Salt Stress-Induced Changes in the Transcriptome, Compatible Solutes, and Membrane Lipids in the Facultatively Phototrophic Bacterium *Rhodobacter sphaeroides*[▽][†]

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Responses to NaCl stress were investigated in phototrophically grown *Alphaproteobacterium Rhodobacter sphaeroides* by transcriptome profiling, mutational analysis, and measurements of compatible solutes and membrane phospholipids. After exposure to salt stress, genes encoding two putative glycine betaine uptake systems, *proVWX* and *betS*, were highly upregulated. Mutational analysis revealed that BetS, not ProVWX, was the primary transporter of this compatible solute. Upon the addition of salt, exogenous glycine betaine was taken up rapidly, and maximal intracellular levels were reached within minutes. In contrast, synthesis of another important compatible solute in *R. sphaeroides*, trehalose, increased slowly following salt stress, reaching maximal levels only after several hours. This accumulation pattern was consistent with the more gradual increase in salt-induced transcription of the trehalose biosynthesis operon *otsBA*. Several genes encoding putative transcription factors were highly induced by salt stress. Multiple copies of one of these factors, *crpO* (RSP1275), whose product is a member of the cyclic AMP receptor protein/fumarate and nitrate reduction regulator (CRP/FNR) family, improved NaCl tolerance. When *crpO* was provided in multicopy, expression of genes for synthesis or transport of compatible solutes was unaltered, but the membrane phospholipid composition became biased toward that found in salt-stressed cells. Collectively, this study characterized transcriptional responses to salt stress, correlated changes in transcription with compatible solute accumulation rates, identified the main glycine betaine transporter and trehalose synthase, characterized salt-induced changes in phospholipid composition, and uncovered a transcription factor associated with changes in phospholipids. These findings set the stage for deciphering the salt stress-responsive regulatory network in *R. sphaeroides*.

Microorganisms adapt to fluctuations in medium osmolarity via two common strategies. One involves reestablishing osmotic balance by accumulation, through import from the medium or through synthesis, of intracellular osmoprotectants known as compatible solutes. The second common strategy involves altering membrane composition to better cope with the changed turgor pressure. Compatible solutes used by various microorganisms include, among others, the amino acids glutamate and proline, the amino acid derivatives glycine betaine ([GB] *N,N,N*-trimethyl glycine) and proline betaine, and the sugar trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) (6, 14). Alterations to the membrane include both changes in

fatty acid saturation and phospholipid composition (reviewed in reference 34).

We are interested in salt stress responses in the remarkably metabolically versatile facultatively phototrophic *Alphaproteobacterium Rhodobacter sphaeroides* 2.4.1. That this freshwater bacterium actually requires sodium for growth was an interesting characteristic established over 40 years ago (38). *R. sphaeroides* 2.4.1 can adapt to concentrations of NaCl up to approximately 0.4 M, but higher concentrations prevent cell growth (1).

Another freshwater strain closely related to strain 2.4.1, *R. sphaeroides* f. sp. *denitrificans*, synthesizes trehalose as a compatible solute in response to salt stress. The roles of trehalose biosynthesis and degradation in adaptation of this bacterium to salt stress have been described (24). *R. sphaeroides* f. sp. *denitrificans* also accumulates potassium cations to counter increased sodium levels brought about by salt stress. However, the organism apparently does not respond by synthesizing GB or amino acids to an appreciable degree (43). With respect to *R. sphaeroides* 2.4.1, Abee et al. reported that GB, when present in the medium, can be taken up for osmoprotection, whereas exogenous proline is ineffective (1). Also, Catucci et al. reported that the relative cardiolipin content of *R. sphaeroides* 2.4.1 membranes increases in response to osmotic stresses (7).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description[a]	Source or reference
Strains		
R. sphaeroides		
2.4.1	Wild type	W. Sistrom
RWAE	2.4.1 Δ*proXW*::Ω(Sm^r/Sp^r)	This study
RBCCT	2.4.1 *betS*::pRBCCT	This study
E. coli		
JM109 λ*pir*	JM109 lysogenized with λ*pir*	25, 44
S17-1 λ*pir*	Tp^r Sm^r *hsdR pro recA* λ*pir* RP4-2-Tc::Mu-Km::Tn7	37
DH5α	λ$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA hsdR17*(r$_K^-$ m$_K^-$) *supE44 thi-1 gyrA relA1*	18
DH5αphe	DH5α *phe*::Tn*10*d; Cm^r	11
HB101	F$^-$Δ(*gpt-proA*)62 *leuB6 supE44 ara-14 galK2 lacY1*; used in triparental matings	9
XL1-Blue	[F′::Tn*10* (Tc^r) *proAB lacI^q*Δ(*lacZ*)M15] *recA1 endA1 gyrA96 thi-1 supE44 relA1 lac*	Agilent Technol., Inc.
Plasmids		
pJP5603	Km^r; R6K-based suicide vector	29
pRWAE001	948-bp PCR product containing RSP3058 (*proW*) upstream sequences in pJP5603	This study
pRWAE002	872-bp PCR product containing RSP3059 (*proX*) downstream sequences in pRWAE001	This study
pRWAE003	Ω(Sm^r/Sp^r) inserted into the SmaI site of pRWAE002[b]	This study
pHP45	Source of Ω(Sm^r/Sp^r) cassette	33
pRBCCT	675-bp PCR product containing RSP3080 (*betS*) sequences in pJP5603[b]	This study
pBSIISK$^+$	ColE1; Ap^r	Agilent Technol., Inc.
pUI1087	pBSIISK$^+$ with modified polylinker; Ap^r	46
pCrpO	PCR-amplified *crpO* DNA inserted into MscI-EcoRV-restricted pUI1087; Ap^{rc}	This study
pBBR1MCS-2	Broad-host-range plasmid, Mob$^+$ Kn^r	21
pBBR-CrpO	HindIII-XbaI fragment from pCrpO inserted into pBBR1MCS-2[c]	This study
pCrpO′	Truncated *crpO′* following oligonucleotide-directed mutagenesis, in pUI1087[c]	This study
pBBR-CrpO′	HindIII-StuI fragment from pCrpO′ inserted into HindIII-EclI136II-restricted pBBR1MCS-2[c]	This study
pRK2013	IncP1 ColE1 Tra$^+$ of RK2 Kn^r; used as a helper plasmid in triparental matings	9, 12

[a] Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Ap, ampicillin; Tc, tetracycline.
[b] See Fig. S1 in the supplemental material.
[c] See Fig. S2 in the supplemental material.

The aims of the present study were (i) to obtain a more comprehensive view of salt stress responses in *R. sphaeroides* 2.4.1, (ii) to investigate the dynamics of engagement of known salt stress systems following exposure to NaCl, and (iii) to identify and begin characterizing potential regulators of NaCl stress. For transcriptome profiling, the *R. sphaeroides* 2.4.1 whole-genome GeneChip described by Pappas et al. (28) was used. The experimental setup involved subjecting phototrophically grown cells to a significant increase in NaCl concentration, i.e., from 85 mM Na$^+$ in the growth medium to a final concentration of 265 mM Na$^+$ (described in the text as an addition of 1.5% NaCl). The response was examined at two time points. The first time point, 7 min after the NaCl addition, was chosen to capture the earliest transcriptional responses, whereas the later time point, 45 min, was chosen to identify longer-term adaptation mechanisms. This range of time points has proven informative in our transcriptome analyses of responses to other environmental stressors in *R. sphaeroides* 2.4.1 (5, 47).

Following transcriptome profiling, the transcriptional data were correlated with transport and synthesis of known compatible solutes. Mutational analysis was then used to investigate the relative importance of several genes predicted to be involved in compatible solute metabolism. Changes in membrane lipid composition in response to salt stress were also investigated, and a transcription factor designated CrpO that controls these changes was identified.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Growth was measured by determining the change in optical densities (ODs; measured as the OD at 600 nm [OD$_{600}$] or by Klett meter) versus time. For microarray experiments, *R. sphaeroides* 2.4.1 was grown in Sistrom's minimal medium A containing succinate as a carbon source (38). The 60-ml cultures were grown in 100-ml glass tubes with continuous vigorous sparging with a gas mixture containing 95% N$_2$ and 5% CO$_2$. The tubes were placed in a transparent water bath (30°C) and exposed to white light at 10 W m^{-2}. Salt stress was induced by the addition to the mid-exponential-growth-phase cultures of an anoxic NaCl solution amounting to an addition of 1.5% NaCl, which increased the Na$^+$ concentration in the growth medium from 85 to 265 mM. Cultures were collected at 0, 7, and 45 min following addition of NaCl.

For compatible solute analyses, *R. sphaeroides* was grown in tightly capped bottles in the same medium as described earlier (24). Conditions used to obtain cells subjected to lipid analysis are provided below. Multicopy analysis of NaCl tolerance of *R. sphaeroides* was performed by monitoring growth of cultures inoculated with equivalent numbers of cells (OD$_{600}$ of 0.17) in filled screw-cap tubes containing kanamycin (final concentration, 50 μg ml^{-1}) for plasmid maintenance and 0% (final concentration, 85 mM), 1.5% (final concentration, 265 mM), or 3.0% (final concentration, 445 mM) additional NaCl.

Genetic manipulations. Standard recombinant DNA techniques were used (36). Plasmids were transferred from *Escherichia coli* into *R. sphaeroides* cells by conjugation as described earlier (29, 37), and recombinants were selected using appropriate antibiotics. The correctness of the mutant strains was verified by Southern blot analysis (36).

The *R. sphaeroides betS* (RSP3080) gene was inactivated in strain RBCCT by insertion of the Km^r suicide vector pRBCCT (Table 1; see also Fig. S1 in the supplemental material), derived from pJP5603 (29). The Δ(*proW proX*) double mutant strain RWAE was constructed by replacing an internal fragment of the genes with an Ω(Sm^r/Sp^r) cassette (33) using plasmid pRWAE3 (Table 1; see also Fig. S1). The primer sequences and schematics of the plasmids used in the construction of these mutants are provided in Fig. S1.

Plasmid pCrpO (Table 1) was constructed by amplifying the *crpO* sequences from genomic DNA using PCR and ligating the product into pUI1087 (46). A DNA fragment carrying *crpO* was then isolated from pCrpO and inserted into plasmid pBBR1MCS-2 (21) to create pBBR-CrpO (Table 1; see also Fig. S2 in the supplemental material). To construct pBBR-CrpO′ (Table 1; see also Fig. S2), a QuikChange II Mutagenesis Kit (Agilent Technologies, Inc.) and mutagenic oligonucleotides were used to delete the 3′ portion of the *crpO* gene in plasmid pCrpO while also creating a new StuI restriction site. The truncated gene was moved into pBBR1MCS-2, which added DNA in frame with CrpO′ coding for five amino acids (LQFAL) followed by a nonsense codon. Further cloning details, oligonucleotide sequences, and plasmid maps are provided in Fig. S2.

RNA extraction, DNA microarrays, and quantitative real-time reverse transcription-PCR (RT-PCR). RNA extraction was performed as described earlier (28). Briefly, rifampin (final concentration, 200 μg ml^{-1}) was added to *R. sphaeroides* cultures to halt transcription initiation, and cells were immediately collected into centrifugation bottles containing shaved ice, followed by brief centrifugation. The pellets were then resuspended in RNeasy Midikit (Qiagen) buffer. Cells were disrupted using a Mini-BeadBeater (Biospec Products), and RNA was extracted using the RNeasy Midikit. The absence of genomic DNA contamination was verified by quantitative real-time PCR using the SYBR green method and iCycler iQ Real Time PCR Detection System (Bio-Rad), as described earlier (28).

High-density oligonucleotide *R. sphaeroides* GeneChips (Affymetrix, Inc.) corresponding to the whole 4.6-Mb genome were used for transcriptome profiling. The construction and performance analysis of the *R. sphaeroides* GeneChip were described earlier (28). The quality and quantity of RNA and cDNA at all steps were monitored using capillary gel electrophoresis (BioAnalyzer, Agilent Technologies, Inc.).

DNA microarray data analysis. Transcriptome profiles from three independently grown cultures exposed to NaCl for 7 or 45 min, as well as five controls at 0 min, were recorded and examined. GeneChip data analysis was carried out using the R statistical software environment (http://www.r-project.org) enhanced with specialized microarray-related software packages from the Bioconductor project (http://www.bioconductor.org). The expression values were obtained using robust multiarray analysis with quantile normalization (16) using the Affy package (15) from Bioconductor. A parametric empirical Bayes approach was applied as described by Kendziorski et al. (19), using the EBarrays Bioconductor package (45). The Pearson correlation between replicate arrays was high (>0.97), which suggested a high level of interreplicate reproducibility. As a criterion for differential gene expression between the growth conditions, we used a combination of a false discovery rate (FDR) cutoff of 0.05 and a median expression ratio (fold change) cutoff of 2 in either direction (i.e., ≤0.5 or ≥2). For the purposes of the current analysis, the genes with changes in expression levels that did not satisfy these criteria were considered not changed (NC).

The expression ratios are shown in the text in parentheses preceded by the locus (RSP) numbers or their corresponding genes. When expression of several genes is discussed, the lower and upper limits of expression changes are given, e.g., a range of 2.0- to 5.0-fold increases.

Trehalose and GB measurements. In order to determine intracellular levels of trehalose and GB, *R. sphaeroides* cells were harvested by centrifugation. For trehalose measurements, cell pellets were resuspended in 80% (vol/vol) aqueous solution of acetonitrile (5 ml per g of wet mass) and then ultrasonically homogenized. The suspension was heated for 5 min at 100°C followed by centrifugation at 18,500 × *g* for 5 min. Subsequently, the supernatant was filtered through a Minisart-RC 4 filter (Sartorius, Germany), and the filtrate was subjected to high-performance liquid chromatography (HPLC) analysis on a Shodex sugar SZ5532 column (6 mm by 150 mm) at a flow rate of 1 ml of 80% acetonitrile min^{-1}. Trehalose concentrations were determined by using refractive index (RI) monitor RI-930 (JASCO, Japan), and a standard curve was generated by using standard solutions of trehalose ranging from 0.1 to 10 mM.

For GB measurements, cell pellets were resuspended in 70% ethanol (10 ml per g of wet mass) and ultrasonically homogenized. The mixture was heated for 5 min at 100°C and centrifuged at 18,500 × *g* for 5 min. The supernatant was evaporated to dryness, and the residue was solubilized in 0.5 volume of distilled water. Esterification of GB was performed as described earlier (25). Briefly, *p*-bromophenacyl bromide (20 mg ml^{-1} in acetonitrile) and reaction buffer (100 mM KHCO$_3$ · KH$_2$PO$_4$ · acetonitrile at 1:1:4, vol/vol) were added to the solution, and the entire contents was then heated at 80°C for 90 min. The heat-treated solution was evaporated to dryness. Subsequently, 300 μl of 100 mM NaH$_2$PO$_4$, pH 3.0, was added to the pellet, which was mixed and then centrifuged at 18,500 × *g* for 5 min. The supernatant was applied to a CAPI-3300 (Otsuka Electronics, Japan) capillary electrophoresis system, and esterified GB

was detected at 254 nm. Concentrations were determined based on a standard curve generated using GB solutions of 0.01 to 10 mM.

Extraction of phospholipids. Resting cells subjected to lipid analysis were prepared essentially as described by De Leo et al. (8) using a mid-exponential-phase phototrophic *R. sphaeroides* culture (grown under the same conditions as in the microarray experiments), except that the culture was divided equally before the cells were pelleted. Potassium phosphate (0.1 M; pH 7.4) was used to wash the cells from the residual medium, and one pellet of washed cells was resuspended in 50 ml of the same buffer with chloramphenicol at a final concentration of 0.3 mg ml^{-1} and the other in buffer alone. Salt was added to the "resting" cell suspensions to achieve either the same concentration present in culture medium (85 mM) or to a final concentration of 0.8 M in order to replicate the experimental conditions described by De Leo et al. (8). The resuspended cells were transferred to 250-ml Erlenmeyer flasks, which were then incubated with gentle rotation at 30°C overnight.

For all other phospholipid analyses, after a 45-min incubation following the addition of 3% NaCl to phototrophic cultures of *R. sphaeroides*, chloramphenicol was added to a final concentration of 0.3 mg ml^{-1} to halt further protein synthesis.

In all cases, the cells were pelleted in 50-ml conical tubes by centrifugation. Cell pellets were then resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and lipid extraction was performed using standard protocols (4, 8). Following evaporation of the solvents used in extraction, the dry weights of the extracts were determined. The lipids were then dissolved in chloroform-methanol (1:1, vol/vol) to a final concentration of approximately 20 μg μl^{-1}. All phospholipid samples were stored at −20°C.

TLC analysis. The extracted lipid samples, together with a standard solution of 10 μg μl^{-1} each of cardiolipin (CL), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Sigma-Aldrich) in chloroform-methanol (1:1, vol/vol), were applied to thin-layer chromatography (TLC) silica gel plates (layer thickness, 0.2 mm; Sigma-Aldrich) that had been washed twice with chloroform-methanol (1:1, vol/vol) and then activated by placing the plates in a dry oven for 30 min at 120°C. The development conditions that were used were previously described (7, 8). Visualization of the phospholipids was achieved by exposing the charred TLC plates to UV light. Digital images were captured using a DC290 zoom digital camera (Eastman Kodak Company). Phospholipid ratios were determined using ImageJ software (http://rsb.info.nih.gov/ij).

Chemical determination of polar phospholipid concentrations. The concentrations of polar phospholipids were determined using a colorimetric method based on the formation of a complex between phospholipids and ammonium ferrothiocyanate as described previously (39). Absorption spectroscopy was performed using a Hitachi U-2010 UV/visible light (Vis) spectrophotometer. A standard curve was generated using a PC solution (39; http://www.cyberlipid.org).

Microarray data accession number. The expression data obtained here were deposited in the Gene Expression Omnibus (GEO) database of NCBI (www.ncbi.nlm.nih.gov/projects/geo/), platform GPL162, series GSE8082.

RESULTS AND DISCUSSION

Scope of transcriptome changes in response to salt stress. The growth of *R. sphaeroides* 2.4.1 under anoxic phototrophic conditions is inhibited by high-salt concentrations (Fig. 1). In the presence of 1.5% added NaCl, the generation time is approximately 1.8-fold longer than that in the standard medium. Growth in the presence of 3% added NaCl was inhibited further and was generally less reproducible (data not shown), suggesting that the population accumulated spontaneous, salt-tolerant mutants. Therefore, for transcriptome analysis, we exposed phototrophic cultures to an additional 1.5% NaCl; however, for other types of analyses we often used both 1.5% and 3% additional NaCl.

Whole-genome transcription profiling revealed that, compared to the untreated culture (0 min), expression of approximately 900 genes changed at least under one time point following exposure to additional 1.5% NaCl, according to the criteria described in Materials and Methods. The numbers of up- and downregulated genes were approximately the same,
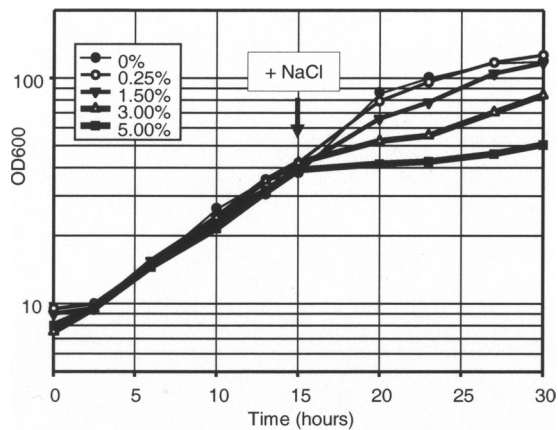
FIG. 1. Effect of added NaCl on anoxic phototrophic growth of *R. sphaeroides* 2.4.1. The arrow indicates the time point at which the NaCl was added. Representative growth curves are shown.

i.e., approximately 450 genes. All of the genes that passed our selection criteria are listed in Table S1 in the supplemental material.

The large number of affected genes suggests that both salt-specific and nonspecific stress responses were triggered.

For example, among the nonspecific responses were the upregulation at 7 min of the SOS response gene *lexA* (RSP1997; 2.3-fold) and the downregulation at 45 min of the σ^{70} gene (RSP0395; 0.43-fold) together with a number of genes encoding ribosomal proteins (RSP0021-RSP0022, RSP1048, RSP1700, RSP1709-RSP1710, RSP1716 to RSP1718 [RSP1716-RSP1718], RSP1720-RSP1733, and RSP2860; 0.30- to 0.48-fold), the latter trend being consistent with the decreased growth rate of the culture in the presence of additional 1.5% (Fig. 1). However, the prototypical osmotic stress responses could also be discerned by changes in expression of genes responsible for compatible solute synthesis and transport (Table 2). These changes are described below.

Compatible solute transport. GB is known to be an important compatible solute for *R. sphaeroides* (1). Three sets of genes (apparently organized in operons) are annotated as encoding components of proline or GB ABC-type transporters. The *proXWV* genes are likely to be important for coping with the NaCl stress since at 45 min they were among the most highly upregulated genes (RSP3057-RSP3059; 17.4- to 56.4-fold) (Table 2). The genes predicted to encode the other two proline/GB ABC-type transporters, RSP3998-RSP4000 and RSP2179-RSP2181, were much less responsive or nonresponsive (Table 2). A *betS* gene (RSP3080) encoding a putative

TABLE 2. Expression of the most relevant salt-stress response systems in *R. sphaeroides* 2.4.1

Function and locus	Annotated product[a]	Fold change at:[b]	
		7 min	45 min
Compatible solute synthesis			
RSP0948-RSP0949	OtsB, trehalose phosphatase	6.5	11.8
	OtsA, trehalose 6-phosphate synthase	2.8	16.2
RSP2446	TreS, trehalose hydrolase	NC	3.5
RSP2450, RSP2452	TreZ, maltooligosyltrehalose trehalohydrolase	NC	NC
	TreY, maltooligosyltrehalose synthase	NC	NC
Compatible solute transport			
RSP3057-RSP3059	ProX, ABC-type glycine betaine/L-proline transporter, periplasmic substrate binding protein	NC	17.4
	ProW, transporter permease protein	3.7	56.4
	ProV, transporter inner membrane ATPase protein	8.9	53.5
RSP3998-RSP4000	ProV1, ABC-type glycine betaine/L-proline transporter, inner membrane ATPase protein	3.2	NC
	ProW1, transporter permease protein	NC	NC
	ProX1, transporter periplasmic substrate binding protein	NC	NC
RSP2179-RSP2181	ProX2W2V2, ABC-type glycine betaine/L-proline transporter	NC	NC
RSP3080	BetS, BCCT family choline/carnitine/betaine transporter	NC	4.0
Cation (potassium) uptake			
RSP1265-RSP1269	KdpABC, K+-transporter	NC	NC
RSP1853-RSP1854, RSP2841-RSP2842	TrkH2H3, TrkA, TrkH1, Trk-type K+ transporter	NC	NC
Additional candidate salt stress tolerance genes			
RSP0697	UspA, universal stress response protein A	NC	2.5
RSP2380	CatC, catalase	NC	2.9
RSP2395	BCCP, cytochrome *c* peroxidase	5.6	5.7
RSP0910-RSP0912	DctP, TRAP dicarboxylate family transporter substrate-binding protein	NC	0.06
	DctQ, transporter subunit	NC	0.04
	DctM, transporter subunit	NC	0.06
RSP0634-RSP0636	DctP-type subunit, TRAP dicarboxylate family transporter	NC	12.1
	DctQ-type transporter subunit	NC	17.7
	DctM-type transporter subunit	NC	6.7

[a] As annotated at NCBI (http://www.ncbi.nlm.nih.gov).
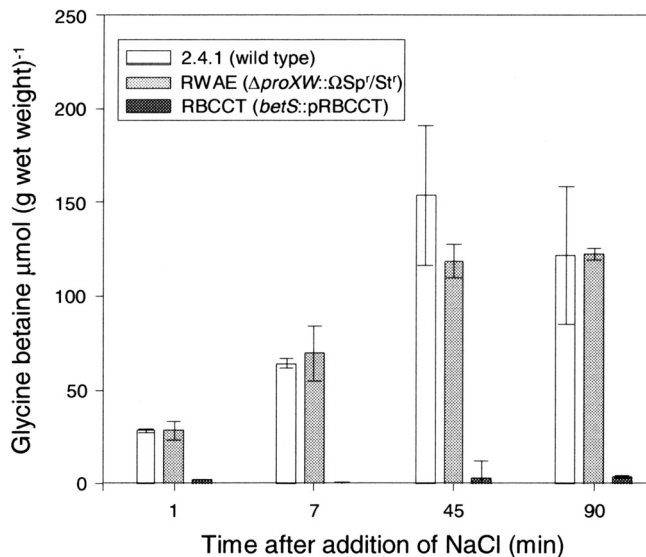[b] NC, no change.

FIG. 2. Dynamics of intracellular GB accumulation in salt-stressed *R. sphaeroides* wild-type 2.4.1 and mutants RWAE (*proXWV*) and RBCCT (*betS*). Strains were grown under anoxic phototrophic conditions in medium containing 1 mM GB. Salt stress was imposed by the addition of 3% NaCl at time zero to exponentially growing cells (OD_{600} of 0.4). Values are the averages of three biological replicates, and the error bars represent the standard deviations.
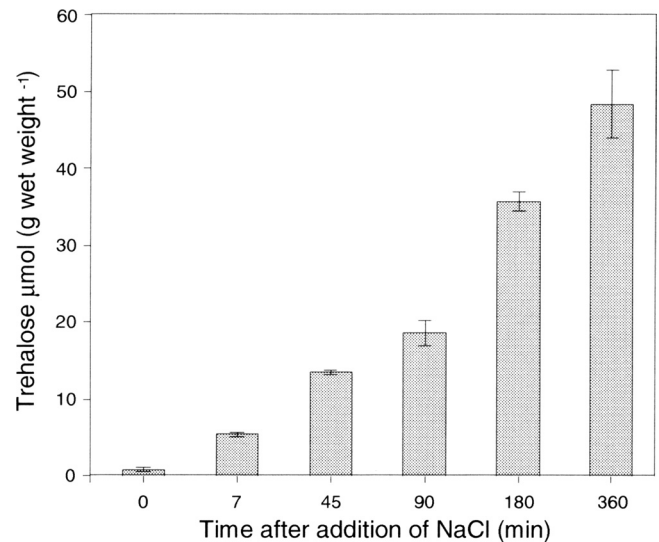

FIG. 3. Dynamics of intracellular trehalose accumulation in salt-stressed *R. sphaeroides* 2.4.1 cells grown under anoxic phototrophic conditions. Salt stress was imposed by the addition of 1.5% NaCl at time zero to exponentially growing cells (OD_{600} of 0.4). Values are the averages of three biological replicates, and the error bars represent the standard deviations.

betaine/carnitine/choline transporter, which can potentially be involved in GB uptake, is present in the *R. sphaeroides* 2.4.1 genome, and its mRNA level was increased in the presence of added salt (4.0-fold) (Table 2).

In order to evaluate the significance of the observed transcriptional responses of the potential GB transporter genes and to decipher their roles in NaCl stress tolerance, the dynamics of GB uptake were examined. Intracellular GB accumulation was monitored in cultures that were grown in the presence of 1 mM GB and exposed to 3% NaCl. Already at 7 min postexposure to NaCl, intracellular GB levels had increased by approximately 36-fold from an average value of 1.6 ± 1.2 preexposure to an average value of 56.9 ± 3.1 μmol of GB (g of wet weight)$^{-1}$ postexposure, based on three biological replicates. Such rapid and robust GB uptake suggests that the GB transporter must be present in *R. sphaeroides* prior to NaCl stress. These results are consistent with the report by Abee et al. (1) that GB uptake was unhindered by the addition of the protein synthesis inhibitor chloramphenicol. Since in the absence of added NaCl *betS* transcripts were present at much higher absolute levels than the absolute levels of *proVWX* transcripts, we hypothesized that GB transport relies primarily upon BetS.

To further evaluate the relative contributions of *proVWX* and *betS* to GB transport, null mutations in *proVWX* and *betS* were constructed (Table 1, strains RWAE and RBCCT, respectively), and GB accumulation rates in these strains following NaCl stress were measured. While GB levels in cells lacking the ProVWX transporter did not differ from those of the wild type, cells lacking BetS were impaired in GB accumulation. For example, at 90 min the GB concentrations in the wild-type strain 2.4.1 and mutant RWAE were 119.6 and 121.9 μmol/g

of wet weight, respectively, whereas the GB concentration in mutant RBCCT was 4.4 μmol/g of wet weight (Fig. 2).

These data suggest that, unlike the situation in enteric bacteria, where a ProVWX homolog is the main GB transporter (reviewed in reference 32), in *R. sphaeroides* BetS fulfills this role. Because the *proVWX* gene products were highly upregulated in response to salt stress and yet play no apparent role in GB uptake, we suggest that the ProVWX transporter is involved in the uptake of an as yet unknown compatible solute, which was not tested in our experiments.

Compatible solute synthesis. Trehalose is the main compatible solute synthesized in response to osmotic stress in the close relative of *R. sphaeroides* 2.4.1, *R. sphaeroides* f. sp. *denitrificans* IL106 (43). The microarray data revealed that the *otsBA* genes (RSP0948-RSP0949) encoding the main trehalose biosynthesis operon in strain 2.4.1 (24) responded rapidly to added NaCl; i.e., the *otsBA* mRNA levels had increased at 7 min (2.8- to 6.5-fold) (Table 2) and remained among the most highly upregulated genes at 45 min (11.8- to 16.2-fold). In contrast, the baseline expression of the second operon predicted to be involved in trehalose synthesis, the *treYZ* genes (RSP2450-RSP2452), was relatively low, and these genes were not induced in response to NaCl stress (Table 2). The *treS* gene (RSP2446), whose product is involved in trehalose turnover (24), was upregulated at a later (45 min) but not earlier (7 min) time point (Table 2).

While the transcription response of the *otsBA* genes to added NaCl was rapid, the baseline expression of the genes was relatively low. This suggested that trehalose synthesis might require a significant amount of time before reaching osmoprotective levels. To investigate the dynamics of trehalose synthesis, intracellular trehalose concentrations in cells exposed to NaCl stress were measured (Fig. 3). At 7 min following the addition of 1.5% NaCl, the intracellular trehalose concentra-

TABLE 3. NaCl-induced changes in phospholipid composition in phototrophic *R. sphaeroides* 2.4.1 resting cells

Incubation condition(s)[a]	Phospholipid ratio[b]	
	CL/PC	PG/PC
0.85 mM NaCl	0.57	0.59
0.8 M NaCl	0.65	0.79
0.8 M NaCl, chloramphenicol[c]	0.85	0.30

[a] Phospholipids were measured in *R. sphaeroides* 2.4.1 cells resting from phototrophic growth.
[b] CL, cardiolipin; PC, phosphatidylcholine; PG, phosphatidylglycerol.
[c] Final concentration, 0.3 mg ml^{-1}.

tion had increased approximately 7-fold (5.3 versus 0.6 μmol of trehalose g of wet weight^{-1}), but it also continued to rise for many hours, reaching a concentration that was approximately 80-fold higher than that prior to induction (47.7 versus 0.6 μmol of trehalose g of wet weight^{-1}).

These results confirm that trehalose is important as a long-term osmoprotectant in *R. sphaeroides* 2.4.1 and reveal a pivotal role of induced *otsBA* expression in trehalose synthesis. They are also consistent with the hypothesis that OtsBA (and not TreYZ) play the primary role in trehalose synthesis in the *R. sphaeroides* species (24).

Membrane lipid alterations. In several bacteria, osmotic stressors have been shown to evoke changes in the degree of saturation in membrane fatty acids and to increase the proportion of anionic phospholipids (reviewed in reference 34). An increase in the relative amount of CL has been documented in *R. sphaeroides* (7).

To identify the reasons underlying changes in membrane lipid composition, the expression of lipid biosynthesis genes was closely examined. Genes whose products are involved in CL production, i.e., *pgsA* (RSP1073) encoding phosphatidyl-glycerol (PG) 3-phosphate synthase (10), *pgpA* (RSP2834) encoding PG 3-phosphate phosphatase, and *cls* (RSP0473) encoding a CL synthase (40), were not responsive to the addition of NaCl. In fact, the transcript levels of only one gene involved in fatty acid biosynthesis, *acpP* (RSP2463), changed (decreased 0.45-fold) in response to added salt (see Table S1 in the supplemental material). While the present knowledge of fatty acid and lipid metabolism in *R. sphaeroides* is incomplete (40) and while some genes involved in these processes may not yet have been identified, the transcriptome analysis suggests that the membrane lipid changes are achieved primarily by posttranscriptional regulation.

To test this conclusion, we performed an experiment with resting cells that had been used previously by De Leo et al. (8) to demonstrate that CL was induced by NaCl in *R. sphaeroides*. We modified the experimental setup in order to compare the NaCl-induced changes in the phospholipid profile when protein synthesis was either permitted or halted (by chloramphenicol). PC was used as an internal reference in measuring relative abundances of CL and PG since its concentration does not change in response to osmotic stress (7). As already reported by De Leo et al. (8), upon NaCl stress both the CL/PC and PG/PC ratios increased under the conditions of permitted protein synthesis. However, when protein synthesis was halted, the CL/PC ratio still increased while the PG/PC ratio decreased (Table 3). Since CL is formed from two molecules of PG, the

data suggest that NaCl stress increases the activity of CL synthase, whereas new protein synthesis is required to replenish PG. Overall, these experiments confirm the primarily posttranscriptional regulation of NaCl-induced changes in membrane lipid composition.

Other features of the salt response transcriptome. In addition to the components of the salt stress response described above, both potassium and its counter ion glutamate have been reported to play roles in bacterial tolerance to salt stress (42). *R. sphaeroides* f. sp. *denitrificans* accumulates K$^+$ in proportion to the levels of exogenous NaCl (43). Therefore, the expression data of genes for K$^+$ uptake and for synthesis and transport of glutamate were examined. They indicated that mRNA levels of neither the *kdp*-type (RSP1265-RSP1269) nor the *trk*-type (RSP2041, RSP1853-RSP1854, and RSP2842) K$^+$ uptake genes were affected by NaCl stress (Table 2). While it is possible that *R. sphaeroides* 2.4.1 differs from *R. sphaeroides* f. sp. *denitrificans* with respect to the role of K$^+$, it is more likely that in both species K$^+$ accumulation is controlled posttranscriptionally.

With respect to glutamate uptake, Jacobs et al. (13) have described a tripartite ATP-independent periplasmic (TRAP)-type glutamate transporter that is detected only in *R. sphaeroides* exposed to osmotic stress (17). The identities of the genes encoding that transporter are not yet known. Curiously, a putative TRAP transporter encoded by RSP0634-RSP0636 is greatly upregulated at 45 min (6.7- to 17.7-fold) (Table 2). Furthermore, salt stress is the only condition out of many for which DNA microarray data are available when this transporter is upregulated (26). Therefore, it is possible that RSP0634-RSP0636 encode the osmotic shock-induced glutamate transporter reported by Jacobs et al. (17).

Alternatively, RSP0634-RSP0636 may play a different role, i.e., that of a succinate uptake system. The *dctPQM* genes (RSP0910-RSP0912), which encode a known TRAP-type transporter of succinate (13), were strongly downregulated following the addition of NaCl (0.04- to 0.06-fold at 45 min) (Table 2). Succinate is the main carbon source in the growth medium, and it is required for growth. According to Jacobs et al. (17), succinate uptake is stimulated, not inhibited, by NaCl. Together, these observations argue that an alternative succinate uptake system is engaged during NaCl stress, and it is possible that the RSP0634-RSP0636 TRAP transporter fulfills this role. These predictions originating from the NaCl stress transcriptome analysis have yet to be tested experimentally.

Other strongly upregulated genes that may contribute to increased salt stress tolerance include RSP0697 encoding a putative universal stress response protein (2-fold at 45 min) (Table 2), catalase *catC* (RSP2380; 2.9-fold at 45 min), and cytochrome *c* peroxidase *ccp* (RSP2395; 5.6-fold at 7 min). RSP0697 must be somewhat specific to salt stress because it was not upregulated upon exposure to oxidative stresses, unlike *catC* and *ccp* (5, 47).

NaCl-responsive transcription factor genes. Over 80 putative transcriptional regulator genes were activated or repressed by NaCl stress under phototrophic conditions (see Table S2 in the supplemental material), suggesting that many regulatory networks were affected. Among these, five σ-factor genes were strongly upregulated (RSP0527, RSP0601, RSP1272, RSP2681, and RSP3606; >3-fold), and three (RSP0032 and
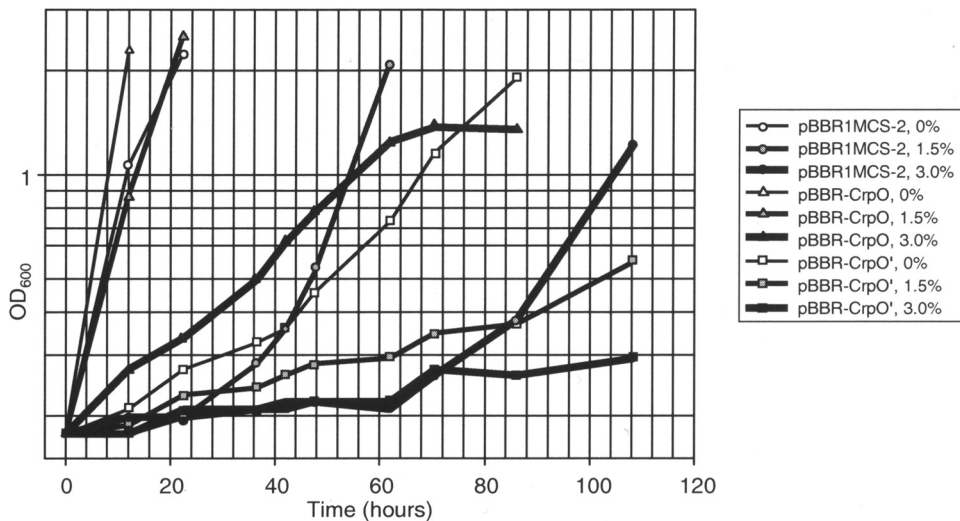
FIG. 4. Effect of the phospholipid regulator CrpO on salt tolerance. Anoxic phototrophic cultures of *R. sphaeroides* 2.4.1 containing the plasmids indicated were grown in the presence of added NaCl (0%, 1.5%, or 3%). Plasmids are as follows: ●, pBBR1MCS-2 (vector); ▲, pBBR-CrpO expressing *crpO*; and ■, pBBR-CrpO′ expressing truncated *crpO*, i.e., *crpO′*.

RSP0068 at < 0.2-fold and RSP0395 at 0.43-fold) were strongly downregulated, including the main σ^{70} factor (RSP0395).

The upregulated σ-factor genes include a previously characterized oxidative stress response σ-factor gene, *rpoH2* (RSP0601), which is a member of the heat shock family (3, 5, 48), and two σ^{54} factors, RSP0527 and RSP3606 (30, 31). One of the strongly downregulated σ-factor genes, RSP0068, also belongs to the σ^{54} family. Differential regulation of the members of this family reinforces the conclusions from earlier studies that the four σ^{54} factors of *R. sphaeroides* 2.4.1 have different functional roles (30, 31). The second significantly downregulated gene encoding a σ-factor, *fliA* (RSP0032), is involved in flagellar gene-specific transcription (reviewed in reference 2). The decreased expression of *fliA* is probably responsible for the reduced transcription of flagellar and motility genes in response to NaCl (see Table S1 in the supplemental material). A decrease in flagellar gene expression has been observed in diverse salt-stressed bacteria, e.g., *E. coli*, *Shewanella oneidensis*, *Sinorhizobium meliloti*, and *Desulfovibrio vulgaris* (23, 27, 35, 41), suggesting that this phenomenon is a common bacterial response to salt stress.

The two remaining upregulated σ-factor genes, RSP2681 and RSP1272, have no known functions. These were upregulated already at 7 min following NaCl addition, and the extent of their upregulation was the highest under salt stress of all other conditions tested by microarrays thus far (26). Therefore, we expect RSP2681 and RSP1272 to play important roles in salt tolerance, a prediction that remains to be tested.

The transcription factor CrpO is important for NaCl tolerance. To begin exploring the contributions to salt tolerance of various regulatory systems, we selected four upregulated genes encoding putative transcription factors of unknown function. Among these genes, RSP1435 was the most highly upregulated (38.8-fold at 7 min and 5.6-fold at 45 min) (see Table S2 in the supplemental material). RSP1945 had a similar pattern (11.3-fold at 7 min and 6.5-fold at 45 min) to that of RSP1435, which

is characterized by higher expression at 7 min and somewhat lower expression at 45 min. The mRNA levels of two other transcription factor genes, RSP0018 (2.5-fold at 7 min and 8.6-fold at 45 min) and RSP1275 (3.1-fold at 7 min and 4.6-fold at 45 min), increased more gradually.

To test whether increased expression of these four putative transcription factors contributes to salt tolerance, all were cloned into a 4- to 6-copy number vector under their native promoters, and salt tolerance of strain 2.4.1 containing the expression plasmids was compared to strain 2.4.1 containing the empty vector, pBBR1MCS-2. The relative growth rate of strain 2.4.1 expressing RSP1435, RSP1945, or RSP0018 in multicopy was not improved in the presence of added salt (data not shown). However, one of four genes in multicopy, RSP1275, greatly improved salt tolerance for 2.4.1(pBBR-CrpO) (Fig. 4). The RSP1275 gene product belongs to the Crp-Fnr regulatory protein family (reviewed in reference 20). Since the effector domain of the protein encoded by RSP1275 lacks cysteine residues involved in iron-sulfur cluster formation, which is characteristic of the Fnr homologs, we designated it CrpO (where O stands for osmotic stress).

TABLE 4. Changes in the CL/PC phospholipid ratio in
R. sphaeroides 2.4.1 with multiple copies of *crpO*

Strain and condition[a]	CL/PC ratio[b]
pBBR1MCS-2	
No NaCl	0.55
+ NaCl	0.75
pBBR-CrpO	
No NaCl	0.72
+ NaCl	0.85

[a] Phototrophically growing *R. sphaeroides* 2.4.1 containing the empty vector, pBBR1MCS-2, or pBBR-CrpO(pBBR1MCS-2::*crpO*).
[b] Phospholipid ratios in cells following a 45-min incubation with 3% added NaCl versus no added NaCl. Values are averages of three biological replicates. Standard deviations did not exceed 10% of the average for all measurements.
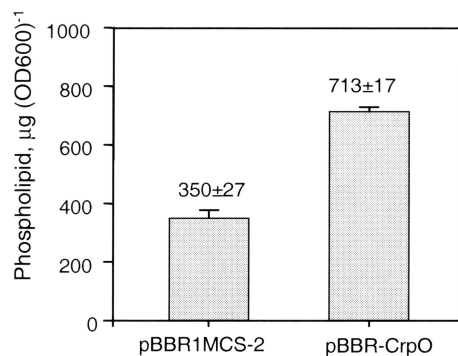
FIG. 5. Changes in phospholipid concentrations induced by CrpO. Shown are phospholipid levels in phototrophic *R. sphaeroides* 2.4.1 with the empty plasmid vector pBBR1MCS-2 or plasmid pBBR-CrpO. Values are averages of a total of four data points consisting of duplicate assays of two independently grown replicates, and error bars indicate standard deviations.

Our attempts to construct a *crpO* null mutant by allele replacement were unsuccessful, suggesting that *crpO* may be essential. To investigate CrpO function, we constructed a dominant negative allele. Because Crp-Fnr proteins are composed of an N-terminal effector domain linked by a dimerization region to a C-terminal DNA binding domain, we expected that a truncated CrpO lacking the DNA-binding domain, CrpO′, would be able to dimerize with the wild-type protein and that CrpO-CrpO′ heterodimers would be impaired in DNA binding. In agreement with these expectations, in the presence of 1.5% added NaCl, CrpO′ inhibited phototrophic growth of the wild type; e.g., by the time 2.4.1(pBBR1MCS-2) reached stationary phase, the cell density of 2.4.1(pBBR-CrpO′) had not yet doubled (Fig. 4).

CrpO affects membrane lipids. Measurements using transcriptional fusions revealed that CrpO in multicopy does not affect expression of genes associated with compatible solute transport and synthesis (data not shown). Therefore, we investigated the involvement of CrpO in changing membrane lipid composition. In 2.4.1(pBBR-CrpO), even in the absence of added NaCl, the CL/PC ratio was found to be similar to the ratio observed in the wild type, 2.4.1(pBBR1MCS-2), experiencing salt stress. Addition of NaCl further increased the CL/PC ratio in 2.4.1(pBBR-CrpO) (Table 4). These observations strongly suggest that CrpO primarily contributes to increased salt tolerance via changes in membrane lipids.

Mukhopadhyay et al. observed that polar lipid content in salt-stressed *D. vulgaris* was increased in response to salt stress (27). Likewise, the total phospholipid amount in 2.4.1(pBBR-CrpO) was approximately 2-fold higher than in 2.4.1(pBBR1MCS-2) (713 versus 350 $\mu g/OD_{600}$ unit) (Fig. 5). This result suggests that CrpO may contribute to increased salt tolerance by both increasing the percentage of CL among the membrane phospholipids and elevating the overall phospholipid levels. How CrpO function mechanistically remains to be explored.

In summary, this study correlated the changes in the NaCl stress-induced transcriptome with the dynamics of transport and synthesis of key compatible solutes in *R. sphaeroides* and identified or confirmed genes responsible for compatible sol-

ute transport and synthesis. This work also characterized NaCl-induced changes in phospholipids and identified an apparently essential transcription factor, CrpO, as an important contributor to salt tolerance that affects membrane lipids. Other known or putative transcription factors that were up- or downregulated by NaCl stress represent potential new regulators of salt tolerance whose analysis will help decipher the salt stress-responsive regulatory network in *R. sphaeroides*. Finally, this study may shed light on the adaptation of freshwater bacteria to a seawater environment since a recently sequenced genome of the seawater strain KD131 of *R. sphaeroides* has remarkably high sequence identity with the genome of strain 2.4.1 (22).

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