RESEARCH LETTER

Host-specific symbiotic requirement of BdeAB, a RegR-controlled RND-type efflux system in *Bradyrhizobium japonicum*

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Abstract

Multidrug efflux systems not only cause resistance against antibiotics and toxic compounds but also mediate successful host colonization by certain plant-associated bacteria. The genome of the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum* encodes 24 members of the family of resistance/nodulation/cell division (RND) multidrug efflux systems, of which BdeAB is genetically controlled by the RegSR two-component regulatory system. Phylogenetic analysis of the membrane components of these 24 RND-type transporters revealed that BdeB is more closely related to functionally characterized orthologs in other bacteria, including those associated with plants, than to any of the other 23 paralogs in *B. japonicum*. A mutant with a deletion of the *bdeAB* genes was more susceptible to inhibition by the aminoglycosides kanamycin and gentamicin than the wild type, and had a strongly decreased symbiotic nitrogen-fixation activity on soybean, but not on the alternative host plants mungbean and cowpea, and only very marginally on siratro. The host-specific role of a multidrug efflux pump is a novel feature in the rhizobia–legume symbioses. Consistent with the RegSR dependency of *bdeAB*, a *B. japonicum regR* mutant was found to have a greater sensitivity against the two tested antibiotics and a symbiotic defect that is most pronounced for soybean.

Introduction

Multidrug resistance (MDR) efflux systems are ubiquitous and important means by which living cells cope with toxic compounds in their environment (Higgins, 2007; Blair & Piddock, 2009). These efflux systems have been classified into five families, whose members recognize and extrude a battery of structurally dissimilar compounds from the cell (Saier & Paulsen, 2001). Transport systems of the resistance/nodulation/cell division (RND) family are the major cause of antibiotic resistance in clinically relevant Gram-negative bacteria (Piddock, 2006). The well-studied RND-type drug export system of *Escherichia coli* consists of the AcrB transport protein, localized in the cytoplasmic membrane, the membrane fusion protein AcrA, and the outer membrane protein TolC (Nikaido & Zgurskaya, 2001). The physiological role of MDR efflux systems is not only restricted to antibiotic resistance, but may also enhance the virulence of animal- and human-pathogenic bacteria (Piddock, 2006; Martinez *et al.*, 2009).

Plant roots produce and secrete a large diversity of secondary metabolites into the rhizosphere, several of which possess bioactive potential and play important roles in the interaction of plants with soil microorganisms. For example, phytoalexins form a central component of the plant defense system (Hammerschmidt, 1999; Grayer & Kokubun, 2001), and flavonoids serve as crucial signaling compounds in the symbiotic interaction between nitrogen-fixing rhizobia and their host plants (Long, 2001; Gibson *et al.*, 2008). In phytopathogenic bacteria, MDR efflux systems were shown to contribute to the successful interaction with host plants. Their loss by mutation compromised the bacteria strongly in virulence and in their capability to extrude antibiotics and phytoalexins (see Martinez *et al.*, 2009, and references therein). By contrast, little is known about the role of MDR efflux pumps in rhizobia. Mutants of the bean symbiont

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Rhizobium etli that lack the RmrAB efflux pump (a member of the major facilitator superfamily) are more sensitive to phytoalexins and are impaired in root-nodule formation (Gonzalez-Pasayo & Martinez-Romero, 2000). In *Sinorhizobium meliloti*, the NolGHI proteins belonging to the RND-type efflux family are possibly involved in the export of nodulation signals (Saier *et al.*, 1994), although this was disputed more recently (Hernandez-Mendoza *et al.*, 2007).

We had previously performed microarray analyses with the objective to identify genes whose expression depends on the RegSR two-component regulatory system in the soybean symbiont *Bradyrhizobium japonicum* (Lindemann *et al.*, 2007). The RegSR system has long been known to activate the transcription of the *nifA* gene that encodes the key regulator for nitrogen-fixation genes in *B. japonicum* (Bauer *et al.*, 1998). Among the novel RegR target genes, we identified a putative operon (blr1515–blr1516) that encodes a predicted multidrug efflux system. Here, we report the characterization of a mutant lacking this predicted transport system, now designated BdeAB, and demonstrate that it confers antibiotic resistance and is required for an efficient symbiosis specifically with soybean.

Materials and methods

Bacterial strains, media, and growth conditions

Bradyrhizobium japonicum strains were routinely cultivated in a peptone–salts–yeast extract (PSY) medium supplemented with 1 g L^{-1} L-arabinose as described elsewhere (Regensburger & Hennecke, 1983; Mesa *et al.*, 2008). Alternatively, we used a modified Vincent's minimal medium (Vincent, 1970; Becker *et al.*, 2004) that was supplemented with 3 g L^{-1} L-arabinose, 10 mM MOPS (final pH of medium adjusted to 6.8 with 2 M NH_3), and trace elements as described elsewhere (Bishop *et al.*, 1976). When appropriate, antibiotics were used at the following concentrations (μg mL^{-1}): spectinomycin, 100; streptomycin, 50; tetracycline, 50 (solid media) or 25 (liquid media); and cycloheximide, 100. *Bradyrhizobium japonicum* strain 110*spc*4 was used as the wild type (Regensburger & Hennecke, 1983). Mutant derivatives relevant for this work were strain 2426 (Δ*regR*::Ω; Bauer *et al.*, 1998); strain 9589 (Δ*bdeAB*::Ω; see below); and strain 9589-38 (strain 9589 complemented with chromosomally inserted wild-type *bdeAB* genes; see below). *Escherichia coli* strains were grown in Luria–Bertani medium (Miller, 1972) containing the following concentrations of antibiotics for plasmid selection (μg mL^{-1}): ampicillin, 200; streptomycin, 50; and tetracycline, 10. Strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) was the host for cloning, and S17-1 (Simon *et al.*, 1983) for the conjugation of plasmids into *B. japonicum*.

Plant growth

Sterilization of seeds of soybean [*Glycine max* (L.) Merr. cv. Williams], cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*), and mungbean (*Vigna radiata*), plant growth conditions, and measurement of nitrogenase activity were performed as described previously (Göttfert *et al.*, 1990; Gourion *et al.*, 2009; Koch *et al.*, 2010). At least 10^7 cells of *B. japonicum* were added as inoculum. For bacteroid isolation, all nodules from individual soybean plants infected by either the wild type or the Δ*bdeAB* strain 9589 were collected and weighed. Nodule material was then crushed in PSY medium, and serial dilutions of the bacteroid suspension were spotted in four parallels on PSY agar plates containing spectinomycin and cycloheximide. After a 1-week incubation at 30 °C, the number of CFU per milligram of nodule wet weight was determined. In total, nodule material was collected from 10 individual plants ($n = 10$) per strain. For statistical analysis, the SPSS statistical package, version 16.0, was used.

Construction of a Δ*bdeAB* (blr1515–blr1516) deletion mutant

Deletion mutagenesis of blr1515 and blr1516 was performed by marker exchange as indicated in Fig. 1. The 5′ and 3′ flanking regions of *bdeA* were PCR amplified using appropriate primers and subcloned in the pBluescript SK(+) vector (Stratagene, La Jolla, CA). Sequences of all primers used in this work are available from the authors upon request. A streptomycin-resistance cassette (Ω) excised from pBSL15Ω was inserted in between the two *B. japonicum* DNA fragments, yielding plasmid pRJ9587. pBSL15Ω had been constructed by cloning of the Ω cassette from pHP45Ω (Prentki & Krisch, 1984) on a 2.1-kb EcoRI fragment into the 2.6-kb backbone of EcoRI-digested plasmid pBSL15 (Alexeyev, 1995). The insert of pRJ9587 was cloned into the vector pSUP202pol6K (Zufferey *et al.*, 1996), and the resulting plasmid pRJ9589 was then mobilized by conjugation from *E. coli* S17-1 (Simon *et al.*, 1983) into *B. japonicum* strain 110*spc*4 (wild type) for marker replacement, yielding mutant strain 9589.

Construction of a complemented Δ*bdeAB* strain

A 6.1-kb *B. japonicum* genome fragment containing the *bdeAB* genes including the flanking regions was PCR amplified with appropriate primers using the Phusion™ DNA Polymerase (Finnzymes, BioConcept, Allschwil, Switzerland). The PCR fragment was digested using intrinsic restriction sites for PstI and EcoRI (Fig. 1), subcloned for verification by sequencing, and eventually cloned into pSUP202pol6K. The resulting plasmid pRJ9638 was mobilized by conjugation into the *B. japonicum* mutant strain

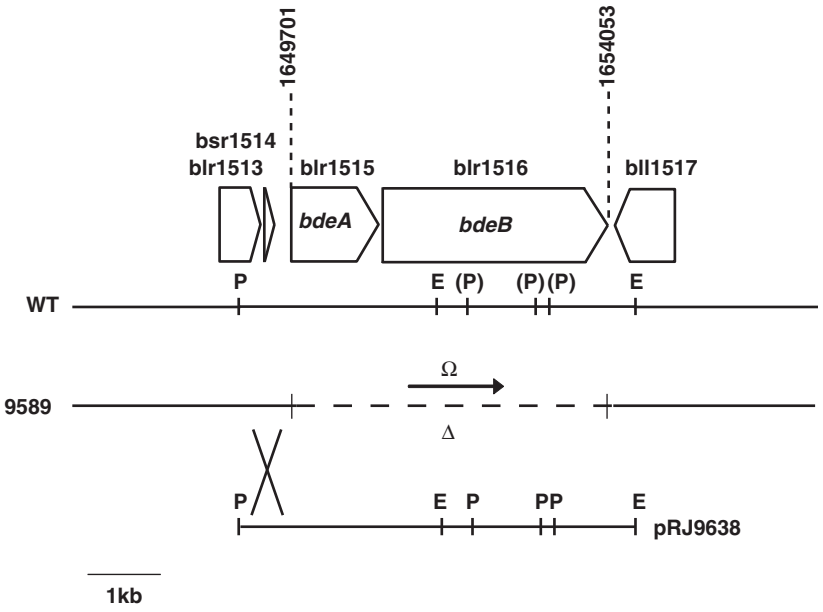

Fig. 1. Physical map of the *bdeAB* locus and genetic constructions relevant for this work. Numbers on top refer to nucleotide positions in the *Bradyrhizobium japonicum* genome database (http://genome.kazusa.or.jp.rhizobase). The genotype of mutant 9589 shows the deleted DNA (Δ) plus the orientation of the inserted Ω cassette (horizontal arrow). The cloned insert of plasmid pRJ9638, which was used for complementation of strain 9589 in *cis*, is also displayed. The complemented strain 9589-38 emerged after a single recombination event upstream of the Ω cassette. Restriction sites used during construction work are abbreviated: P, PstI; E, EcoRI; those in parentheses were not used.

9589, yielding strain 9589-38. Candidates with a single-recombination event were picked, and the correct integration of pRJ9638 upstream of the Ω cassette present in strain 9589 was verified by Southern blot analysis of genomic DNA.

Drug sensitivity assays

Potential growth-inhibitory compounds and antimicrobial peptides were purchased from Sigma Aldrich Co. (Buchs, Switzerland) and screened first for growth inhibition using the gradient agar plate technique (Szybalski & Bryson, 1952).

For agar plate diffusion assays, 15 mL of 0.9% PSY agar was warmed to 42 °C, inoculated with bacterial cell suspensions to 5×10^6 CFU mL^{-1} and quickly poured into round Petri dishes. Holes were punched into each plate using the end of a Pasteur glass pipette, and 15 μL of the test compound was pipetted into each hole. After 2 days of incubation at 30 °C, test plates were monitored for zones of growth inhibition on the bacterial lawn. The assays were repeated at least three times.

Results

The blr1515–blr1516 genes encode a putative RND-type multidrug efflux pump

Based on annotations compiled in the *B. japonicum* genome sequence database (http://bacteria.kazusa.or.jp/rhizobase/), genes blr1515 and blr1516 code for components of a multidrug efflux system, and their genomic organization (Fig. 1) suggests that they form an operon. Gene blr1515 is predicted to encode a protein of 397 amino acids (aa) with sequence similarity to *Pseudomonas aeruginosa* MexC (43%) and *E. coli* AcrA (40%) (Poole *et al*., 1993; Ma *et al*., 1995), for example. These proteins form the membrane fusion protein of tripartite RND-type multidrug efflux systems. Gene blr1516 is predicted to encode a protein of 1050 aa, with 11 predicted transmembrane helices and significant sequence similarity to the RND-type transporters MexD of *P. aeruginosa* (54%) and AcrB of *E. coli* (44%), for example. Based on these structural features and the functional data described below, genes blr1515 and blr1516 were termed *bdeA* and *bdeB*, respectively, acronyms of the *Bradyrhizobium* drug exporter.

Database searches revealed that, in addition to BdeAB, the *B. japonicum* genome encodes 23 further putative RND-type efflux pumps, which are potentially involved in multidrug export. We determined the phylogenetic relationship between these paralogous transporters in *B. japonicum*, and compared them with prototypic RND-type transporters of known substrates (deposited in the Transport Classification Database at http://www.tcdb.org/; Saier *et al*., 2006, or described in the literature as being present in phytopathogenic bacteria). Phylogenetic analysis revealed that the BdeB membrane transporter is more closely related to orthologs from other Gram-negative bacteria than to any of its 23 paralogs (see Supporting Information, Fig. S1). BdeB clustered with MexD and MexY of *P. aeruginosa*, AmrB of *Burkholderia pseudomallei* and MtrD of *Neisseria gonorrhoeae*. MexD and MexY have a common basic substrate profile [quinolones, macrolides (e.g. erythromycin), tetracycline, chloramphenicol, and certain β-lactams] that is extended by novobiocin (MexD) and aminoglycosides

(MexY) (Masuda *et al.*, 2000; Jeannot *et al.*, 2005). Aminoglycosides and erythromycin are also exported by AmrB (Moore *et al.*, 1999), whereas MtrD was reported to export mainly fatty acids and bile salts (Hagman *et al.*, 1997).

Phenotypic characterization of the Δ*bdeAB* mutant

Colonies formed by the Δ*bdeAB* mutant (strain 9589) on plates were more mucous as compared with those formed by the wild type. Cultures used for all assays performed in this work were inoculated from second-generation precultures in order to minimize the potential risk of exopolysaccharide interference with OD measurements. Heterotrophic growth of the Δ*bdeAB* mutant cultivated under oxic and micro-oxic conditions in a complex medium was indistinguishable from that of the wild type, and so was growth in minimal medium under oxic conditions (data not shown). The potential susceptibility of the Δ*bdeAB* strain 9589 to various antimicrobial compounds was tested qualitatively in gradient plate assays (not shown) or, more quantitatively, using agar plate diffusion assays. The deletion of *bdeAB* resulted in a marked and significant increase of sensitivity to the aminoglycosides kanamycin and gentamicin as compared with the wild type (1.7- and 5.5-fold difference, respectively, based on the size of the inhibition zone; Fig. 2). The complemented strain 9589-38 showed wild-type resistance levels and a largely normal colony morphology. Because the transcription of the *bdeAB* genes strongly depends on activation by the response regulator RegR (Lindemann *et al.*, 2007), we rationalized that a Δ*regR* strain might exhibit an aminoglycoside susceptibility profile similar to the *bdeAB* knockout strain. Our data showed, indeed, that the Δ*regR* mutant was at least as sensitive to kanamycin and gentamicin as the Δ*bdeAB* strain (Fig. 2).

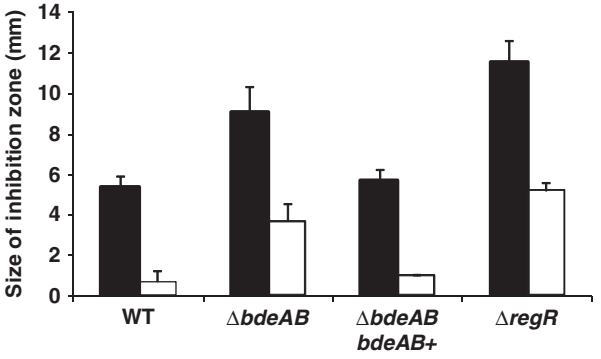

Fig. 2. Susceptibility of *Bradyrhizobium japonicum* toward aminoglycoside antibiotics determined using agar plate diffusion assays. The following strains were examined: wild type (WT), mutant strain 9589 (Δ*bdeAB*), the complemented strain 9589-38 (Δ*bdeAB*, *bdeAB*$^+$), and the *regR* mutant strain 2426 (Δ*regR*). The compounds tested were kanamycin (black columns) and gentamicin (white columns), both applied at a concentration of 100 μg mL^{-1}. Indicated is the distance (mm) of the growth zone around the rim of the drug-containing hole measured after incubation of the PSY agar plates for 2 days at 30 °C. At least three independent experiments were performed, of which one representative data set is shown. Error bars reflect SDs deduced from a total of six measurements per experiment. The error bar is missing in one case because the six measurements yielded identical results.

No significant difference was observed between the wild-type and the mutant strains when they were tested for their sensitivity against additional selected antibiotics from different classes, flavonoids, heavy metals, and detergents, among others. For a complete list of tested compounds, see Table S1.

Symbiotic properties of the Δ*bdeAB* strain

The identification of genes for a functional MDR pump, which are coregulated with symbiotically relevant genes by RegR (Lindemann *et al.*, 2007), raised the attractive hypothesis that BdeAB might be involved in the formation of an effective symbiosis of *B. japonicum* with its host plants. Soybean plants infected with the Δ*bdeAB* strain perhaps had a marginally increased number of nodules compared with plants infected by the wild type, but the nodule dry weight was within the wild-type range (Table 1). This shows that the mutant is not affected in its ability to nodulate. However, symbiotic nitrogen-fixation activity of the mutant was strongly decreased (Table 1), which was further manifested by the pale green-to-yellowish color of soybean leaves, a typical sign of nitrogen starvation (not shown). The symbiotic defect of the mutant was maintained after prolonged plant growth for up to 5 weeks, which speaks against a delayed phenotype. Chromosomal integration of wild-type *bdeAB* genes into the Δ*bdeAB* mutant almost restored a wild-type level of nitrogen-fixation activity (Table 1).

Confocal microscopy imaging of 3-week-old nodules elicited by the Δ*bdeAB* strain revealed that, while infected plant cells were densely packed with bacteroids, there was larger number of uninfected cells as compared with nodules infected by the wild type (not shown). To follow up on this observation, bacteroids were reisolated from 3-week-old nodules, with the result that, on average, a 10-fold lower number of viable cells were recovered from nodules infected by the Δ*bdeAB* strain as compared with nodules infected by the wild type (Fig. 3).

The Δ*bdeAB* strain was also tested for its symbiotic properties on other *B. japonicum* host plants such as cowpea, mungbean, and siratro. Surprisingly, in contrast to soybean, the nitrogen-fixation activity of the Δ*bdeAB* strain was not decreased on cowpea and mungbean, and was only marginally lower on siratro, as compared with the wild type (Fig. 4). It was shown previously that the Δ*regR* mutant had a strong symbiotic defect on soybean (Bauer *et al.*, 1998);

Table 1. Symbiotic phenotype of the Δ*bdeAB* mutant (9589) and the complemented strain (9589-38) on soybean 3 weeks post inoculation*

Strain	Relevant genotype	Nodule number	Nodule dry weight (mg per nodule)	Nitrogenase activity (% of wild type)
110*spc*4	Wild type	22 ± 5	0.64 ± 0.13	100 ± 13
9589	Δ*bdeAB*	27 ± 6	0.54 ± 0.06	27 ± 13
9589-38	Δ*bdeAB*, *bdeAB*$^+$	23 ± 7	0.58 ± 0.1	85 ± 25

*The symbiotic phenotype was determined in four independent soybean (*Glycine max*) infection experiments, of which one representative data set is shown. At least six plants were measured per strain, and the mean values ± SDs are shown.

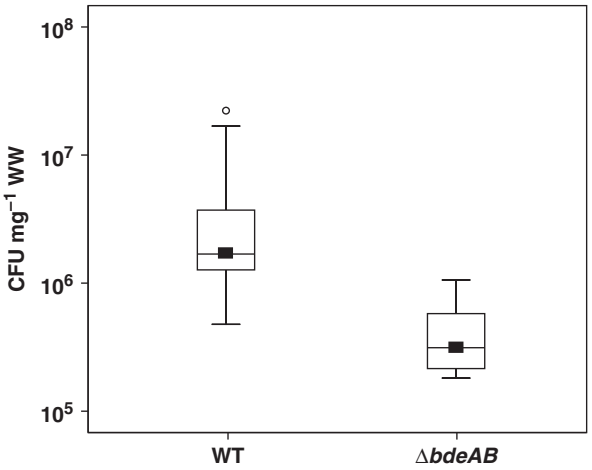

Fig. 3. Number of viable cells reisolated from individual pools of soybean nodules that had been infected by the wild type or the Δ*bdeAB* strain 9589. The Mann–Whitney *U*-test was used for analysis of statistical significance. The box plot shows the median, 10th, 25th, 75th, and 90th percentiles of the number of CFU per milligram of nodule wet weight (WW). The dot indicates an outlier in the data set of the wild-type strain.

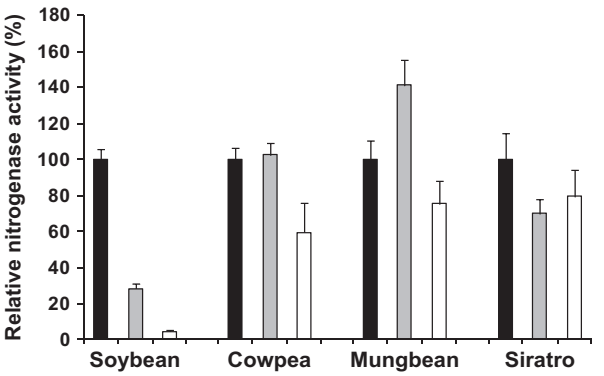

Fig. 4. Symbiotic nitrogen-fixation activity in *Bradyrhizobium japonicum*-induced root nodules on four different host plants (soybean, cowpea, mungbean, and siratro). The following strains were inoculated: wild type (black columns), Δ*bdeAB* strain 9589 (gray columns), and the Δ*regR* strain 2426 (white columns). Measurements were performed 3 weeks after infection, except for siratro (4 weeks). At least five plants were measured per strain, and average values ± SE from at least two independent plant tests are shown.

however, other host plants had never been tested. While the strong symbiotic defect on soybean was confirmed, the nitrogen-fixation activity of the Δ*regR* mutant was far less affected on the other three hosts (Fig. 4). Notably, the deletion of *regR* had more severe consequences on symbiotic nitrogen fixation than elimination of the BdeAB efflux system when respective mutants were tested on soybean, cowpea, and mungbean.

Discussion

In several recent studies, MDR efflux pumps of phytopathogenic bacteria were shown to be involved in the extrusion of plant-derived antimicrobial metabolites, which promotes host colonization and enhances virulence (Martinez *et al*., 2009, and references therein). Plant-associated soil bacteria are challenged in several ways, for example by abiotic environmental stresses or competing organisms and their metabolic products. At least conceptually, symbiotic and phytopathogenic bacteria appear to initiate similar programs for invasion and colonization (Soto *et al*., 2006; Deakin & Broughton, 2009). Therefore, the expression of efflux proteins seems to be a useful common trait of these bacteria that allows them to cope with the toxic compounds that they may encounter during infection.

In this work, we have characterized an RND-type multidrug efflux system, termed BdeAB, in the legume symbiont *B. japonicum*. Another putative efflux pump, RagCD, was described previously in *B. japonicum* (Krummenacher & Narberhaus, 2000). However, *ragCD* mutants did not differ from the wild type in their antibiotic susceptibility profile and in their symbiotic phenotype. By contrast, we have shown here that the loss of the BdeAB proteins increases the susceptibility toward aminoglycoside antibiotics, supporting the idea that these proteins principally function as a drug efflux pump. Unlike the RmrAB efflux pump of the bean symbiont *R. etli*, which was shown to be required for nodulation (Gonzalez-Pasayo & Martinez-Romero, 2000), the *B. japonicum bdeAB* mutant was not affected in nodule formation. However, soybean nodules elicited by this strain contained fewer bacteroids as compared with nodules formed by the wild type. The impaired colonization by the

Δ*bdeAB* strain might account for the decreased nitrogen-fixation activity in these nodules.

It is known that legumes synthesize phytoalexins not only in response to a pathogenic attack but also in the presence of rhizobia (see the review by Baron & Zambryski, 1995, and references therein). In fact, the RmrAB efflux pump confers tolerance to plant-derived antimicrobial compounds (Gonzalez-Pasayo & Martinez-Romero, 2000). Recently, another example of the importance of export proteins in plant–microorganisms interactions was reported. In *Mesorhizobium tianshanense*, a LysE-family exporter for the antimetabolite canavanine was identified, which helps those rhizobia to survive in a canavanine-rich legume rhizosphere (Cai *et al*., 2009). It is tempting to speculate that the BdeAB system provides a similar advantage to *B. japonicum*, perhaps coping with an as yet unidentified soybean-derived compound. The observation that symbiosis of the *B. japonicum bdeAB* mutant with cowpea and mungbean was not negatively affected is suggestive of a higher abundance of noxious BdeAB substrates in soybean (and to a small extent possibly also in siratro) than in the other hosts.

Even if one considers the large size of the *B. japonicum* genome (9.1 Mbp), the number of 24 putative RND-type transporters it encodes is notably high. Yet, none of the defects in the Δ*bdeAB* strain could apparently be rescued by its paralogs, suggesting a rather specific substrate profile of the BdeAB exporter. This is in line with the fact that BdeB clusters phylogenetically with its orthologs from other bacteria rather than with its paralogs.

Based on 14 aa involved in *E. coli* AcrB ligand binding, Hernandez-Mendoza *et al*. (2007) defined 16 groups of different ligand preferences among 47 RND proteins. While the phylogenetic similarity between BdeB and the four RND transporters MexD, AmrB, MexY, and MtrD is well reflected at the critical amino acid positions (two to four identical positions among the five most informative amino acids), a correlation with substrate preferences is less obvious because individual BdeB orthologs have relatively broad substrate ranges that may (MexD, AmrB, and MexY) or may not (MtrD) include aminoglycosides. In any case, it appears that apart from extruding antimicrobial compounds, efflux systems may also contribute to the export of intrinsic, potentially harmful molecules, for example those generated under oxidative or membrane stress conditions (Poole, 2007).

Inner membrane export systems require the presence of an outer membrane factor (OMF) in order to channel the substrate to the extracellular space (Koronakis *et al*., 2004). The OMF structural gene often maps in close vicinity to the genes for the transport system (Poole *et al*., 1993). Yet, inspection of the genomic region around *bdeAB* did not reveal a gene that might code for an OMF. According to the Transport Database (Ren *et al*., 2007), the *B. japonicum* genome encodes nine putative OMFs. It is conceivable that any one of these OMFs may form a tripartite efflux pump together with BdeAB. Intriguingly, however, none of them seems to belong to the RegR regulon (Lindemann *et al*., 2007).

Originally, the symbiotic defect of the Δ*regR* mutant was solely explained by the fact that RegR in the wild type activates the essential nitrogen-fixation regulatory gene *nifA* (Bauer *et al*., 1998). However, subsequent microarray experiments revealed that the RegR regulon comprises numerous NifA-independent genes, which, in principle, may contribute to symbiosis (Lindemann *et al*., 2007). The key finding of this study, i.e., mutation of the RegR-controlled *bdeAB* genes causes a symbiotic defect, demonstrates that such genes indeed exist. Moreover, the defect is observed primarily on soybean and much less so on the other hosts tested (cowpea, mungbean, and siratro). Interestingly, based on recent proteomics data, RegR and, although marginally, also BdeA are more abundant in soybean than in cowpea and siratro. Moreover, *regR* transcription was sixfold higher in soybean nodules than in those of cowpea or siratro (Koch *et al*., 2010; mungbean was not included in that study). This may explain why the deletion of these genes had more severe consequences on the interaction with soybean than with the other two hosts.

In three of the four hosts, we noticed a more severe symbiotic phenotype for the Δ*regR* strain as compared with the Δ*bdeAB* mutant. Given the large regulon of RegR, this difference could be readily explained by the simultaneous downregulation of several symbiotically relevant genes in the *regR* mutant. One of them is *nifA*, and thus one may wonder why a *regR* mutant is able to fix nitrogen at all. This is explained by the fact that a low, but significant level of *nifA* gene expression is uncoupled from RegR (Bauer *et al*., 1998; Lindemann *et al*., 2007) and that NifA protein synthesized under low-oxygen conditions activates its own transcription (Thöny *et al*., 1989; Barrios *et al*., 1995). Therefore, it is likely that the nodule environment allows for a sufficiently high RegR-independent NifA synthesis and subsequent *nifA* autoactivation in bacteroids. In conclusion, the RegR-dependent, but NifA-independent expression of *bdeAB* has emerged from this work as a novel, important facet in the root-nodule symbiosis of *B. japonicum* with soybean.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Unrooted phylogenetic tree based on amino acid sequence similarities of the membrane transporter component of 24 RND-type efflux transporters of *Bradyrhizobium japonicum* (Bj) and several other RND-type transporters according to the Transport Classification Database (Saier *et al*., 2006).

Table S1. Compounds tested in drug sensitivity assays.

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