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Regulation of *Rhizobium meliloti exo* Genes in Free-Living Cells and In Planta Examined by Using Tn*phoA* Fusions

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The *exo* loci of *Rhizobium meliloti* are necessary for the production of an acidic exopolysaccharide, EPS I, that is needed for alfalfa nodule invasion by strain Rm1021. We have isolated and characterized alkaline phosphatase fusions made with Tn*phoA* in several *exo* loci of *R. meliloti* and used these fusions to examine the subcellular localization of *exo* gene products and the regulation of *exo* genes in free-living cells and in planta. In the course of this work, we isolated a new *exo* locus, *exoT*. We have obtained evidence that several of the *exo* loci may encode membrane proteins. The activity of Tn*phoA* fusions in several *exo* loci is increased two- to fivefold in the presence of the regulatory mutations *exoR95* and *exoS96*. While examining the regulation of the *exo* genes by *exoR95* and *exoS96*, we found that certain classes of *exo* mutations are lethal in an *exoR95* or *exoS96* background unless a plasmid complementing the *exo* mutation is present. This result has possible implications for the role of these *exo* loci in EPS I biosynthesis. We have developed a method for staining nodules specifically for the alkaline phosphatase activity present in the inducing bacteria and used this method to show that an *exoF*::Tn*phoA* fusion is expressed mainly in the invasion zone of the nodule.

Rhizobium meliloti fixes nitrogen in symbiotic association with alfalfa. The bacteria induce nodules on the plant root, a process which requires a novel signal molecule produced by the *nod* genes (21). They then invade the nodules through tubes called infection threads, are enclosed in a plant-derived peribacteroid membrane, are released into plant cells, and then differentiate into nitrogen-fixing bacteroids (for reviews, see references 24 and 30). Alfalfa produces cylindrical nodules which are indeterminate (retain a distal meristem [14, 30]) and are pink due to the presence of leghemoglobin. New plant cells are continuously being produced and then invaded by bacteria, resulting in an early symbiotic or invasion zone behind the meristem. Farther toward the root is the late symbiotic zone, where plant cells are packed with mature, nitrogen-fixing bacteroids. In older nodules, there is a root-proximal senescent zone, where the bacteroids have degenerated (14).

We have previously shown that an acidic exopolysaccharide produced by strain Rm1021, EPS I, which fluoresces under long-wave UV light when bound to the dye Calcofluor White, is necessary for nodule invasion (11, 20, 22). Mutants defective in the synthesis of this exopolysaccharide fail to fluoresce under UV light on medium containing Calcofluor and elicit round, white nodules which do not contain bacteroids. A cluster of *exo* genes on the second of two symbiotic megaplasmids present in this strain, pRmeSU47b, is necessary for the production of this exopolysaccharide. Mutations in *exoA*, *exoB*, *exoF*, *exoL*, *exoM*, *exoP*, and *exoQ* were found to abolish the production of EPS I entirely (23). *exoH* mutants fail to succinylate EPS I and induce Fix$^-$ nodules (19). *exoG* and *exoJ* mutants produce less EPS I and invade nodules at reduced efficiency. *exoN* mutants also produce less EPS I but invade nodules normally. *exoK* mutations decrease EPS I production and cause a delay in the appearance of a halo of fluorescence on Calcofluor plates but do not affect nodulation (23).

Two loci that regulate the synthesis of EPS I have been described, *exoR* and *exoS* (9). Strains carrying transposon Tn*5*-generated mutations in these loci produce greatly increased amounts of EPS I and form very mucoid colonies. *exoS* mutants induce normal nodules. *exoR* mutants, however, are Fix$^-$. When plants are inoculated with *exoR* mutants, some Fix$^+$ nodules are produced, but all bacteria isolated from these nodules have acquired unlinked suppressors which reduce the amount of EPS I produced by the strains (9). This implies that some regulation of EPS I synthesis is needed for effective nodulation.

We have previously described the use of Tn*phoA* to produce translational gene fusions to alkaline phosphatase in *R. meliloti* (22). Tn*phoA* is a Tn*5* derivative carrying an *Escherichia coli* alkaline phosphatase gene lacking a signal sequence (26). Alkaline phosphatase is inactive in the cytoplasm, apparently because it must dimerize to be active, and the environment in the cytoplasm of the cell is too reducing for the formation of the disulfide bond necessary for dimerization. Therefore, active fusions are obtained only to membrane or periplasmic proteins. In a previous screen of strains carrying active Tn*phoA* fusions for mutants defective in symbiosis (22), Tn*phoA* fusions to two *exo* genes were isolated. In this work we have isolated and characterized Tn*phoA*-generated fusions to several *exo* genes and used these fusions to study the cellular localization of *exo* gene products and *exo* gene regulation in free-living cells and in planta.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains, plasmids, and phage strains are listed in Table 1.

Media. Bacteria were grown in LB medium (25), with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ added for *R. meliloti* cultures. Antibiotics were used at the following concentrations for *R. meliloti*: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; tetracycline, 10 μg/ml; gentamicin, 20 μg/ml; spectinomycin, 100 μg/ml. For *E. coli*, the following concentrations were used: chloramphenicol, 20 μg/ml; kanamycin, 25

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or phage	Relevant genotype or characteristics	Source or reference
R. meliloti		
Rm1021	SU47 Sm^r	F. Ausubel
Rm7031	Rm1021 *exoA31*::Tn*5*	20
Rm7055	Rm1021 *exoF55*::Tn*5*	20
Rm7095	Rm1021 *exoR95*::Tn*5*	9
Rm7096	Rm1021 *exoS96*::Tn*5*	9
Rm8002	Rm1021 Pho$^-$	22
Rm8264	Rm8002 *exoA264*::Tn*phoA*	This work
Rm8265	Rm8002 *exoF265*::Tn*phoA*	This work
Rm8266	Rm8002 *exoF266*::Tn*phoA*	This work
Rm8267	Rm8002 *exoF267*::Tn*phoA*	This work
Rm8268	Rm8002 *exoF268*::Tn*phoA*	This work
Rm8269	Rm8002 *exoP269*::Tn*phoA*	This work
Rm8270	Rm8002 *exoP270*::Tn*phoA*	This work
Rm8271	Rm8002 *exoP271*::Tn*phoA*	This work
Rm8272	Rm8002 *exoP272*::Tn*phoA*	This work
Rm8273	Rm8002 *exoQ273*::Tn*phoA*	This work
Rm8274	Rm8002 *exoT274*::Tn*phoA*	This work
Rm8295	Rm8002 *exoR95*::Tn*5-233*	This work[a]
Rm8296	Rm8002 *exoS96*::Tn*5-233*	This work[a]
Rm8302	Rm1021 *exoG302*::Tn*5*	23
Rm8319	Rm1021 *exoJ319*::Tn*5*	23
Rm8332	Rm1021 *exoQ332*::Tn*5*	23
Rm8347	Rm1021 *exoB347*::Tn*5*	23
Rm8359	Rm8002 *exoB359*::Tn*phoA*	23
Rm8365	Rm8002 *exoP365*::Tn*phoA*	22
Rm8366	Rm8002 *exoP366*::Tn*phoA*	22
Rm8369	Rm8002 *exoF369*::Tn*phoA*	22
Rm8416	Rm1021 *exoN416*::Tn*5*	23
Rm8428	Rm1021 *exoH428*::Tn*5*	23
Rm8431	Rm1021 *exoL431*::Tn*5*	23
Rm8439	Rm1021 *exoP439*::Tn*5*	23
Rm8442	Rm1021 *exoP442*::Tn*5*	23
Rm8449	Rm1021 *exoP449*::Tn*5*	23
Rm8457	Rm1021 *exoM457*::Tn*5*	23
Rm8468	Rm1021 *exoP468*::Tn*5*	23
Rm8476	Rm1021 *exoK476*::Tn*5*	23
Plasmids		
pRK600	pRK2013*npt*::Tn*9*	12
pRK609	pRK600ΩTn*phoA*	22
pD56	Tc^r pLAFR1 cosmid complementing *exoB*, *exoQ*, and *exoF*	23
pEX154	Tc^r pLAFR1 cosmid complementing *exoA* and *exoP*	23
pEX312	Tc^r pLAFR1 cosmid complementing *exoP*, *exoA*, *exoT*, *exoF*, and *exoQ*	23
pEX20	Tc^r pSUP104-based subclone complementing *exoA* and *exoP*	23
pEX31	Tc^r pRK404-based subclone complementing *exoB*	23
pRmT8	Tc^r pLAFR1 cosmid complementing *dctA*	12, 39
Bacteriophages		
ϕM-12	General transducing phage for *R. meliloti*	10
ϕM-1, ϕM-5, ϕM-6, ϕM-7, ϕM-9, ϕM-10, ϕM-11, ϕM-14	*R. meliloti* bacteriophages	E. Signer

[a] *exoR95*::Tn*5-233* and *exoS96*::Tn*5-233* were constructed by replacing the Tn*5* insertions in *exoR95* and *exoS96* with Tn*5-233* by recombination. Tn*5-233* is a gentamicin/kanamycin- and streptomycin/spectinomycin-resistant derivative of Tn*5* (7).

μg/ml. Calcofluor White M2R (Cellufluor; Polysciences, Warrington, Pa.) and XP (5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt; Sigma, St. Louis, Mo.) were added to agar buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 7.4 to concentrations of 200 and 40 μg/ml, respectively.

Genetic manipulations. Transductions with ϕM12 were performed as described previously (10). Mobilization of pLAFR1-derived cosmids and *exo* subclones into *R. meliloti* was performed by triparental matings with pRK600 to provide transfer functions as described before (20). Tn*phoA* mutagenesis of *R. meliloti* was performed by mating the transposon-containing plasmid pRK609, which cannot replicate in *Rhizobium* spp., into Rm8002 and selecting Nm^r transconjugants as described by Long et al. (22). Tn*phoA* mutagenesis of plasmids in *E. coli* was performed by using λTn*phoA* to infect *E. coli* carrying the plasmid as described before (22). Insertions on plasmids were homogenotized into the *R. meliloti* genome by mating an incompatible plasmid into the strain as described before (31).

Enzyme assays, plant nodulation assays, and phage sensitivity tests. Alkaline phosphatase assays were performed by the method of Brickman and Beckwith (3) as modified by Long et al. (22). *Medicago sativa* cv. Iroquois was obtained from Agway, Inc. (Plymouth, Ind.). Plant nodulation assays were performed as described before (20). Phage sensitivity was determined by spot tests (15), in which samples (5 μl) of lysates of the *R. meliloti* phages ϕM-1, ϕM-5, ϕM-6, ϕM-7, ϕM-9, ϕM-10, ϕM-11, ϕM-12, and ϕM-14 were spotted onto a lawn of an *R. meliloti* strain.

DNA manipulations and Southern hybridization. Chromosomal DNA was isolated from *R. meliloti* by the method of Marmur (27). Restriction enzyme digests were performed according to the specifications of the supplier (New England BioLabs, Beverly, Mass.). The DNA probes were labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.) with a nick translation kit (Bethesda Research Laboratories, Gaithersburg, Md.). Southern blotting and hybridization to GeneScreen Plus (New England Nuclear, Boston, Mass.) were performed according to the manufacturer's instructions.

Cell fractionation. The method of de Maagd and Lugtenberg (6) as modified by Long et al. (22) was used for cell fractionation.

Isolation of bacteroids. Peribacteroid membrane-enclosed bacteroids were isolated by a modification of the method of Verma et al. (37). Sterile alfalfa seeds (4 g) were planted in Perlite saturated with Jensen's medium (38) in a plastic dishpan (30 by 34 cm). The seedlings were inoculated 1 week after planting with bacteria from a 200-ml late-log-phase or saturated culture which had been washed with Jensen's medium and resuspended in 500 ml of Jensen's medium. The plants were kept covered with Saran Wrap through 1 week after inoculation and then left uncovered. Nodules were harvested 27 to 29 days after inoculation into ice-cold 10 mM HEPES (pH 7.0), drained, and gently crushed in 8 ml of buffer A (16% sucrose, 10 mM HEPES [pH 7.0]) with a mortar and pestle. The resulting solution was filtered through Miracloth (Calbiochem, San Diego, Calif.), and the filtrate was centrifuged at 6,000 × *g* for 10 min at 4°C. The pellet was resuspended in 2 ml of buffer A and layered over a step sucrose gradient containing 3 ml of 60%, 4 ml of 45%, and 3 ml of 34% (wt/vol) sucrose in 10 mM HEPES, pH 7.0. The gradients were centrifuged at 150,000 × *g* for 60 min at 4°C in a swinging-bucket rotor. Peribacteroid membrane-enclosed bacteroids were then recovered from the 45-60% sucrose interface with a Pasteur pipette. The solution con-

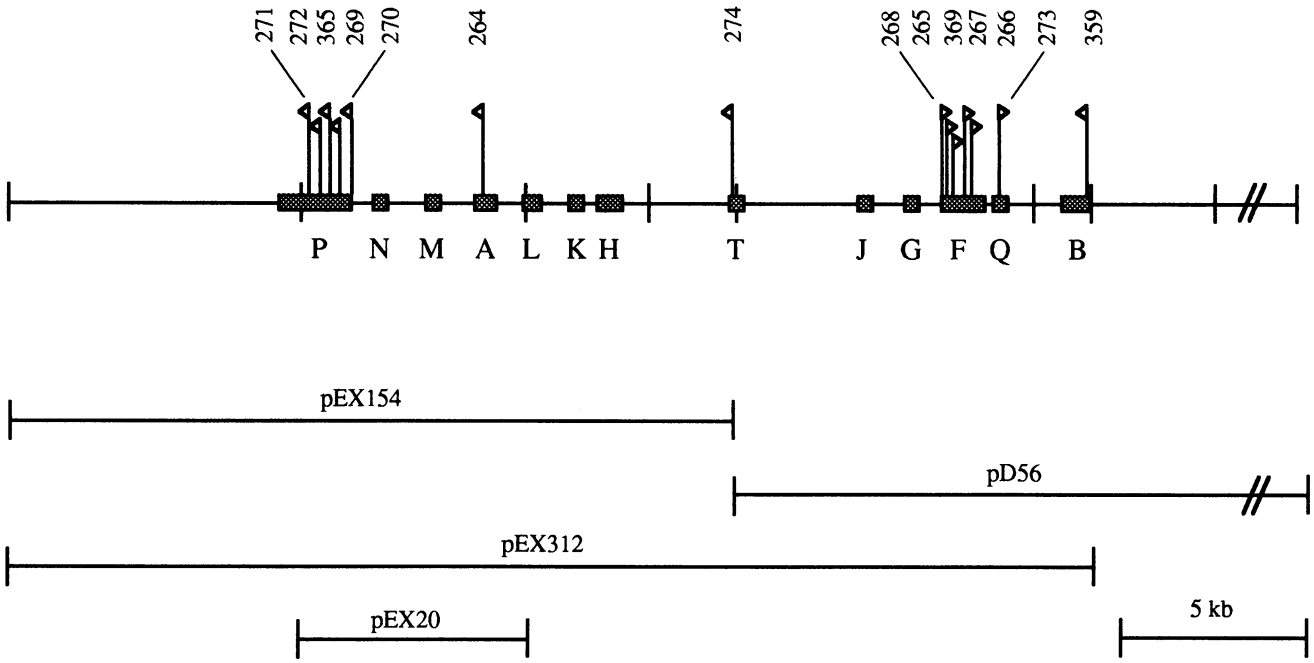

FIG. 1. *exo* region of pRmeSU47b and relevant plasmids. Tn*phoA* insertions are denoted by arrows, with the allele numbers given above. pD56 extends for approximately 19 kb past *exoB*.

taining the bacteroids was diluted by 50% with ice-cold 10 mM HEPES and centrifuged at 6,000 × *g* for 10 min at 4°C. The bacteroid pellet was then washed briefly in 1 M Tris, pH 8.0, and resuspended in this solution for alkaline phosphatase assays. To reduce the background alkaline phosphatase activity present in Pho$^-$ bacteroids, bacteroid samples were sometimes heated to 65°C for 10 min before they were assayed. After being heated, they were quickly cooled on ice to room temperature before disruption with sodium dodecyl sulfate (SDS) and chloroform for the assay.

Histochemical staining of nodules. Nodules were harvested 3 to 4 weeks after inoculation of plants and fixed on ice in a solution containing 3.5% glutaraldehyde, 1.5% paraformaldehyde (EM grade; Polysciences), and 0.1 M cacodylate, pH 7.2, for 2 to 18 h. The nodules were rinsed twice with 0.1 M Tris (pH 9.0)–1 mM $MgCl_2$ for 30 min per rinse. The nodules were then affixed to a carrot slice with cyanoacrylate adhesive (Crazy Glue) and sectioned on a Polaron H1200 vibrating microtome (Bio-Rad Microscience Division, Cambridge, Mass.). Sections (50 μm thick) were stained overnight at 37°C in a solution containing 0.1 M Tris (pH 9.0), 1 mM $MgCl_2$, and 1.6 mg of XP per ml (20 μl of an 80-mg/ml stock in dimethyl sulfoxide per ml of Tris).

RESULTS

Isolation of *exo*::Tn*phoA* fusions and identification of a new *exo* locus. The previously described alkaline phosphatase gene fusions to *exoF* and *exoP* (22) represented only 2 of the 11 known *exo* loci on the second symbiotic megaplasmid. In an attempt to isolate Tn*phoA* fusions to other *exo* loci, we mutagenized a Pho$^-$ derivative of Rm1021 with Tn*phoA* and screened the resulting neomycin-resistant colonies for lack of fluorescence under UV light on medium containing Calcofluor. Nonfluorescent strains were then examined for alkaline phosphatase activity, as indicated by blue color on medium containing XP. Of 4 × 10^5 neomycin-resistant colonies, 347 were nonfluorescent on Calcofluor, and of these, 10 independent mutants carried active Tn*phoA* fusions to *exo* loci.

In order to assign these insertions to the various *exo* complementation groups we have described previously (22), cosmids encompassing the *exo* region of the second symbiotic megaplasmid (Fig. 1) and derivatives of these cosmids carrying insertions in *exo* genes were introduced into each strain, and the resulting merodiploids were examined for Calcofluor fluorescence. By this procedure we were able to assign 9 of the 10 fusions to known *exo* complementation groups: one fusion to *exoA*, three to *exoF*, four to *exoP*, and one to *exoQ*. We also established the map positions of the insertions by Southern hybridization analysis. As shown in Fig. 1, the insertions all mapped to positions corresponding to their assigned complementation groups.

The 10th *exo*::Tn*phoA* fusion was not assignable to any of the previously described complementation groups, but mapped near the center of the *exo* region. It was complemented by pEX312 but not by pD56 or pEX154, suggesting that it lies in a locus that spans the adjacent *Eco*RI site (Fig. 1). This location at the junction of pD56 and pEX154 could explain why this locus was missed in our previous mutagenesis of the *exo* region (22), which was performed by homogenotizing Tn*5* insertions made in the cosmids pD56 and pEx154 into the *R. meliloti* genome. The new mutant resembled previously described Exo$^-$ strains: it was nonfluorescent ("dark") under UV light when grown on medium containing Calcofluor and produced round, white, empty, Fix$^-$ nodules on alfalfa. Thus, we designated this locus *exoT*. Because *exoB* mutants have an altered pattern of phage sensitivity, which is probably due to cell surface alterations (11, 20), the *exoT* strain was checked for resistance to nine *R. meliloti* phages. It was found to be sensitive to all phages tested, as are all *exo* mutants except *exoB* mutants.

TABLE 2. Alkaline phosphatase activities of strains carrying *exo*::Tn*phoA* fusions

Strain	Relevant genotype	Alkaline phosphatase activity[a] (U)
Rm8002	Rm1021 Pho$^-$	1.4
Rm8264	*exoA264*::Tn*phoA*	14
Rm8359	*exoB359*::Tn*phoA*	4.0
Rm8265	*exoF265*::Tn*phoA*	25
Rm8266	*exoF266*::Tn*phoA*	20
Rm8267	*exoF267*::Tn*phoA*	30
Rm8268	*exoF268*::Tn*phoA*	27
Rm8369	*exoF369*::Tn*phoA*	27
Rm8269	*exoP269*::Tn*phoA*	11
Rm8270	*exoP270*::Tn*phoA*	10
Rm8271	*exoP271*::Tn*phoA*	13
Rm8272	*exoP272*::Tn*phoA*	25
Rm8365	*exoP365*::Tn*phoA*	16
Rm8273	*exoQ273*::Tn*phoA*	6.3
Rm8274	*exoT274*::Tn*phoA*	5.4

[a] Alkaline phosphatase activity is expressed as arbitrary units; 1 U = (OD_{420}/min · ml · OD_{600}) × 10^3.

TABLE 3. Distribution of alkaline phosphatase activity in strains carrying *exo*::Tn*phoA* fusions[a]

Fusion	Alkaline phosphatase activity[b] (U/mg of protein)	% of activity in:		
		Periplasm	Membrane	Cytoplasm
exoA264	161	32	64	4
exoF369[c]	739	56	28	16
exoP369[c]	286	53	44	3
exoQ273	108	51	43	6
exoT274	91	70	4	26
exoT274 exoR	130	88	6	6

[a] Alkaline phosphatase activity and activity distribution values are averages of three experiments.
[b] Defined as (OD_{420}/min · ml · mg of protein) × 10^3.
[c] Data from Long et al. (22).

In an independent attempt to isolate fusions to other *exo* genes, λTn*phoA* was used to mutagenize subcloned *exo* genes that were present on high-copy-number plasmids in *E. coli*. These plasmids were then transferred to *R. meliloti* by conjugation and screened for alkaline phosphatase activity. Although we did obtain some additional *exoF* and *exoP* fusions and a weakly expressed fusion to *exoB* by this method, it proved more difficult than the direct Tn*phoA* mutagenesis of *R. meliloti* described above. Exo$^+$ *R. meliloti* strains carrying Tn*phoA* fusions on these high-copy plasmids grew poorly, and variants expressing altered levels of alkaline phosphatase accumulated in cultures. The strains were stable when the fusions were homogenotized into the megaplasmid, suggesting that the instability is due to the high copy number of the plasmids encoding the fusion proteins. Overproduction of fusion proteins may be deleterious to the cell, causing selection against expression of the fusion proteins. The *exoF* and *exoP* fusions obtained by this method were not characterized further.

In total, we isolated Tn*phoA* fusions to six *exo* loci. Table 2 shows the levels of alkaline phosphatase activity in strains carrying the *exo*::Tn*phoA* fusions isolated in this work and also that in the strains carrying the *exoF369*::Tn*phoA* and *exoP365*::Tn*phoA* fusions previously described by Long et al. (22). Strains carrying fusions in different *exo* loci showed different ranges of alkaline phosphatase activity. In general, strains carrying the *exoF*::Tn*phoA* fusions had the highest activity, those carrying the *exoP* and *exoA* fusions showed intermediate levels of activity, and those carrying the *exoT*, *exoQ*, and *exoB* fusions showed very low activity. With the exception of a single *exoP*::Tn*phoA* fusion, *exoP272*, the levels of alkaline phosphatase activity were relatively consistent among strains with different Tn*phoA* fusions in the same *exo* locus.

Localization of *exo* gene products. Translational fusions made by using Tn*phoA* can be used to gain information about the subcellular locations of the gene products by assaying alkaline phosphatase activity in cell fractions (22). We have previously shown that strains carrying *exoF369*::Tn*phoA* or *exoP365*::Tn*phoA* fusions show a distribution of activity between the membrane and periplasmic fractions, with the greater portion in the membrane (22) (Table 3). These results are consistent with the hypothesis that the *exoF* and *exoP* products are membrane proteins, because it has been shown that Tn*phoA* fusions are vulnerable to cleavage near the fusion joint, which releases a protein near in size to intact *E. coli* alkaline phosphatase (47 kDa) into the periplasm (22, 26). A band of this size that cross-reacted with antibody to *E. coli* alkaline phosphatase was seen in Western immunoblots of the *exoF369* and *exoP365* mutants (22). We found that in *exoA264* and *exoQ273* strains there was also a distribution of alkaline phosphatase activity between the membrane and periplasmic fractions, with an average of 64% of the total recovered activity in the membrane fraction for the *exoA264* strain and 43% for the *exoQ273* strain. It seems likely, therefore, that these proteins could also be membrane proteins. In the *exoT274* strain, however, there was a preponderance of activity in the periplasmic fraction, with less than 5% associated with the membrane fraction. Because the low alkaline phosphatase activity of the *exoT274* strain made it difficult to recover sufficient activity during fractionation to accurately determine the localization of the activity, the fractionation was repeated with an *exoT274 exoR95* strain to increase the recoverable activity. This strain has higher alkaline phosphatase activity (see below), but most of this activity was still localized in the periplasmic fraction. This observation might suggest that the *exoT* product is located in the periplasm. This result must be interpreted with caution, however, because of the cleavage of fusion proteins discussed above.

The *exoA264* strain showed a 47-kDa cleavage product that cross-reacted with antibody to *E. coli* alkaline phosphatase in Western blots, as well as a 70-kDa fusion product (data not shown). Strains containing the *exoT274* and *exoQ273* fusions also showed a band at 47 kDa, but no fusion products were visible. This could indicate that the fusion products are rapidly cleaved to release alkaline phosphatase activity into the periplasm. It is also possible that the fusions are near the 5′ ends of their respective genes, and therefore the fusion protein is not significantly larger than the alkaline phosphatase moiety. Nothing is yet known about the size of the *exoT* locus because it is defined by a single Tn*phoA* insertion. It is not yet possible, therefore, to determine where the fusion lies in the locus, and we cannot make a conclusion about the localization of the *exoT* gene product. The *exoQ* locus is less than 0.6 kb long (23). Because the *exoQ273* strain did give activity in the membrane fraction when fractionated, it is possible that the *exoQ* fusion protein is not significantly larger than alkaline phosphatase. Because of the extremely low activity of the *exoB359* fusion, we were

TABLE 4. Viability of *exo* mutants upon introduction of *exoR95* and *exoS96*

Mutation	Calcofluor phenotype	Viability[a] in combination with *exoR95* and *exoS96*	
		Uncomplemented	Complemented
exoA31	Dark	+	+
exoB347	Dark	+	+
exoF55	Dark	+	+
exoL431	Dark	−	+
exoM457	Dark	−	+
exoP468	Dark	−	+
exoQ332	Dark	−	+
exoT274	Dark	−	+
exoG302	Dim	+	ND
exoJ319	Dim	+	ND
exoN416	Dim	+	ND
exoH428	Haloless	+	ND
exoK476	Delayed halo	+	ND

[a] Symbols: +, viable; −, not viable; ND, not determined.

unable to recover sufficient activity to determine the location of its gene product.

Certain classes of Exo⁻ mutations are lethal in *exoR95* or *exoS96* backgrounds. The isolation of fusions to *exo* genes makes it possible to examine their regulation in free-living cells and in planta. We have previously reported that the expression of the *exoF369*::Tn*phoA* and *exoP365*::Tn*phoA* fusions is increased in a strain containing either *exoR95* or *exoS96*, which cause overproduction of EPS I (9). These double mutant strains were constructed by transducing the *exoR95*::Tn*5-233* or *exoS96*::Tn*5-233* mutation into a strain carrying the *exoF*::Tn*phoA* or *exoP*::Tn*phoA* fusion and a plasmid complementing the *exoF* or *exoP* mutation and subsequently curing the complementing plasmid. To examine the regulation of the new Tn*phoA* fusions, we attempted to construct double mutants more directly by transducing the *exoR95*::Tn*5-233* or *exoS96*::Tn*5-233* mutations into uncomplemented *exo*::Tn*phoA* strains. However, we were unable to construct these double mutant strains with strains carrying fusions to *exoQ273* and *exoT274*, although they were easily constructed with strains carrying fusions to *exoA264* and *exoB359*. We were also unable to transduce the *exoQ273* and *exoT274* fusions into strains carrying the *exoR95*::Tn*5-233* or *exoS96*::Tn*5-233* mutation.

Two hypotheses might account for these observations: (i) certain of the chimeric proteins encoded by *exo*::Tn*phoA* fusions are lethal when overproduced, as hypothesized for the plasmids carrying *exo*::Tn*phoA* fusions, or (ii) blocking EPS I synthesis at certain stages is lethal in an *exoR95* or *exoS96* background, perhaps due to toxic accumulation of intermediates. To distinguish between these possibilities, we transduced *exoR95*::Tn*5-233* and *exoS96*::Tn*5-233* mutations into strains carrying Tn*5* mutations in various *exo* genes and into the *exoT274*::Tn*phoA* strain. The results (Table 4) show that *exo* mutations are divided into two classes based on viability in combination with *exoR95* and *exoS96*. *exoR95* and *exoS96* were lethal in combination with *exoL431*, *exoM457*, *exoP468*, *exoQ332*, and *exoT274*, all of which exhibit a Calcofluor-dark phenotype and fail to synthesize EPS I (23). In contrast, they were not lethal in combination with the other Calcofluor-dark mutations *exoA31*, *exoB347*, and *exoF55*, nor were they lethal in combination with the *exoG302*, *exoJ319*, and *exoN416* mutations, which cause a "dim" phenotype on Calcofluor because of EPS I underproduction (23); the *exoH428* mutation, which causes failure to succinylate EPS I (19); or the *exoK476* mutation, which causes a delayed-halo phenotype (23).

The fact that some *exo*::Tn*5* mutations were also lethal in combination with *exoR95* and *exoS96* suggests that overproduction of Tn*phoA* fusion proteins was not responsible for the observed lethality. However, it was still possible that truncated *exo* proteins produced in the Tn*5* mutants could be toxic at high levels. Therefore, we attempted to transduce *exoR95*::Tn*5-233* and *exoS96*::Tn*5-233* into various *exo*::Tn*5* and *exo*::Tn*phoA* strains carrying a cosmid complementing the *exo*::Tn*5* or *exo*::Tn*phoA* mutation. In all cases, the *exoR95 exo* and *exoS96 exo* double mutant strains could be constructed in the presence of a complementing plasmid (Table 4). Thus, the block in EPS I biosynthesis rather than accumulation of abnormal proteins is responsible for lethality.

Two classes of *exoP* alleles. The result that the *exoP468* mutation was lethal in combination with *exoR95* and *exoS96* was unexpected because, as described above, an *exoP365 exoR95* double mutant was previously constructed and cured of the plasmid which complemented the *exoP365* mutation (9). To test the hypothesis that there might be two classes of *exoP* alleles, we examined a number of *exoP*::Tn*5* and *exoP*::Tn*phoA* insertions. We found that the *exoP* alleles tested did fall into two groups, as shown in Fig. 2. A cluster of insertions in the proximal portion of the *exoP* locus were lethal in combination with *exoR95* or *exoS96*, but three other insertions in the more distal end of the locus were not. Interestingly, all *exoP* mutations are complemented by a subclone, pEx20 (Fig. 1), containing only the portion of *exoP* to the right of the *Eco*RI site (23). It is possible that the insertions to the left of the *Eco*RI site allow some gene function. The mutant carrying *exoP442*, one of the *exoP*::Tn*5* mutations that was not lethal in an *exoR95* or *exoS96* background, was previously shown to have material in the culture supernatant that reacts with the hexose-detecting reagent anthrone (23). No Calcofluor fluorescence, however, was observed in these strains or in the *exoP442 exoR95* and *exoP442 exoS96* double mutants. Surprisingly, the *exoP365* mutation was lethal in combination with *exoR95* and *exoS96*. We therefore suspect that the previously described *exoP365 exoR* and *exoP365 exoS* double mutants (9) have acquired another mutation which allows survival without a complementing plasmid.

Coordinate regulation of *exo* genes. We used the *exo*::Tn*phoA exoR95* and *exo*::Tn*phoA exoS96* strains we had constructed to examine the regulation of the *exo* genes by *exoR* and *exoS* by comparing the alkaline phosphatase activity of the *exo*::Tn*phoA* fusions in wild-type, *exoR95*, and *exoS96* backgrounds. To be consistent, all *exoR95* and *exoS96* derivatives contained a cosmid complementing the *exo*::Tn*phoA* mutation whether it was necessary for survival of the strain or not. As shown in Table 5, all *exo*::Tn*phoA* fusions except that to *exoB* showed a two- to fivefold increase in alkaline phosphatase activity in an *exoR95* background and a two- to fourfold increase in alkaline phosphatase activity in an *exoS96* background.

It is perhaps not surprising that *exoB* is not regulated by *exoR* and *exoS* because *exoB* mutants are also defective for the production of a second exopolysaccharide, EPS II (13, 41), and show lipopolysaccharide alterations (4, 18). Recently it has been shown that the *exoB* gene encodes UDP-galactose epimerase, which converts UDP-glucose to UDP-galactose (3a). (It should be noted that in *R. meliloti*, UDP-galactose epimerase is not involved in galactose catab-

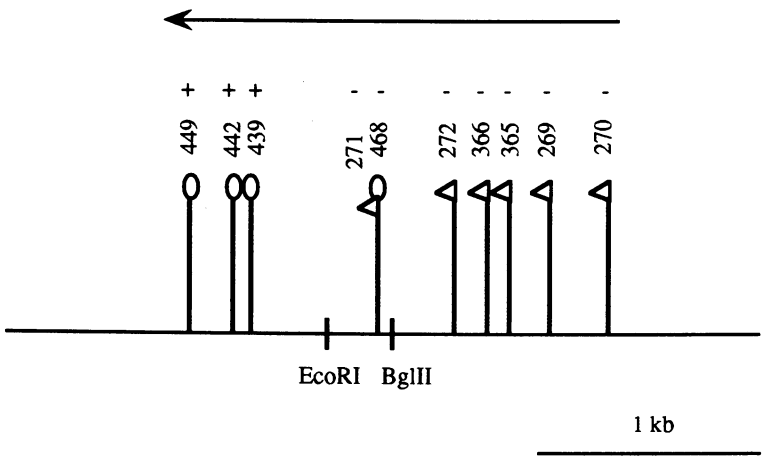

FIG. 2. Viability of *exoP* alleles in *exoR95* and *exoS96* backgrounds. A circle denotes a Tn*5* insertion, and an arrowhead denotes a Tn*phoA* insertion. Direction of transcription is from right to left, as indicated by the large arrow. Symbols: +, viability in an *exoR95* or *exoS96* background; −, inviability.

olism [1], and therefore *exoB* mutants are not galactose sensitive.) Thus, *exoB* has a role in synthesizing other polymers and would not necessarily be expected to be regulated in the same manner as the other *exo* genes. None of the other *exo* loci on the second symbiotic megaplasmid are necessary for EPS II production (28a), and none have been shown to have lipopolysaccharide alterations. Therefore, these data suggest that the expression of at least five of the *exo* loci which are specific to EPS I production is controlled by some type of regulatory circuit involving the ExoR and ExoS proteins.

***exo* fusions are expressed at low levels in bacteroids.** EPS I is known to be needed for invasion of nodules by bacteria. However, it is not known whether there is a further requirement for EPS I at later stages of nodulation. Keller et al. (16) have reported that *lacZ* fusions to some *exo* genes produce activity in a crude bacteroid preparation. However, it is unclear whether contaminating undifferentiated bacteria were removed by their procedure. Also, the recovered activity was expressed only as units per milligram of nodule wet weight, and therefore it cannot be compared with the activity present in free-living cells. We investigated the expression of *exo*::Tn*phoA* fusions in purified bacteroids. Plants were inoculated with *exoF369*, *exoP365*, or *exoA264* mutant strains containing appropriate complementing cosmids so that they could form effective nodules. Rm8002, the Pho$^-$ strain in which the *exo*::Tn*phoA* fusions were constructed (22), served as a negative control for alkaline phosphatase activity. Rm8384, which contains a Tn*phoA* fusion to *dctA* (22), served as a positive control, because DctA is known to be needed in the nodule. Peribacteroid membrane-enclosed bacteroids were isolated from nodules approximately 28 days after inoculation and purified on a sucrose gradient, and alkaline phosphatase activity was assayed.

Bacteroids derived from the Pho$^-$ strain Rm8002 exhibited a low level of alkaline phosphatase activity, 12 U/mg of protein, presumably from residual *R. meliloti* alkaline phosphatase or contaminating plant activity. We were able to reduce this background activity to 3 U/mg of protein by taking advantage of the heat stability of *E. coli* alkaline phosphatase and heating the bacteroids to 65°C for 10 min before assaying alkaline phosphatase. In free-living cells, *exo*::Tn*phoA* fusions retained greater than 90% of their alkaline phosphatase activity after this treatment, while *R. meliloti* alkaline phosphatase was inactivated. As shown in Table 6, the *exo*::Tn*phoA* fusions were expressed in the isolated bacteroids, but only at levels ranging from 1 to 4% of their level of expression in free-living cells when normalized to the amount of protein. However, it is possible that this represents an underestimate of the level of *exo* gene expression in bacteroids since the level of expression of the fusion to *dctA*, which is known to be needed in the nodule, was only 5.5% of its level of expression in free-living cells.

TABLE 5. Alkaline phosphatase activity of *exo*::Tn*phoA* fusions in *exoR95* and *exoS96* backgrounds

Fusion	Activity[a] (U) in background:		
	Rm8002 (Pho$^-$)	Rm8295 (*exoR95* Pho$^-$)	Rm8296 (*exoS96* Pho$^-$)
exoA264::Tn*phoA*	14	86	61
exoB359::Tn*phoA*	4.0	3.8	3.7
exoF369::Tn*phoA*	27	110	92
exoP365::Tn*phoA*	16	57	50
exoQ273::Tn*phoA*	6.3	20	20
exoT274::Tn*phoA*	5.4	8.8	12

[a] Units of activity are defined in Table 2, footnote *a*. *Values are averages of five determinations.*

TABLE 6. Alkaline phosphatase activity in bacteroids and free-living cells

Mutation and plasmid	Activity[a] (U/mg of protein)	
	Bacteroids[b]	Free-living cells
exoA264(pD34)	8.8	161
exoF369(pD56)	12	739[c]
exoP365(pD34)	14	286[c]
dctA384(pRmT8)	67	1,157[c]

[a] Values are averages of three determinations. Activity was determined as described in Table 3, footnote *b*.

[b] The background level of phosphate activity found in Rm8002 bacteroids, 3 U/mg of protein, has been subtracted.

[c] Data from Long et al. (22).

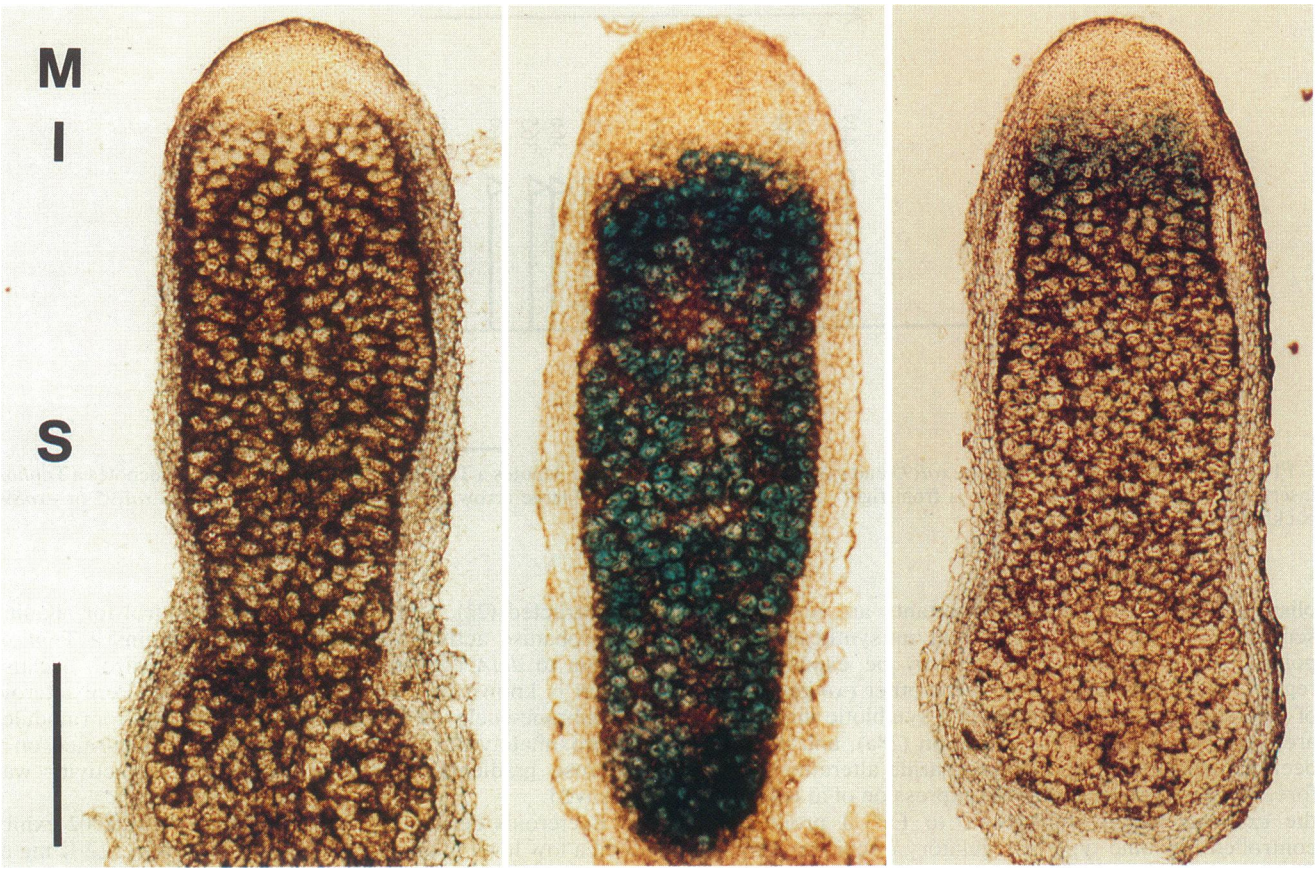

FIG. 3. Nodules stained for alkaline phosphatase activity present in the inducing bacteria. From left to right: a nodule induced by Rm8002, a Pho$^-$ strain; a nodule induced by Rm8384(pRmT8), a strain carrying a Tn*phoA* fusion to *dctA*; and a nodule induced by Rm8369(pD56), a strain carrying a Tn*phoA* fusion to *exoF*. M, Meristematic zone of the nodules; I, invasion zone; S, late symbiotic or bacteroid zone. Bar, 0.5 mm.

Histochemical staining shows *exo*::Tn*phoA* fusions are active primarily in the early symbiotic zone of the nodule. To gain a better understanding of the nature of *exo* gene expression during nodulation, we used histochemical staining. We first attempted to use β-glucuronidase gene fusions to *exo* genes for this purpose, but we found that these fusions were very poorly expressed in *R. meliloti* cells and therefore were not useful. However, we were able to develop a system to stain nodules specifically for alkaline phosphatase activity present in the inducing bacteria. Plant cells contain a high level of endogenous phosphatase activity, and when nodules are stained at pH 8, the pH at which assays of alkaline phosphatase of free-living cells are usually performed, the background obscures any signal. Heating the nodules to 65°C before sectioning and staining reduced the background significantly, but a high level of staining remained in the vascular bundles. Staining nodule sections at pH 9 without heating, however, eliminated staining in nodules induced by the Pho$^-$ control strain almost entirely (Fig. 3), with only occasional faint background in the vascular bundles. Free-living cells assayed at pH 9 retained approximately 40 to 50% of the activity they showed at pH 8.

Nodules induced by *exoF369*, *exoP365*, *exoA264*, and *dctA384* mutant strains containing appropriate complementing cosmids were stained at pH 9. The *exoF369* strain showed activity primarily in the early symbiotic or invasion zone of the nodule, where the bacteria are invading the plant cells (Fig. 3). In the late symbiotic zone, which contains mature bacteroids, little activity was seen. Occasionally, staining was seen near the base of the nodule. Nodules induced by *exoP365* and *exoA264* strains showed little staining, but faint staining of the invasion zone was seen after long incubations. The alkaline phosphatase activity of both these fusions was fairly low (16 and 14 U, respectively, in free-living cells). The *dctA384* strain showed staining throughout the late symbiotic zone of the nodule, as expected for a gene which is expressed in bacteroids (Fig. 3).

DISCUSSION

In this work we have used Tn*phoA*-generated fusions to *exo* genes in *R. meliloti* to analyze the regulation of these genes in the free-living cells and in planta and to examine the subcellular location of the *exo* gene products. In the course of screening for fusions, we isolated a new *exo* locus, *exoT*.

We have shown that five *exo* genes which are specific to EPS I production, *exoA*, *exoF*, *exoP*, *exoQ*, and *exoT*, are coordinately regulated by the products of the *exoR* and *exoS* loci. This regulation is probably at the level of transcription or mRNA stability, because it has been shown (28a) that there is an approximately fivefold increase in mRNA levels from several genes of the *exo* region in an *exoR95* background, which agrees well with the two- to fivefold increase in alkaline phosphatase activity observed in this back-

ground. It is probable, therefore, that most if not all of the EPS I-specific *exo* genes are coordinately regulated in an *exoR*- and *exoS*-dependent fashion.

The observation that at least several of the *exo* loci are coordinately regulated in free-living cells raises the possibility that they are also regulated together in planta. We have shown that the *exoF* gene has a characteristic pattern of expression in the nodule. There is high expression in the early symbiotic or invasion zone of the nodule, and relatively little or no detectable expression in the late symbiotic or bacteroid zone. A similar pattern of expression was seen with *exoA* and *exoP* mutants after long staining, although the lower activity of these fusions made detection more difficult. If the levels of the *exo* gene products in the bacteroids are accurately reflected by the level of alkaline phosphatase activity in the *exo*::Tn*phoA* derivatives, this suggests that little, if any, EPS I synthesis is necessary in later stages of nodulation. It is even possible that inhibition of EPS I synthesis is necessary in later stages of nodulation, because *exoR95* strains induce Fix$^-$ nodules unless they acquire suppressing mutations which reduce the level of EPS I synthesis of the strain (9). Thus, it is possible that *exoR* is involved in suppressing EPS I synthesis in the nodule.

Another regulatory locus, *psi*, in *R. leguminosarum* has been described (2), which inhibits exopolysaccharide production in free-living cells when present in multiple copies and is thought to be involved in repressing exopolysaccharide production in the nodule. *psi* mutants are Fix$^-$ on *Phaseolus* spp. As hypothesized for *exoR95* strains, inability to turn off polysaccharide production in the nodule may be responsible for the Fix$^-$ phenotype. Recently, an analog of *psi*, *exoX*, has been identified in *R. meliloti* (28a, 40). However, *exoX* mutants of *R. meliloti* form normal nodules. If *exoX* is involved in the regulation of *exo* gene expression seen in the nodule by histochemical staining, this regulation must not be necessary for normal nodulation. In free-living cells, the presence of *exoX* in multiple copies has little or no effect on the activity of *exo* gene fusions, although exopolysaccharide production is greatly reduced (28a, 40). Therefore, because *exoX* does not seem to exert its effects on EPS I synthesis through the regulation of *exo* gene expression in free-living cells, it seems unlikely that it is involved in regulating the expression of *exo* genes in the nodule. Experiments are under way to determine whether any of the known regulators of EPS I synthesis in the free-living state affect the expression of the *exo* genes in the nodule. It is also quite possible that a separate system of regulation exists that is active in planta.

The result that the *exo* genes seem to be expressed specifically in the invasion zone of the nodule suggests that they have a specific role at that stage of nodulation. This correlates well with the phenotype of *exo* mutants, which are blocked in nodulation at bacterial invasion. It is possible that EPS I functions as a signal to the plant. We and others (17a, 35a) have found that a low-molecular-weight fraction from *R. meliloti* supernatant related to EPS I can partially suppress the symbiotic deficiencies of *exo* mutants on plants, as has been reported previously for *Rhizobium* sp. strain NGR234 and *R. trifolii* (8). It is known that oligosaccharides can function as signal molecules in plants (28). Furthermore, it has recently been shown that the product of the *nod* genes of *R. meliloti* is a sulfated and acylated oligosaccharide (21). Other possible roles for EPS I include serving as part of the matrix of the infection thread or masking the bacteria to prevent induction of plant defense responses.

Certain of the observations we report in this article have implications for EPS I biosynthesis. We have obtained evidence that several of the *exo* genes seem to encode membrane proteins. This is consistent with the model that EPS I biosynthesis occurs in the cytoplasmic membrane. Sugar transferases are generally membrane-bound enzymes (32). Also, some *exo* gene products may be involved in the export of polysaccharide from the periplasmic space, as was found for the K antigen genes of *E. coli* (17, 29). Such proteins might be expected to be associated with the inner or outer membranes.

We have shown that blocking EPS I synthesis at certain stages is lethal in an *exoR95* or *exoS96* background and that this lethality is not due to the overproduction of abnormal proteins. It seems likely that the buildup of EPS I biosynthetic intermediates is responsible for the lethality. It has previously been shown that certain *cps* mutations in *Erwinia stewartii* are detrimental to cell growth (5) and that the *gumJ* and *gumE* mutations in *Xanthomonas campestris* are lethal unless a mutation in sugar nucleotide biosynthesis is present in the strain (35b, 36). It is known that the biosynthesis of EPS I takes place on polyprenyl lipid carriers in the cytoplasmic membrane (34, 35). The repeating octasaccharide subunits are built up on these carriers from nucleotide sugar precursors, beginning with a galactose residue, and are then polymerized. The same lipid carriers are used for lipopolysaccharide and peptidoglycan biosynthesis (33). It is possible that the buildup of unpolymerizable lipid-linked EPS I intermediates could diminish the pool of lipid carriers available for the biosynthesis of these vital polymers to the extent that the cells would die. The possibility that the availability of lipid carriers regulates the biosynthesis of polymers has been discussed by Sutherland (33). It is interesting that the *exoB* mutation, which has been reported to inactivate UDP-galactose epimerase (3a) and therefore should block the addition of the first galactose residue of the octasaccharide subunit to the lipid carrier, is not lethal in combination with *exoR* or *exoS*. We have begun experiments to define the biochemical blocks of the *exo* mutants.

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