Involvement of the SmeAB Multidrug Efflux Pump in Resistance to Plant Antimicrobials and Contribution to Nodulation Competitiveness in *Sinorhizobium meliloti*[▽][†]

Shima Eda,* Hisayuki Mitsui, and Kiwamu Minamisawa

Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan

The contributions of multicomponent-type multidrug efflux pumps to antimicrobial resistance and nodulation ability in *Sinorhizobium meliloti* were comprehensively analyzed. Computational searches identified genes in the *S. meliloti* strain 1021 genome encoding 1 pump from the ATP-binding cassette family, 3 pumps from the major facilitator superfamily, and 10 pumps from the resistance-nodulation-cell division family, and subsequently, these genes were deleted either individually or simultaneously. Antimicrobial susceptibility tests demonstrated that deletion of the *smeAB* pump genes resulted in increased susceptibility to a range of antibiotics, dyes, detergents, and plant-derived compounds and, further, that specific deletion of the *smeCD* or *smeEF* genes in a Δ*smeAB* background caused a further increase in susceptibility to certain antibiotics. Competitive nodulation experiments revealed that the *smeAB* mutant was defective in competing with the wild-type strain for nodulation. The introduction of a plasmid carrying *smeAB* into the *smeAB* mutant restored antimicrobial resistance and nodulation competitiveness. These findings suggest that the SmeAB pump, which is a major multidrug efflux system of *S. meliloti*, plays an important role in nodulation competitiveness by mediating resistance toward antimicrobial compounds produced by the host plant.

One of the major mechanisms of bacterial multidrug resistance is the active extrusion of drugs from cells by multidrug resistance efflux pumps (MDR pumps) (1, 29). Five families of MDR pumps have been described (51), including the ATP-binding cassette (ABC) family (55), the major facilitator superfamily (MFS) (15), the resistance-nodulation-cell division (RND) family (42), the small multidrug resistance (SMR) family (54), and the multidrug and toxic compound extrusion (MATE) family (27). The SMR and MATE pumps are single-component systems, whereas the RND pumps are multicomponent systems. Pumps in the remaining two families, the ABC family and MFS, occur as either single-component or multicomponent systems. In Gram-negative bacteria, which have cell walls made up of two membranes, multicomponent-type MDR pumps play a key role in multidrug resistance (41). Multicomponent MDR pumps consist of an inner membrane transporter (ABC family, MFS, or RND family transporter), a membrane fusion protein (MFP) (66), and an outer membrane factor (OMF) (46). These three components are essential for pump function and are assembled to form a large complex spanning both the inner and outer membranes (38, 65).

Because the emergence of bacterial pathogens with multidrug resistance poses an enormous threat to public health, the occurrence and role of MDR pumps have been exclusively studied in clinically relevant bacteria. However, recent whole-genome sequence analyses have revealed that MDR pumps are present in almost all bacteria and are generally chromosomally encoded (33, 45, 51). Therefore, it is now recognized that MDR pumps are evolutionarily ancient and might play a general role in enabling bacteria to circumvent the effects of naturally occurring toxic compounds in their own habitats (35, 49).

It is well known that plants produce a diverse array of antimicrobial secondary metabolites to protect themselves against pathogen infection (11). Several studies have demonstrated the involvement of MDR pumps in phytopathogenic bacteria in overcoming the chemical barriers formed by host plants (2, 5, 7, 34, 50). Barabote et al. showed that loss of TolC, an efflux pump protein from the OMF family, causes growth impairment of *Erwinia chrysanthemi* in witloof chicory leaves (5). Maggiorani Valecillos et al. demonstrated that two RND (Acr1AB and Acr2AB) pumps and one MFS (Emr2AB) pump are important for the growth of *E. chrysanthemi in planta* (34). The RND-type AcrAB-TolC pump of *Erwinia amylovora* and the MexAB-OprM pump of *Pseudomonas syringae* were shown by Ullrich et al. to be required for bacterial multiplication in apple rootstock and on pea leaves, respectively (2, 7). The importance of TolC in the growth of *Xylella fastidiosa* was demonstrated by Reddy et al. when a *tolC* mutant strain of this bacterium was not recovered after its inoculation into grape xylem (50). In all of these studies, efflux pump deletion mutants exhibited enhanced sensitivity to plant-derived antimicrobials, suggesting that efflux-mediated resistance generally contributes to the ability of pathogenic bacteria to colonize their host plants.

In plant-symbiotic bacteria, such as rhizobia, the role of MDR pumps is relatively unclear. The RmrAB pump identified in *Rhizobium etli* is a flavonoid-inducible MFS pump, and *rmrAB* mutants have an increased sensitivity to flavonoids and form fewer nodules on bean roots (19). Unlike with the *R. etli* RmrAB pump, loss of the RND-type RagCD pump in *Brady-*

rhizobium japonicum has no effect on the sensitivity of this symbiotic bacterium to antimicrobials or on its nodulation behavior in soybean roots (26). Very recently, Lindemann et al. reported that another *B. japonicum* RND-type pump, BdeAB, was not required for nodule formation on soybean roots but was essential for effective symbiotic nitrogen fixation in soybean nodules (31). In other host plants such as mung bean, cowpea, and siratro, nodules elicited by the *bdeAB* mutant fixed nitrogen efficiently, suggesting the host-specific role of the pump.

In *Sinorhizobium meliloti*, a symbiont of alfalfa, the OMF family protein TolC has recently been characterized by Cosme et al. (8). A *tolC* mutant strain of *S. meliloti* was highly sensitive to antibiotics, detergents, and plant-derived compounds and formed no, or very few, nodules on alfalfa roots. However, several phenotypes of this mutant could not be explained by efflux pump defects alone. The *tolC* mutant showed increased sensitivity to osmotic and oxidative stress and, moreover, showed greatly reduced production of succinoglycan, an exopolysaccharide that plays a crucial role in establishing successful symbiosis with the host plant. Thus, the contribution of MDR pumps to the nodulation ability of *S. meliloti* remains to be clarified.

In this study, we screened the genome of *S. meliloti* strain 1021 for all genes encoding transporter and MFP component proteins, as well as for those encoding OMF component proteins, and examined the effect of the loss of these genes on the bacterium's susceptibility to antimicrobials and on its symbiotic phenotype. By this comprehensive approach, an MDR pump system that contributes to antimicrobial resistance and competitive nodulation ability was identified.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α and MT616 were grown in Luria-Bertani medium (LB) (37). *S. meliloti* Rm1021 and its isogenic mutants were grown at 30°C in LB/MC (LB supplemented with 2.5 mM MgCl and 2.5 mM CaCl$_2$) or tryptone-yeast (TY) medium (23). Antibiotics at the following final concentrations for the culture of *S. meliloti* were used: streptomycin, 250 μg ml^{-1}; gentamicin, 50 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}.

Bioinformatics. DNA and amino acid sequences were analyzed with programs available on the Internet. Database searches with the BLAST program were performed at the genome database for rhizobia, RhizoBase (Kazusa DNA Research Institute [http://genome.kazusa.or.jp/rhizobase/]). Protein domain and motif analysis was done with the InterPro database (European Bioinformatics Institute [http://www.ebi.ac.uk/interpro]). InterPro integrates other protein signature databases, including ProSite, HAMAP, and Pfam.

General DNA manipulation. Standard molecular biology methods were used, as described previously (52). PCRs were performed with the high-fidelity polymerase PrimeStar (Takara Bio, Shiga, Japan). DNA sequencing was performed with an ABI Prism BigDye v3.1 terminator kit and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

Construction of mutants. All the PCR primer sets and restriction enzymes used for construction and confirmation of mutants are listed in Table S1 in the supplemental material. The transporter/MFP components and repressor deletion strains were constructed as follows. Efflux system genes together with flanking regions were amplified from the genomic DNA of *S. meliloti* by PCR and cloned into pTAC-1 (BioDynamics Laboratory, Tokyo, Japan). The resultant plasmids were digested with the appropriate restriction enzymes to remove the desired segments of the genes and then self-ligated. The DNA fragments containing deletions were transferred to the suicide vector pGMS1 (36). The resulting plasmid constructs were mobilized to *S. meliloti* by triparental mating, using *E. coli* MT616 as a helper strain. Integration of the plasmids introduced into the chromosome by a single-crossover event was tested by selection of the recombinants on LB/MC plates containing 50 μg ml^{-1} gentamicin or 5% sucrose. For deletion of the target genes, colonies showing both gentamicin resistance and

sucrose sensitivity were grown overnight in liquid LB/MC and spread on LB/MC plates containing 5% sucrose. Selected sucrose-resistant and gentamicin-sensitive strains were then tested for the deletion by PCR.

The OMF component deletion strains were constructed as follows. The SMa1037 and SMc02281 genes were amplified by PCR and cloned into the BamHI sites of pK18*mob* (53). The resulting plasmids were digested with the appropriate restriction enzymes and ligated with the tetracycline resistance cassette, excised from p34S-Tc (10) with SmaI, yielding pSE1037 and pSE02281. The replacement of the *tolC* gene with the gentamicin resistance cassette was done by using a modified insertion/deletion PCR method (18). First, a megaprimer was amplified from the plasmid pMS246 (6) by using primers Mega-1 and Mega-2. Next, the whole sequence of the plasmid pTAC-tolC, which contains the *tolC* gene and its flanking regions, was amplified with the megaprimer. At this step, the *tolC* gene was replaced by a tetracycline resistance cassette. The resultant plasmid was digested with HindIII, and the fragment containing the tetracycline-resistant cassette was cloned into the corresponding sites of pK18*mob*, yielding pSEtolC. Plasmids pSE1037, pSE02281, and pSEtolC were mobilized to *S. meliloti* as described above. To select for double-crossover mutants, transconjugants were patched onto LB/MC plates containing 200 μg ml^{-1} of neomycin. The tetracycline-resistant and neomycin-sensitive cells, as well as the gentamicin-resistant and neomycin-sensitive cells, were tested for the deletion by PCR.

Construction of a *smeAB*-expressing plasmid. The *smeAB* genes and their putative promoter regions were amplified by using primers 5′-ATTCTAGAGC CTTGGTGATCTTCACTT-3′ and 5′-ATGGATCCGTTCGGCGCATTTATT CAA-3′, which introduced XbaI or BamHI restriction sites (underlined). The amplified fragment was cleaved with XbaI and BamHI and then cloned into the corresponding sites of pFAJ1702, a stable RK2-derived cloning vector (12), yielding plasmid pSE344.

Antimicrobials and susceptibility determination. Erythromycin, tetracycline, chloramphenicol, nalidixic acid, neomycin, kanamycin, vancomycin, polymyxin B, acriflavine, crystal violet, sodium dodecyl sulfate, deoxycholate, and *p*-coumaric acid were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan); rifampin, novobiocin, naringenin, and berberine were obtained from Sigma-Aldrich (St. Louis, MO); genistein was obtained from Extrasynthese (Genay, France); and luteolin and chrysin were obtained from LKT Laboratories (St. Paul, MN). The MICs of the antimicrobials and NaCl were determined by 2-fold serial agar dilution, according to the method recommended by the Japanese Society of Chemotherapy, with some modifications (20). In brief, serial 2-fold dilutions of the antimicrobials in TY agar were spotted with 5 μl of culture suspension (10^6 cells ml^{-1}) of the test strains. After incubation of the samples at 30°C for 48 h, the MIC was determined by observing the lowest concentration of the antimicrobial in which bacterial growth was inhibited.

H$_2$O$_2$ sensitivity test. The zone inhibition assay for determination of H$_2$O$_2$ sensitivity was performed as follows. TY plates were streaked with a cotton swab soaked in *S. meliloti* cell suspension (10^7 cells ml^{-1}). Sterile paper disks (8 mm in diameter) were placed on the plate, and 20 μl of 100 mM H$_2$O$_2$ was applied to the disks. After incubation of the samples at 30°C for 48 h, the sizes of the inhibitory zones were measured.

Plant growth conditions. Seeds of alfalfa (*Medicago sativa* 'Du Puits') were surface sterilized in a 1% (wt/vol) sodium hypochlorite solution and then rinsed in sterilized water. They were germinated on 1% water-agar for 2 days at 25°C and then transplanted to a Leonard jar (59) containing sterile vermiculite and Jensen's nitrogen-free nutrient solution (62). The seeds were then inoculated with *S. meliloti* cells and grown in a growth chamber for 4 weeks at 25°C, with a photoperiod regime of 16 h of illumination (80 μmol m^{-2} s^{-1} photosynthetically active radiation) and 8 h of darkness.

Competition assay for nodulation. Late logarithmic cultures of the wild-type and mutant strains were washed twice with sterile water, suspended, and mixed at wild type to mutant ratios of 9:1, 1:1, and 1:9 [for Δ*smeAB*, Δ*smeAB* (*smeAB*$^+$), and Δ*smeR* strains] or 1:1 (for other mutants). Seedlings were inoculated with 1 ml of each mixture containing 10^6 cells. Nodulated root systems were sterilized with 70% ethanol for 30 s and a 1% (wt/vol) sodium hypochlorite solution for 3 min, followed by rinsing with sterilized water three times. At least 96 nodules from at least 24 separate plants in each test were aseptically cut off from the roots and crushed in 50 μl of sterilized water with a sterilized toothpick. This toothpick was then streaked across an LB/MC plate. The resultant colonies were examined for their susceptibility to antibiotics or for their genotypes by PCR. The antibiotics used and final concentrations were as follows: erythromycin, 10 μg ml^{-1}, and tetracycline, 1 μg ml^{-1} or 10 μg ml^{-1}. The competition assay was performed three times, and the significance of differences was analyzed by the chi-square test.

TABLE 1. Predicted multicomponent-type MDR pump proteins in *S. meliloti*

Transporter or channel	Assigned family	Cognate MFP	Operon structure	Replicon	Location of[a]:	
					Operon	Deletion
SMc04351	ABC	SMc04350	MPF-ABC	Chromosome	2232766–2235983	2233004–2235261
SMb20698	MFS	SMb20699	MFP-MFS	pSymB	1501447–1498701	1499762–1501113
SMc00563	MFS	SMc00564	MFS-MFP	Chromosome	1233181–1235861	1234064–1235310
SMc03167	MFS	SMc03168	MFP-MFS	Chromosome	3129921–3127155	3127691–3129698
SMa0875 (NolG)	RND	SMa0876 (NolF)	MFP-RND	pSymA	489965–485661	485857–489957
SMa1662	RND	SMa1664	MFP-RND	pSymA	932189–927892	929122–932006
SMa1884	RND	SMa1885	MFP-RND	pSymA	1074193–1069938	1070528–1073525
SMb20345	RND	SMb20346	MFP-RND	pSymB	354680–350348	350810–353988
SMb21498	RND	SMb21497	MFP-RND	pSymB	1418498–1422856	1419045–1422491
SMc01095 (SmeD)	RND	SMc01094 (SmeC)	MFP-RND	Chromosome	465625–461103	461790–465428
SMc01457	RND	SMc01458	MFP-RND	Chromosome	2305172–2300845	2301195–2304578
SMc01829 (SmeF)	RND	SMc01828 (SmeE)	MFP-RND	Chromosome	2651785–2656299	2652284–2656291
SMc02867 (SmeB)	RND	SMc02868 (SmeA)	MFP-RND	Chromosome	224287–219907	221106–223242
SMc03971	RND	SMc03972	MFP-RND	Chromosome	2968834–2964504	2964584–2968500
SMa1037	OMF			pSymA	570509–571975	570687–571679
SMc02082 (TolC)	OMF			Chromosome	1629672–1631042	1629672–1631042
SMc02281	OMF			Chromosome	653617–655062	654037–654784

[a] Numbers represent the nucleotide positions in each replicon.

Real-time RT-PCR. *S. meliloti* strains were grown to an optical density at 600 nm (OD_{600}) of 0.6 or 1.7 in TY medium. Total RNA was purified by using the RNeasy minikit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions, and then treated with the Turbo RNase-free DNase (Ambion, Austin, TX) to remove the residual genomic DNA. cDNA was synthesized from 0.5 μg of total RNA with a random 6-mer primer using the PrimeScript reverse transcription (RT) reagent kit (Takara Bio), according to the manufacturer's instructions. Quantitative real-time PCR was performed in a final volume of 25 μl containing 2× SYBR Premix EX *Taq* (Takara Bio), 0.3 μM forward and reverse primers, and 2 μl of cDNA from the RT reaction mixture. The sequences of the primers used are listed in Table S2 in the supplemental material. Amplification was performed with an iCycler iQ apparatus (Bio-Rad Laboratories, Tokyo, Japan) using the following protocol: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The comparative cycle threshold method was used to quantify the abundance of target transcripts, and changes in expression level were calculated after normalization to the internal control, SMc00128 (25).

RESULTS

Identification of multicomponent-type MDR pump genes. To comprehensively survey the effect of the loss of multicomponent-type MDR pump genes on susceptibility to antimicrobials and on phenotypes relevant to symbiosis, we searched the *S. meliloti* 1021 genome for the genes encoding proteins with sequence similarity to, and signature motifs for, well-known efflux pump components. By means of a BLAST search and subsequent domain analysis, 1 ABC-type, 3 MFS-type, and 10 RND-type systems and 3 OMF proteins were identified (Table 1).

A protein encoded by the SMc04351 gene showed highly significant similarity to MacB (E value of 1e−156), a transporter component of the *E. coli* ABC-type MacAB-TolC pump (24). The SMc04351 protein was also assigned with high probability by the protein database HAMAP to be a MacB family protein (family ID, MF_01720). MacB family proteins contain two domains, an ATPase domain (InterPro accession no. IPR003593) and an FtsX-like permease domain (InterPro accession no. IPR003838). Because no other ABC proteins with the FtsX-like permease domain were found in the *S. meliloti* 1021 genome, we concluded that SMc04351 is only one transporter component of the ABC-type multicomponent MDR

pump in this organism. The genes encoding transporter components of multicomponent MDR pumps occur mostly in tandem with the gene encoding the cognate MFP component. Indeed, an MFP gene (SMc04350) is located adjacent to SMc04351.

MFS, one of the largest families of membrane transporters, transports a variety of small organic molecules, such as sugars, amino acids, and drugs (44). Although several InterPro signatures for MFS drug transporters (IPR001411, IPR001958, IPR004734, IPR004751, and IPR004821) have been described, the sequence signature specific for multicomponent-type MFS drug transporters is not yet known. In this study, the occurrence of MSF and MFP genes in tandem organization was adopted as a criterion for selecting multicomponent-type MFS drug transporters, because all known genes encoding multicomponent-type MFS transporters are accompanied by MFP genes (19, 28, 32, 34, 63). A BLAST search with *R. etli* RmrB (19) returned 22 sequences with BLAST E values of less than 1.0. Among the genes encoding these sequences, three genes (SMb20698, SMc00563, and SMc03167) were associated with MFP genes.

RND superfamily transporters also have diverse substrate specificity (60). Within this superfamily, members of the hydrophobe/amphiphile efflux 1 (HAE-1) family extrude multiple drugs, those of the heavy metal efflux (HME) family export monovalent or divalent heavy metal cations, and those of the SecDF family are involved in protein translocation. A BLAST search with *E. coli* AcrB, an HAE-1 family transporter component of the AcrAB-TolC pump (16), returned 12 sequences with BLAST E values of less than 1.0. The top 10 BLAST matches showed highly significant E values (lower than 2e−36). The two sequences with relatively insignificant E values, those of SMb20882 (1e−4) and SMc02057 (0.23), were excluded from further analysis because the polypeptide length of SMb20882 (149 aa) was much shorter than that of AcrB (1,049 aa) and because SMc02057 showed highly significant similarity to *E. coli* SecD (E value of 3e−55). A domain-based search identified 12 sequences containing the acriflavine resis-

tance protein domain (InterPro accession no. IPR001036). Two of them, the sequences of SMb20882 (see above) and SMc02265 (another SecD homolog), were excluded from further analysis, and the remaining 10 sequences were identical to those identified by the BLAST homology search. Because two amino acid residues, D408 and K940 (positions referred to *E. coli* AcrB), which are conserved in the HAE-1 family but not in the HME family (48, 60), were found in all 10 sequences, we concluded that *S. meliloti* 1021 has 10 RND-type drug transporters. The MFP genes were located adjacent to all of the RND genes.

A computational search for the OMF family proteins of *S. meliloti* identified SMc02082 (designated TolC) (8). This study also identified two sequences, designated SMc1037 and SMc02281, encoding the outer membrane efflux protein motif (Pfam ID PF02321), but these sequences were not analyzed because they displayed low similarity to *E. coli* TolC and each had a possible lipid attachment site, unlike TolC (8). However, OprM, a well-characterized OMF family protein of *Pseudomonas aeruginosa*, is a lipoprotein with a palmitoyl moiety modification at its N terminus (39). Thus, in the present study, SMc1037 and SMc02281 were further analyzed. In certain bacterial species, such as *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*, the OMF components are usually specific for a particular efflux pump, and the genes encoding them form a gene cluster together with cognate MFP and transporter component genes (48, 56). In contrast, in the *Enterobacteriaceae*, such as *E. coli* and *Salmonella*, TolC is used by many efflux pumps as the common outer membrane component, and the *tolC* gene is not linked to other component genes (22, 43). In *S. meliloti* 1021, we found that the genes encoding OMF proteins were located at positions distant from the MFP and transporter component genes.

The SmeAB-TolC, SmeCD-TolC, and SmeEF-TolC systems are responsible for resistance to toxic substances. To identify the pump components responsible for resistance to toxic substances, mutants containing deletions in each of the 14 transporter/MFP gene pairs were constructed, and their susceptibilities to 20 compounds were tested. Strain SE122 (ΔSMc02868-SMc02867) was the only transporter/MFP deletion mutant to exhibit an altered susceptibility to the tested compounds (Table 2; see also Table S3 in the supplemental material). The deletion mutation in SMc02868-SMc02867 resulted in a 2- to 64-fold decrease in resistance to a diverse array of compounds, including antibiotics, detergents, dyes, and plant-derived chemicals. When SMc02868-SMc02867 genes were expressed in *trans* in the mutant strain, the resistance to these compounds was completely restored (strain SE536) (Table 2). These results suggested that the RND-type SMc02868-SMc02867 pump, which we designated SmeAB (*Sinorhizobium* multidrug efflux protein AB), plays a major role in the extrusion of toxic substances in *S. meliloti* 1021.

Next, a series of double mutants between *smeAB* and other transporter/MFP pairs were constructed because the presence of a major efflux pump can mask the effect of the absence of a minor efflux pump when the pumps have overlapping substrate profiles (57, 58). Deletions of the two RND-type pumps, SMc01094-SMc01095 (*smeCD*) or SMc01828-SMc01829 (*smeEF*), in a ΔsmeAB background resulted in a further increase in susceptibility to chloramphenicol and nali-

TABLE 2. Susceptibility of *S. meliloti* efflux pump gene deletion strains to antimicrobial compounds

Strain[a]	Relevant genotype	MIC (µg/ml)[b]																			
		ERM	TET	CAM	NAL	RIF	NEO	KAM	NOV	VAN	PXB	ACR	CV	SDS	DOC	Gen	p-CA	Nar	Ber	Lut	Chr
1021	Wild type	100	0.39	12.5	200	3.13	6.25	50	>100	50	1.56	100	400	400	>800	100	400	200	1,600	>50	>25
SE122	ΔsmeAB	**1.56**	**0.1**	**6.25**	**100**	**1.56**	6.25	50	>100	50	1.56	**50**	400	**200**	>800	**50**	400	**100**	**400**	50	25
SE536	ΔsmeAB smeAB+	100	50[c]	12.5	200	**6.25**	6.25	50	>100	50	1.56	**200**	400	400	>800	100	400	200	1,600	>50	>25
SE404	ΔsmeAB ΔsmeCD	1.56	0.1	**3.13**	**50**	1.56	6.25	50	>100	50	1.56	50	400	200	>800	50	400	100	400	>50	25
SE272	ΔsmeAB ΔsmeEF	**0.78**	**0.05**	6.25	100	1.56	6.25	50	>100	50	1.56	50	400	200	>800	50	400	100	400	50	25
SE569	ΔsmeAB ΔsmeCD ΔsmeEF	**0.78**	**0.05**	**3.13**	**50**	1.56	6.25	50	>100	50	1.56	50	400	200	>800	50	400	100	400	50	25
SE424	ΔtolC	**0.2**	**0.03**	**0.78**	**12.5**	**0.78**	**1.56**	**12.5**	**0.39**	**12.5**	**0.39**	**3.13**	**3.13**	**6.25**	**25**	**6.25**	**200**	**25**	**50**	**12.5**	**6.25**
SE466	ΔsmeR	>100	**1.56**	**25**	**400**	**12.5**	6.25	50	>100	50	1.56	**400**	400	400	>800	**200**	400	>200	**3,200**	>50	>25

a Only strains that showed a change in sensitivity are shown.
b MIC determination was performed at least three times. For single mutants, values in boldface differ from the 1021 (wild-type) control strain value by ≥2-fold. For double and triple mutants, values in boldface differ from the SE122 (ΔsmeAB) control strain value by ≥2-fold. Abbreviations: ERM, erythromycin; TET, tetracycline; CAM, chloramphenicol; NAL, nalidixic acid; RIF, rifampin; NEO, neomycin; KAM, kanamycin; NOV, novobiocin; VAN, vancomycin; PXB, polymyxin B; ACR, acriflavine; CV, crystal violet; SDS, sodium dodecyl sulfate; DOC, deoxycholate; Gen, genistein; *p*-CA, *p*-coumaric acid; Nar, naringenin; Ber, berberine; Lut, luteolin; Chr, chrysin.
c Tetracycline resistance was conferred by the antibiotic resistance marker.

dixic acid or to erythromycin and tetracycline, respectively (Table 2; see also Table S3 in the supplemental material).

Consistent with the findings of Cosme et al. (8), deletion of the *tolC* gene resulted in increased susceptibility to antibiotics, detergents, dyes, and plant-derived chemicals (Table 2). Deletions in the remaining two OMF genes, SMa1037 and SMc02281, had no effect on antimicrobial susceptibility in both wild-type and Δ*tolC* backgrounds (see Table S3 in the supplemental material), indicating that the SMa1037 and SMc02281 proteins are not involved in the efflux of toxic substances tested. Thus, we concluded that TolC is a common OMF component shared by multiple MDR pump systems and that, at least under the conditions tested, three complexes, SmeAB-TolC, SmeCD-TolC, and SmeEF-TolC, are responsible for resistance to toxic substances in *S. meliloti* 1021.

Notably, the MIC values of tested compounds for the *tolC* mutant were mostly lower than those for the triple mutant strain SE569, in which all three active pumps were knocked out (Δ*smeAB* Δ*smeCD* Δ*smeEF*) (Table 2). Furthermore, the *tolC* mutant was sensitive to several compounds that were not extruded by the three pumps (i.e., neomycin, kanamycin, novobiocin, vancomycin, polymyxin B, crystal violet, deoxycholate, and *p*-coumaric acid). These results suggest that the increased susceptibility of the *tolC* mutant was caused not only by the loss of the TolC-dependent efflux pump functions but also by an unknown multidrug-efflux-independent mechanism.

Deletions in transporter/MFP genes had no effect on sensitivity to osmotic and oxidative stress and production of succinoglycan. Cosme et al. reported that the *tolC* mutant exhibited increased sensitivity to osmotic and oxidative stress and a loss of succinoglycan production (8). To examine whether these impairments are caused by the loss of the TolC-dependent MDR pump functions, the growth of the transporter/MFP deletion mutants (single, double, and triple mutants) and the *tolC* mutant in the presence of NaCl or H_2O_2 was examined first. The MICs of NaCl for all transporter/MFP deletion mutants were comparable to that of the wild-type strain (0.75 M), whereas the MIC of the *tolC* mutant was 2-fold lower than that of the wild-type strain (0.375 M). When the strains were exposed to disks containing 100 mM H_2O_2, the wild type and all transporter/MFP deletion mutants had almost the same-sized inhibitory zones (14 ± 1 mm in diameter), but the *tolC* mutant had one that was larger (17 ± 1 mm in diameter). Next, the succinoglycan production ability of the mutants was tested by using plates containing calcofluor white, on which succinoglycan-producing strains form fluorescent colonies (14). The wild-type strain and all transporter/MFP-deletion mutants formed bright fluorescent colonies on these plates (see Fig. S1 in the supplemental material). In contrast, the *tolC* mutant colonies were dark. Taken together, these results suggest that none of the transporter/MFP pairs are involved in osmotic and oxidative stress resistance or in succinoglycan production.

smeAB **deletion weakens the competitive nodulation ability of *S. meliloti*.** To examine the role of the multicomponent-type MDR pumps in nodulation, a single-inoculation experiment was performed first. Alfalfa seedlings were inoculated with the wild-type strain, 17 single mutants (14 transporter/MFP deletion mutants and 3 OMF deletion mutants), and a triple mutant (strain SE569), and after 4 weeks of cultivation under nitrogen-limited conditions, plant growth and nodule forma-
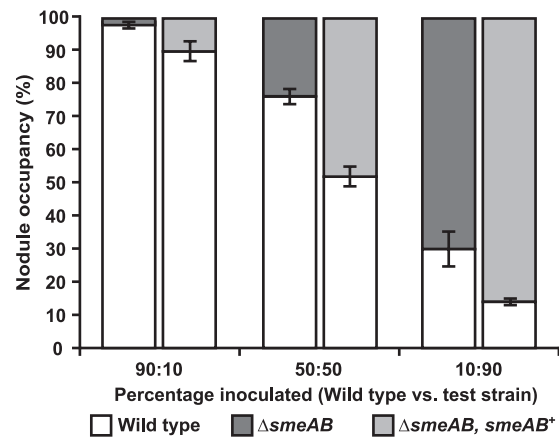

FIG. 1. Competitive nodule occupancies of the *smeAB* mutant and the complemented strains in coinoculations with the wild-type strain. Each test strain was mixed with the wild-type strain in three different ratios and inoculated onto alfalfa roots. Four weeks after inoculation, bacteria were reisolated from nodules, and the percent recovery of each strain was calculated. The experiment was performed three times, and data are presented as the means ± standard deviations (SD). The difference between the expected and observed percentages was evaluated by the χ^2 test. In each repetition of the experiment, the *smeAB* mutant was less competitive than the wild-type strain ($P < 0.01$) at all inoculation ratios.

tion were investigated. All mutants, except the *tolC* mutant, formed normal numbers of nitrogen-fixing nodules on alfalfa roots under the conditions tested (data not shown). Consistent with the report by Cosme et al., the *tolC* mutant elicited no, or only a few, non-nitrogen-fixing nodule-like structures (data not shown) (8).

Because the extrusion of toxic substances by MDR pumps might benefit bacterial cells in environments where these substances are present, it is tempting to speculate that losses of multidrug efflux pumps would affect the competitive nodulation ability of *S. meliloti*. To test this notion, we performed coinoculation experiments in which the wild-type strain and one of each single mutant (except the nodulation-deficient *tolC* mutant) were combined at a 1:1 ratio and then applied to the plants. Whereas most mutants were equally competitive with the wild-type strain in nodule occupancy, the *smeAB* mutant was less competitive than the wild-type strain, as the observed proportion of nodule occupancy by the *smeAB* mutant was lower than the expected proportion of nodule occupancy by the mutant (Fig. 1). The same trends were observed when the wild-type and mutant strains were coinoculated at ratios of 9:1 and 1:9 (Fig. 1). The introduction of a plasmid carrying *smeAB* into the mutant restored competitiveness to almost wild-type levels (Fig. 1), confirming the contribution of the SmeAB pump to competitive nodulation ability.

smeR **deletion results in increased resistance to toxic substances and competitiveness in nodulation.** Immediately downstream of the *smeB* gene, there is an open reading frame (SMc02866) corresponding to 216 amino acids, which is predicted to be a TetR family transcription regulator (Fig. 2A). A mutant with a deletion of SMc02866 showed significantly higher levels of *smeA* and *smeB* mRNA than the wild-type strain, regardless of growth phase (Table 3), indicating that
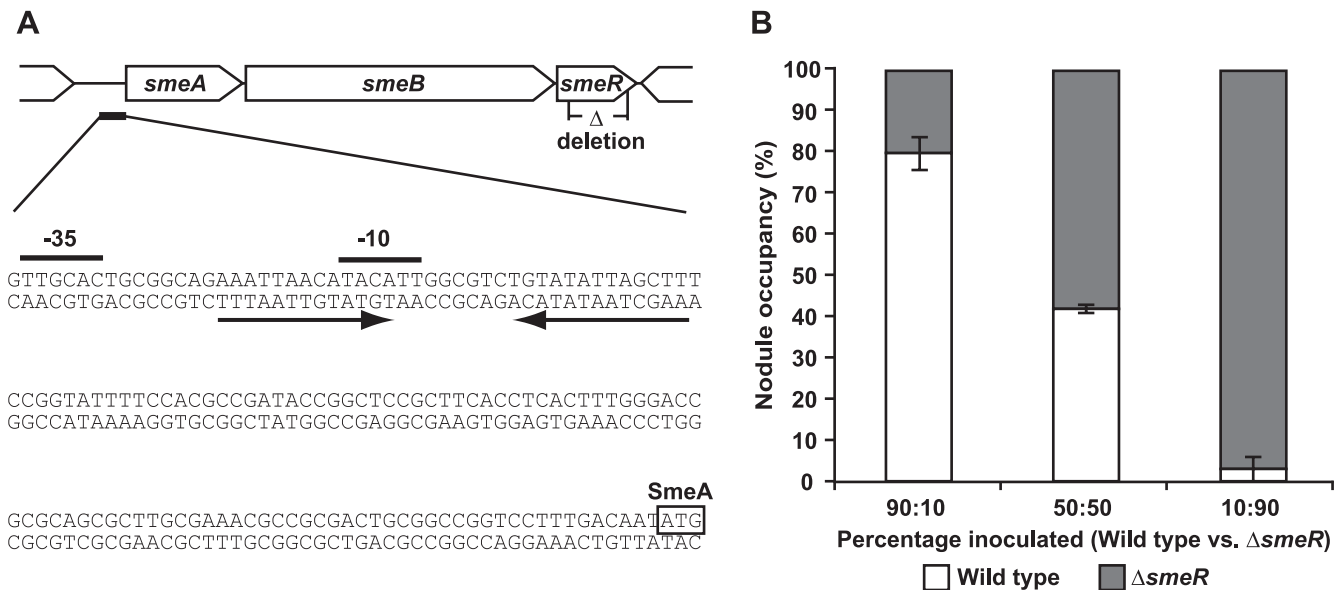
A

B

FIG. 2. Characterization of the *smeR* mutant strain. (A) Schematic representation of the putative *S. meliloti smeABR* operon region. The deleted region of the *smeR* mutant is indicated by the "Δ" symbol. The start codon (ATG) of the *smeA* gene is boxed. The predicted promoter sequence (−35 and −10) is overlined. An inverted repeat corresponding to a potential regulatory site is shown by arrows. (B) Competition for nodule occupancy between the Δ*smeR* mutant and the wild-type strains. See the legend to Fig. 1 for details. In each repetition of the experiment, the *smeR* mutant was more competitive than the wild-type strain ($P < 0.01$) at all inoculation ratios.

SMc02866 negatively regulates *smeAB* expression. Therefore, the SMc02866 gene was designated *smeR*. The *smeR* deletion mutant exhibited a higher level of resistance to nine compounds that were substrates of the SmeAB pump than that exhibited by the wild-type strain (Table 2). Subsequent coinoculation experiments demonstrated that the *smeR* mutant was consistently more competitive than the wild-type strain in nodule occupancy at all inoculum combinations tested (Fig. 2B).

DISCUSSION

The present study revealed that, of the 14 efflux systems, the SmeAB RND-type pump makes a major contribution to the antimicrobial resistance of *S. meliloti*, whereas the other two RND-type pumps, SmeCD and SmeEF, make only minor contributions. This is consistent with the observations that *E. coli* and *P. aeruginosa* have multiple multicomponent-type MDR pumps, and AcrAB of *E. coli* and the MexAB of *P. aeruginosa* play major roles in making these bacteria intrinsically resistant to most classes of antibiotics (29, 30, 57). Therefore, it is assumed that, even in bacteria with large numbers of multicomponent-type MDR pumps, only a very limited number of

pumps contribute to intrinsic antimicrobial resistance. Because the bacteria with the largest numbers of multicomponent-type MDR pumps are found in the soil or in association with plants (45), it might be interesting to investigate whether this is generally true in such organisms.

In contrast to the strains with knockouts of the three pumps mentioned above, those with knockouts of the remaining 11 pumps showed no increase in antimicrobial susceptibility. There are several possible explanations for these observations that lie outside the possibility that these pumps are dysfunctional systems. First, these 11 pumps might extrude compounds that were not used in this study. Although we tested compounds representative of several different classes, they might not have been able to detect the contributions of all pumps. Second, the 11 pumps might have been poorly expressed, or not expressed at all, under the conditions tested, so that their deletion would have had very little effect on antimicrobial susceptibility. Third, these pumps might not be involved in antimicrobial resistance but might play other roles. Several recent studies have revealed the physiological roles of multicomponent-type MDR pumps (35, 49). For example, the MexAB-OprM system of *P. aeruginosa* is involved in the translocation of quorum sensing signals (13, 47). If efflux pumps transport nontoxic compounds that serve physiological functions, their activities cannot be detected by the antimicrobial susceptibility test.

MDR pump operons often contain a physically linked regulatory gene (21). In *S. meliloti*, six multicomponent-type MDR systems are associated with putative regulator genes (see Table S4 in the supplemental material). In this study, a TetR family transcription regulator, named SmeR, was shown to negatively regulate the expression of *smeAB* genes. Because most of the TetR family regulators recognize and bind to DNA

TABLE 3. Effect of the *smeR* deletion on *smeAB* expression

Gene	Fold change at indicated phase (OD$_{600}$ value)^a	
	Log phase (0.6)	Stationary phase (1.7)
smeA	10.2 ± 2.7	8.1 ± 2.9
smeB	9.0 ± 1.2	7.6 ± 0.9

^{a} The relative change in mRNA expression is calculated from the cycle threshold values obtained by real-time PCR analysis. Positive values indicate higher expression in the *smeR* mutant. The means ± standard deviations from three independent experiments are shown.

sequences with inverted repeats (palindromic sequences) (64), it is speculated that the negative regulation of *smeAB* expression is mediated by the binding of SmeR to an inverted repeat found in the putative *smeAB* promoter region (Fig. 2A). One other common feature of TetR family regulators is that their DNA-binding activity is abrogated by the interaction with ligand molecules (64). Thus, it is also speculated that transcription from the *smeAB* promoter is induced by the presence of SmeAB pump substrates. Experiments designed to examine these speculations are under way.

The RND-type NolFG pump (SMa0876-SMa0875) is thought to be involved in nodulation, because a Tn*5* insertion in any one of the genes of the *nodM-nolFG-nodN* operon results in delayed nodulation and reduction in the number of nodule of alfalfa (3). In a further study, this group suggested that Tn*5* insertions in the *nolFG* genes lower the transcript level of the downstream *nodN* gene, which encodes a protein required for the efficient production of the Nod factor (4). Because our markerless *nolFG* deletion mutant showed no altered symbiotic phenotypes, it is highly likely that the Tn*5*-induced mutations in the *nolFG* genes caused a downstream polar effect on expression of the *nodN* gene, which in turn caused the impaired nodulation phenotype. In addition to the NolFG pump, the remaining 13 pumps were not essential for the establishment of symbiosis with alfalfa. However, the *smeAB* mutant, which exhibited increased sensitivity to several plant-derived compounds, was consistently less competitive than the wild-type strain for nodule occupancy in coinoculation experiments, indicating the importance of efflux-mediated antimicrobial resistance in the nodulation competitiveness of *S. meliloti*. This is consistent with the notion suggested by González-Pasayo and Martínez-Romero (19) that MDR pumps prevent the accumulation of toxic plant compounds within bacterial cells and thereby confer advantages to rhizobia for nodulation. The observation that the *smeR* mutant, which overexpressed the *smeAB* genes, exhibited increased resistance to antimicrobial agents and was more competitive than the wild-type strain is also consistent with this notion. At this stage, however, it cannot be excluded that derepression of other SmeR-controlled genes, if any, causes an increase in nodulation competitiveness.

Recently, Lindemann et al. have characterized an efflux pump (BdeAB) of the RND family from *B. japonicum* (31). A mutant with deletion of the *bdeAB* genes showed an increased susceptibility to aminoglycoside antibiotics and had strongly decreased symbiotic nitrogen fixation activity on the soybean. The nodules formed on the mung bean, cowpea, and siratro by the *bdeAB* mutant fixed nitrogen efficiently, suggesting the host-specific role of the pump. Although SmeB is the closest ortholog of *B. japonicum* BdeB in *S. meliloti* and shares high similarity (64.1% identity) with BdeB, alfalfa plants inoculated with the *smeAB* mutant did not show nitrogen starvation symptoms. In addition, the other 13 MDR pump systems also appeared not to be required for symbiotic nitrogen fixation. Because *S. meliloti* establishes symbiosis not only with *M. sativa* (alfalfa) but also with other *Medicago* species, such as *Medicago truncatula*, it might be worthwhile to determine whether *S. meliloti* efflux pump mutants exhibit host-specific symbiotic phenotypes.

The results from investigations into the *tolC* mutant suggest that the outer membrane integrity of this mutant is compromised. The *tolC* mutant was highly sensitive to novobiocin (>256-fold), crystal violet (128-fold), deoxycholate (DOC; >32-fold), vancomycin (4-fold), and polymyxin B (4-fold). By comparison, none of the transporter/MFP deletion mutants showed increased sensitivity to the compounds. All of these compounds are commonly used to assess alterations in the cell envelopes of Gram-negative bacteria, and the increased sensitivity of bacteria to these compounds serves as an indicator for the loss of outer membrane integrity (9, 17). In addition, the MIC values of compounds tested with the *tolC* mutant were consistently lower than those tested with the triple-pump mutant (strain SE569). The outer membrane constitutes a permeability barrier that usually limits antibiotic access to intracellular targets (40), and therefore, its destruction generally results in increased sensitivity to antibiotics. The *tolC* mutant exhibited increased sensitivity not only to antimicrobials but also to osmotic and oxidative stress. Because some rhizobial mutants exhibiting loss of outer membrane also show increased sensitivity to osmotic and/or oxidative stress (9, 61), the defects in osmotic and oxidative stress resistance in the *tolC* mutant might be a consequence of the loss of outer membrane integrity. It is also possible that the defect in succinoglycan production in the *tolC* mutant is caused by impairment of outer membrane integrity, because outer membrane integrity must be maintained to ensure the proper function of membrane machineries, such as the polysaccharide export complex. Further experiments are required to examine these hypotheses and other possibilities.

In conclusion, the primary outcome of this study was the observation that the SmeAB system, which is the main MDR pump responsible for resistance to antimicrobial plant compounds, contributed to competitive nodulation ability in *S. meliloti* 1021. This finding has extended our knowledge of the roles of multicomponent-type MDR pumps in nodulation and symbiosis. We also revealed that none of the transporter/MFP deletion mutants showed increased osmotic and oxidative stress resistance or the loss of succinoglycan production ability. This finding led us to conclude that the defects observed in the *tolC* mutant are independent of the MDR pump. Future work will concentrate on whether the outer membrane disorganization resulting from the loss of TolC contributes to the symbiotic defect phenotype of the *tolC* mutant.

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