Studies of Symbiotically Important *Rhizobium meliloti* Exopolysaccharides EPSII and Succinoglycan

by

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B.S. Biology
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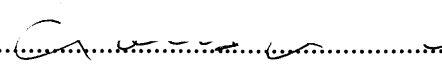SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGEREE OF

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Science

TABLE OF CONTENTS

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by

Louis LeCour

Submitted to the Department of Biology
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Requirements for the Degree of
Masters of Science in Biology

Rhizobium meliloti needs to be able to synthesize either succinoglycan or EPS II in order to invade developing nodules on alfalfa. There is interest, therefore in 1.) the genetics of the assembly, export, and processing of EPS II, and 2.) how the secretion of ExoK (endo-1,3-1,4-β-glycanase homolog), which degrades the succinoglycan polymer to the biologically active trimer form, is regulated. The investigation of EPS II began with the assigning of functions to open reading frames from the *exp* region. The genes examined are hypothesized to deal with EPS II subunit assembly and putative transport of polymer. The function of each gene disrupted was sought through *in vitro* radiolabeling of *exp* mutants. UDP-[^{14}C]-Gal was introduced into *R. meliloti* cells by a freeze-thaw protocol or by electroporation and the resulting radiolabeled oligosaccharides were examined by thin-layer chromotography (TLC) and P4 gel filtration. The electroporated *expR101 exoY exoB* had material in their supernatant found in the void volume of the P4 column. These same cells produced other oligosaccharides (visible on TLC) that could be organically extracted, indicating that EPS II was assembled on a lipid carrier. In addition, all of the *exp* mutations transduced into the *expR101 exoY exoB* (later exposed to electroporation and labeling), except for the *expD* mutation, diminished or eliminated the appearance in the culture supernatant of material that appeared in the void volume during P4 gel filtration. This investigation also looked for gene(s) responsible for the control of the molecular weight distribution of EPS II. A screen was designed to restore the ability of *mucR* to produce low molecular weight EPS II by conjugal transfer of a *R. meliloti* cosmid library. The purpose of this was to find the library plasmid responsible and to obtain a subcloned fragment of DNA.

Previous work had shown that ExoK [produced by Rm1021(*exoY*)] accumulated much less in GMS supernatants than it did in MGS supernatants. This observation generated interest first in a study to determine what conditions regulated extracellular/intracellular

ExoK accumulation. Individual changes in the composition of GMS and MGS liquid media were made to find out what restricted or encouraged the extracellular/intracellular accumulation of ExoK [produced by Rm1021(exoY)]. These modifications revealed that the presence of trace minerals (3), as well as the reduction of phosphate concentration, in GMS diminished extracellular ExoK accumulation. This observation led to the question of which trace minerals reduced extracellular Exok accumulation. Individual salts contained in the trace minerals were excluded from GMS experiments or included in MGS in order to identify the salt(s) affecting extracellular ExoK accumulation. These variations showed that $FeCl_3$, $CoCl_2$, and $ZnSO_4$ had the most negative effect on extracellular ExoK accumulation. The next study tested whether pH affected extracellular accumulation of ExoK. Rm1021(exoY) was grown in MGS of the pH range 6.0-8.0, resulting in the observation that this range did not diminish ExoK accumulation in the supernatant at all. Finally, there is interest as to what gene(s) is/are involved in the restriction of extracellular ExoK accumulation in GMS. The restriction of ExoK extracellular accumulation in GMS raises the possibility of a delayed halo phenotype in Rm1021 on solid GMS media. If this phenotype is confirmed, then random Tn5-233 mutagenesis of Rm1021(exsH) could be used to screen for a positive/negative regulator of ExoK.

Acknowledgments

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I would like to thank my friends Brian, Sandra, Hugh, Roland, and Peter for their much-needed support and encouragement. Their presence was never in the way.

I would like to thank my family very much for the unlimited encouragement, love, and support they gave me at my asking (or when I would never ask!)

Most of all, I would like to thank Almighty God for my existence, many triumphs, his unlimited forgiveness, and for my peace of mind.

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Chapter 1

Analyzing the Genetic Basis for EPS II Assembly, Export, and Processing in *Rhizobium meliloti.*

Abstract

There is interest in the study of the genetics of the assembly, export, and processing of EPS II in the nitrogen-fixing bacterium *Rhizobium meliloti*. This study began with the assigning of functions to open reading frames in the *exp* region. The *exp* genes whose roles were examined are hypothesized to deal with EPS II subunit assembly and putative transport of polymer. *In vitro* radiolabeling of *exp* mutants was used to identify the function of the gene disrupted. UDP-[^{14}C]-Gal was introduced into *R. meliloti* cells by a freeze-thaw protocol or by electroporation and the resulting radiolabeled oligosaccharides were examined by thin-layer chromotography (TLC) and P4 gel filtration. The electroporated *expR101 exoB exoY* cells had material in their supernatant that appeared in the void volume of the P4 column. From these same cells oligosaccharides (visible on TLC) could be organically extracted, indicating that EPS II was made on a lipid carrier. Additionally, all of the *exp* mutations transduced into the *expR101 exoB exoY* cells (prior to electroporation and labeling), with the exception of the *expD* mutation, severely impaired the appearance of material in the void volume of the P4 column. This study also looked for gene(s) responsible for the control of the molecular weight distribution of EPS II. A screen was planned to restore the ability of *mucR* to produce low molecular weight EPS II by conjugal transfer of a *Rhizobium* cosmid genomic library. The purpose of this was to find the library plasmid responsible and to obtain a subcloned fragment of DNA.

<u>Introduction</u>

Rhizobium meliloti is a gram-negative soil bacterium that lives in a symbiotic relationship with alfalfa (*Medicago sativa*) (1, 2). *R. meliloti* is chemotactically attracted to a root hair of alfalfa and produces a chemical known as Nod factor which stimulates pre-invasion nodule development of the root hair. The bacteria then invade the developing nodule through invaginations known as infection threads, and are subsequently enclosed in a plant-derived membrane as they enter individual plant cells in a process similar to endocytosis. Established inside the plant cells, the bacteria differentiate into alternative morphological forms known as bacteroids, which fix nitrogen inside of the fully formed nodules. This is the outcome of the symbiosis between the two species.

In this process, chemical communication between the plant and the bacteria is absolutely vital. Should the plant fail to signal *R. meliloti*, or the bacterium secretes no Nod factor, the symbiotic relationship would not develop. Furthermore, it is now known that *Rhizobium meliloti* requires at least one of two excreted acidic exopolysaccharides in order to invade the developing nodule properly. Mutants of R. *meliloti* strain Rm1021 that do not make either exopolysaccharide (Succinoglycan or EPS II) cannot invade (1, 2, 3). Alfalfa inoculated with such *R. meliloti* mutants form nodules, but these nodules are small and devoid of bacteroids.

Succinoglycan (**Figure 1-1**) has been shown to be a polymer of octasaccharide subunits composed of one galactose and seven glucose moieties (4). The octasaccharide subunit contains three chemical modifications: acetyl (on the third sugar/second glucose residue), succinyl (on the seventh sugar/6th glucose residue), and pyruvyl (on the eighth sugar/7th glucose residue). This polysaccharide is responsible for the blue-green fluorescence exhibited by *R. meliloti* when grown on solid media containing the dye Calcofluor (1, 2) and irradiated with ultraviolet (UV) light. This dye was used to develop a facile screen for mutants defective in succinoglycan production (specifically, the *exo* mutants, most of which map to the second of the two megaplasmids possessed by *R. meliloti* [5]). Insertion mutations in the *exoA, exoB, exoF, exoL, exoM, exoP*, and *exoY* genes completely abolish succinoglycan production, fluorescence on Calcofluor plates, and the effective nodule phenotype (6, 7, 8, 9, 10). Null mutations in *exoG, exoJ, exoK*, or *exoN* exhibit "dim" (reduced) fluorescence on Calcofluor media and produce less exopolysaccharide than wild-type, yet can still form effective nodules on alfalfa (sometimes with reduced efficiency) (7). A strain carrying a null mutation in *exoH* forms

colonies that fluoresce on Calcofluor plates but that lack the fluorescent halo produced by wild type *R. meliloti*. It produces a derivative of succinoglycan that lacks the succinyl modification and is defective in nodule invasion (7, 11). The fluorescent halo seen around wild type *R. meliloti* colonies has been shown to be the low molecular weight form of succinoglycan, resulting from succinyl-group-dependent degradation, and this low molecular weight form is believed vital in making invasion of the developing nodule possible by the bacterium (12).

EPS II (galactoglucan) expression has been found in strains designated *expR101* (3) or *mucR* (13, 14). These mutants were identified by their extremely mucoid but non fluorescent phenotype on Calcofluor plates. In an *exoA* mutant (unable to add the second sugar, glucose, in the formation of EPS I) background, the EPS II can be isolated as a polymer consisting of the repeating unit (-3)-Glc-β(1,3)-Gal-α(1,-) with a pyruvyl modification attached to Gal, and an acetyl to Glc (13, 14, 15, 16 and **Figure 1-1**). When rendered unable to produce succinoglycan by most *exo* mutations (except for *exoB*), *expR101* derivatives can form effective nodules on alfalfa roots, but not on some other plants that wild-type *Rhizobium* (Rm1021) can nodulate effectively (13). *expR101 exoY exoB* strains, which do not produce either succinoglycan or EPS II, are Fix$^-$. The known genes required for the synthesis of this exopolysaccharide are located in the 29 kb *exp* region on the second megaplasmid (pSymB). This region contains 6 putative complementation groups (deduced by Tn5 mutagenesis) (3), all required for the expression of EPS II and encompassing a region recently sequenced by the Pühler Lab (discussed below). 24 ORFS were uncovered, via sequencing, in this region. Most of the ORFS have been assigned possible functions on the basis of homology, and half were found to be previously disrupted by various transposon insertions used to assign 6 complementation groups to the region (3). Three putative galactosyltransferase genes, three putative glucosyltransferase genes, and two genes making up a single putative ATP-binding cassette (ABC) transporter are the most conspicuous of the ORFS. None of the putative galactosyltransferases show any homology to *exoY, gumD, rfbP*, or *cpsD*, genes coding for enzymes to transfer the first sugar-1-phosphate from a UDP-sugar to the undecaprenol phosphate lipid carrier. I have been attempting to develop the techniques necessary to ultimately assign functions to the putative *exp* glycosyltransferases and export proteins. The production of this polysaccharide also requires *exoB* to provide UDP-galactose.

In my work, the two aspects of EPS II (galactoglucan) synthesis explored are 1) biosynthesis and export, and 2) processing of completed exopolysaccharide.

<u>Methods and Materials</u>

Assigning Functions.

Strain Construction. I constructed the following strains: *expR101 exoY210 exoB24* and a set of derivatives of this strain, each of which carried a different *exp* mutation. I also acquired the following strains: *exoY210 exoB24 exoR395* , *exoB24 exoR395*, and *exoL431 exoB294 exoR395* (10). The first strain and its derivatives were made via ØM12 phage transduction of insertions, selected for by drug resistance, and were confirmed by complementation analysis with plasmids containing wild-type versions of the genes disrupted. In a single step, I transduced *exoY210 exoB24* into *expR101* and *expR101 exp*. I selected for the presence of *exoY* via drug resistance, and screened for the presence of *exoB* by selecting colonies still non-fluorescent (on Calcofluor media) after *exoY* complementation.

Freeze-Thaw Protocol. As described by Reuber and Walker (10), the strains were grown in LB/MC to an OD_{600} of ~1, washed with 70 mM Tris buffer (pH 8.2), concentrated each 100X in 70 mM Tris-EDTA, and each preparation was freeze-thawed three times. The cells were labeled with ~2 mM UDP-[^{14}C]-Gal (specific activity of 309 mCi/mmol) + 35.7 mM UDP-Glc, incubated 1/2 hr at 10°C, and organically extracted with 1:2:0.3 chloroform-methanol-water. 1/10 of the organic extract was counted for activity of lipid-linked sugars, while the rest was treated for removal of putative lipid carrier and analyzed by thin-layer chromatography.

Electroporation Protocol (17, 18). The same strains used in the freeze-thaw protocol were grown to an OD_{600} of 0.6-0.8. The strains were then washed and concentrated ~307X in 5% glycerol. They were then electroporated (1.5 kV, 25 mF, 400 Ω) with UDP-[^{14}C]-Gal (11.6 mM; specific activity of 309-332 mCi/mmol) and 35.7 mM UDP-Glc, and incubated 1 hr at 30°C. Post-incubation, the cells were washed with Tris buffer (70 mM, pH 8.2) to remove putative EPS polymers, and were subsequently extracted with 1:2:0.3 chloroform-methanol-water to remove putative lipid-linked EPS "subunits." The Tris buffer washes were combined and run on a Biogel P4 column for separation into 1.25 ml fractions. The organic extracts were dried down, and treated with trifluoroacetic acid (separates lipid carriers from succinoglycan sugar subunits [10]). Following a treatment with Sigma bovine alkaline phosphatase (removes pyrophosphate from succinoglycan sugar subunits), the samples were then run on TLC plates.

<u>Results and Discussion</u>

Radiolabeling EPS II by the freeze-thaw protocol

In order to gain information about the various genes known to play roles in EPS II production, I decided to examine the intermediates in EPS II biosynthesis. To introduce radiolabeled UDP-[^{14}C]-Gal into the rhizobia, I initially used the same freeze-thaw protocol employed by Lynne Reuber in her study of succinoglycan biosynthesis (10). The *expR101* mutation, as indicated earlier, is necessary for EPS II production. The *exoB* (UDP-glucose-4-epimerase) mutation prevents the production of endogenous UDP-Gal, which would hinder incorporation of UDP-[^{14}C]-Gal. The *exoY* (galactose-1-phosphate transferase) mutation prevents the initiation of succinoglycan octasaccharide subunit production. The *exoL* mutation prevents the addition of the third sugar to the developing succinoglycan octasaccharide. The *exoR* mutation upregulates succinoglycan biosynthesis.

Given the nature of the above mutations, a given combination was expected to show a particular phenotype. Upon labeling with UDP-[^{14}C]-Gal, the *expR101 exoY210 exoB24* strain was expected to produce labeled EPS II subunits. Due to disruption of the *exp* region, derivatives of this strain were expected to produce no subunits. The *exoY210 exoB24 exoR395* was known to produce no EPS (10). The *exoL431 exoB294 exoR395* and the *exoB24 exoR395* strains were known to produce labeled disaccharide and octasaccharide subunits of succinoglycan, respectively.

At the time these experiments were performed, the *exp* region was known to consist of 6 complementation groups, so it was of interest to know how this region was needed for the biosynthesis of EPS II. Namely, it was of interest to know 1)*if EPS II biosynthesis relies on the use of an undecaprenol lipid carrier, and 2) what role each of the exp gene products plays in the process of EPS II biosynthesis*. To address these two items of interest, I employed a protocol used by Lynne Reuber (10). In her protocol, Reuber first freeze-thawed different *Rhizobium meliloti exo* mutants. She then labeled them with UDP-[^{14}C]-Gal + UDP-Glc to isolate biosynthetic intermediates from each mutant, and finally characterized these with thin-layer chromatography and gel-filtration chromatography. She wanted to assign functions to each *exo* gene in succinoglycan biosynthesis. In the case of succinoglycan, previous work from another lab had already established that succinoglycan subunits were synthesized on an undecaprenol lipid

carrier. Therefore, the biosynthetic intermediates were organically extracted with 1:2:0.3 chloroform-methanol-water. Such an extraction was done on an EPS II-producing strain to determine if the exopolysaccharide subunits were made on a lipid carrier.

The negative control *exoY210 exoB24 exoR395* did not produce lipid-linked products on thin-layer chromatography (TLC). This was expected due to the strain's deficiency in succinoglycan and EPS II production. Generally, the *expR exoY exoB* strain apparently could not produce lipid-linked products visible on TLC, even though minimal activity above background (extract from *exoY exoB exoR* strain) was seen in the organic extract. The positive controls (*exoB exoR* and *exoL exoB exoR*), on the other hand, often yielded lipid-linked sugars seen on TLC. These findings suggested to me that either EPS II was not produced on a lipid carrier, or that the freeze-thaw process of permeabilized cells was impairing the ability of EPS II-producing cells to produce sufficient amounts of lipid-linked subunits. Interestingly, it was observed by Reuber that her protocol did not allow for the polymerization of succinoglycan octasaccharide subunits beyond the size of a dimer in *exoB exoR* strain. Therefore, I proceeded to seek an alternate means of extracting EPS II from the *expR exoY exoB* strain, whether its subunits were lipid-linked or not.

Radiolabeling EPS II by the electroporation protocol

I decided to try a new protocol, developed by Carlos Semino (17, 18), to yield polymers of EPS from *Acetobacter*. In this procedure, the radiolabelled nucleotide sugars were introduced by electroporation. Post-incubation, the cells were washed with Tris buffer (70 mM, pH 8.2) to remove putative EPS polymers, and were subsequently extracted with 1:2:0.3 chloroform-methanol-water to remove putative lipid-linked EPS "subunits." The Tris buffer washes were combined and run on a Biogel P4 column for separation into 1.25 ml fractions. The organic extracts were dried down, and treated with trifluoroacetic acid (separates lipid carriers from succinoglycan sugar subunits [10]). Following a treatment with Sigma bovine alkaline phosphatase (removes pyrophosphate from succinoglycan sugar subunits), the samples were then run on TLC plates.

Analysis of oligosaccharides from the organic extraction by thin layer chromatograpy

The thin-layer chromatography (TLC) data of organic isolate from *expR101 exoY210 exoB24* indicated the presence of subunits in the apparent form of mostly

13

monosaccharides and disaccharides, but other slower-running moities (as well as spots which migrate faster than galactose) are present and yet to be characterized. The possibility that EPS II subunits are attached to a lipid carrier cannot be excluded.

The TLC data of organic isolate from *exoB294 exoR95* displayed oligosaccharides which are intermediates in the pathway of octasaccharide biosynthesis. This observation was made previously by Lynne Reuber (10). The TLC data of organic isolate from *exoY exoB exoR* showed no apparent subunits. This was expected, for this strain produces no succinoglycan or EPSII.

As noted above, the TLC data for the *expR101 exoY210 exoB24* organic isolates was not completely characterized. What remains to be done is the removal of the acetyl and pyruvyl substituents (on the glucose and galactose, respectively) before proper characterization of the EPS II oligosaccharide subunits can take place. Treatment of the subunits with 10 mM KOH (room temperature, 5 hr), and then with 50 mM oxalic acid (100°C, 90 min), should remove the acetyl and pyruvyl groups respectively (10). Once the decorations are removed, the apparent size of the subunits can be seen on the TLC autoradiograms. Afterwards, an effort should be made to assign EPS II assembly functions to the putative glycosyltransferase genes (*expA2, expA3, expC, expE2, expE4, expE7*) and to genes apparently involved in EPS II polymer export (the ABC transporter homologues *expD1* and *expD2*, the putative exported Ca^{2+} binding protein *expE1*). Puhler's lab is in possession of nonpolar *lacZ*-Gm interposon insertions in these reading frames. The mutations each need to be transduced into an *expR101 exoY210*::Tn5 *exoB24*::Tn5-Tp background, and selected for gentamycin resistance. Once the integrity of each new strain is proven, it will be necessary to *in vitro*-label them with 11.6 mM UDP-[^{14}C]-Gal according the Carlos Semino's protocol, and to extract the putative lipid-linked sugar subunits and analyze them by TLC. Also, the aqueous washes will be separated on the Biogel P4 to assay for EPS II polymer.

Additional characterization of the apparent polymeric and lipid-linked subunit forms of the radiolabeled EPS II (from *expR101 exoY210 exoB24)* could be carried out. For example, one could label *expR101 exoY210 exoB24* with 11.6 mM UDP-[^{14}C]-galactose and 11.6 mM UDP-[^{14}C]-glucose simultaneously. This way, products would be produced for acid-hydrolysis (to yield monosaccharide units) and TLC analysis to quantitatively confirm that galactose and glucose are in a 1:1 ratio (normal for EPS II). The determination of the ratio on TLC would take place via densitometry of the labeled

sugars on the autoradiogram (at positions where the galactose and glucose from the hydrolyzed EPS II sample are expected to run). Such an analysis would allow one to better ascertain the sizes and structure of the lipid-linked sugar subunits removed from *expR101 exoY210 exoB24*.

Separation of ^{14}C-labelled fractions by P4 gel filtration

As shown in **Figure 1-2**, the *expR101 exoY210 exoB24* strain (incapable of producing succinoglycan, needs exogenous UDP-Gal to produce EPS II), when labeled with 11.6 mM UDP-[^{14}C]-Gal, produced a major peak in the void volume. This was followed by three smaller peaks separated into increasing fraction numbers. The major peak may represent HMW EPS II, and the smaller peaks may represent EPS II of lower molecular weights. This separation was apparently unique for the *expR101 exoY210 exoB24* strain.

The positive control strain *exoB294*::Tn5-Tp *exoR95*::Tn5-233 (needs endogenous UDP-Gal to produce succinoglycan), when labeled with 11.6 mM UDP-[^{14}C]-Gal, displayed a major peak in the void volume (up to about 60,000 cpm), in addition to two smaller peaks (at about fraction #46, up to about 17,000 cpm; and at about fraction #57, up to about 26,000 cpm). The major peak may represent HMW succinoglycan polymer, the peak at fraction #46 may represent trimers and tetramers of succinoglycan, and the peak at about fraction #57 may represent octasaccharide units of succinoglycan.

The negative control strain *exoY210 exoB24 exoR395* produced no material in the void volume or larger, prior to the salt volume (between fractions #56-#74). This indicates that this strain produces no EPS.

As shown in **Figure 1-3**, *expR101 exoY210 exoB24* strains with Tn5 insertions in *expA125, expC156, expD109, expE177, expF111, and expG222* were labeled as above with 11.6 mM UDP-[^{14}C]Gal, and washed. The washes were chromatographed on the P4 matrix column to separate out any potential EPSII polymer. As shown, all of these *exp* mutations (except for *expD109*) severely disrupted the synthesis of potential polymer. This provides evidence that the product in the void volume, made by the *expR101 exoY210 exoB24* strain, is EPS II polymer labeled with [^{14}C]Gal.

Control of the Molecular Weight of EPS II.

expR101 versus mucR12. *expR101* and *mucR12*, both capable of producing EPS II, differ because *expR101* produces high molecular weight EPS II and lower molecular weight oligomers, while *mucR12* produces only high molecular weight EPS II (19); it is known that the production of low molecular weight EPS II by *expR101 exo* strains renders this strain Fix$^+$, and the lack thereof renders *mucR exo* strains Fix$^-$. The possibility of a glycanase being involved is unlikely because there is no indication of a glycanase homologue in the newly sequenced *exp* region (Puhler, personal communication). However, the possible involvement of a particular gene product active in the *expR101* strain in realizing the production of low molecular weight EPS II cannot be discounted. The *expR101 mucR* double mutant is indistinguishable in appearance from *expR101* (Gonzales, personal observations). It is possible that the putative product is negatively regulated (directly or indirectly) by the wildtype version of the gene(s) nulled completely in *expR101*. Alternatively, this putative product could be positively regulated by an inducible gene(s) constitutively expressed in *expR101*. I developed a plan to screen a *Rhizobium* cosmid genomic library in *mucR12*, in an effort to identify gene(s) responsible for production of low molecular weight EPS II.
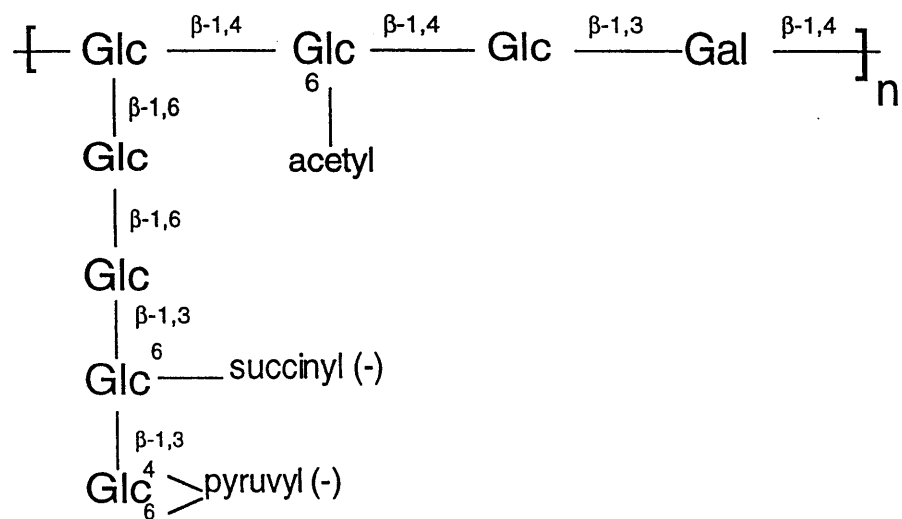
Increasing copies of putative gene in mucR. Predicting that the putative protein responsible for molecular weight control of EPS II is somehow repressed in the *mucR12* strain, I plan to increase the copy number of this unknown gene in hopes of titrating out any repressor. I will do this by transconjugating the *Rhizobium* cosmid genomic library into *mucR12* and screening for recipients which display more mucoidy than the original *mucR12* strain. I will then isolate the cosmids from each of these recipients and subject them to restriction analysis to find a common fragment. I will clone this fragment and try to find the gene(s) responsible within this fragment.

Mode of Regulation of Responsible Gene. I speculated above that the putative gene responsible for molecular weight variance of EPS II is regulated by ExpR (directly or indirectly). At what level is this gene regulated: transcriptional or post-transcriptional? I plan to address this problem by generating a *lacZ* fusion within the gene. I will mutagenize the gene with Tn3hohoKm (confers neomycin [Nm] resistance in Rhizobia) within an *expR101* background (to obtain active transcriptional fusions by selecting bluish mutants from an LB/Nm/X-gal plate). I will then transduce the active fusion into *expR101 exoY210, mucR12 exoY210,* and *exoY210,* and do the β-gal assay. Should the

gene be transcriptionally regulated by ExpR, the β-galactosidase activity of the *lacZ*-fused gene should be much higher in the *expR101 exoY210* background than in the *mucR12 exoY210* and *exoY210* backgrounds.

Succinoglycan

$$
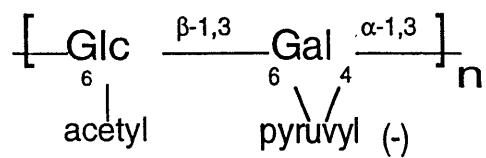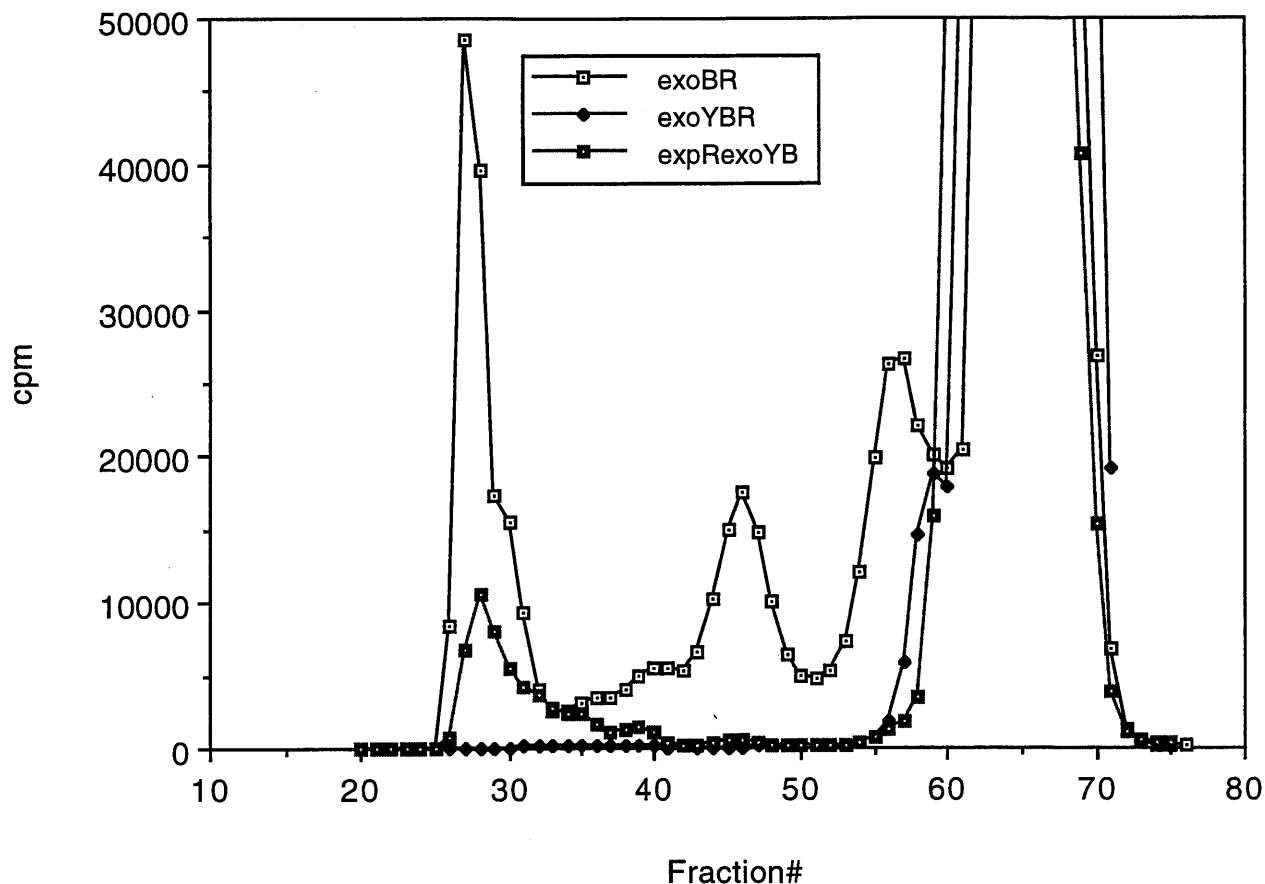\left[\text{Glc} \xrightarrow{\beta\text{-1,4}} \overset{6}{\text{Glc}} \xrightarrow{\beta\text{-1,4}} \text{Glc} \xrightarrow{\beta\text{-1,3}} \text{Gal} \xrightarrow{\beta\text{-1,4}} \right]_n
$$

Glc $\mid$ β-1,6
Glc — acetyl (under second Glc position 6)

Glc $\mid$ β-1,6

Glc $\mid$ β-1,3

$\overset{6}{\text{Glc}}$ —— succinyl (-) $\mid$ β-1,3

$\overset{4}{\underset{6}{\text{Glc}}}$ >pyruvyl (-)

EPS II

$$
\left[\overset{6}{\text{Glc}} \xrightarrow{\beta\text{-1,3}} \overset{6}{\underset{4}{\text{Gal}}} \xrightarrow{\alpha\text{-1,3}} \right]_n
$$

acetyl   pyruvyl (-)

Figure 1-1. Structures of succinoglycan and EPS II.

Strains + 11.6 mM of UDP-[14C]-galactose

Fig. 1-2. P4 gel filtration of radiolabeled material from culture supernatants of *exoR exoB*, *exoR exoY exoB, and expR exoY exoB* strains that have been electroporated with 11.6 mM UDP-[14C]-galactose and 35.7 mM UDP-glucose, and incubated at 30°C for 1 hr. Each reaction was pelleted, and washed three times with 70 mM Tris (pH 8.2). The supernatant and washes were combined for each reaction, and run on the P4. 1.25 ml fractions were collected. The counts per minute (cpm) of each strain was derived from the average of two experiments.

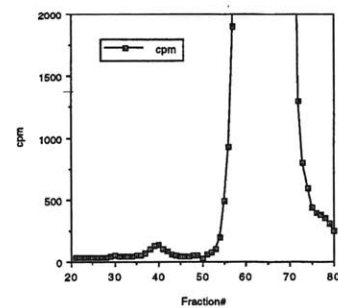expRexpAexoYexoB + 11.6 mM UDP-[14C]-galactose
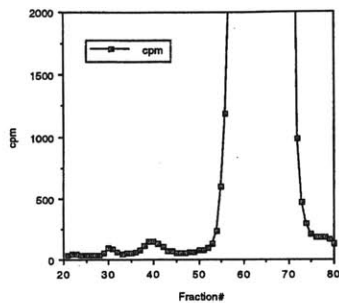
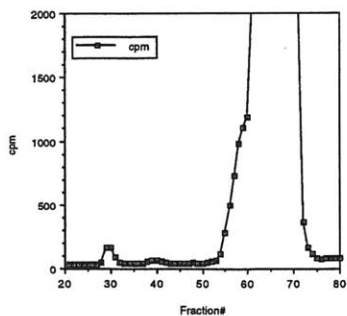expRexpCexoYexoB + 11.6 mM UDP-[14C]-galactose
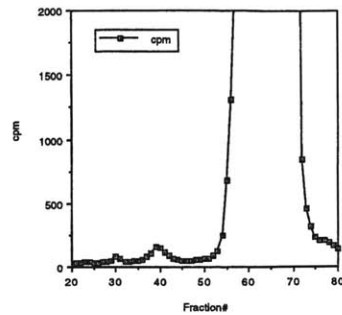
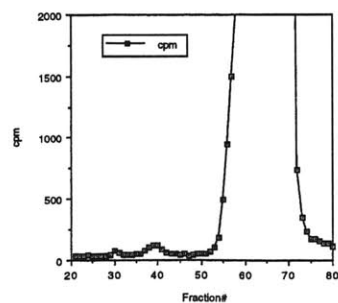expRexpDexoYexoB + 11.6 mM UDP-[14C]-galactose
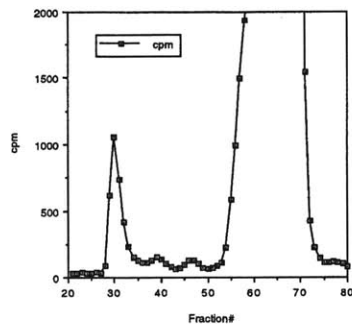
expRexpEexoYexoB + 11.6 mM UDP-[14C]-galactose

expRexpFexoYexoB + 11.6 mM UDP-[14C]-galactose

expRexpGexoYexoB + 11.6 mM UDP-[14C]-galactose

Fig. 1-3. P4 data of *expR101 exoY210 exoB24 exp* derivatives that had been electroporated with 11.6 mM UDP-[14C]-galactose and 35.7 mM UDP-glucose, and incubated at 30ºC for 1 hr. Each reaction was pelleted, and washed three times with 70 mM Tris (pH 8.2). The supernatant and washes were combined for each reaction, and run on the P4. 1.25 ml fractions were collected. The counts per minute (cpm) of each strain was derived from the average of two experiments.

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Chapter 2

Regulation of Extracellular ExoK Accumulation in *Rhizobium meliloti*

Abstract

   The exoK gene product has homology to known endo-1,3-1,4-β-glycanases and is capable of degrading succcinoglycan (1). York and Walker observed that ExoK [produced by Rm1021(*exoY*)] accumulated much less in GMS supernatants than it did in MGS supernatants (2). Interest in this observation resulted in a study to determine what conditions regulated extracellular/intracellular ExoK accumulation. Individual changes in the composition of GMS and MGS liquid media were made to find out what restricted or encouraged the extracellular/intracellular accumulation of ExoK [produced by Rm1021(*exoY*)]. These adjustments revealed that the presence of trace minerals (3), as well as the reduction of phosphate concentration, in GMS resulted in diminished extracellular ExoK accumulation. The next question addressed was which trace minerals reduced extracellular ExoK accumulation. Individual salts composing the trace minerals were excluded from GMS experiments or included in MGS in order to identify the salt(s) affecting extracellular ExoK accumulation. These variations showed that $FeCl_3$, $CoCl_2$, and $ZnSO_4$ had the most negative effect on extracellular ExoK accumulation. The next study tested whether pH affected extracellular accumulation of ExoK. Rm1021(*exoY*) was grown in MGS of varying pH to find out if pH (range 6.0-8.0) affects extracellular ExoK accumulation. It was found that this range had no effect on ExoK accumulation in the supernatant. Finally, there is interest as to what gene(s) is/are involved in the restriction of extracellular ExoK accumulation in GMS. The restriction of ExoK extracellular accumulation in GMS raises the possibility of a delayed halo phenotype in Rm1021 on solid GMS media. If this phenotype is confirmed, then random Tn5-233 mutagenesis of Rm1021(*exsH*) can be used to screen for a positive/negative regulator of ExoK.

Introduction

Rhizobium meliloti requires a low molecular weight (LMW) form of succinoglycan to successfully invade developing nodules of alfalfa (4, 5, 6). It is believed that a trimer of the basic octasaccharide subunit is active in invasion. One of the ways LMW succinoglycan could be produced is through enzymatic action. ExoK and ExsH are two proteins produced by genes belonging to the family of those encoding endo-1,3-1,4-β-glycanases (7, 8, 9). These proteins are believed at least partly responsible for LMW succinoglycan production for two other reasons.

The first is that the Rm1021(*exoK*) mutant requires six days instead of four to produce a fluorescent halo (under ultraviolet light) on MGS solid Calcofluor media (9). The Rm1021(*exoK exsH*) mutant produces no halo at all on MGS (9). Rm1021(*exoH*) is incapable of producing both a Calcofluor halo and LMW succinoglycan (6). Based on this, and the observation that Rm1021(*exoK*) and Rm1021(*exoK exsH*) strains produce less LMW than Rm1021 (9), the Calcofluor halo is associated with the capacity to produce LMW succinoglycan.

The second reason is that York and Walker (2) showed that ExoK and ExsH are able to cut succinoglycan while it is <u>actively</u> being produced from Rm1021(*exoK exsH*). In an *in vivo* experiment, York and Walker found that succinoglycan in cell-free supernatants from 3-day old GMS cultures cannot be cut by either enzyme. However, succinoglycan produced by the cells from those cultures can be cut if either enzyme is present during the 24 hr period of the experiment (2).

In addition, York has reported that ExoK produced by Rm1021(*exoY*) accumulates extracellularly in much less concentration in 3-5-day-old GMS cultures than in 3-5-day-old MGS cultures. Two possibilities were proposed: either ExoK was hardly ever secreted, or ExoK was quickly destroyed outside of the cells. If the former is assumed, then the condition(s) must be found in GMS that restricts ExoK secretion, as well as the gene(s) responding to those condition(s) by impeding secretion.

Methods and Materials

Media conditions regulating extracellular ExoK accumulation. The original media compositions were as follows: MGS [39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4), 55 mM mannitol, 5 mM monosodium glutamate (MGS), and 8 mM sodium chloride]; and GMS [6 mM potassium phosphate dibasic, 27.5 mM mannitol, 5 mM glutamic acid, and trace minerals (3), adjusted to pH 7.0 with NaOH]. MGS and GMS were supplemented with $MgSO_4$ (1 mM), $CaCl_2$ (0.25 mM), biotin (0.01 mg/ml), and thiamine (0.1 mg/ml) after autoclaving of media.

The GMS media adjustments were as follows: 1.) no NaCl->8 mM NaCl, 2.) trace minerals ->no trace minerals, 3.) 27.5 mM->55 mM mannitol, 4.) 6 mM potassium phosphate dibasic-> 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4), 5.) 5 mM glutamic acid-> 5 mM MSG, 6.) normal GMS, 7.) no NaCl->8 mM NaCl, trace minerals->no trace minerals, 8.) no NaCl->8 mM NaCl, 27.5 mM->55 mM mannitol, 9.) no NaCl->8 mM NaCl, 6 mM potassium phosphate dibasic-> 39 mM potassium phosphate monobasic, and 10.) no NaCl->8 mM NaCl, 5 mM glutamic acid-> 5 mM MSG.

The MGS media adjustments were as follows: 1.) 8 mM NaCl->no NaCl, 2.) no trace minerals->trace minerals, 3.) 55 mM->27.5 mM mannitol, 4.) 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4)->6 mM potassium phosphate dibasic, 5.) 5 mM MSG-> 5 mM glutamic acid, 6.) normal MGS, 7.) 8 mM NaCl->no NaCl, no trace minerals->trace minerals, 8.) 8 mM NaCl->no NaCl, 55 mM->27.5 mM mannitol, 9.) 8 mM NaCl->no NaCl, 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4)->6 mM potassium phosphate dibasic, 10.) 8 mM NaCl->no NaCl, 5 mM MSG-> 5 mM glutamic acid.

The volume of each adjusted medium was 5 ml. This was inoculated with 100 ml of saturated Rm1021(*exoY*), and incubated at 30°C for 72 hrs. 1 ml of each culture was centrifuged, and the supernatants were separated from the cells. The cells were resuspended in 0.5 ml of 0.85% saline. 5 ml of supernatant/pellet sample was mixed with 5 ml of 2X sample buffer (with 20% v/v β-mercaptoethanol), and run on 10% PAGE. Western transfers and blots were performed (with the use of ExoK polyclonal antibodies). Kodak XOMAT film was used for exposure of blots.

The resuspended pellet samples were then centrifuged, and the supernatants were removed. Saline was added to each pellet in a volume proportional to the original OD_{600} of its culture. These resuspended samples were run on 10% SDS-PAGE, and Western transfer and blots were performed.

What salts reduce extracellular ExoK accumulation? It is known that ExoK [expressed from Rm1021 (*exoY*)] accumulates extracellularly much more in MGS than in GMS. Recent experiments indicate that the presence of trace minerals (3) reduces considerably the extracellular accumulation of ExoK.

The trace minerals in GMS (3) are composed of 2.5 μg/ml $FeCl_3$, 0.01 μg/ml H_3BO_3, 0.01 μg/ml $CuSO_4$, 0.01 μg/ml $CoCl_2$, 0.01 μg/ml $ZnSO_4$, and 0.1 μg/ml $MnCl_2$. Before a screen can be done for the gene(s) in Rm1021 responding to the trace minerals, the individual salt(s) affecting extracellular ExoK accumulation must be found.

The original media compositions were as follows: MGS [22 mM potassium phosphate monobasic + 77 mM Na_2HPO_4 (pH 7.4), 55 mM mannitol, 5 mM monosodium glutamate (MGS), and 8 mM sodium chloride]; and GMS [6 mM potassium phosphate dibasic, 27.5 mM mannitol, 5 mM glutamic acid, and trace minerals (3), adjusted to pH 7.0 with NaOH]. MGS and GMS were supplemented with $MgSO_4$ (1 mM), $CaCl_2$ (0.25 mM), biotin (0.01 mg/ml), and thiamine (0.1 mg/ml) after autoclaving of media.

The GMS media variables were as follows: 1.) normal GMS, 2.) $FeCl_3$->no $FeCl_3$, 3.) H_3BO_3->no H_3BO_3, 4.) $CuSO_4$->no $CuSO_4$, 5.) $CoCl_2$->no $CoCl_2$, 6.) $ZnSO_4$->no $ZnSO_4$, 7.)$MnCl_2$->no $MnCl_2$.

The MGS media variables were as follows: 1.) normal MGS, 2.) no $FeCl_3$->$FeCl_3$, 3.) no H_3BO_3->H_3BO_3, 4.) no $CuSO_4$->$CuSO_4$, 5.) no $CoCl_2$->$CoCl_2$, 6.) no $ZnSO_4$->$ZnSO_4$, 7.) no $MnCl_2$->$MnCl_2$.

The volume of each variable was 5 ml. This was inoculated with 100 ml of saturated Rm1021(*exoY*) culture, and incubated at 30°C for 72 hrs. 1 ml of each culture was centrifuged, and both the supernatants and the cell pellets were kept. The cells were frozen at -20°C. The supernatant of each variable was lyophilized, and resuspended in a

volume of dH_2O proportional to the original OD_{600} of the culture. This was run on 10% PAGE, and Western transfer and blots were performed.

Effect of pH on extracellular ExoK accumulation. Because MGS is more heavily buffered than GMS, GMS is more vulnerable to pH change with increasing culture OD_{600}. This factor should be considered when determining why extracellular accumulation of ExoK is considerable reduced in GMS as opposed to MGS. Therefore, varying the pH of MGS media seemed an attractive posibility.

The original composition of MGS medium is: 22 mM potassium phosphate monobasic + 77 mM Na_2HPO_4 (pH 7.4), 55 mM mannitol, 5 mM monosodium glutamate (MGS), and 8 mM sodium chloride. MGS is supplemented with $MgS0_4$ (1 mM), $CaCl_2$ (0.25 mM), biotin (0.01 mg/ml), and thiamine (0.1 mg/ml) after autoclaving of media. The pH variables will be made by adjusting the concentrations of potasium phosphate monobasic and Na_2HPO_4.

The MGS media variables were as follows: 1.) 22 mM potassium phosphate monobasic + 77 mM Na_2HPO_4 (pH 7.4)->87.7 mM KH_2PO_4 + 12.3 mM Na_2PO_4 (pH 6.0), 2.) 22 mM KH_2PO_4 + 77 mM Na_2HPO_4 (pH 7.4)->68.5 mM KH_2PO_4 + 31.5 mM Na_2PO_4 (pH 6.5), 3.) 22 mM KH_2PO_4 + 77 mM Na_2HPO_4 (pH 7.4)->39 mM KH_2PO_4 + 61 mM Na_2PO_4 (pH 7.0), 4.) Normal MGS (pH 7.4), 5.) 22 mM KH_2PO_4 + 77 mM Na_2HPO_4 (pH 7.4)->16 mM KH_2PO_4 + 84 mM Na_2HPO_4 (pH 7.5), 6.) 22 mM KH_2PO_4 + 77 mM Na_2HPO_4 (pH 7.4)->5.3 mM KH_2PO + 94.7 mM Na_2HPO_4 (pH 8.0).

The volume of each variable was 5 ml. This was inoculated with 100 ml of saturated Rm1021(*exoY*) culture, and incubated at 30°C for 72 hrs. 1 ml of each culture was centrifuged, and both the supernatants and the cell pellets were retained. The cellular portion was frozen at -20°C. The supernatant of each variable was lyophilized, and resuspended in a volume of dH_2O proportional to the original OD_{600} of the culture. This was run on 10% PAGE, and Western transfer and blots were performed.

<u>Results</u>

Media conditions regulating extracellular ExoK accumulation. It was known that ExoK [expressed from Rm1021(*exoY*)] accumulates extracellularly much more in MGS than in GMS. Why this happens is related to the difference in composition between MGS and GMS. My aims were to 1.) change the molarity of a shared component to that of the other medium, and 2.) to remove one component from one medium, and place it (at its original molarity) in the other. The amount of ExoK secreted into each type of medium was determined by subjecting the culture supernatants to SDS-PAGE, transferring the proteins to a membrane and probing them with anti-ExoK antibody. The Western blots of cultures from adjusted MGS liquid media suggest that ExoK accumulation in the experiment is reduced considerably by 1.) 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4) ->6 mM potassium phosphate dibasic, and 2.) no trace minerals->trace minerals. The Western blot of cultures from adjusted GMS liquid media suggests that ExoK accumulation is possible only when 6 mM potassium phosphate dibasic->39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4).

The amount of ExoK in the cells grown in each medium was determined by centrifuging the cells, resuspending them in saline, subjecting them to SDS-PAGE, transferring the proteins to a membrane, and probing with anti-ExoK antibody. The first Western blot of cultures from adjusted MGS liquid media suggests that intracellular ExoK accumulation is somewhat reduced when 39 mM potassium phosphate monobasic + 61mM Na_2HPO_4 (pH 7.4) ->6 mM potassium phosphate dibasic. The second blot of the same cultures, however, shows equivalent intracellular ExoK accumulation (the signal on this blot was too strong for comparison). The first Western blot of cultures from adjusted GMS liquid media suggests that intracellular ExoK accumulation is somewhat increased when 6 mM potassium phosphate dibasic->39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4). The second blot of the same cultures, however, shows equivalent ExoK accumulation inside of cells (the signal on this blot was also too strong for comparison).

Effect of individual trace mineral salts on extracellular ExoK accumulation. It was determined that trace mineral salts diminished the extracellular accumulation of ExoK. From this there was interest into finding out which salt(s) negatively affected extracellular ExoK accumulation.

The Western blot of cultures from adjusted GMS liquid media suggests that ExoK accumulation is seen only when all trace minerals were removed. The removal of individual salts produced no visible ExoK accumulation.

The Western blot of cultures from adjusted MGS liquid media suggests that ExoK accumulation is reduced somewhat in the presence of H_3BO_3 and $CuSO_4$ salts. ExoK accumulation is almost absent in the presence of all trace minerals, $FeCl_3$, $CoCl_2$, and $ZnSO_4$. The absence of trace elements and the presence of $MnCl_2$ permitted the normal level of accumulation of ExoK.

Effect of pH on extracellular ExoK accumulation in MGS supernatants. The pH range from 6.0 to 8.0 had no effect on ExoK accumulation. ExoK accumulation for this pH range was at the level seen for MGS at pH 7.4.

Idea for the screen and the test of its feasibility. ExoK accumulates in much less concentration in GMS than in MGS. Therefore, Rm1021 may give a more delayed halo phenotype on solid GMS/Calcofluor media than on solid MGS/Calcofluor media. If this is true, then screening for one or more putative ExoK regulators is possible.

To see if the screen works, I planned to streak Rm1021, Rm1021 (*exsH*), and Rm1021(*exsH exoK*) on MGS/ Calcofluor and GMS/Calcofluor (both at pH of 7.0). Rm1021 (*exsH*::Tn5) is the strain of choice for the screen, because I expect it to be haloless on GMS Calcofluor media. Once I confirm that Rm1021 (*exsH*) has distinct phenotypes on MGS/Calcofluor and GMS/Calcofluor, then I will start the screen. I will first randomly mutagenize a population of Rm1021 with Tn5-233. I will then transduce pools of these insertion mutations into a population of Rm1021(*exsH*). These transductants will be selected on LB/Nm/Gm/Sp/Calcofluor plates, and individually streaked on MGS/Calcofluor and GMS/Calcofluor media. Mutations in a negative regulator may result in a normal halo phenotype on both MGS and GMS. Mutations in a positive regulator may result in a haloless phenotype on both MGS and GMS.

I have observed that Rm1021 and Rm1021(*exsH*) acquired halos within 48 hrs after streaking, both on GMS and MGS. Rm1021(*exoK*) acquired a halo within 5 days on MGS but not on GMS (through the seventh day of observation). Rm1021(*exoK exsH*) never acquired a halo throughout the entire 7 days of observation.

Rm1021 and Rm1021(*exsH*) display same-size haloes on both MGS and GMS from the second to the fifth day. For these strains halo size reaches maximum on the 5th day for GMS, but increases considerably for MGS from the 6th through the seventh day.

Discussion

Media conditions regulating extracellular ExoK accumulation. Results presented above indicate that extracellular accumulation of ExoK apparently requires the presence of 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4) in GMS and MGS liquid media, as well as the absence of trace elements in MGS. Above results also indicate that intracellular accumulation of ExoK apparently requires the presence of 39 mM potassium phosphate monobasic + 61 mM Na_2HPO_4 (pH 7.4) in GMS and MGS liquid media. In the future, more care will be taken to acquire a less heavy exposure of these blots on Kodak XOMAT film, making qualitative comparison of ExoK concentration from each medium adjustment possible.

Why do most of the trace minerals reduce or almost eliminate extracellular ExoK accumulation? Most of the minerals which restrict extracellular ExoK accumulation are found in Jensen's agar in much higher concentrations. Do these minerals, in addition to supplementing the plant, serve to regulate the ratio of high molecular weight to low molecular weight succinoglycan, in order to optimize nodule invasion? Plant inoculation experiments restricting or permitting the access of Rm1021 to these trace elements are feasible and must be performed.

pH (in a physiologically tolerable range for Rm1021) is not a factor in affecting extracellular ExoK accumulation.

Is a screen for gene(s) regulating ExoK extracellular accumulation possible? Rm1021(*exsH*) was not haloless on GMS as expected, but showed halos on both MGS and GMS. However, whereas the halo on GMS reached maximum size on the 5th day, the halo on MGS grew considerably in size through the 7th day. This suggests a somewhat distinct halo phenotypes for Rm1021(*exsH*) on GMS and MGS that can be screened for. This halo examination needs to repeated, but small patches of strains should be used instead of streaks.

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