Role of Potassium Uptake Systems in Sinorhizobium meliloti

Osmoadaptation and Symbiotic Performance

Ana Domínguez-Ferreras, Socorro Muñoz, José Olivares, María J. Soto and Juan Sanjuán*

Departamento de Microbiología del Suelo y Sistemas Simbióticos. Estación Experimental del Zaidín, CSIC, Granada, Spain

Short title: K⁺-uptake systems in S. meliloti

* Corresponding author. Mailing address: Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, E-18008 Granada, Spain. Telephone: +34-958-181600 Ext. 259; Fax +34-958-129600; E-mail: Juan.Sanjuan@eez.csic.es
Abstract

Stimulation of potassium uptake is the most rapid response to an osmotic upshift in bacteria. This cation accumulates by a number of different transport systems whose importance has not been previously addressed in rhizobia. *In silico* analyses reveal the presence of genes encoding four possible potassium uptake systems in the genome of *Sinorhizobium meliloti* 1021: Kup1, Kup2, Trk and Kdp. The study of the relevance of these systems in a number of different growth conditions as well as in symbiosis showed that the integrity of Kup1 or Trk is essential for growth under laboratory conditions even in osmotically balanced media and the absence of both systems leads to a reduced infectivity and competitiveness of the bacteria in alfalfa roots. Trk is the main system involved in the accumulation of potassium after an osmotic upshift and the most important system for growth of *S. meliloti* in hyperosmotic conditions. The other three systems, specially Kup1, are also relevant during the osmotic adaptation of the cell and the relative importance of the Kdp system increases at low potassium concentrations.
INTRODUCTION

Rhizobia are gram-negative soil bacteria able to establish nitrogen-fixing symbiosis with leguminous plants under conditions of nitrogen deprivation. During this process, an exchange of molecular signals occurs between the two partners, leading to the formation of the root nodule where biological nitrogen fixation takes place (12).

Nearly 40% of the world’s land surface can be categorized as having potential salinity problems (38). Most crops are sensitive to relatively low levels of salinity (36), and in the case of legumes, there is an additional problem since not only the plant but also the symbiotic bacteria are sensitive to salinity both at the free-living stage and during the symbiotic process (22). The *Rhizobium*-legume symbiosis is highly sensitive to salt or osmotic stress since these conditions may inhibit the initial steps of the symbiotic interaction (root colonization, nodule infection and nodule development) and also have a depressive effect on nitrogen fixation (38). It has been observed that *Rhizobium* mutants affected in adaptation to high salinity present deficiencies in their symbiotic capacity (25). These results emphasize the importance of studying the adaptation mechanisms of rhizobia to changes in the osmotic conditions of the soil environment.

Response and adaptation to environmental stresses is probably a complex phenomenon involving many physiological and biochemical processes that likely reflect changes in gene expression and in the activity of enzymes and transport proteins (6, 7, 23). A rise in the external salinity and osmolarity triggers the outflow of water from the cell, resulting in a reduction in turgor and dehydration of the cytoplasm, causing a decrease in cytoplasm volume and thus an increase in ion concentration in the cytosol (33). A recent transcriptomic study in *Sinorhizobium meliloti* has revealed that the response of this bacterium to ionic and non-ionic compounds is very similar and includes the induction of
many genes of unknown function suggesting the existence of still unexplored osmoadaptation mechanisms (6). However, rhizobia are known to use distinct mechanisms for long-term adaptation to hyperosmotic stress, such as intracellular accumulation of low-molecular-weight organic solutes (amino acids, sugars and polyamines), changes in cell morphology and size or modifications in the pattern of extracellular polysaccharides (20, 22, 34, 38). Nevertheless, the most rapid response to an osmotic upshift, a sudden increase in external osmolarity, is the stimulation of potassium uptake (33).

Potassium (K\(^+\)), being the most abundant intracellular cation, makes a major contribution to the turgor pressure of the cells playing important roles in bacterial osmoadaptation, pH regulation, gene expression and activation of cellular enzymes (8). The elevated K\(^+\) levels after an osmotic upshift act as a second messenger to turn on other osmotically activated responses (2).

K\(^+\) uptake after an osmotic upshift is achieved by bacteria through specialized transport systems which have been well studied in certain bacteria (8, 17). Three different types of K\(^+\) transporters have been involved in this process: Trk, Kdp and Kup. Trk is a multicomponent complex widespread in bacteria and archea. Trk systems have a moderate affinity for K\(^+\), are generally expressed constitutively and are probably energized by taking up K\(^+\) in symport with a proton. They consist of a transmembrane protein named TrkH or TrkG, which is the actual K\(^+\)-translocating subunit, and a cytoplasmic membrane surface protein TrkA, which is a NAD\(^+\) binding protein required for the system’s activity (33). Kdp is an inducible system with high affinity and specificity for K\(^+\), found in *Escherichia coli* and many other bacteria. Kdp is a P-type ATPase expressed only when the needs for K\(^+\) are not satisfied by other transport systems, and thus plays a vital role when the ion is present at concentrations too low to be efficiently taken up by the other K\(^+\)-transport systems, and at
higher K⁺ concentrations when other K⁺-transport systems are abolished by mutation (8). Kdp is the only bacterial K⁺-uptake system whose expression is strongly regulated at the transcriptional level, which is mediated by the KdpD sensor kinase and the KdpE response regulator (reviewed in 8). Finally, Kup is a constitutive K⁺-uptake system of modest affinity found in some bacteria. It is a single membrane protein that probably functions by proton symport. Kup activity in E. coli is more important at low pH where its maximum rate exceeds that of Trk (35).

Although it has been described that the mutation of a K⁺-uptake system of Rhizobium tropici causes a decrease in the osmotic tolerance and the symbiotic competence of the strain (25), the role of K⁺ transport systems has not been comprehensively studied in rhizobia. In this work we have addressed a genetic and functional characterization of K⁺ uptake systems in S. meliloti. We obtained clear evidence that the presence of either Trk or Kup1 is required for bacterial growth even in osmotically balanced conditions. Furthermore, K⁺ accumulation after an osmotic upshift is mainly triggered by the Trk system. Nevertheless, Kup1, Kup2 and Kdp are also involved in the osmoadaptation process and the relative importance of the Kdp system increases in media containing low K⁺ concentrations. The relevance of the different K⁺ uptake systems was also studied during establishment of symbiosis with alfalfa.
MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Sinorhizobium meliloti strains were grown at 30°C in TY complex medium (4) or in minimal medium (MM) containing glutamate (1.1 g/l), mannitol (10 g/l), and mineral salts (K$_2$HPO$_4$ 0.3 g/l, KH$_2$PO$_4$ 0.3 g/l, MgSO$_4$$\cdot$7H$_2$O 0.15 g/l, CaCl$_2$$\cdot$2H$_2$O 0.05 g/l, FeCl$_3$ 0.006 g/l and NaCl 0.05 g/l) (29). The K$^+$-free medium for S. meliloti was MM in which K$^+$ phosphates were substituted by Na$_2$HPO$_4$/NaH$_2$PO$_4$ 0.5 mM. When a specific pH was required, the MM was buffered with Tris/MES 5 mM. The K$^+$ content of the MM used was adjusted when required by the addition of KCl from a 3 M stock solution. Escherichia coli strains were propagated on Luria-Bertani (LB) medium (30). When required, antibiotics were added at the following final concentrations: for E. coli, streptomycin (Sm) 50 µg/ml, spectinomycin (Sp) 50 µg/ml, kanamycin (Km) 50 µg/ml, gentamicin (Gm) 20 µg/ml, tetracyclin (Tc) 10 µg/ml and ampicillin (Ap) 200 µg/ml; for S. meliloti, streptomycin (Sm) 200 µg/ml, spectinomycin (Sp) 100 µg/ml, kanamycin (Km) 200 µg/ml, gentamicin (Gm) 15 µg/ml and tetracyclin (Tc) 5 µg/ml. When osmotic stress conditions were imposed, the osmolarity of the medium was increased by the addition of appropriate amounts of NaCl from a 4 M stock solution (made in MM) or sucrose. Growth in the selected medium was followed measuring the absorbance at 600 nm in cultures incubated in a giratory shaker or by comparative analysis on solid medium.

DNA sequence analysis and construction of S. meliloti mutant strains

DNA sequence analysis was performed with the program BLAST from the network service at the NCBI (1). Standard techniques were used for DNA manipulations (30).
To construct plasmids for the disruption of the different genes, we first amplified the corresponding regions from the *S. meliloti* genome by polymerase chain reaction using custom-synthesized DNA primers (Table 2). After cloning the amplicons in appropriate vectors, we deleted part of the gene sequence by endonuclease digestion, and inserted antibiotic resistance cassettes to obtain each mutant construct *in vitro*. The constructs were then subcloned into the appropriate shuttle vector and the plasmids obtained were transferred by conjugation to the wild-type strain 1021. Transconjugants were selected on medium containing the appropriate antibiotics. In the case of pK18mobsacB, the use of sucrose addition during selection as described by Schäfer et al. (31) was avoided since its osmotic action could affect the obtention of osmosensitive mutants. Double and triple mutants were obtained by transduction with φM12 phage as described by Finan et al. (11). Disruption of the genes of interest were then confirmed by Southern hybridization with specific probes.

**Determination of cell K⁺ content**

Cells were grown in MM to exponential phase (O.D.₆₀₀nm=0.6) and NaCl was added to a final concentration of 0.3 M to induce an osmotic shock. Cells were then incubated under standard growth conditions and aliquots were collected after 5, 30 and 60 minutes. An additional aliquot was collected right before NaCl addition to determine cell K⁺ content just before the osmotic upshift.

For each time point, 10 ml of cultured cells were harvested by centrifugation at room temperature (12000 r.p.m., 2 minutes), the media was removed carefully, and the ionic content of the cells was extracted with 1 volume of HNO₃ 0.1% at room temperature during 20 hours. The cellular debris was then removed by centrifugation and the K⁺ content...
determined in the supernatant obtained using an IRIS Intrepid II XDL inductively coupled plasma-optical emission spectrometer (ICP-OES).

**Plant assays**

Alfalfa (*Medicago sativa* L. cv. Aragon) seeds were sterilized and germinated as described by Olivares et al. (26). Germinated seeds were then transferred to tubes or Leonard jars for growth.

To test the infectivity of the rhizobial strains, 24 individual plants grown in tubes were inoculated with each rhizobial suspension ($10^6$ cfu/plant). After inoculation, the number of nodulated plants and the number of nodules per plant were recorded daily.

To determine the competitive ability, 12 plants grown in tubes were inoculated with a mixture of 1021 (pGUS3) and each strain tested using a ratio of 1:1. The plasmid pGUS3 contains the marker gene coding for $\beta$-glucuronidase (GUS). Nodule occupancy was determined 12 days after inoculation. Roots were collected, briefly washed with water and incubated overnight in the dark at 37ºC in 1 mM X-Gluc (5-bromo-4-chloro-3 indolyl-$\beta$-D-glucuronide; Apollo Scientific) in 50 mM sodium phosphate buffer (pH 7.5) with 1% SDS. Those nodules occupied by 1021 (pGUS3) stain blue, so nodule occupancy could be determined by counting blue and white nodules. All the nodules from the 12 inoculated plants were screened ( aproximately 100 nodules). The results for any given strain were compared to those obtained with the wild type strain 1021 in the same experiment.

To study the symbiotic effectiveness of the strains, 32 individual plants grown in Leonard jars (8 plants per jar) were inoculated with each rhizobial suspension ($10^9$ cfu/plant). 7 weeks after inoculation the plants were collected and the extent of nitrogen fixation was assessed by measurement of the nitrogen content of the aerial part (shoot) (24) and comparison of the shoot dry weight.
RESULTS

In silico analysis of putative K⁺ uptake systems genes in Sinorhizobium meliloti

In silico analysis of the S. meliloti 1021 genome revealed the presence of genes coding for proteins homologous to the components of 3 K⁺-uptake systems: Trk, Kdp and Kup (13). Genes homologous to trkA and trkH are present in the chromosome of S. meliloti 1021 (SMc01046 and SMc00936, respectively), together with a gene located in the pSymA and described as trkH-like (SMa1691). All these genes code for proteins with high sequence identity (30-40%) to the corresponding components of the Escherichia coli Trk system. The presence of a Trk system seems to be an exception among rhizobia since trk-like genes could only be detected in S. meliloti, S. medicae and Mesorhizobium sp. BCN1 genomes and appear to be missing in Rhizobium etli CFN42, R. leguminosarum bv. viciae 3841, M. loti MAFF303099, Bradyrhizobium sp. BTAi1, Bradyrhizobium sp. ORS278 and B. japonicum USDA110 genomes. The situation is very different regarding putative Kdp systems since kdpABC genes are annotated in all sequenced rhizobial genomes forming putative operons with at least two other genes homologous to those coding for the two-component system KdpDE in E. coli. The Kdp systems are conserved among rhizobia and show significant identities with other known Kdp P-type ATPases. In S. meliloti, the products of the kdpABC genes (SMa2333, SMa2331 and SMa2329) show 51%, 63% and 47% sequence identity with the E. coli KdpA, KdpB and KdpC subunits, respectively. Furthermore, the products of S. meliloti genes SMa2327 and SMa2325, which form a putative operon with kdpABC, display 39% and 48% identity with the E. coli KdpD and KdpE subunits of the two-component regulator controlling kdp expression, respectively. On the other hand, we could not identify in the S. meliloti 1021 genome a gene homologous to kdpF, encoding the fourth component of the Kdp system in E. coli. Finally, there is a
surprisingly high number of annotated \textit{kup}-like genes in rhizobial genomes. Several species harbour three or even four genes coding for probable Kup systems. There are two annotated \textit{kup} genes in \textit{M. loti} MAFF303099 (18), three in \textit{R. etli} CFN42 (16), \textit{R. leguminosarum} bv. viciae 3841 (37), \textit{B. japonicum} USDA110 (19) and \textit{Bradyrhizobium} sp. ORS278 (15) and up to four in \textit{Bradyrhizobium} sp. BTAi1 (15). \textit{S. meliloti} 1021 harbours two putative \textit{kup} genes: \textit{kup1} (SMc00873) and \textit{kup2} (SMa1798), whose products show 42% and 41% identity, respectively, with the Kup protein from \textit{E. coli}.

\textbf{Construction of \textit{S. meliloti} mutants in K\textsuperscript{+} uptake systems}

To study the importance of each of the four possible K\textsuperscript{+} uptake systems identified in \textit{S. meliloti}, we constructed several 1021-derivative mutant strains: \textit{10tAG}, \textit{10KdpSS}, \textit{10K1K} and \textit{10K2SS} harbouring interrupted versions of the genes \textit{trkA}, \textit{kdpA}, \textit{kup1} and \textit{kup2}, respectively (Fig. 1). We also obtained a double mutant (\textit{10K2K1}) lacking both Kup transporters, three double mutants (\textit{10tAK1}, \textit{10tAK2} and \textit{10tAKdp}) lacking TrkA and either Kup1, Kup2 or Kdp, respectively, and two triple mutants (\textit{10tAK21} and \textit{10tAKK}) lacking TrkA, Kup1 and either Kup2 or Kdp, respectively. The growth of these 10 different mutants was tested in TY complex medium and in defined minimal medium (MM). In these media, all mutants grew like the wild type, except strains \textit{10tAK1} (Trk\textsuperscript{−}, Kup1\textsuperscript{−}), \textit{10tAK21} (Trk\textsuperscript{−}, Kup2\textsuperscript{−}, Kup1\textsuperscript{−}) and \textit{10tAKK} (Trk\textsuperscript{−}, Kdp\textsuperscript{−}, Kup1\textsuperscript{−}), in which both the Trk and Kup1 systems were missing (Fig. 2A and B). The ability of the \textit{10tAK1} (Trk\textsuperscript{−}, Kup1\textsuperscript{−}) mutant to grow in TY or MM could be restored by complementation of the mutant with either the \textit{kup1} region (pJB3K1) or the \textit{trkA} region (pJB3tA) (data not shown).

It has been described that growth rates of \textit{E. coli} defective in K\textsuperscript{+}-uptake systems depend on the K\textsuperscript{+} content of the media. In fact, some of these mutants were isolated taking advantage
of their inability to grow at low K\(^+\) concentrations. An *E. coli* triple mutant lacking Trk, Kdp and Kup systems achieves a half-maximal growth rate at a K\(^+\) concentration of 20 mM (27). Therefore, we decided to explore the possibility that the growth defects of *S. meliloti* mutants lacking Trk and Kup1 could be relieved by externally added KCl. The growth ability of these mutants was determined on solid MM amended with different concentrations of KCl (2 mM, 5 mM, 10 mM and 20 mM) and we observed that 10 mM KCl was able to restore the growth of these mutants to a wild type extent (Fig. 2). These results suggest that Trk and Kup1 are the main transport systems involved in K\(^+\) homeostasis during growth in osmotically balanced media.

**Role of K\(^+\)-uptake systems in *S. meliloti* osmoadaptation**

To study the involvement of the different transport systems in osmoadaptation, we first determined the growth ability of the wild type strain in MM supplemented with NaCl (0.2 M, 0.3 M, 0.4 M, 0.5 M and 0.6 M) or sucrose (0.3 M, 0.5 M, 0.7 M and 0.9 M) (data not shown). We defined a concentration range of 0.3 M to 0.5 M NaCl and 0.5 M to 0.7 M sucrose to be used for the determination of the osmoadaptive ability of the mutants. The growth curves of all mutants but 10tAK1 (Trk\(^-\), Kup1\(^-\)), 10tAKK (Trk\(^-\), Kdp\(^-\), Kup1\(^-\)) and 10tAK21 (Trk\(^-\), Kup2\(^-\), Kup1\(^-\)), were determined in liquid MM containing different concentrations of NaCl (0.3 M, 0.4 M or 0.5 M) or sucrose (0.5 M or 0.7 M). Only results obtained with the highest concentrations of osmolytes are presented in Figure 3. Among the single mutants only the one lacking Trk displayed a delayed growth both in the presence of NaCl or sucrose. The effect of NaCl addition was stronger than that of sucrose addition. The delay in the mutant growth was clear in the three NaCl concentrations used, however, sucrose addition only produced a slight delay at the highest concentration tested. This behavior points out the importance of the Trk system for osmoadaptation in *S. meliloti*. 
However, the growth defects were stronger in trk-kup2 and trk-kdp double mutants, suggesting an involvement of both Kup2 and Kdp systems in the osmoadaptation process which is revealed only in the absence of Trk (Fig. 3).

Influence of pH in S. meliloti K⁺-uptake during osmoadaptation

Among the conditions that determine the importance of different K⁺ uptake systems during osmoadaptation in bacteria, the pH and the K⁺ content of the media are the most relevant ones (8). The Kup system has been reported to be the main system involved in osmoadaptation in E. coli at low pH, when Trk importance for K⁺ uptake is clearly reduced (35).

To establish the possible influence of pH on the relative importance of the different K⁺ transport systems of S. meliloti during osmotic adaptation, we used MM plates buffered at pH 6, pH 6.5 and pH 7 and supplemented with different concentrations of NaCl (0.3 M, 0.4 M or 0.5 M) or sucrose (0.5 M, 0.6 M or 0.7 M). The parental strain 1021 was unable to grow on media containing the highest concentrations of NaCl or sucrose at pH 6. Similar results were obtained in the remaining conditions, (illustrative examples are presented in Fig. 4 and Fig. 5). The only single mutant affected by the addition of solutes was 10tAG (Trk⁻). Although NaCl addition caused a stronger effect than sucrose addition, we could observe the same trend in both cases and regardless the pH or the osmolyte concentration.

The ability of the 10tAG (Trk⁻) mutant to grow in NaCl added media could be restored to the wild type levels by complementation with the trkA region (pJB3tA) (data not shown). The growth ability of the 10tAK1 (Trk⁻, Kup1⁻) mutant was strongly reduced by the addition of osmolytes to the media (Fig. 4 and Fig. 5). This reduction could be relieved by complementation of the mutant with either the kupl region (pJB3K1), which restored its growth ability to the levels of the single mutant 10tAG (Trk⁻), or the trkA region (pJB3tA),
which restored its growth ability to the levels of the single mutant 10K1K (Kup1⁺) (data not shown). The double mutant 10tAKdp (Trk⁻, Kdp⁻) also displayed a reduction in its growth ability with respect to 10tAG (Trk⁻) but only when challenged with high external NaCl (Fig. 4).

In contrast to the reported function of the Kup system at low pH in E. coli (35), we could not appreciate differences in the growth of the mutants 10K1K (Kup1⁻), 10K2SS (Kup2⁻) or 10K2K1 (Kup2⁻, Kup1⁻) compared to the parental strain 1021 in any of the conditions tested.

**Influence of the external K⁺ in the osmotolerance of S. meliloti K⁺-uptake mutants**

The deficient growth displayed by mutants lacking Trk and Kup1 systems in osmotically balanced media (Fig. 2), hampered the study of the possible involvement of the Kup1 system in osmoadaptation in the absence of Trk. This deficiency was more severe under abiotic stresses such as low pH or high osmolyte concentrations (Fig. 4 and Fig. 5). Nevertheless, the actual involvement of Kup1 in the osmotolerance of strains lacking Trk could not be assessed since the growth of these strains was not comparable to that of the wild type in non stressing media. As previously described, the addition of KCl to the media releaved the growth deficiencies caused by the lack of Trk and Kup1 (Fig. 2). Therefore, we tested the osmoadaptation ability of these mutants in the presence of 10 and 20 mM KCl. The experiments were performed on solid MM added with different concentrations of NaCl (0.3 M or 0.4 M). The results for the different conditions tested were highly consistent, so we present only an illustrative example in Figure 6. Even in these conditions a reduction in the growth ability of 10tAG (Trk⁻) was observed upon NaCl addition, confirming a role of the Trk system in the osmoadaptation process. With these assays, we could as well demonstrate a role of Kup1 during osmoadaptation in the absence of a
functional Trk system. Furthermore, strain 10tAK21 (Trk’\textsuperscript{−}, Kup2’\textsuperscript{−}, Kup1’\textsuperscript{−}) presented even more difficulties to grow in the presence of NaCl than 10tAK1 (Trk’\textsuperscript{−}, Kup1’\textsuperscript{−}), thus revealing an involvement of the Kup2 system during adaptation to hyperosmotic conditions, but only when Trk and Kup1 are absent. On the other hand, we did not notice any effect of the Kdp mutation on the growth of the 10tAKK (Trk’\textsuperscript{−}, Kdp’\textsuperscript{−}, Kup1’\textsuperscript{−}) mutant during osmoadaptation in these conditions. This result was not unexpected since the Kdp system is known to be inhibited by high K\textsuperscript{+} concentrations (8). In fact, Kdp is believed to act in the K\textsuperscript{+} uptake process when the concentration of this cation is too low to be effectively accumulated by other systems. To test the functionality of the Kdp system of \textit{S. meliloti} in response to the addition of NaCl or sucrose at low K\textsuperscript{+} concentrations we used K\textsuperscript{+}-free MM (described in the Material and Methods section) (Fig. 7). The only single mutant affected by NaCl addition was 10tAG (Trk’\textsuperscript{−}), although the defect was not as severe as in K\textsuperscript{+}-containing MM. On the other hand, the addition of NaCl to the K\textsuperscript{+}-free medium almost abolished 10tAKdp (Trk’\textsuperscript{−}, Kdp’\textsuperscript{−}) growth (Fig. 7). Even sucrose addition in these conditions resulted in a decreased growth of 10tAKdp (Trk’\textsuperscript{−}, Kdp’\textsuperscript{−}), not observed in normal K\textsuperscript{+}-containing MM (Fig. 5). These results corroborate the higher importance of the Kdp system for osmoadaptation of \textit{S. meliloti} at low K\textsuperscript{+} concentrations. Nevertheless, even in such conditions, Trk is still the main system involved in the osmoadaptation process and the importance of Kdp can only be assessed in the absence of Trk.

\textbf{Potassium accumulation}

To test whether the growth deficiencies in hyperosmotic conditions observed in some K\textsuperscript{+}-uptake mutants were related with defects in K\textsuperscript{+} accumulation, the levels of this cation present in the different mutants were determined after an osmotic upshift caused by addition of 0.3 M NaCl. In these assays we used those mutants able to grow in liquid MM
at a rate similar to the parental strain. Therefore we could not test the mutants in which both Trk and Kup1 were absent. Among the strains tested, only those lacking the Trk system displayed accumulation kinetics different from the parental strain. We observed that the K\(^+\) accumulation process was significantly delayed in these mutants and seemed even slightly slower in 10tAKdp (Trk\(^-\), Kdp\(^-\)) than in 10tAG (Trk\(^-\)) (Fig. 8). These results correlate well with the observed implication of these systems during adaptation to hyperosmotic conditions suggesting that the osmoadaptation deficiencies are indeed due to the reduced rate of K\(^+\) accumulation in these mutants.

**Importance of K\(^+\) transport systems for symbiosis**

All K\(^+\)-uptake mutants obtained were able to induce nitrogen-fixing root nodules on alfalfa plants and most of them presented nodulation kinetics comparable to that of the parental strain 1021 (data not shown). Nevertheless, the mutants lacking both Trk and Kup1 systems (10tAK1, 10tAK21 and 10tAKK) exhibited delayed nodulation (Fig. 9A). This phenotype was not due to loss of cell viability, since the number of cfu determined in the hydroponic solution was comparable for all strains (data not shown). Nevertheless, 10tAK1 (Trk\(^-\), Kup1\(^-\)) induced the development of a significantly lower number of nodules per plant than the parental strain (as determined by ANOVA tests) until 11 days after inoculation. In the case of the triple mutants, the number of nodules per plant did not reach that of the parental strain until 22 days after inoculation.

The lack of different K\(^+\) uptake systems had little effect on the competitiveness of most strains. However, a significant reduction in nodule occupancy was associated to the lack of Trk and Kup1 systems and, to a lesser extent, to the lack of Trk and Kup2 systems (Fig. 9B). These results strengthened again the importance of Trk and Kup1 in *S. meliloti*. We also studied the symbiotic effectiveness of the single mutants 10tAG (Trk\(^-\)) and 10K1K
Nevertheless, there were not significant differences in the nitrogen content nor the shoot weights of the plants inoculated with the mutants or the wild type 1021 (data not shown).
DISCUSSION

In this work we present the construction and functional characterization of *Sinorhizobium meliloti* 1021 mutants lacking each of the four possible K⁺-uptake systems identified in the genome sequence of this bacterium, as well as double and triple mutants in which several K⁺ transport systems were simultaneously inactivated. The lack of Trk and Kup1 caused a decrease in the viability of the cells during growth in osmotically balanced conditions (Fig. 2). Such phenotype could be reverted by the addition of KCl to the culture media, suggesting that the observed deficiencies were due to a reduction in the availability of K⁺ to these strains. Therefore, Trk and Kup1 would be the main systems involved in K⁺ homeostasis during *S. meliloti* growth. A similar phenotype has been described in *Escherichia coli* mutants in which all three Trk, Kup, and Kdp systems were inactivated by mutation (8).

We could determine the active implication of all four K⁺ transport systems of *S. meliloti* in osmoadaptation studying the growth of the mutants in hyperosmotic media. In contrast to the situation in other bacteria (35), the Trk system seems to be the most important K⁺ importer involved in the osmoadaptation of *S. meliloti* regardless of the pH, the osmolyte added or the K⁺ content of the medium. Only in the absence of a functional Trk system could we establish the implication of the other K⁺ uptake systems in osmotolerance. The involvement of Kup1 in the adaptation to hyperosmotic conditions was only revealed after assaying the behavior of the mutants lacking both Trk and Kup1 at high external K⁺ concentrations (Fig. 6). Furthermore, these experiments evidenced a role of the Kup2 system in osmoadaptation, although its implication in this process could only be assessed in the absence of both Trk and Kup1. It is well known that the presence of a K⁺-uptake system can mask a phenotype associated to the absence of other K⁺ transporter. For example, in *E.
coli the Trk system could only be identified after inactivating the Kdp system (28), while in
Bacillus subtilis the transcription of each of the two Ktr K⁺-uptake systems is elevated
when the other is abolished by mutation (17).

Our results indicate that the Kdp system is inactive at high K⁺ concentrations in S. meliloti
and its importance in osmoadaptation increases as the K⁺ content of the medium diminishes
(Fig. 7), which agrees with observations with all Kdp systems studied so far. Nevertheless,
we could detect an involvement of the Kdp system in osmoadaptation even in K⁺-
containing MM, albeit only in the absence of a functional Trk system and only in response
to NaCl addition and not to sucrose addition (Fig. 4 and Fig. 5). It has been previously
reported that the Kdp response to an osmotic upshift depends on the nature of the osmolyte
utilized in other bacteria. For example, the induction of the kdp operon in Salmonella is
much more sensitive to ionic solutes than to nonpolar ones (2). This could explain the
stronger effect of NaCl compared to sucrose on the osmosensitivity of strains lacking a Kdp
system in our experiments. Nevertheless, the differential effect caused by NaCl or sucrose
on the osmoadaptive ability of the mutants lacking Trk (considered a constitutively
expressed system in other bacteria; 8) suggests that a similar regulation might control as
well the transport activity of these systems.

Although all the 1021-derivatives obtained were able to nodulate alfalfa plants, the mutants
unable to grow at rates comparable to that of the parental strain in standard media presented
delayed nodulation kinetics (Fig. 9). This delay was not due to lost of viability and this fact,
together with the observation that the absence of Trk and Kup1 caused a clear reduction of
the competitiveness of the strain (Fig. 9), suggests that the growth defects of these mutants
would affect their ability to colonize roots during symbiosis establishment. On the other
hand, the study of the effectiveness of the symbiosis established by 10tAG (Trk⁻) and
10K1K (Kup1−) with alfalfa plants did not reveal significant differences with the wild type strain 1021. This observation differs from the report by Nogales et al. (25) who observed that a mutation in a kup gene in R. tropici caused a reduction in the symbiotic effectiveness of the strain together with a deficiency in adaptation to hyperosmotic conditions. These results point out the differences in the involvement of K⁺ transport systems in the symbiosis establishment by different rhizobial species. Furthermore, the genomic sequences of rhizobia reveal clear divergences with respect to the number and type of K⁺ uptake systems harboured by these bacteria. It would be necessary to widen our understanding of K⁺ transporters functionality in other rhizobia to draw conclusions on the importance of these systems for the symbiotic interaction.
ACKNOWLEDGMENTS

This work was supported by grants AP2000-3118, I3P-postgrado-2006 to ADF, and BOS2002-04182-C02-01 and BIO2005-08089-CO2-01 to JS.
REFERENCES


Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1021</td>
<td>SU47 str-21 (wild type), Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>10K1K</td>
<td>1021 (Δ<em>kup1::Km</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10K2SS</td>
<td>1021 (Δ<em>kup2::Sm/Sp</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAG</td>
<td>1021 (Δ<em>tkrA::Gm</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10KdpSS</td>
<td>1021 (Δ<em>kdpA::Sm/Sp</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10K2K1</td>
<td>10K2SS (Δ<em>kup1::Km</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAK1</td>
<td>10tAG (Δ<em>kup1::Km</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAK2</td>
<td>10tAG (Δ<em>kup2::Sm/Sp</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAKdp</td>
<td>10tAG (Δ<em>kdpA::Sm/Sp</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAK21</td>
<td>10tAK2 (Δ<em>kup1::Km</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>10tAKK</td>
<td>10tAKdp (Δ<em>kup1::Km</em>), Sm&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>supE44</em>, ΔlacU169, f80, lacZΔM, recA1, endA1, gyrA96, thi1, relA1, 5<em>hsdR171</em></td>
<td>Bethesda Research Lab®</td>
</tr>
<tr>
<td>S17.1</td>
<td><em>thi</em>, <em>pro</em>, <em>recA</em>, <em>hsdR</em>, <em>hsdM</em>, Rp4Tc::Mu, Km::Tn7; Tp&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript II KS</td>
<td>Cloning vector; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Vector/Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>pGEM-T easy</td>
<td>PCR cloning vector; Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pH45Ω</td>
<td>pBR322 derivative with a streptomycin / spectinomycin resistance cassette; Ap&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>pH45Ω-Km</td>
<td>pBR322 derivative with a kanamycin resistance cassette; Ap&lt;sup&gt;f&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>pMS255</td>
<td>pUC8 derivative with a gentamycin resistance cassette; Ap&lt;sup&gt;f&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>Suicide plasmid; Mob&lt;sup&gt;r&lt;/sup&gt;, sacB; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>pSUP202Pol4</td>
<td>Suicide plasmid; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>pGUS3</td>
<td>pmfeD::gusA translational fusion in pBl101 (Clontech); Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>14</td>
</tr>
<tr>
<td>pJB3Tc19</td>
<td>IncP cloning vector; Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>pJB3K1</td>
<td>pJB3Tc19 derivative containing a PCR amplified fragment including the kup1 gene from <em>S. meliloti</em> 1021; Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pJB3tA</td>
<td>pJB3Tc19 derivative containing a PCR amplified fragment including the trkA gene from <em>S. meliloti</em> 1021; Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

1<sup>a</sup> Sm<sup>r</sup>, streptomycin resistance; Km<sup>r</sup>, kanamycin resistance; Sp<sup>r</sup>, spectinomycin resistance;
2<sup>a</sup> Gm<sup>r</sup>, gentamicin resistance; Tp<sup>r</sup>, trimethoprim resistance; Ap<sup>f</sup>, ampicillin resistance; and
3<sup>a</sup> Tc<sup>r</sup>, tetracyclin resistance.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward/reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trkA (SMc01046)</td>
<td>TAATCTAGACGATCATATCGACC/\textit{XbaI}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGATCCATACATACATGACC/\textit{XbaI}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGATCATCATCATGACC/\textit{XbaI}</td>
</tr>
<tr>
<td>kdpA (SMa2333)</td>
<td>TAAGAATTGCATACCTGACTGACGAGAG/\textit{EcoRI}</td>
</tr>
<tr>
<td></td>
<td>TAAGAATTGCATACATCGAGAAGAGAG/\textit{EcoRI}</td>
</tr>
<tr>
<td></td>
<td>TAAGAATTGCATACATCGAGAAGAGAG/\textit{EcoRI}</td>
</tr>
<tr>
<td>kup1 (SMc00873)</td>
<td>TAAGAGCTCGCTACGACATCTCTCCAGTCAT/\textit{SacI}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGATCCATACATACATGACC/\textit{XbaI}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGATCCATACATACATGACC/\textit{XbaI}</td>
</tr>
<tr>
<td>kup2 (SMa1798)</td>
<td>TAATCTAGACGCTGTGACGACGATCTAGCTACG/\textit{EcoRV}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGCTGTGACGACGATCTAGCTACG/\textit{EcoRV}</td>
</tr>
<tr>
<td></td>
<td>TAATCTAGACGCTGTGACGACGATCTAGCTACG/\textit{EcoRV}</td>
</tr>
</tbody>
</table>

Underlined sequences denote the action sites of the endonucleases used for cloning the mutated gene versions in the shuttle vector.
**FIGURE LEGENDS**

**Figure 1.** Construction of *S. meliloti* 1021-derivative mutants affected in the putative K⁺ uptake systems Trk (A), Kup1 (B), Kup2 (C) and Kdp (D). The wild type version of the amplified region obtained by PCR is represented and the mutated version is shown next. E, *EcoRI*; Sp, *SphI*; S, *SmaI*; EV, *EcoRV*; K, *KpnI*; B, *BamHI*; X, *XbaI*; H, *HindIII*

Gm<sup>R</sup>, gentamicin resistance cassette; Km<sup>R</sup>, kanamycin resistance cassette; Sm<sup>R</sup>/Spc<sup>R</sup>, streptomycin / spectinomycin resistance cassette.

**Figure 2.** Ability of *S. meliloti* 1021-derivative mutants to grow in TY (A), MM (B), MM supplemented with 5 mM KCl (C) and MM supplemented with 10 mM KCl (D). In each line, drops contained approximately the number of cfu indicated on the left. A representative example of at least 2 experiments is shown. Pictures were taken after 2-4 days of incubation in the indicated conditions.

**Figure 3.** Growth curves of *S. meliloti* 1021-derivative strains in MM (A), MM supplemented with 0.5 M NaCl (B) or MM supplemented with 0.7 M sucrose (C). Only the growth curves of mutants significantly different from the wild type are shown. 1021 (wild type; black squares), 10tAG (Trk<sup>−</sup>; black triangles), 10tAK2 (Trk<sup>−</sup>, Kup2<sup>−</sup>; white diamonds) and 10tAKdp (Trk<sup>−</sup>, Kdp<sup>−</sup>; asterisks). Data are representative from at least two replicate experiments. OD<sub>600</sub>, optical density at 600 nm.

**Figure 4.** Ability of *S. meliloti* 1021-derivative mutants to grow in MM supplemented with 0.4 M NaCl at pH 7 (A), pH 6.5 (B) or pH 6 (C). In each line, drops contained approximately the number of cfu indicated on the left. A representative example of at least
2 experiments is shown. Pictures were taken after 4-6 days of incubation in the indicated conditions.

**Figure 5.** Ability of *S. meliloti* 1021-derivative mutants to grow in MM supplemented with 0.6 M sucrose at pH 7 (A), pH 6.5 (B) or pH 6 (C). In each line, drops contained approximately the number of cfu indicated on the left. A representative example of at least 2 experiments is shown. Pictures were taken after 4-6 days of incubation in the indicated conditions.

**Figure 6.** Ability of *S. meliloti* 1021-derivative mutants to grow in MM supplemented with 10 mM KCl (A), 10 mM KCl and 0.3 M NaCl (B), 20 mM KCl (C) or 20 mM KCl and 0.3 M NaCl (D). In each line, drops contained approximately the number of cfu indicated on the left. A representative example of at least 2 experiments is shown. Pictures were taken after 4-6 days of incubation in the indicated conditions.

**Figure 7.** Ability of *S. meliloti* 1021-derivative mutants to grow in K⁺-free MM at pH 7 (MM-K) supplemented with 0.4 M NaCl (A) or 0.6 M sucrose (B). In each line, drops contained approximately the number of cfu indicated on the left. A representative example of at least 2 experiments is shown. Pictures were taken after 4 days of incubation in the indicated conditions.

**Figure 8.** K⁺ accumulation kinetics of *S. meliloti* 1021-derivative strains after the addition of 0.3 M NaCl to mid-exponential cultures (D.O₆₀₀nm = 0.6) growing on MM. 1021 (wild type; black squares), 10tAG (Trk⁻; black triangles), 10tAK2 (Trk⁻, Kup²⁻; white diamonds)
and 10tAKdp (Trk^−, Kdp^−; asteriscs). Only the kinetics of mutants able to grow in MM at a rate similar to that of the parental strain and showing differences in K^+ accumulation with that of the wild type are shown. Time 0 corresponds to the point of application of the osmotic upshift. A representative example of 2 experiments is shown.

**Figure 9.** Symbiotic phenotype of *S. meliloti* mutants in K^+ uptake systems

A) Nodulation kinetics of alfalfa plants inoculated with strains 1021 (wild type; black squares), 10tAK1 (Trk^−, Kup1^−; black triangles), 10tAKK (Trk^−, Kdp^−, Kup1^−; black dots) and 10tAK21 (Trk^−, Kup2^−, Kup1^−; asteriscs). The bars indicate standard errors.

B) Competition assays. Data represent the percentage of white nodules occupied by the wild type (1021), 10tAG (Trk^−, tAG), 10tAK1 (Trk^−, Kup1^−; tAK1), 10tAK2 (Trk^−, Kup2^−; tAK2) and 10tAKdp (Trk^−, Kdp^−; tAKdp), after inoculation with 1:1 mixtures of each strain and 1021 (pGUS3). A representative example of 2 experiments is shown. Bars correspond to standard error and different letters indicate significative differences according to an ANOVA test.
A) MM pH 7

B) MM pH 6.5

C) MM pH 6
A) MM pH 7

B) MM pH 6.5

C) MM pH 6
A) MM + 10 mM KCl

B) MM + 10 mM KCl + 0.3 M NaCl

C) MM + 20 mM KCl

D) MM + 20 mM KCl + 0.3 M NaCl