Osmotic Adaptation of *Thermus thermophilus* RQ-1: Lesson from a Mutant Deficient in Synthesis of Trehalose

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Strains of *Thermus thermophilus* accumulate primarily trehalose and smaller amounts of mannosylglycerate in response to salt stress in yeast extract-containing media (O. C. Nunes, C. M. Manaia, M. S. da Costa, and H. Santos, Appl. Environ. Microbiol. 61:2351-2357, 1995). A 2.4-kbp DNA fragment from *T. thermophilus* strain RQ-1 carrying *otsA* (encoding trehalose-phosphate synthase [TPS]), *otsB* (encoding trehalose-phosphate phosphatase [TPP]), and a short sequence of the 5′ end of *treS* (trehalose synthase [TreS]) was cloned from a gene library. The sequences of the three genes (including *treS*) were amplified by PCR and sequenced, revealing that the genes were structurally linked. To understand the role of trehalose during salt stress in *T. thermophilus* RQ-1, we constructed a mutant, designated RQ-1M6, in which TPS (*otsA*) and TPP (*otsB*) genes were disrupted by gene replacement. Mutant RQ-1M6 accumulated trehalose and mannosylglycerate in a medium containing yeast extract and NaCl. However, growth in a defined medium (without yeast extract, known to contain trehalose) containing NaCl led to the accumulation of mannosylglycerate but not trehalose. The deletion of *otsA* and *otsB* reduced the ability to grow in defined salt-containing medium, with the maximum salinity being 5% NaCl for RQ-1 and 3% NaCl for RQ-1M6. The lower salt tolerance observed in the mutant was relieved by the addition of trehalose to the growth media. In contrast to trehalose, the addition of glycine betaine, mannosylglycerate, maltose, and glucose to the growth medium did not allow the mutant to grow at higher salinities. The results presented here provide crucial evidence for the importance of the TPS/TPP pathway for the synthesis and accumulation of trehalose and the decisive contribution of this disaccharide to osmotic adaptation in *T. thermophilus* RQ-1.

Thermophilic organisms, like the vast majority of other microorganisms, accumulate compatible solutes in response to water stress imposed by salt. However, the compatible solutes of thermophilic and hyperthermophilic prokaryotes are generally different from those of their mesophilic counterparts (34), and some compatible solutes, namely, di-*myo*-inositol-phosphate, di-mannosyl-di-*myo*-inositol-phosphate, diglycerol phosphate, and mannosylglyceramide, are confined to organisms that grow at extremely high temperatures. Mannosylglycerate is also a common compatible solute of thermophiles and hyperthermophiles (21, 28, 35). Despite this association with organisms that grow at extremely high temperatures, mannosylglycerate was initially identified in red algae of the order *Ceramiales* (4, 15). Trehalose, a canonical compatible solute of mesophiles, also accumulates in a few thermophilic and hyperthermophilic organisms, where it appears to serve as a compatible solute (18, 21, 35). This nonreducing disaccharide has also been implicated in several stress responses in prokaryotes and eukaryotic microorganisms (10, 32, 36, 37) and also serves as an intermediate in the synthesis of glycolipids, sulfolipids, and lipooligosaccharides in mycobacteria (3).

The most common pathway for the synthesis of trehalose in bacteria involves trehalose-phosphate synthase (TPS), encoded by the gene *otsA*, which converts UDP-glucose and glucose-6-phosphate to trehalose-6-phosphate. This intermediate is subsequently dephosphorylated to yield trehalose via a specific trehalose-6-phosphate phosphatase (TPP), encoded by *otsB* (12). Another pathway converts maltose to trehalose via a trehalose synthase encoded by *treS*. Homologues of *treS* are known to occur in a *Pimelobacter* sp. (38), *Mycobacterium tuberculosis* (8), *Thermus thermophilus* AT-62 and GK24 (16, 39), and *Deinococcus radiodurans* (40). A third pathway, found in several bacteria, such as *Arthrobacter* and *Rhizobium* spp. (22–24), *M. tuberculosis* (8), and the hyperthermophilic archaeon *Sulfolobus acidocaldarius* (25), converts the terminal α(1→4)-linked unit of a glucose polymer to an α(1→1) linkage by maltooligosyltrehalose synthase encoded by *treY*. The terminal disaccharide is then cleaved by a maltooligosyltrehalose trehalohydrolase, encoded by *treZ*, to yield free trehalose. Organisms may possess one, two, or even all three of these pathways (8).

The species of the genus *Thermus* have an optimum growth temperature of 70 to 75°C, and the majority, being exclusively isolated from continental hydrothermal springs venting fresh water, do not grow in media containing more than 1% (wt/vol) NaCl. Strains of *T. thermophilus*, however, are commonly isolated from marine hot springs and are capable of growing in media containing up to about 5% NaCl (7). Trehalose is the major compatible solute of the strains of *T. thermophilus* grown

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in yeast extract-tryptone-NaCl medium although these organisms also accumulate lower levels of mannosylglycerate (28). The accumulation of trehalose, during osmotic stress, by *T. thermophilus* in yeast extract-containing medium is, as in many other organisms, most likely due to its uptake from the medium (18, 27). The role of compatible solutes in the osmotic adjustment of thermophilic and hyperthermophilic organisms remains largely unknown because we lack genetic tools for the manipulation of the organisms and defined media to examine the events leading to the accumulation of compatible solutes under conditions of osmotic stress. However, genetic tools have been developed for *T. thermophilus* strains that can aid our understanding of osmotic adjustment in this organism (11, 19). In this study we identified and sequenced the complete *otsA*, *otsB*, and *treS* genes of *T. thermophilus* RQ-1, deleted the *otsA* and *otsB* genes for the synthesis of trehalose in strain RQ-1, developed a defined medium lacking yeast extract, examined the effect of these deletions on the osmotic adjustment of the mutant, and examined the effect of the addition of organic solutes on the growth and accumulation of compatible solutes during salt stress.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. RQ-1 (DSM 9247) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *T. thermophilus* strains were grown in *Thermus* medium, containing tryptone (1.0 g/liter) and yeast extract (1.0 g/liter) as source of growth factors, carbon, and nitrogen (41). *Escherichia coli* DH5α (2) and *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA2 lac* [F′ *proAB lacIqZ*ΔM15 Tn*10*(Tet′)]) were used as hosts for the cloning vectors. *E. coli* was grown in YT medium containing, per liter, 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl. Ampicillin was added to the medium at a final concentration of 100 μg/ml, and kanamycin was added at a final concentration of 30 μg/ml for selection of plasmids. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-thio-β-D-galactoside (IPTG) were added to the medium at final concentrations of 80 μg/ml and 0.5 mM, respectively. Plasmid pKT1 (19) was kindly provided by José Berenguer (Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain).

Construction of a *T. thermophilus* gene library. The following degenerate oligonucleotides were designed based on conserved regions of several mesophilic trehalose-phosphate synthase genes available from public databases: forward primer 5′-TGG(A/G)TCCA(C/T)GACTA(C/T)CAC-3′ and reverse primer 5′-AC(G/C)A(A/G)GTTCAT(G/C)CCGTC-3′. A 0.720-kbp PCR fragment was amplified from *T. thermophilus* RQ-1 with these primers. This fragment was subsequently used as a probe for the isolation of the complete gene from a genomic library.

Total DNA was isolated from *T. thermophilus* RQ-1 as described by Marmur (20). A partial genomic library from RQ-1, containing DNA fragments selected by Southern blot analysis of genomic DNA with the 0.720-kbp fragment probe, was obtained by complete digestion of total DNA with restriction enzyme *Hin*dIII, followed by ligation of purified fragments of around 2.5 kbp into the *Hin*dIII site of pUC18 (Invitrogen) and subsequent transformation of *E. coli* XL1-Blue with the ligation mixture. Positive clones were detected by colony hybridization with a digoxigenin-labeled probe (Roche Molecular Biochemicals). Nucleotide sequences were determined by AGOWA (Berlin, Germany). Inserts of positive pUC18 clones and pGEM-T Easy (Promega) clones were sequenced in both orientations by using vector- and insert-specific oligodeoxynucleotide primers. Nucleic acid and protein sequence analyses were performed with programs of the Wisconsin Genetics Computer Group software package (9). The European Bioinformatics Institute databases (www.ebi.ac.uk) and functionally annotated genomes in the Kyoto Encyclopedia of genes and genomes (www.genome.ad.jp) were screened for homology by using the (T) FASTA (29, 30) and (T) BLAST (1) algorithms.

DNA amplification and analysis. DNA manipulation techniques used in this study were carried out as described by Sambrook et al. (33). For sequencing, recombinant plasmid DNAs were prepared with plasmid kits (Roche Molecular Biochemicals). PCR amplifications were carried out in a Perkin-Elmer Gene

Amp PCR system 2400 in reaction mixtures (50 μl) containing 100 ng of genomic *T. thermophilus* DNA, 200 ng of each primer, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.5 U of *Taq* or *Pfu* DNA polymerase, and 0.2 mM concentrations of each deoxynucleoside triphosphate (Amersham Biosciences). The mixture was preincubated for 5 min at 94°C and then subjected to 25 cycles of denaturation at 94°C for 1 min. Annealing was performed at 48°C for 1 min, and primer extension was at 72°C for 1 min. The extension reaction in the last cycle was prolonged for 7 min. Amplification products were purified from agarose gels for use as hybridization probes and for cloning into the pGEM-T Easy vector (Promega). Southern blots of restriction endonuclease-cleaved genomic DNA and colony blots were hybridized with DNA probes labeled with a digoxigenin DNA labeling and detection kit (Roche Molecular Biochemicals).

Amplification of *treS* from RQ-1 genomic DNA was performed with a GC-rich PCR system (Roche), with primers based on the AT-62 *treS* gene sequence. Two separate amplifications were necessary to obtain the complete gene. The first PCR product was a 1.482-kbp fragment and was obtained using the forward primer 5′-GTGGACCCCCTCTGGTACAAG-3′ and the reverse primer 5′-GGCCTGGGTGTAGCG-3′. The second PCR product was a 1.683-kbp fragment obtained by amplification with the forward primer 5′-GCCTTCTCCCGCGCC-3′ and the reverse primer 5′-CTAGGCTTTTCCGGCCTTGGCC-3′. Both fragments were cloned into pGEM-T Easy vector and were sequenced in both orientations, as described above.

Construction of *T. thermophilus* otsA/otsB deletion mutants. A 691-bp fragment corresponding to the 5′ region of *otsA* (*otsA′*) was amplified from the chromosomal DNA of strain RQ-1, using the forward primer 5′-GCG<u>GGTACC</u>ATGG GGCTAATCATCG-3′ and the reverse primer 5′-GCG<u>AAGCTT</u>GCGCCCCG TGTCTATCC-3′. Restriction sites (underlined) for *Kpn*I and *Hin*dIII, respectively, were added to the primers to clone this fragment, immediately upstream of the *pslA-kat* cassette, into the pKT1 vector (19), yielding pAKT1. Using a similar strategy, a 492-bp fragment corresponding to the 3′ region of *otsB* (′*otsB*) was amplified. The forward primer used was 5′-GCG<u>GAATTC</u>GGACA AGGGCTTCG-3′ and the reverse primer was 5′-GCG<u>GCGGCCGC</u>CTAAAG GCTAGTGGGTCG-3′. Restriction sites (underlined) for *Eco*RI and *Not*I, respectively, were introduced. The PCR-amplified 3′ region of *otsB* (′*otsB*) was cloned into *Eco*RI/*Not*I restriction sites of the TOPO 2.1 cloning vector (Invitrogen), prior to cloning into pAKT1. ′*otsB* was removed from the TOPO vector by *Eco*RI/*Not*I digestion followed by digestion of vector DNA with *Rsa*I and *Bsp*MI, to eliminate possible contaminations with TOPO cloning vector in the subsequent steps. Then, ′*otsB* was cloned into pAKT1, to yield pAKBT1. Vector pAKBT1 was used to transform *T. thermophilus* RQ-1. The method used for plasmid transformation was essentially that of Koyama et al. (17). For this purpose, the recipient strain was grown at 65°C in a transformation medium which contained, per liter of water, 4 g of tryptone (Difco), 2 g of yeast extract (Difco), 1.5 g NaCl, 1 mM MgCl₂, and 0.5 mM CaCl₂ (pH 7.5). Samples (1 ml) of the culture with a turbidity of 0.3 (610 nm) were transferred to 5-ml sterile tubes, and 20 μg of DNA was added. After 1.5 h of incubation at 65°C under strong aeration, cells were directly plated onto each of three agar *Thermus* agar plates containing 30 μg of kanamycin per ml and incubated at 55, 60, and 65°C.

Development of a defined medium lacking yeast extract and growth of the organisms. A defined medium lacking yeast extract (which contains trehalose) was developed to examine growth parameters in the presence or absence of putative exogenous compatible solutes. The defined medium (TD medium) was composed of tryptone (2 g/liter) and a vitamin solution containing thiamine, riboflavin, pyridoxine, biotin, folic acid, inositol, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, and cyanocobalamin (Sigma-Aldrich) at a final concentration of 40 μg/liter. This medium was supplemented with NaCl to a final concentration of 1 to 6% (wt/vol) to test the effect of salinity on growth and on the accumulation of organic solutes. Growth of the organisms was performed in 1-liter metal-capped Erlenmeyer flasks containing 200 ml of medium in a reciprocal-water bath shaker (120 rpm) at 70°C. For the cultivation of mutants, the culture medium did not contain kanamycin, but their ability to grow in the presence and absence of kanamycin was always tested on solid medium during or after cultivation. The effect of organic solutes on growth and compatible solute accumulation was examined by the addition of filter-sterilized (Gelman type GN-6; pore size, 0.45 μm; diameter, 47 mm) trehalose, maltose, glucose, glycine betaine, and potassium mannosylglycerate to the culture medium at a final concentration of 0.26 mM.

Overexpression of *otsA* and *otsB* genes in *E. coli*. The *otsA* gene was amplified by PCR with the forward primer 5′-GCG<u>GAATTC</u>ATGGGGCTCATCATC G-3′ and the reverse primer 5′-GCG<u>CTGCAG</u>TCATCCCTCCTCCAAGGAG GC-3′. The *otsB* gene was amplified with the forward primer 5′-GCG<u>GAATT C</u>ATGAGGGCGGAAAACCCCG-3′ and the reverse primer 5′-GCG<u>CTGCA G</u>CTAAAGGCTAGTGGGTCG-3′. The forward primers contained an additional

*Eco*RI sequence (underlined), immediately upstream of the ATG codon, and the reverse primers had an additional *Pst*I recognition site (underlined), directly behind the TGA stop codon. The PCR products were purified after digestion with *Eco*RI and *Pst*I and ligated into the corresponding sites into the pKK223-3 expression vector (Amersham Biosciences) to obtain pKKTPS and pKKTPP, respectively. Each construction was transformed into *E. coli* XL1-Blue cells. Host cells containing pKKTPS or pKKTPP were grown to mid-exponential growth phase, induced with IPTG, and grown further for 6 to 8 h.

Preparation of cell extracts. *T. thermophilus* RQ-1, *T. thermophilus* RQ-1M6, and transformed *E. coli* cells were harvested by centrifugation ($7,000 \times g$, 10 min, 4°C), and the cell pellet was resuspended in 20 mM Tris-HCl (pH 7.6) containing (per milliliter of suspension) MgCl$_2$ (25 mM), DNase I (10 μg), and the protease inhibitors phenylmethylsulfonyl fluoride (80 μg), leupeptin (20 μg), and antipain (20 μg). Cells were disrupted with a sonicator (Labsonic U; B. Braun, Melsungen, Germany), followed by centrifugation ($13,000 \times g$, 1 h, 4°C). The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.6) to remove endogenous low-molecular-weight compounds (including trehalose) prior to examination of cell extracts for enzyme activity.

Enzymatic assays. Cell extracts of *E. coli* XL1-Blue expressing *otsA* from pKKTPS or *otsB* from pKKTPP were denatured at 70°C for 20 min and centrifuged ($13,000 \times g$, 2 min) to remove precipitated proteins. The supernatant solutions (10 μl) were incubated with the TPS substrates UDP-glucose (Sigma-Aldrich) and glucose-6-phosphate (Sigma-Aldrich) or the TPP substrate trehalose-6-phosphate (Sigma-Aldrich), at a final concentration of 2 mM, in 20 mM Tris-HCl (pH 7.6) with 25 mM MgCl$_2$. The reaction mixtures were incubated at 65°C for 1 h. The product of the TPS reaction was incubated for an additional hour with 2 U of alkaline phosphatase (Roche) at 37°C to form trehalose. *T. thermophilus* RQ-1 and RQ-1M6 cell extracts were directly incubated with UDP-glucose and glucose-6-phosphate at 65°C for 1 h. To visualize trehalose, samples were spotted onto thin-layer chromatography (TLC) plates.

TreS activity was assessed in crude cell extracts obtained from cultures of RQ-1 and RQ-1M6 grown in *Thermus* medium until late exponential phase of growth (turbidity of 0.6 at 610 nm). The cell extracts obtained by sonication were dialyzed against 25 mM MOPS (morpholinepropanesulfonic acid), pH 7.6. Samples (50 μl) of these extracts were incubated with [^{14}C]maltose or [^{14}C]trehalose at a final concentration of 1.35 μM. Aliquots (10 μl) were removed at different times (0.5, 1.0, 1.5, 2.0, and 10.0 min) and extracted with 5 μl of 12% trichloroacetic acid. After incubation on ice (10 min) and centrifugation (2 min, $13,000 \times g$), the supernatants were applied to TLC plates as described above. The solvent system was composed of butanol-pyridine-water (7:3:1 [vol/vol/vol]). After drying, the TLC plates were autoradiographed for 4 days. [^{14}C]maltose and [^{14}C]glucose were obtained from Amersham Biosciences, and [^{14}C]trehalose was prepared as described previously (13)

Extraction and determination of intracellular solutes. Wild-type (RQ-1) and mutant (RQ-1M6) cells were harvested by centrifugation ($7,000 \times g$, 10 min, 4°C) during mid-exponential-phase growth and washed twice with a NaCl solution identical in concentration to that of the growth medium. Cell pellets were extracted twice with boiling 80% ethanol as described previously (31). The protein content of the cells was determined by the Bradford assay (5), after sonication of cells, from an aliquot of the suspension before extraction of compatible solutes.

TLC. TLC was performed on Silica Gel 60 plates (Merck) with a solvent system composed of butanol-ethanol-water (5:3:2 [vol/vol/vol]). The sugars and sugar derivatives were visualized by spraying with α-naphtholsulfuric acid solution followed by charring at 120°C (14). Authentic standards of trehalose, UDP-glucose, and glucose-6-phosphate were used for comparative purposes.

Quantification of organic solute pools by NMR spectroscopy. Freeze-dried extracts were dissolved in D$_2$O and analyzed by nuclear magnetic resonance (NMR). NMR spectra were acquired in a Bruker AMX300 spectrometer using a 5-mm inverse detection probe head at 25°C, with presaturation of the water signal, a 60° flip angle, and a repetition delay of 60 s. A known amount of sodium formate was added and used as a concentration standard.

Nucleotide sequence accession number. The sequences of *otsA*, *otsB*, and *treS* reported in this paper have been deposited in GenBank under accession number AY275558.

RESULTS

Sequence analysis of trehalose-synthesizing genes. A 2.4-kbp fragment carrying the *T. thermophilus* RQ-1 *otsA* (encoding TPS) and *otsB* (encoding TPP) genes was cloned from a gene library, and its complete sequence was determined. The deduced amino acid sequence of TPS had a calculated molecular mass of 52.64 kDa and had significant similarities with several TPS proteins from other organisms, including 35% identity with *E. coli* TPS (GenPept AAC74966) (Fig. 1). The deduced amino acid sequence of TPP from strain RQ-1, with a calculated molecular mass of 26.91 kDa, also had significant similarities to several TPP proteins. The *otsB* sequence from strain RQ-1 was identical to the plasmid-borne homologue from *T. thermophilus* strain GK24 (AF135796). Immediately downstream of the *otsB* gene we found a sequence for *treS* (trehalose synthase). The product of *treS*, a 965-amino-acid protein, had 98 and 99% amino acid identity to the corresponding proteins from *T. thermophilus* AT-62 and GK24, respectively (16, 39). The activity of the recombinant TPS or TPP of strain RQ-1, overexpressed in *E. coli*, was confirmed by TLC using UDP-glucose and glucose-6-phosphate as substrates for TPS and trehalose-phosphate for TPP (results not shown).

Construction and isolation of *T. thermophilus* RQ-1 *otsA*/*otsB* deletion mutants. *T. thermophilus* mutants, derived from the parental strain RQ-1, were constructed by homologous recombination using a plasmid-encoded deletion between *otsA*′ and ′*otsB* (Fig. 2). Sixty kanamycin-resistant colonies were obtained after transformation at 55°C in *Thermus* medium plates with yeast extract (a source of trehalose) without the addition of NaCl.

All colonies were plated on *Thermus* medium plates with kanamycin and NaCl up to 4% for 48 h. None of the colonies grew on the plates containing 4% NaCl and 12 strains did not grow in medium with 3% NaCl. Three mutants that did not grow in medium with 3% NaCl, designated RQ-1M3, RQ-1M6, and RQ-1M8, were randomly chosen. Southern blotting experiments performed with genomic DNA of these kanamycin-resistant mutants that was digested with *Bam*HI, *Pst*I, and *Acc*I confirmed that the construction was properly inserted into the chromosome. A 2.05-kbp fragment corresponding to *otsA* and *otsB* genes and a 0.78-kbp fragment corresponding to *kat* gene were used as probes to distinguish between the normal wild-type and insertional forms of the genes in the mutants (Fig. 2).

Digestion of the wild-type strain DNA with *Bam*HI followed by Southern blot hybridization with an *otsA*/*otsB* probe produced two bands, while the mutant strains produced three bands that confirm the complete insertion of the *kat* gene. These results show that an extra *Bam*HI restriction site, 273 bp downstream of the original wild-type restriction site, was responsible for the additional band in the mutants (Fig. 2A). These results were confirmed with *Acc*I and *Pst*I. The restriction site present near the amino-terminal end of the *otsB* gene in the wild-type strain, demonstrated by the two-band pattern (Fig. 2A), was not present in the mutants, demonstrating that the required 213 bp of this region of *otsB* gene was removed and replaced by the *pslpA-kat* cassette. *Pst*I sites are not present in *otsA* or *otsB* genes of the wild-type strain; however, the correct insertion of the *pslpA-kat* cassette led to the introduction of a *Pst*I restriction site shown by the two-band pattern in the mutants. These results show that the replacement of 659 bp at the C-terminal end of *otsA* and 213 bp at the N-terminal end of *otsB* by the *pslpA-kat* cassette occurred via double-recombination events. Probing with the *kat* gene indicated its presence in the mutant strains alone (Fig. 2B).

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Tther RQ-1     1   ----------------MG---LIIVANRAPFRLT--PEG---LVPAVGGLATALLPVLER  36
Styph          1   -----------------MSRLVVVSNRIAPPD---------NKGGAGGLAVGVLGALKA  33
Rhisp.         1   ------------------MSRLVIVSNRVPVPDKG-------GIAPAGGLAVALKVALEE  35
Mtube          1   MAPSGGQEAQICDSETFGDSDFVVVANRLPVDLERLPDGSTTWKRSPGGLVTALEPVLRR  60
Rsola          1   ------------------MSRLIVVSNRVAPIAE--------GQASAGGLAVGVYDALRD  34
Ecoli          1   ------------------MSRLVVVSNRIAPPDE--------HAASAGGLAVGILGALKA  34

Tther RQ-1    37   RHG-VWVAAGEWREKGQAATP---R----ESQVRLEQVFLPEKEWQGYYGGFSNRVLWPL  88
Styph         34   AGG-LWFGWSGETG--NEDEP---LKKVTKGNITWASFNLSEQDYEDYYCQFSNAVLWPA  87
Rhisp.        36   QGGGIWMGWSGKSSGEDEPAP---LAQLQQGNITYALTDLTDTDVEEYYHGFANRVLWPI  92
Mtube         61   RRG-AWVGWPGVNDDGAEPDLHVLDGPIIQDELELHPVRLSTTDIAQYYEGFSNATLWPL 119
Rsola         35   TGG-VWFGWSCEIAP-GASGE---PSIAAKGAITFATVGLSRRDYDQYYRGFANATLWPV  89
Ecoli         35   AGG-LWFGWSGETG--NEDQP---LKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPA  88

Tther RQ-1    89   CHYFLER-----------VEL-RRFRE--------GDTVFVQDYHLLLLPRLLFERIP-A 127
Styph         88   FHYRLDLVQFQRPAWEGYMRVNALLADKLLPLIKENDIIWVHDYHLLPFASELRKRGVNN 147
Rhisp.        93   CHYRLDLAEYGRKEMAGYFRVNRFFAHRLAPLVKPDDVIWVHDYPLIPLAAELRQMGLEN 152
Mtube        120   YHDVIVKPLYHREWWDRYVDVNQRFAEAASRAAAHGATVWVQDYQLQLVPKMLRMLRPDL 179
Rsola         90   FHYRVDLARYERQEYHGYRRVNTQFAHQLKALVQPDDILWVHDYHLIPFAAECRALGLRN 149
Ecoli         89   FHYRLDLVQFQRPAWDGYLRVNALLADKLLPLLQDDDIIWIHDYHLLPFAHELRKRGVNN 148

Tther RQ-1   128   KIGFFFHIPWPSSGVFRILPWGRALVEGVLGADLIGFHTPEYVENELR-TAAYYGYQVEE 186
Styph        148   RIGFFLHIPFPTPEIFNALPPHDELLEQLCDFDLLGFQTENDRLAFLDSLSSQTR--VTT 205
Rhisp.       153   RIGFFLHIPWPPADVLFTMPVHEEIMRGLSHYDVVGFQTDHDLENFASCLRREG---IGD 209
Mtube        180   TIGFFLHIPFPPVELFMQMPWRTEIIQGLLGADIVGFHLPGGAQNELILSRRLVGTDTSR 239
Rsola        150   RIGFFLHIPFPSPEILTTIPPHEELMRALCAYDLLGFQTETDRVAFYDYIEREARGYIEN 209
Ecoli        149   RIGFFLHIPFPTPEIFNALPTYDTLLEQLCDYDLLGFQTENDRLAFLDCLSNLTR--VTT 206

Tther RQ-1   187   NRVRV---------GERWVRVEAHPLGIDTGRFGELAQDPHIGLHARALKRLAGAER--- 234
Styph        206   RSGK------QHIAWGKDFQTEVYPIGIEPD---EIALQAAGPLPPKLAQLKAELKN--- 253
Rhisp.       210   ALGGG-----RLSAYGRIFKGGVYAIGIETAAFAEFAKKASTNSTVKKARESIERRS--- 261
Mtube        240   GTVGVRSRFGAAVLGSRTIRVGAFPISVDSGALDHAARDRNIRRRAREIRTELGNPRK-- 297
Rsola        210   KEHNG-----PVHAYGNTLRAEVYPIGVHPD---EIARQAVSSLARRNPFAREADASGGR 261
Ecoli        207   RSAK------SHTAWGKAFRTEVYPIGIEPK---EIAKQAAGPLPPKLAQLKAELKN--- 254

Tther RQ-1   234   ---LILGVDRLDYTKGILERLIAYERLLQSYPQWRGRVAFFQIATPSRTSVHAYRELKRR 291
Styph        253   -VKNIFSVERLDYSKGLPERFLAYEALLENYPQHRGKIRYTQIAPTSRGEVQAYQDIRHQ 312
Rhisp.       261   ---LIIGVDRLDYSKGLTQRIEAFERFILANPAQRGRVTYLQITPKSRSEVPEYEAMQRT 318
Mtube        297   ---ILLGVDRLDYTKGIDVRLKAFSELLAEGRVKRDDTVVVQLATPSRERVESYQTLRND 354
Rsola        262   PLKLIMSVDRLDYSKGLPERFRAFEQLLDDFPDHRRHVTFVQIAPTSRQDVQSYQQIRQR 321
Ecoli        254   -VQNIFSVERLDYSKGLPERFLAYEALLEKYPQHHGKIRYTQIAPTSRGDVQAYQDIRHQ 313

Tther RQ-1   292   VDEVVGRIMCSFLREDWVPLRYFYQTYTQEELAAFYRAADVALIITPLRDGMNLVAMEYAY 351
Styph        313   LETEAGRINCKYGQLGWTPLYYLNQHFDRKLLMKIFRYSDVGLVTPLRDGMNLVAKEEVA 372
Rhisp.       319   VAEQAGRVNCALGAVDWVPMRYINRSVGRRVLAGLYRLGKVGLVTPLRDGKNLVAKEYVA 378
Mtube        355   IERQVGHINCEYGEVGHPVVHYLHRPAPRDELIAFFVASDVMLVTPLRDGMNLVAKEYVA 414
Rsola        322   LEAESGRINCKHSELDWTPIRYINKQYDRRMLMALFRASHIGYVTPLRDGMNLVAKEYVA 381
Ecoli        314   LENEAGRINCKYGQLGWTPLYYLNQHFDRKLLMKIFRYSDVGLVTPLRDGMNLVAKEYVA 373

Tther RQ-1   352   TTED---GVLVLSNIAGAAEYLKEALLVNPYDLDGMAQALDRALRMPGGERRERLSALKA 408
Styph        373   AQDPANPGVLVLSQFAGAANELTSALIVNPYDRDDVAAALNRALTMPLAERISRHAEMLD 432
Rhsp.        379   AQDPDDPGVLVLSRFAGAAREELQGALLVNPYDIEGTANAMARSLSMPLEERQERWTTMMD 438
Mtube        415   CRSDLG-GALVLSEFTGAAAELRHAYLVNPHDLEGVKDGIEEALNQTEEAGRRRMRSLRR 473
Rsola        382   AQDPEAPGVLVLSRFAGAARELDAALIVNPYDTRGMAEALNRALTMPLEERKARHAHMMD 441
Ecoli        374   AQDPANPGVLVLSQFAGAANELTSALIVNPYDRDEVAAALDRALTMSLAERISRHAEMLD 433

Tther RQ-1   409   RIEALDVQGWAERFIASLEEG------------------- 429
Styph        433   VIVKNDINRWQERFIHDLKEVTPRSPERQQQNNVATFPKLA 473
Rhisp.       439   QLLEHDVSRWCRDFLNDLTASSDRCG--------------- 464
Mtube        474   QVLAHDVDRWAQSFLDALAGAHPRGQG-------------- 500
Rsola        442   RLRAADLTAWRERFIADLRGASAR---------------- 465
Ecoli        434   VIVKNDINHWQECFISDLKQIVPRSAESQQRDKVATFPKLA 474
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FIG. 1. Alignment of TPS of *T. thermophilus* RQ-1. The deduced amino acid sequence of *otsA* of RQ-1 was aligned with those of homologous proteins: *Styph*, *Salmonella enterica* serovar Typhimurium (GenPept AF213176); *Rhi*sp., *Rhizobium* sp. (GenPept AAB91813); *Mtube*, *M. tuberculosis* (GenPept CAB08713); *Rsola*, *Ralstonia solanacearum* (GenPept CAD17882); *Ecoli*, *E. coli* (GenPept AAC74966). Black shading represents identity, and gray shading represents conservative changes.
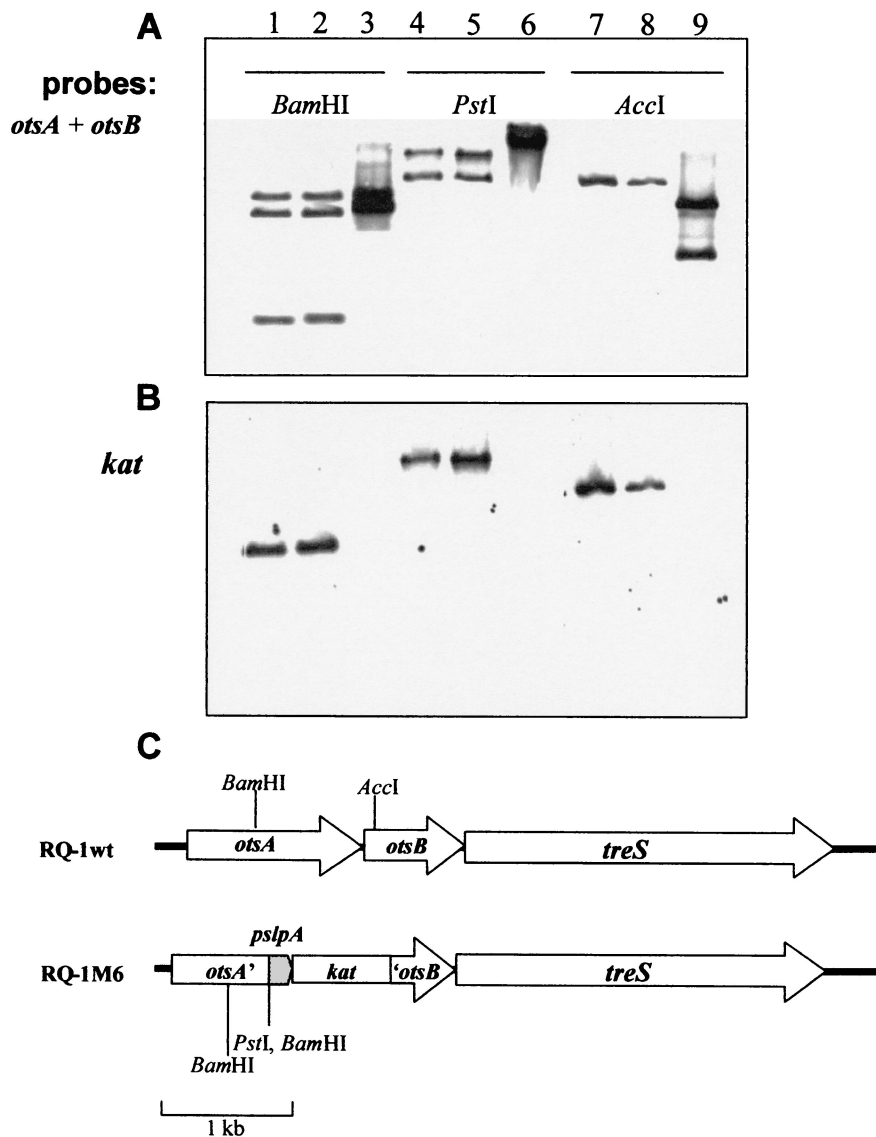
FIG. 2. Verification of the internal deletion in *otsA* and *otsB*. Southern blots of genomic DNA from *T. thermophilus* RQ-1 and two kanamycin-resistant clones (RQ-1M6 and RQ-1M8) are shown. Bands were identified by hybridization with a 2.05-kbp fragment corresponding to *otsA* and *otsB* (A) or *kat* (B). Lanes 1, 4, and 7, RQ-1M6; lanes 2, 5, and 8, RQ-1M8; lanes 3, 6, and 9, RQ-1. (C) Genetic organization of trehalose-synthesizing genes in the chromosomes of the wild type and an RQ-1 deletion mutant.

In contrast to the wild-type RQ-1 strain, the extracts of the partial *otsA* and *otsB* deletion mutants did not, as assessed by TLC, produce trehalose from UDP-glucose and glucose-6-phosphate. The conversion of maltose to trehalose, indicative of TreS activity, could not be detected in crude dialyzed extracts of RQ-1 and RQ-1M6. The reverse reaction could also not be detected (results not shown). Southern blot analysis of the transformants and preliminary salt tolerance tests showed that all three strains (RQ-1M3, RQ-1M6, and RQ-1M8) were identical. RQ-1M6 was chosen for further examination.

Growth of strains RQ-1 and RQ-1M6 in defined medium with different NaCl levels. Growth of RQ-1 and RQ-1M6 was similar in the defined TD medium (without an external source of trehalose) without NaCl or containing 1% (wt/vol) NaCl (Fig. 3A and C). The growth rate of RQ-1M6 decreased con-

siderably in the medium containing 2% NaCl and was insignificant in TD medium containing 3% NaCl. The growth rate of RQ-1M6 was 7.3 times lower at this salinity than that of the wild-type strain, and the cell yield was about half that of the parental strain. RQ-1 grew consistently in TD medium containing 5% NaCl but did not grow in the medium containing 6% NaCl (Fig. 3A).

Effect of exogenous trehalose on growth. The addition of trehalose to the defined medium without NaCl had no effect on the growth rate or the cell yield of the wild type or the mutant. However, the addition of trehalose favored the growth of the wild-type strain and the mutant under salt stress conditions (Fig. 3B and D). Exogenous trehalose caused an increase in the growth rate of the wild-type strain in TD medium containing 5% NaCl from 0.06 to 0.12 h^{-1} and allowed growth in
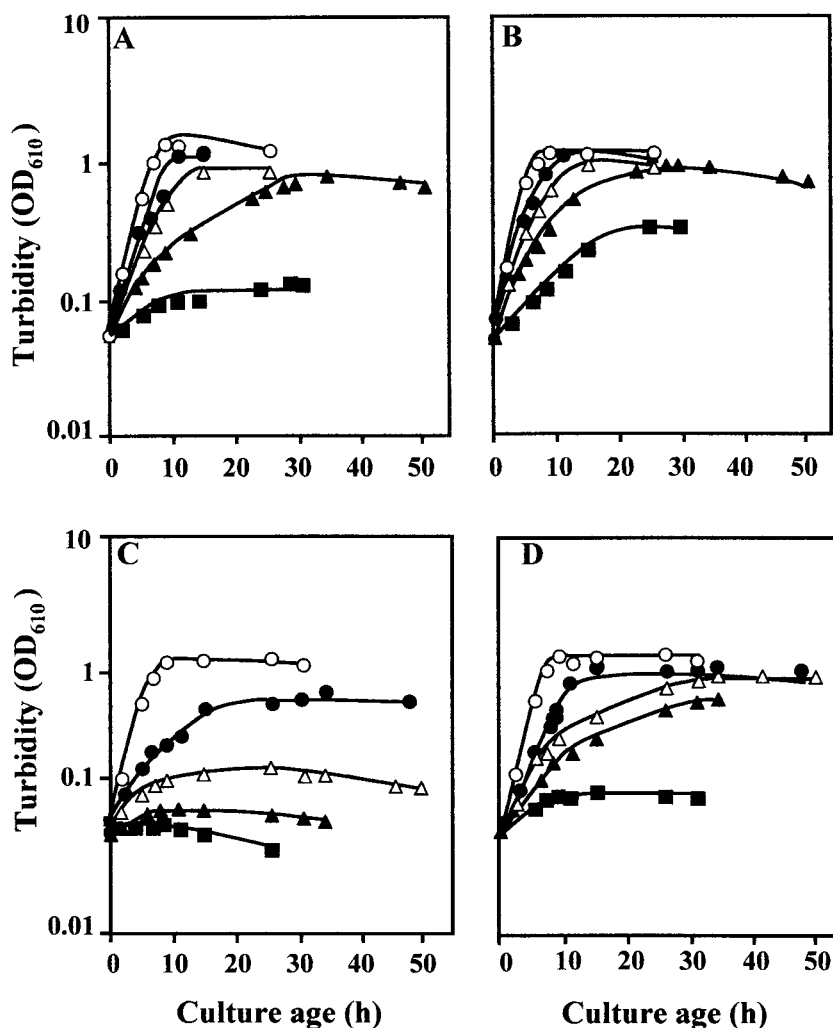
FIG. 3. Growth of wild-type and mutant *T. thermophilus* strains in TD medium with 0% (○), 3% (●), 4% (△), 5% (▲), and 6% (■) NaCl. RQ-1 (A and B) and RQ-1M6 (C and D) were grown in TD medium in the absence (A and C) or presence (B and D) of exogenous trehalose. OD$_{610}$, optical density at 610 nm.

medium with 6% NaCl. We observed no alteration in the final yield after growth in TD medium containing 3 and 4% NaCl and a slight increase of 0.19 and 0.22 optical density unit in medium containing 5 and 6% NaCl, respectively (Fig. 3B).

The effect of exogenous trehalose on the growth of RQ-1M6 was more pronounced. The maximum NaCl concentration for growth of the mutant in TD medium increased from 3 to 5% with the addition of trehalose. The growth rate of the mutant was lower than that of the wild type in trehalose-containing defined medium with 3 and 4% NaCl, but the final yield was similar. The mutant also grew in TD medium containing 5% NaCl, although the growth rate and the final yield were lower than those of the wild type. However, the mutant did not grow, even in the presence of trehalose, in medium containing 6% NaCl (Fig. 3D).

Effect of exogenously provided mannosylglycerate, glycine betaine, maltose, and glucose on growth. The addition of the solutes mannosylglycerate, glycine betaine, maltose, and glucose to medium containing 4, 5, and 6% NaCl had no effect on

the growth of the mutant. We observed, however, that maltose and glucose, like trehalose, increased the growth rate of the wild-type strain, and glycine betaine caused a slight increase in the growth rate, while mannosylglycerate did not (results not shown). Maltose and glucose also increased the cell yield of the wild-type strain, but betaine had only a very slight effect on the cell yield of this strain, while mannosylglycerate had no effect (Fig. 4).

Accumulation of organic osmolytes by RQ-1 and RQ-1M6. Growth of the wild-type strain in defined TD medium containing 3% NaCl led to the accumulation of trehalose and low levels of mannosylglycerate. The addition of trehalose or maltose led to a slight increase in the levels of intracellular trehalose. There was, however, no increase in the intracellular levels of mannosylglycerate; glucose and maltose were never detected (Fig. 5).

Growth of the mutant in TD medium led to the progressive accumulation of mannosylglycerate in medium containing 1 to 3% NaCl, but trehalose was not detected. On the other hand,
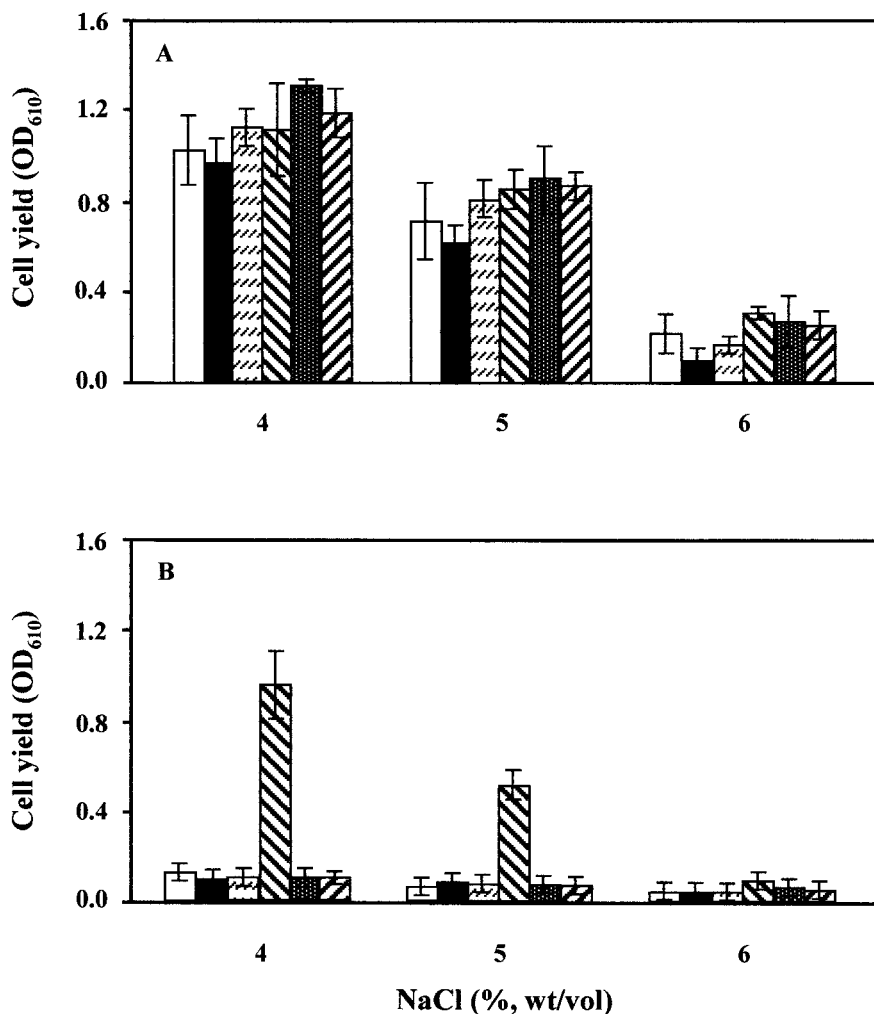
FIG. 4. Effect of the presence of exogenous organic solutes on the cell yield of RQ-1 and RQ-1M6. RQ-1 (A) and mutant RQ-1M6 (B) were grown in TD medium containing 4, 5, and 6% NaCl. Within each group, bars represent (from left to right) TD medium without exogenous solutes and TD medium supplemented with mannosylglycerate, glycine betaine, trehalose, maltose, and glucose. OD_{610}, optical density at 610 nm.

growth of the mutant in TD medium containing 1 to 3% NaCl with exogenous trehalose led to the accumulation of progressively larger amounts of the disaccharide and smaller amounts of mannosylglycerate. At the same salt concentration, the level of mannosylglycerate in these cells was always lower than in TD medium without trehalose (Fig. 6). The addition of maltose or glucose to TD medium containing 3% NaCl did not lead to their intracellular accumulation or the conversion of these sugars into trehalose. Glycine betaine was never detected in cell extracts (Fig. 5).

DISCUSSION

Trehalose is a very common solute of bacteria, yeasts, fungi, and archaea that appears to play several roles related to stress protection (6, 8). The protecting role of trehalose in osmotic adjustment, heat stress, and dehydration has been amply demonstrated, although under some experimental conditions, the

precise role of this sugar is unclear because it may have several roles in the same organism (36, 37).

T. thermophilus strains generally grow in media containing NaCl at concentrations as high as 5 to 6%. In media containing yeast extract, the major compatible solute is trehalose, while mannosylglycerate is found in smaller amounts (28). Under these conditions, trehalose could be synthesized de novo or could be taken up from the growth media, because the disaccharide is present in the yeast extract (42).

Here we show that strain RQ-1 possesses the pathway for the synthesis of trehalose that proceeds via trehalose-6-phosphate. Cloning and functional overexpression of *otsA* and *otsB* from *T. thermophilus* RQ-1 in *E. coli* showed that these genes code for the enzymes responsible for the two-step pathway leading to the synthesis of trehalose. However, we were unable to demonstrate TreS activity in crude extracts of *T. thermophilus* RQ-1 or RQ-1M6. We cannot explain these negative results, although the *treS* gene product of strain RQ-1 shares 98 and 99% identity with its homologue in *T. thermophilus* AT-62
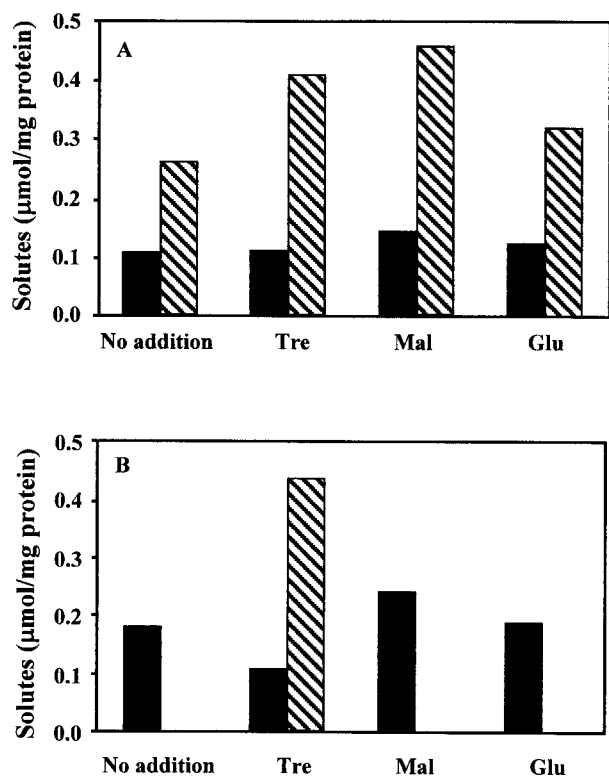
FIG. 5. Accumulation of compatible solutes by RQ-1 and RQ-1M6. RQ-1 (A) and RQ-1M6 (B) were grown in TD medium containing 3% NaCl without the addition of exogenous organic solutes (no addition) or with trehalose (Tre), maltose (Mal), or glucose (Glu). Bars represent intracellular concentrations of mannosylglycerate (■) and trehalose (▨).

or may not be organized in an operon-like arrangement, since obvious promoter sequences could not be recognized. However, these promoter sequences are not easily identified in *T. thermophilus* (26). The presence of a partial *otsB* sequence and a complete *treS* sequence in *T. thermophilus* strain AT-62 (39) as well as a complete *otsB* and *treS* sequence in strain *T. thermophilus* GK-24 (AF135796) had already been reported, but this is the first report of the presence of *otsA* in *T. thermophilus*. It should also be noted that several wild-type *T. thermophilus* strains lack *otsA* while others lack *otsA*, *otsB*, and *treS*. None of the organisms grow in defined medium containing more than 2% NaCl, confirming the results obtained with RQ-1M6. Moreover, none of the strains examined possess *treY* or *treZ* (S. Alarico, Z. Silva, A. Henne, H. Santos, and M. S. da Costa, unpublished results).

The role of trehalose in osmotic adjustment of *T. thermophilus* RQ-1 was demonstrated by deletion of *otsA* and *otsB*, because the mutant lacking these genes was unable to accumulate this compatible solute in a defined medium deprived of the disaccharide or to grow with NaCl above 3%. The accumulation of trehalose was never detected in the mutant when maltose or glucose was supplied to this strain, indicating that maltose could not be converted to trehalose via *treS* or that glucose, derived from the hydrolysis of maltose, could not be converted to trehalose via *otsA* and *otsB*. On the other hand, the accumulation of trehalose by the mutant in trehalose-containing medium showed that it was taken up from the medium. This uptake system has been identified as an ATP binding cassette-type transporter that also takes up maltose (Z. Silva, M. Sampaio, M. S. da Costa, and H. Santos, unpublished results). We are therefore led to conclude that *T. thermophilus* RQ-1 can synthesize trehalose in a medium lacking trehalose or can take up trehalose from a medium containing yeast extract. Compatible solute uptake is well documented and is preferred to de novo synthesis in many organisms (6, 34).

Exogenous glycine betaine did not serve as a compatible solute in *T. thermophilus* RQ-1, despite the earlier observation that trace levels of this solute accumulate in a few other strains of *T. thermophilus* (28). Glycine betaine did not improve growth of mutant RQ-1M6 in media containing salt, nor did it accumulate during salt stress in glycine betaine-containing medium. This observation indicates that one of the most common

and GK-24, respectively, where maltose-converting activity was detected by using recombinant proteins (16, 39). Our inability to detect TreS activity in the crude extracts of the wild type or the mutant may be due to lack of expression of the gene under the conditions examined.

Sequence analysis showed that *otsB* was located immediately downstream of *otsA*, and *treS* is located immediately downstream of *otsB*. The three genes are structurally linked and may
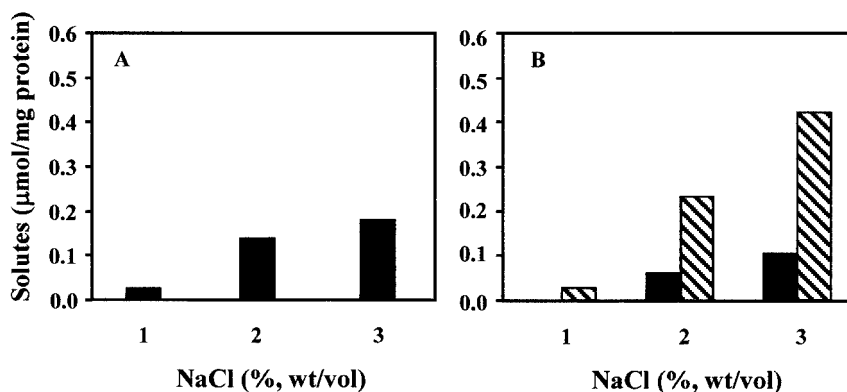

FIG. 6. Effect of salinity on the accumulation of solutes by RQ-1M6. RQ-1M6 was grown in TD medium without (A) or with (B) exogenous trehalose. Bars represent intracellular concentrations of mannosylglycerate (■) and trehalose (▨).

compatible solutes of mesophilic prokaryotes does not serve as a compatible solute in *T. thermophilus* or, for that matter, in any of the (hyper)thermophiles examined thus far. We speculate that glycine betaine does not meet the requirements of thermophiles or hyperthermophiles for osmotic adjustment, perhaps because it is not taken up from the environment due to the lack of the appropriate transporters or is unstable at high growth temperatures (34). The accumulation of glucose and maltose did not, as expected, occur when these sugars were added to the medium, since these sugars are only rarely used as compatible solutes in prokaryotes (6).

The inability of the mutant to grow in TD medium with more than 3% NaCl leads us to view the accumulation of trehalose as evidence that this disaccharide serves as a compatible solute. The mutant accumulates progressively higher levels of mannosylglycerate as the salt concentration increases up to 3%, but osmotic adjustment at higher salt concentrations is not possible unless trehalose is taken up by the organism. Growth is restored in defined trehalose-containing medium with 4 or 5% NaCl, although growth is slower than in the parental strain. Moreover, trehalose accumulates to levels as high as those in the wild-type strain. Under these conditions the levels of intracellular mannosylglycerate in the wild type are lower than in the mutant in defined medium without the addition of trehalose.

Mannosylglycerate serves as the sole or major compatible solute under salt stress in other thermophiles and hyperthermophiles in yeast extract media (34), but *T. thermophilus* RQ-1 appears to use mannosylglycerate only under low-level osmotic adjustment in media containing less than about 3% NaCl. Above these salt levels, trehalose is required for growth and the accumulation of mannosylglycerate becomes less important for osmotic adjustment. The accumulation of mannosylglycerate alone, even during low-level osmotic adjustment, does not fulfill the requirement for trehalose accumulation, since the growth rates and the final yields are lower, at the corresponding salinity, than after uptake of trehalose.

The results presented here show that trehalose plays, under the conditions examined, an important role in osmotic adjustment of *T. thermophilus* RQ-1. The role of trehalose in the osmotic adjustment of this organism was demonstrated by the inability of the mutant to grow in a defined medium without yeast extract containing more than 3% NaCl. This is the first study using genetic deletion mutants showing that an organic solute is a de facto compatible solute in a thermophilic organism.

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