RESEARCH LETTER

Characterization of the quaternary amine transporters of *Rhizobium leguminosarum* bv. *viciae* 3841

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Abstract

Rhizobium leguminosarum bv. *viciae* 3841 contains six putative quaternary ammonium transporters (Qat), of the ABC family. *Qat6* was strongly induced by hyperosmosis although the solute transported was not identified. All six systems were induced by the quaternary amines choline and glycine betaine. It was confirmed by microarray analysis of the genome that pRL100079–83 (*qat6*) is the most strongly upregulated transport system under osmotic stress, although other transporters and 104 genes are more than threefold upregulated. A range of quaternary ammonium compounds were tested but all failed to improve growth of strain 3841 under hyperosmotic stress. One Qat system (*gbcXWV*) was induced 20-fold by glycine betaine and choline and a Tn*5::gbcW* mutant was severely impaired for both transport and growth on these compounds, demonstrating that it is the principal system for their use as carbon and nitrogen sources. It transports glycine betaine and choline with a high affinity (apparent K_m, 168 and 294 nM, respectively).

Introduction

Several transporters for quaternary amines have now been examined in detail in *Sinorhizobium meliloti*, including Hut, Cho, BetS and most recently Prb (Boncompagni *et al.*, 2000; Boscari *et al.*, 2002; Dupont *et al.*, 2004; Alloing *et al.*, 2006). Hut and Cho are ABC systems from the quaternary amine transporter (Qat) subfamily; Hut transports histidine as well as proline and proline betaine (stachydrine) (Boncompagni *et al.*, 2000), while Cho is specific for choline (Dupont *et al.*, 2004). BetS is a secondarily coupled system that is energized by the sodium gradient and transports glycine betaine and proline betaine (Boscari *et al.*, 2002). It is constitutively expressed but is rapidly activated upon osmotic upshift. Prb is an ABC transporter from the Opp (oligopeptide) subfamily that transports proline betaine, as well as choline and glycine betaine, but shows no specificity for or inhibition by oligopeptides (Alloing *et al.*, 2006). Expression of the *prb* operon is induced by both NaCl and proline betaine, suggesting that it has a role in both proline betaine catabolism and its use as an osmoprotectant. Protection of *S. meliloti* from osmotic stress by proline betaine is entirely due to transport by Prb and BetS, while protection by glycine betaine uses BetS, Prb and other unknown systems (Alloing *et al.*, 2006). While this detailed picture has emerged for *S. meliloti* little is know about the mechanisms responsible for transport of quaternary amines in other rhizobia. This study was therefore initiated to gain an understanding of the role of multiple Qat systems in *Rhizobium leguminosarum* and whether they respond to hyperosmotic stress or have a role in catabolism of quaternary ammonium compounds. This is particularly interesting because key systems such as BetS are not present in *R. leguminosarum*.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are detailed in Table 1. *Rhizobium leguminosarum* bv. *viciae* strains were grown at 28 °C in acid minimal salts medium (AMS) (Poole *et al.*, 1994) with 10 mM D-glucose and 10 mM ammonium chloride as the sole source of carbon and nitrogen, unless otherwise stated. Antibiotics were used at the following concentrations ($\mu g\, mL^{-1}$); gentamicin, 20; kanamycin, 40; neomycin, 80; and streptomycin, 500.

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Transport assays

Metabolite uptake was assayed by the rapid-filtration method as previously described (Poole *et al*., 1985). The final concentration of solute was 25 μM [0.125 μCi (4625 Bq) of ^{14}C-labelled solute] and competing solutes were added at 500 μM. The kinetics of solute uptake were determined by using various ^{14}C-solute concentrations in standard uptake assays. All cultures were grown on AMS as described in the text.

Genetic modification of bacterial strains

Transposon mutants were located in a Tn*5* library of strain 3841 using PCR primers complementary to the end of IS*50* and the gene of interest (Table 1). Other mutants were isolated by amplifying a 700–900-bp internal fragment of a selected gene and cloning this into pK19mob as described previously (Prell *et al*., 2002). Plasmids were conjugated into *R. leguminosarum*, integrated into the chromosome, and neomycin-resistant colonies isolated. The correct location of each insertion was confirmed by PCR using a primer complementary to the vector (pK19/18A) and a primer complementary to a region of the genome that is downstream of the cloned fragment.

Promoter analysis

Regions of DNA predicted to contain the promoters of the qat operons were amplified by PCR using primers indicated in Table 1 and inserted into pRU1097/D-TOPO according to Invitrogen's instructions for TOPO reactions (Mauchline *et al*., 2006). This resulted in the transcriptional fusion of each putative promoter region to *gfp-mut 3.1*. Each plasmid was then conjugated into *R. leguminosarum* 3841, as previously described (Simon *et al*., 1983). Strains were grown in 48-well microtitre plates and their $OD_{600\,nm}$ and fluorescence (485 nm excitation; 510 nm emission) measured at intervals over 3 days. Final specific fluorescence values are for cultures in midexponential growth at an $OD_{600\,nm}$ of 0.4–0.6.

RNA isolation and microarray analysis

Rhizobium leguminosarum 3841 strains were grown (50 mL) overnight in 250-mL flasks at 225 r.p.m. to midlog phase ($OD_{600\,nm}$ 0.4–0.6). Cultures (12 mL) were added directly to RNA later (24 mL) and centrifuged at 26 000***g*** at 4 °C in a Sorvall SS34 rotor centrifuge. RNA was isolated as previously described (Karunakaran *et al*., 2006).

A whole genome DNA Oligo array for *R. leguminosarum* 3841 was constructed from the genome sequence published by Young *et al*. (2006). Unique 70-mer oligonucleotides were synthesized for all 7344 genes of strain 3841 by Operon Biotechnologies and printed with Pronto buffer on Corning Ultragaps slides at the Functional Genomics and Proteomics Laboratory, University of Birmingham, UK. Microarray slides were blocked for 15 min in sodium borohydride [sodium borohydride 20 mM, succinic anhydride 140 mM, dissolved in 1-methyl-2-pyrrolidinone (90% v/v)], washed in deionized water and dried.

Total RNA was labelled and purified with the Cyscribe Post-Labelling kit (GE Healthcare) according to the manufacturer's instructions. Equal amounts (30–35 pmol) of Cy5- and Cy3-labeled cDNA were mixed and dried (5–10 μL). Hybridization solution (90 μL), containing 25% formamide, 5 × saline sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) and calf thymus DNA (9 μg), was added to the labeled DNA and heated at 95 °C for 2 min. The mixture was incubated at room temperature for 2 min and centrifuged for 2 min. Hybridization was carried out at 42 °C for 16 h using an Array Booster (Implen, UK). Microarray slides were then washed in 2 × SSC, 0.1% SDS for 8 min at 42 °C, twice in 0.2 × SSC, 0.1% SDS for 5 min at 42 °C, twice in 0.2 × SSC for 4 min, at room temperature and 1 min in 0.1 × SSC. Slides were then washed with MilliQ water (5 s), isopropanol (5 s) at room temperature, dried and scanned for fluorescence intensity using a GenePix 4000A microarray scanner at a resolution of 10 μm (Axon Instruments, Union City, CA). Spot recognition was performed with Bluefuse (BlueGnome Limited, Cambridge, UK) and data were imported into GeneSpring 7.2 (Silicon Genetics, Redwood, CA). The local background value was subtracted from the intensity of each spot and a Lowess normalization applied to the slide. The normalized expression ratio of experimental sample to control was calculated by taking the natural log of the ratio for each replicate experiment, averaging this for all replicates and then calculating the natural antilog. Arrays were conducted on three independent cultures of strain 3841 grown on AMS with glucose (10 mM), ammonium (10 mM) either with 100 mM NaCl (experimental) or without NaCl (control). Samples were harvested in midexponential growth at an $OD_{600\,nm}$ of 0.6. The array data were deposited in MIAMExpress with an accession number of E-MEXP-1041.

Plant growth

Rhizobium leguminosarum bv. *viciae* strains were used to inoculate surface-sterilized pea seeds (*Pisum sativum* cv. Avola) at the time of sowing. Plants were grown in a growth room (16-h light 22 °C) in 2-L flasks filled with a sterile vermiculite mixture and watered with a nitrogen-free nutrient solution as previously described (Poole *et al*., 1994). Acetylene reduction was measured at flowering (21–23 days) in peas as previously described (Allaway *et al*., 2000).

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Table 1. Bacterial strains, plasmids and primers used in this study

	Description	References
Strains		
3841	*Rhizobium leguminosarum* bv. *viciae* Sm^r derivative of strain 300	Johnston & Beringer (1975)
RU2192	Insertion mutant of *qat6V*	This work
RU2410	3841 *gbcW*::Tn*5*, Kn^r	This work
RU2411	3841 *qat2W*::Tn*5*, Kn^r	This work
RU2412	3841 *qat5W*::Tn*5*, Kn^r	This work
RU2496	Insertion mutant of *qat3W*	This work
RU2497	Insertion mutant of *qat4V*	This work
Plasmids		
PK19mob	Used to generate insertion mutants in 3841 containing *lacZ* reporter, Nm^r	Schafer *et al*. (1994)
pRU1097/D-TOPO	Promoter probe vector with *gfpmut3.1* reporter, Gm^r	Mauchline *et al*. (2006)
pRU1450	pK19mob containing internal fragment of *qat6V*	This work
pRU1614	*qat6* promoter in pRU1097/D-TOPO	This work
pRU1700	*gbc* promoter in pRU1097/D-TOPO	This work
pRU1758	*qat2* promoter in pRU1097/D-TOPO	This work
pRU1760	*qat3* promoter in pRU1097/D-TOPO	This work
pRU1763	*qat4* promoter in pRU1097/D-TOPO	This work
pRU1764	*qat5* promoter in pRU1097/D-TOPO	This work
pRU1800	pK19mob containing internal fragment of *qat3V*	This work
pRU1801	pK19mob containing internal fragment of *qat4V*	This work
Primers	Target	Sequence
IS50 Downie	IS*50* end used to map Tn*5* mutants	GAACGTTACCATGTTAGGAGGT
p469	Forward primer for internal region of *qat6V* for insertional mutagenesis in pK19mob	GATCATGCCGTGATAGGTCT
p470	Reverse primer for internal region of *qat6V* for insertional mutagenesis in pK19mob	GATCATGCCGTGATAGGTCT
p663	Forward primer for promoter region of RL3533 (*gbc*)	CACCGAAGGCTGCGATCAGTTGCA
p664	Reverse primer for promoter region of RL3533 (*gbc*)	CCTCTAGAAAGCGGACGGTAGTGCAGTT
p694	Forward primer for promoter region of RL0512 (*qat2*)	CACCAACGTCATCGCCAGTTCGGT
p695	Reverse primer for promoter region of RL0512 (*qat2*)	CCCAAGCTTCGTTGCCGAGCAGCGTGCCT
p698	Forward primer for promoter region of pRL120516 (*qat3*)	CACCTTGAAACCTTTGTCGGCTAT
p699	Reverse primer for promoter region of pRL120516 (*qat3*)	CCCAAGCTTACCTCCGCACTCTGCCAGTT
p704	Forward primer for promoter region of pRL120531 (*qat4*)	CACCAGCCCCGAACTCGACCGATA
p705	Reverse primer for promoter region of pRL120531 (*qat4*)	CCCAAGCTTCGTCAGGTCCTCCCACGACA
p706	Forward primer for promoter region of pRL120750 (*qat5*)	CACCGACGCCATAGGAGGTCTCGA
p707	Reverse primer for promoter region of pRL120750 (*qat5*)	TTTAAGCTTGCCAGGAAACGCTCCTCGAC
p718	Forward primer for internal region of *qat3W* for insertional mutagenesis in pK19mob	CGAAAGCCTCTTCTCCCCGC
p719	Reverse primer for internal region of *qat3W* for insertional mutagenesis in pK19mob	CCGATCATCGAAGCGACGAC
p793	Forward primer for internal region of *qat4V* for insertional mutagenesis in pK19mob	TTCAACTGCGTCGTCGGCGTGTCC
p794	Reverse primer for internal region of *qat4V* for insertional mutagenesis in pK19mob	CAGCGAGGCGATGTCGGAATTCG

Results

Bioinformatic analysis of the Qat loci

Approximately 10 000 colonies from a library of strain 3841 genomic DNA containing random promoter fusions to the reporter gene *gfp-UV* were screened for fluorescence on 100 mM sucrose. The brightest colony on agar plates was of a strain which DNA sequencing showed contains a *gfp-UV* fusion to pRL100079. This gene codes for a protein with 56% identity over 224 residues to ProV from *Escherichia coli*. ProV is the ATP-binding protein of the Qat for glycine betaine. It was confirmed in liquid culture that this fusion is induced strongly by 100 mM sucrose (data not shown). BLAST analysis of the genome of *R. leguminosarum* bv. *viciae* strain 3841 using the sequence of pRL100079 allowed the identification of six operons with similarity to the QAT family and the system containing pRL100079 was named

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Qat6 (Fig. 1). In all cases the proteins coded for by these six operons had highest identity to characterized glycine betaine transporters such as ProV from *E. coli* and *Salmonella typhimurium*. When all the ABC transport systems of *R. leguminosarum* were aligned using CLUSTALW the six putative QAT systems formed a tight clade (data not shown). While their sequence identity is highest to transporters of the Qat family of ABC transporters (Saier, 2000) this is not intended to indicate that they must transport a quaternary ammonium compound. Four of the remaining systems were named Qat2–5. The proteins of the final Qat system have high sequence identity to the Cho system in *S. meliloti* (Dupont *et al*., 2004) and this operon was named gbc, for glycine betaine choline, because we demonstrate below that it has a much wider solute specificity than the *S. meliloti* system. Gbc comprises a solute-binding protein, GbcX (88% similar to ChoX), an integral membrane protein, GbcW (79% similar to ChoW) and an ATP-binding cassette protein, GbcV (90% similar to ChoV) (Fig. 1).

Transcriptional analysis of Qat systems

Promoter fusions for each putative *qat* operon were cloned into pRU1097/D-TOPO (Fig. 1) and *gbc* was induced at least 20-fold when strain 3841 was grown with choline or glycine betaine (Fig. 2). In addition, *qat2* and *qat3* were induced up to fivefold, *qat4* up to threefold and *qat5 and qat6* up to twofold. These data show that all six systems were induced by glycine betaine and choline; consistent with them transporting quaternary amines (Fig. 2). However, the system most strongly induced by hyperosmosis (100 mM NaCl) was *qat6* although *gbc* was also induced 2.9-fold (Fig. 2).

During the later part of this project the complete sequence of *R. leguminosarum* bv. *viciae* 3841 became available so it became possible to design a microarray for the genome. This enabled an analysis of the Qat systems within the context of the global response to osmotic stress of this organism. Overall, there were 104 genes threefold or more upregulated and 73 genes threefold or more downregulated when 100 mM NaCl was included in the growth medium (supporting Tables S1 and S2). In agreement with the Gfp fusion results, the most strongly upregulated transport operon in the whole genome was Qat6. Qat6V, Qat6W and Qat6X and were up regulated five-, two- and sevenfold, respectively. None of the other Qat systems identified here were upregulated by NaCl addition. Because *gbc* was slightly upregulated by osmotic stress when measured by Gfp fusion the absence of its detectable upregulation in microarrays may be due to the lack of sensitivity of two-colour micro-arrays to small transcriptional changes. In addition, two genes downstream of *qat6*, pRL100082 and pRL100083 were also four- and 10-fold upregulated by high osmotic pressure and may be part of the same operon. Both of these genes code for small putative proteins of 96 and 144 amino acids, respectively, and are of unknown function, although pRL100082 contains a putative helix-turn-helix that suggests a role in regulation.

Another ABC system found on the sym plasmid, pRL100462–pRL100464, was upregulated three- to fourfold by osmotic stress but this system belongs to the carbohydrate uptake class 2 (CUT2) of sugar transporter (Saier, 2000). Interestingly two genes upstream of this are also upregulated threefold, with the first (pRL100466) being *otsB*, which codes for trehalose phosphatase, while pRL10465 codes for a putative AraC transcriptional regulator. OtsB catalyses the last step in trehalose synthesis, while OtsA is a trehalose-6-phosphate synthase that is found on the chromosome (RL0503) rather than the sym plasmid (pRL10). However, the ABC transporter genes (pRL100462–pRL100464) do not show particularly high identity to the known trehalose transporter of *S. meliloti* (SMb20325–SMb20328), which belongs to the CUT1 family (Jensen *et al*., 2002). Instead the highest identity of the solute-binding protein (pRL100464) is with SMc02021, which was induced fivefold by butyric acid and valeric acid (Mauchline *et al*., 2006). There is also another ABC operon (pRL120351–pRL120353) that was upregulated threefold by osmotic stress and belongs to the CUT1 family but it shows little similarity to the trehalose transporter.

Mutation of Qat systems

To define the function of the Qat transporters, insertional mutations were isolated for each of them. In the case of *gbc, qat2* and *qat5*, Tn*5* insertional mutations in their cognate *W* genes were identified by PCR from a transposon mutant library, using oligonucleotides that bind to IS*50* and downstream of the gene of interest. Mutations in *qat3W*, *qat4V* and *qat6V* were obtained by recombining into the genome internal fragments of these genes cloned in pK19mob (Prell *et al*., 2002). The '*V*' and '*W*' genes encode the ATP-binding cassette and the permease, respectively, and which one was chosen depended on the ease of cloning as mutation of either class of gene is almost always completely disruptive to the function of ABC transporters (Fig. 1).

The six mutants and wild type were grown on acetylcholine, betaine aldehyde, choline, glycine betaine, histidine, proline or proline betaine as the sole carbon/nitrogen sources; while glucose/ammonia was used as a control. The *gbcW* mutant was unable to grow on betaine aldehyde, choline or glycine betaine, but its growth was similar to that of wild type on the other tested carbon/nitrogen sources listed above. Choline is an alcohol that is directly oxidized to the betaine aldehyde, which is in turn oxidized to the carboxylic acid glycine betaine (Pocard *et al*., 1997). The inability of the *gbcW* mutant to grow on any of these three

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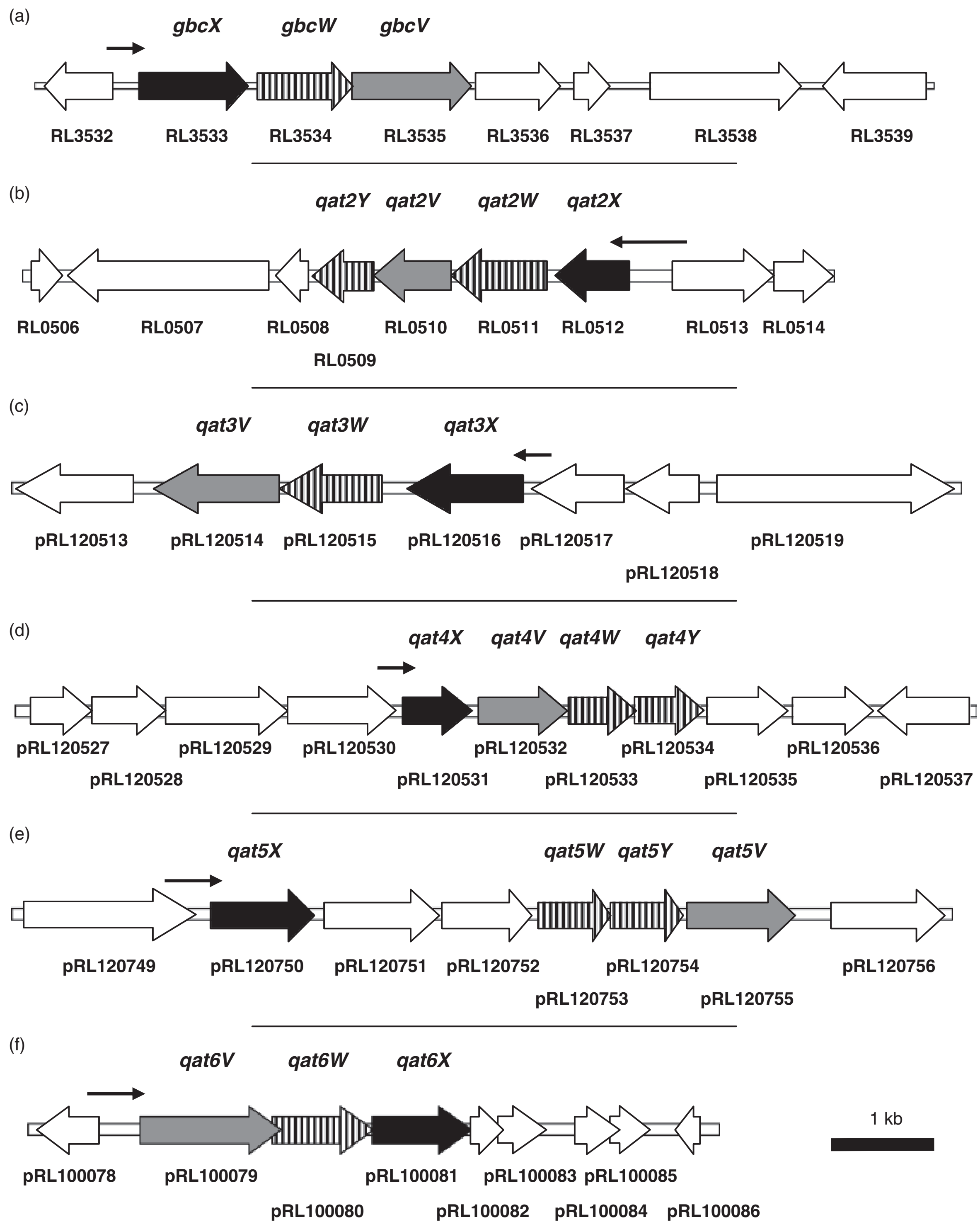

Fig. 1. *Qat* operons. The *qat* operons and neighbouring genes are shown to scale (size of 1 kb indicated). Genes coding for solute-binding proteins are shown in black, integral membrane proteins have vertical lines and ATP-binding cassette are grey. The systematic identifiers for each gene are shown below each arrow. Small arrows above the genes show the locations of regions used for promoter analysis. (a) Gbc system, (b) Qat2 system, (c) Qat3 system, (d) Qat4 system, (e) Qat5 system, (f) Qat6 system.

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compounds is consistent with them being transported by Gbc. All the other mutants grew as well as the wild type on all of the tested carbon/nitrogen sources listed above. These analyses show that *gbc* was induced both by choline and glycine betaine as well as being required for growth on them as carbon sources.

While the *gbc* mutant was impaired for growth on choline and glycine betaine as carbon sources none of the Qat mutants were altered in their rate of growth relative to the wild type on AMS glucose (10 mM) and ammonium (10 mM) with 125 mM NaCl. A transport system for a compatible solute normally only improves growth under osmotic stress in the presence of the solute. Therefore, a number of potential compatible solutes (carnitine, choline, ectoine, glycine betaine, dimethylsulfoniopropionate (DMSP), proline betaine, proline and trehalose) were added to the growth medium at 1 mM but none of them significantly altered the mean generation time of strain 3841 grown in glucose (10 mM) and ammonia (10 mM) with either 125 mM NaCl or sucrose up to 800 mM (data not shown). Furthermore, none of the compounds enabled any growth of strain 3841 over 72 h when incubated under the same conditions, but with NaCl increased to 200 mM.

Transport measurements on the Qat systems

Choline and glycine betaine uptake experiments were carried out on all six *qat* mutants grown on glucose and ammonia (Table 2). Choline transport was only dramatically reduced in the *gbc* mutant. However, after growth on glucose and ammonia transport of glycine betaine was reduced most significantly in the *qat3* mutant. The *qat3* mutant was not affected in growth on either choline or glycine betaine, which suggests it is not the main inducible system required for catabolism of these compounds. As indicated above by the induction and growth data *gbc* is most likely to have this role in strain 3841 and therefore this was tested below.

To measure induced transport rates for choline and glycine betaine, strain 3841 was grown overnight in AMS with choline, glycine betaine or glucose/ammonia as the

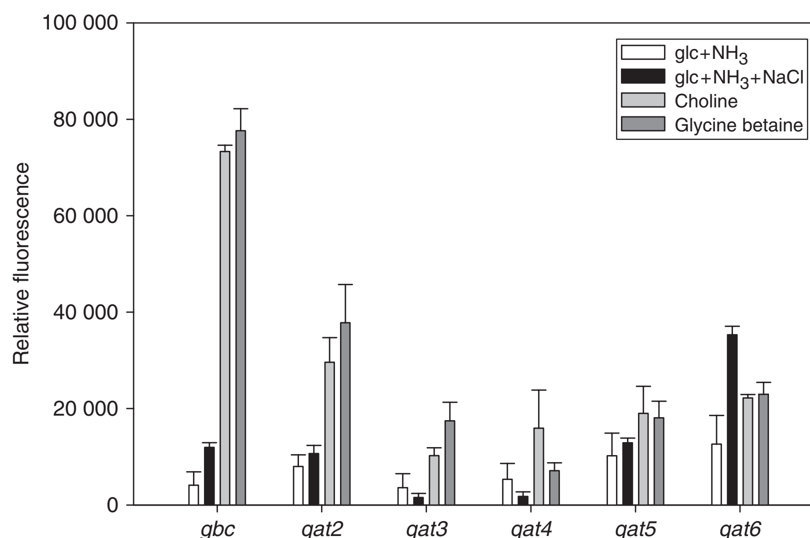

Fig. 2. Induction of *qat gfp* fusions. Relative fluorescence (absolute fluorescence divided by the $OD_{600\,nm}$) of strain 3841 carrying a Gfp fusion to each of the six *qat* promoter regions. Growth substrates shown in each group left to right were glucose/ammonia (white bars); glucose/ammonia plus NaCl (black bars); choline (light grey bars); glycine betaine (dark grey bars). Inositol/ammonia, histidine and pyruvate/ammonia were also tested but there were no significant changes and they have been omitted for clarity. All solutes were 10 mM, except for NaCl, which was 125 mM and pyruvate, which was 20 mM. Results are the mean of three independent experiments plus and minus the SEM.

Table 2. Transport of glycine betaine and choline by various strains

Strain (genotype)	Grown on	Glycine betaine transport	Choline transport
3841	Glucose	3.8 ± 0.3	1.4 ± 0.1
RU2410 (*gbcW*::Tn*5*)	Glucose	3.0 ± 0.53	0.37 ± 0.25
RU2411 (*qat2W*::Tn*5*)	Glucose	3.51 ± 0.09	1.26 ± 0.12
RU2496 (*qat3W*::pK19)	Glucose	1.92 ± 0.12	1.42 ± 0.27
RU2497 (*qat4V*::pK19)	Glucose	3.2 ± 0.05	1.73 ± 0.27
RU2412 (*qat5W*::Tn*5*)	Glucose	2.9 ± 0.38	1.3 ± 0.05
RU2192 (*qat6V*::pK19)	Glucose	4.0 ± 0.29	1.18 ± 0.19
3841	Choline	29.5 ± 3.0	11.5 ± 1.3
3841	Glycine betaine	40.1 ± 1.8	21.5 ± 1.8

Cells were either grown overnight in glucose (10 mM)/ammonia (10 mM) or choline (10 mM) or glycine betaine (10 mM) then washed and resuspended in minimal salts before transport was measured. Rates are nmol min^{-1} mg^{-1} protein and are $\pm$ SEM from four independent replicates. Glycine betaine and choline were at 25 μM in transport assays.

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carbon and nitrogen sources. A low level of glycine betaine and choline transport was measured in 3841 grown on glucose/ammonia (Table 2). However, when 3841 was grown on choline the rates of transport of glycine betaine and choline were induced eightfold compared with cells grown on glucose/ammonia. Similarly, when 3841 was grown on glycine betaine the rates of transport of glycine betaine and choline were induced 11–15-fold compared with cells grown on glucose/ammonia (Table 2). The magnitude of these increases in transport rate is similar to that for induction of *gbc* after growth on choline or glycine betaine.

To test whether Gbc is the main uptake system required for catabolism of choline and/or glycine betaine, 3841 and RU2410 (*gbcW*::Tn*5*) were grown on AMS with glucose and ammonia, as RU2410 cannot grow on choline. Cells were then washed and resuspended in AMS with 20 mM choline and incubated for 6 h, to induce glycine betaine and choline transport. After incubation in choline, strain 3841 transported choline and glycine betaine at 10.3 ± 0.9 and 14.9 ± 1.7 nmole min^{-1} mg^{-1} protein, respectively. However, strain RU2410 only transported choline and betaine at 0.1 ± 0.05 and 1.9 ± 0.02 nmole min^{-1} mg^{-1} protein, respectively. These data are consistent with *gbc* being the principal inducible system required for catabolism of choline and glycine betaine, while the retention of a low level of glycine betaine transport in RU2410 is consistent with its transport by Qat3.

Inhibitors of transport by Gbc

The specificity of choline transport in the wild-type strain was assessed by assays performed in the presence of a 20-fold excess of potential competitors (Fig. 3a). Inhibition of glycine betaine uptake by betaine aldehyde, choline and glycine betaine was also tested on these cells (Fig. 3b). ^{14}C-choline uptake was only significantly inhibited by unlabelled choline and betaine aldehyde. Radioactive glycine betaine uptake was significantly inhibited by unlabelled glycine betaine, betaine aldehyde and choline. Strain 3841 grown on AMS with 20 mM choline as the sole carbon source had an apparent K_m and apparent V_{max} for uptake of choline of 294 nM ($\pm$19 SEM; $n=3$) and 20.9 nmol mg^{-1} min^{-1}, respectively ($\pm$2.4 SEM; $n=3$), while for glycine betaine the apparent K_m and apparent V_{max} were 168 nM ($\pm$18 SEM; $n=3$) and 23.8 nmol mg^{-1} min^{-1} ($\pm$4.2 SEM; $n=3$), respectively.

Plant phenotypes

All the *qat* mutants and wild type were inoculated onto pea plants and acetylene reduction was determined on 21-day-old plants. The results for wild type, *gbc, qat2, qat3, qat4, qat5* and *gat6* were 2.8 ± 0.18, 4.3 ± 0.27, 2.99 ± 0.29, 3.61 ± 0.56, 3.64 ± 0.49, 2.62 ± 0.23 and 3.75 ± 0.62 µmol h^{-1} per plant ($\pm$ SEM; $n=6$), respectively. This shows that none of the mutants are impaired in N_2 fixation. Consistent with this all six *qat* mutants and wild type were inoculated onto peas and after 6 weeks of growth all plants were healthy and green with red nodules, while uninoculated plants lacked nodules and were yellow and stunted.

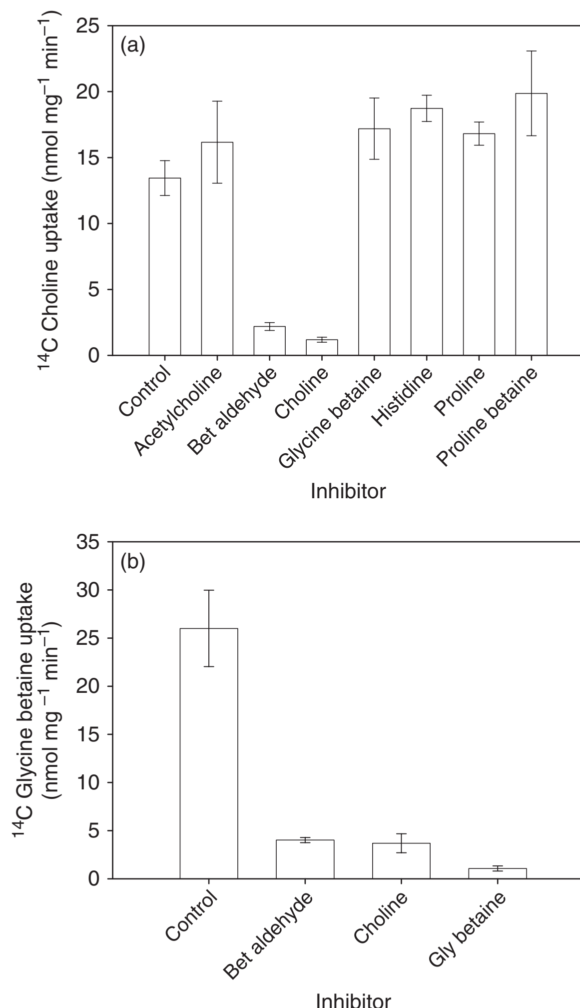

Fig. 3. Inhibition of quaternary ammonium compound uptake by 3841 grown on choline. (a) Uptake of choline (25 µM) over 4 min with 500 µM of inhibitor added as indicated on the *x*-axis. (b) Uptake of glycine betaine (25 µM) over 4 min with 500 µM of inhibitor added as indicated on the *x*-axis. All values are the mean of at least four independent experiments plus and minus SEM. Rates are given as nmol min^{-1} mg^{-1} protein. Bet aldehyde, betaine aldehyde.

Discussion

As a soil bacterium, *R. leguminosarum* encounters severe competition for nutritional sources as well as being subjected to environmental stress. Perhaps in response to this its

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genome has a very large number of transport systems (*c.* 15%), as shown by the annotated sequence of 3841 (Young *et al.*, 2006). In this study we identified six Qat-like transporters in *R. leguminosarum*, one of which, Gbc, appears to be the sole transporter of choline and the principal transporter for uptake of glycine betaine.

Out of the six Qat transporters, only *qat6* was strongly induced by hyperosmotic stress (Fig. 3), although we were unable to identify the solute that it transports. However, it has recently been shown that ABC transporters are inhibited by hyperosmosis, leading to a paradox of how they function for the transport of compatible solutes under hyperosmotic stress (Fox *et al.*, 2006). The proline betaine transporter (Prb) of *S. meliloti* is induced by both proline betaine and high osmolarity but this belongs to the oligopeptide class of transporters, although transport of proline betaine uptake by Prb is not inhibited by oligopeptides (Alloing *et al.*, 2006). Although both *qat6* and *prb* are induced by osmotic stress they are not similar to each other.

Although six Qat-like transporters are present in the 3841 genome, no transporters similar to BetS from *S. meliloti* (Boscari *et al.*, 2002) could be identified by sequence comparison. The absence of a BetS like system in *R. leguminosarum* may be important in the sensitivity of this species to NaCl stress. This sensitivity of all three biovars of *R. leguminosarum* to NaCl stress and the inability of choline and glycine betaine to protect against it has been observed previously (Boncompagni *et al.*, 1999). In this study a much wider range of compatible solutes were tested but again none were effective. While no solutes have been identified that protect against osmotic stress in *R. leguminosarum* the endogenous synthesis of trehalose by *R. leguminosarum* bv. *trifolii* strain NZP561 has been shown to be important in desiccation resistance (McIntyre *et al.*, 2007).

The Gbc system in *R. leguminosarum* was induced six- to eightfold by growth on either choline or glycine betaine and was required for growth on either compound as well as betaine aldehyde as the sole carbon/nitrogen source. However, there is at least one other constitutive transporter for glycine betaine in *R. leguminosarum*. It seems likely that Qat3 transports glycine betaine because a mutation in it reduced glycine betaine transport by 50% in cells grown on glucose and ammonia and the operon was induced 4.8-fold by growth on glycine betaine. Mutation of the *qat2* operon did not reduce glycine betaine transport in cells grown on glucose ammonia but the system was induced 4.7-fold after growth on glycine betaine so it may be a third transporter for glycine betaine. However, double- and even triple- *gbc*/*qat* mutants would be needed to investigate this because the loss of low-level glycine betaine transport by one of the *qat* mutants may have been masked by the presence of a functional Gbc. While the Qat systems are induced to varying extents by glycine betaine and choline they are likely to transport different sub-classes of quaternary amine as their principal solute. Although Gbc has strong sequence similarity to Cho from *S. meliloti*, it differs in being the sole transporter of choline in 3841, while Cho is one of three transporters in *S. meliloti* (Dupont *et al.*, 2004). Hence a *cho* mutant of *S. meliloti* can grow on choline, while a *R. leguminosarum gbc* mutant cannot. Also *cho* appears to be induced by choline alone (Dupont *et al.*, 2004), whereas *gbc* is induced by glycine betaine and choline.

In summary, the genome of *R. leguminosarum* bv. *viciae* 3841 is rich in quaternary amine transporters. As a group they all appear to be induced to varying extents by quaternary amines, with Gbc required for growth on glycine betaine and choline. However, only *qat6* was strongly induced by hyperosmosis although the solute transported remains unknown.

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Supporting Information

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

Table S1. Genes whose expression was upregulated 3 or more fold after addition of 100 mM NaCl.

Table S2. Genes whose expression was downregulated 3 or more fold after addition of 100 mM NaCl.

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