RpoE2 of *Sinorhizobium meliloti* is necessary for trehalose synthesis and growth in hyperosmotic media

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Adaptation to osmotic stress can be achieved by the accumulation of compatible solutes that aid in turgor maintenance and macromolecule stabilization. The genetic regulation of solute accumulation is poorly understood, and has been described well at the molecular level only in enterobacteria. In this study, we show the importance of the alternative sigma factor RpoE2 in *Sinorhizobium meliloti* osmoadaptation. Construction and characterization of an *S. meliloti* *rpoE2* mutant revealed compromised growth in hyperosmotic media. This defect was due to the lack of trehalose, a minor carbohydrate osmolyte normally produced in the initial stages of growth and in stationary phase. We demonstrate here that all three trehalose synthesis pathways are RpoE2 dependent, but only the OtsA pathway is important for osmoinducible trehalose synthesis. Furthermore, we confirm that the absence of RpoE2-dependent induction of *otsA* is the cause of the osmotic phenotype of the *rpoE2* mutant. In conclusion, we have highlighted that, despite its low level, trehalose is a crucial compatible solute in *S. meliloti*, and the OtsA pathway induced by RpoE2 is needed for its accumulation under hyperosmotic conditions.

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INTRODUCTION

Living cells adapt to an increase of external osmolarity by accumulating organic osmolytes in the cytoplasm. These compounds are accumulated at high intracellular concentration, and they allow turgor recovery. Indeed, their presence at high intracellular concentration must not be deleterious for metabolism. So, only members of a limited number of families of molecules are used as organic osmolytes by living cells (Yancey *et al.*, 1982). These osmolytes are members of the polyols, amino acids (and their derivatives) and quaternary ammonium amines (da Costa *et al.*, 1998). These compounds are synthesized by osmotically stressed cells, and hereafter they are referred to as endogenous osmolytes. Bacteria synthesize one or various endogenous osmolytes (Roessler & Müller, 2001). If present in the surrounding medium, osmolytes can also be accumulated by active transport (Sleator & Hill, 2002), and these are called osmoprotectants.

In addition to their contribution to turgor recovery, organic osmolytes have other roles in the cell (Yancey,

2005). They are, in some instances, called chemical chaperones, since they have a similar action to genuine chaperones, both *in vitro* and *in vivo* (Bourot *et al.*, 2000; Crowe, 2007; Diamant *et al.*, 2001). Osmolytes are also active cytoprotectants (Singer & Lindquist, 1998; Yancey, 2005). When various osmolytes are present within the cell, it is difficult to understand their specific roles; some might be involved in turgor maintenance only, while others might have a minor effect on turgor but a great effect on macromolecular protection (Crowe, 2007; Yancey, 2005).

Adaptation of *Sinorhizobium meliloti* to an increase in medium osmolarity is achieved by the accumulation of three organic osmolytes: glutamate and the dipeptide *N*-acetylglutaminyglutamine amide (NAGGN) are the main solutes present during the exponential growth phase (Smith & Smith, 1989), and trehalose has been described as a minor osmolyte present during the exponential growth phase, but its concentration increases at the end of exponential growth (Gouffi *et al.*, 1999). Osmoprotectants glycine betaine (Talibart *et al.*, 1997), homobetaine (HB) (Barra *et al.*, 2006) and dimethylsulfoniopropionate (Pichereau *et al.*, 1998) are accumulated within the cell to act as substitutes for endogenous osmolytes. In contrast with other bacteria, some osmoprotectants, such as ectoine (Talibart *et al.*, 1994), and disaccharides, such as sucrose (Gouffi *et al.*, 1999), exert a strong osmoprotective effect, while they are never accumulated. In *S. meliloti*, these non-accumulated compounds

Abbreviations: ABTS, azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HB, homobetaine; LB, Luria–Bertani; NAGGN, *N*-acetylglutaminyglutamine amide.

A supplementary figure showing the localization and nucleotide sequence of DNA fragments used for the promoter assays is available with the online version of this paper.

allow a strong increase of glutamate and NAGGN synthesis, while they do not affect the trehalose level.

In bacteria, three major pathways of trehalose synthesis have been described (De Smet *et al.*, 2000; Wolf *et al.*, 2003). Trehalose-6-phosphate synthase (OtsA) allows the conversion of UDP-glucose and glucose-6-phosphate into trehalose-6-phosphate, which is dephosphorylated in a second step by trehalose-6-phosphate phosphatase (OtsB). Trehalose can also be synthesized from malto-oligosaccharides by a mechanism involving two enzymes: malto-oligosyl trehalose synthase (TreY), which converts $\alpha(1\rightarrow4)$ bonds of reducing extremities of maltodextrins into $\alpha(1\rightarrow1)$ bonds, and malto-oligosyl trehalose trehalohydrolase (TreZ), which cleaves the resulting terminal trehalose moiety. Finally, trehalose synthase (TreS) catalyses the conversion of maltose into trehalose, and is also able to function in the opposite direction according to stoichiometric conditions (Cardoso *et al.*, 2007). The regulation of trehalose synthesis is RpoS-dependent in *Escherichia coli* (Hengge-Aronis *et al.*, 1991) and *Salmonella typhimurium* (Fang *et al.*, 1996), but the regulatory mechanisms controlling its synthesis have not been identified in other bacteria.

In *S. meliloti*, enzymic activities corresponding to TreYZ pathways have been described in strains 102F34, 1005 and 1011 (Streeter & Bhagwat, 1999). OtsAB activity has been described in GR4 strain (Streeter & Gomez, 2006), but the corresponding genes and their regulation have not been characterized. The RpoS factor is absent in *S. meliloti* (Galibert *et al.*, 2001), suggesting that other factors control trehalose synthesis in this bacterium. This regulation could be performed by RpoE factors. *rpoE2*, one of the 11 predicted *rpoE* genes (Galibert *et al.*, 2001), was proposed as a general stress-response regulator when 44 RpoE2-dependent genes were identified by a transcriptomic analysis (Sauviac *et al.*, 2007). Moreover, *rpoE2* is induced by osmotic stress (Sauviac *et al.*, 2007), and this suggests that it could be involved in osmotic adaptation.

In this study, we observed a growth defect for an *rpoE2* strain under salt stress, and showed that this defect resulted from the involvement of *rpoE2* in the synthesis of the compatible solute trehalose.

METHODS

Bacterial strains and media. *S. meliloti* and *E. coli* strains are listed in Table 1; these were grown in Luria–Bertani (LB) medium (Miller, 1972). *S. meliloti* was also grown in GAS medium, which consists of S medium (Gouffi *et al.*, 1999) supplemented with 10 mM galactose and 10 mM aspartate, as carbon and nitrogen sources, respectively. Cells grown in LB were harvested, washed in minimal medium (S medium), and inoculated at an OD₅₇₀ of 0.1. Osmoprotectants were added at a final concentration of 1 mM. *S. meliloti* and *E. coli* strains were grown aerobically at 30 and 37 °C, respectively. When appropriate, antibiotics were added: neomycin (50 µg ml⁻¹), gentamicin (5 µg ml⁻¹) and tetracycline (10 µg ml⁻¹) for *E. coli*, and neomycin (50 µg ml⁻¹), gentamicin (25 µg ml⁻¹) and tetracycline (5 µg ml⁻¹) for *S. meliloti*.

DNA manipulations. Chromosomal and plasmid DNA isolations were carried out according to standard procedures (Sambrook *et al.*, 1989). All cloning steps were performed in *E. coli* DH5 α .

Inactivation of trehalose biosynthesis genes. A DNA fragment bearing *otsA* or *treY* was amplified by PCR from Rm1021 genomic DNA using the oligonucleotides described in Table 2, and cloned into the pGEM-T Easy vector (Promega). An antibiotic resistance cassette (Tc^R and Gm^R for *otsA* and *treY*, respectively) was introduced in a unique restriction site (*EcoRI* and *SmaI* for *otsA* and *treY*, respectively) of the target gene, and the mutated gene was transferred into pK18mobsacB. The recombinant suicide plasmid was introduced into the Rm1021 recipient strain by triparental mating, using strain MT616 for its mobilization. *S. meliloti* recombinants were selected on LB medium containing 0.3 M sucrose, and either tetracycline or gentamicin. Colonies were screened for neomycin sensitivity.

For *treS* inactivation, an internal fragment of the ORF was amplified by PCR, cloned into the pGEM-T Easy vector, and introduced in pK18mob. A Gm^R cassette was introduced into the unique *XhoI* site located in the *treS* ORF. The resulting recombinant suicide plasmid was transferred into Rm1021 recipient strain by triparental mating. *S. meliloti* recombinants were selected on LB medium containing neomycin.

For all three genes, insertion was confirmed by PCR. Mutations were periodically transduced to the wild-type background by using Φ M12 (Finan *et al.*, 1984).

Transcriptional fusions with *uidA*. Promoter regions of *otsA*, *treY* and *treS* genes were amplified by PCR (primers are described in Table 2; the sequence of the amplified region, and its characteristics, are described in Supplementary Fig. S1, available with the online version of this paper). The amplified fragments were introduced in the *uidA* promoterless vector pD6796. This plasmid was constructed by insertion of a 2 kb *PstI*–*KpnI* fragment of pTH1522 (Cowie *et al.*, 2006), bearing the promoterless *uidA* gene, between the *NsiI* and *KpnI* sites of pBBRI-MCS2 (a low-copy-number plasmid that is able to replicate in *S. meliloti*). Recombinant plasmids were transferred to *S. meliloti* wild-type and the *rpoE2* mutant by triparental mating.

Enzyme assays. *S. meliloti* cells were grown in GAS medium, and GAS medium supplemented with 0.5 M NaCl (0.5 M NaCl GAS). Cells were collected by centrifugation (5000 g, 10 min). Protein concentration was determined by the Bradford method, and β -glucuronidase activity was measured as described previously (Bardonnnet & Blanco, 1992). The results are the means of at least three independent experiments.

Extraction of solutes, and NMR spectroscopy. Major intracellular compounds were extracted and identified by ¹³C NMR, as described previously (Pichereau *et al.*, 1998).

Assays for compatible solutes. Glutamate and NAGGN were quantified as described previously (Gouffi *et al.*, 1998). For the trehalose assay, cells were grown in GAS medium, collected by centrifugation, and extracted with 80% ethanol. Extracts were dried under vacuum, resuspended in 0.3 ml 135 mM citrate buffer, pH 5.7, and 0.1 ml of the extract was blended with 0.4 ml citrate buffer containing 10 mU trehalase from porcine kidney (Sigma). The mixture was incubated at 37 °C for 2 h, and neutralized by the addition of 0.5 ml 500 mM Tris buffer, pH 7.5. A 0.3 ml volume of the trehalase reaction mixture was added to 0.3 ml 1 mM azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 50 mM sodium phosphate buffer (pH 7.2) and 0.3 ml of glucose oxidase/ peroxidase reagent (Sigma), and the mixture was incubated at 37 °C for 20 min. HCl, at a final concentration of 40 mM, was added, and the absorbance of oxidized ABTS was measured at 410 nm. The

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype	Origin
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argG)U169 deoR</i>	Hanahan (1983)
MT616	MM294 pRK600 Cm ^R	Finan <i>et al.</i> (1985)
<i>S. meliloti</i>		
Rm1021	SU47, Sm ^R	Meade <i>et al.</i> (1982)
R641	Rm 1021, <i>rpoE2::Gm^R</i>	Flechard <i>et al.</i> (2009)
R635	Rm 1021, <i>rpoE2⁺ rpoE2::uidA</i>	Flechard <i>et al.</i> (2009)
R826	Rm 1021, <i>otsA::Tc^R</i>	This study
R779	Rm 1021, <i>treY::Gm^R</i>	This study
R950	Rm 1021, <i>treS::Gm^R</i>	This study
Plasmids		
pGEM-T Easy	pUC derivative cloning vector, Ap ^R	Promega
pK18mob	Mobilizable pUC derivative	Schafer <i>et al.</i> (1994)
pBBRI-MCS3	Broad-host-range replicating mobilizable vector, Tc ^R	Kovach <i>et al.</i> (1995)
pBBRI-MCS2	Broad-host-range replicating mobilizable vector, Nm ^R	Kovach <i>et al.</i> (1995)
pK18mobsacB	Mobilizable pUC derivative, <i>sacB</i> Nm ^R	Schafer <i>et al.</i> (1994)
pAB2001	pUC18 derivative bearing a <i>lacZ-Gm^R</i> cassette	Becker <i>et al.</i> (1995)
pHP45 Ω Tet	ori ColEI Ap ^R Ω Tc ^R cassette	Fellay <i>et al.</i> (1987)
p34SGm	ori ColEI Ap ^R Gm ^R cassette	Dennis & Zylstra (1998)
pUIDK ₃	pUC18 derivative bearing a ' <i>uidA</i> -Neo ^R cassette	Bardonnet & Blanco (1992)
pD6796	pBBRI-MCS2, <i>uidA</i> promoterless	This study
pD3532	pK18mobsacB <i>rpoE2::lacZ-Gm^R</i>	This study
pD9186	pK18mob <i>treS</i>	This study
pD7548	pK18mobsacB <i>treY::Gm^R</i>	This study
pD7539	pK18mobsacB <i>otsA::Tc^R</i>	This study
pD7474	pD6796 <i>treS-uidA</i>	This study
pD7463	pD6796 <i>treY-uidA</i>	This study
pD6951	pD6796 <i>otsA-uidA</i>	This study
pE219	pBBRI-MCS2, <i>otsA</i> transcribed from <i>lac</i> promoter	This study

glucose concentration was determined according to a calibration performed with a glucose standard solution (Sigma), which was treated as described above. The amount of glucose present in cellular extracts was determined using the protocol described above, with the omission of the trehalase action. The results are the means of three independent experiments.

Complementation of *rpoE2* mutant with *otsA* of *S. meliloti*.

Using the primers *otsAD* and *otsAR* (Table 2), the *otsA* gene was amplified as a 1.4 kb DNA fragment containing the *otsA* ORF with its ribosome-binding site, but without its promoter. The amplicon was cloned in pGEM-T Easy. The insert was cleaved as an *EcoRI* fragment, and cloned into pBBRI-MCS2 into the unique *EcoRI* restriction site.

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5'→3')	Gene description
MutotsAG	TGGTCTTCGACGGCTCGGA	<i>otsA</i>
MutotsAD	CATGGCTTCGTATTCGGGCAC	
MuttreYG	GACGTGCTTGAAGAGGCCG	<i>treY</i>
MuttreYD	GGTGTCGTGCGTGGAGGTAG	
MuttreSintG	GATGAAAGAGGCGCCGTGG	<i>treS</i>
MuttreSD2	GGCTGCAAAGGTTTCCTCTC	
grOTSA5	CGTCCACGCCCCCGAATC	Promoter region of <i>otsA</i>
grOTSA3	AGGGACGGGCACACGATTGG	
b20574G	GAATTCTCGCTTTAGCTCCCCGTG	Promoter region of <i>treY</i>
b20574D	AAGCTTCATGGCGGTCTTGACAC	
opb20099G	GAATTCTCCTATCCCGTACGTGGC	Promoter region of <i>treS</i>
opb20099D	AAGCTTCATATTTGCCCTCCTCC	
otsAD	CTTGAATGAAAGGAATTCGGC	Promoterless <i>otsA</i> ORF
otsAR	GCATGATGCTGGCAGGCAG	

The orientation allowing *otsA* transcription from the *lac* promoter in the plasmid was selected, and the plasmid was named pE219. This plasmid was transferred into *S. meliloti* by triparental mating, and clones containing the plasmid were selected as Sm^R, Nm^R colonies.

RESULTS

Osmotic behaviour of the *rpoE2* mutant

Wild-type and *rpoE2* strains were inoculated into GAS medium and 0.5 M NaCl GAS medium (Fig. 1). In non-salted GAS medium, *rpoE2* growth was identical to that of the parental strain. In contrast, in 0.5 M NaCl GAS medium, *rpoE2* cells showed a reduced growth rate and growth yield compared with the wild-type strain. This defect was linked to osmolarity, since when 0.5 M NaCl was substituted with 1 M galactose (which develops the same osmotic strength), the same difference in growth rate and yield was observed for *rpoE2* and the wild-type (data not shown).

Influence of osmoprotectants on the growth of the *rpoE2* mutant in medium of high osmotic strength

In 0.5 M NaCl GAS medium, the growth of Rm1021 was improved when an osmoprotectant (sucrose, ectoine or HB) was added. Addition of sucrose or ectoine resulted in a slight improvement in the growth of the *rpoE2* strain in 0.5 M NaCl GAS medium, but the growth rate and growth

yield were lower than those obtained with the parental strain (Fig. 1). In contrast, addition of HB improved the growth of *rpoE2* to levels that were similar to those of the wild-type (Fig. 1). Thus, HB was a powerful osmoprotectant for *rpoE2*, while the efficiency of the non-accumulated osmoprotectants ectoine and sucrose was reduced in *rpoE2*.

Growth phase and osmotic regulation of *rpoE2*

The regulation of *rpoE2* was analysed in the R635 strain carrying a wild-type copy of *rpoE2* and a *rpoE2-uidA* fusion in the chromosome (Flechar *et al.*, 2009). Expression of the fusion was analysed throughout growth in GAS medium. β -Glucuronidase activity was low and constant during the exponential growth phase [the amount of substrate hydrolysed was $5 \pm 1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$], but it increased as soon as cells entered the stationary growth phase, when substrate hydrolysis reached a level of $30 \pm 2 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (Fig. 2); these results were in accordance with previous observations (Flechar *et al.*, 2009; Sauviac *et al.*, 2007). When cells were inoculated into 0.5 M NaCl GAS medium, β -glucuronidase activity increased immediately from $5 \pm 1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ to $10.0 \pm 1.5 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, and it remained stable during exponential growth. The activity increased again at the end of exponential phase to reach $28.7 \pm 2.2 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. Addition of 1 mM sucrose to 0.5 M NaCl GAS medium resulted in improved growth, but it did not modify the β -glucuronidase activity pattern (Fig. 2). In contrast, addition of 1 mM HB to 0.5 M NaCl GAS medium abolished the osmotic induction of *rpoE2* during exponential growth, and drastically reduced its expression during the stationary growth phase (Fig. 2). These results suggest that *rpoE2* is an osmo-responsive gene.

Osmolyte content of *rpoE2* mutant

In *S. meliloti*, osmoprotection by HB results from its accumulation (Barra *et al.*, 2006). In contrast, in the absence of osmoprotectants, or in the presence of non-accumulated osmoprotectants, osmoprotection is achieved by the synthesis and accumulation of glutamate, NAGGN and trehalose (Gouffi *et al.*, 1999; Jebbar *et al.*, 2005). The growth of the *rpoE2* mutant is affected in hyperosmotic medium deprived of osmoprotectants or containing non-accumulated osmoprotectants, suggesting that synthesis of glutamate, NAGGN or trehalose is affected.

Wild-type and *rpoE2* strains were grown in 0.5 M NaCl GAS medium, and the osmolyte content was analysed by ¹³C NMR (Fig. 3). Cells were collected during exponential growth (OD₅₇₀ 0.4 and 0.2 for parental and *rpoE2* strains, respectively) and at the end of exponential growth (OD₅₇₀ 0.9 and 0.4 for wild-type and *rpoE2* strains, respectively). Glutamate and NAGGN were observed in ethanolic extracts of both strains in the two conditions analysed (Fig. 3). The amount of glutamate and NAGGN was

Fig. 1. Influence of osmoprotectants on growth of Rm1021 (wild-type, WT) and the *rpoE2* mutant. The strains were inoculated into GAS medium ($\diamond$), 0.5 M NaCl GAS medium ($\square$), and 0.5 M NaCl GAS medium supplemented with an osmoprotectant (1 mM): sucrose ($\circ$), ectoine ($\bullet$) or homobetaine ($\blacktriangle$). Error bars, SD.

Fig. 2. Influence of medium osmolarity and osmoprotectants on *rpoE2* expression. Growth of R635 (*rpoE2*⁺ *rpoE2*::*uidA*; open symbols) and β -glucuronidase activity [measured as the amount of substrate hydrolysed, $\mu\text{mol min}^{-1}$ (mg protein^{-1}); closed symbols] were analysed in (a) GAS medium (diamonds) and 0.5 M NaCl GAS medium (squares), and (b) 0.5 M NaCl GAS medium containing 1 mM HB (triangles) or 1 mM sucrose (circles). The results are the means ($\pm$ SD) of three independent experiments.

identical in both strains [620 ± 35 and 160 ± 22 nmol (mg protein^{-1}) for glutamate and NAGGN, respectively, in early exponential growth, and 440 ± 28 and 230 ± 32 nmol (mg protein^{-1}) for glutamate and NAGGN, respectively, in late exponential growth]. In Rm1021, trehalose was observed during exponential growth as a minor solute, and its abundance increased at the end of exponential growth (Fig. 3). In contrast, trehalose was not detected in the extracts of *rpoE2* strain either during or at the end of exponential growth (Fig. 3).

The trehalose content was quantified in cells grown in 0.5 M NaCl GAS medium. In the wild-type strain, it increased continuously throughout growth to reach its maximal level after growth for 48 h [140 ± 11 nmol (mg protein^{-1}); this value is similar to the level found in strain 102F34 in the same experimental conditions (Gouffi *et al.*, 1999; Jebbar *et al.*, 2005; Talibart *et al.*, 1994). Trehalose was not detected in the *rpoE2* mutant in the same experimental conditions. In non-salted GAS medium, trehalose was not detected during exponential growth of

Fig. 3. Identification of compatible solutes accumulated in medium of high osmotic strength. Wild-type (WT) and *rpoE2* strains were grown in 0.5 M NaCl GAS medium. Cells were collected during exponential growth (OD_{570} 0.4 and 0.2 for WT and *rpoE2*, respectively) and at the end of exponential growth (OD_{570} 0.9 and 0.4 for WT and *rpoE2*, respectively), and ethanolic extracts of the cells were analysed by ^{13}C NMR. Peaks corresponding to trehalose (t), and glutamate and NAGGN (g+n), are indicated.

Rm1021 and the *rpoE2* mutant, but it reached a level of 0.23 ± 0.03 nmol (mg protein)⁻¹ in the stationary growth phase for Rm1021, while it was not detected in the *rpoE2* strain. Thus, *rpoE2* affects trehalose synthesis during osmotic adaptation, and also during the stationary growth phase.

Trehalose biosynthesis genes are under the control of *rpoE2*

BLAST analysis revealed that Sma0233 and Smb20574 had 78 and 48% identity to OtsA and TreY, respectively, of *Rhizobium leguminosarum* (McIntyre *et al.*, 2007). Smb20099 has 39% identity with TreS from the alpha-proteobacterium *Rhodobacter sphaeroides* sp. *denitrificans*. Sma0233, Smb20574 and Smb20099 are therefore referred to as OtsA, TreY and TreS, respectively, hereafter. These three ORFs are induced by osmotic stress, as described in the transcriptomic study by Domínguez-Ferreras *et al.* (2006). A sequence close to the consensus of the *rpoE2*

promoter (ggAAC-16–17 nt-gcgTTt) proposed by Sauviac *et al.* (2007) is located upstream of the operon they proposed, suggesting a putative *rpoE2*-dependent transcript (Supplementary Fig. S1).

To analyse the regulation and *rpoE2* dependence of *otsA*, *treS* and *treY* of *S. meliloti*, transcriptional fusions with *uidA* were constructed on a low-copy-number plasmid, and introduced into Rm1021 and *rpoE2* strains. β -Glucuronidase activity was assayed during growth in GAS medium (Fig. 4). β -Glucuronidase activity was not observed when cells containing the empty vector were grown in GAS medium or 0.5 M NaCl GAS medium. Expression of *treY-uidA* and *treS-uidA* fusions was strongly induced in the wild-type strain during the stationary growth phase, while activity of the *otsA-uidA* fusion was very low throughout growth. Stationary-growth-phase induction of *treY* and *treS* was abolished in the *rpoE2* mutant (Fig. 4). These results suggest that *rpoE2* is required for *treY* and *treS* induction during the stationary growth phase. When wild-type cells containing

Fig. 4. Influence of *rpoE2* on the growth phase and osmotic dependence of *otsA*, *treS* and *treY* expression. Rm1021 (closed symbols) and *rpoE2* (open symbols) containing pD6951 (*otsA-uidA*) (a, d), pD7463 (*treY-uidA*) (b, e) or pD7474 (*treS-uidA*) (c, f) were grown in GAS medium (a, b, c) or 0.5 M NaCl GAS medium (d, e, f). Growth, represented by OD₅₇₀ (triangles), and β -glucuronidase specific activity [measured as the amount of substrate hydrolysed, $\mu\text{mol min}^{-1}$ (mg protein)⁻¹] (squares) were determined at time intervals. The results are the means ($\pm$ SD) of three independent experiments.

these plasmids were grown in 0.5 M NaCl GAS medium, expression of *otsA*, *treY* and *treS* fusions increased continuously during the exponential growth phase to reach maximal activity at the end of the growth phase (6-, 20- and 40-fold increases in induction, respectively). This induction by NaCl was abolished in the *rpoE2* mutant (Fig. 4), suggesting that *rpoE2* is necessary for osmotic induction of *otsA*, *treY* and *treS* genes.

The osmotic induction of *otsA*, *treY* and *treS* is in agreement with the results obtained in transcriptomic work by Domínguez-Ferreras *et al.* (2006). Our results show that the expression of *treS* and *treY* was under the control of *rpoE2* during the stationary growth phase in non-stressing medium. Moreover, the osmotic induction of *otsA*, *treY* and *treS* during the exponential growth phase was *rpoE2* dependent.

Trehalose content and growth behaviour of *S. meliloti otsA*, *treY* and *treS* mutants

In order to confirm the role of *otsA*, *treS* and *treY* in trehalose synthesis during growth in GAS medium and 0.5 M NaCl GAS medium, growth parameters and trehalose content were analysed in the wild-type, and in *rpoE2*, *otsA*, *treY* and *treS* mutants. Mutations in *rpoE2*, *otsA*, *treY* or *treS* did not affect growth in GAS medium. Trehalose was not detected in any of the mutants or the wild-type during the exponential growth phase. It accumulated in Rm1021 during the stationary growth phase [0.23 ± 0.03 nmol (mg protein) $^{-1}$]; this level was not affected by *otsA* and *treS* mutations. In contrast, trehalose was not detected in *rpoE2* and *treY* strains collected in the stationary growth phase in GAS medium, suggesting that stationary-growth-phase accumulation of trehalose in non-stressing conditions results from TreY activity.

Growth of *rpoE2* and *otsA* strains in 0.5 M NaCl GAS medium was reduced in comparison with the wild-type strain (Fig. 5). In contrast, the *treY* and *treS* mutants grew to levels that were almost identical to the parental strain (Fig. 5). The intracellular content of trehalose increased in the wild-type strain, and *treS* and *treY* strains, throughout growth, to reach a maximal level in the middle of the exponential growth phase that was almost identical for each of the three strains (Fig. 5). The trehalose content was negligible in *rpoE2* and *otsA* mutants during all phases of growth (Fig. 5). The trehalose content and the ability to grow in hyperosmotic media were correlated, suggesting that *S. meliloti* needs to accumulate trehalose for optimal growth in medium containing 0.5 M NaCl.

The *rpoE2* osmotic phenotype is complemented by the *S. meliloti otsA* gene

Osmotic induction of *otsA* was RpoE2 dependent; therefore, to analyse whether the *rpoE2* defect results from the absence of transcription of the *otsA* gene only, the *rpoE2* mutant was complemented by pE219 expressing a

Fig. 5. Growth and trehalose content of *otsA*, *treY* and *treS* mutants in medium of high osmotic strength. Wild-type Rm1021 (●), *rpoE2* (○), *otsA* (■), *treY* (△) and *treS* (□) were inoculated into 0.5 M NaCl GAS medium. (a) Growth (OD₅₇₀), and (b) trehalose content [nmol (mg protein) $^{-1}$] were measured periodically. The results are the means ($\pm$ SD) of three independent experiments.

promoterless *otsA* gene from the *lac* promoter of pBBRI-MCS2. Wild-type and *rpoE2* strains bearing pBBRI-MCS2 behave similarly to the corresponding bacteria without the plasmid. Growth patterns of the wild-type strain containing pE219 did not show a significant difference compared with the strain containing the empty vector (Fig. 6). In contrast, introduction of pE219 into the *rpoE2* strain abolished the growth defect in 0.5 M NaCl GAS medium (Fig. 6). Thus, the expression of *otsA* from the *lac* promoter suppressed the osmotic phenotype of *rpoE2* mutant, demonstrating that this phenotype was caused by a lack of *otsA* transcription.

DISCUSSION

The ability of *S. meliloti* to adapt to hyperosmotic media is linked to the synthesis and accumulation of organic osmolytes. Glutamate and NAGGN are the major solutes present during exponential growth; they have been described as the main factors affecting turgor (Gouffi *et al.*, 1999; Talibart *et al.*, 1997), and their levels are not affected by the *rpoE2* mutation. The glutamate level increases, and reaches a maximum level a few minutes after exposure to high osmolarity; it then decreases as the levels of NAGGN and trehalose increase (Gouffi *et al.*, 1998). NAGGN and trehalose reach their maximal level in the middle of exponential growth, and NAGGN is fivefold more abundant than trehalose. The level of NAGGN then decreases, while that of trehalose remains constant (Gouffi *et al.*, 1998). While trehalose appears as a minor solute during growth (Gouffi *et al.*, 1998; Talibart *et al.*, 1994,

Fig. 6. Complementation of the *rpoE2* mutation with the *S. meliloti* *otsA* gene. Wild-type (WT) and the *rpoE2* mutant containing pBBR1-MCS2 empty vector (open symbols) or pE219 (closed symbols) were grown in 0.5 M NaCl GAS medium. The results are the means ($\pm$ SD) of three independent experiments.

1997), we show in this study that trehalose synthesis is crucial for growth in hyperosmotic media.

Three pathways of trehalose synthesis have been described in *S. meliloti* (Streeter & Bhagwat, 1999; Streeter & Gomez, 2006). We did not characterize all the genes corresponding to these pathways, but we analysed the expression of a specific gene in each pathway (*treS*, *treY* and *otsA*). These three genes are regulated by *rpoE2*. Inactivation of *treS* did not affect trehalose synthesis in GAS medium; as described in *Propionibacterium freudenreichii*, TreS might be involved in trehalose degradation (Cardoso *et al.*, 2007). The *treY* mutation suppressed trehalose synthesis in the low-osmolarity medium during the stationary growth phase. Only the *otsA* mutation abolished osmoregulated trehalose synthesis, and affected growth in hyperosmotic medium. Osmoregulated trehalose synthesis is mediated by TreYZ in *Corynebacterium glutamicum* (Wolf *et al.*, 2003). Nevertheless, in most bacteria possessing the three trehalose pathways, OtsAB is the main pathway for osmoregulated trehalose synthesis, as observed in *Mycobacterium tuberculosis* (Murphy *et al.*, 2005), *Rhodobacter sphaeroides* (Makihara *et al.*, 2005) and *P. freudenreichii* (Cardoso *et al.*, 2007).

Osmotic stress is a component of other phenomena, such as desiccation. The *rpoE2* mutation drastically reduces survival during desiccation (Humann *et al.*, 2009). Trehalose accumulation is involved in desiccation resistance of *Rhizobium leguminosarum*; mutants that do not accumulate trehalose are sensitive to drying (McIntyre *et al.*, 2007). Desiccation tolerance is improved when cells

are collected during the stationary growth phase (Vriezen *et al.*, 2007). This corresponds to maximal induction of *rpoE2* and trehalose accumulation by *S. meliloti*. Our results showed that RpoE2 is required for trehalose synthesis, suggesting that the *rpoE2* defect in desiccation tolerance is due to the control of trehalose biosynthesis by *rpoE2*.

In this study, we have shown that trehalose is essential for optimal growth in hyperosmotic media, despite its low level during the initial stages of growth compared with the two other organic osmolytes produced by *S. meliloti*. This suggests that trehalose participation in osmoadaptation is not only limited to turgor recovery but also involves protective properties of trehalose against macromolecule denaturation (Yancey, 2005). This is consistent with accumulation of trehalose during the stationary growth phase when turgor is not essential. The important role of trehalose during desiccation is also consistent with its protective role on macromolecules (Humann *et al.*, 2009). This cytoprotective role of trehalose and its dependence on RpoE2 might explain, in part, the importance of RpoE2 as a general stress factor (Sauviac *et al.*, 2007), even though specific *rpoE2*-dependent genes have not been identified for each stress.

The regulation of trehalose synthesis has been studied at the molecular level in *E. coli* and *Salmonella typhimurium* only. In these two related bacteria, trehalose is exclusively produced by the OtsBA pathway. The *otsBA* operon is regulated by RpoS, which has been identified as a general stress factor (Hengge-Aronis, 2002). RpoS allows the transcription of the *otsBA* operon in stationary phase and also in osmotically stressed cells during exponential growth (Kaasen *et al.*, 1992). The trehalose level is very low in *E. coli* during the stationary phase in low-osmotic media; trehalose synthase is activated by potassium glutamate, allowing a high trehalose level during osmotic stress (Glaeser *et al.*, 1988). The *rpoS* gene is not present in the *S. meliloti* genome (Galibert *et al.*, 2001), but trehalose synthesis has similar traits in *S. meliloti* and *E. coli*. It is produced at a low level during stationary phase in non-stressing media, and its synthesis is activated by osmotic stress. In both *S. meliloti* and *E. coli*, and in all growth conditions, trehalose synthesis is controlled by an alternative sigma factor (RpoS for enterobacteria and RpoE2 for *S. meliloti*). These sigma factors have a more general role in stress regulation, since they control genes involved in adaptation to other stresses (Flechar *et al.*, 2009; Hengge-Aronis, 2002; Sauviac *et al.*, 2007). In this respect, RpoE2 has a role that is very similar to that of *E. coli* RpoS. In this study, we observed that *S. meliloti* *otsA* is not induced in stationary phase in low-osmotic media, but during osmotic stress only; in contrast, *treY* and *treS* are induced in both conditions. This suggests that transcription of *otsA* is not only RpoE2 dependent but that it is also affected by another regulatory process. In *E. coli*, the transcription of some osmoregulated genes, such as *proP* and *otsA*, requires not only RpoS, but also transcriptional and post-transcriptional regulators (Kunte *et al.*, 1999; McLeod *et al.*, 2000). In *S. meliloti*, RpoE2 participates in osmoregulation;

however, much work is necessary to identify the molecular mechanisms of its action.

The osmotic induction of *treS* and *treY* suggests that these two genes play a role in osmoadaptation that was not revealed under our experimental conditions. A recent study (Domínguez-Ferreras *et al.*, 2009) performed in a different growth medium showed OtsA to be the main factor for trehalose synthesis under salt stress conditions, but it also showed participation of TreS and TreY in trehalose synthesis under stress conditions. The influence of medium composition on trehalose synthesis has been described in *S. meliloti* (Streeter, 1985) and other micro-organisms (Seto *et al.*, 2004). The three pathways use different precursors for trehalose synthesis; the availability of precursors depends on medium composition and affects the pathway used for trehalose biosynthesis (Carpinelli *et al.*, 2006). TreS uses maltose as substrate; the medium used in our study did not contain maltose, hence TreS was not relevant. A similar situation has been described in *C. glutamicum*, where TreS-mediated trehalose synthesis occurred only when maltose was used as the carbon source (Wolf *et al.*, 2003). The TreY pathway is dependent on glycogen availability (Carpinelli *et al.*, 2006). Significant differences in glycogen biosynthesis have been shown as a function of the metabolic state of the cells and the carbon source (Encarnación *et al.*, 2002; Tavernier *et al.*, 1998). Precursors not only affect metabolic flux (Carpinelli *et al.*, 2006) but also are input signals in regulatory circuits (Pinedo *et al.*, 2008). Glycogen metabolism is connected to other major cellular processes by a complex regulatory network (Eydallin *et al.*, 2007). Many global regulators that sense medium status are also involved in *glg* gene regulation in *E. coli* (Montero *et al.*, 2009). Sauviac *et al.* (2007) showed that glycogen biosynthesis genes in *S. meliloti* are induced by starvation, and are RpoE2-dependent. The regulatory proteins mediating signal transduction to *rpoE2* promoters remain to be identified.

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