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.

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*-acetyl-glutaminylglutamine 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 dimethylsulfoxipropionate (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...
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). Trehalase can also be synthesized from maltoligosaccharides by a mechanism involving two enzymes: maltoligosyl trehalose synthase (TreY), which converts \( \alpha(1 \rightarrow 4) \) bonds of reducing extremities of maltodextrins into \( \alpha(1 \rightarrow 1) \) bonds, and maltoligosyl 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 (Streeeter & Bhagwat, 1999). OtsAB activity has been described in GR4 strain (Streeeter & 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 RpoE-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.

*Salinibacterium meliloti* and *E. coli* strains are listed in Table 1; these were grown in Luria–Bertani (LB) medium (Miller, 1972). *Salinibacterium 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 OD<sub>570</sub> of 0.1. Osmoprotectants were added at a final concentration of 1 mM. *Salinibacterium meliloti* and *E. coli* strains were grown aerobically at 30 and 37 °C, respectively. When appropriate, antibiotics were added: neomycin (50 µg ml<sup>-1</sup>), gentamicin (5 µg ml<sup>-1</sup>) and tetracycline (10 µg ml<sup>-1</sup>) for *E. coli*, and neomycin (50 µg ml<sup>-1</sup>), gentamicin (25 µg ml<sup>-1</sup>) and tetracycline (5 µg ml<sup>-1</sup>) for *Salinibacterium 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* DH5α.

#### 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 (T<sup>C</sup> and Gm<sup>R</sup> for *otsA* and *treY*, respectively) was introduced in a unique restriction site (EcoRI and Smal for *otsA* and *treY*, respectively) of the target gene, and the mutated gene was transferred into pK18mob sacB. 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<sup>R</sup> cassette was introduced into the unique Xhol 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 ΦMd12 (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 *Pet–KpnI* fragment of pTH1522 (Cowie et al., 2006), bearing the promoterless *uidA* gene, between the *Nsi*I and *Kpn*I sites of pBRII-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.

*Salinibacterium 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 (Bardonnet & 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 <sup>13</sup>C 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 µM 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
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 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype</th>
<th>Origin</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA–argF)U169 deoR</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>MT616</td>
<td>MM294 pRK600 CmR</td>
<td>Finan et al. (1985)</td>
</tr>
<tr>
<td>S. meliloti Rm1021</td>
<td>SU47, SmR</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>R641</td>
<td>Rm 1021, rpoE2::GmR</td>
<td>Flechard et al. (2009)</td>
</tr>
<tr>
<td>R635</td>
<td>Rm 1021, rpoE2::uidA</td>
<td>Flechard et al. (2009)</td>
</tr>
<tr>
<td>R826</td>
<td>Rm 1021, otsA::TcR</td>
<td>This study</td>
</tr>
<tr>
<td>R779</td>
<td>Rm 1021, treY::GmR</td>
<td>This study</td>
</tr>
<tr>
<td>R950</td>
<td>Rm 1021, treS::GmR</td>
<td>This study</td>
</tr>
</tbody>
</table>

| Plasmids | pUC derivative cloning vector, ApR | Promega |
| pK18mob | Mobilizable pUC derivative | Schafer et al. (1994) |
| pBBRI-MCS3 | Broad-host-range replicating mobilizable vector, TcR | Kovach et al. (1995) |
| pBBRI-MCS2 | Broad-host-range replicating mobilizable vector, NmR | Kovach et al. (1995) |
| pK18mobsacB | Mobilizable pUC derivative, sacB NmR | Schafer et al. (1994) |
| pAB2001 | pUC18 derivative bearing a lacZ–GmR cassette | Becker et al. (1995) |
| pHP45Tet | ori CoEl Ap R tetC cassette | Fellay et al. (1987) |
| p34sGm | ori CoEl Ap R GmR cassette | Dennis & Zylstra (1998) |
| pUIDK3 | pUC18 derivative bearing a uidA–NeoR cassette | Bardonnet & Blanco (1992) |
| pD6796 | pBBRI-MCS2, uidA promoterless | This study |
| pD3532 | pK18mobsacB rpoE2::lacZ–GmR | This study |
| pD9186 | pK18mob treS | This study |
| pD7548 | pK18mobsacB treY::GmR | This study |
| pD7539 | pK18mobsacB otsA::TcR | This study |
| pD7474 | pD6796 treS–uidA | This study |
| pD7463 | pD6796 treY–uidA | This study |
| pD6951 | pD6796 otsA–uidA | This study |
| pE219 | pBBRI-MCS2, otsA transcribed from lac promoter | This study |

Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Gene description</th>
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<tbody>
<tr>
<td>MutotsAG</td>
<td>TGGTCTTGCAGCGGCTCGGA</td>
<td>otsA</td>
</tr>
<tr>
<td>MutotsAD</td>
<td>CATGCTTCGTAATCGGCCAC</td>
<td>treY</td>
</tr>
<tr>
<td>MuttreyG</td>
<td>GACGTCTTTGAAGCCGGC</td>
<td>treS</td>
</tr>
<tr>
<td>MuttreyY</td>
<td>GGTGTCGTGGCAGGGTAG</td>
<td></td>
</tr>
<tr>
<td>MuttreySintG</td>
<td>GATGAAAGAGGGCCGCTGG</td>
<td></td>
</tr>
<tr>
<td>MuttreySD2</td>
<td>GGTCGCAGAAAGGTTCCTCTC</td>
<td></td>
</tr>
<tr>
<td>grOTSA5</td>
<td>CGTCCACGCCCCTCGGATT</td>
<td>Promoter region of otsA</td>
</tr>
<tr>
<td>grOTSA3</td>
<td>AGGACAGGGACACAGTTG</td>
<td></td>
</tr>
<tr>
<td>b20574G</td>
<td>GAATCTCGTCAATATTAGGGTCGGTG</td>
<td>Promoter region of treY</td>
</tr>
<tr>
<td>b20574D</td>
<td>AGGTCTTCGATGGCGTCTTGG</td>
<td></td>
</tr>
<tr>
<td>opb20099G</td>
<td>GAATCTCGTCAATATTAGGGTCGGTG</td>
<td>Promoter region of treS</td>
</tr>
<tr>
<td>opb20099D</td>
<td>AGGTCTTCGATGGCGTCTTGG</td>
<td></td>
</tr>
<tr>
<td>otsAD</td>
<td>CTTGAAATGAAAAAGGATCG</td>
<td>Promoterless otsA ORF</td>
</tr>
<tr>
<td>otsAR</td>
<td>GCATGATGCTGGCAGGA</td>
<td></td>
</tr>
</tbody>
</table>
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 SmR, NmR 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 (Flechard 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 ± 1 μmol min⁻¹ (mg protein)⁻¹], but it increased as soon as cells entered the stationary growth phase, when substrate hydrolysis reached a level of 30 ± 2 μmol min⁻¹ (mg protein)⁻¹ (Fig. 2); these results were in accordance with previous observations (Flechard 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 ± 1 μmol min⁻¹ (mg protein)⁻¹ to 10.0 ± 1.5 μmol min⁻¹ (mg protein)⁻¹, and it remained stable during exponential growth. The activity increased again at the end of exponential phase to reach 28.7 ± 2.2 μmol min⁻¹ (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 osmoreponsive 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...
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

![Graph](image)

**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, μmol 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 (±sd) of three independent experiments.

![Graph](image)

**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$_{670}$ 0.4 and 0.2 for WT and rpoE2, respectively) and at the end of exponential growth (OD$_{670}$ 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 alphaproteobacterium *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-ggcTTt) 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

![Graphs showing β-glucuronidase activity and OD values over time](http://mic.sgmjournals.org)
these plasmids were grown in 0.5 M NaCl GAS medium, expression of \textit{otsA}, \textit{treY} and \textit{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 \textit{rpoE2} mutant (Fig. 4), suggesting that \textit{rpoE2} is necessary for osmotic induction of \textit{otsA}, \textit{treY} and \textit{treS} genes.

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

**Trehalose content and growth behaviour of \textit{S. meliloti} \textit{otsA}, \textit{treY} and \textit{treS} mutants**

In order to confirm the role of \textit{otsA}, \textit{treS} and \textit{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 \textit{rpoE2}, \textit{otsA}, \textit{treY} and \textit{treS} mutants. Mutations in \textit{rpoE2}, \textit{otsA}, \textit{treY} or \textit{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 $[0.23 \pm 0.03 \text{ nmol (mg protein)$^{-1}$}]; this level was not affected by \textit{otsA} and \textit{treS} mutations. In contrast, trehalose was not detected in \textit{rpoE2} and \textit{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 \textit{TreY} activity.

Growth of \textit{rpoE2} and \textit{otsA} strains in 0.5 M NaCl GAS medium was reduced in comparison with the wild-type strain (Fig. 5). In contrast, the \textit{treY} and \textit{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 \textit{treS} and \textit{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 \textit{rpoE2} and \textit{otsA} mutants during all phases of growth (Fig. 5). The trehalose content and the ability to grow in hyperosmotic media were correlated, suggesting that \textit{S. meliloti} needs to accumulate trehalose for optimal growth in medium containing 0.5 M NaCl.

**The \textit{rpoE2} osmotic phenotype is complemented by the \textit{S. meliloti} \textit{otsA} gene**

Osmotic induction of \textit{otsA} was \textit{RpoE2} dependent; therefore, to analyse whether the \textit{rpoE2} defect results from the absence of transcription of the \textit{otsA} gene only, the \textit{rpoE2} mutant was complemented by pE219 expressing a promoterless \textit{otsA} gene from the lac promoter of pBBRI-MCS2. Wild-type and \textit{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 \textit{rpoE2} strain abolished the growth defect in 0.5 M NaCl GAS medium (Fig. 6). Thus, the expression of \textit{otsA} from the lac promoter suppressed the osmotic phenotype of \textit{rpoE2} mutant, demonstrating that this phenotype was caused by a lack of \textit{otsA} transcription.

**DISCUSSION**

The ability of \textit{S. meliloti} to adapt to hyperosmotic media is linked to the synthesis and accumulation of organic osmoles. 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 \textit{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, 1999; Talibart et al., 1994).
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 (Giaever 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 (Flechard et al., 2009; Hengge-Aronis, 2000; 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;

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**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 (±sd) of three independent experiments.
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 ggl 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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