Article

Programmed Lab Experiments for Biochemical Investigation of Quorum-Sensing Signal Molecules in Rhizospheric Soil Bacteria

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Abstract

Biochemistry courses in the Department of Molecular Biology at the National University of Río Cuarto, Argentina, are designed for undergraduate students in biology, microbiology, chemistry, agronomy, and veterinary medicine. Microbiology students typically have previous coursework in general, analytical, and organic chemistry. Programmed sequences of lab experiments allow these students to investigate biochemical problems whose solution is feasible within the context of their knowledge and experience. We previously designed and reported a programmed lab experiment that familiarizes microbiology students with techniques for detection and characterization of quorum-sensing (QS) and quorum-quenching (QQ) signal molecules. Here, we describe a sequence of experiments designed to expand the understanding and capabilities of biochemistry students using techniques for extraction and identification of QS and QQ signal molecules from peanut rhizospheric soil bacteria, including culturing and manipulation of bacteria under sterile conditions. The program provides students with an opportunity to perform useful assays, draw conclusions from their results, and discuss possible extensions of the study. © 2016 by The International Union of Biochemistry and Molecular Biology, 44:256–262, 2016.

Keywords: *biochemistry; quorum sensing; AHL signals; soil bacteria; biosensor*

Introduction

Bacterial quorum sensing (QS) is a genetic regulatory phenomenon whereby cells excrete or secrete a chemical signal into the surrounding environment. At sufficient concentration the signal alters the expression of specific genes [1, 2]. Many Gram-positive and Gram-negative bacteria use QS signal circuits to coordinate a diverse array of physiological behaviors, including symbiosis, competence, virulence, conjugation, antibiotic production, motility, and biofilm development [3, 4]. Most Gram-negative bacteria use LuxI/LuxR type QS, based on synthesis of diffusible signal molecules identified as *N*-acyl-homoserine lactone (AHLs), to coordinate physiological behaviors [5]. QS in Gram-positive bacteria is mediated primarily by processed oligopeptides [6]; however, species of the genus *Exiguobacterium* were identified recently as AHL producers [7].

AHL-mediated QS function requires three major components: an AHL signal molecule, an AHL synthase protein to make the AHL signal (LuxI), and a regulatory protein that responds to the surrounding concentration of AHL (LuxR) (Fig. 1). At low cell densities, the *luxI* gene is constitutively expressed at a low, basal level, and signal molecules are produced by a *luxI*-encoded AHL synthase at low concentration and accumulate in the surrounding environment [8]. Thus, the AHL signal is distributed in and around the cell. At high AHL concentration, the sigma-receptor protein (LuxR) forms a complex with AHL and becomes activated. The activated complex in turn forms dimers or multimers with other activated AHL-LuxR complexes, which function as transcriptional regulators controlling expression of QS-regulated target genes. Each individual QS-regulated gene has a specific quorum size at which it becomes activated; there is no single population density at which all the genes are activated [9].

Screening studies of Gram-negative bacteria have revealed that AHLs are produced by numerous genera found in diverse environments [10]. These AHL-producing genera include

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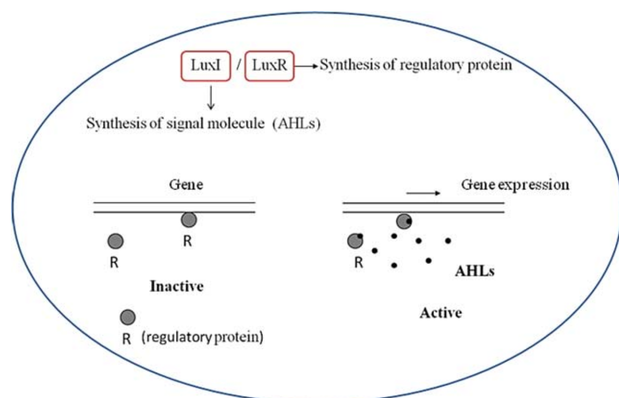

FIG 1 *Schematic representation of LuxI/LuxR QS circuits in Gram-negative bacteria. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

Agrobacterium, *Sinorhizobium*, *Serratia*, *Vibrio*, *Chromobacterium*, *Bradyrhizobium*, *Pseudomonas,* and *Burkholderia*. Several methods have been developed for detection of AHL-producing bacteria. A commonly used method involving biosensor strains is sensitive and allows real-time detection of AHLs [11]. The biosensor strains contain QS regulatory promoters fused to *lux* operon or *lacZ*, and lack AHL synthase enzyme. The strains cannot produce AHLs, but promoter activity may be induced by exogenous QS signals, in which case the receptor is activated, binds to its cognate *luxI* promoter, and initiates expression of specific genes [12]. Expression of the genes results in phenotypes such as β-galactosidase production by *Agrobacterium tumefaciens* NTL4 (pZLR4) [13], violacein pigmentation by *Chromobacterium violaceum* CV026 [14], green fluorescent protein production by *Vibrio fischeri* [15], and bioluminescence by *Pseudomonas putida* 117 [16]. Each biosensor strain detects a narrow range of AHLs; therefore, testing the wide range of AHLs produced by a single bacterium requires the use of multiple biosensor strains.

The most commonly used biosensor strain, *A. tumefaciens* NTL4 (pZLR4) (*TraG*::*lacZ*), contains a *lacZ* fusion in the *traI* gene of pTiC58, resulting in production of blue color from hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside by β-galactosidase activity, in response to a broad range of AHLs, *e.g.*, 3-oxo-AHLs with side acyl chains from C4 to C12, 3-unsubstituted AHLs with side acyl chains from C6 to C12, and 3-hydroxy-AHLs with side acyl chains from C8 to C10 [17, 18]. A second class of biosensor strains used to identify short-chain AHLs with acyl chains from C4 to C6 is represented by *C. violaceum* CV026. This is a mini-Tn5 mutant of ATCC31532 containing a LuxR homologue CviR that regulates production of the purple pigment violacein when induced by short-chain exogenous AHLs [19]. Several other classes of biosensors have also been developed [20–23].

The programmed lab experiments described here are designed to introduce undergraduate students to biological techniques for detection of bacterial signal molecules, and to familiarize them with some simple but useful laboratory tools. With a similar objective, we previously described a lab experiment designed to familiarize microbiology students with techniques for isolation and detection of ribosomal RNA [24], and a set of experiments whereby students elucidate the properties of the *expR* gene responsible for exopolysaccharide biosynthesis in *Sinorhizobium meliloti* [25]. The new set of lab experiments presented here allows students in our biochemistry courses to detect and characterize QS signal molecules in microorganisms isolated from rhizospheric soil of cultivated peanut (*Arachis hypogaea* L.), an important commercial crop in Argentina.

TABLE 1 *Biosensor strains used*

Strain	*Reference*
A. tumefaciens NTL4	Ref. [13]
A. tumefaciens NTL4 (ptiΔ*acc*R)	Ref. [13]
A. tumefaciens NTL4 (pZLR4)	Ref. [13]
C. violaceum 026	Ref. [14]

Materials and Methods

Bacterial Strains

Two bacterial strains from peanut rhizospheric soil, identified as *Bradyrhizobium* P8A and *Bradyrhizobium* P5 [26], are grown on nutritive agar (AN) medium at 28°C with rotary shaking (Model SI4-2 Shel Lab, 12 mm orbit, Sheldon Manufacturing, Cornelius, OR, USA) at 150 rpm. Biosensor strains (Table 1) *C. violaceum* CV026 and *A. tumefaciens* NTL4, and their transconjugants *A. tumefaciens* NTL4 (pZLR4) and *A. tumefaciens* NTL4 (pTiC58Δ*acc*R), are grown on LB medium and agrobacterium medium (AB),

TABLE 2 *Organization of laboratory classes*

Day	*Activity*
1	Theoretical explanation. Growth of bacterial strains. Preparation of plates.
2	Extraction of culture supernatants. Preparation of plates. TLC analysis.
3	Observation of plates. General discussion.

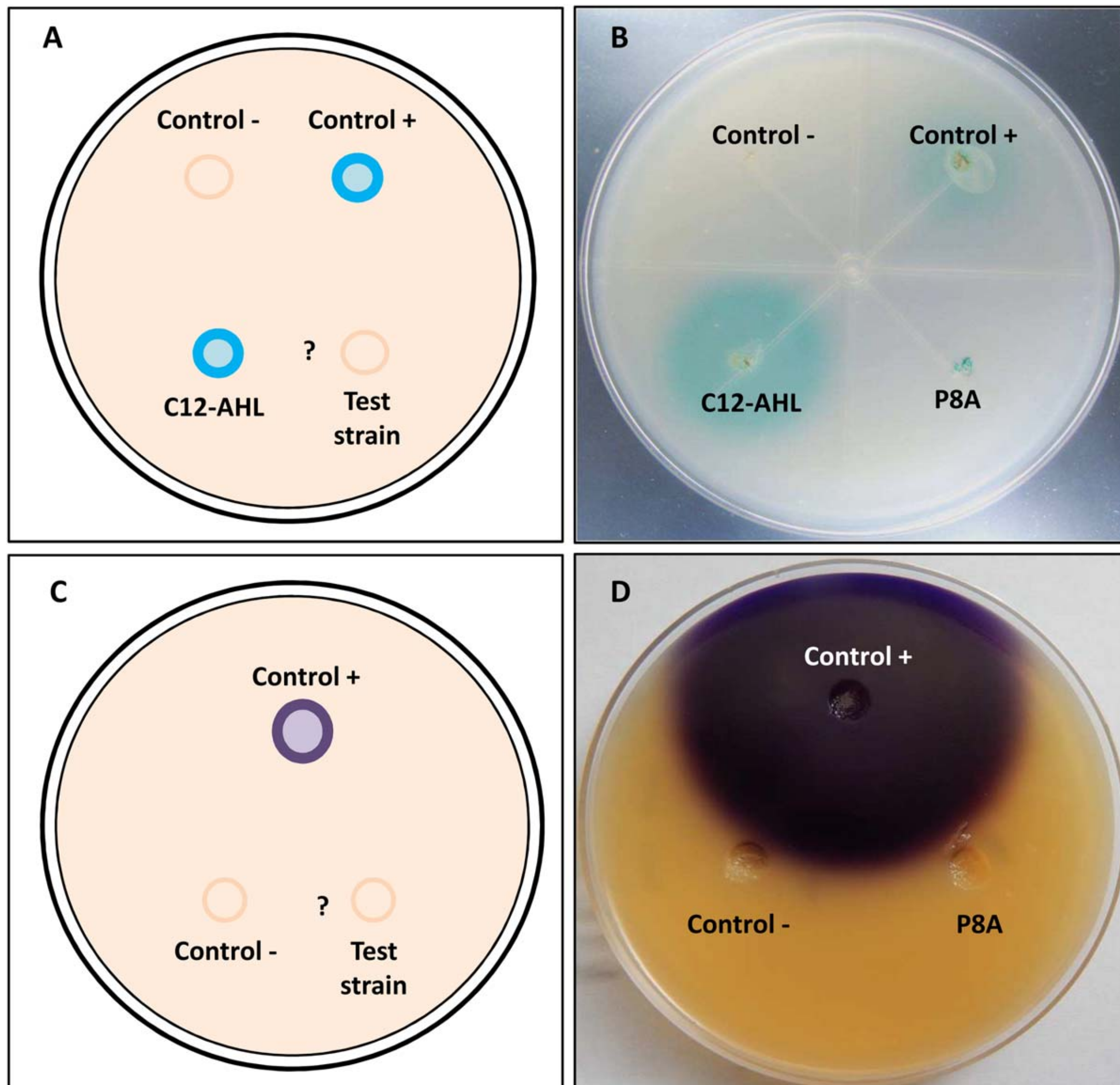

FIG 2 *(a) Hypothetical bioassay results for detection of long-chain AHLs by biosensor strain A. tumefaciens NTL4. (b) Actual detection of long-chain AHLs in Bradyrhizobium strain P8A. C+ (positive control): A. tumefaciens NTL4 pTiC58ΔaccR. C– (negative control):* A. tumefaciens *NTL4. (c) Hypothetical bioassay results for detection of short-chain AHLs by biosensor strain C. violaceum CV026. (d) Actual detection of short-chain AHLs in strain P8A. C+ (positive control): N-hexanoyl-DL-homoserine lactone (C6). C– (negative control): P. fluorescens WCS17. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

respectively, at 28°C with rotary shaking at 150 rpm. Antibiotics added, and their final concentrations, are cycloheximide (50 μg mL^{-1}), vancomycin (4 μg mL^{-1}), and/or gentamicin (30 μg mL^{-1}).

Bioassays

C. violaceum CV026 is used for determination of AHL-like molecules with short acyl chains (C4–C8) and molecules with quorum quenching (QQ) activity for AHL short chain, as described by ref. 14. This strain is a mini-Tn5 double mutant defective in synthesis of the violacein pigment. Production of this pigment *in vitro* is activated by AHLs with short acyl chains. Petri dishes used for this assay contain solid LB medium in two layers: a lower layer with 1.5% soft agar and an upper layer with 0.6% agar. The upper layer is added with biosensor strain (50 μL per 5 mL medium), using a punch, then inoculated with culture supernatant of the tested strain, and dishes are incubated overnight at 30°C. Inhibition of violacein production, reflecting QQ activity, is indicated by a clear halo around the well [14].

To assay QQ activity, the upper layer is added with biosensor and standard short-chain (C6) AHLs (5 μM solution), inoculated with culture supernatant of the test strain, and incubated overnight at 30°C. A positive result is appearance of a white halo around the colony, indicating production of lactonases for short-chain AHLs.

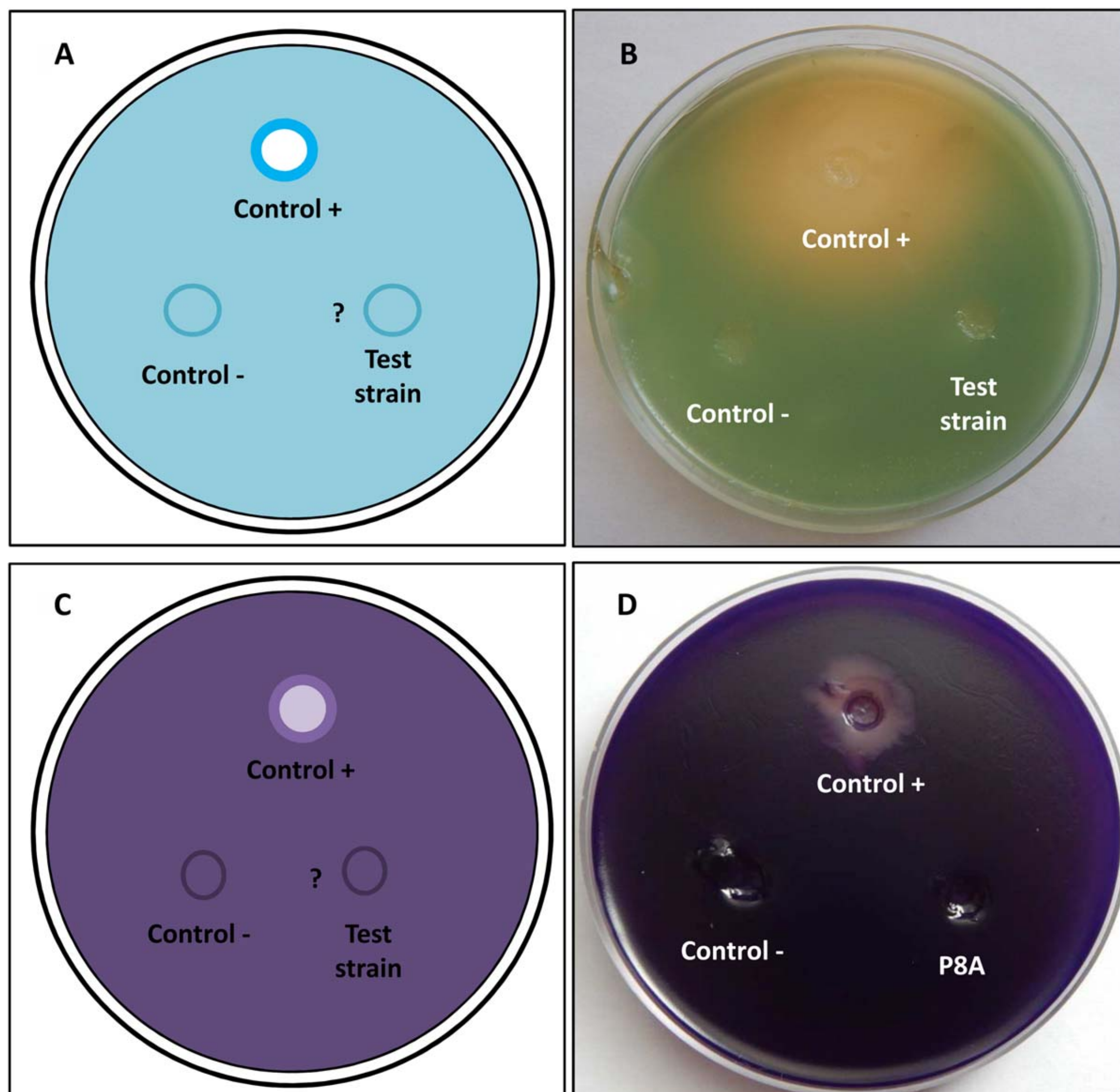

FIG 3 (a) Hypothetical bioassay results for detection of QQ activity toward long-chain AHLs. (b) Actual detection of QQ activity toward long-chain AHLs in Bradyrhizobium strain P8A. C+ (positive control) and C– (negative control): native strains from peanut rhizospheric soil. (c) Hypothetical bioassay results for detection of QQ activity toward short-chain AHLs. (d) Actual detection of QQ activity toward short-chain AHLs in strain P8A. C+ (positive control): N-(3-oxodecanoyl)-L-homoserine lactone (C10). C– (negative control): native strain from peanut rhizospheric soil. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A. tumefaciens NTL4 (pZLR4) is used to detect long-acyl-chain AHLs and molecules with QQ activity for this autoinducer. This strain carries the plasmid pZLR4, which contains a *traG::lacZ* fusion and *traR*. In the presence of long-chain AHLs, TraR protein is activated, transcription of the *traG::lacZ* fusion is initiated, and LacZ (β-galactosidase) activity serves as a reporter of *traG* transcription. Petri dishes used for this assay contain solid AB medium in two layers: a lower layer with 1.5% agar and 40 μg/mL X-Gal and an upper layer with 0.7% agar and 40 μg/mL X-Gal. The upper layer is added with biosensor (50 µL per 5 mL medium), inoculated with test strain, and incubated 24–48 h at 30°C. A positive result is appearance of a blue halo, indicating production of long-chain AHLs and hydrolysis of X-Gal.

To assay QQ activity [lactonase activity toward long-chain (with 3-oxo substitution) AHLs], the upper layer is added with biosensor and various long-chain (3-oxo-C10, 3-oxo-C12, 3-oxo-C14) AHL standards using a punch, inoculated with test strain culture supernatant, and incubated 24–48 h at 30°C. A positive result is appearance of a clear halo [27].

Preparation of AHL Extracts

AHL extracts are prepared as described by [17]. In brief, soil rhizospheric bacteria are grown to early stationary

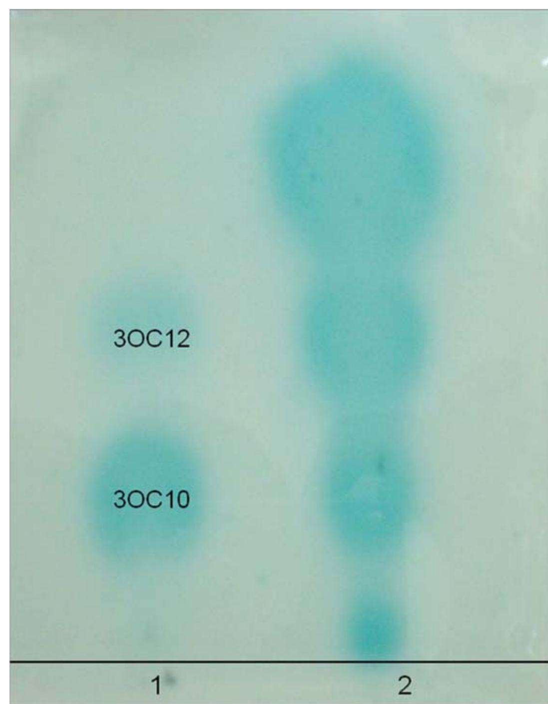

FIG 4 TLC plate overlaid with biosensor strain A. tumefaciens NTL4 (pZLR4). Lane 1: long-chain 3-oxo-AHL standards. Lane 2: ethyl acetate extract (10 μL) from supernatant of liquid culture of P8A cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

phase, and cells are removed from 25 mL medium by centrifugation (12,000 × *g*, 15 min, 4°C). AHLs are extracted from culture supernatants with three equal volumes of ethyl acetate, and the extracts are dried by rotary evaporation and resuspended in 500 μL ethyl acetate.

Thin-Layer Chromatography (TLC)

Aliquots (10 μL) of AHL extracts and 5-μM AHL standards (C6, 3-oxo-C10, 3-oxo-C12, 3-oxo-C14) are analyzed by reverse-phase C18 TLC using methanol/water 60:40 (v/v) as the mobile phase. Plates are air-dried, overlaid with AB medium, 0.7% agar containing 40 μg/mL X-Gal, and biosensor *A. tumefaciens* NTL4 (pZLR4), and incubated overnight at 30°C as described by Cha et al. [27].

Hazard Avoidance

For TLC analysis, the mobile phase is methanol/water 60:40 (v/v). When working with methanol solvent, students are instructed to always use gloves, goggles, and a fume hood to prevent release of solvent vapor.

Results and Discussion

Students are divided into working groups of four. The experiments are designed to be performed during three laboratory classes (Table 2).

On Day 1, the instructor explains the theoretical background and organization of the experiments. Petri dishes are available that contain rhizospheric strains to be tested or biosensor strains. Groups of students grow the bacterial strains in appropriate media for 48 h at 30°C. Students are instructed how to work under sterile conditions, and begin preparing media for bioassays.

On Day 2 (part 1), students (i) check the growth of bacterial colonies; (ii) prepare extracts for assaying production of signal molecules (short- and long-chain AHLs); (iii) obtain culture supernatants for detection of QQ molecules (e.g. lactonases); (iv) finish preparing plates for bioassays and extracts of culture supernatants; (v) incubate cells 24–48 h at 30°C.

On Day 2 (part 2), students perform TLC analysis. Ten-μL aliquots of AHL extracts are placed on TLC plates containing AHL standards that approximate the type of structure (if any) of signal molecules produced by the test strains. Plates are then added with biosensor *A. tumefaciens* NTL4 (pZLR4) and incubated overnight at 30°C.

On Day 3, students examine plates to determine production of short- and long-chain AHLs, and QQ activity for AHLs. Production of long-chain AHLs is indicated by a blue halo resulting from hydrolysis of X-Gal (Figs. 2A and 2B). Production of short-chain AHLs is indicated by production of violacein pigment (Figs. 2C and 2D). QQ activities toward long-chain (with 3-oxo substitution) and short-chain AHLs are illustrated in Figs. 3A and 3B and Figs. 3C and 3D, respectively. TLC plates are analyzed (Fig. 4), and students estimate the type of structure of signal molecules by comparison with Rf values of AHL standards.

For a long series of research studies in our laboratory, we have used nitrogen-fixing bacteria (rhizobia) as a model system, because such bacteria are symbiotic partners of

TABLE 3 *Model chart for summary of experimental results*

Strain	*AHLs long-chain*	*AHLs short-chain*	*QQ AHLs long-chain*	*QQ AHLs short-chain*	*TLC analysis*
Test strain					

several economically important legume crops in the agricultural region of Argentina where our university is located. However, the techniques used in these lab experiments are not specific to rhizobia; other model bacteria may be readily used depending on the environmental, social, and economic interests of the country or region where the educational institution is located, and the methodology adapted accordingly. Regardless of the model bacterium used, the students will complete a summary chart of their results (model shown as Table 3) after completing the experiments, with the purpose of encouraging their observational skills, logical thinking, and skill in data analysis. At the conclusion of the final class, using their acquired knowledge and experimental results, students will be able to assign the correct phenotype to each strain tested. The series of experiments provides students an opportunity to perform useful assays, draw conclusions from their results, and discuss possible ways to extend the study.

We have used and refined this program many times in our microbiology and biochemistry courses, and found that the experiments are reasonably simple and reproducible. Inclusion of a lab exercise of this kind in the course structure introduces students to modern experimental techniques that are likely to be useful in their future careers. The sequence of lab procedures and experiments can be performed during three periods of 3–4 h each, on successive days or weeks, and the program can be adapted for graduate or postgraduate-level courses. The instructor provides the necessary reference materials, which may include book chapters, original research articles, and protocols related to practical topics. Following completion of the experiments, the students discuss their results (with one another and the teacher) and prepare a summary report structured like a scientific publication.

The undergraduate microbiology program in our university has a duration of five years. During the last two years, students choose specific courses from a range of electives. One of these electives, titled "Experimental biochemistry", is a 70-h course involving a large proportion of experimental work on biochemistry of prokaryotic and eukaryotic model systems. The number of students taking the course ranges from 5 to 15. The programmed lab experiments described here are part of the course. Students graduating from the microbiology program typically look for employment soon afterward, in related fields in the industrial, business, or educational sectors. Feedback from our former students indicates that the lab skills and reasoning ability they acquire in this and other courses enhance their competitiveness in the job market.

By performing these experiments and then analyzing, discussing, and reporting their results, students learn the underlying concepts, significance, and techniques for biochemical determination of QS and QQ signals in bacterial strains that originate from natural environments and have agricultural and/or ecological importance.

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