

# The Cation-Responsive Protein NhaR of *Escherichia coli* Activates *pgaABCD* Transcription, Required for Production of the Biofilm Adhesin Poly- $\beta$ -1,6-*N*-Acetyl-D-Glucosamine<sup>∇</sup>

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**The *pgaABCD* operon of *Escherichia coli* is required for production of the biofilm adhesin poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine (PGA). We establish here that NhaR, a DNA-binding protein of the LysR family of transcriptional regulators, activates transcription of this operon. Disruption of the *nhaR* gene decreased biofilm formation without affecting planktonic growth. PGA production was undetectable in an *nhaR* mutant strain. Expression of a *pgaA'*-*lacZ* translational fusion was induced by NaCl and alkaline pH, but not by CaCl<sub>2</sub> or sucrose, in an *nhaR*-dependent fashion. Primer extension and quantitative real-time reverse transcription-PCR analyses further revealed that NhaR affects the steady-state level of *pga* mRNA. A purified recombinant NhaR protein bound specifically and with high affinity within the *pgaABCD* promoter region; one apparent binding site overlaps the  $-35$  element, and a second site lies immediately upstream of the first. This protein was necessary and sufficient for activation of *in vitro* transcription from the *pgaA* promoter. These results define a novel mechanism for regulation of biofilm formation in response to environmental conditions and suggest an expanded role for NhaR in promoting bacterial survival.**

Bacteria in the natural environment often form complex communities of cells associated with a surface or interface and containing a polysaccharide matrix (8, 13, 19, 30). This physiological state, known as a biofilm, provides an environment that can facilitate horizontal gene transfer and promote survival under hostile conditions (13, 15, 66). Biofilm-associated cells are generally more resistant to antimicrobial treatments than free-living bacteria (17, 24, 44). In addition, pathogenic organisms growing as biofilms are protected from attack by the immune system, and these resilient communities of microbes can lead to chronic infections (15, 24, 30, 31), resulting in medical complications and substantial economic losses (14, 47, 56). An understanding of the factors that regulate the colonization process is important for developing approaches to combat and control biofilm formation.

Biofilm development is a complex process, initiated by cell attachment to a surface and formation of microcolonies (14, 47). A temporary attachment step often precedes “permanent” attachment (2, 3, 9, 34) and may be aided by motility (53, 78). In various strains of *Escherichia coli*, attachment and microcolony formation are facilitated by proteinaceous adhesins and polysaccharides such as cellulose and poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine (PGA) (16, 53, 55, 58, 74, 77, 80). The latter polymer is involved in both cell-cell adhesion and attachment to certain abiotic surfaces by *E. coli* K-12, and it also stabilizes biofilm structures of other gram-negative bacteria and staphylococci (reference 36 and references therein). Its production

depends upon the *pgaABCD* operon, which encodes a GT-2 family vectorial glycosyltransferase (PgaC) that synthesizes this polymer and other proteins thought to be involved in PGA export and localization. Quorum-sensing molecules and other polysaccharides influence the further maturation of various biofilms (18, 28, 52). Eventually, planktonic cells are released from the biofilm, completing the developmental cycle and leading to colonization elsewhere (47). Thus, biofilm formation may be viewed as a flexible or dynamic developmental process involving sequential gene expression patterns that are influenced by environmental cues (6, 25, 47).

Several regulatory systems affect *E. coli* biofilm formation (see, e.g., references 1, 12, 20, 37, 38, 54, and 74). The RNA-binding protein CsrA regulates biofilm formation primarily by binding to the untranslated leader and proximal coding region of *pgaABCD* mRNA, which blocks *pgaA* translation and destabilizes this transcript (37, 38, 59, 76). Thus, a *csrA* mutant overproduces PGA and exhibits a dramatic increase in biofilm formation. CsrA activity in the cell is controlled by two non-coding RNAs, CsrB and CsrC, which bind to and sequester multiple copies of CsrA and thus activate *pga* expression and biofilm formation (see, e.g., reference 76). EnvZ/OmpR, H-NS, and the Cpx and Rcs systems all appear to affect biofilm formation by regulating the production of curli fimbriae in response to osmotic conditions (16, 39, 54, 67). The Cpx system also may mediate surface sensing to activate biofilm formation (42, 48).

Living cells not only respond to changing osmotic conditions but must also maintain an externally directed sodium gradient and a relatively constant intracellular pH (51). Na<sup>+</sup>/H<sup>+</sup> antiporters, membrane proteins that exchange Na<sup>+</sup> (or Li<sup>+</sup>) for H<sup>+</sup>, play important roles in these processes. In *E. coli*, NhaA is the key antiporter that protects against sodium stress, and it is essential for growth in the presence of high sodium concen-

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TABLE 1. Strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or phage	Description or genotype	Source or reference
<i>E. coli</i> K-12 strains		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> ) <i>hsdR17 relA1</i> <i>recA1 endA1 gyrA96 thi-1</i>	
MG1655	F <sup>-</sup> $\lambda$ <sup>-</sup>	Michael Cashel, NICHD, National Institutes of Health, Bethesda, Maryland
TRMG	MG1655 <i>csrA::kan</i>	61
CF7789	MG1655 $\Delta$ <i>lacI-Z</i> (MluI)	Michael Cashel
TRCF7789	CF7789 <i>csrA::kan</i>	69
DJ25	TRMG $\Delta$ <i>motB</i> <i>uvrC279::Tn10</i> $\Delta$ <i>fimB-H</i>	37
XWMGDABCD	MG1655 $\Delta$ <i>pgaABCD</i>	77
TRXWMGDABCD	TRMG $\Delta$ <i>pgaABCD</i>	77
NM81	<i>nhaA::kan</i>	Etana Padan, Hebrew University of Jerusalem, Israel
96B10 <sup>a</sup>	DJ25 <i>nhaR::cam</i> at +432 relative to initiation codon	This study
CGMR	MG <i>nhaR::cam</i>	This study
CGTR	TRMG <i>nhaR::cam</i>	This study
XWZ4	CF7789 <i>pgaA'-lacZ</i>	76
TRXWZ4	XWZ4 <i>csrA::kan</i>	76
CGCFR	XWZ4 <i>nhaR::cam</i>	This study
CGTRCFR	TRXWZ4 <i>nhaR::cam</i>	This study
<i>S. epidermidis</i> strains		
1457	PIA positive	Dietrich Mack, Universitätsklinikum, Hamburg-Eppendorf, Germany
1457-M10	PIA negative; Ery <sup>r</sup> Cipro <sup>r</sup> Gm <sup>r</sup>	Dietrich Mack
Plasmids		
pKK223-3	Cloning vector; Ap <sup>r</sup>	Pharmacia Corp.
pUC19	Cloning vector; Ap <sup>r</sup>	
pCR 2.1 TOPO	Cloning vector; Ap <sup>r</sup> Kn <sup>r</sup>	Invitrogen
pCRnhaAR	<i>nhaAR</i> operon in pCR2.1	This study
pNhaR	<i>nhaR</i> in pKK223-3	This study
pNhaR-His <sub>6</sub>	C-terminally hexahistidine-tagged NhaR for protein purification	This study
pPGA372	<i>pgaABCD</i> in pUC19	77
Bacteriophage P1vir	Strictly lytic P1	Carol Gross, University of California, San Francisco

<sup>a</sup> Original transposon mutant.

trations, while NhaB becomes essential only in the absence of NhaA (49, 50). The *nhaA* gene is located in a two-gene operon, *nhaAR*, which is induced by the presence of monovalent cations. The gene *nhaR* of this operon encodes an autoregulatory protein that activates *nhaAR* transcription and is homologous to the LysR-OxyR family of prokaryotic transcriptional regulators (57, 64). NhaR also activates *osmC* transcription, which is required for resistance to organic peroxides and long-term survival in stationary phase (29, 43, 68, 70).

LysR-type transcriptional regulators (LTTRs) respond to low-molecular-weight coinducer molecules, although coinducer binding often has been indirectly inferred by isolating mutants that fail to respond to, or have altered specificity for, the coinducer (64, 73, 75). Coinducers typically do not increase promoter affinity but instead activate transcription via a conformational change in the LTTR-DNA complex (11, 35, 46, 64). While NhaR activates gene expression in vivo in response to Na<sup>+</sup>, K<sup>+</sup>, or Li<sup>+</sup>, this cation response has not been reconstituted in vitro (10, 22, 23, 70).

Here we establish that NhaR stimulates *pga* transcription, and thus biofilm formation, in response to monovalent cations and alkaline conditions. We propose that this represents a

novel means by which NhaR promotes survival of *E. coli* and perhaps other enteric bacteria in response to environmental conditions. Despite its regulatory role in the cell, NhaR-dependent in vitro transcription did not respond to monovalent cations, suggesting that additional factors might be involved in this regulation.

## MATERIALS AND METHODS

**Bacterial strains, phage, plasmids, and growth conditions.** All *E. coli* strains, phage, and plasmids used in the present study are listed in Table 1. Unless otherwise indicated, bacteria were routinely grown at 37°C in Luria-Bertani medium (LB) (pH 7.4) (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter) with shaking at 250 rpm. L broth was identical to LB but lacked NaCl. Biofilms were grown in 96-well flat-bottom polystyrene microtiter plates (Corning Inc., Life Sciences, Acton, MA) at 26°C for 24 h under static conditions. Colonization factor antigen medium (1% Casamino Acids, 0.15% yeast extract, 0.005% MgSO<sub>4</sub>, and 0.0005% MnSO<sub>4</sub>, pH 7.4) was used for initial isolation of biofilm mutants. Media were supplemented with antibiotics as needed at the following final concentrations: ampicillin, 100  $\mu$ g/ml; chloramphenicol, 25  $\mu$ g/ml; kanamycin, 100  $\mu$ g/ml; and tetracycline, 10  $\mu$ g/ml.

**Molecular biology and genetics.** Standard procedures were used for isolation of supercoiled plasmids, restriction digests, ligations, transformation, and P1vir transduction of antibiotic markers, including *nhaR::cam* (45, 63).



lyzed on a 6% polyacrylamide sequencing gel containing 6 M urea, which was dried and subjected to autoradiography using a phosphorimager (Storm 860; Amersham Bioscience). Two different concentrations of RNA were examined to ensure that the signal was limited by the RNA transcripts and not the primer concentration.

**q-RT-PCR analysis of *pgaA* mRNA.** For q-RT-PCR, *csrA* wild-type (MG1655) and mutant (TRMG) strains containing a wild-type or mutant *nhaR* gene were grown at 26°C in LB to transition phase. Total RNA was isolated and treated to remove DNA, and the steady level of *pgaL-A* was determined with the primer pair PGART1/PGART2 (Table 2), using the iScript one-step RT-PCR Kit with SYBR green (Bio-Rad, Hercules, CA) according to the manufacturer's guidelines. Reactions were performed in duplicate using 100 ng RNA template. A reaction lacking reverse transcriptase was included for each sample as a control for DNA contamination. Reactions were conducted using the iCycler iQ real-time system (Bio-Rad) under the following conditions: 65°C for 5 min; 53°C for 60 min; 95°C for 10 min, and 35 cycles of 95°C for 30 s, 57°C for 1 min, and 68°C for 1 min. The difference in cycle threshold between samples from wild-type and mutant strain pairs was calculated. The PCR product identities were confirmed by agarose gel electrophoresis, and product uniformity was determined using melting curves (iCycler instruction manual; Bio-Rad). These experiments were conducted three times with similar results, and the mean values from the three experiments were determined. A standard curve for each experiment was constructed using a plasmid template to ensure linearity of the reactions under our experimental conditions. For normalization of *pgaA* RNA levels, the primer pair 16S-4/16S-5 (Table 2) was used to amplify 99 bp of 16S rRNA, using 1- and 10-ng samples of total RNA and a 20-min reverse transcription time.

**Purification of NhaR-His<sub>6</sub>.** NhaR-His<sub>6</sub> was purified as described previously, with some modification (10). An overnight culture of *E. coli* DH5α(pNhaR-His<sub>6</sub>) was used (20 ml/liter) to inoculate 1 liter of LB, and the culture was grown at 37°C with aeration to an *A*<sub>600</sub> of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) (2 mM) was added to the culture, and growth was continued for 3 h before cells were harvested by centrifugation and stored at -80°C. Frozen cells were thawed and resuspended (2 g of cells per ml) in lysis buffer containing 1 mg/ml of lysozyme, 4 mM imidazole, 20 mM Tris-HCl (pH 7.9), 500 mM KCl, and 5 mM β-mercaptoethanol (BME). After incubation on ice for 30 min, cells were disrupted by sonication. NhaR-His<sub>6</sub> was then purified by affinity chromatography using a His-Trap column (QIAexpressionist; QIAGEN), as recommended by the manufacturer. The binding buffer consisted of 4 mM imidazole, 500 mM KCl, 20 mM Tris-HCl (pH 7.9), and 5 mM BME. Wash and elution buffers were identical to binding buffer except that they contained 60 and 400 mM imidazole, respectively. This single chromatographic step provided an almost homogeneous preparation of NhaR-His<sub>6</sub> with greater than 98% purity, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining. Fractions containing the peak concentration of NhaR-His<sub>6</sub> were pooled and dialyzed overnight at 4°C against 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM EDTA (pH 8.0), 15 mM BME, and 10% glycerol. Glycerol was added to 10%, and aliquots (100 μl) were stored at -80°C. This procedure yielded ~0.54 mg of His-tagged NhaR per liter of culture. For experiments designed to examine cation dependency, the protein was extensively dialyzed against this buffer lacking the KCl.

**MALDI-TOF mass spectrometry.** NhaR-His<sub>6</sub> was confirmed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. Spectra were acquired in a linear mode by using a Voyager STR (Applied Biosystems, Foster City, CA) and a nitrogen laser (λ = 337 nm). One hundred to 600 laser pulses were used to obtain the spectra. The matrix was a saturated solution of 1,4-hydroxyphenylazobenzonic acid in 50% acetonitrile and 1% trifluoroacetic acid and was mixed in a 10:1 ratio with sample. One microliter of the mixture was transferred to the sample plate and allowed to dry. The predicted mass of recombinant NhaR-His<sub>6</sub> is 35,110 Da, and the experimental mass was determined to be 35,168 Da. The Voyager MALDI-TOF mass spectrometer with internal calibration is sensitive to ±0.05% (±20 Da). The larger experimental mass was suggestive of a K<sup>+</sup> (40-Da) ion associated with the protein.

**Gel mobility shift assay with purified NhaR-His<sub>6</sub> and DIG-labeled *pga* DNA.** A 132-bp PCR product containing the promoter region of *pgaABCD* was amplified using primers PgaA GS FWD and PgaA GS REV (-115 to +18) (Table 2) and *Taq* polymerase (Promega). This product was 3' end labeled with digoxigenin (DIG)-11-ddUTP (DIG gel shift kit, second generation; Roche Diagnostics, Indianapolis, IN) as suggested by the manufacturer. Purified recombinant NhaR (NhaR-His<sub>6</sub>, 1.17 μM) was added to DNA binding reaction mixtures (10 μl) containing 50 mM KCl, 20 mM Tris-HCl, (pH 7.9), 1 mM dithiothreitol, 10% glycerol, 125 μg/ml bovine serum albumin, and 1.38 fmol of DIG-labeled PCR product. Reactions were carried out for 20 min at 25°C. Loading dye (1.6 μl) containing bromophenol blue and glycerol, supplied with the labeling kit (Roche

Diagnostics), was immediately added and gently mixed, and reactions were subjected to native gel electrophoresis (5% acrylamide, 0.5× TBE [50 mM Tris, 40 mM boric acid, 0.5 mM EDTA]) at room temperature. The DNA was electroblotted (400 mA, 12 V, 45 min) onto positively charged nitrocellulose membranes (Roche Diagnostics). Membranes were rinsed for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and nucleic acids were cross-linked (UV Stratalinker 1800; Stratagene, La Jolla, CA). The signal was detected by chemiluminescence as recommended by the manufacturer (Wash and Block buffer set; Roche Diagnostics), using a Bio-Rad ChemiDoc system.

A nonspecific control DNA (*pgaB-C*) with a size and G+C content similar to those of the promoter DNA was amplified from the region overlapping *pgaB* and *pgaC* by using the primer set PgaB-C FWD/PgaB-C REV (Table 2). Quantity One (Bio-Rad) software was used to analyze the blots and quantify the bound and unbound probe. An apparent equilibrium binding constant (*K<sub>d</sub>*) for the NhaR-*pgaA* DNA complex was calculated using GraphPad Prism (Graph Pad) according to a previously described cooperative binding equation (79), adapted as follows:  $Y_{\text{max}} = Y_{\text{max}} \times [(\text{NhaR}_f/K_d)^n]/(1 + [(\text{NhaR}_f/K_d)^n])$ . In this equation, *Y<sub>max</sub>* is the maximum possible bound fraction (100%) of *pgaA* probe DNA (*Y<sub>max</sub>*), and *K<sub>d</sub>* is the concentration of free protein (NhaR<sub>f</sub>) at which *Y<sub>max</sub>* reaches 50% bound. Isolated NhaR protein was assumed to be 100% active for the calculations. The cooperativity of binding is described by the Hill coefficient (*n*).

**DNase I footprint of NhaR-His<sub>6</sub> on a linear DNA fragment of *pgaA*.** Oligonucleotides PgaA GS FWD and PgaA GS REV (100 pmol) were individually end labeled with [γ-<sup>32</sup>P]ATP (6,000 Ci/mmol; Perkin-Elmer) using T4 polynucleotide kinase (Epicenter) as per the manufacturer's instructions. Each labeled primer (20 pmol) was then used with the corresponding unlabeled primer (20 pmol) to amplify the promoter region of the *pgaABCD* operon from pPGA372 plasmid DNA with Platinum *Taq* polymerase (Invitrogen). The final PCR products corresponded to a 132-bp fragment containing the promoter region of *pgaA* (-115 to +18) and were gel purified and cleaned with QIAGEN QuickSpin columns.

Footprinting reaction mixtures contained 0.67 pmol (≈10,000 cpm) of the end-labeled DNA and were incubated with increasing concentrations of NhaR-His<sub>6</sub>. The 20-μl reaction mixtures contained 33 mM Tris acetate (pH 8.0), 0.15 mg/ml bovine serum albumin, 10 mM Mg acetate, 1 mM dithiothreitol, and 10% glycerol. The reaction mixtures were incubated at 25°C for 15 min before addition of DNase I (2 μl of a 0.01-mg/ml solution; 51.8 units). After 5 min at 25°C, the digests were terminated by addition of 200 μl of stop buffer (570 mM ammonium acetate, 80% ethanol). The DNA in each reaction was precipitated with ethanol, using Pellet Paint coprecipitant (Novagen, San Diego, CA). The same labeled primer and plasmid template pPGA372 were used to generate a DNA sequencing ladder with the SequiTherm EXCEL II DNA sequencing kit (Epicenter). Products were separated on 6 M urea-8% polyacrylamide sequencing gels. Gels were dried under vacuum, and the labeled fragments were detected using a Storm 860 phosphorimager (Amersham Bioscience). Footprinting reactions were conducted in the presence or absence of added sodium chloride. The experiment was conducted four times.

**In vitro transcription.** For in vitro transcription reactions, supercoiled plasmid pPGA372 (1 μg) and His-tagged NhaR (455 nM) were mixed in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 10% glycerol and incubated at 25°C for 20 min. *E. coli* σ<sup>70</sup>-RNA polymerase holoenzyme (1.25 U; Sigma-Aldrich Corp., St. Louis, MO) was added, and the mixtures were incubated for 10 min at 37°C. For transcription, 3.5 μl of a mixture of the four nucleoside triphosphates (2.5 mM each) and 40 U of Supersasin RNase inhibitor (Ambion, Austin, TX) was added, and the solution was incubated for 30 min at 37°C. The potassium and sodium contents of the reaction mixtures were estimated to be no greater than 1.1 mM. Transcription reactions were terminated by incubation with DNase I (5 units; Epicenter) for 15 min. The mixtures were extracted twice with phenol-chloroform and three times with chloroform, and RNA was precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water (10 μl).

The *pgaA* transcript was detected by primer extension. PEXT3-labeled primer (3.5 pmol) was added to in vitro-transcribed RNA (5 μl). For comparison, total RNA (25 μg) from the indicated strains was analyzed as stated above (see "Primer extension analysis"), except that Supersasin (Ambion) was added to the reaction mixture instead of the RNase inhibitor supplied with the reverse transcriptase.

**Microscopy.** Sterile borosilicate coverslips were placed in 15-cm petri dishes containing 25 ml of a freshly inoculated (1:100) overnight culture. The petri dishes were incubated at room temperature, and coverslips were removed at various times and rinsed gently with water. Each coverslip was inverted over a Parafilm gasket on a microscope slide, and images were obtained 10 min thereafter, as described previously (2, 3). Adherent cells were viewed by transmitted

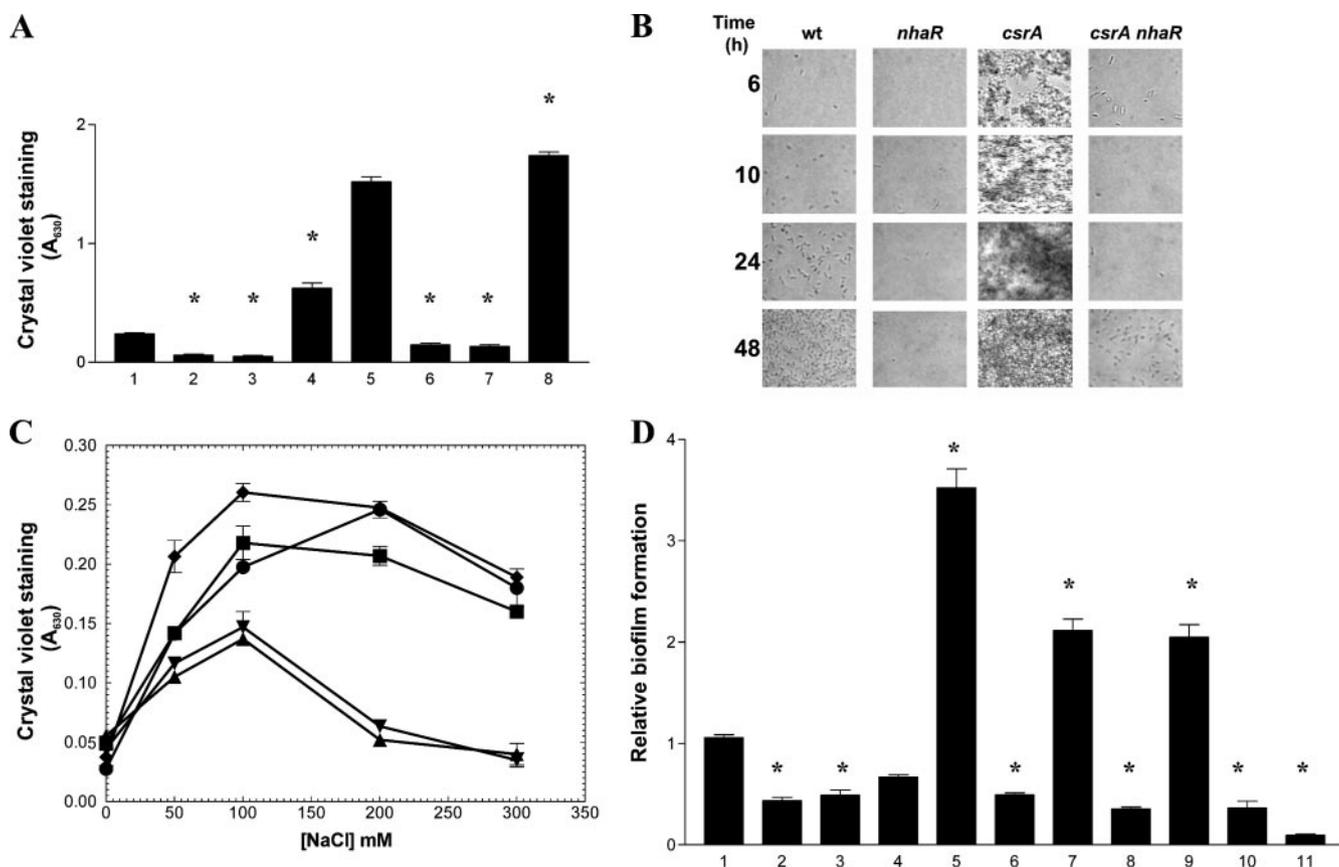


FIG. 1. Disruption of *nhaR* of *E. coli* affects biofilm formation. (A) Cultures were grown in microtiter plates in LB (pH 7.4) for 24 h at 26°C, and biofilm formation was assessed by crystal violet staining (see Materials and Methods). Bars depict the results obtained with a series of isogenic strains: 1, MG1655 (wild type); 2, *nhaR::cam*; 3, *nhaR::cam*(pKK223-3); 4, *nhaR::cam*(pNhaR); 5, *csrA::kan*; 6, *csrA::kan nhaR::cam*; 7, *csrA::kan nhaR::cam*(pKK223-3); 8, *csrA::kan nhaR::cam*(pNhaR). Error bars indicate standard errors of the means. (B) Time course of adherence to coverslips by isogenic *E. coli* K-12 MG1655 strains (described in Materials and Methods). Representative fields are shown. wt, wild type. (C) Effect of sodium chloride on biofilm formation. Cultures were grown in L broth (LB without NaCl) supplemented with the indicated concentrations of NaCl. Symbols represent the results obtained with a series of isogenic strains: ■, MG1655 (wild type); ▲, *nhaR::cam*; ▼, *nhaR::cam*(pKK223-3); ◆, *nhaR::cam*(pNhaR); ●, *nhaR::cam*(pNhaR-His<sub>6</sub>). Bars represent the averages for two wells, and error bars correspond to the standard errors of the means. (D) Epistasis analyses of *nhaR* and *pgaABCD* on biofilm formation. Biofilm formation in the following isogenic strains was assessed as for panel A: 1, MG1655; 2,  $\Delta$ *pgaABCD*; 3, *nhaR::cam*; 4, MG1655(pUC19); 5, MG1655(pPGA372) (contains *pgaABCD*); 6, *nhaR::cam*(pUC19); 7, *nhaR::cam*(pPGA372); 8,  $\Delta$ *pgaABCD*(pUC19); 9,  $\Delta$ *pgaABCD*(pPGA372); 10,  $\Delta$ *pgaABCD*(pKK223-3); 11,  $\Delta$ *pgaABCD*(pNhaR). Bars represent the averages for 16 wells normalized to the biofilm formed by MG1655, and error bars correspond to the standard errors of the means. The asterisks denote significant differences relative to the corresponding parent strain ( $P < 0.001$  [Tukey's multiple-comparison test]).

light with an Olympus 1X71 microscope (40 $\times$  objective lens with a 1.6 $\times$  selector; Olympus, Thornwood, NY). The images were captured by using a charge-coupled device camera (COHU-4915; COHU, Inc., Florence, KY) connected to a frame-grabber board installed in a personal computer and processed using Image Pro-Plus 4.5 software (Media Cybernetics, Silver Spring, MD).

**Bioinformatics.** The phylogenetic distribution of NhaR was assessed using BLAST analyses (4) at the NCBI website, using the *E. coli* protein as the query sequence.

## RESULTS AND DISCUSSION

***nhaR* is needed for optimal biofilm formation.** In order to identify genes that affect biofilm formation in *E. coli* K-12 (DJ25), Wang et al. conducted a random transposon mutagenesis and screened for altered biofilm phenotypes (77). This screen yielded an insertion in the *nhaR* open reading frame (*nhaR::cam*), which was determined by transduction to be linked to the biofilm-deficient phenotype of this strain (96B10). The gene product of *nhaR* is a 301-amino-acid DNA-binding

protein of the LysR family, which is involved in adaptation to elevated Na<sup>+</sup> and alkaline pH and activates expression of the *nhaAR* operon (10, 22, 49, 57). Transduction of the *nhaR::cam* mutation into both MG1655 and its isogenic *csrA::kan* mutant caused growth inhibition under the combination of high sodium (0.6 M NaCl) and high pH (8.5) on solid media, but growth was normal on LB. The growth defect was restored by complementation with pNhaR, a plasmid clone of *nhaR* (data not shown). The *nhaR* mutant exhibited normal motility (data not shown).

Disruption of *nhaR* significantly decreased biofilm formation in both MG1655 (4-fold) (Fig. 1A, compare bars 1 and 2) and its isogenic *csrA* mutant (>10-fold) (compare bars 5 and 6). A plasmid containing *nhaR* (pNhaR) complemented the biofilm defect of *nhaR::cam* mutants (Fig. 1A and data not shown), confirming the role of this gene in biofilm formation. In fact, this plasmid enhanced biofilm formation beyond that formed

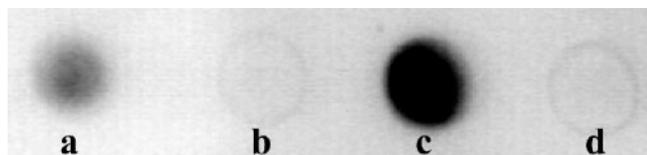


FIG. 2. Effect of *nhaR* mutation on accumulation of PGA. Cell lysates were prepared from a series of isogenic strains and analyzed by immunoblotting with an anti-PIA antiserum (see Materials and Methods). Sample identities are as follows: (a) parent (TRMG); (b) *nhaR::cam*; (c) *nhaR::cam(pNhaR)*; (d)  $\Delta$ *pgaABCD*. This experiment was repeated in entirety three times, with essentially identical results. A representative blot is shown.

by the wild-type strain (Fig. 1A, compare bars 1 and 4). Disruption of *nhaR* also compromised the ability of these strains to adhere to borosilicate glass coverslips, even after 60 h of incubation (Fig. 1B and data not shown), demonstrating that these mutants are not simply delayed in biofilm development. Multilayered biofilms were not formed by *nhaR* mutants, and these strains were defective in making the transition from temporary to permanent attachment (data not shown), as described previously (2). Consistent with the known response of NhaR to sodium levels, biofilm formation in MG1655 was inducible (~5-fold) by the addition of NaCl (100 mM) to L broth (Fig. 1C). The *nhaR* mutant showed more modest effects (~2.5-fold), which were complemented in *trans* by pNhaR or a similar plasmid expressing a hexahistidine-tagged NhaR protein (Fig. 1C). Because *nhaR* was expressed from a *tac* promoter in the latter example, it is clear that activation via NaCl does not simply reflect increased *nhaAR* expression by this autoregulatory protein. This *nhaR* mutation caused strains to become defective for biofilm formation at 26°C or 37°C in colonization factor antigen medium, in unbuffered LB, or in LB buffered to pH 6.4, 7.4, or 8.4 (data not shown). The altered attachment behavior of the *nhaR* mutant was similar to that of *pga* mutants, which do not produce the polysaccharide adhesin PGA (2, 77).

**Regulation of biofilm formation by NhaR depends on the *pgaABCD* operon.** Next, we conducted experiments to gain insight into the mechanism by which NhaR affects biofilm formation. Plasmidic expression of *nhaR* in an *nhaA* mutant strain increased biofilm, establishing that NhaR effects on biofilm were mediated independently of the  $\text{Na}^+/\text{H}^+$  antiporter NhaA (data not shown). The biofilm defect of an *nhaR* mutant was restored by ectopic expression of *pgaABCD* from a multi-copy plasmid (Fig. 1D, compare bars 3 and 7). However, biofilm formation in a strain with the *pgaABCD* operon deleted was not affected by ectopic expression of *nhaR* (data not shown). These results are consistent with the possibility that *nhaR* might be needed for the production of PGA.

**An *nhaR* mutant does not accumulate PGA.** To examine the effect of NhaR on PGA accumulation, relative levels of cell-bound PGA were compared by immunoblot analysis of *nhaR* wild-type and isogenic mutant strains, as well as a control strain with *pgaABCD* deleted. Parental and complemented *nhaR* mutant strains (Fig. 2a and c, respectively) produced anti-PIA-reacting material, whereas *nhaR::cam* and  $\Delta$ *pgaABCD* mutants failed to react with the antiserum (Fig. 2b and d, respectively).

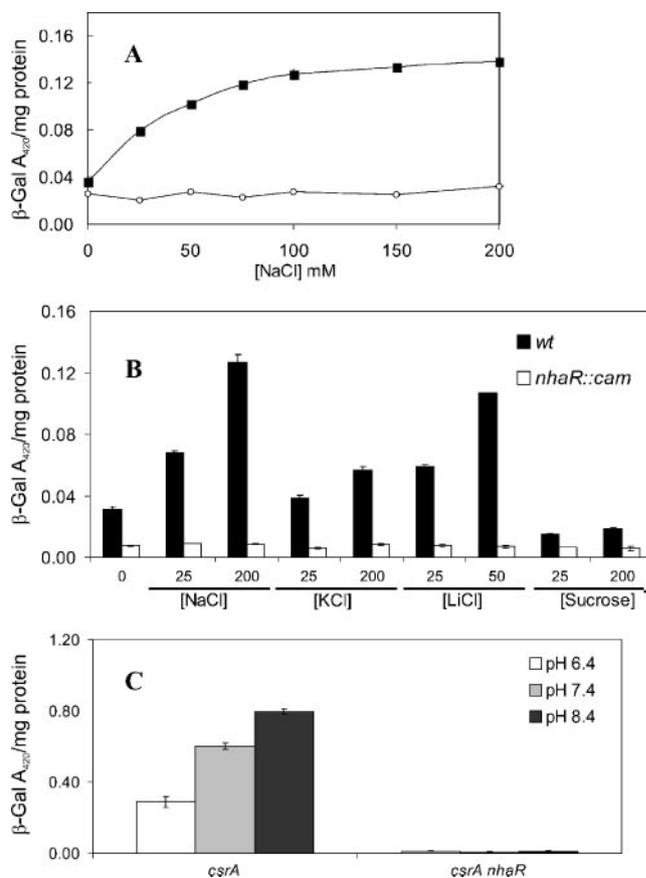


FIG. 3. Effects of *nhaR::cam*, monovalent cations and pH on expression of a *pgaA'*-*lacZ* translational fusion. (A) Activity of the *pgaA'*-*lacZ* chromosomal fusion in XWZ4 (■) and its isogenic *nhaR* mutant (CGCFR) (□) after 14 h of growth at 26°C in L broth (LB) without NaCl or in L broth supplemented with the indicated concentrations of NaCl. (B) Activity of the reporter fusion in L broth containing the indicated concentrations (mM) of NaCl, KCl, LiCl, or sucrose. wt, wild type. (C) Effect of pH on *pgaA'*-*lacZ* expression. Strains were grown for 24 h at 26°C in LB buffered with 60 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane, and the pH was adjusted to the indicated values with HCl. Values represent the means for three separate reactions  $\pm$  standard errors of the means. Error bars smaller than the symbols are not visible. These experiments were repeated at least three times with similar results.

In addition, disruption of *nhaR* prevented the accumulation of PGA in the culture supernatant (data not shown).

**NhaR is required for induction of *pgaA'*-*lacZ* expression by monovalent cations and alkaline pH.** To test whether NhaR regulates the *pgaABCD* operon itself, expression of a chromosomally carried *pgaA'*-*lacZ* translational fusion containing the upstream noncoding region through the initiation codon of *pgaA* (76) was examined in *nhaR* wild-type and mutant strains. This reporter fusion was activated by increasing concentrations of NaCl in the *nhaR* wild-type strain but not in the isogenic mutant (Fig. 3A).

Previously, NhaR was found to activate gene expression in response to increasing [NaCl], [KCl], [LiCl], or pH of the medium (23, 70). We found that increasing [NaCl], [LiCl], and alkaline pH also activated expression of *pgaA'*-*lacZ* in an *nhaR*-dependent fashion (Fig. 3B and C). Addition of KCl or

MgCl<sub>2</sub> at the same osmolarity as NaCl weakly induced *pgaA'*-*'lacZ* expression, whereas sucrose or CaCl<sub>2</sub> did not (Fig. 3B and data not shown), suggesting that this response is not due to increasing osmolarity or Cl<sup>-</sup> concentration.

Previous studies indicated that transcription of *nhaAR* in *E. coli* increases as the pH of the medium is increased from 6.5 to 8.5 (22). Furthermore, exposure of *Shewanella oneidensis* to alkaline pH caused upregulation of *nhaA* and *nhaR* transcripts (41). Thus, we grew cultures at pH 6.4, 7.4, and 8.4 in LB buffered with 60 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (22) and examined the expression of the *pgaA'*-*'lacZ* fusion. This expression increased from pH 6.4 up to 8.4 in a *csrA* mutant strain and was virtually eliminated by disruption of *nhaR* (Fig. 3C). Biofilm formation in the strain increased modestly from pH 6.4 to 7.4 but, in contrast to *pgaA'*-*'lacZ* expression, decreased slightly in medium at pH 8.4 (data not shown). In *csrA* wild-type strains, expression of the fusion also increased modestly from pH 6.4 to 7.4 and then decreased modestly at pH 8.4, more closely reflecting the results of biofilm assays in this strain (data not shown). Expression of *csrA'*-*'lacZ* and *csrB-lacZ* fusions did not vary substantially at these pH levels (data not shown). This suggests that the *csrA* and *csrB* genes, which posttranscriptionally control *pgaABCD* expression, were not responsible for the effects of pH on the *pgaABCD* operon.

**Examination of other possible influences on *pga* expression.** H-NS regulates many stress response genes by acting as a major component of chromatin and by responding to changes in osmolarity (5, 21). Furthermore, *hns* mutants are derepressed for *nhaAR* expression (22). We observed that *pgaA'*-*'lacZ* expression was only slightly increased (25 to 30%) and was still inducible by NaCl in an *hns* mutant strain background (data not shown), suggesting that HN-S is not a major regulatory determinant of *pga* gene expression. Mutations eliminating the stationary-phase sigma factor RpoS and the response regulator OmpR did not affect *pgaA'*-*'lacZ* expression (data not shown). However, OmpR modestly activates biofilm formation in these strains (reference 38 and data not shown). Thus, the modest induction of biofilm formation by NaCl in an *nhaR* mutant (Fig. 1C) may be due to the effect of OmpR. A transposon insertion that was isolated during the course of these studies in the *rscD* (*yojN*) gene of the RcsC-RcsD-RcsB signal transduction system affected biofilm formation but not *pgaA'*-*'lacZ* expression (data not shown), indicating that the Rcs regulatory system, which responds to osmolarity and envelope stress, does not affect *pga* expression. In addition, we previously determined that *cpxR* does not affect biofilm formation by MG1655 or its isogenic *csrA* mutant (3).

***nhaR* affects *pga* transcript levels in vivo.** Our previous studies revealed that (i) the *pgaABCD* genes are cotranscribed as an operon, (ii) the 5' end of the *pgaABCD* transcript corresponds to an A residue 234 nucleotides upstream from the *pgaA* initiation codon, and (iii) all four coding regions of this transcript are elevated in a *csrA* mutant (76). Primer extension analysis (Fig. 4, compare lanes 1 and 5) further demonstrated that this transcript was absent from *nhaR* mutants (lanes 2, 3, 6, and 7) and was restored upon complementation by a multicopy plasmid clone of *nhaR* (lanes 4 and 8). *pga* transcript levels were positively correlated with the amounts of biofilm formed by these strains (Fig. 4 and 1A and data not shown).

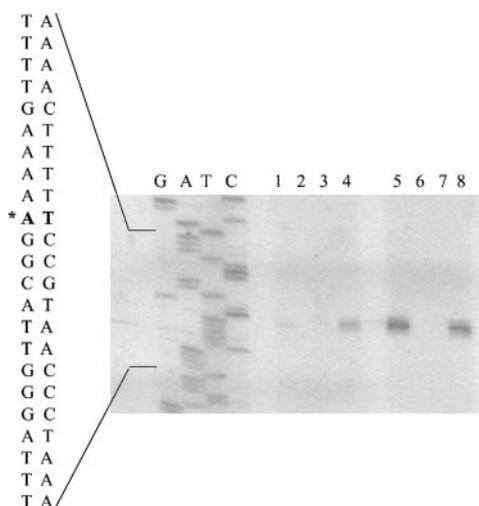


FIG. 4. Primer extension analysis of *pgaA* transcript in *nhaR* wild-type and mutant (*nhaR::cam*) strains. Cultures were grown at 26°C with shaking and harvested for RNA isolation in the late exponential phase of growth. Strain identities are as shown in Fig. 1A. The dideoxy sequencing ladder (lanes G, A, T, and C) was generated with PEXT3, using pPGA372 as a template. The 5' end of the *pgaA* transcript is marked with an asterisk.

q-RT-PCR was used to quantitate the effect of *nhaR* on steady-state levels of the *pgaA* transcript (see Materials and Methods). Disruption of *nhaR* decreased *pgaA* transcript levels 200-fold in both the *csrA* wild-type and mutant backgrounds.

**Purified recombinant NhaR binds specifically to *pgaABCD* promoter DNA.** To determine whether NhaR-His<sub>6</sub> binds to *pgaA* promoter DNA, gel mobility shift assays were conducted using a 132-bp labeled DNA fragment containing the promoter region and putative NhaR binding sites of *pgaA*. Binding produced a single shift with an apparent *K<sub>d</sub>* of 0.21 μM (Fig. 5), which is within the range of at least one other LTTR (7). The Hill coefficient was determined to be 1.28 ± 0.17, suggesting that the binding might be weakly cooperative. Binding was not affected by the presence or absence of 50 and 100 mM NaCl, KCl, or LiCl in the reaction mixtures (data not shown). Competition studies confirmed the specificity of this binding (Fig. 5B).

LTTRs generally bind degenerate inverted repeats that have a T-N<sub>11</sub>-A motif and are found upstream of -35 promoter elements (64). Comparison of the putative *pgaABCD* promoter region with NhaR binding sites of *nhaA* (10) and *osmC* (68) revealed two T-N<sub>11</sub>-A motifs, one overlapping the -35 promoter element and a second further upstream (Fig. 6E and data not shown).

**DNase I footprinting of NhaR binding to *pgaA* promoter DNA.** To more precisely define the NhaR binding sites on the *pgaA* promoter, DNase I footprint analysis was conducted with NhaR-His<sub>6</sub> and a DNA fragment (from -115 to +18 with respect to the 5' end of the *pga* transcript). NhaR protected a region spanning ~60 bp which overlapped and extended upstream from the putative -35 promoter region (Fig. 6). Within this protected region, two apparent binding sites with dyad symmetry were separated by a small region that became hypersensitive to DNase I digestion in the presence of NhaR, suggestive of an altered conformation. The extended region of

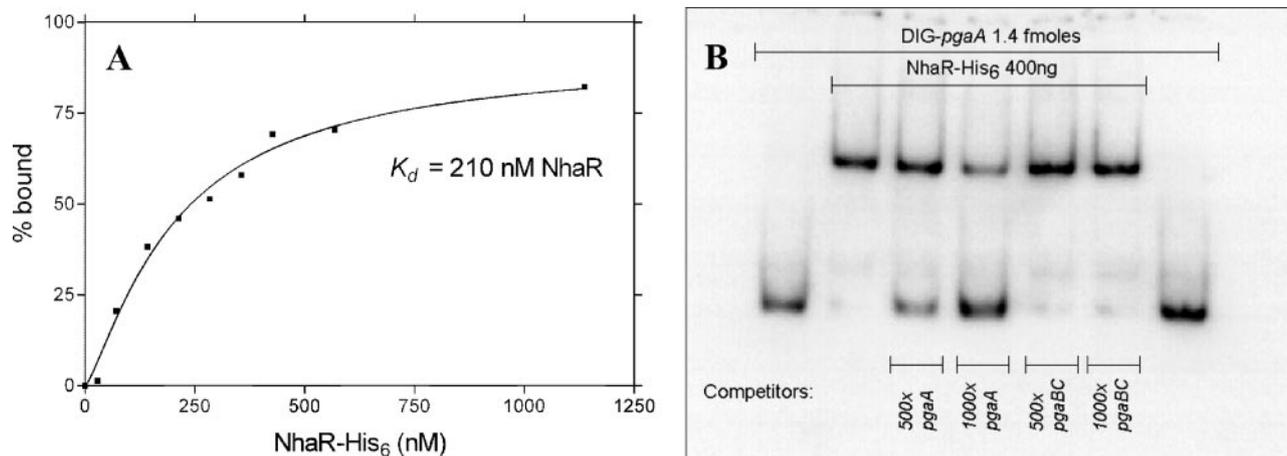


FIG. 5. Purified NhaR-His<sub>6</sub> binds specifically to *pgaABCD* promoter DNA. (A) A 132-bp PCR product containing the promoter region and putative NhaR binding site of *pgaA* was 3' labeled with DIG-11-ddUTP and used for gel mobility shift assays. The apparent equilibrium binding constant ( $K_d$ ) was determined as described in Materials and Methods. (B) Five hundred- and 1,000-fold molar excesses of unlabeled specific DNA (*pgaA*) and nonspecific DNA (*pgaBC*) were used as competitors in the binding reactions.

protection was seen on both strands and is characteristic of LysR-type transcriptional regulators (11, 64, 72). In addition, a hypersensitive region immediately downstream of the -10 element also became evident in the presence of NhaR. Addition

of 50 mM NaCl had no consistent effect on the footprint (compare Fig. 6A and C and Fig. 6B and D).

**NhaR is required for in vitro transcription of *pgaABCD*.** To further examine the role of NhaR in *pga* expression, in vitro

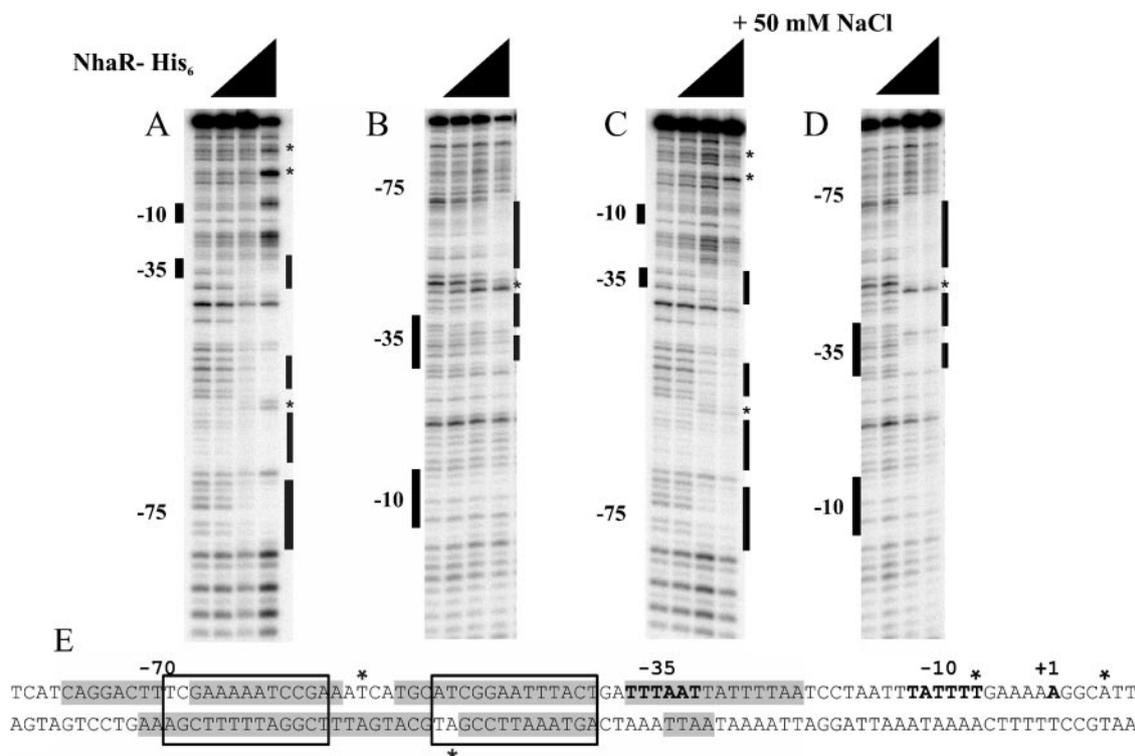


FIG. 6. DNase I protection footprint of NhaR on the *pgaA* promoter. A 132-bp PCR product containing the promoter region of *pgaA* (-115 to +18) was 5' end labeled with <sup>32</sup>P on the top (A and C) or bottom (B and D) strand, as diagrammed in panel E, and subjected to DNase I footprinting in the presence of increasing concentrations of NhaR-His<sub>6</sub> (0, 0.29, 0.57, and 1.14 μM). The binding reaction mixtures contained no NaCl (A and B) or 50 mM NaCl (C and D). Protected regions are marked by vertical lines adjacent to the sequences. Hypersensitive sites are indicated by asterisks. Numbers depict positions relative to the initiating nucleotide (+1). These footprints were repeated four times, with no significant difference in the protection pattern observed in the presence or absence of NaCl. (E) Summary of DNase I footprinting. Protected regions are shaded gray; boxed sequences contain a LysR-type transcriptional regulator binding motif (T-N<sub>11</sub>-A). The -35, -10, and +1 promoter elements are in boldface. Hypersensitive sites are indicated by asterisks.

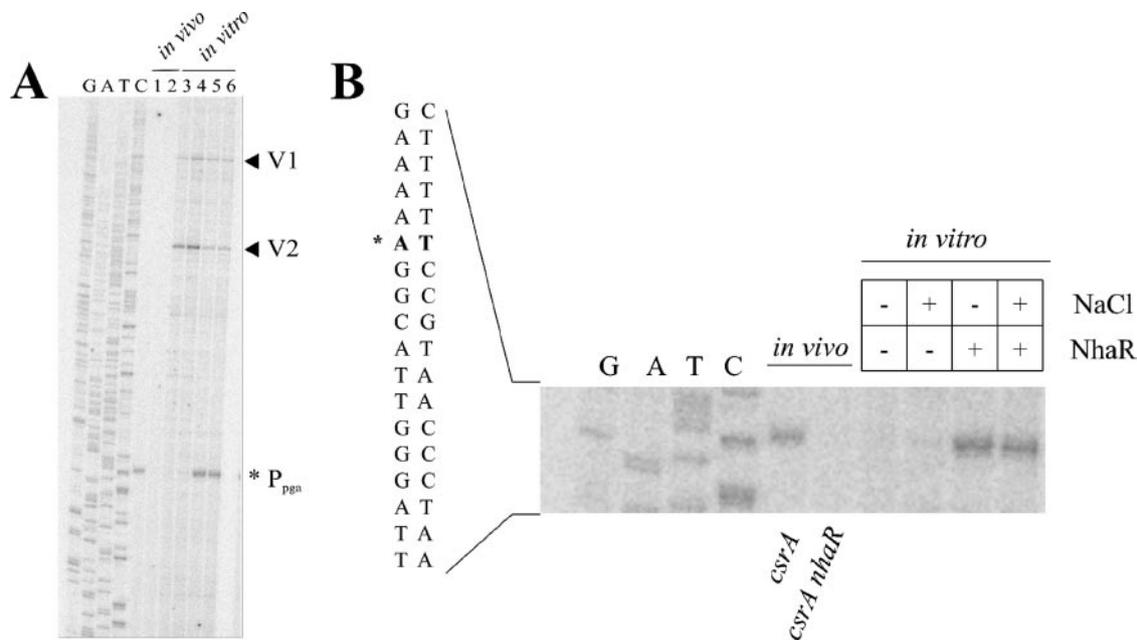


FIG. 7. NhaR-His<sub>6</sub> activates in vitro transcription from the *pgaA* promoter. NhaR-His<sub>6</sub> and  $\sigma^{70}$ -saturated RNA polymerase were used for in vitro transcription of plasmid DNA containing the *pgaABCD* operon (pPGA372). The resulting transcripts were analyzed by primer extension (see Materials and Methods). The full image (A) and a close-up (B) obtained from a single gel are shown. In the full gel image, two transcripts originating from the vector are indicated by arrowheads labeled V1 and V2; an asterisk denotes the 5' end of the transcript originating from *pgaA* (P<sub>pga</sub>). Identical results were obtained when 25 mM KCl was used instead of NaCl in the transcription reaction (data not shown). The first two lanes (in vivo) depict primer extension analysis of total cellular RNAs isolated from the indicated strains. The last four lanes (in vitro) correspond to transcription reactions conducted in the presence or absence of NhaR (455 nM) and NaCl (25 mM).

transcription of a *pgaABCD*-containing supercoiled plasmid template by  $\sigma^{70}$ -saturated *E. coli* RNA polymerase was determined in the presence or absence of NhaR-His<sub>6</sub>. Recombinant NhaR was necessary and sufficient to activate in vitro transcription from the *pga* promoter (Fig. 7). The 5' end of the NhaR-dependent in vitro transcript was identical to that observed in vivo. The intensities of two transcripts originating from vector DNA (V1 and V2) did not increase in the presence of NhaR (Fig. 7A). This indicates that NhaR was not acting as a general activator of transcription. Addition of 25 mM NaCl or KCl did not affect these reactions (Fig. 7 and data not shown).

**NhaR is conserved in the Enterobacteriaceae.** BLAST analyses revealed that homologs of *nhaAR* are present in the genomic sequences of most *Enterobacteriaceae* (82% of total), including pathogens such as *E. coli* O157:H7 (99% NhaR identity), *Yersinia pestis* (79%), and *Erwinia carotovora* (77%), which also contain loci homologous to the *pgaABCD* operon of *E. coli* (77). The genes of the *pgaABCD* operon encode proteins necessary for the synthesis and possibly the subcellular localization of polymeric PGA to the cell envelope (77). This polymer promotes biofilm formation in *E. coli* and several other species (36, 40). Furthermore, PGA-like polysaccharides are important determinants of disease transmission and virulence in *Yersinia pestis* and staphylococci, respectively (cited in reference 77). Additional investigation will be required to determine whether these species, which inhabit distinctly different environmental niches than *E. coli* K-12, utilize similar strategies for regulating PGA production and biofilm formation in response to environmental cues.

**Conclusions.** The present study demonstrates that the LysR-type transcriptional regulator NhaR is necessary and sufficient to activate transcription from the *pgaABCD* promoter of *E. coli* K-12 (Fig. 7) and consequently activates PGA production (Fig. 2) and biofilm formation (Fig. 1). It is evident that NhaR activates expression of its target operons, *nhaAR*, *osmC*, and *pgaABCD*, as a coordinated response to elevated Na<sup>+</sup> and/or pH. Consequently, NhaR is required for survival under high concentrations of NaCl, high pH, and certain oxidative stresses (49, 70). While the full ramifications of our findings remain to be determined, the regulatory role of NhaR in adhesion and biofilm formation is consistent with the idea that biofilm formation itself provides protection against a variety of biological and chemical stresses (13–15, 17, 19) and further suggests that NhaR is a stress response regulator of substantial importance.

These studies also demonstrate that monovalent cation concentrations, distinct from osmolarity, can be an important regulatory cue for biofilm formation by a gram-negative bacterium (Fig. 1 and 3). Surprisingly, activation of *pga* in vitro transcription by NhaR did not require monovalent cations (Fig. 7). As LTTR proteins often bind to their DNA targets in the absence of coinducer ligands (11, 64, 71), it was not surprising that NhaR bound to *pga* promoter DNA without the addition of salts. Likewise, it was not unexpected to find that the DNase I footprint pattern of NhaR was not altered by the addition of salts (Fig. 6) (10, 23). It is tempting to suggest that NhaR might not bind directly to its coinducer cations but might function similarly to the LTTR protein GcvA. In this case, the binding of glycine to GcvR prevents this protein from interacting with

GcvA to block activation (26, 32). Further studies will be required to assess this or other mechanisms that could explain the discrepancy between *in vitro* and *in vivo* cation requirements of NhaR.

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Kane Biotech, Inc., may develop applications related to the findings herein. T. Romeo serves as chief scientific advisor for, owns equity in, and may receive royalties from this company. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict-of-interest policies.

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