. To explicitly test this possibility, clearance of the Impranil DLN substrate was tested using a cell-free filtrate of

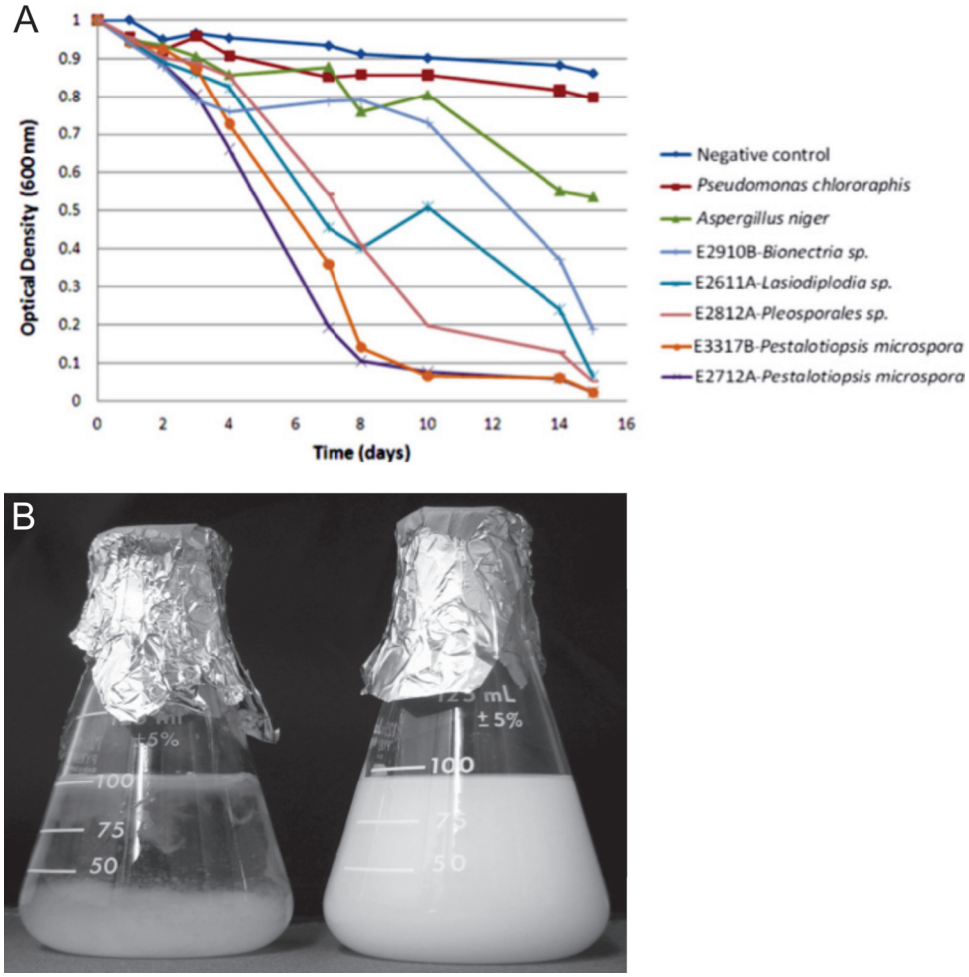

FIG. 4. (A) Degradation of PUR as a sole carbon source by the five most active organisms was monitored over a 16-day time course. Cultures containing PUR-L_{min} medium with Impranil DLN as the sole carbon source were inoculated with washed fungal inoculums. The assay was performed in duplicate, and the values shown represent the averages for the two cultures at each time point. *Aspergillus niger* (green) and *Pseudomonas chlororaphis* (red) served as the positive controls. (B) *Pestalotiopsis microspora* E2712A (left) degrading PUR Impranil DLN as a sole carbon source after a 16-day time course. The remaining material in the flask is the result of fungal growth.

Pestalotiopsis microspora E2712A. Filtrate was prepared from a fungal culture grown in PUR-L_{min} medium for 10 days. The crude filtrate from the PUR culture was able to completely clear the Impranil DLN in 1 h or less (data not shown). The active protein appears to be induced under PUR-L_{min} growth conditions, as cell-free filtrate prepared from equivalent PDB liquid cultures did not exhibit any clearance activity (data not shown). This suggests that the activity is excreted, diffusible, and induced by cellular exposure to the polyurethane substrate.

The filtrate was next tested for the inhibition of the enzymatic activity responsible for the degradation activity using heat and small-molecule treatments. Heating the filtrates at 60°C and 80°C for 15 min had no detectable effect on substrate degradation activity. Treatment at 98°C for 20 min was required to inactivate the enzyme. The demonstration of heat-sensitive PUR biodegradation activity from a 0.22-μm-filtered extracellular extract indicates that a soluble and secreted extracellular protein is responsible for the enzymatic activity.

The enzyme responsible for PUR biodegradation was further characterized through the use of mechanism-based inhibitors. Phenylmethylsulfonyl fluoride (PMSF) specifically inactivates the active-site serine in serine hydrolases by covalent modification. Iodoacetate inactivates the active-site cysteine in cysteine hydrolases by a similar mechanism. EDTA sequesters metals and specifically inhibits the activity of metallohydrolases. PMSF, iodoacetate, and EDTA were used at inhibitory concentrations in cell-free filtrate of *Pestalotiopsis microspora* E2712A to monitor changes in the resulting activity of PUR clearance. Heat-treated extract was used as a negative control. Addition of PMSF, a serine hydrolase inhibitor, resulted in inactivation of the biodegradation activity. Addition of iodoacetate and EDTA had no effect on the rate or extent of activity (data not shown).

The reaction of the crude cell-free filtrate of *Pestalotiopsis microspora* E2712A with serine hydrolase inhibitor probes efficiently labeled one protein in the fraction grown in PUR-L_{min} medium. Specific protein labeling was absent in the cell-free filtrate from PDB liquid culture, the condition in which no PUR degradation activity was observed (Fig. 7B). These re-

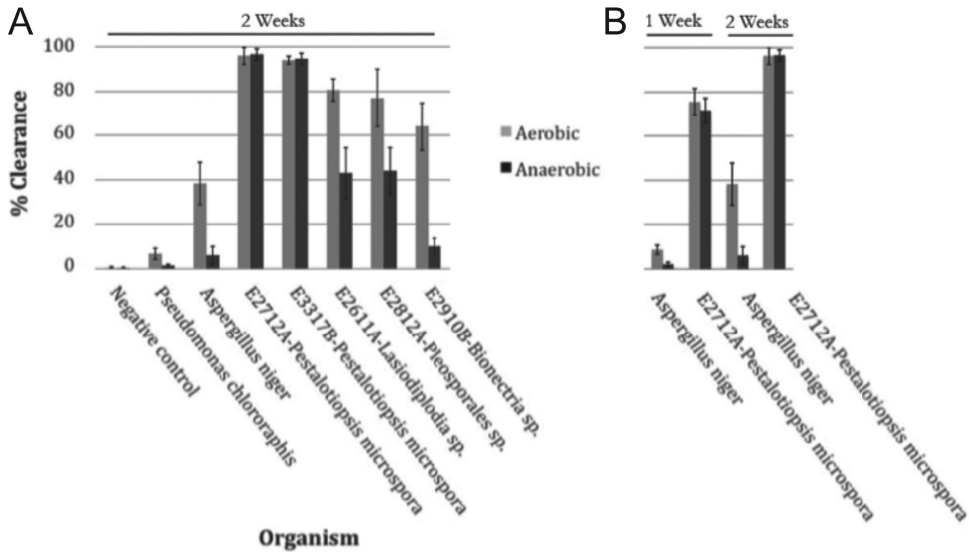

FIG. 5. (A) PUR degradation measurements of fungal cultures grown under aerobic and anaerobic conditions. Triplicate sets of fungal cultures were grown in PUR-L_{min} medium containing Impranil DLN as the sole carbon medium under aerobic or anaerobic conditions. At the end of 2 weeks of growth, samples were removed from the sealed chamber and the percent clearance of the PUR in solution for each set of cultures was measured by decreased light scattering at a wavelength of 600 nm using UV-visible spectrophotometry The results for the controls *Pseudomonas chlororaphis* and *Aspergillus niger* are shown at the left. Two strains of *Pestalotiopsis microspora* (E2712A and E3317B) maintained polyurethanase activity when grown under anaerobic conditions, while *Pseudomonas chlororaphis*, *Aspergillus niger*, *Lasiodiplodia* sp. strain E2611A, *Pleosporales* sp. strain E2812A, and *Bionectria* sp. strain E2910B all showed significant reductions in the magnitude of PUR degradation activity. Error bars represent the standard deviation for each data set. (B) The rates of degradation were compared by measurement in triplicate of cultures of *Aspergillus niger* and *Pestalotiopsis microspora* E2712A after 1 week and 2 weeks of growth. *Pestalotiopsis microspora* E2712A shows equivalent rates of degradation activity under aerobic and anaerobic conditions. Error bars represent the standard deviation for each data set.

sults suggest that an approximately 21-kDa serine hydrolase-like enzyme is responsible for PUR degradation. Furthermore, growth in PUR-L_{min} induced secretion of this enzyme (Fig. 7A). The 21-kDa protein was purified from the crude filtrate to approximately 90% purity, and it retained the ability to degrade PUR (data not shown).

DISCUSSION

Endophytes isolated from Ecuadorian Amazonian plant samples were screened for their ability to degrade polyester polyurethane (PUR). Almost half of the organisms displayed some activity in the initial plate clearance assay. Eighteen active and two inactive endophytes were further characterized. Eight of the most active organisms belonged to the *Pestalotiopsis* genus. The current literature reports some fungi with the ability to degrade PUR (4–6, 8), although these studies have focused primarily on organisms isolated from soil samples. This is the first study that demonstrates PUR degradation by endophytic fungi. The broad distribution of activity suggests that endophytes might be a promising source of biodiversity in which to test for activities important for bioremediation.

All active fungi were identified as *Ascomycota*, with a cluster of organisms belonging to the class *Dothidiomycetes* and the order *Pleosporales*. A large portion of the active fungi belonged to the class *Sordariomycetes*, including those identified as *Pestalotiopsis* sp. strains.

Although robust activity was observed among several *Pestalotiopsis* sp. isolates, not all of them demonstrated equivalent levels of activity. Of the nine isolates tested, three were highly active (E2712A, E3317B, and E2711A), five were moderately active (E3314A, E2520A, E2911H, E3412F, and E2708A), and one was inactive (E4112A). This variability in activity among distinct *Pestalotiopsis microspora* isolates suggests that there are genetic differences among the organisms.

The genus *Pestalotiopsis* is grouped in the *Xylariales* order and comprises several known plant pathogens. The fungus is not host specific and causes rot and disease in a wide variety of plant species (29), although these isolates were all endophytic and the plants showed no pathogenic symptoms. *Pestalotiopsis microspora* isolates have previously been shown to have a propensity for horizontal gene transfer. In one notable case, a *Pestalotiopsis microspora* strain isolated as a fungal endophyte from the taxol-producing plant *Taxus wallachiana* had acquired the ability to synthesize taxol (27). Such a propensity for horizontal gene transfer may have contributed to the ability of a subset of these isolates to degrade polyester polyurethane as a sole carbon substrate, or it may reflect a significant level of phenotypic diversity among the genus.

There are no previous reports of members of the genus *Pestalotiopsis* having biodegradation activity. We found that two isolates of *Pestalotiopsis microspora* (E2712A and E3317B) were able to degrade PUR when grown anaerobically with Impranil DLN serving as the sole carbon source. For these two organisms, the level of activity was the same when grown under either aerobic or anaerobic conditions. This is in contrast to the control fungus *Aspergillus niger*, which showed substantially less activity when grown anaerobically. This observation may have practical significance in that fungal growth on and me-

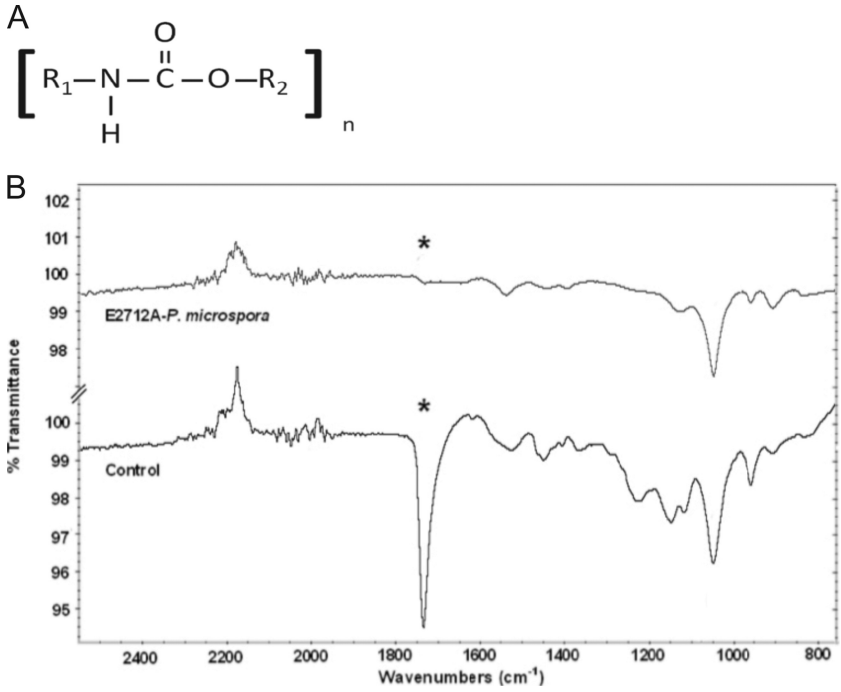

FIG. 6. (A) General chemical structure of the polyurethane molecule. R_1 and R_2 connect subsequent urethane monomers within the polymeric molecule. (B) Infrared spectra of PUR liquid medium containing Impranil DLN taken after 6 days of incubation with the fungus *Pestalotiopsis microspora*. The sample spectrum (top) and control spectrum (bottom) are shown together with a common scale. The strong peak denoted by an asterisk at 1,735 cm^{-1} in the control corresponds to the ester C=O stretch in the ethyl carbamate of the urethane motif. This peak disappears after degradation of the ester linkage by the polyurethanase enzyme.

tabolism of PUR by *Pestalotiopsis microspora* could be used in anaerobic fermentation systems.

The enzyme produced by *Pestalotiopsis microspora* that is responsible for PUR degradation appears to be a member of the serine hydrolase family. Furthermore, activity extended throughout the medium at a distance well removed from the areas of fungal growth. This suggests that the enzyme responsible for degradation is extracellular, secreted, and diffusible. In comparison to an inactive cell-free filtrate from a fungal culture grown in rich medium, we found that the polyurethanase is inducible when *Pestalotiopsis microspora* E2712A is grown in minimal PUR-L_{min} medium containing a suspension of Impranil DLN. By using activity-based probes, the active enzyme was identified as a serine hydrolase with an approximate molecular mass of 21 kDa. The protein was shown to be able to degrade PUR after subsequent purification, showing that activity is independent of other components of the culture filtrate.

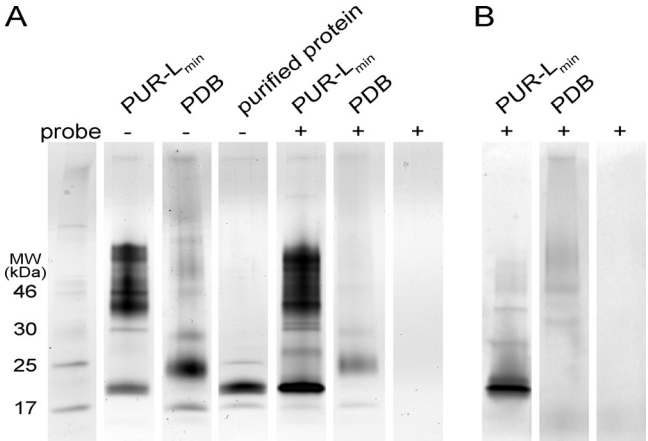

FIG. 7. Probing for serine hydrolases in crude protein from cell-free filtrate. SDS-PAGE analysis of proteins from a crude cell-free filtrate of *Pestalotiopsis microspora* E2712A grown in PUR-L_{min} medium and PDB rich medium is shown. Cell-free filtrates were incubated with serine hydrolase activity-based probes prior to loading. (A) Protein bands were visualized by silver staining. (B) Labeled protein bands were visualized using a fluorescence scanner. This analysis shows that an approximately 21-kDa protein expressed exclusively under the induced PUR-L_{min} growth condition is efficiently labeled by the serine hydrolase activity-based probe.

Polyurethanases have previously been isolated and characterized from protein extracts of several organisms, including the bacteria *Pseudomonas chlororaphis* (24) and *Comamonas acidovorans* (1, 2), as well as the fungus *Candida rugosa* (8). The active enzymes have been classified (17) as esterases (5, 13, 16), lipases (24), and proteases and ureases (19), suggesting degradation of the PUR substrate by cleavage of the ester bond. The IR analysis and molecular inhibition of PUR degradation by *Pestalotiopsis microspora* suggest that ester hydrolysis by a serine hydrolase is responsible for PUR biodegradation.

This investigation established the robust polyurethane degradation activity under anaerobic conditions in which the synthetic polymer served as the only carbon source for the fungus. A cell extract of the active culture containing a critical serine

hydrolase is able to clear the polymer in under 1 h using the PUR concentrations reported here. This work establishes that endophytes are a useful source of biodiversity with potential application for bioremediation. The relative ease with which organisms can be isolated and screened makes this a highly accessible and environmentally relevant project for engaging undergraduate students in scientific research. It is possible that activities against other, more recalcitrant polymers could be discovered using this abundant source of biodiversity.

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