

Characterization of a γ -Aminobutyric Acid Transport System of *Rhizobium leguminosarum* bv. *viciae* 3841[∇]

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Spontaneous mutants of *Rhizobium leguminosarum* bv. *viciae* 3841 were isolated that grow faster than the wild type on γ -aminobutyric acid (GABA) as the sole carbon and nitrogen source. These strains (RU1736 and RU1816) have frameshift mutations (*gtsR101* and *gtsR102*, respectively) in a GntR-type regulator (GtsR) that result in a high rate of constitutive GABA transport. Tn5 mutagenesis and quantitative reverse transcription-PCR showed that GtsR regulates expression of a large operon (pRL100242 to pRL100252) on the Sym plasmid that is required for GABA uptake. An ABC transport system, GtsABCD (for GABA transport system) (pRL100248-51), of the spermidine/putrescine family is part of this operon. GtsA is a periplasmic binding protein, GtsB and GtsC are integral membrane proteins, and GtsD is an ATP-binding subunit. Expression of *gtsABCD* from a *lacZ* promoter confirmed that it alone is responsible for high rates of GABA transport, enabling rapid growth of strain 3841 on GABA. Gts transports open-chain compounds with four or five carbon atoms with carboxyl and amino groups at, or close to, opposite termini. However, aromatic compounds with similar spacing between carboxyl and amino groups are excellent inhibitors of GABA uptake so they may also be transported. In addition to the ABC transporter, the operon contains two putative mono-oxygenases, a putative hydrolase, a putative aldehyde dehydrogenase, and a succinate semialdehyde dehydrogenase. This suggests the operon may be involved in the transport and breakdown of a more complex precursor to GABA. Gts is not expressed in pea bacteroids, and *gtsB* mutants are unaltered in their symbiotic phenotype, suggesting that Bra is the only GABA transport system available for amino acid cycling.

Nitrogen fixation is the reaction whereby prokaryotes reduce N₂ to NH₃ catalyzed by the enzyme nitrogenase, with the consumption of at least 16 molecules of ATP (6). Whereas the ability of free-living prokaryotes to fix nitrogen is reasonably widespread, only a few bacteria have evolved the ability to form nitrogen-fixing symbioses with higher plants. The largest group of such bacteria are members of the family *Rhizobiaceae*, which consist of several genera of gram-negative bacteria belonging to the alpha subdivision of proteobacteria. They are common in soil and induce root nodule formation with leguminous plants in a species-specific manner to facilitate nitrogen fixation. This mutualistic association results in rhizobia differentiating to form membrane-enclosed pleomorphic cells, referred to as bacteroids, that fix N₂ (26). Ammonia is the primary product of nitrogen fixation, and this is released by bacteroids to the plant, where it is assimilated into amino acids. In return, the plant uses photosynthate to provide the bacteroid with a carbon and energy source, principally as C₄-dicarboxylic acids (31).

It has long been known that amino acids supplied to isolated bacteroids stimulate the secretion of aspartate and alanine (1, 33). Furthermore, it was recently proposed that ammonium assimilation may be shut down in *Rhizobium leguminosarum* bacteroids of pea nodules due to the cycling of amino acids between host and symbiont (25). Conclusive evidence for the

importance of amino acid transfer between bacteroids and the plant in pea nodules was demonstrated by mutation of the two principal broad-range amino acid transporters: the general amino acid permease (Aap) and the branched-chain amino acid permease (Bra) (25). Peas inoculated with these double amino acid transport mutants were nitrogen starved, even though bacteroids were fully developed and retained the ability to fix N₂. This suggested a model for nitrogen fixation within legume nodules wherein a C₄-dicarboxylate and an amino acid are supplied to bacteroids, allowing ammonium assimilation to shutdown, with ammonium and another amino acid returned to the plant. Within this model it was suggested that the plant might supply glutamate to the bacteroid. An alternative to glutamate would be its direct decarboxylation product γ -amino butyric acid (4-amino butyric acid [GABA]). However, the identity of any amino acid that moves from the plant to the bacteroid has not been easy to prove because Aap and Bra have a very broad solute specificity (13).

High concentrations of GABA have been reported in isolated bacteroids of *B. japonicum* and *S. meliloti* (27, 38). ¹⁵N nuclear magnetic resonance analysis on detached pea nodules showed GABA to be the second most abundant amino acid (37). This is consistent with an important role for GABA in bacteroid metabolism and a possible role in amino acid cycling. Although it has already been established that GABA can be transported by Bra (13), in the present study a GABA-specific transport system was identified that, while normally cryptic, can be selected for by growth on GABA. The mechanism by which its expression is activated, its solute specificity, and its role in bacteroid metabolism are considered.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
Strains		
3841	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> , Str ^r derivative of strain 300	16
RU1722	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc$	25
RU1736	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101$	This study
RU1816	<i>gsrR102</i>	This study
RU1979	$\Delta aapJ braC::\Omega Spc$	This study
RU2227	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 gtsD::Tn5$ (255923)	This study
RU2228	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 pRL100244::Tn5$ (248940)	This study
RU2229	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 gtsC::Tn5$ (255191)	This study
RU2230	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 pRL100243::Tn5$ (248568)	This study
RU2231	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 pckA::Tn5$ (41043)	This study
RU2232	$\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gsrR101 gtsD::Tn5$ (255562)	This study
RU2396	Rlv3841 <i>gtsC::Tn5</i> (255191)	This study
RU4097	Rlv3841 <i>gtsD::gusA</i>	This study
RU4100	Rlv3841 $\Delta braEF::\Omega Tc \Delta aapJQM::\Omega Spc gtsD::gusA$	This study
LMB147	Rlv3841 <i>gtsR::pK19Nm</i>	This study
Plasmids		
pCR-BluntII	Cloning vector for PCR products	Invitrogen
pRK415	Broad-host-range P-group cloning vector; Tc ^r	21
pK19mob	Used to generate mutants in Rlv3841; Nm ^r	36
pRU877	pK19mob with <i>gusA</i>	24
pRU877D	<i>gtsD::gusA</i> in pRU877	This study
pRU1097/D-TOPO	TOPO-adapted vector containing <i>gfpmut3.1</i> ; Gm ^r	26
pRU1604	<i>gtsABCD</i> from Rlv3841 in pCR-BluntII	This study
pRU1606	<i>gtsABCD</i> from RU1736 in pCR-BluntII	This study
pRU1689	<i>gtsABCD</i> from RU1736 without promoter in pRK415	This study
pRU1702	<i>gtsABCD</i> from Rlv3841 without promoter in pRK415	This study
pRU1703	<i>gtsABCD</i> from Rlv3841 with <i>lacZ</i> promoter in pRK415	This study
pRU1704	<i>gtsABCD</i> from RU1736 with <i>lacZ</i> promoter in pRK415	This study
pRU1815	<i>gtsR</i> from Rlv3841 in pRU1097/D-TOPO	This study
pRU1816	<i>gtsR</i> from RU1736 in pRU1097/D-TOPO	This study
pRU1817	pRU1815 with a HincII deletion in <i>gtsR</i>	This study
pRU2060	<i>gtsD</i> from Rlv3841 in pRU877	This study
pLMB117	518-bp internal fragment of <i>gtsR</i> in pK19mob	This study

^a The location of the Tn5 insertion is given in parentheses as the first base of the transposon 9-bp repeats in the Rlv3841 genome. Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Nm^r, neomycin resistance; Str^r, streptomycin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *R. leguminosarum* bv. *viciae* strains (identified by the prefix Rlv) were grown at 28°C on either TY medium (2) or acid minimal salts medium (AMS) (28) with 10 mM D-glucose and 10 mM ammonium chloride as the sole source of carbon and nitrogen, unless otherwise stated. Mean generation times were determined in three independent cultures and are shown \pm the standard error of the mean (SEM). Antibiotics were used at the following concentrations: ampicillin, 50 μ g ml⁻¹; kanamycin, 40 μ g ml⁻¹; neomycin, 80 μ g ml⁻¹; streptomycin (Str), 500 μ g ml⁻¹; and tetracycline, 2 μ g ml⁻¹ in AMS and 5 μ g ml⁻¹ in TY.

Genetic modification of bacterial strains. Standard protocols were used for DNA manipulations (35). Sequencing was carried out by MWG-Biotech AG. Similarity searches were done by using the Basic Local Alignment Search Tool on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Tn5 insertions were cloned and sequenced as previously described (32). Transposon mutations were transduced by using the phage RL38 as previously described (28).

A 4.2-kb region, encompassing *gtsABCD*, was amplified by PCR with the primers p631 (5'-ACAGCGAGGGATCATTTGAGCTTC-3') and p633 (5'-GCTCGACTATACCCGTACCAAGACG-3'), from both wild type (Rlv3841) and strain RU1736 (*aapJQM braEF gsrR101*). Amplified products were cloned into pCRII-Blunt, producing pRU1604 and pRU1606 (for Rlv3841 and RU1736, respectively) and then into pRK415 in both orientations for each, either as a KpnI-XbaI fragment (pRU1703 and pRU1704 from Rlv3841 and RU1736, respectively) or as a HindIII-XbaI fragment (pRU1702 and pRU1689 from Rlv3841 and RU1736, respectively). Cloning *gtsABCD* as a KpnI-XbaI fragment

ensured expression from the vector's *lacZ* promoter in plasmids pRU1703 and pRU1704.

An integration mutant was made in *gtsR* by PCR amplifying a 518-bp internal fragment with the primers pr0175 (5'-TTTCTAGAGAATTGGCGCTCGACAAGGT-3') and pr0176 (5'-TTTAAGCTTATCTTCGACATCTGCAGCGG-3') containing an XbaI and a HindIII restriction site (indicated in boldface). This was cloned into pK19mob (generating pLMB117) and integrated into the chromosome to produce strain LMB147 by selecting for neomycin resistance. The pK19mob *lacZ* promoter is oriented in the opposite direction from *gtsR* in this construct. The correct insertion was verified by PCR using the primers p1003 (5'-ATACGATTCTTGATTCGGC-3') and M13rev (5'-CAGGAAACAGCTATGACC-3').

To complement strains with *gtsR* from either Rlv3841 or RU1736, a 1.2-kb fragment was amplified by PCR, using the primers p857 (5'-CACCGGATCA GTCAATCACCCTT-3') and p858 (5'-AAAAAGCTTCCAGGGATTCACGACCGC-3'). Amplified products were then cloned into pRU1097/D-TOPO, generating pRU1815 and pRU1816 (for Rlv3841 and RU1736, respectively). An in-frame deletion was made in the *gtsR* of pRU1815 by HincII restriction digestion (yielding pRU1817).

To map *gtsR101* and *gtsR102* mutations, three independent fragments of the *gtsR* genes from Rlv3841, RU1736, and RU1816 were amplified by PCR using the primers p869 (5'-AAGCTTACTGGCTCGTCAACTGCAGGGGAAT-3') and p872 (5'-AGGCTATCCCTCGACAGCATCGCCG-3') and sequenced with these primers, as well as p1003 (5'-ATACGATTCTTGATTCGGC-3'). The sequences were then aligned by using CLUSTAL W at EBI (www.ebi.ac.uk/clustalw/), using the published Rlv3841 sequence (39) as a reference.

To examine the expression levels of *gts* in bacteroids, a 1.6-kb region encoding *gtsC* and part of *gtsD* was cloned as a BamHI fragment from pRU1604 into the

pK19-*gusA* integrating reporter vector pRU877 (24), forming pRU2060. This was integrated into the chromosomes of 3841 and RU1736 by single crossover recombination by selecting for neomycin resistance (RU4097 and RU4100 from Rlv3841 and RU1736, respectively). *GusA* activity was measured as previously described (24).

Isolation of RNA, quantification, and quantitative reverse transcription-PCR (qRT-PCR) was performed as previously described (19). The comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) was used, and C_T values were normalized to the endogenous housekeeping gene *mdh* (4). The primers used were p789 (5'-GTTCTCTTCGACATCGCGGACGGC-3') and p790 (5'-GACCTTGAGGTTTGATGCCGAGAAG-3') for amplification of *mdh*, p821 (5'-CCGACAACATGGTCTTTTCG-3') and p822 (5'-ACGTCCCAGGTGACCTGACC-3') for *gtsA*, p823 (5'-GCAAGGCAACAGGGTTTGGG-3') and p824 (5'-TTGAGATAGGTCGCGGATCC-3') for *gtsB*, p825 (5'-AGGCGAGCCGTGGGGGTGTGC-3') and p826 (5'-TTCGATCAGCCGGTGCC-3') for *gabD4*, p829 (5'-GAAATTCACGCAGGCGCTCC-3') and p830 (5'-CGTGTGGATAAGCGCGGACC-3') for pRL100242, and p831 (5'-ATGAGCGGTGCTCTGAAG-3') and p832 (5'-CAAATGCCTCAGAACCCTC-3') for *gtsR*.

Transport and enzyme assays. *R. leguminosarum* uptake assays were performed by the rapid filtration method as previously described (29). The final concentration of solute was 25 μ M (0.125 μ Ci of 14 C), and competing solutes were added to 0.5 mM. The kinetics of solute uptake by *R. leguminosarum* strains was determined using various 14 C solute concentrations in standard uptake assays. Rates are shown \pm the SEM.

Cultures were grown and harvested, cell extracts were prepared, and succinate semialdehyde dehydrogenase activity was assayed as previously described (30).

Plant growth. *R. leguminosarum* bv. *viciae* strains were used to inoculate surface-sterilized pea seeds (*Pisum sativum* cv. Avola) at the time of sowing. For dry-weight determination, plants were grown as previously described (28) in a growth room in 2-liter flasks filled with a sterile vermiculite mixture, watered with a nitrogen-free nutrient solution, and harvested at 6 weeks. Plants were dried for 48 h at 70°C for 72 h before being weighed, and values are shown \pm the SEM.

RESULTS

Isolation of Gts-expressing strains. It was previously shown that mutation in any of the genes encoding the Bra ABC transporter prevents growth of Rlv3841 on 10 mM GABA, as the sole carbon and nitrogen source (13). However, when strain RU1722 (*ΔbraEF::ΩTc ΔaapJQM::ΩSpc*) was streaked onto agar plates with 10 mM GABA as the sole added carbon source, large mutant colonies became visible among small pin colonies after approximately 8 days. A purified mutant colony (strain RU1736) retained rapid growth on GABA when subcultured on solid or in liquid media. The mean generation time of the wild type (Rlv3841) was slow on 10 mM GABA compared to 10 mM glucose ammonia (15.6 ± 0.9 h versus 5.3 ± 0.1 h, respectively), while the mean generation times of RU1736 were similar on the two carbon sources (6.3 ± 0.2 h versus 5.5 ± 0.3 h, respectively). RU1736 retained its antibiotic resistance markers and did not grow on other amino acids such as glutamate, which is characteristic of the parent strain, RU1722 (13). Thus, RU1736 is unlikely to harbor a spontaneous reversion at the *braEF* loci; instead, it most probably results from selection for expression of a previously unexpressed transport system that may be specific for GABA.

To determine whether the ability to grow rapidly on GABA could be acquired in the wild-type strain, Rlv3841 was cultured on agar plates containing 10 mM GABA as the sole carbon source. After approximately 8 days, colonies with a larger morphology were present, and one was purified and stocked (strain RU1816). Its growth phenotype for all other amino acids matched that of Rlv3841, except for its rapid growth on GABA (mean generation time of 6.6 ± 0.2 h).

To determine whether the increased growth rate of RU1736 and RU1816 on GABA results from increased transport, up-

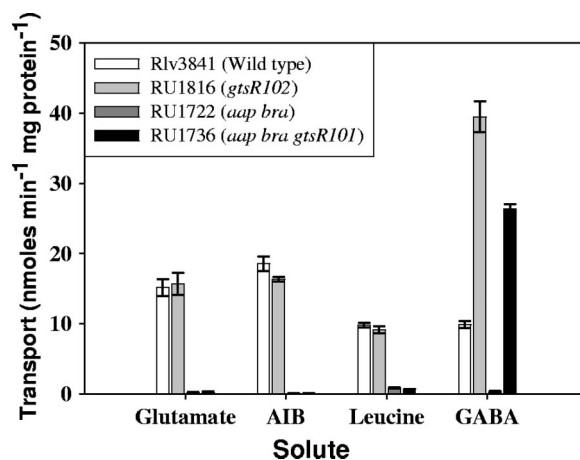


FIG. 1. Amino acid transport for strains showing an increased growth rate on GABA. Cells were grown overnight in 10 mM glucose–10 mM ammonia AMS. The number of independent replicates was ≥ 3 , and values are shown \pm the SEM.

take assays for GABA and other amino acids were performed on cells grown on glucose as the sole carbon source (Fig. 1). The uptake of GABA, but not other amino acids, by RU1736 and RU1816 was increased significantly over that of the parent strains, Rlv3841 and RU1722 (*ΔbraEF::ΩTc ΔaapJQM::ΩSpc*), respectively. The lack of GABA uptake by strain RU1722 is consistent with what was previously reported for a strain mutated in the *bra* operon (13). Since GABA transport is very high in cultures of RU1736 and RU1816 grown on glucose as a carbon source, it appears that expression of the acquired transport system is constitutive. Consistent with this, the rate of GABA uptake in RU1736 was not increased by growth on GABA versus glucose/ammonia (25.2 ± 1.5 versus 29.2 ± 1.9 nmol mg of protein⁻¹ min⁻¹, respectively). Although an increased uptake of GABA was observed for RU1736 and RU1816, there was no increase in the uptake of other common amino acids, suggesting that this transport system is relatively solute specific, unlike the two main amino acid transport systems of *R. leguminosarum* Aap and Bra (Fig. 1). Due to this apparent narrow specificity the permease was termed the GABA transport system (Gts), and the mutant alleles that lead to its constitutive expression in RU1736 and RU1816 were initially named *gts-101* and *gts-102*, respectively. However, as they were later mapped to *gtsR* (see below) they were renamed *gtsR101* and *gtsR102* and, to avoid confusion, these latter designations will be used. The identification of Gts indicates that *R. leguminosarum* encodes at least one other GABA transporter that is not commonly expressed in free-living cells cultured on minimal or complete media but may function in other environments or in symbiosis.

Identification of the operon encoding Gts. To identify the genes that code for the *gts*, approximately 6,000 colonies from a Tn5 mutant library of strain RU1736 (*ΔbraEF::ΩTc ΔaapJQM::ΩSpc gtsR101*) were screened for loss of rapid growth on 10 mM GABA. In all, six mutants were isolated (RU2227 to RU2232) that did not grow on GABA. Five of these (RU2227, RU2228, RU2229, RU2230, and RU2232) also lacked significant GABA uptake (≤ 1.1 nmol mg of protein⁻¹ min⁻¹). The strain RU2231, which was not altered in GABA

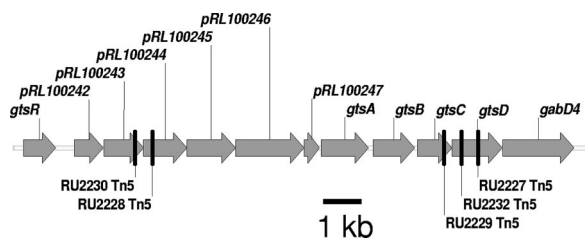


FIG. 2. Map of the *gts* operon. Transposon insertion points are shown as solid vertical lines and are labeled with the strain number below the gene arrows. The precise position of transposon insertions is given in Table 1.

transport, had a Tn5 insertion in phosphoenol pyruvate carboxykinase (*pckA*, RL0037). RU2231 is mutated in the gluconeogenic pathway and only grew after the addition of one of a variety of sugars, including glucose, sucrose, and arabinose. Cloning and sequencing of the transposons from the five strains that lacked significant GABA transport revealed they map within a 12-kb region on the Sym plasmid (pRL10) (Table 1 and Fig. 2). In particular, three transposons are located within genes that encode components of a putative member of the polyamine/opine/phosphonate (POPT; transporter classification 3.A.1.11) ABC transporter family. The genes encoding this putative transport complex were named *gtsABCD*. The predicted proteins of this transport system show greatest homology to the spermidine/putrescine PotABCD ATP-binding cassette (ABC) transport system of *Escherichia coli* (9, 20). GtsA has 28% identity to the solute binding protein, PotD, while GtsB and GtsC have 33 and 29% identity to the integral membrane proteins, PotB and PotC, respectively. GtsD has 40% identity to the nucleotide binding domain protein, PotA. TMPred prediction for GtsB and GtsC indicate both have six putative transmembrane domains, with the N terminus located in the cell cytoplasm.

To confirm that *gtsABCD* encodes a GABA ABC transport system, RU1979 ($\Delta aapJ$ *braC::\Omega Spc*), which lacks significant GABA uptake via the Bra system (0.36 ± 0.18 nmol mg of protein⁻¹ min⁻¹), was complemented in *trans* with Gts. Expression of *gtsABCD* under the control of the *lacZ* promoter, amplified from both Rlv3841 (pRU1703) and the suppressor strain RU1736 (pRU1704), enabled transport of GABA at 44.07 ± 2.49 and 42.04 ± 3.91 nmol mg of protein⁻¹ min⁻¹, respectively, but did not alter the transport of other amino acids. The ability of GtsABCD derived from both Rlv3841 and RU1736 to increase GABA uptake suggests the mutation in RU1736 does not cause a gain of function within *gtsABCD* but is likely to be in a regulatory region that controls its expression. When the same fragment was cloned in the opposite orientation, where the transporter was not expressed from the vector's *lacZ* promoter (pRU1702 and pRU1689), neither plasmid increased GABA uptake significantly (0.74 ± 0.37 and 0.86 ± 0.11 nmol mg of protein⁻¹ min⁻¹, respectively). Furthermore, Rlv3841 containing pRU1703, but not pRU1702, grew rapidly on GABA as the sole carbon source. Thus, Gts is a GABA-specific transporter, whose expression dramatically increases the rate of growth of Rlv3841 on GABA.

Expression of *gtsABCD*, revealed by GABA uptake in RU1979 containing either pRU1703 or pRU1704, relied on

expression from the *lacZ* promoter in the vector, suggesting that its native promoter lies further upstream. This is consistent with transposon insertions in pRL100243 and pRL100244 abolishing GABA uptake, since they are up to 4 kb upstream of *gtsA*. The polarity of these Tn5 insertions on expression of *gtsABCD*, as measured by transport, indicates the whole region forms part of a large operon, including this distal ABC transport system for GABA.

Regulation of the *gts* operon by GtsR. Gts appears to be regulated by a promoter upstream of the Tn5 insertion in pRL100243 (RU2230), which may lie immediately upstream of pRL100242. There is a large intergenic region (123 bp) between pRL100242 and pRL100241 and a putative transcriptional regulator of the GntR-family, from now on named *gtsR*, is encoded by pRL100241. Two ways in which regulation of the *gts* operon could be altered are either a mutation in the promoter upstream of pRL100242 or in the coding region of *gtsR*. To determine whether a mutation in *gtsR* leads to expression of the downstream genes, RU1736 and RU1816 were supplied in *trans* with *gtsR* that had been PCR amplified from either wild-type Rlv3841 or from the *gtsR101* strain RU1736 to give plasmids pRU1815 and pRU1816, respectively. Since these clones carry all of *gtsR*, including intergenic regions before *gtsR* and pRL110242, a 123-bp *HincII* in-frame deletion was introduced in the coding region of *gtsR* in pRU1815, yielding pRU1817. This clone lacks the ability to form a functional GtsR but retains all potential binding motifs in the intergenic regions. RU1736 (*gtsR101*) and RU1816 (*gtsR102*) were prevented from growing on 10 mM GABA as the carbon source when GtsR from Rlv3841 was provided on pRU1815 but grew as well as the plasmid-free strains when provided with GtsR from RU1736 on pRU1816. All strains were unchanged for growth on 10 mM glucose ammonia. In addition, RU1736 and RU1816 were still able to grow on GABA when provided in *trans* with *gtsR* from Rlv3841 containing an internal deletion (pRU1817). These data demonstrate that the mutations in RU1736 (*gtsR101*) and RU1816 (*gtsR102*) result from the loss of function of GtsR. To map the mutations *gtsR101* and *gtsR102*, *gtsR* was amplified from Rlv3841, RU1736, and RU1816 and sequenced. Relative to the Rlv3841 sequence, RU1736 (*aapJQM braEF gtsR101*) has an AC deletion at bp 532 and 533 and RU1816 (*gtsR102*) has a G insertion between bp 610 and 611 in *gtsR*. Both mutations cause frameshifts in the C terminus of GtsR and increase the predicted length of the protein. Thus, as stated above, *gts-101* and *gts-102* were designated *gtsR101* and *gtsR102*.

Control of expression by the GntR family of regulators has been best studied in *B. subtilis* where the first gene of the gluconate operon encodes GntR, which acts as a negative regulator by binding to the DNA at the promoter site, competing with RNA polymerase (8). To examine the regulatory role of GtsR, qRT-PCR was used to measure the expression of *gts* genes in RU1722 (*aapJQM braEF*), RU1736 (*aapJQM braEF gtsR101*), and RU2228 (*aapJQM braEF gtsR101* pRL100244::Tn5) which is unable to transport or grow on GABA. As a control qRT-PCR was also carried out on the *gts* operon of Rlv3841, and all data are expressed relative to this strain (Table 2). Expression of all genes within the putative *gts* operon was similar for Rlv3841 and strain RU1722. However, expression of all genes downstream of *gtsR* in RU1736

TABLE 2. Expression of genes in the *gts* operon of *R. leguminosarum* mutant strains relative to Rlv3841 as measured by qRT-PCR

Strain ^a (genotype)	Mean fold expression \pm SEM relative to Rlv3841 ^b				
	<i>gtsR</i>	pRL100242	<i>gtsA</i>	<i>gtsB</i>	<i>gabD4</i>
RU1722 (<i>aapJQM braEF</i>)	0.7 \pm 0.6	1.3 \pm 0.3	2.1 \pm 0.1	0.9 \pm 0.2	1.6 \pm 0.2
RU1736 (<i>aapJQM braEF gtsR101</i>)	4.4 \pm 0.1	61.9 \pm 0.1	80.4 \pm 0.2	33.8 \pm 0.2	16.2 \pm 0.2
RU2228 (<i>aapJQM braEF gtsR101</i> , pRL100244::Tn5)	3.0 \pm 0.1	75.7 \pm 0.1	4.8 \pm 0.1	2.2 \pm 0.1	1.1 \pm 0.4

^a Cells were grown overnight in 10 mM glucose–10 mM ammonia AMS.

^b qRT-PCR was performed on three independent cultures.

(*aapJQM braEF gtsR101*) showed a dramatic increase in expression over the parent strain RU1722. It also showed that *gabD4*, which encodes a putative succinate semialdehyde dehydrogenase, is highly upregulated and likely to be part of the *gts* operon. This increase in expression was also observed for genes upstream of the transposon insertion point in strain RU2228 (*aapJQM braEF gtsR101* pRL100244::Tn5). However, genes downstream of the Tn5 insertion in pRL100244 were substantially reduced in expression compared to their immediate parent RU1736, confirming the polarity of this insertion. It was also shown by qRT-PCR that expression of *gtsR* was increased fourfold in RU1736 (*aapJQM braEF gtsR101*) relative to Rlv3841, while expression of all other genes of the *gts* operon was increased 16- to 80-fold (Table 2). There is a large intergenic region (123 bp) between *gtsR* and pRL100242, and expression of the latter increased 61-fold in RU1736 compared to 4-fold for *gtsR*. The absolute expression levels of *gtsR*, as measured by C_T values, in RU1722 and RU1736 were 19.0 ± 0.5 versus 16.7 ± 0.1 , while the absolute expression levels of pRL100242 were 21.1 ± 0.4 and 15.6 ± 0.1 in RU1722 and RU1736, respectively. Thus, transcription of pRL100242 is weaker than that of *gtsR* in RU1722 (i.e., 21.1 versus 19.0 or \sim 4-fold weaker) but stronger in RU1736 (i.e., 15.6 versus 16.7 or \sim 2-fold stronger). This suggests that the main regulated promoter for this operon lies immediately upstream of pRL100242, with the likelihood of a weaker promoter upstream of *gtsR*. An insertion mutation in *gtsR* resulted in rapid growth of strain LMB147 on GABA, a finding consistent with GtsR acting as a repressor binding to the promoter region upstream of pRL100242.

Since the qRT-PCR data indicates that *gabD4* is expressed as part of this operon, succinate semialdehyde dehydrogenase was assayed. In glucose/ammonia-grown cultures of Rlv3841 it was undetectable, while in RU1736 it was 268 nmol mg of protein⁻¹ min⁻¹, thus confirming *gabD4* expression in a *gtsR101* background. This indicates that GtsR (pRL100241) regulates a large operon beginning with a conserved hypothetical (pRL100242), a putative hydrolase (pRL100243), a putative flavin mono-oxygenase (pRL100244), a putative mono-oxygenase (pRL100245), a putative aldehyde dehydrogenase (pRL100246), a conserved hypothetical (pRL100247), GtsA (pRL100248), GtsB (pRL100249), GtsC (pRL100250), GtsD (pRL100251), and *GabD4* (pRL100252). The expression of genes downstream of *gabD4* was not examined, although there are only two small conserved hypothetical genes before an ABC operon on the opposite strand of the DNA. Syntenous genes for most of this operon are present in *Rhizobium etli* CFN42, *Sinorhizobium meliloti* 1021, *Sinorhizobium medicae* WSM419, and *Agrobacterium tumefaciens* C58

but not in *Bradyrhizobium japonicum* USDA110 or *Mesorhizobium loti* MAFF303099. However, *S. meliloti* 1021 lacks a complete copy of *gtsR* and in *A. tumefaciens* C58 a gene homologous to *gtsR* lies further upstream. It is interesting that in *S. meliloti* 1021 the *gts* operon is on pSymA, very close to *nifB* and the rest of the main *nif* operon.

In summary, these data indicate that GtsR likely represses the *gts* operon by binding in the intergenic region between *gtsR* and pRL100242. Spontaneous mutations in *gtsR*, giving alleles *gtsR101* and *gtsR102* in RU1736 and RU1816, respectively, result in a loss of repressor function and lead to constitutive expression of the *gts* operon. It is possible that the laboratory strain Rlv3841 has a mutation in *gtsR* that locks it in the off-form. There are two nucleotide changes in *gtsR* between Rlv3841 and *R. leguminosarum* bv. trifolii WSM1325 resulting in D86G and A225G. However, since both *A. tumefaciens* and *R. etli* CFN42, like Rlv3841, encode residues D86 and A225, this suggests these changes are unlikely to result in nonfunctional repressor molecules.

The *gts* operon might be induced by a solute other than GABA, so to test this *gtsD* was fused to *gusA* in an integrating vector (pRU2060), and this was recombined into the chromosome of Rlv3841 (RU4097) and RU1736 (RU4100). Although RU4100 had constitutive GusA activity, RU4097 displayed no detectable GusA activity on a wide variety of media, including TY and minimal medium supplemented with the common amino acids alanine, aspartate, glutamate, asparagine, and glutamine; the polyamines putrescine and spermidine; butyric acid and aminoisobutyric acid; and the aromatic compounds 3-piperidinecarboxylic acid, 3-aminobenzoic acid, and 4-aminobenzoic acid.

Ligand recognition properties of Gts. The increased transport rate of GABA, but not other amino acids, in RU1736 was confirmed by examining the inhibition of GABA uptake by a range of amino acids (Fig. 3). Only unlabeled GABA inhibited the uptake of ¹⁴C-labeled GABA (¹⁴C-GABA). Compared to other proteobacterial transport systems GtsABCD is closest in homology to the POPT family putrescine/spermidine PotA-BCD transport system of *E. coli*. GABA is an open-chain four-carbon molecule with carboxyl and amino groups at distal ends, while putrescine is also an open-chain four-carbon molecule but with amino groups at both ends. Spermidine is an open-chain seven-carbon molecule with amino groups at both ends and between carbons four and five. However, the rate of GABA uptake by RU1736 was not inhibited by either putrescine or spermidine (Fig. 4). The inability of putrescine to inhibit GABA uptake via Gts suggests that compounds must contain one carboxyl group and one amino group. Consistent with this, butyric acid, which, like GABA, has four carbon

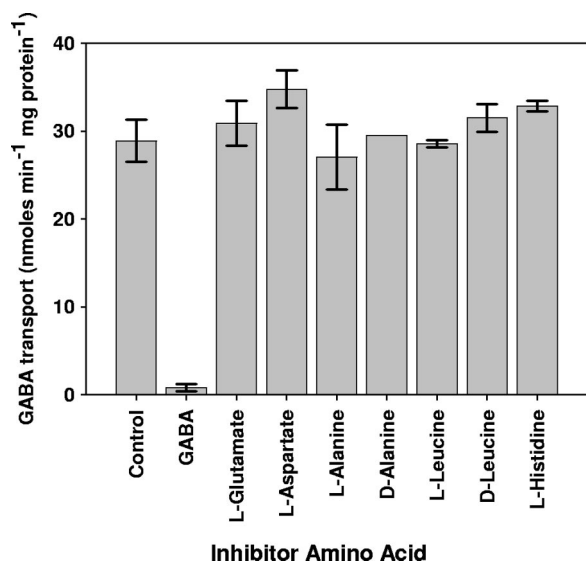


FIG. 3. Inhibition of ¹⁴C-GABA uptake in RU1736 (*aapJQM braEF gtsR101*) with a range of common amino acids. Cells were grown overnight in 10 mM glucose–10 mM ammonia AMS. The number of independent replicates was ≥ 3 , and values are shown \pm the SEM. The concentration of GABA was 25 μ M and that of each competitor was 0.5 mM.

atoms but no amino group, did not inhibit GABA uptake. Neither the L- nor the D-isomer of 2-aminobutyric acid inhibited GABA uptake, indicating the spacing between the carboxyl and amino group is crucial. Consistent with this, it was

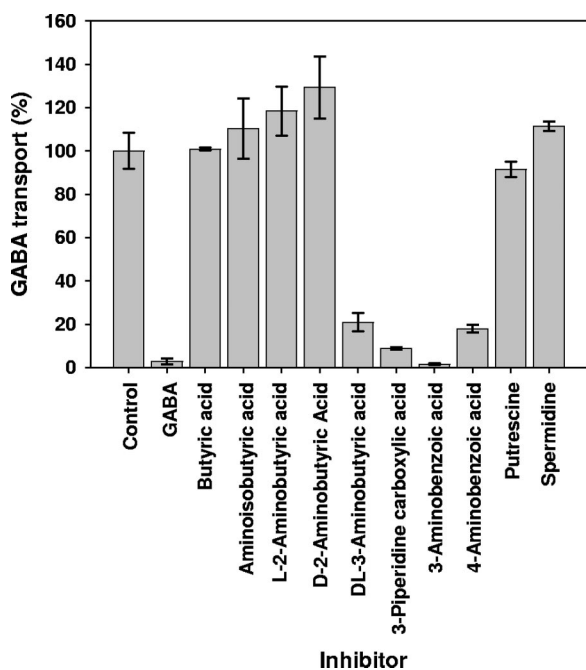


FIG. 4. Inhibition of ¹⁴C-GABA uptake in RU1736 (*aapJQM braEF gtsR101*) by a number of GABA analogs. Cells were grown overnight in 10 mM glucose–10 mM ammonia AMS. The number of independent replicates was ≥ 3 , and values are shown \pm the SEM. Values are given as the percentage of the absolute rate of transport in RU1736 (28.9 ± 2.4 nmol mg of protein⁻¹ min⁻¹). The concentration of GABA was 25 μ M, and that of each competitor was 0.5 mM. The control has no competitor added.

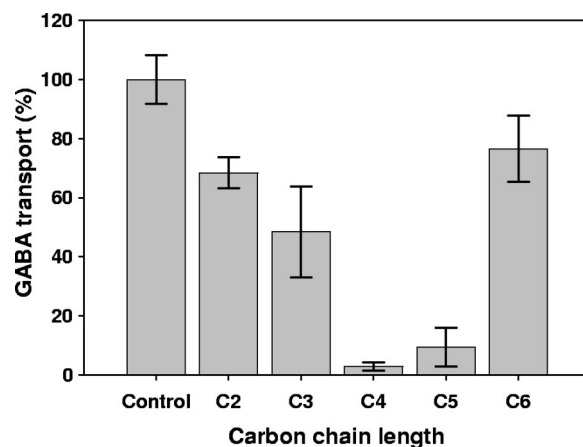


FIG. 5. Inhibition of ¹⁴C-GABA uptake in RU1736 (*aapJQM braEF gtsR101*) by open-chain amino acid compounds of differing carbon chain lengths. Cells were grown overnight in 10 mM glucose–10 mM ammonia AMS. The number of independent replicates was ≥ 3 , and values are given \pm the SEM. Control, no inhibitor; C₂, glycine; C₃, β -alanine; C₄, GABA; C₅, 5-aminopentanoic acid; C₆, 6-aminohexanoic acid. Values are given as the percentage of the absolute rate of transport in RU1736 (28.9 ± 2.4 nmol mg of protein⁻¹ min⁻¹). The concentration of GABA was 25 μ M, and that of each competitor was 0.5 mM.

found that DL-3-aminobutyric acid did inhibit GABA uptake, although not to the same degree as GABA itself. This suggests that Gts transports compounds with the carboxyl group at one end and the amino group at least three carbon atoms distant. This was further investigated by varying the open-chain length of compounds with terminal amino and carboxyl groups used as competitors for GABA uptake (Fig. 5). GABA (C₄) and 5-aminopentanoic acid (C₅) were both strong inhibitors of uptake, whereas glycine (C₂), β -alanine (C₃), and 6-aminohexanoic acid (C₆) were weak inhibitors. This demonstrates that the best inhibitors have an open-chain length between four and five carbon atoms.

Several cyclic compounds containing conformationally constrained amino and carboxyl groups inhibited uptake of GABA (Fig. 4). The ability of 3-piperidinecarboxylic acid, 3-aminobenzoic acid, and 4-aminobenzoic acid to inhibit GABA uptake suggests that free rotation of bonds is not essential for ligand recognition. As expected, 3-aminobenzoic acid had the greatest inhibitory effect on GABA uptake since the amino and carboxyl groups are separated by four carbon atoms. The kinetics of GABA transport by Gts were determined in RU1736 grown on glucose ammonia: $K_m = 8.45 \pm 1.36$ μ M and $V_{max} = 42.6 \pm 2.1$ nmol mg of protein⁻¹ min⁻¹.

Symbiotic properties of Gts. The expression level of the *gts* operon was measured in bacteroids containing *gusA* integrated into the chromosome in Rlv3841 (RU4097 *gtsD::gusA*) and RU1736 (RU4100 *gtsD::gusA*). At 21 days after inoculation, nodules were harvested, and bacteroids were assayed for GusA-activity. There was no difference in GusA activity in pea bacteroids of Rlv3841, with or without an integrated *gtsD::gusA* fusion (3.9 ± 1.8 or 6.2 ± 3.6 nmol mg of protein⁻¹ min⁻¹ respectively), indicating no significant expression of *gts* in nodules. The expression of *gtsD::gusA* in bacteroids of RU4100 was

very high (GusA activity = 196.3 ± 5.9 nmol mg of protein⁻¹ min⁻¹).

The *gtsC* transposon mutation of strain RU2229 was transduced into wild-type Rlv3841 to generate RU2396 (*gtsC::Tn5*). Plants inoculated with either Rlv3841 or RU2396 and grown in nitrogen-free medium were green and healthy with red nodules, while uninoculated control plants were not nodulated, stunted and yellow. Furthermore, plants inoculated with RU2396 or Rlv3841 were not significantly different in dry weight (RU2396, 1.26 g per plant \pm 0.11; $n = 15$; Rlv3841, 1.58 g per plant \pm 0.09; $n = 15$) after 6 weeks growth, while uninoculated plants were significantly different ($0.52 \text{ g} \pm 0.10$; $n = 15$). This shows that RU2396, with a mutation in *gtsC*, is unimpaired in nitrogen fixation.

When inoculated onto peas, RU1736 produced severely nitrogen-starved plants with pale pink nodules that were indistinguishable from plants inoculated with the parent strain RU1722. This demonstrates that constitutive expression of *gtsABCD*, leading to a high rate of GABA transport, cannot overcome the loss of the broad solute range amino acid transporters Aap and Bra.

DISCUSSION

The cytosolic concentration of GABA from soybean nodules was measured as $71 \mu\text{g g}^{-1}$ (fresh weight), while nuclear magnetic resonance analysis of pea nodules revealed a GABA concentration of $5.72 \mu\text{mol g}^{-1}$ (fresh weight) (37, 38). High GABA concentrations have also been reported in isolated bacteroids of *S. meliloti* ($41 \text{ nmol mg of protein}^{-1}$) (27), suggesting it has an important role in bacteroid metabolism. GABA can be made by many bacteria from glutamate by glutamate decarboxylase in a pathway known as the GABA shunt. One of the main roles of this pathway is to enable glutamate metabolism to bypass the 2-oxoglutarate dehydrogenase complex, which is proposed to be limited by the low redox potential within the nodule (7, 34). However, glutamate decarboxylase activity is barely detectable in bacteroids from nodules of both snake bean and soybean (12, 15, 27, 34). A gene with significant identity to known glutamate decarboxylases is also absent from the published genome sequences of *M. loti*, *S. meliloti*, *B. japonicum*, *R. etli*, and *R. leguminosarum* (10, 11, 17, 18, 39). These observations make it highly unlikely that rhizobia have a classical GABA shunt, since they appear unable to convert glutamate to GABA via glutamate decarboxylase. This suggests that the GABA present in bacteroids is likely to be of plant origin. The presence of plant-derived GABA in bacteroids is also indicated by the high level of expression of enzymes for the metabolism of GABA in bacteroids. For example, the 2-oxoglutarate-dependent GABA transaminase, GabT, which removes the amino group from GABA to form glutamate, is highly expressed in pea bacteroids (30). The semialdehyde released from GABA when it acts as a transamination donor is succinate semialdehyde, and the enzyme succinate semialdehyde dehydrogenase is also highly expressed in bacteroids (30). These conclusions are also supported by ¹⁵N₂ labeling, enzyme activity, and genetic analysis of the GABA catabolic pathway in pea bacteroids (J. Prell et al., submitted for publication).

The data in the present study indicate Gts is an ABC trans-

porter of the POPT family with homology to the PotABCD putrescine/spermidine transport system in *E. coli*. Putrescine and spermidine are natural polyamines that are ubiquitous in almost all prokaryotic cells. Polyamines are known to be involved in the biosynthesis of nucleic acids and proteins, as well as to mediate cell growth and proliferation (14). However, Gts appears to be specific for GABA and does not transport polyamines or common amino acids. The solute specificity of Gts is typical of that observed for the secondary APC transporter GabP in *E. coli* (3, 22, 23). Gts was determined to have a K_m of $8.45 \pm 1.36 \mu\text{M}$ and a V_{max} of $42.6 \pm 2.1 \text{ nmol mg of protein}^{-1} \text{ min}^{-1}$ for GABA. The affinity of Gts for GABA is significantly lower than that of Bra, the only other identified transporter in *R. leguminosarum* with specificity for GABA (13). However, the V_{max} is significantly higher, explaining why strains mutated in *bra* but expressing *gts* have a faster growth rate than Rlv3841.

Inhibition studies showed that Gts recognizes compounds that have a backbone of four or five carbon atoms with carboxyl and amino groups at either end. Four carbon open-chain compounds, such as putrescine and butyric acid, which lack the carboxyl and amino groups, respectively, do not inhibit GABA uptake. These compounds may be bound and transported by Gts but, if they are, it would be with much lower affinity than for GABA. 3-Piperidinecarboxylic acid also inhibited GABA uptake, indicating that Gts may also allow the uptake of large aromatic compounds. In addition, the putative protein products of the genes upstream of *gts* (pRL100243 to pRL100246) encoding a hydrolase, a flavin-dependent oxygenase, a mono-oxygenase, and an aldehyde dehydrogenase suggest that they may be involved in the release and catabolism of side chains and breakage of the aromatic ring. It might be that succinate semialdehyde, or a compound similar to it, is finally released for oxidation by GabD4. However, several aromatic compounds did not induce a *gtsD::gusA* fusion, so at present it is only possible to say that *gts* was cryptic under the conditions tested.

Transposon mutagenesis revealed Gts is encoded by four genes that form part of a large operon, of ~ 12 kb, under the control of GtsR, which belongs to the GntR family of negative regulators. Mutants derived from Rlv3841 or RU1722 (RU1736 and RU1816, respectively) that grow rapidly on GABA were easily detected. Sequence analysis of *gtsR* PCR amplified from RU1736 and RU1816 showed that they have different point mutations, both of which cause a frameshift leading to a GstR with an extended C terminus. Both of these mutations in *gtsR* allow constitutive expression of the distal genes *gtsABCD*, encoding the transport complex. Although *gtsR* has its own promoter, it can be inferred from qRT-PCR analysis, as well as from an insertional mutation in *gtsR*, that there is likely to be a promoter in the intergenic region between *gtsR* and pRL100242. This mode of regulation would be similar to the classical model of the gluconate operon in *B. subtilis* (8). If this is correct, GtsR would bind to its own promoter region and to that of pRL100242, through its helix-turn-helix motif, thus abolishing transcription of the downstream genes until a ligand sequesters or inactivates the repressor protein. Confirmation of this would require much closer examination of the promoters of the *gts* operon and physical analysis of binding to them by GtsR. However, this is

peripheral to the present study and likely to be similar to the situation for GntR in *B. subtilis*.

Overall, it is clear that Rlv3841 has a cryptic GABA transporter that is normally only expressed in laboratory culture after mutation and is not significantly expressed in pea bacteroids. However, the presence of such a large cryptic operon that also includes genes that might be involved in catabolism of a more complex compound suggests the role of this operon may not be confined to GABA catabolism. It is notable that the quorum signaling homoserine lactones (AHL) are catabolized to succinate semialdehyde, although this normally occurs via the products of the *attKLM* operon (5). AttM is a lactonase that opens the AHL ring, releasing γ -hydroxybutyrate. A γ -hydroxybutyrate dehydrogenase (AttL) oxidizes this to succinate semialdehyde, which is oxidized to succinate by AttK. AttK (GabD1) is one of six putative succinate semialdehyde dehydrogenases in Rlv3841, which includes GabD4 of the Gts operon. It has been shown in *A. tumefaciens* that GABA induces the *aatKLM* operon, and this leads to increased breakdown of *N*-(3-oxooctanoyl) homoserine lactone. Rlv3841 also has an *aatKLM* operon (pRLL10134-136). Plants synthesize GABA as a natural part of their wound response, and this appears to attenuate bacterial virulence by enhancing AHL degradation. It has been observed that *A. tumefaciens* has a transport system with high identity to Rlv3841 Bra, and mutation of this system increased bacterial virulence, presumably by reducing GABA-dependent induction of *attKLM* (5). The Gts has some interesting parallels to this in that it may break down a complex metabolite and release GABA. It is intriguing that induction of Gts might result in a highly avirulent phenotype in plant pathogens, particularly since it has a much higher transport rate for GABA than Bra does. Whether plants are capable of inducing Gts in an organism such as *A. tumefaciens*, which has a putative Gts system, is of course pure speculation. We have only been able to artificially select for *gts* expression, and its natural induction remains illusive.

One of the principal reasons for examining Gts in depth is the possible role of GABA in amino acid cycling, which is essential for effective nitrogen fixation in pea nodules (25, 31). However, it is clear that *gts* is not expressed in nodules, which leaves the Bra as the only known system capable of GABA transport in Rlv3841 bacteroids. Although we cannot strictly exclude the possibility of there being other GABA transporters, microarray studies in our laboratory have not revealed any obvious candidates expressed in bacteroids (R. Karunakaran, unpublished data). Furthermore, since plants grown on nitrogen-free medium and inoculated with single mutants in the Bra system are not impaired in growth or N_2 fixation, this suggests that GABA transport is not essential for bacteroid function. GABA might of course contribute to amino acid cycling in the wild type, but in *bra* mutants other amino acids must be sufficient.

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