Characterisation of SalRAB a Salicylic Acid Inducible Positively Regulated Efflux System of *Rhizobium leguminosarum* bv *viciae* 3841

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Abstract

Salicylic acid is an important signalling molecule in plant-microbe defence and symbiosis. We analysed the transcriptional responses of the nitrogen fixing plant symbiont, *Rhizobium leguminosarum* bv *viciae* 3841 to salicylic acid. Two MFS-type multicomponent efflux systems were induced in response to salicylic acid, rmrA/B and the hitherto undescribed system salRAB. Based on sequence similarity salA and salB encode a membrane fusion and inner membrane protein respectively. salAB are positively regulated by the LysR regulator SalR. Disruption of salA significantly increased the sensitivity of the mutant to salicylic acid, while disruption of rmrA did not. A salA/rmrA double mutation did not have increased sensitivity relative to the salA mutant. Pea plants nodulated by salA or rmrA strains did not have altered nodule number or nitrogen fixation rates, consistent with weak expression of salA in the rhizosphere and in nodule bacteria. However, BLAST analysis revealed seventeen putative efflux systems in Rlv3841 and several of these were highly differentially expressed during rhizosphere colonisation, host infection and bacteroid differentiation. This suggests they have an integral role in symbiosis with host plants.

Introduction

Plants produce and secrete a diverse number of compounds into the rhizosphere. These include a myriad of phytoalexins and signalling molecules which not only mediate plant defences but orchestrate plant microbial interactions including symbiosis with nitrogen fixing symbiotic rhizobia [1,2]. One key molecule in the response of plants to microbes is salicylic acid, which is a phenolic hormone with varied roles in plant metabolism and physiology including plant defence [3]. When a plant recognises a biotrophic pathogen, salicylic acid regulates the specific and localised Hypersensitive Response (HR) leading to cell death at the site of infection [4]. Salicylic acid is also involved in the longer lasting systemic protection of the plant against a range of pathogens, termed Systemic Acquired Resistance (SAR) [5]. Plants and microbes have evolved complex signal interactions in order to distinguish friend from foe. In the case of symbioses with rhizobia plants do not usually elicit a defence response [6], although salicylic acid may be important in controlling host range and regulating nodule formation. A number of studies have investigated salicylic acid levels and nodule formation. Exogenous application of salicylic acid has been shown to decrease or inhibit nodule formation when *Bradyrhizobium japonicum* or *Rhizobium leguminosarum* are grown on Soybean and Vetch respectively [7,8]. Similarly, decreasing endogenous levels of salicylic acid in *Lotus japonicus* led to increased nodule numbers when inoculated with *Mesorhizobium loti* [6].

One mechanism to circumvent the toxic effects of compounds that microbes encounter in the environment is their extrusion via efflux systems. These systems are ubiquitous in bacteria and comprise five superfamilies [9]. The first belong to the ATP Binding Cassette (ABC) family, which are primary transporters using ATP hydrolysis to drive efflux. The other four, The Major Facilitator Superfamily (MFS), the Resistance Nodulation Division (RND), the Multi Antimicrobial Extrusion (MATE) and Drug Metabolite Transporters (DMT)/SMR are secondary H⁺ or Na⁺ antiporters. In gram negative bacteria some ABC, MFS and all RND are multicomponent tripartite systems spanning both inner and outer membranes. They comprise an inner membrane transporter, a membrane fusion protein and a TolC like outer membrane factor [10]. Usually, the inner membrane transport and membrane fusion protein are located together however, cells generally have several TolC like proteins that act with a number of different efflux systems [10].

Successful phytopathogenic bacteria must export or detoxify plant phytoalexins, with disruption of two RND type efflux systems in *Erwinia amylovora* (ArcAB) and *Pseudomonas syringae* (MexAB) reducing virulence on apple trees and bean leaves respectively [11,12]. The role of these systems in promoting pathogenesis is clear but efflux systems are also common in plant symbiotic bacteria such as the nitrogen fixing rhizobia. Disruption of the multicomponent MFS efflux pump (*rmrAB*) in *Rhizobium etli* CEN42 increased phytoalexin sensitivity and led to impaired nodule formation. [13]. Likewise, nitrogen fixation was impaired...
and antimicrobial sensitivity increased when the RND family bdeAB efflux system was disrupted in the soybean symbiont *Bradyrhizobium japonicum* 110fcc4 [14]. Nodulation, competitiveness and toxin sensitivity were also affected when the RND smeAB pump was disrupted in *Sinorhizobium meliloti* 1021 [15].

We investigated the transcriptional responses of *Rhizobium leguminosarum* bv *viciea* 3841 to salicylic acid, coupled with a genome screen to identify putative multicomponent efflux systems. We investigated the contribution of these systems to salicylic acid resistance and their induction during plant colonisation and nodulation.

**Results**

*R. leguminosarum* 3841 transcriptional responses to salicylic acid

In response to the addition of salicylic acid (0.72 mM) a total of 21 genes were up-regulated more than two fold (t-test p≤0.05) compared to free living cells (Table 1). These responses can be broadly classified into those likely to be involved in salicylic acid export or its catabolism. Two genes RL1329 and RL1330 encoding putative efflux pump components were upregulated 18.1 and 2.2 fold respectively, with RL1329 being the most highly elevated gene in Rlv3841 in response to salicylic acid. Upstream of these genes is a putative LysR like transcriptional regulator, RL1328. It is proposed these genes be designated salR (RL1326), salA (RL1329) and salB (RL1330) (Figure 1). Based on homology with known proteins it is proposed salRAB is a MFS family multicomponent efflux system, where salA is putative membrane fusion protein and salB is an inner membrane transporter. In addition rmrA (pRL90059), which has 0.5% identity to the membrane fusion protein of the characterised MFS family efflux pump in *R. etli* CFN42 [13], was also upregulated 4.1 fold.

**Mutation of the sal genes**

To determine if disruption of the *salRAB* operon in *R. leguminosarum* 3841 alters sensitivity to salicylic acid a *salA* deletion mutant was isolated (LMB519) and growth assayed at varying salicylic acid concentrations. In addition, as loss of *rmrA* has been shown to increase salicylic acid sensitivity in *R. etli* CFN42 [13], a Rv3841 rmrA (pRL90059) deletion mutant previously isolated [16] was also tested. Salicylic acid (2 mM) significantly impaired growth of the salA mutant compared to controls (Figure 2A). In contrast disruption of *rmrA* led to no detectable difference in growth compared to wild type controls (Figure 2B). Furthermore a double salA/rmrA (LMB523) mutant was not more sensitive to salicylic acid than the single salA mutant. In this instance the appropriate wild type control for comparison to the salA/rmrA (LMB523) mutant was RU2223 [16] which contains a ΔSp and pK19mob mutation in genes unrelated salRAB. This was so because antibiotic selection could be used in the media and any differences in growth due to the presence of the spectinomycin cassette or pK19mob insertion could be discounted.

In order to complement the salA gene disruption the whole Sal operon (salRAB) was cloned into the stable low copy number plasmid pJP2 [17] forming pSal. When not induced with salicylic acid carriage of pSal had no effect on growth of either salA::ΔSp (LMB519) or WT control nifH::ΔSp (RU3940) compared to pJP2 parent plasmid containing strains (LMB641 and LMB640 respectively). A nifH mutant was used as a control instead of Rlv3841 because it enabled spectinomycin to be included in all media, ensuring growth differences were not due to the presence of a ΔSp cassette or spectinomycin. When induced with salicylic acid, instead of complementing the salA::ΔSp mutation (LMB519) pSal reduced growth relative to a strain (LMB641) containing the parent plasmid pJP2 alone. Similarly, pSal in the wild type control RU3940 (nifH::ΔSp) also reduced growth relative to pJP2 containing strain (LMB640) when induced with salicylic acid (Figure 3A). Since salicylic acid is required for induction of the sal operon (see below) this suggests over-expression of the salAB operon from a low copy number plasmid is inhibitory and reduces growth. The problem of over expression of a membrane transporter leading to reduced growth is not uncommon. Since complementation proved problematic in order to confirm that the disruption of the salA was responsible for increased sensitivity to salicylic acid, independent pK19mob insertion mutants were isolated for each gene of the Sal operon. In the absence of salicylic acid there was no growth difference between wild type and mutant strains (Doubling times (h ± standard error of the mean for three triplicates); RU4062 (nifH::pK19mob) 8.3±0.017, LMB455 (salR::pK19mob) 8.2±0.06, LMB415 (salA::pK19mob) 8.2±0.2, LMB409 (salB::pK19mob) 8±0.03). With the addition of 1.45 mM salicylic acid all three sal mutants had reduced growth compared to wild type strain (nifH::pK19mob) (Figure 3B) as was observed with LMB519 (salA::ΔSp) (Figure 2A).

**Plant symbiosis and antimicrobial sensitivity**

The disruption of multicomponent efflux systems in other rhizobia has been shown to affect sensitivity to toxins, nodule formation and/or nitrogen fixation activity [13–15]. To ascertain if disruption of either the salRAB or rmrAB affected plant nodulation and nitrogen fixation the salA (LMB319), rmrA (RU4314) and double salA/salR (LMB523) mutants were inoculated on pea seeds. After 21 days growth the number of nodules were recorded and nitrogen fixing activity assessed by acetylene reduction assays. Compared to wild type none of the mutants differed in either the number of nodules formed (Rlv3841...
Table 1. Rlv3841 genes above two fold upregulated in response to 0.72 mM salicylic acid.

| Gene id [31] | Fold induction | P-value | Gene name | Product description [31] |
|--------------|----------------|---------|-----------|-------------------------|
| pRL110531    | 4.2            | 0.0012  | hypothetical protein |
| pRL110532    | 6.9            | 0.0009  | conserved hypothetical protein |
| pRL110534    | 5.4            | 0.0598  | putative two-component sensor kinase regulator |
| pRL110535    | 2.2            | 0.1101  | putative two-component response regulator |
| pRL110536    | 2.9            | 0.0055  | putative haloalkane dehalogenase |
| pRL120214    | 2.0            | 0.0002  | putative dioxygenase |
| pRL120215    | 2.1            | 0.1313  | tfTE1 putative maleylacetate reductase |
| pRL120216    | 6.0            | 0.0231  | putative amidohydrolase |
| pRL120218    | 2.3            | 0.1362  | putative flavin reductase |
| pRL120616    | 2.1            | 0.0081  | putative reductase |
| pRL90059     | 4.1            | 0.0233  | rmrA putative type I HlyD transporter |
| RL0272       | 4.7            | 0.0017  | putative aldo-keto reductase/oxidoreductase |
| RL0577       | 3.8            | 0.0001  | putative transmembrane protein |
| RL1329       | 18.1           | 0.0051  | salA putative HlyD family efflux pump protein |
| RL1330       | 2.2            | 0.0189  | salB putative transmembrane efflux pump protein |
| RL1507       | 2.2            | 0.0288  | conserved hypothetical protein |
| RL1860       | 8.6            | 0.0000  | phHA putative phenylalanine-4-hydroxylase |
| RL1910       | 2.2            | 0.0549  | conserved hypothetical protein |
| RL1911       | 7.7            | 0.0312  | putative arylsulfatase |
| RL1917       | 2.7            | 0.0718  | conserved hypothetical protein |
| RL1918       | 4.3            | 0.0859  | putative exported arylsulfatase protein |
| RL1924       | 3.4            | 0.1454  | conserved hypothetical protein |
| RL1925       | 4.1            | 0.1013  | conserved hypothetical protein |
| RL1966       | 4.6            | 0.0977  | aldA alanine dehydrogenase |
| RL2029       | 2.2            | 0.0056  | hypothetical protein |
| RL2670       | 2.9            | 0.0482  | putative oxidoreductase |
| RL2726       | 2.5            | 0.0003  | ribA1 putative riboflavin biosynthesis protein |
| RL3172       | 2.9            | 0.0061  | hypothetical protein |
| RL3286       | 3.3            | 0.0003  | putative ABC transporter component, pseudogene |
| RL3297       | 2.0            | 0.0067  | lpxC putative UDP-3-O-[3-hydroxyxymyristoyl] N-acetylgalcosamine deacetylase |
| RL4612       | 2.3            | 0.0006  | putative transmembrane MF5 family transport |

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Salicylic Acid Efflux

To examine the regulation of the salRAB operon two transcriptional reporter plasmids were constructed. Plasmid pLMB537 contains the putative intergenic promoter region between salR and salA (salAp) (Figure 1) upstream of a promoterless gfpmut3.1 reporter. The second plasmid (pLMB357) contains the promoter region as well as the complete salR gene (salAp and salR) (Figure 1). The plasmids were introduced into the mutant strain LMB455 (salR::pK19mob) and Rlv3841. In the absence of salicylic acid there was no detectable induction of salA (Figure 4). When Rlv3841 (WT) carrying pLMB537 (salA p and salR) or pLMB557 (salAp only) were incubated with 0.72 mM salicylic acid salA was induced (Figure 4A). However, salA was not induced in the salR insertion mutant (LMB455) carrying pLMB557 (salAp only) (Figure 4B). This indicates that the salRAB operon is positively regulated by SalR. This was confirmed by restoration of salA induction in the salR insertion mutant (LMB455) when carrying pLMB537, which contains salAp and a full length copy of salR (Figure 4B).

Induction specificity of the salRAB operon

It is proposed that salicylic acid is synthesised in plants via two enzymatic pathways from the primary metabolite chorismate, either via phenylalanine, cinnaamate and benzoate intermediaries, or by a second pathway from isochorismate [18]. To test the
specificity of the Sal operon several of these proposed intermediates and derivatives were used in induction assays with Rlv3841 containing the reporter plasmid pLMB557 (LMB475). These included cinnamate, benzoate and phenylalanine as well as the volatile ester of salicylic acid, methyl salicylate (wintergreen oil), a plant defence signalling molecule [19]. In addition catechol, p-hydroxybenzoate, protocatechuic acid and acetylsalicylic acid (aspirin) that have a similar structure to salicylic acid were also tested. Apart from salicylic acid itself all of these compounds failed to induce pLMB557, except for acetylsalicylic acid which showed some induction after 24 hours. This slow and weak induction may be the result of spontaneous hydrolysis of acetylsalicylic acid releasing salicylic acid.

Other multicomponent efflux systems of Rlv3841

To put the MFS type salRAB and rmrAB in context of the total multicomponent efflux systems encoded by Rlv3841, the genome was screened for proteins with sequence similarity to characterised systems. In total 17 systems were identified, including salRAB and rmrAB (Table 2). Eleven of the systems belong to the RND family, four to the MFS family and two to the ABC families. Fifteen of the systems were encoded on the chromosome and one each on pRL9

![Figure 2. Salicylic acid sensitivity assays. A, Single salA mutant, salA::pSp (LMB519) and wild type control nifH::pSp (RU3940) grown in AMS and 2.0 mM salicylic acid. B, Single rmrA mutant, rmrA::pK19mob (RU4314) and wild type control, nifH::pK19mob (RU4062) grown in AMS and 2.0 mM salicylic acid. Data are shown as the mean ± standard error of the mean (SEM) for triplicate cultures. doi:10.1371/journal.pone.0103647.g002](image)

![Figure 3. Complementation assay and salRAB independent insertion mutations. A, Growth of nifH::pSp (RU3940) and salA::pSp (LMB519), in AMS and 2.0 mM salicylic acid, when carrying either pJP2 control or pSal containing the full salRAB operon. B, Independent pK19 insertion mutations of the three genes of the salRAB operon compared to wild type control (nifH::pK19mob) when grown for 48 hours in AMS and 1.45 mM salicylic acid. Data are shown as the mean ± standard error of the mean (SEM) for triplicate cultures. doi:10.1371/journal.pone.0103647.g003](image)
and pRL10. In addition several TolC like homologs were also identified (RL3876, pR100291 and pR100178).

We have collected a large dataset (73 growth conditions) of the transcriptional response of Rlv3841 to different environments and inducers, most of which has been published previously, including colonisation of different plant rhizospheres [20] and in bacteroid development [16]. This allowed analysis of the expression of the efflux transporters when interacting with host plants, as well as in response to salicylic acid (Figure 5). In early seven day bacteroid development many of the systems were highly upregulated compared to free living cells and in comparison to 21 day bacteroids (Figure 5B). In total five systems show an above five-fold elevation at seven days with \textit{rmrA} the highest at 27.5 fold up. In contrast \textit{salA} has a modest 1.5 fold change. On colonisation of the rhizosphere of seven-day old pea, alfalfa and sugar beet (Figure 5C) both \textit{rmrA} and \textit{salA} showed transcriptional increases of 5.5,3.5,2.5- and 1.8,2,2.7-fold, respectively. By far the largest increase at 135-fold was that of RL4274, a putative RND type efflux pump. How the efflux systems are differentially expressed when Rlv3841 colonises Pea plants of different ages is given in Figure 5D. Thus \textit{salRAB} is only weakly induced in the plant rhizosphere and during nodule formation explaining the lack of a phenotype in a \textit{salA} mutant.

\section*{Discussion}

Multidrug efflux systems are important to plant pathogens and symbions alike. Regardless of lifestyle soil organisms must overcome both abiotic and biotic stresses, including antimicrobials derived from plants and microbial competitors. In this study we investigated the global transcriptional response of Rlv3841 to salicylic acid, and identified two efflux systems significantly unregulated. The first of these was the hitherto uncharacterised \textit{salRAB} and the second \textit{rmrAB}, which has 85\% (\textit{rmrA}) and 99\% (\textit{rmrB}) amino acid identity to the characterised efflux system of \textit{R. etli} CFN42 [13].

Disruption of \textit{salRAB} in Rlv3841 led to significantly increased sensitivity to salicylic acid. However, it did not increase sensitivity to other antimicrobials tested or affect nodulation and nitrogen fixation. In \textit{R. etli} CFN42, a close relative of Rlv3841 which also shows a high degree of sequence similarity to the \textit{salRAB} system of Rlv3841 (RHE\textunderscore CH01191, 90%; RHE\textunderscore CH01192, 82% and RHE\textunderscore CH01193 93\% amino acid similarity to \textit{salRAB} respec-
Salicylic Acid Efflux

| Designated family | IMT        | MFP        | Replicon | Sequence position |
|-------------------|------------|------------|----------|-------------------|
| MFS               | pRL90060 (rmeB) | pRL90059 (rmeA) | pRL9      | 61508.64307       |
| RND               | RL3875 (rmel)  | RL3874 (smeA) | Chromosome | 4098266.4102636   |
| RND*              | pRL100286    | pRL100287   | pRL10    | 295910.300156     |
| MFS               | RL1330 (salB) | RL1329 (salA) | Chromosome | 1392779.1395765   |
| RND*              | RL1453       | RL1454      | Chromosome | 1516274.1520694   |
| ABC*              | RL2365       | RL2364      | Chromosome | 2485492.2487513   |
| RND               | RL2666       | RL2667      | Chromosome | 2814549.2818924   |
| RND*              | RL3725       | RL3724      | Chromosome | 3921000.3925529   |
| RND*              | RL3774       | RL3775      | Chromosome | 3978570.3983080   |
| RND               | RL4223       | RL4224      | Chromosome | 4475300.4479612   |
| RND*              | RL4225       | RL4274      | Chromosome | 4531632.4536139   |
| RND               | RL3787       | RL3786      | Chromosome | 3992927.3997287   |
| RND               | RL3783       | RL3784      | Chromosome | 3989164.3991792   |
| RND               | pRL120698    | pRL120696/pRL120697 | Chromosome | 752595.757818     |
| ABC               | RL3029       | RL3030      | Chromosome | 3193055.3194982   |
| RND*              | RL3269       | RL3270      | Chromosome | 3421636.3425996   |
| MFS               | RL4179       | RL4180      | Chromosome | 4430233.4430370   |

Abbreviations: IMT, inner membrane protein; MFP, membrane fusion protein. * No adjacent transcriptional regulator.

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In this study we identified 17 putative efflux systems in Rhv3841 and interrogated transcriptional data previously produced from our lab to gain insight into the induction of these systems during rhizosphere colonisation, host infection and bacteroid differentiation during nodulation. In seven day bacteroids over one third of all the putative encoded efflux systems were induced above three-fold, after 21 days only one system was induced above three-fold. The involvement of so many systems is testament to the complex and temporal physiological conditions encountered during nodule development. It is also possible that these systems overlap in specificity and thus offer an explanation why disruption of rmrA, the most upregulated system in seven-day bacteroids, had no effect on nodulation and nitrogen fixation. Furthermore, many of these systems may be expressed constitutively resulting in significant background resistance to antimicrobials.

Similarly, during plant colonisation of pea, alfalfa and sugar-beet a large number of different efflux systems were upregulated including salRAB and rmrAB. In addition most respond similarly to the different rhizospheres i.e. are generalists. Although, one system (RND type RL4274/4275), while upregulated in all three rhizospheres, was most highly induced by pea (Figure 5C), suggesting specific induction for this system. Indeed in a previous study mutation of RL4274 decreased the competitiveness compared to Rhv3841 in the pea rhizosphere [20].

In this study we have demonstrated that salAB is positively regulated by a LysR family transcriptional type regulator SalR. Induction of this operon is highly specific to salicylic acid. Salicylic acid is known to be instrumental in nodule development such that in Rhizobium leguminosarum bv viciae strains RBL 5525 and 248 exogenous application of 10^{-4} M salicylic acid completely inhibited nodule formation on vetch [8]. Moreover, Stacey et al., 2006 [6] demonstrated that reducing the endogenous levels of salicylic acid by transgenic expression of salicylate hydroxylase (NahG) in Lotus japonicus correlated with an increase in nodule number when inoculated with Mesorhizobium loti. As salRAB confers increased resistance at high levels of salicylic acid (above

Table 2. Putative multicomponent efflux systems of R. leguminosarum bv viciae 3841.

- The table lists putative efflux systems identified in Rlv3841,
- It includes the designated family, IMT, MFP, replicon, and sequence position for each system.
- The table highlights the importance of these systems in conferring resistance to antimicrobials.
- The table also notes the specific induction of certain systems in different rhizospheres, indicating their role in adaptation.
- The table provides a foundation for understanding the complex mechanism of resistance and adaptation in R. leguminosarum bv viciae 3841.
1.45 mM) it can be hypothesised salRAB confers a fitness advantage to Rlv3841 by eliminating the inhibitory effects of salicylic acid through expulsion from the cell. However, the Sal system was only weakly expressed in the rhizosphere and in nodule bacteria explaining the lack of effect of mutation in salA on nodulation and N₂-fixation.

Materials and Methods

Strains, plasmids and culture conditions

The strains and plasmids used in this study are detailed in Table 3. All Escherichia coli strains were grown at 37°C in Lennox (L) broth or L agar. R. leguminosarum strains were grown at 28°C in Tryptone-yeast extract [23] or Acid Minimal Salts (AMS) [24] supplemented with 30 mM Pyruvate and 10 mM ammonium chloride and agitated at 220 rpm. Antibiotics were used in the following concentrations (µg ml⁻¹) unless otherwise stated. Gentamicin, 20 (10 in E. coli); Kanamycin, 20; Neomycin, 20; Spectinomycin, 50; Streptomycin 500; Tetracycline (2 in AMS, 5 in TY).

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All general DNA cloning was as described [25]. PCR amplification, unless otherwise stated, was performed in 100 µl volumes using 2.5 units GoTaq (Promega), 1 µM primer and 0.2 mM dNTPs. Cycling conditions were: One cycle of 95°C for 2 minutes, 30 cycles of 95°C for 45 s, 57°C for 45 s, 72°C for 3 minutes with a final extension of 10 minutes at 72°C. All constructs were confirmed using Sanger sequencing. Plasmids were conjugated from E. coli into R. leguminosarum strains using the helper plasmid pRK2013 as previously described [26]. PCR primers used in this study are given in Table 4.

RNA isolation and microarray analysis

R. leguminosarum 3841 was grown in triplicate in 100 ml AMS to mid-log growth phase before the cells were harvested and washed twice in AMS and resuspended in AMS with or without 0.72 mM salicylic acid (i.e. 100 µg ml⁻¹). Cultures were induced for three hours before RNA was extracted, amplified and hybridised as previously described [16]. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2187.

Figure 5. Transcriptional responses of Rlv3841 putative tripartite efflux systems to different environments determined by microarray analysis. Efflux systems are in order as given in Table 2. Fold changes are given for the membrane fusion protein (MFP) of each efflux system. A, Addition of 0.72 mM salicylic acid. B, Bacteroids isolated from 7 and 21 day nodules [16]. C, Rlv3841 isolated 7 day post inoculation from 7 day old pea (Pisum sativum), alfalfa (Medicago sativa) and sugar beet (Beta vulgaris) after 7 day post inoculation [20]. D, Rlv3841 isolated 1 day post inoculation from 7, 14 and 21 day old peas [20].

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**Table 3. Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|--------------------|
| **Plasmid**       |             |                    |
| pHP45S2           | pBR322 derivative carrying ΩpHP45 replicon; Ap<sup>+</sup> Sp<sup>+</sup> | [27] |
| pET1.2 Blunt     | PCR product cloning vector; Ap<sup>+</sup> | Thermo Scientific |
| pJP2             | Stable broad host range cloning vector; Tc<sup>+</sup> | [17] |
| pJQ2005K         | Suicide vector sacB gene; Gm<sup>+</sup> | [28] |
| pK19mob          | mob<sup>+</sup>; Km<sup>+</sup> | [32] |
| pLMB474          | pK19 containing internal fragment of salB/RL1330; Km<sup>+</sup> | This work |
| pLMB492          | pK19 containing internal fragment of salA/RL1329; Km<sup>+</sup> | This work |
| pLMB537          | pRU1097 parent transcriptional fusion WITH regulator salR/RL1328 and salA promoter region (salAp and salR); Gm<sup>+</sup> | This work |
| pLMB546          | pK19 containing internal fragment of salA/RL1328; Km<sup>+</sup> | This work |
| pLMB557          | pRU1097 parent transcriptional fusion WITHOUT regulator salR/RL1328 only the salA promoter region (salAp); Gm<sup>+</sup> | This work |
| pLMB607          | pr1310-pr1311 PCR product in pET1.2 blunt; Ap<sup>+</sup> | This work |
| pLMB608          | ΔSp cassette cloned into EcoRI site of pLMB607; Ap<sup>+</sup> Sp<sup>+</sup> | This work |
| pLMB611          | XbaI/Xhol fragment from pLMB608 cloned into pJQ2005K; Ap<sup>+</sup> Sp<sup>+</sup> | This work |
| pRK2013         | ColE1 replicon with RK2 tra genes, a helper plasmid used for mobilising plasmids; Km<sup>+</sup> | [33] |
| pRU1097         | Promoterless gfpmut3.1 cloning vector; Gm<sup>+</sup> | [34] |
| pSal            | pJP2 parent, complete salRAB operon (RL1328-1330); Tc<sup>+</sup> | This work |
| **R. leguminosarum** |             |                    |
| Rlv3841          | Str<sup>+</sup> derivative of R. leguminosarum 300 | [35] |
| LMB409           | Rlv3841 salB/RL1330::pK19mob (pLMB492) | This work |
| LMB415           | Rlv3841 salA/RL1329::pK19mob (pLMB546) | This work |
| LMB455           | Rlv3841 salR/RL1328::pK19mob (pLMB474) | This work |
| LMB475           | Rlv3841 with pLMB557 | This work |
| LMB519           | Rlv3841 sal::ΔSp cassette | This work |
| LMB523           | LMB519 (salA/RL1329::ΔSp cassette); rmrA::pK19mob | This work |
| LMB635           | RU3940 (nifH::ΔSp cassette) with pSal | This work |
| LMB636           | LMB519 (salA/RL1329::ΔSp cassette) with pSal | This work |
| LMB637           | LMB523 (salA/RL1329::ΔSp cassette; rmrA::pK19mob) with pSal | This work |
| LMB640           | RU3940 (nifH::ΔSp cassette) with pJP2 | This work |
| LMB641           | LMB519 (salA/RL1329::ΔSp cassette) with pJP2 | This work |
| LMB649           | Rlv3841 with pLMB537 | This work |
| LMB650           | LMB455 (salR/RL1328::pK19mob) with pLMB537 | This work |
| LMB651           | LMB455 (salR/RL1328::pK19mob with pLMB557 | This work |
| RU3940           | Rlv3841 nifH::ΔSp cassette | [16] |
| RU4062           | Rlv3841 nifH::pK19mob | [16] |
| RU4223           | Double glucose transporter mutant | [16] |
| RU4314           | Rlv3841 rmrA::pK19mob | [16] |

Abbreviations: Ap<sup>+</sup>, ampicillin; Gm<sup>+</sup>, gentamicin; Km<sup>+</sup>, Kanamycin; Sp<sup>+</sup>, spectinomycin; Str<sup>+</sup>, streptomycin; Tc<sup>+</sup>, tetracycline.

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**Induction reporter assays**

Induction assays were performed in 100 ml volumes. Cells were grown to mid log growth phase before candidate inducers were added to the media to a final concentration of 0.72 mM. Assays were performed in triplicate and GFP fluorescence was detected with a Tecan GENios fluorometer (excitation 485 nm, emission 510 nm).

**Cloning and mutant isolation**

A stable mutation in salA (RL1329) was made by amplifying a 2.9 kb fragment from Rlv3841 using primers pr1310/pr1311 and blunt cloning into pET1.2 blunt (Thermo Scientific), yielding pLMB607. An omega spectinomycin resistance cassette from pH45 [27] was ligated into the EcoRI site of pLMB607 yielding pLMB608. The salA::ΔSp resistance cassette XhoI/Xbal from pLMB608 was cloned into the suicide vector pJQ2005K [28] forming pLMB611. This was conjugated into Rlv3841 and cells plated on AMS agar supplemented with 10% sucrose, spectino-
mycin, 10 mM NH₄Cl to select for gene replacement. LMB519 was confirmed to contain the insertion by PCR mapping using primers pr1312/pOTfarForward. The double mutant (salA::VSp/rmrA::pK19mob) was isolated by using the general transducing phage RL38 [29] to lye LMB519 (salA::VSp) and transduce the spectinomycin cassette into RU4314 (rmrA::pK19mob) as previously described [29].

Single crossovers were made independently in each gene of the salRAB operon (RL1328-30) of Rlv3841. Internal fragments of each gene were PCR amplified using primers RL1328_BD_F/RL1328_BD_R, RL1329_BD_F/RL1329_BD_R, and RL1330_BD_F/RL1330_BD_R, respectively. The fragments were cloned into the HindIII digested pK19 utilising BD in-fusion cloning (Clontech) to give plasmids pLMB546, pLMB492 and pLMB474 that were introduced into Rlv3841. Cells were plated on TY with neomycin (80 μg ml⁻¹) to select for plasmid integration. The correct single cross-over integration in LMB455 (salR/RL1328::pK19mob) LMB415 (salA/RL1329::pK19mob) and LMB409 (salB/RL1330::pK19mob) were confirmed by PCR with primers RL1328_BD_map, RL1329_BD_map, and RL1330_BD_map and pK19mob specific primers pK19A and B.

To construct the complementing plasmid pSal, salRAB was amplified using primers pr1394/pr1395 (containing KpnI and BamHI) respectively on the 5′ ends) and blunt cloned into pJET1.2 blunt (Thermo Scientific). Digestion with KpnI/BamHI enabled ligation into KpnI/BamHI digested pJP2.

### Table 4. Primers used in this study.

| Primer name | Sequence | Function |
|-------------|----------|----------|
| RL1328_BD_F | TGATTACGCCAAGCTCCGAACTCGATGAGGCGGAC | To amplify internal fragment of salR (RL1328) |
| RL1328_BD_R | GCAGGCCAGCTGGGTGCGGAGGGTGGTGTCG | To amplify internal fragment of salR (RL1328) |
| RL1328_MAP  | TACATTGTCGTTGACGAGCAC | salR (RL1328) pK19 mapping primer |
| RL1329_BD_F | TGATTACGCCAAGCTCCGAACTCGATGAGGCGGAC | To amplify internal fragment of salA (RL1329) |
| RL1329_BD_R | GCAGGCCAGCTGGGTGCGGAGGGTGGTGTCG | To amplify internal fragment of salA (RL1330) |
| RL1329_MAP  | GCAGGCCAGCTGGGTGCGGAGGGTGGTGTCG | To amplify internal fragment of salB (RL1330) |
| RL1330_BD_F | TGATTACGCCAAGCTCCGAACTCGATGAGGCGGAC | To amplify internal fragment of salB (RL1330) |
| RL1330_BD_R | GCAGGCCAGCTGGGTGCGGAGGGTGGTGTCG | To amplify internal fragment of salB (RL1330) |
| RL1330_MAP  | GCAGGCCAGCTGGGTGCGGAGGGTGGTGTCG | To amplify internal fragment of salB (RL1330) |
| pK19A       | ATCAGATCTTGATCCCCTGCA | pK19 mapping primer |
| pK19B       | GCACGAGGGAGCTCCAGGAGG | pK19 mapping primer |
| Pr1310      | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salRAB operon |
| Pr1311      | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salRAB operon |
| Pr1312      | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salRAB operon |
| Pr1394      | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salRAB operon |
| Pr1395      | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salRAB operon |
| PotFarForward | TTTTCTAGAATAGACCTTCAGCGTGCCCC | Sequencing primer to confirm spectinomycin cassette insertion in salA (RL1329) |
| E6_RL1328_for | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salR (RL1328) gene and promoter region for creating pLMB537 |
| E6_RL1328_rev | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify salR (RL1328) gene and promoter region for creating pLMB537 |
| E6_P_For   | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify promoter region upstream of salA (RL1329) for creating pLMB557 |
| E6_P_rev   | TTTTCTAGAATAGACCTTCAGCGTGCCCC | To amplify promoter region upstream of salA (RL1329) for creating pLMB557 |

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Transcriptional reporter plasmids pLMB537 (containing the salR-salA intergenic region, salAp) and pLMB537 (containing salAp as well as salR) were isolated by PCR amplification of fragments from Rhizobium Labilis strains of N. Braun and R. Rodriguez. The correct insertions were confirmed by sequencing and conjuncting into R. leguminosarum strains.

Sensitivity assays

Cells were harvested from TY slopes and adjusted to an OD600 of 0.2. Aliquots of 100 μl were mixed into 3 ml soft TY agar (0.7%) and overlaid on TY plates. Sterile filter discs (6 mm diameter) were placed on the top agar and 5 μl of the test compound was added per disc. A two-fold dilution series was used for each compound giving test concentration ranges of Tetracycline (0.31–5 μg ml⁻¹), Nahidacid (0.13–2 μg ml⁻¹), Naringenin (0.013–5 mg ml⁻¹), Genistein (0.013–5 mg ml⁻¹), Berberine (0.31–5 mg ml⁻¹). Each compound at each different concentration was tested in triplicate. After 72 hours growth at 28°C the size of the zone of inhibition was measured.

Identification of putative multicomponent efflux pumps

Genes encoding transporter and membrane fusion proteins in Rhizobium were identified by BLAST sequence/search experiments with the NCBI NR database and uniprotkb database, as well as searches with characterised systems including: mrmb (MFS) from R. etli CNF42, ermAB (MFS) and macAB (ABC) from Escherichia coli K12 MG1655, bldAB (RND) from Bradyrhizoillum japonicum USDA110 and smeAB (RND) of Sinorhizobium meliloti 1021. Motif and protein domain analysis was performed with InterPro (http://www.ebi.ac.uk/interpro/).

Author Contributions

Conceived and designed the experiments: AJT PSP. Performed the experiments: AJT RK. Analyzed the data: AJT PSP. Contributed reagents/materials/analysis tools: AJT RK. Wrote the paper: AJT PSP.

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