The Succinoglycan Endoglycanase Encoded by *exoK* Is Required for Efficient Symbiosis of *Sinorhizobium meliloti* 1021 with the Host Plants *Medicago truncatula* and *Medicago sativa* (Alfalfa)

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Submitted 26 March 2013. Accepted 30 April 2013.

The acidic polysaccharide succinoglycan produced by the nitrogen-fixing rhizobial symbiont *Sinorhizobium meliloti* 1021 is required for this bacterium to invade the host plant *Medicago truncatula* and to efficiently invade the host plant *M. sativa* (alfalfa). The β -glucanase enzyme encoded by *exoK* has previously been demonstrated to cleave succinoglycan and participate in producing the low molecular weight form of this polysaccharide. Here, we show that *exoK* is required for efficient *S. meliloti* invasion of both *M. truncatula* and alfalfa. Deletion mutants of *exoK* have a substantial reduction in symbiotic productivity on both of these plant hosts. Insertion mutants of *exoK* have an even less productive symbiosis than the deletion mutants with the host *M. truncatula* that is caused by a secondary effect of the insertion itself, and may be due to a polar effect on the expression of the downstream *exoLAMON* genes.

Sinorhizobium meliloti 1021 is a soil bacterium that establishes a nitrogen-fixing symbiosis with the host plants *Medicago truncatula* ‘Jemalong A17’, *M. sativa* (alfalfa), and other species of the *Medicago* and *Melilotus* genera (Gibson et al. 2008; Jones et al. 2007; Oldroyd et al. 2011; Udvardi and Poole 2013). These *Medicago* spp. are both agriculturally important forage crops and key model organisms for studying the symbiotic interaction between rhizobial bacteria and legume plant hosts. Under nitrogen-limiting conditions, *S. meliloti* induces the formation of nodules on host plant roots, invades and colonizes these nodules (Jones et al. 2007; Oldroyd et al. 2011), and then begins to convert or “fix” dinitrogen gas to ammonia, a form that the plant can use (Udvardi and Poole 2013). The invasion of host plant roots by rhizobial bacteria requires that multiple signals be exchanged between these symbiotic partners. Host plants produce flavonoid compounds that signal *S. meliloti* to produce a lipochitooligosaccharide called Nod factor (NF) (Peters et al. 1986). NF induces multiple responses in host plants, including curling of root hairs that

encloses bacterial cells within the curl and the induction of cell division in the root cortex which initiates the formation of the nodule primordium (Gage 2004; Oldroyd and Downie 2006). The bacteria invade and colonize the roots through tubules called infection threads that originate from microcolonies of bacteria trapped in the curls of the root hair cells (Jones et al. 2007; Timmers et al. 1999). These infection threads are progressive ingrowths of plant cell membrane containing a matrix composed of bacterial exopolysaccharides (EPS) and plant cell wall material (Gage 2004). Infection thread initiation and development require that *S. meliloti* propagate in the infection thread and produce both the EPS succinoglycan and NF (Klein et al. 1988). The infection threads in root hair cells are extended to the base of these cells, and new infection threads initiate at each cell layer, eventually delivering the bacteria to proliferating cells of the inner root cortex (Timmers et al. 1999; Voroshilova et al. 2009).

Succinoglycan is required for *S. meliloti* 1021 to induce infection thread development on its host plants, because it is the only symbiotically active EPS that this strain produces in sufficient quantities to mediate infection thread formation (Glazebrook and Walker 1989; Leigh and Lee 1988). *S. meliloti* 1021 strains that do not produce succinoglycan, such as the *exoY* mutant (Reed et al. 1991), are able to colonize plant surfaces and become enclosed within curled root hairs but they fail to initiate infection threads, and the few infection threads that are initiated are aborted (Cheng and Walker 1998). These aborted infections are associated with gene expression responses (Jones et al. 2008) and cytological responses (Niehaus et al. 1993) characteristic of plant defense reactions. It has not yet been determined how succinoglycan influences the conditions within the colonized curled root hair or why it is required for infection thread initiation and progression.

The structure of succinoglycan has been determined (Reinhold et al. 1994) and the biosynthesis of succinoglycan by *S. meliloti* 1021 has been studied extensively (Jones et al. 2007). The succinoglycan monomer is an octasaccharide composed of one galactose and seven glucose residues, with acetyl, succinyl, and pyruvyl modifications (Reinhold et al. 1994). The carboxylate groups of the succinyl and pyruvyl moieties render the polysaccharide acidic. The structure of the succinoglycan monomer along with the identity of the gene product that catalyzes each step in the biosynthetic pathway is shown in Figure 1A (Glucksmann et al. 1993a and b; Reinhold et al. 1994; Reuber and Walker 1993a and b). The undecaprenyl-phosphate galac-

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tose phosphotransferase encoded by *exoY* and the glycosyltransferases encoded by *exoA*, *exoL*, *exoM*, *exoO*, *exoU*, and *exoW* are required for production of complete succinoglycan (Glucksmann et al. 1993a and b; Long et al. 1988; Reed et al. 1991; Reuber and Walker 1993a and b). The *exoH* gene product transfers the succinyl substituents to the oligosaccharide (Leigh et al. 1987) while *exoV* and *exoZ* add the pyruvyl and acetyl groups, respectively (Glucksmann et al. 1993b; Reuber and Walker 1993a and b). The glycanases encoded by *exoK* and *exsH* cleave the succinoglycan polymer to produce the low molecular weight (LMW) fraction of succinoglycan (York and Walker 1998a). The gene organization of the *exo* genes on the *S. meliloti* pSymB plasmid is shown in Figure 1B (Finan et al. 2001).

Other rhizobial strains and species also produce acidic EPS that facilitate infection thread formation. Galactoglucan (EPSII), produced by *S. meliloti* SU47 and *S. meliloti* 8530 (*S. meliloti* 1021 carrying the *expR101* mutation), is able to substitute for succinoglycan in facilitating infection thread formation on the host plant alfalfa but not on the host *M. truncatula* (Glazebrook and Walker 1989; Zhan et al. 1989). The acidic EPS produced by *Rhizobium leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* is required for infection thread formation by these symbionts on their plant hosts (Borthakur et al. 1986; Diebold and Noel 1989; Laus et al. 2005; van

Workum et al. 1995). It has also recently been demonstrated that *Mesorhizobium loti* mutants with defects in the later stages of EPS biosynthesis have greatly reduced efficiency in their symbiotic interaction with plant hosts of the genus *Lotus* (Kelly et al. 2013). In *S. fredii* NGR234, strains that do not produce symbiotic EPS are impaired in the interaction with the plant host *Leucaena leucocephala* (Staehelin et al. 2006). The symbiotic EPS produced by all of these rhizobial species are acidic, due to the presence of succinyl or pyruvyl modifications or the presence of glucuronic acid in the oligosaccharide chain (Aman et al. 1981; Djordjevic et al. 1986; Gil-Serrano et al. 1990; Jansson et al. 1977; Laus et al. 2005; O'Neill et al. 1991; Reinhold et al. 1994; Robertsen et al. 1981; Zhan et al. 1989).

The LMW fraction of *S. meliloti* succinoglycan has been proposed to be critical for this bacterium to initiate and maintain infection thread formation on its plant hosts (Battisti et al. 1992; Urzainqui and Walker 1992). In the studies that have reported rescue of host plant invasion by *S. meliloti* EPS-deficient mutants by coinoculation with purified EPS, this rescue has been associated with the LMW fraction of the EPS (Battisti et al. 1992; Urzainqui and Walker 1992). The LMW fraction is generated by cleavage of the succinoglycan chain to form exclusively monomers, dimers, and trimers of the octasaccharide repeating unit (Wang et al. 1999). Uncleaved, high molecular weight (HMW) succinoglycan is a very viscous material com-

Fig. 1. A, Structure of the succinoglycan monomer, showing the sugar linkages and the positions of the acetyl, succinyl, and pyruvyl substituents (Glucksmann et al. 1993a and b; Reinhold et al. 1994; Reuber and Walker 1993a and b). A second succinylation site is marked with an asterisk (Wang et al. 1999). Glycanases encoded by the *exoK* and *exsH* genes (glycosyl hydrolase family 16) cleave HMW succinoglycan into the LMW form (York and Walker 1997, 1998a). The glycosyltransferase encoded by the *exoL* gene adds the second glucose residue onto the growing oligosaccharide (Reuber and Walker 1993b). **B,** Organization of the succinoglycan biosynthesis genes on the *S. meliloti* 1021 pSymB plasmid (Galibert et al. 2001).

posed of chains of hundreds of the octasaccharide repeating unit (Wang et al. 1999). Succinoglycan cleavage is performed by two β -glucanases of glycosyl hydrolase family 16 (GH16), encoded by the *exoK* and *exsH* genes (Glucksmann et al. 1993b; York and Walker 1997, 1998a). *S. meliloti* 1021 strains carrying transposon Tn5 or antibiotic-resistance cassette insertions in the *exoK* (Becker et al. 1993; Long et al. 1988; York and Walker 1997) and *exsH* (York and Walker 1997) genes produce a smaller amount of succinoglycan in the LMW form than does *S. meliloti* 1021 wild type (Sm1021[w.t.]). These *S. meliloti* *exoK* (Becker et al. 1993; Long et al. 1988; York and Walker 1997) and *exsH* (York and Walker 1997) insertion mutants have previously been reported to produce functional, nitrogen-fixing nodules on the host plant alfalfa but the symbiotic phenotypes of these mutants were not quantified in these studies. In contrast, an *S. fredii* NGR234 strain carrying an Ω -cassette-insertion in the *exoK* gene, which encodes a β -glucanase 83% identical at the amino acid level to the one encoded by *S. meliloti* *exoK* (Altschul et al. 1997), forms no functional nodules and has an ineffective symbiosis with several of NGR234's plant hosts (Staehelin et al. 2006). Another set of observations may also provide evidence for the importance of LMW succinoglycan in host plant invasion. An *exoH* mutant, which produces succinoglycan without the succinyl modification, is completely blocked in host invasion (Cheng and Walker 1998) and cannot engage in a productive symbiosis (Leigh et al. 1987). This unsuccinylated succinoglycan produced by *exoH* transposon-insertion mutants cannot be cleaved by the ExoK and ExsH glycanases (York and Walker 1998b), and almost all of the polysaccharide produced by *exoH* mutants is in the HMW form. Because *exoH* mutants produce succinoglycan that is both unsuccinylated and exclusively in the HMW form, it is not clear at this point whether the symbiotic defects are due to the absence of the succinyl modification or to the absence of the LMW fraction of succinoglycan.

Because of the profound symbiotic defect of an *S. fredii* NGR234 *exoK* Ω -cassette-insertion mutant, the demonstrated role of the *exoK*-encoded glycanase in generating LMW succinoglycan, and the evidence suggesting a critical role for the LMW fraction of succinoglycan in *S. meliloti* invasion of its hosts we have further examined the role of *exoK* in *S. meliloti* 1021 interaction with its host plants. We have determined the symbiotic phenotypes of the *S. meliloti* *exoK445::Tn5* transposon insertion mutant and new deletion mutants of *exoK* on the model host plant *Medicago truncatula* A17 and the host plant alfalfa. Analysis of the deletion mutants demonstrates that loss of *exoK* in *S. meliloti* 1021 confers a significant loss of symbiotic efficiency.

RESULTS

Symbiotic phenotypes of the *exoK445::Tn5* mutant.

The alfalfa symbiotic phenotype of the *exoK445::Tn5* mutant was originally reported in Long and associates (1988) and confirmed as Fix⁺ by York and Walker (1997) but the symbiotic phenotype was not characterized further. We have found that the *exoK445::Tn5* mutant has a severe symbiotic defect on the plant host *M. truncatula* A17 and a moderate symbiotic defect on the plant host alfalfa. The symbiotic productivity measured by i) shoot length and ii) shoot fresh weight of both *M. truncatula* and alfalfa plants inoculated with the *exoK445::Tn5* mutant compared with the Sm1021(w.t.) and with uninoculated plants is shown in Figure 2. The shoot weight of *M. truncatula* plants inoculated with the *exoK445::Tn5* mutant is approximately 30% that of plants inoculated with Sm1021(w.t.) (Fig. 2B). The alfalfa symbiotic productivity with *exoK445::Tn5* is also lower than with Sm1021(w.t.) (Fig. 2A and B), although

the effect is not as large as that seen with *M. truncatula*. The *exoK445::Tn5* mutation also reduced the development of functional root nodules on *M. truncatula*, as shown by a decrease in the number of pink nodules (Fig. 2C). Pink color of the root nodules induced by rhizobial infection is due to the production of leghemoglobin by the host plant and is indicative of a functional symbiosis (Johnson and Vance 1981; Pladys and Vance 1993; Vance et al. 1980; Vasse et al. 1993). The larger number of white nodules on *M. truncatula* roots inoculated with the *exoK445::Tn5* mutant indicates that nodule primordia are induced by this strain but the nodules are unable to develop and mature. In contrast, there is not a significant difference in the number of pink nodules formed on alfalfa roots by the *exoK445::Tn5* mutant versus Sm1021(w.t.). This suggests that the symbiosis with *M. truncatula* is more sensitive to defects of the *exoK445::Tn5* strain than is the symbiosis with alfalfa.

The symbiotic defects of the *exoK445::Tn5* mutant are not complemented by expression of *exoK* alone.

Our next goals were to determine whether the *M. truncatula* symbiotic defect of the *exoK445::Tn5* strain could be complemented by expression of *exoK*, and whether overexpression of *exoK* in Sm1021(w.t.) could lead to improved symbiotic productivity of this strain. There was a slight but significant increase in shoot growth and development of pink nodules by plants inoculated with Sm1021(w.t.) cells carrying the pExoK::MSO4 overexpression construct relative to those carrying the negative control plasmid pMSO4 (Fig. 3A and B). However, we found that expression of *exoK* alone from the *trp* promoter in plasmid pMSO4 (Barnett et al. 2000) was not able to complement the *M. truncatula* shoot growth defect or the nodule development defect of the *exoK445::Tn5* mutant (Fig. 3A and B). To determine whether the pExoK::MSO4 plasmid was expressing ExoK protein, we performed a Western blot with α -ExoK antisera (provided by Graham C. Walker, Massachusetts Institute of Technology) (Fig. 3C). Both the Sm1021(w.t.) and *exoK445::Tn5* strains carrying the pExoK::MSO4 plasmid produced a large quantity of ExoK protein when grown in MGS medium (York and Walker 1997) (Fig. 3C, lanes 3 and 5). The Sm1021(w.t.) strain carrying the negative control plasmid produced a moderate amount of ExoK protein (lane 2), while the *exoK445::Tn5* mutant carrying the negative control plasmid produced no ExoK protein (lane 4). This indicates that ExoK protein is produced by the pExoK::MSO4 plasmid, at least in the free-living state, and that the lack of complementation of the *exoK445::Tn5* symbiotic phenotypes is not due to a nonfunctional complementation construct. A second *exoK* expression construct was made in the stable, low-copy, pLAFR1-based plasmid pRF771 (Chen et al. 2008; Egelhoff and Long 1985; Wells and Long 2002). The resulting plasmid, pExoK::RF771, also failed to complement the *exoK445::Tn5* defects in symbiotic productivity (Fig. 3D) and in nodule development (Fig. 3E). The pExoK::RF771 plasmid did not confer enhanced symbiotic phenotypes on the Sm1021(w.t.) strain.

Expression of *exoK* complements the delayed Calcofluor-halo phenotype of the *exoK445::Tn5* mutant strains.

S. meliloti strains with mutations in *exoK* are distinguished by delayed formation of a "halo" on agar medium containing the fluorescent dye Calcofluor (Becker et al. 1993; Long et al. 1988; York and Walker 1997). Calcofluor is used as an indicator of succinoglycan production because it fluoresces when bound to this polysaccharide (Wood 1980). LMW succinoglycan is able to diffuse away from growing *S. meliloti* cells into the surrounding Calcofluor-agar medium, producing a fluorescent halo around the cells, whereas HMW succinoglycan does not readily

diffuse and remains closely associated with the growing cells (Leigh et al. 1987; Long et al. 1988). Because *exoK* mutants produce less LMW succinoglycan than does Sm1021(w.t.) (Becker et al. 1993; York and Walker 1997), there is a delay in

the development of a Calcofluor halo around the *exoK* mutant cells (Long et al. 1988). We tested both *exoK445::Tn5* strains and Sm1021(w.t.) strains expressing *exoK* from the pExoK::RF771 plasmid to determine whether ExoK expression is suffi-

Fig. 2. The *exoK445::Tn5* strain has reduced symbiotic productivity on *Medicago truncatula* and alfalfa, measured by **A**, shoot length or **B**, shoot fresh weight. **C**, Degree of nodule development, shown as the number of pink, functional nodules; white, nonfunctional nodules; and brown, necrotic nodules. *M. truncatula* A17 plants were grown for 7 weeks and alfalfa plants for 5 weeks. Asterisk (*) denotes statistically significant difference from that plant species inoculated with the *Sinorhizobium meliloti* 1021 wild type reference strain. Data are averaged from two separate experiments for alfalfa and three separate experiments for *M. truncatula*.

Fig. 3. ExoK expression alone does not complement the *exoK445::Tn5* mutant symbiotic defect on *Medicago truncatula* A17. **A**, Plant height in centimeters and **B**, number of pink, functional nodules demonstrate that *exoK445::Tn5* is not complemented by expression of the *exoK* open reading frame. **C**, Western blot probed with α -ExoK antiserum (provided by G. C. Walker, Massachusetts Institute of Technology) showing production of ExoK protein under the control of the *trp* promoter of the pMSO4 plasmid in *Sinorhizobium meliloti* strains. Expression of *exoK* alone in a second complementation construct (the pExoK::RF771 plasmid) also does not complement the **D**, reduced shoot weight or **E**, reduced number of pink, functional nodules of *exoK445::Tn5* mutant strains. Values for independently isolated strains of each genotype are grouped together (e.g., 1021 + pExoK::RF771 "1" and "2"). Asterisk (*) denotes statistically significant difference from plants inoculated with *S. meliloti* 1021 wild type. Plant phenotype data are averaged from three separate experiments.

cient to complement the *exoK445::Tn5* delayed-halo phenotype in free-living cells. Development of the Calcofluor halo around Sm1021(w.t.) and *exoK445::Tn5* cells imaged at 3-day intervals is shown in Figure 4A to D. As a comparison, the Calcofluor-dark phenotype of an *exoY::Tn5* mutant is shown in Figure 4E. A magnified view of a representative Calcofluor halo outlined with a circle is shown in Figure 4F. The difference in Calcofluor-halo phenotypes between the strains is particularly evident after 9 days of growth (Fig. 4C). Sm1021(w.t.) cells with no plasmid and those carrying the pRF771 negative control plasmid have a prominent Calcofluor halo (Fig. 4C), whereas *exoK445::Tn5* cells and *exoK445::Tn5* + pRF771 cells have not yet developed a halo. Complementation of the delayed-halo phenotype of *exoK445::Tn5* by the pExoK::RF771 plasmid is evident in the right-hand side of the same panel. Both Sm1021(w.t.)

Fig. 4. Expression of *exoK* alone in pExoK::RF771 is sufficient to complement the delayed Calcofluor-halo phenotype of the *exoK445::Tn5* mutant grown on LBMC (Luria-Bertani Miller medium supplemented with MgSO_4 and CaCl_2). After **B**, 6 and **C**, 9 days, *Sinorhizobium meliloti* 1021 wild type (Sm1021[w.t.]) and Sm1021(w.t.) carrying the negative control construct pRF771 have a significant halo of Calcofluor-stained LMW succinoglycan diffusing away from the spot of growing cells. Both Sm1021(w.t.) and *exoK445::Tn5* carrying the pExoK::RF771 expression plasmid also have well-developed LMW halos at this time point. **D**, Uncomplemented *exoK445::Tn5* mutant strains do not develop a significant halo until 12 days after inoculation, demonstrating the delayed and reduced production of LMW succinoglycan in these strains. **E**, For comparison, the same time points are shown for the Calcofluor-dark, succinoglycan-deficient *exoY::Tn5* mutant. **F**, Magnified view of a single spot of growing *S. meliloti* cells with the margins of the Calcofluor halo outlined. All images were 0.25-s exposures of 365-nm UV light.

and *exoK445::Tn5* have a more pronounced halo at 9 days' growth when they are overexpressing *exoK* from the pExoK::RF771 plasmid (Fig. 4C). This indicates that the reduction in LMW succinoglycan production in the *exoK445::Tn5* mutant is complemented by the pExoK::RF771 plasmid, suggesting that the failure of this plasmid to complement the symbiotic defects of *exoK445::Tn5* is not due to an absence of succinoglycan cleavage by ExoK.

A secondary effect of the *exoK445::Tn5* insertion is responsible for the lack of *exoK* complementation of the symbiotic defects of the *exoK445::Tn5* mutant strains.

We tested complementation by the pEX154 cosmid to confirm that the symbiotic defect of the *exoK445::Tn5* strain is caused by a lesion in the same region of the *S. meliloti* genome that contains *exoK* (Fig. 5). The pEX154 cosmid carries an approximately 19.8-kb fragment of pSymB DNA that extends from the *exoT* gene through the SMb20967 ORF (Long et al. 1988) (Figs. 1B and 6A). This cosmid has previously been demonstrated to complement the symbiotic defects of transposon insertion mutations in the *exoH* (Leigh et al. 1987), *exoL*, *exoA*, *exoM*, *exoN*, and *exoP* genes (Long et al. 1988). We found that this cosmid was able to complement the shoot growth (Fig. 5A) and nodule development (Fig. 5B) defects of the *exoK445::Tn5* mutant, whereas the negative control construct pLAFR3 was not able to complement. (In sequencing the region surrounding the Tn5 insertion in the *exoK445::Tn5* strain, we have not detected any additional mutations that could lead to complementation by pEX154 but not by *exoK* alone. This suggests that all of the defects in the *exoK445::Tn5* strain are caused by the Tn5 insertion itself.)

The most likely explanation for the complementation results described above is that insertion of the transposon in *exoK445::Tn5* has a deleterious effect on expression of one or more of the downstream *exo* genes (*exoLAMON*) (Fig. 1B), and that this is responsible, in part, for the symbiotic phenotype. Schlüter and associates (2013) have reported a strong transcription start site 76 bp upstream of the annotated start site of *exoH* (pSymB position 1181162; transcript ID SmelB108) detected in an RNA pool that included *S. meliloti* grown in the presence of the plant flavonoid luteolin (Schlüter et al. 2013). This study also identified four additional, weaker transcription start sites within *exoH* and one within *exoK* (Schlüter et al. 2013). No transcription start sites were identified in the 495 bp upstream of the *exoL* start codon (Schlüter et al. 2013), suggesting that most transcriptions of *exoL* (and likely the downstream *exo* genes as well) are dependent on read-through from the *exoHK* region.

To directly test the effect of the *exoK445::Tn5* insertion on the expression of downstream genes, we performed quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) on the *exoL* gene in Sm1021(w.t.) cells, the original *exoK445::Tn5* mutant, and two *exoK445::Tn5* strains generated by transduction of this insertion (Fig. 7). We found that *exoL* is expressed at low levels (compared with the *trp* promoter) in free-living cells grown in Luria-Bertani (LB) Miller medium supplemented with 2.5 mM MgSO_4 and 2.5 mM CaCl_2 (LBMC) and that this level of expression is not dramatically altered in the *exoK445::Tn5* mutant (Fig. 7A). We also tested the expression of *exoL* in these strains grown in Jensen's plant growth medium supplemented with 5 mM glutamic acid sodium salt, 12.5 mM mannitol, and biotin at 1 $\mu\text{g}/\text{ml}$ (JMG). Under these conditions, expression of *exoL* is extremely low and variable, and there are no significant differences in expression between Sm1021(w.t.) versus the *exoK445::Tn5* strains (Fig. 7B). This is in agreement with the data of Becker and associates (1993), which showed that

integration of the *exoK* disruption/reporter fusion plasmid pVXB1 did not prevent expression of the *exoL* gene by free-living *S. meliloti* (Becker et al. 1993).

Although there do not appear to be any obvious effects of the *exoK445::Tn5* insertion on the expression of downstream genes in the free-living state, it is possible that the *exoL* expression pattern in *S. meliloti* during symbiotic interaction with host plants is altered in the *exoK445::Tn5* mutant strains. We have found that a β -glucuronidase (GUS)-reporter fusion to *exoL* (*exoHKL::JH104*) is expressed very strongly by *S. meliloti* in the *M. truncatula* nodule epidermis (Fig. 8A to D) at 10 days postinoculation and in nodules (Fig. 8E to H) at 17 days postinoculation. These strains carry a duplication of a 3.4-kb region of pSymB extending from the 3' end of SMB20952 to the 3'

end of *exoL*, and place the inserted GUS reporter under the same transcriptional controls as *exoL*. The transposon insertion in the *exoK445::Tn5* mutant separates the *exoLAMON* genes from all but one of the transcription start sites identified by Schlüter and associates (2013), and polar effects of this insertion on symbiotic expression of these genes may explain the lack of complementation of the *exoK445::Tn5* mutant by the *exoK* gene alone.

A nonpolar deletion of *exoK* has a small but significant loss of symbiotic productivity on both the host *M. truncatula* and the host alfalfa.

In order to dissect the effects of deletion and modification of the *exoK* and *exoH* genes on symbiosis and succinoglycan func-

Fig. 5. Cosmid pEX154 complements the *exoK445::Tn5* symbiotic defects in *Medicago truncatula* A17: **A**, shoot fresh weight and **B**, number of pink, functional nodules. The negative control construct pLAFR3 does not complement the defect. This is consistent with a secondary effect of the *Tn5* insertion being at least partially responsible for the symbiotic growth defect of the *exoK445::Tn5* mutant. Values for independently isolated strains of each genotype are grouped together (e.g., 1021 + pEX154 "5" and "6"). Asterisk (*) denotes statistically significant difference from plants inoculated with the *Sinorhizobium meliloti* 1021 wild-type reference strain.

tion without deleterious polar effects on the *exoLAMON* operon, we constructed strains that place the *exoLAMON* genes under the control of a constitutive promoter and isolate this new artificial *trp-exoLAMON* operon from the *exoHK* genes. The goal was to generate strains in which the *exoLAMON* genes are under identical regulatory control despite alterations to the upstream *exoHK* region. The construction of these strains is shown in Figure 6B. The *trpexoL* strains are identical to Sm1021(w.t.) except that the *trp* promoter drives expression of the *exoLAMON* genes and a neomycin/kanamycin-resistance cassette (Ω Nm/Km) separates these genes from *exoHK* (Fig. 6A and B). The Kdel-*trpexoL* strains are the same as the *trpexoL* strains except for the deletion of *exoK* (Fig. 6B). Expression of the *exoL* gene in i) LBMC medium and ii) JMG medium for two separately isolated *trpexoL* strains and three separately isolated Kdel-*trpexoL* strains is shown in Figure 7. The expression of *exoL* in these strains is ≥ 30 fold more than that of Sm1021(w.t.) in LBMC medium, and ≥ 200 fold more than that of Sm1021(w.t.) in JMG medium (Fig. 7). This demonstrates that we have produced strains that have strong, approximately equivalent expression of *exoL* regardless of the presence or absence of the *exoK* gene.

The symbiotic productivity (Fig. 9A) and nodule development (Fig. 9B) phenotypes of the *trpexoL* strains are not significantly different from those of Sm1021(w.t.) on the plant host *M. truncatula*. This demonstrates that there are no adverse effects on the symbiosis of placing the *exoLAMON* genes under the control of the *trp* promoter and placing the Ω Nm/Km-resistance cassette between the *exoHK* genes and the *trp-exoLAMON* operon. In contrast, the Kdel-*trpexoL* strains have a small but significant defect in both symbiotic productivity (Fig. 9A) and nodule development (Fig. 9B). The symbiotic defect of the Kdel-*trpexoL* strains on *M. truncatula* (shoot weight 60 to 70% that of Sm1021[w.t.]) is not as severe as the defect of the *exoK445::Tn5* strain (40% that of Sm1021[w.t.]). This suggests that the *exoK445::Tn5/M. truncatula* symbiotic defects are due to the additive effect of the loss of *exoK* and a secondary effect of this transposon insertion. In the symbiosis with alfalfa, there is no significant difference in symbiotic productivity between the Kdel-*trpexoL* strains and the *exoK445::Tn5* strain (Fig. 9C). Alfalfa plants inoculated with any of the *exoK* mutants have a shoot weight that is 54 to 69% that of Sm1021(w.t.) (Fig. 9C). This indicates that both plant hosts require *exoK* function for optimal symbiotic productivity. This also suggests that the difference in the extent of the symbiotic defect of the *exoK445::Tn5* mutant on the two plant hosts is due to a

secondary effect of the Tn5 insertion, which is more deleterious to the symbiosis with *M. truncatula* than alfalfa.

Unlike *exoK445::Tn5*, the defect in *M. truncatula* symbiotic productivity of the Kdel-*trpexoL* strains can be complemented by expression of *exoK* alone from the pExoK::RF771 plasmid (Fig. 10A), confirming that there are no secondary effects of the *exoK* deletion in the Kdel-*trpexoL* strains. This result is in striking contrast to the data in Figure 3E and F, showing that the symbiotic defects of the *exoK445::Tn5* mutant are not complemented by the pExoK::RF771 plasmid. These results also show that overexpression of *exoK* in Sm1021(w.t.) or in the *trpexoL* strains does not enhance symbiotic productivity with *M. truncatula*.

The Calcofluor-halo phenotype of the *trpexoL* strains is similar to that of the Sm1021(w.t.) strain when these strains are grown on LBMC medium (Fig. 11A). Compared with these strains, at 6 and 9 days of growth, the Kdel-*trpexoL* strains have a delay in halo formation similar to that of the *exoK445::Tn5* strains (Fig. 11A). In JMG plant medium, the delay in halo formation of the *exoK445::Tn5* strains and the Kdel-*trpexoL* strains relative to Sm1021(w.t.) and the *trpexoL* strains is less obvious but it can be observed at 6 days of growth (Fig. 11B). This indicates that the loss of *exoK* has a similar effect on the production of LMW succinoglycan in the Kdel-*trpexoL* strains and in the *exoK445::Tn5* strains.

DISCUSSION

We have shown that the loss of the *exoK* glycanase in *S. meliloti* 1021 results in reduced symbiotic efficiency on both *M. truncatula* and alfalfa. We have also shown that the *exoK445::Tn5* mutant strain has a more pronounced symbiotic defect on *M. truncatula* than on alfalfa, whereas the nonpolar Kdel deletion strains have a similar reduction in symbiotic productivity on both of these hosts. This indicates that the reduction in the amount of LMW succinoglycan due to the loss of *exoK* leads to a similar loss in symbiotic efficiency on both hosts, and that it is a secondary effect of the Tn5 insertion in the *exoK445::Tn5* strain that leads to the more pronounced symbiotic defect with *M. truncatula*. This secondary effect can be complemented by the pEX154 cosmid, which carries the pSymB gene region from *exoT* to SMb20967 (Fig. 1B). This is consistent with the secondary effect of the Tn5 insertion producing a polar effect on the downstream *exoLAMON* genes that is not apparent in the free-living state but causes a defect in the symbiotic interaction with *M. truncatula*. This is also consistent

Fig. 6. A, Arrangement of the *exoHKLAMON* genes on the pSymB plasmid of *Sinorhizobium meliloti* 1021. The extent of the region carried on the pEX154 cosmid is also shown. B, Construction of the *trpexoL* and Kdel-*trpexoL* strains in order to eliminate polar effects on the *exoLAMON* genes. In all of these strains, the *exoLAMON* genes are isolated from the upstream *exoHK* genes by an Ω Nm^r cassette carrying neomycin and kanamycin resistance. In these strains, the *trp* promoter drives the expression of *exoLAMON*.

with results from Jones (2012) demonstrating that increasing succinoglycan production increases the efficiency of the symbiosis with *M. truncatula* but has no effect on the symbiosis with alfalfa (Jones 2012). Perhaps any perturbation of succinoglycan production due to negative effects on *exoLAMON* expression pushes the succinoglycan level below a critical threshold level for successful symbiosis with *M. truncatula*.

We have also shown that there is strong expression of *exoL* by *S. meliloti* during invasion of *M. truncatula* and in nodules, despite weak expression in Jensen's medium. Succinoglycan production has been shown to be increased by high phosphate concentration (Mendrygal and González 2000) and it is possible that phosphate is involved in regulation of *exoHKLAMON* expression on plants, because phosphate levels are usually higher on plant roots (Bielecki 1973; Israel 1987) than the 8.5 mM found in Jensen's medium (Jones 2012; Mendrygal and González 2000). It is also possible that other compounds produced by host plants, such as flavonoids (Peck et al. 2006), affect expression of the *exoHKLAMON* genes.

In another rhizobial symbiont–host plant pair, there is an apparent contrast to our current results. An *exoK* Ω -cassette insertion mutant of *S. fredii* NGR234 produces no pink, functional nodules on the host plant *L. leucocephala* (Staehelin et al. 2006). However, complementation of this mutant with a plasmid carrying the NGR234 *exoK* gene produces an intermediate phenotype: 3.1 ± 0.8 pink nodules were produced per plant relative to the 10.3 ± 1.5 pink nodules/plant produced by plants inoculated with the *S. fredii* NGR234 wild-type strain carrying the *exoK* plasmid (Staehelin et al. 2006). In *S. fredii* NGR234, the order of the *exoKLAMON* genes is the same as in *S. meliloti* 1021 (Schmeisser et al. 2009). One possibility is that the intermediate phenotype of the NGR234 *exoK* Ω -cassette insertion + p*ExoK* strain is due a lack of complementation by *exoK* of a secondary effect of the insertion, analogous to the lack of complementation of the *S. meliloti* *exoK445::Tn5* symbiotic phenotype by expression of *exoK* in *S. meliloti*.

There are several critical questions for future study. For example, does the intermediate symbiotic phenotype caused by

Fig. 7. Expression of the *exoL* gene in **A**, LBMC (Luria-Bertani Miller medium supplemented with MgSO₄ and CaCl₂) and **B**, JMG (Jensen's plant growth medium supplemented with glutamic acid sodium salt, mannitol, and biotin) relative to 16S rRNA expression (measured by quantitative reverse-transcriptase polymerase chain reaction and expressed in arbitrary units multiplied by 10⁷). In both LBMC and JMG, *exoL* expression under the control of the *trp* promoter is much stronger than expression from the native promoter in *Sinorhizobium meliloti* 1021 wild type (Sm1021[w.t.]). Free-living bacteria in both LBMC and JMG have low-level expression of *exoL* in Sm1021(w.t.) and in the *exoK445::Tn5* strains. Inset in B is an adjusted-scale view of *exoL* expression in 1021 and *exoK::Tn5* 1, 1.5, and 6.2. The only significant difference in *exoL* expression from Sm1021(w.t.) by an *exoK445::Tn5* strain is in *exoK::Tn5* 6.2 in LBMC medium. The only significant difference in *exoL* expression among the *trpexoL* and *Kdel-trpexoL* strains is in *Kdel-trpexoL* 3.3 in JMG medium (B).

Fig. 8. **A to D**, Expression of an *exoHKL*:: β -glucuronidase (GUS) reporter fusion on the *Medicago truncatula* developing nodule epidermis at 10 days postinoculation and **E to H**, in nodules at 17 days postinoculation. The GUS reporter in the inserted plasmid pJH104 is under the transcriptional control of a duplicated 3.4-kb region of pSymB extending from the 3' end of SMb20952 to the 3' end of *exoL*. Five independently isolated fusion strains are shown: **A**, *exoHKL*::JH104.1C14; **B** and **E**, *exoHKL*::JH104.2C10; **C** and **D**, *exoHKL*::JH104.2B6; **F**, *exoHKL*::JH104.1B6; **G** and **H**, *exoHKL*::JH104.2C12. **I**, Negative control, *S. meliloti* 1021 without a GUS fusion (17 days postinoculation). **J**, Positive control for strong GUS expression from the SMc00911::JH104 reporter fusion (Queiroux et al. 2012) (17 days postinoculation). In all panels, the bar corresponds to 100 μ m. Roots were stained for 23 to 24 h (**A** to **C** and **E** to **J**) or 47 h (**D**).

Fig. 9. Symbiotic performance of *Medicago truncatula* plants inoculated with two independently isolated *trpexoL* strains is not significantly different from those inoculated with the *Sinorhizobium meliloti* 1021 wild type (Sm1021[w.t.]), measured by **A**, shoot fresh weight or **B**, number of pink, functional nodules. In contrast, the *Kdel-trpexoL* strains carrying a deletion of the *exoK* gene have a significantly reduced **A**, shoot weight and **B**, number of pink, functional nodules relative to those inoculated with the *S. meliloti* Sm1021(w.t.). However on *M. truncatula*, the symbiotic defects of the *Kdel-trpexoL* strains are not as severe as those of the *exoK445::Tn5* mutant. On the plant host alfalfa, the **C**, symbiotic productivity and **D**, nodule development of the *Kdel-trpexoL* and *exoK445::Tn5* strains are not significantly different from one another. Values for independently isolated strains of each genotype are grouped together (e.g., *trpexoL* “3Xs3” and “16Xs4”). Asterisk (*) denotes statistically significant difference from plants inoculated with the *S. meliloti* Sm1021(w.t.) reference strain. Data are averaged from five separate experiments.

loss of *exoK* in the *S. meliloti* Kdel-*trpexoL* strains mean that LMW succinoglycan is beneficial but not required for host invasion, or are there alternate mechanisms for producing LMW succinoglycan during host invasion? It is known that the glycanase encoded by *exsH* cleaves succinoglycan in vitro and that it contributes to the production of LMW succinoglycan by *S. meliloti* in the free-living state (York and Walker 1997, 1998a). However, it is not clear that the *exsH*-encoded glycanase makes any contribution to the host invasion efficiency of *S. meliloti*. A double mutant in *exoK445::Tn5/exsH* was found to be capable of symbiosis with the host plant alfalfa but the symbiotic phenotypes were not quantified (York and Walker 1997). It may be that another succinoglycan glycanase contributes to the production of LMW succinoglycan during host invasion or that the LMW fraction of succinoglycan is beneficial but not required for host invasion. We are currently determining the molecular weight distribution of succinoglycan produced by the Kdel-*trpexoL*/*exsH* double mutants under free-living conditions that mimic as closely as possible those the bacteria en-

counter on host plants to determine whether any LMW succinoglycan can be detected in these strains. We are also determining whether other putative glycanases encoded in the *S. meliloti* 1021 genome can contribute to the production of LMW succinoglycan, and whether these glycanases can complement the reduced symbiotic efficiency of the Kdel-*trpexoL* mutant strains.

Acidic rhizobial EPS are required for host invasion or contribute to host invasion efficiency in many different symbiont–host pairs (Borthakur et al. 1986; Diebold and Noel 1989; Kelly et al. 2013; Laus et al. 2005; Staehelin et al. 2006; van Workum et al. 1995). A role for these rhizobial EPS in induction of infection thread formation via a signaling pathway is possible; however, it is also possible that these compounds act by altering the conditions within the infection thread. Acidic EPS produced by plant-pathogenic bacteria are also important determinants of virulence in plant disease. For example, in *Xanthomonas campestris* pv. *campestris*, production of the polysaccharide xanthan enhances bacterial infection and tissue damage of host plants (Newman et al. 1994; Yun et al. 2006).

Fig. 10. *Medicago truncatula* symbiotic defects of the Kdel-*trpexoL* strains are complemented by expression of *exoK* alone. **A**, Shoot fresh weight and **B**, number of pink, functional nodules of plants inoculated with the complemented strains are not significantly different from those inoculated with *Sinorhizobium meliloti* 1021 wild type (Sm1021[w.t.]) or the *trpexoL* strains. Values for independently isolated strains of each genotype are grouped together (e.g., *trpexoL* “3Xs3” and “16Xs4”). Asterisk (*) denotes statistically significant difference from plants inoculated with the *S. meliloti* Sm1021(w.t.) reference strain. Data are averaged from three separate experiments.

Xanthan and other bacterial polysaccharides have been demonstrated to suppress plant defense responses by binding Ca^{2+} and preventing the activation of these responses by Ca^{2+} -signaling (Aslam et al. 2008). Also, the acidic EPS alginate exacerbates disease symptoms caused by *Pseudomonas syringae* on host plants (Yu et al. 1999). It has been proposed that both alginate and xanthan aid in host infection by absorbing water and creating a hydrated microenvironment around the invading bacteria (Beattie 2011; Yu et al. 1999). It is possible that bacterial acidic EPS facilitate these very different symbiotic and pathogenic bacteria–host interactions by performing similar chemical or mechanical functions. During infection thread formation by symbiotic rhizobia, acidic EPS might be involved in modulating Ca^{2+} levels or in hydration of the infection thread matrix. Cleavage of EPS into smaller LMW units would be expected to raise the osmolarity (Buchanan et al. 2000) and, thus, the osmotic potential of the solution in the expanding portion of the infection thread, which might facilitate infection thread extension.

We have determined that *S. meliloti* 1021 strains in which the loss of *exoK* is the sole genetic defect have reduced symbiotic productivity on both the plant hosts *M. truncatula* and alfalfa. The *exoK* glycanase is known to contribute to the production of LMW succinoglycan under free-living conditions. These results suggest that LMW succinoglycan is beneficial but not absolutely required for this rhizobial species to establish a symbiosis with its host plants, or that *exsH* or another one of the putative glycanases being investigated can partially replace the function of *exoK* during symbiosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

S. meliloti 1021 strains (Supplementary Table 1) (Meade et al. 1982) were grown at 30°C in either LBMC medium or JMG (Jones 2012; Leigh et al. 1985; Vincent 1970). Bacterial plates contained 1.5% Bacto agar (BD, Franklin Lakes, NJ, U.S.A.). Calcofluor polysaccharide indicator plates contained 0.02% Calcofluor white M2R (fluorescent brightener 28; Sigma-Aldrich, St. Louis) (Leigh et al. 1985). *S. meliloti* 1021 was grown with streptomycin at 500 µg/ml. *S. meliloti* strains carrying pLAFR-based cosmids and pRF771-based plasmids were also grown with tetracycline at 10 µg/ml. *S. meliloti* strains carrying pMSO4-based plasmids were also grown with spectinomycin at 100 µg/ml. *S. meliloti* strains carrying transposon Tn5, the Nm/Km Ω-cassette, or pJH104 insertions were also grown with neomycin at 200 µg/ml. *Escherichia coli* strains were grown at 37°C in LB medium (Sambrook et al. 1982), with appropriate antibiotics used at the following concentrations: kanamycin at 50 µg/ml, chloramphenicol at 10 µg/ml, tetracycline at 10 µg/ml, and spectinomycin at 100 µg/ml.

Construction of *S. meliloti* mutant strains.

All restriction enzymes and Phusion High-Fidelity polymerase were obtained from New England Biolabs (Ipswich, MA, U.S.A.). All primers were obtained from Eurofins MWG Operon, (Huntsville, AL, U.S.A.). Plasmids pExoK::MSO4 and pExo::RF771 that constitutively express *exoK* were constructed by amplifying the *exoK* open reading frame (ORF) with primers

Fig. 11. A, *Kdel-trpexoL* strain has a delayed Calcofluor-halo phenotype similar to those of the *exoK445::Tn5* strains on LBMC (Luria-Bertani Miller medium supplemented with MgSO_4 and CaCl_2). The delay in halo formation by these strains relative to *Sinorhizobium meliloti* 1021 wild type (Sm1021[w.t.]) and the *trpexoL* strains is most obvious after 9 days of growth. **B,** On Jensen's medium, the difference in halo formation is less apparent but, at both 6 and 9 days of growth, the *exoK445::Tn5* and *Kdel-trpexoL* strains have a fainter halo than Sm1021(w.t.) and the *trpexoL* strains. All exposures were 0.25 s with 365-nm UV light illumination.

exoK.rbs.fwd.Pst and exoK.rev.BamHI, using Phusion polymerase, and cloning into pCR-Blunt II-TOPO using the Zero-TOPO-Blunt cloning kit (Life Technologies, Carlsbad, CA, U.S.A.), creating plasmid exoK.Pst/Bam/TOPO.1. The ribosome binding site used for translation of *exoK* (Gage et al. 1996) was incorporated into the exoK.rbs.fwd.Pst primer. The *exoK* ORF *PstI/BamHI* fragment from exoK.Pst/Bam/TOPO.1 was cloned into plasmid pMSO4 (Barnett et al. 2000) to make plasmid pExoK::MSO4.PB, placing *exoK* under the control of the *trp* promoter, which is constitutively active in *S. meliloti* (Egelhoff and Long 1985). This fragment was also cloned into plasmid pRF771 (Wells and Long 2002) to make plasmid pExoK::RF771. Plasmids were mobilized into *S. meliloti* by triparental conjugation, as described (Backman and Boyer 1983; Finan et al. 1986; Glazebrook and Walker 1991). *S. meliloti* exconjugants carrying pMSO4 or pExoK::MSO4.PB were selected on LBMC medium containing spectinomycin at 100 µg/ml and streptomycin at 1,000 µg/ml. Exconjugants carrying pRF771, pExoK::RF771, pLAFR3 (Staskawicz et al. 1987), or pEX154 (Leigh et al. 1987) were selected on LBMC medium containing tetracycline at 10 µg/ml and streptomycin at 1,000 µg/ml.

S. meliloti 1021 *exoHKL::JH104* GUS-reporter insertion strains were constructed by amplifying a 3.4-kb product from within the 3' end of SM_b20952 to the 3' end of *exoL* using primers exoH5'gus.rep.fwd and exoL3'gus.rep.rev and cloning the amplified product into the plasmid pJH104 (Ferguson et al. 2005). The resulting plasmid, pexoHKL::JH104, was conjugated into *S. meliloti* 1021 and exconjugants were selected on LBMC medium containing neomycin at 200 µg/ml and streptomycin at 1,000 µg/ml. The exconjugants *exoHKL::JH104.1B6*, *exoHKL::JH104.1C14*, *exoHKL::JH104.2B6*, *exoHKL::JH104.2C10*, and *exoHKL::JH104.2C12* were confirmed to have a complete duplication of the region from the 3' end of SM_b20952 to the 3' end of *exoL*, generating a GUS reporter fusion to *exoL*.

S. meliloti strains *trpexoL3Xs3* and *trpexoL16Xs4*, in which the *exoLAMON* operon is expressed under the control of the *trp* promoter and is separated from the *exoHK* genes by a Ω Nm/Km-resistance cassette (Fellay et al. 1987), were constructed using the plasmid pK3.trpexoKm2. This plasmid was constructed as follows. The *trp* promoter was amplified from pMSO4 (Barnett et al. 2000) and was fused with a 0.97-kb fragment of *exoL* (beginning with the start codon) using primers trp/exoL.up1.SalI, trp/exoL.dwn1, trp/exoL.up2 and trp/exoL.dwn2.XhoI. The fusion primers trp/exoL.dwn1 and trp/exoL.up2 also introduced a ribosome-binding site and spacer between the *trp* promoter and the *exoL* start codon. This fragment was cloned into the *SalI* and *XhoI* sites of the *sacB* gene-containing suicide plasmid pJQ100SK (Quandt and Hynes 1993). The resulting plasmid was then digested with *BamHI* and ligated with the *BamHI* Nm/Km-resistance cassette fragment from pHP45 Ω -Nm/Km (Fellay et al. 1987) to generate plasmid ptxoL- Ω Km. This plasmid was digested with *SacI* and *XbaI* and ligated with a 0.8-kb *SacI/XbaI* fragment that includes the 3' end of the *exoK* gene (amplified with primers exoK.fwd.SacI and exoK.rev.XbaI) to generate plasmid pK3.trpexoKm2. This plasmid was conjugated into *S. meliloti* 1021 to generate strains in which the Nm/Km-resistance cassette has been inserted immediately 3' of *exoK*, followed by the *trp* promoter driving expression of the *exoLAMON* operon (Fig. 6, diagram). Insertion-carrying exconjugants were selected on neomycin at 200 µg/ml and streptomycin at 1,000 µg/ml. Double-recombinant gene-replacement strains *trpexoL3* and *trpexoL16* were selected for the loss of the *sacB* gene carried by pJQ100SK on 1/10 LBMC + 7% sucrose medium, with neomycin at 200 µg/ml and streptomycin at 1,000 µg/ml. The Nm/Km-resistance markers from *trpexoL3* and *trpexoL16* were transduced into *S. meliloti* 1021 using the phage ϕ M12 (Finan et al.

1984) to generate strains *trpexoL3Xs3* and *trpexoL16Xs4*. The presence of the Nm/Km-resistance cassette and the *trp* promoter between *exoK* and *exoL* was confirmed by PCR.

The strains Kdel-*trpexoL.1-1*, Kdel-*trpexoL.2-1*, and Kdel-*trpexoL.3-3* were constructed using the plasmid Kdelp.txoL. This plasmid was constructed from plasmid ptxoL- Ω Km (discussed above). Plasmid ptxoL- Ω Km was digested with *Eco53kI* and *XbaI* and was ligated with a 0.9-kb *EcoRV/XbaI* fragment that includes the sequence immediately 5' of the *exoK* gene (amplified with primers Kdel.trpexo1fwd and Kdel.trpexo1rev). This generated plasmid Kdelp.txoL, which was conjugated into *S. meliloti* 1021 to generate strains Kdel-*trpexoL.1*, Kdel-*trpexoL.2*, and Kdel-*trpexoL.3*, in which the Nm/Km-resistance cassette has been inserted immediately 5' of *exoK*, deleting *exoK*, and followed by the *trp* promoter driving expression of the *exoLAMON* operon (Fig. 6, diagram). Exconjugants were selected as described above for strains *trpexoL3* and *trpexoL16*. The Nm/Km-resistance markers from strains Kdel-*trpexoL.1*, Kdel-*trpexoL.2*, and Kdel-*trpexoL.3* were ϕ M12-transduced into *S. meliloti* 1021 to generate strains Kdel-*trpexoL.1-1*, Kdel-*trpexoL.2-1*, and Kdel-*trpexoL.3-3*.

Plant nodulation assays.

The host plant *Medicago sativa* (alfalfa) 'Iroquois' was prepared for inoculation with *S. meliloti* as described by Leigh and associates (1985) with modifications: seed were sterilized for 5 min in 50% bleach, rinsed in sterile water, and germinated for 3 days on plant cell culture-tested 1.1% (wt/vol) agar-water (Sigma-Aldrich). Seedlings were then moved to individual 100-by-15-mm Jensen's medium microcosm plates (Vincent 1970) and inoculated with 100 µl of *S. meliloti* of the appropriate strain at an optical density of 0.05 at wavelength 600 nm. Plants were grown in a Percival AR-36L incubator (Perry, IA, U.S.A.) at 21°C with 60 to 70% relative humidity and light at 100 to 175 µmol m⁻² s⁻¹ for 5 weeks. The host plant *M. truncatula* (barrel medic) Jemalong A17 was prepared for inoculation with *S. meliloti* by scarifying seed in concentrated sulfuric acid for 10 to 12 min, rinsing eight times in sterile water, soaking overnight at 4°C, and germinating for 3 days on plant cell culture-tested 1.1% (wt/vol) agar-water (Penmetsa and Cook 2000). Seedlings were then treated, inoculated, and maintained as for alfalfa (discussed above) for 7 weeks.

Western blotting.

Each stationary phase culture (20 µl) grown in MGS medium (York and Walker 1997) was added to 105 µl of sodium dodecyl sulfate (SDS) lysis buffer (final concentration 5% β -mercaptoethanol, 2.3% SDS, 8% glycerol, 42 mM Tris [pH 6.8], and 0.005% Bromphenol blue) and boiled for 5 min. Each cleared lysate (12 µl) was loaded onto a 12% SDS polyacrylamide gel electrophoresis gel (Bio-Rad mini-PROTEAN gel apparatus; Bio-Rad, Hercules, CA, U.S.A.). Approximately equivalent quantities of total protein were loaded, as measured by Bradford assay (Bradford, 1976). The gel was then blotted to polyvinylidene difluoride for 1 h in Tris/glycine/methanol buffer according to the manufacturer's recommendations (Bio-Rad mini Trans-Blot). The blot was blocked with 3% gelatin and incubated with α -exoK antisera (provided by G. Walker, Massachusetts Institute of Technology) (York and Walker 1998a) at a 1:400 dilution for 3 h at room temperature. Anti-rabbit immunoglobulin G secondary antibody and alkaline phosphatase substrate were used according to the manufacturer's suggestions (Bio-Rad Immun-blot AP colorimetric kit).

qRT-PCR assays.

RNA was isolated from 1.2 to 1.25 ml of culture from triplicate samples of each *S. meliloti* strain grown in either LBMC

or JMG medium. RNA was isolated using the Ambion Totally RNA kit (Life Technologies, Carlsbad, CA, U.S.A.). RNA was treated with DNase I from the Zymo DNA-free clean-up kit (Zymo Research, Irvine, CA, U.S.A.), and test qPCR tests performed to ensure that no DNA remained. Total RNA (225 µg) was reverse transcribed with random hexamers using Superscript III, followed by RNase H treatment (Life Technologies). qPCR of *exoL* was performed in a Bio-Rad CFX96 real-time cycler (conditions: 98°C for 2 min; 39 cycles of 98°C for 5 s and 65°C for 5 s; and melt curve of 65 to 95°C in 0.5°C increments for 5 s each) with 2× iQ SYBR green mix (Bio-Rad) with primers *exoL*-JGfwd versus *exoL*-JGrev (Glenn et al. 2007) on 3 µl of a 1/10 dilution of each RT reaction. qPCR of 16S rRNA was performed under the same conditions with primers JG16Sfwd versus JG16Srev (Gurich and Gonzalez 2009) on a 1/50 dilution of each RT reaction. Negative controls were 3 µl of a 1/10 dilution of each mock no-RT control reaction for *exoL* and 3 µl of a 1/50 dilution of each mock no-RT control reaction for 16S. Expression was quantified by qPCR with each primer set against a standard dilution curve of an RT reaction performed on *S. meliloti* 1021 total RNA. *exoL* expression was normalized to 16S rRNA expression and the resulting value multiplied by 10⁷ for presentation.

Detection of GUS activity and imaging of roots and nodules.

GUS expression by bacteria on root surfaces and within nodules was detected by staining whole roots in X-gluc buffer (1 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt; 0.02% SDS; and 50 mM Na-phosphate, pH 7) (Jefferson et al. 1987) for 23 to 47 h. Whole roots were imaged on an AZ100 Multi-Zoom Microscope equipped with a DS-Fi1, 5 Megapixel color camera (Nikon Instruments U.S., Melville, NY, U.S.A.).

ACKNOWLEDGMENTS

We thank G. C. Walker (Massachusetts Institute of Technology) for his kind gift of the α-ExoK antisera and the reviewers for their comments and suggestions. This work was funded by the United States Department of Agriculture National Institute of Food and Agriculture, Agriculture and Food Research Initiative grant 2010-65108-20582 to K. M. Jones. H. C. Mendis, C. Queirox, and B. K. Washburn constructed strains, performed experiments, analyzed data, and critically reviewed the manuscript; T. E. Brewer and O. M. Davis constructed strains; and K. M. Jones designed experiments, constructed strains, performed experiments, analyzed data, and wrote the manuscript.

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