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Laboratory Exercises

PCR Analysis of *expR* Gene Regulating Biosynthesis of Exopolysaccharides in *Sinorhizobium meliloti*

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Exopolysaccharide (EPS) production by the rhizobacterium *Sinorhizobium meliloti* is essential for root nodule formation on its legume host (alfalfa), and for establishment of a nitrogen-fixing symbiosis between the two partners. Production of EPS II (galactoglucan) by certain *S. meliloti* strains results in a mucoid colony phenotype. Other strains that are unable to produce EPS II display a dry phenotype, due to the presence of an insertion element in the gene *expR*, a key regulator involved in many important cellular processes, including production of low-molecular-weight EPS II. We describe a series of three programmed undergraduate biochemistry laboratory classes teaching PCR and electrophoresis procedures to detect non-functional *expR* loci in *S. meliloti*.

Keywords: *expR* gen, PCR, exopolysaccharides, rhizobacteria.

We describe here a set of biochemistry experiments designed for students. Biochemistry courses at Universidad Nacional de Río Cuarto are aimed at undergraduate students majoring in Chemistry, Biology, Microbiology, Agronomy, or Veterinary Medicine. Microbiology students, in particular, have previously taken courses in general, analytical, and organic chemistry. A laboratory experiment for these students is therefore based on a protocol to investigate a defined, small problem whose solution is feasible within the context of their knowledge and experience. We previously designed and reported a lab experiment to familiarize Microbiology students with techniques for isolation and detection of ribosomal RNA [1]. We now describe a newly-designed set of experiments to further expand the students' understanding and capabilities in biochemistry, using techniques for microorganism manipulation, sterile conditions, DNA extraction, and polymerase chain reaction (PCR) assay to detect *expR* gene in the symbiotic bacterium *Sinorhizobium meliloti*.

BACKGROUND

S. meliloti is a well-studied soil bacterium (rhizobacterium) which forms a symbiotic association with the agriculturally important legume *Medicago sativa* (alfalfa), and converts (fixes) atmospheric nitrogen into forms that the plant is able to utilize. The process of nitrogen fixation is carried out in specialized structures, termed nodules, formed in the roots of the plant. Interactions between rhizobacteria and plants show a high degree of host specificity [2] and successful colonization (infection) of plant roots by rhizobacteria dependent on a reciprocal molecular "dialogue" between the bacterium and its host [3].

Exopolysaccharides (EPSs) are abundant extracellular products accumulated on the bacterial cell surface and secreted into the surrounding microenvironment. They are involved in several nonspecific functions, including protection against environmental stress factors, attachment of bacteria to surfaces, and nutrient uptake. In particular, EPSs on the surface of rhizobacteria play important roles in symbiosis with legume host plants, and formation of active nodules [4]. Rhizobacterial mutants defective in production of EPSs, lipopolysaccharides, and capsular polysaccharides typically display reduced induction of active nodules, and reduced ability to infect host plants through infection threads [5].

S. meliloti produces two symbiosis-promoting EPSs: succinoglycan (also known as EPS I) and galactoglucan (or EPS II). EPS I consists of a repeated octasaccharide structure containing one galactose and seven glucose units, with succinyl, acetyl, and pyruvyl modifications [6]. EPS II consists of a repeated disaccharide containing an acetylated glucose and a pyruvylated galactose unit [7].

A group of 32-kb genes, termed *exp* genes (including *expA*), is responsible for synthesis of EPS II. ExpR is a LuxR-type positive regulator of EPS II synthesis. The presence of a functional *expR* open reading frame on a plasmid, or in the genome, is sufficient to promote production of symbiotically active EPS II (Fig. 1), for example, in *S. meliloti* strain Rm8530, which has an intact, functional *expR* locus and is therefore termed $expR^+$ [8]. Rm8530, which has a mucoid phenotype, forms a highly

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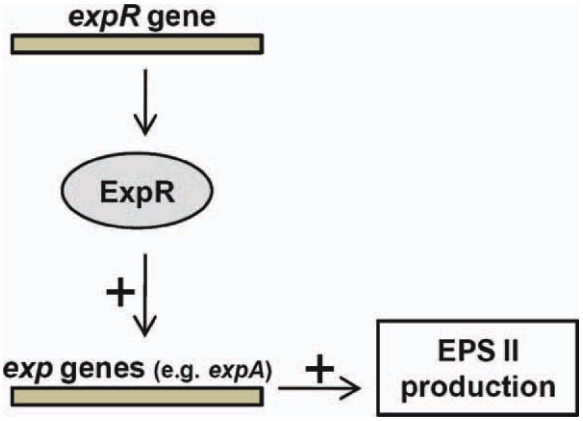

FIG. 1. **Role of *expR* gene in EPS II synthesis in *S. meliloti*.** [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

structured biofilm, in contrast to the unstructured one formed by non-EPS II-producing strain 1021 [9]. Wild-type *S. meliloti* reference strains that carry non-functional *expR* loci, and are therefore unable to synthesize EPS II, do not display autoaggregation, and form only a small biomass adhered to plastic surfaces. These non-functional loci can be detected by PCR analysis. The PCR product from Rm8530 (termed Rm1021 *expR*$^{+}$; formerly *expR101*) has a size of 0.9 kb, whereas the PCR product from wild-type Rm1021 has a size of 2.2 kb. Sequence analysis of the 2.2 kb PCR product indicates that the *expR* open reading frame (ORF) is disrupted in Rm1021 by a copy of IS*Rm*2011-1, a previously-described 1,319-bp insertion sequence (IS) element [10, 11].

The purpose of the study (set of lab experiments) described here is to elucidate the properties of the *expR* gene responsible for EPS biosynthesis in *S. meliloti*.

MATERIALS AND METHODS

Experimental Procedures

Bacterial Strains—The strains used are listed in Table I, and were grown in tryptone yeast extract (TY) on a rotator shaker (200 rpm) at 30 °C. The antibiotics used, and their final concentrations, were: streptomycin (500 μg/mL); neomycin (200 μg/mL); gentamycin (40 μg/mL). For maintenance of strains, and testing on plates, the medium was solidified using 1.5% Bacto-agar (Difco Laboratories Inc, Detroit, MI, USA).

DNA Extraction—Bacterial colonies in a petri dish were suspended in 200 μL TE (Tris-EDTA) buffer, pH 8.0. DNA extraction was performed using Genomic DNA Purification Kit no. K0512 (Fermentas Life Sciences, Glen Burnie, MD, USA), following the manufacturer's instructions. This particular extraction kit does not contain phenol (see "Potential laboratory hazards" section, below). The extraction product was used as bacterial DNA template for PCR reaction.

Diagnostic PCR Analysis of expR gen—PCR was performed as described by Pellock *et al.* (2002) [10], with minor modifications. Two primers were used to amplify the *expR* region: Rm*ndvA*5′out (5′-GCGAGGAGATCCT GCCCGAG-3′), and Rm*pyc*5′out (5′AGAGTGGCGTGAA CATTCGG-3′). One microliter of DNA preparation template in 20 μL PCR mixture containing 2.5 U Taq polymerase (Invitrogen, Buenos Aires, Argentina) was used, following the manufacturer's recommended buffer conditions. Primers and deoxynucleoside triphosphates were used at concentrations of 1 μM and 200 μM, respectively. The PCR program was: 1) 95 °C for 5 min; 2) 94 °C for 30 second; 3) 65 °C for 30 second; 4) 72 °C for 5 min; 5) hold at 4 °C. Steps 2–4 were repeated 29 times. Final reaction volume was 20 μL. The PCR product was analyzed by electrophoresis in 0.8% (w/v) agarose gel using TAE (Tris-acetate-EDTA) buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0), with ethidium bromide (0.05 μg/mL), at 90 V for 60 min. 6× Loading Dye Solution (10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA) (Fermentas Life Sciences) was used to prepare DNA markers and samples for loading on agarose gel.

Potential Laboratory Hazards

Although the DNA extraction kit used for these lab experiments is phenol-free, some reagents used for DNA isolation contain phenol, which is toxic. When working with such reagents, use gloves and eye protection, work in a chemical fume hood, and avoid breathing the vapor.

Ethidium bromide is a potential mutagen. Use gloves when handling this fluorescent compound. There are alternatives to ethidium bromide which are advertised as being less dangerous and having better performance. For example, SYBR Green or similar reagents are used by some laboratories for development of agarose gel electrophoresis.

Wear a face shield to avoid exposure of the skin, particularly the eyes, to hazardous, high-intensity UV light from the trans-illuminator.

RESULTS

The experimental methodology described here is designed to be performed in a series of three laboratory classes.

In the first class, the lab instructor explains the theoretical background, and the strategy of the experiments to be performed. The students are organized into working groups (3 or 4 students per group). Each group begins growing the three rhizobacterial strains (Rm1021, Rm8530, and Rm8530 *expA*) in a petri dish containing

TABLE I
Bacterial strains used

Strain	Genotype	Reference
S. meliloti		
Rm1021	SU47 *str21 expR102*::IS*Rm*2011-1	Meade *et al.*, 1982 [12]
Rm8530	SU47 *str21 expR101* (*expR* $^{+}$)	Glazebrook & Walker, 1989 [8]
Rm8530 *expA*	*expA3*::Tn5-233	Sorroche *et al.*, 2010 [13]

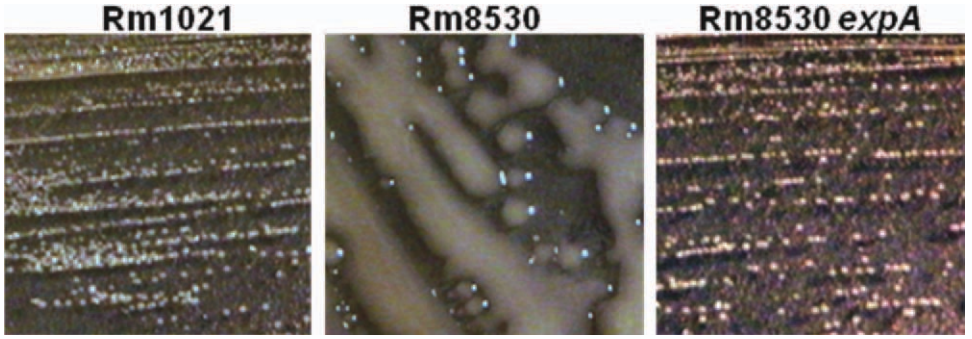

FIG. 2. **Colony phenotypes of *S. meliloti* strains.** Rm1021 (*expR*-) and Rm8530 *expA (*expR+, *expA*−) do not produce EPS II, and therefore develop dry colonies. Rm8530 (expR+) produces EPS II, and therefore develops highly mucoid colonies. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TY solid medium. The dishes are incubated for 48 hour at 30 °C.

In the second class, students check the growth of their three strains, and note the phenotype (mucoid or dry colony form) of each. Typical phenotypes of the three strains are shown in Fig. 2. ExpR is a LuxR-homologue, whose function includes activation of EPS II production in the presence of *N*-acyl-homoserine lactone (AHL), which is produced by the *sinR*/*sinI* system in *S. meliloti*. Rm1021 therefore appears dry (not mucoid) since it cannot produce EPS II, due to the presence of an insertion element in the chromosomal gene *expR*. In contrast, EPS II-producing Rm8530 displays a highly mucoid phenotype. Rm8530*expA* (which carries an intact copy of *expR* gene) has a dry phenotype. This mutant is defective in function of *expA3* gene (involved in EPS II biosynthesis), confirming that mucoid phenotype in *S. meliloti* results from production of EPS II. Students take samples from colonies of each strain, perform DNA extraction, and then use the extracted DNA as template for PCR analysis of each strain, as described in Materials and Methods section.

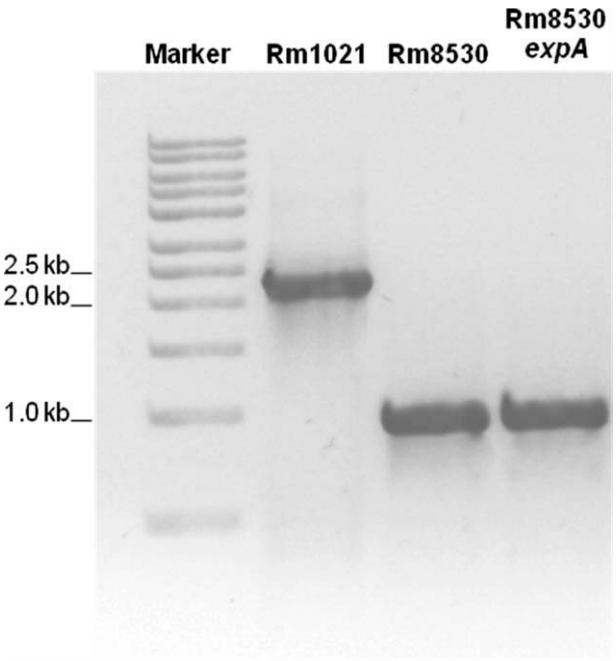

FIG. 3. **Analysis of the *expR* locus.** PCR amplicons are separated in 0.8% agarose gel. Lane "Marker" lane contains a 1 kb molecular marker ladder (Promega). Lane "Rm1021" contains 2.2 kb amplicon (IS-interrupted *expR* locus). Lanes "Rm8530" and "Rm8530 *expA*" contain 0.9 kb product (intact *expR* gene).

In the third class, students take the PCR product and subject it to electrophoresis in agarose gel (provided by the instructor). As shown in Fig. 3, the four lanes of the gel correspond to molecular weight markers (Promega ladder), and to the three strains: Rm1021, Rm8530, and Rm8530 *expA.* Strains Rm8530 and Rm8530 *expA* harbor an intact (not interrupted) copy of the *expR* gene (0.9 kb PCR product). In contrast, strain Rm1021 yields a larger amplicon (2.2 kb PCR product), since the *expR* ORF is disrupted by a copy of IS*Rm*2011-1, a 1,319-bp IS element.

The three strains are coded as no. 1, no. 2, and no. 3 so that the students are not aware of their identities during the experiments. This is to encourage the students' observational skills, logical thinking, and experimental data analysis. A chart as below is used. At the conclusion of the third class, students are able to assign the correct genotype to each coded strain.

Strain	Colony phenotype	*expR* product size	Strain genotype
No. 1			
No. 2			
No. 3			

DISCUSSION

Results shown in Figs. 2 and 3 indicate that an intact *expR* gene is required for active EPS II synthesis in *S. meliloti*. An insertion element in the *expR* regulator (detectable by PCR) abolishes EPS II synthesis. It is possible to block EPS II not only at this regulatory level, but also by mutation of *expA*, a structural gene involved in EPS II synthesis. In general, rhizobacterial cell surface components such as EPSs, in combination with bacterial functional signals, are essential for the processes of autoaggregation [13], and biofilm formation [9]. Both processes play important ecological roles for survival of rhizobacteria in their natural soil environment, and probably for the nitrogen-fixing symbiosis occurring within root nodules, in which EPSs are essential for early stages of infection [4].

TABLE II
Organization of programmed classes no. 1, no. 2, no. 3

Day	Activity
1	Theoretical explanation. Preparation of plates.
2	Observation of plates. DNA extraction. PCR experiment.
3	Agarose gel experiment. General discussion.

Since the *expR* gene has been sequenced for strains Rm8530 and Rm1021, a bioinformatic analysis can be performed as an optional addition to the three laboratory classes described above. The *expR* gene from Rm8530 has been deposited in the GenBank nucleotide sequence database (accession number DQ366275.1). Students can take this nucleotide sequence from the database to perform a BLAST search (http://blast.ncbi.nlm.nih.gov). Results of this analysis will reveal homologies between the Rm1021 and Rm8530 sequences. Consistent with findings from the agarose gel experiment, the analysis will show the presence of a 1,319-bp insertion sequence in Rm1021, since the *expR* ORF is disrupted by a copy of ISRm2011-1 [10].

These experiments provide opportunity for discussion of theoretical concepts such as EPS metabolic pathways and functions of regulatory genes, as well as practical procedures for PCR and gel electrophoresis.

CONCLUSIONS

These interesting experiments are simple and reproducible. The kits and reagents are typically available within the country where the teaching institution is located, and therefore inexpensive. This sequence of lab procedures can be performed during three periods of 3–4 hour each (Table II). The sequence can be adapted for postgraduate courses, possibly with two lab periods of 5–6 hour each. The three classes may be on successive days or weeks, depending on the students' course schedule. In either case, samples can be stored at 4 °C until the next class.

By performing, analyzing, and discussing these experiments, students learn the concepts, detection techniques, and biochemical significance of gene alterations in bacterial genomes. In our experience, undergraduate students show genuine interest in the experiments, and gain important understanding of phenotypic consequences resulting from altered structure of a regulatory gene.

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