Isolation and Characterization of NaCl-Sensitive Mutants of *Caulobacter crescentus*

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An attempt to characterize *Caulobacter crescentus* genes important for the response to high concentrations of NaCl was initiated by the isolation of mutants defective in survival in the presence of 85 mM NaCl. A transposon Tn5 library was screened, and five strains which contained different genes disrupted by the transposon were isolated. Three of the mutants had the Tn5 in genes involved in lipopolysaccharide biosynthesis, one had the Tn5 in the *nhaA* gene, which encodes a Na⁺/H⁺ antiporter, and one had the Tn5 in the *ppiD* gene, which encodes a peptidyl-prolyl *cis-trans* isomerase. All the mutant strains showed severe growth arrest in the presence of 85 mM NaCl, but only the *nhaA* mutant showed decreased viability under these conditions. All the mutants except the *nhaA* mutant showed a slightly reduced viability in the presence of 40 mM KCl, but all the strains showed a more severe reduction in viability in the presence of 150 mM sucrose, suggesting that they are defective in responding to osmotic shock. The promoter regions of each disrupted gene were cloned in *lacZ* reporter vectors, and the pattern of expression in response to NaCl and sucrose was determined; this showed that both agents induced *ppiD* and *nhaA* gene expression but did not induce the other genes. Furthermore, the *ppiD* gene was not induced by heat shock, indicating that it does not belong to the σ^{32} regulon, as opposed to what was observed for its *Escherichia coli* homolog.

Free-living bacteria must survive dramatic changes in extracellular osmolality. To respond to osmotic stress, they have developed some very specific mechanisms that allow the immediate restoration of the osmotic balance. When exposed to osmotic upshifts, bacteria respond in three overlapping phases: dehydration, adjustment of cytoplasmic solvent composition and rehydration, and cellular remodeling (49).

The cytoplasmic membrane is highly permeable to water, and there are also water-specific porins (aquaporins) and other sorts of membrane solute transporters that play a major role in the adaptation to high osmolality (8, 11, 46, 50). In *Escherichia coli*, the immediate response to osmotic stress is K⁺ uptake through the Kdp and Trk systems (5, 42) followed by uptake of compatible solutes, which also depends on outer membrane porins (16).

Bacteria also have to cope with variable levels of environmental solutes that, besides causing osmotic stress, are also toxic to the bacterial cell, as is the case of Na⁺ ions. The Na⁺ intracellular concentration has to be kept at tolerable levels, and bacteria use several Na⁺ efflux systems to attain sodium homeostasis. These systems may carry out active transport of the ion by using energy driven by ATP, coupled to metabolic enzyme transport or to respiration, or by catalyzing the efflux of Na⁺ in exchange for external H⁺ (reviewed in references 12 and 37).

When *E. coli* cells are exposed to high osmolality, the general stress factor σ^s is highly induced, driving the transcription of many genes involved in osmoprotection, including *osfBA*,

treA (*osmA*), *osmB*, *proU*, and *proP* (23). Furthermore, osmotic shock transiently induces heat shock genes belonging to the σ^{32} and σ^E regulons that are necessary for protein folding in the cytoplasm and cell envelope (7, 20, 33).

In *Bacillus subtilis*, the σ^B regulon mediates the general stress response, recognizing environmental signals such as those resulting from salt stress, heat shock, or ethanol (1, 22). Besides the general response, a more specific response may be achieved via other sigma factors. Horsburgh and Moir showed that the extracytoplasmic-function (ECF) sigma factor σ^M is required for growth and survival after salt stress (24). Although both σ^B and σ^M are important for the salt stress response in *Bacillus*, their major contributions are made in different phases of the cell cycle. Expression of σ^M is highest during early exponential and mid-exponential growth, and its transcription is reduced in post-exponential growth, whereas σ^B activity rises at the end of exponential growth before falling slowly as the cells enter the stationary phase (24, 47).

Caulobacter crescentus is one of the major models for studying cellular differentiation in prokaryotes, and its molecular mechanisms have been extensively characterized (27, 35). The complete genome of *C. crescentus* is now available (34), which allows the evaluation of most of its metabolic capabilities. However, the responses of *C. crescentus* to environmental stimuli are poorly characterized, except for the heat shock response (3, 6). The presence of a significant number of sigma factors that belong to the extracytoplasmic family and the absence of a sigma S homolog suggests that the stress responses in this bacterium are probably more specific than in *E. coli* (34).

As an aquatic bacterium, *Caulobacter* faces different stresses in its environment and must maintain an adequate intracellular steady state at all situations. To investigate how *Caulobacter* responds to osmotic stress, particularly to high NaCl concen-

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TABLE 1. Identification of the Tn5 insertion sites and similarity to ORF sequences from the database

Mutant strain	Gene name	Disrupted ORF ID	Putative ORF function	Closest similarity (% identity/% similarity) ^a	E-value
SP1503	<i>gmhB</i>	AAK25578	D-Glycero-D-manno-heptose 1,7-bisphosphate phosphatase	<i>Aneurinibacillus thermoaerophilus</i> AAK27853 (36/61)	2E-26
SP1803	<i>nhaA</i>	AAK22437	Na ⁺ /H ⁺ antiporter 1	<i>Brucella melitensis</i> AAL52716 (64/76)	1E-104
SP4104	<i>ppiD</i>	AAK23869	Peptidyl-prolyl <i>cis-trans</i> isomerase D	<i>Agrobacterium tumefaciens</i> strain C58 AAL42686 (26/46)	3E-47
SP4202	<i>rfa</i>	AAK23035	Heptosyltransferase	<i>Fusobacterium nucleatum</i> subsp. <i>nucleatum</i> AAL94742 (21/38)	7E-11
SP4703	<i>manA</i>	AAK25579	Mannose-6-phosphate isomerase	<i>Brucella melitensis</i> AAL52575 (43/54)	3E-79

^a Numbers indicate identity/similarity obtained by the BLASTP program using a low-complexity filter and the matrix BLOSUM 62.

trations, we have identified and characterized five genetic loci that are important for its growth and survival under these conditions. Moreover, we have determined that NaCl and sucrose induce transcription of the *ppiD* and *nhaA* genes. This is a first step toward understanding the regulatory network involved in the osmotic stress response in *Caulobacter*.

MATERIALS AND METHODS

Bacterial strains, growth conditions and genetic procedures. *C. crescentus* NA1000 (15) was used as the wild type for all experiments. The bacteria were grown at 30°C in peptone yeast extract (PYE) medium or minimal M2 glucose medium (14) supplemented with kanamycin (5 µg/ml), tetracycline (1 µg/ml), or nalidixic acid (25 µg/ml) as necessary. *E. coli* DH5α (Life Technologies) was used in the cloning procedures. *E. coli* was grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 µg/ml) or tetracycline (12.5 µg/ml) as necessary. Plasmids were introduced into *Caulobacter* by conjugation with *E. coli* strain S17-1 (44).

Isolation of NaCl-sensitive strains from a *C. crescentus* transposition library. We used a previously constructed *C. crescentus* Tn5(Kan) transposition library of 5,000 clones (25). Screening was performed by growing the cells in 96-well plates at 30°C followed by plating in PYE plus kanamycin containing 85 mM NaCl. Five mutant strains that showed no growth and were also unable to grow in plates containing 50 mM NaCl, clones SP1503, SP1803, SP4104, SP4202, and SP4703, were selected.

Cell growth and survival tests. The strains were grown in PYE plus kanamycin to mid-log phase; the cultures were then divided into 5-ml aliquots, and one of the following agents was added to an aliquot: 85 mM NaCl, 40 mM KCl, and 150 mM sucrose. The cells were further incubated at 30°C with agitation, and the growth of each culture was determined by measuring the optical density at 600 nm at different times during the incubation. A survival test was performed for each osmotic agent tested, by adding the agent to exponential cultures and taking aliquots at different times. The cells were subjected to serial dilutions in PYE without addition of osmotic agents and plated in PYE medium to determine the number of CFU.

Determination of the Tn5 insertion sites. The Tn5 insertion sites of the strains were determined by reverse PCR as described previously (25). The sizes of the bands obtained for each strain were as follows: SP1503, 1.0 kb; SP1803, 1.6 kb; SP4104, 0.7 kb; SP4703, 1.2 kb; and SP4202, two bands (1.3 and 1.0 kb). The DNA fragments were gel purified and used for automatic DNA sequencing with BigDye terminators on an ABI Prism 377 sequencer (AP Biosystems).

Cloning of the promoter regions and gene expression analysis. The complete sequence of the regions flanking the disrupted genes was obtained from the *C. crescentus* genome sequence (34). Oligonucleotides 15-3AN (CAATGGATCC ATGGAAGGGGCGAGTGG) and 15-3A2 (CAATGAATTCTAGGCGGACAG GAAGTAG) were used for PCR amplification of the putative promoter region in strain SP4703, generating a 675-bp fragment. Oligonucleotides 15-3A1 (CA ATGGATCCGATCGATATTGAGCACAC) and 15-3A2 were used for PCR amplification of the putative promoter region in strain SP1503, generating a 450-bp fragment. Oligonucleotides 41-4E1 (CAATGAATTCTGGCTCAGACG ATGCAAC) and 41-4E2 (CAATGGATCCTCGATCGCGCTCATACTG) were used for PCR amplification of the putative promoter region in strain SP4104, generating a 470-bp fragment. Those fragments were cloned into pBlue-script SK (Stratagene). To amplify the putative promoter of the disrupted gene in strain SP4202, oligonucleotides 42-2F1 (CAATGGATCCTCGGAATTTCG

GATCACC) and 42-2F2 (CAATGAATTCGTCATACTCCGAGGGCC) were used, and for strain SP1803 oligonucleotides 18-3B1 (CAATGAATTCGGGCT GGTCAATGAACCG) and 18-3B2 (CAATGGATCCAGTTCAGCGGTATA GAAC) were used, generating 980- and 1,065-bp fragments respectively. The amplified fragments were cloned into the TOPO vector from the TOPO TA cloning kit for sequencing (Invitrogen) and confirmed by DNA sequencing.

PCRs were carried out with 1.5 µg of *C. crescentus* chromosomal DNA, 50 pmol of each set of oligonucleotides (described above), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 2.5 U of *Taq* DNA polymerase (Invitrogen) and 1× PCR buffer (supplied with the enzyme). The PCR conditions were 5 min at 94°C followed by 40 cycles of 90 s at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final cycle of 7 min at 72°C.

All the fragments obtained were cloned into pRKLacZ290 (17), and promoter activities were determined by immunoprecipitation assays with monoclonal anti-β-galactosidase antibody (Sigma) and by β-galactosidase activity assays. Cells were grown to mid-log phase in M2 glucose medium supplemented with tetracycline (1 µg/ml); the cultures were then divided into aliquots, and one of the following agents was added to an aliquot: 85 mM NaCl and 150 mM sucrose. For the immunoprecipitation experiments, cells were incubated further at 30°C with agitation and proteins were pulse-labeled with 10 µCi of [³⁵S]methionine (NEN) per ml for 5 min at different times during the incubation. Equal counts of labeled proteins (10⁶ cpm) were immunoprecipitated as described previously (18). For the β-galactosidase activity assays, the cultures were incubated at 30°C after addition of the agents and aliquots were taken at several time points to determine enzyme activity as described by Miller (31).

For the heat shock experiment, the NA1000 cells harboring construct *ppiD/lacZ lacZ* were incubated either at 30 or at 42°C and aliquots were pulse-labeled with [³⁵S]methionine as above. The proteins were then immunoprecipitated with antisera anti-β-galactosidase and anti-GroEL (6).

RESULTS AND DISCUSSION

Isolation of mutants sensitive to NaCl. To identify genes necessary for the adaptation of *C. crescentus* to high salt concentrations, we screened a Tn5 insertion library of 5,000 clones for mutants that showed no growth in plates containing 85 mM added NaCl. Previous experiments showed that this is the maximal concentration of NaCl that *Caulobacter* can withstand without affecting growth or viability (data not shown).

These mutants isolated were further tested for growing in plates containing 50 mM added NaCl. The presence of Tn5 was confirmed in five strains that were unable to grow even at this lower concentration of NaCl (Table 1). It should be noticed that transposon insertion was found only once in each gene, suggesting that the genome is not saturated with mutations, and therefore there still could be other genes involved in adaptation to high NaCl concentration that were not identified.

Interestingly, three of these mutants were disrupted in genes probably involved in lipopolysaccharide (LPS) biosynthesis (*gmhB*, *rfa*, and *manA*). Analysis of each Tn5 insertion region

FIG. 1. Growth of the parental and mutant strains under different osmotic challenges. Strains NA1000, SP1503, SP1803, SP4104, SP4202, and SP4703 were grown in PYE medium to mid-log phase, and after 3 h the culture was divided into aliquots and the following solutes were added to the respective final concentrations: 85 mM NaCl (black squares), 40 mM KCl (black triangles), and 150 mM sucrose (white triangles). As a control, one aliquot was left without additions (white squares). Growth was determined by measuring the absorbance at 600 nm.

showed that the *manA* and *rfa* are probably not cotranscribed with any other downstream gene. The *rfa* gene product belongs to the heptosyltransferase family and has a putative function of LPS core biosynthesis. In *E. coli*, the two heptoses are linked to the 3-deoxy- α -D-manno-oct-2-ulopyranosonic acid (KDO) by the action of two heptosyltransferases, WaaC (RfaC) and WaaF (RfaF), and heptose III is transferred to heptose II by WaaQ (RfaQ) (19). The disrupted gene in SP4703 (*manA*)

encodes a mannose-6-phosphate isomerase and is transcribed divergently to the gene disrupted in mutant SP1503. The *manA* gene product is involved in the conversion of fructose-6-phosphate to mannose-6-phosphate in a reversible reaction, and mannose is then incorporated into the outer core of the *Caulobacter* LPS structure (38, 45). The *gmhB* gene encodes a conserved hypothetical protein, which has 36% identity to a D-glycero-D-manno-heptose 1,7-bisphosphate phosphatase, and

FIG. 2. Viability test of the wild-type and mutant strains to osmotic agents. Strains NA1000, SP1503, SP1803, SP4104, SP4202, and SP4703 were grown in PYE medium to mid-log phase, and the following agents were added to the cultures at time zero: 85 mM NaCl (A), 40 mM KCl (B), or 150 mM sucrose (C). At intervals after addition of the salt, aliquots were removed and plated for counting colonies, and survival was determined relative to the culture without addition (time zero). The results are the average of three independent results. The key for each curve depicted in panel A is the same for all graphs.

its last codon overlaps the first codon of a downstream gene encoding a dTDP-4-dehydroramnose reductase. The *gmhB* gene product in *Aneurinibacillus thermoaerophilus* is necessary in the conversion of D- α -D-heptose 1,7-bisphosphate to D- α -D-heptose 1-phosphate (29).

The LPS structure of *C. crescentus* consists of an inner core region containing three molecules of KDO, two α -L-glycero-D-mannoheptose and one α -D-glycero-D-mannoheptose; an outer core region of α -D-mannose, α -D-galactose, and α -D-glucose (probably phosphorylated); and a lipid A moiety containing 3-OH-dodecanoic acid attached to a backbone with an undetermined structure (40). The structure of LPS O antigen is not yet completely clarified, but in a recent paper Awram and Smit proposed that it is composed of a single 4,6-dideoxy-4-aminohexose-*N*-acetylperosamine (4).

The LPS structures produced by these three strains are probably defective in the core sugar composition. The inner core sugars seem to be essential for the correct trimerization of porins in the outer membrane of *Salmonella enterica* serovar Typhimurium, since a deep rough LPS has a very low percentage of assembly of trimeric OmpF porin (43). In the mutants isolated in this work, the LPS would have only the two initial α -L-glycero-D-mannoheptoses (*gmhB* and *rfa*), or extend further to the α -D-glycero-D-mannoheptose (*manA*), and therefore they are probably affected in the assembly of outer membrane protein complexes.

Strain SP1803 is disrupted in an open reading frame (ORF) that encodes a homolog of the *E. coli* NhaA protein, involved in the adaptation to Na⁺ and alkaline pH (in the presence of Na⁺). This class of antiporters catalyzes efflux of Na⁺ in exchange for external H⁺ and plays a major role in Na⁺ resistance. Despite the coexistence of several antiporter systems in the bacterial cell, a single mutation or deletion in one of these antiporter-encoding genes may produce a sodium-sensitive phenotype (9, 21, 26, 36, 48).

The gene disrupted in SP4104 (*ppiD*) encodes a putative rotamase with 26% identity to PpiD from *Agrobacterium tumefaciens*, and downstream of it lies the *trpE* gene, which codes for anthranilate synthase component I and probably forms an operon with *ppiD*. PpiD is a peptidyl-prolyl *cis-trans* isomerase of the parvulin class, which catalyze *cis-trans* isomerization around X-Pro peptide bonds and are present both in the cytoplasm and in the periplasmic space of the cell. There are two characterized enzymes of the parvulin family in the periplasm, SurA and PpiD. SurA helps in the maturation and folding process of outer membrane protein monomers, and *surA* mutants have a defective cell envelope as well as increased sensitivity to hydrophobic agents (30, 32, 41). *ppiD* is a suppressor of *surA* mutants in *E. coli*, and it is able to restore the ability of outer membrane protein folding when overexpressed (10). Although *C. crescentus* also has a homolog of the SurA protein, the *ppiD* knockout in strain SP4104 caused the cells to be highly sensitive to osmotic stress, indicating that a deficient periplasmic protein folding prevents a proper response.

Effect of osmotic agents in growth and viability of the mutants. The inability of the mutants to grow in plates containing NaCl could be an effect of either growth impairment or loss of viability. To assess if NaCl causes growth arrest in the strains selected, growth curves were determined for cultures to which NaCl was added to yield a final NaCl concentration of 85 mM (Fig. 1). As can be seen, all the cultures showed growth inhibition after addition of the salt, with a more pronounced effect after 6 h, except for mutant SP1803, which stopped growing immediately when exposed to NaCl.

To determine whether the addition of NaCl was also affect-

FIG. 3. Determination of promoter activity of the transcriptional fusions. The promoter regions of the genes disrupted in mutants SP4104 and SP1803 were cloned in the transcriptional reporter plasmid pRKlacZ290 (17), generating fusions *ppiD-lacZ* (A and B) and *nhaA-lacZ* (C and D), respectively. Mid-log-phase cells were incubated with either 85 mM NaCl (A and C) or 150 mM sucrose (B and D), and β -galactosidase (β -gal) activity was assayed at different time points after addition. The background value of β -galactosidase activity given by pRKlacZ290 was subtracted from the results. (E) Expression of the *ppiD-lacZ* fusion under heat shock. The proteins were labeled with [35 S]methionine for 5 min at several time points after incubation at 42°C, equal counts were immunoprecipitated with both anti- β -galactosidase and anti-GroEL antisera, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ing the viability of the cells, a viability test was performed by determining the number of CFU after incubation with the salt (Fig. 2A). The experiment showed that viability was not affected in four of the mutants, but the *nhaA* strain suffered a drastic reduction in viability after 3 h in the presence of NaCl,

which agrees with the growth arrest observed for this mutant. This loss of viability is not accompanied by lysis of the cells, as judged by microscopy and absorbance of the *nhaA* strain after 24 h in the presence of NaCl. These results indicate that the other four mutants, despite being affected, could still resume

growth after removal of the salt (when plated for the CFU counts), suggesting that in these mutants the internal Na^+ concentration has not irreversibly damaged any vital functions.

The mutant strains were then evaluated for their ability to respond to other osmotic agents, such as KCl and sucrose. As can be seen in Fig. 1, 40 mM KCl caused a more severe reduction in growth of the *ppiD* and *manA* strains while *nhaA*, *gmhB*, and *rfa* showed a less strong effect. Accordingly, the *ppiD*, *manA*, *gmhB*, and *rfa* strains showed a more significant decrease in viability while *nhaA* was not affected, as expected (Fig. 2B). These results suggested that with exception of *nhaA*, the deficient response to NaCl could be a result of a broader defect in the cells, possibly involving a deficiency in the osmotic stress response. To test this hypothesis, we tested the effect of a nonionic high-osmolarity shock, by growing the cells in the presence of 150 mM sucrose (Fig. 1 and 2C). The results showed a decrease in absorbance in the presence of high sucrose concentrations for all mutants except the *nhaA* strain. However, this strain showed a similar decrease in viability in the presence of sucrose to that shown by the others, which suggested that the increase in absorbance was a result of an alteration in the cell volume instead of an increase in cell number. Microscopy observation showed that this was really the case; the cells of the *nhaA* strain become filamentous in the presence of sucrose, causing an increase in absorbance. The reason for the loss of viability of the *nhaA* strain in 150 mM sucrose is not clear, but it could be that in the absence of Na^+ efflux the cell cannot achieve osmotic balance under high-osmolarity conditions.

The reasons for the impaired osmotic stress response of the defective-LPS strains and the *ppiD* mutant could be that the proteins responsible for the cell adaptation to high osmolarity are not properly folded in the outer compartment of the cell. Further data that corroborate this idea are that overproduction of PpiD complements the defects of the *htrM* (*rfaD*) mutation, where the gene *htrM* encodes an ADP-1-glycol-D-mannoheptose-6 epimerase, which is needed for LPS biogenesis (39).

Transcriptional regulation in response to osmotic stress. To analyze whether the disrupted genes could be regulated by osmotic upshift, the putative promoter regions of each gene were cloned into a *lacZ* reporter vector and introduced into *C. crescentus* NA1000 strain. The cells were incubated with either 85 mM NaCl or 150 mM sucrose, and transcription was measured both by pulse-labeling the proteins with [^{35}S]methionine for 5 min at several time points and immunoprecipitating the β -galactosidase and by performing β -galactosidase activity assays. The promoter fusions of *manA*, *gmhB* and *rfa* showed no detectable induction in the presence of either osmotic agent (not shown).

Figure 3A shows that transcription driven from the promoter of *ppiD* is increased from 60 min after addition of NaCl and from 30 min after addition of sucrose, indicating that this gene is induced by high-osmolarity conditions. This higher level of expression of *ppiD* in the presence of the osmotic agents is maintained for at least 5 h, showing a 2-fold induction by NaCl and a 2.2-fold induction by sucrose. The *Caulobacter* *nhaA* homolog showed a small induction by NaCl in β -galactosidase activity assays (1.6-fold induction [Fig. 3C]), in contrast to the *E. coli* *nhaA* gene, whose transcription is highly induced by Na^+ and Li^+ (5- to 10-fold after 60 minutes) but

not by increases in osmolarity and ionic strength (13, 28). A very small increase in β -galactosidase activity (1.2-fold) was observed after incubation with 150 mM sucrose, but whether this fact has any physiological relevance remains to be determined.

In *E. coli*, *ppiD* is induced by high temperature, and its transcription is dependent on σ^{32} -RNA polymerase. PpiD is so far the only periplasmic chaperone that has shown regulation by the heat shock sigma factor σ^{32} , and is also regulated by the two-component system CpxR-CpxA (10). σ^{32} from *Caulobacter* has a well-known consensus-binding site, and it regulates specific heat shock genes (2, 3, 6). The putative promoter region of *ppiD* in *Caulobacter* does not have any sequence resembling the σ^{32} consensus, and transcription is not induced by heat shock (Fig. 3E). These results indicate that in *C. crescentus*, *ppiD* transcription is dependent on other regulatory mechanisms, being induced by osmotic upshift but not by heat shock. Since *Caulobacter* has 13 sigma factors of the ECF family (34), one of these alternative factors could regulate *ppiD* expression, possibly together with a two-component regulatory system. In any case, further investigation of the regulatory regions involved in the osmotic shock induction will allow us to determine the pathways used by *Caulobacter* to respond to this stress condition.

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