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Bar-Coded Enterobacteria: An Undergraduate Microbial Ecology Laboratory Module

Thao Le, James Trexel, Alexandra Brugler, Kristina Tiebel, Yukari Maezato, Brian Robertson, Paul Blum*

School of Biological Sciences, University of Nebraska, Lincoln NE, USA
*Corresponding author: pblum1@unl.edu

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Abstract Microbial community ecology is an area of rapid growth centered within the larger discipline of microbiology. Newly developed research methods using molecular strategies have transformed this area into an accessible research topic. Despite such advances, transmission of this topic into pedagogical form has lagged behind. To improve this situation, an undergraduate research team created an artificial microbial community for class room use. They used color-coded enterobacteria taxa transformed with broad-host range plasmids that encoded green fluorescent protein color variants. Using this instructional tool, a class room teaching module was developed about microbial fitness. Over a multi-semester period, the module was introduced into a conventional microbiology curriculum and refined. The learning outcomes for this module include; understanding community composition, that the members of a community can respond in different ways to external events and, that these responses can be used to measure fitness. Learning outcomes were measured through pre and post testing and indicated a gain in understanding about microbial communities.

Keywords*: Microbial Ecology, Laboratory Exercise, Enterobacterial, Bar-coding, GFP*

1. Introduction

Microbes live in communities not pure cultures. Environmental samples probed with nucleic acid “stains” indicate that only 1% or less of resident cells can be cultured [1,4,8]. This means that culture-based methods for evaluating community structure are biased towards organisms that can be readily grown. Consequently, the use of pure cultures and culture-based methods dominate national microbiology laboratory curricula.

Currently, students enrolled in general microbiology laboratory courses are left with little information about microbial community structure or function. However, culture-independent methods can be used to identify the unculturable majority in a class room setting. Woese recognized that small subunit ribosomal RNA (16SrRNA) sequence comparisons could be used to conduct microbial systematics [9], and thus provided the impetus for Pace and coworkers to apply this technology to environmental sample composition [2,7]. The basic approach continues to rest on PCR amplification of 16SrRNA genes present within environmental DNA extracts. The resulting PCR products are cloned into plasmids and individually characterized by DNA sequencing or other methods permitting identification of microbial community composition.

Culture bias has profound implications for understanding how microbes impact the world. Despite the emerging importance of this topic, most college microbiology laboratory courses and published laboratory text books skip this topic. To remedy this omission, class room laboratory modules that focus on microbial communities and culture-independent techniques are needed.

The microbiology laboratory instructional program within the School of Biological Sciences at the University of Nebraska-Lincoln serves over 400 students per year at three instructional levels. Students come from a broad area including Nebraska, North and South Dakota, Iowa, Kansas and Missouri. Many of these students are representative of underserved rural populations as defined by US federal poverty guidelines. The School of Biological Sciences at the University of Nebraska-Lincoln is responsible for all introductory instruction in microbiology at this institution (24,000 undergraduates).

At UNL, the general microbiology laboratory course, BIOS314, attracts primarily upper division students and meets once per week. Students are self identified as pre-med, pre-pharmacy, biology majors and biochemistry majors. The first half of the semester is used to teach student techniques in microscopy, cultivation and physiology. There are two exercises on genetics, one on mutagenesis and the second on genetic exchange notably transformation and conjugation. During the last four weeks of class (15 week semester) students attempt to identify two "unknown" organisms using metabolic phenotypes. As this type of a course relies entirely on pure cultures and students do not use microbial communities, there is a specific need to integrate the use of ecology-based microbial concepts.

The goal of this project was engage undergraduate students in an effort to develop an ecology-based laboratory module and introduce the module into the established course. One important consideration was to minimize cost such as necessitated by molecular biology methods and time due to class room teaching constraints. The approach taken here was to use engineered bacteria expressing color variants of green fluorescent protein that when mixed and cultured on a solid medium produced artificial communities of colorized colonies. Changes in community structure could then be created by environmental challenge before plating and then equated to differences in biological fitness through the enumeration of colony types.

Intended audience. The laboratory exercise presented here is intended for students enrolled in a general microbiology laboratory course. This could include Microbiology/Biology majors, Allied health majors, Biotechnology majors and Science education majors.

Learning time. The laboratory exercise is divided between two successive laboratory class meetings. The first meeting includes time to perform the procedure, the second meeting includes time to analyze and interpret the results from the procedure. The time required to understand the concepts inherent to this exercise are met within a single three hour laboratory teaching period.

Prerequisite learning knowledge. Necessary laboratory skills need for this exercise include aseptic technique for use of solid and liquid microbial media and have an understanding of the structure and function of a prokaryotic cell.

Learning objectives. The primary learning objectives are to understand that the microbial world consists largely of communities of species, that species within communities interact, and that biological fitness of a species within a community is a reflection of both its genetics and its environment. At the completion of this exercise, students will have measured the relative fitness of specific microbial species. They will be able to state which species is more or less resistant to selected stresses and using this information draw conclusions about how this might influence the activity of the species within the environment. An assessment of this ability would be gained by responses to questions about the apparent fitness of species tested within the artificial microbial community.

2. Materials and Methods

2.1. Procedure

2.1.1 Construction of Fluorescent Enterobacterial taxa

Fluorescent proteins (FP) included; green FP (GFP), blue FP (BFP), enhanced green FP (eGFP), yellow FP (YFP), and cyan FP (CYP) all either natural or synthetic variants originally from Aequorea victoria along with monomeric red fluorescent protein (RFP) from Discosoma. FP genes were obtained from laboratory stocks and were cloned into a plasmid vector prior to introduction into selected bacterial species. Molecular biology methods were as described [3,5,6]. All reagents were obtained from common suppliers. Transformation studies using plasmid pUC19 and selection for ampicillin resistance (bla) demonstrated this ColEI-derived vector was able to replicate in diverse genera belonging to the Enterobacteriacaea. Maximum colony fluorescence was achieved by cloning fluorescent protein coding sequences in-frame with the 5' region of lacZ resident in both vectors and resulting in protein fusion to the seven amino acid (MTMITPS) N-terminus of beta-galactosidase. Fluorescent protein synthesis was therefore dependent on transcription driven by the lacZ promoter and translation initiating at the lacZ start codon. PCR primers encoding HinDIII (forward) and BamHI (reverse) restriction sites and complementary sequences to fluorescent protein genes were used to amplify and clone each color variant into the pUC19 multiple cloning site. PCR and recombinant DNA techniques were performed using standard methods. Ligation reactions were transformed into E. coli DH5alpha and ampicillin resistant colonies were screened by colony PCR followed by restriction analysis of purified plasmid DNA. Fluorescence yield was also evaluated in lacI+ E. coli strain BL21DE3. Both E. coli strains were obtained from laboratory stocks.

2.1.2. Artificial Microbial Communities

Enterobacterial transformants synthesizing single fluorescent proteins were combined into artificial communities prior to laboratory class meetings typically in the early morning using pure liquid culture samples. Propagation of pure and mixed cultures in a liquid complex medium consisting of tryptic soy broth (TSB) at 0.2% (w/v) required inclusion of ampicillin added after sterilization to retain selective pressure for plasmids at a concentration of 100 μg/ml. Ampicillin stocks were prepared at a concentration of 100 mg/ml of dimethylsulfoxide (DMSO) and were stored at -20℃ for up to 4 months. The simplest community involved one fluorescent taxa and one nonfluorescent taxa. A more complex community involved three fluorescent taxa including Salmonella enterica (Typhimurium); Escherichia coli, and Citrobacter freundii. Other mixtures of taxa were also produced and tested.

2.1.3. Fitness Assays

Relative species fitness was determine by challenging artificial communities with various environmental challenges including dilute bleach (sodium hypochlorite) and dilute hydrochloric acid. Chemical stocks were provided to students in prediluted form to minimize hazardous risk. Fitness was measured by plating serial dilutions of cultures on a solid medium containing 0.2% (w/v) TSB, 1.5% (w/v) agar and 100 μg/ml ampicillin added after autoclaving followed by incubation at 37℃ for 1 to 2 days.

2.1.4. Fluorescent Light Sources

Fluorescent proteins have specific excitation and emission light wavelength maxima. It is conventional to use broad wavelength hand-held ultra violet light sources

for excitation. Shorter wave lengths (254 nm – 300 nm) produce the most energetic light and the greatest excitation of fluorescent proteins. This produces the most fluorescent colonies. However such light sources are dangerous and direct eye exposure must be avoided. Ultraviolet emitting photodiodes offer less expensive excitation light sources that use longer UV wavelengths. For this reason, UV photodiodes also are more safe for use in the class room setting. These were obtained from various sources identified through the internet.

2.1.5. Items per Student or Group of Students

First meeting period items include: i) premixed samples of selected fluorescent enterobacterial taxa prepared in advance of the class meeting; ii) LB ampicillin containing petri plates, (4) each; iii) glass beaker with 70% (v/v) ethanol and spreader; iv) bunsen burner; v) pipetors (100 and 1000 ul); vi) sterile glass tubes, pipetor plastic tips, sterile LB medium to prepare serial dilutions of the artificial community; vii) stressor such as ethanol, soap, antimicrobial to be combined with artificial community. Second meeting period items include: i) hand held ultra violet light source (long wave length, > 300 nm); ii) marking pen to count colonies; iii) paper to score counts.

3. Results

3.1. Bar Color-coded Enterobacteria

Cell lines producing GFP variants were constructed using several species belonging to different genera within the Enterobacteriaceae. These included Escherichia coli (K12), Salmonella enterica (Typhimurium), Klebsiella pneumoniae and Citrobacter freundii. Transformation studies using both ColEI replicons (pUC19) and P15A (pACYC184) replicons encoding various antibiotic resistant determinants demonstrated both types of replicons functioned within diverse enterobacterial genera. Subsequent studies used the ColEI system encoding ampicillin resistance (bla) for fluorescent protein production.

An initial assessment of colony fluorescence found that GFP transformed cells yielded more light than other color variants. Despite expression using a vector with identical copy number, this could have been due to differences in relative fluorescent protein abundance arising from differences in DNA sequence between the plasmid encoded promoter (*lacZp*) and the start points of transcription for the fluorescent protein genes. Alternatively, different N-terminal fluorescent protein sequences could influence translation efficiency and or protein stability and thereby fluorescence yield. Assessment of these differences indicated that differences in the identity of the N-terminal sequence of the fluorescent protein were critical. Fusion of the N-terminus of LacZ (beta-galactosidase) to GFP and all GFP variants greatly improved fluorescence yield resulting brighter colonies (Figure 1). This type of protein fusion was used in all classroom constructs.

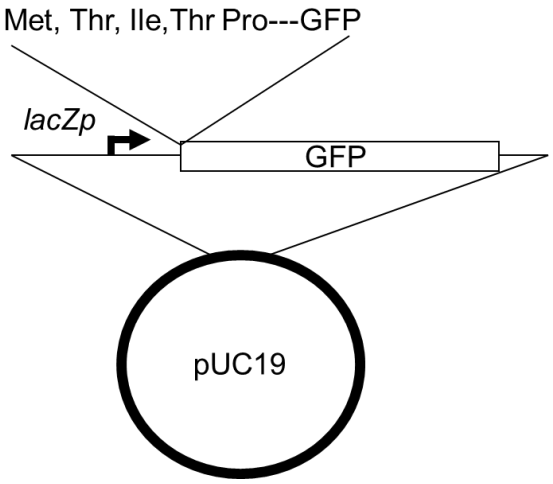

Figure 1. Map of pUC19 with fluorescent protein gene cloned in frame with *lacZ*

3.2.Artificial Community and SpeciesFitness

The goal of this project was to provide laboratory class room students with an artificial microbial community that could be used to study biological fitness in a microbial community setting within classroom time constraints. Artificial communities consisted of mixtures of separately cultivated fluorescent strains (Figure 2) combined at equal cell densities prior to classroom use. The simplest community involved one fluorescent strain and one nonfluorescent strain. The most complex community involved four fluorescent strains including Salmonella enterica (Typhimurium), Escherichia coli, Klebsiella pneumoniae, and Citrobacter freundii.

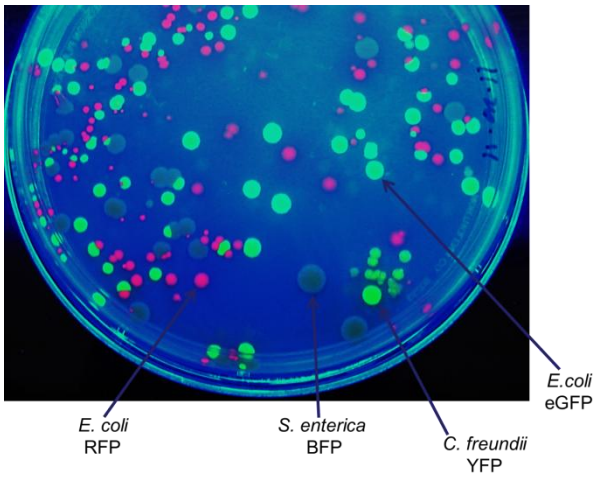

Figure 2. Artificial community sample. A mixture of four types of fluorescent enterobacterial colonies viewed under long wave fluorescent light

Biological fitness was assessed by treating the community with selected environmental challenges including oxidants and acid. Untreated and treated samples were then plated on a solid medium containing antibiotic and incubated to allow colony formation. Following incubation, plates were examined using

ultraviolet lights to excite the fluorescent proteins and produce fluorescing colonies. The relative fitness of individual species within the community setting was then scored by counting the numbers of colonies of each color and comparing them before and after the treatment regimen.

The results obtained by one laboratory class of twenty four students using a two species community are shown (Table 1). To produce these data, students worked in pairs to perform the procedures. They were provided with a premixed liquid culture artificial community. Students treated the cell suspensions with specific chemical for set times followed by plating and incubation. Community composition was recorded as total counts for each species and the percentage of the total comprised of S. enterica. The data indicate that in a community setting S. enterica is less fit than E. coli when challenged by hypochloric acid but has equivalent fitness when challenged by bleach. Data interpretation consisted of class discussion about the mechanisms underlying resistance to acid and bleach along with why these organisms might be different.

4. Discussion

Though microbes can manifest distinctive cell morphologies that can be viewed using a light microscope, diverse species often exhibit identical shapes. For example almost all 64 genera of the Enterobacteriaceae are rod shaped. PCR-based molecular methods could be used as an alternative to distinguish species identity but are both too expensive and too slow to fit laboratory classroom time constraints. Bar coding of selected enterobacterial species using fluorescent proteins provides a simple and rapid method for distinguishing species identity.

The fluorescent artificial community system has been used by students to evaluate relative species fitness within a community setting. Fitness was measured as culturability and the formation of colonies. Many types of variables have been tested that could impact fitness. These included chemical challenges (bleach, acid, base, salt), antibiotics, bacteriophage, starvation, stationary phase, and the introduction of non-enterobacterial species. Of greatest interest to students was the use of the system to explore and then discuss the concept of biological fitness along with mechanisms that might control and or predict this trait.

The system was introduced initially as a new one week module in a conventional upper level microbiology laboratory course with 24 students in one section that met twice a week. Subsequently, the system was modified for use in class that met only once a week but included five sections of 24 students each. Modifications included identifying appropriate doses of challenge chemicals to avoid excessive or insufficient amounts and adjusting artificial community cell densities to ensure appropriate serial dilution prior to plating. Since its inception over 800 students have used this system.

This project resulted from the leadership efforts of undergraduate researchers and as undergraduate teachers. Following a training period to provide instruction on molecular cloning, students created the appropriate expression vectors, transformed strains and developed the laboratory protocols. Learning-based teaming strategies were key and arose from integrating undergraduate efforts with graduate students and laboratory staff. Examples of outcomes resulting from teaming were best illustrated by ideas for new ways to use the class room module. These included measuring the impact of : i) hand sanitizers; ii) sugars versus artificial sweeteners; iii) virus (phage); and iv) grazers (rotifers).

The effectiveness of the instructional module on student learning was assessed through a comparison of pretest and posttest results using a cohort of 110 students. Preconceptions about microbial ecology typically consisted of a tendency to think about species as isolated entities devoid of the ability to interact and or impact neighbor species. This was particularly the case when the species were related. Misconceptions were that biodiversity of microbial species in natural samples is small, that the definition of a species derives from that used for eukaryotes and, that communities are the same as populations. Following exposure to the class room module, learning gains indicated by testing revealed a change in perception of species interactions. Students indicated they now viewed a microbial community as a complex and interactive entity where fitness was a driving evolutionary force. A frequent comment about the class room modules was student excitement about the use of mixed fluorescence because it provided an immediate sense of experimental outcome.

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Table 1. Relative Fitness Following Oxidant and Acid Challenge

				post bleach	post bleach	post bleach
	Control Salmonella	Control E.coli	% Salmonella	Salmonella	E.coli	% Salmonella
Section 1	25	21	0.54	0	14	0.00
"	15	16	0.48	2	10	0.17
"	21	20	0.51	15	11	0.58
"	20	15	0.57	8	10	0.44
Section 4	107	190	0.36	42	31	0.58
"	150	179	0.46	19	22	0.46

"	118	260	0.31	11	28	0.28
Section 2	8	10	0.44	1	5	0.17
Section 5	124	88	0.58	68	65	0.51
"	88	292	0.23	48	384	0.11
"	56	137	0.29	60	206	0.23
Section 6	60	187	0.24	49	25	0.66
	148	148	0.50	194	0	1.00
	157	117	0.57	37	20	0.65
Average %			0.43			0.42
				post HCL	**post HCL**	**post HCL**
	Control *Salmonella*	**Control *E.coli***	***% Salmonella***	***Salmonella***	***E.coli***	***% Salmonella***
Section 1	22	17	0.56	0	10	0.00
"	132	96	0.58	0	62	0.00
Section 4	150	150	0.50	3	97	0.03
"	130	160	0.45	20	42	0.32
"	140	190	0.42	10	30	0.25
Section 2	17	17	0.50	0	5	0.00
"	76	7	0.92	1	6	0.14
"	12	16	0.43	0	2	0.00
Section 5	82	142	0.37	77	123	0.39
"	92	123	0.43	12	180	0.06
"	292	46	0.86	0	135	0.00
Average %			0.54			0.11

Footnote: To determine the relative fitness of each bacteria in response to the environmental stress, efficiency of plating (EOP) before and after the stress treatment were determined. Change in EOP was used to compare and determine the relative fitness of each speci

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