JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 589–594
0021-9193/90/020589-06$02.00/0

Vol. 172, No. 2

Expression of the *Pseudomonas aeruginosa toxA* Positive Regulatory Gene (*regA*) in *Escherichia coli*

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Received 12 June 1989/Accepted 23 October 1989

The *regA* gene is a positive regulatory gene that regulates toxin A production in *Pseudomonas aeruginosa* at the transcriptional level. The product of the *regA* gene was examined in *Escherichia coli* with the expression vector pT7-7. A 1.3-kilobase *AvaI-HindIII* fragment containing the *regA* gene was cloned into the pT7-7 vector. A recombinant plasmid (pAH1) encoded a 29-kilodalton protein. The molecular weight of this protein correlated closely with the predicted molecular weight of the RegA protein. Production of the RegA protein in *E. coli* required both an *E. coli* promoter and an *E. coli* ribosome-binding site. Two in-frame deletion derivatives in which certain regions of the *regA* gene were expressed from the T7 promoter encoded 26- and 18-kilodalton fusion proteins, respectively. The RegA protein and the two fusion proteins were localized to the inner membrane of *E. coli*. Neither RegA protein nor the two fusion proteins showed DNA-binding activity to the 410-base-pair fragment containing the upstream region of *toxA* when synthesized in *E. coli*.

Toxin A is one of several extracellular virulence factors produced by *Pseudomonas aeruginosa* (41). The 66-kilodalton (kDa) mature protein is an ADP-ribosyl transferase (14). It catalyzes the transfer of the ADP-ribosyl moiety of oxidized NAD onto elongation factor 2 of eucaryotic cells, causing the inhibition of protein synthesis (14).

Toxin A production in *P. aeruginosa* is controlled by different factors, including the level of iron in the growth medium, the growth temperature of the culture, and the presence or absence of certain nucleotides in the growth medium (17). Maximum level of toxin production is obtained when *P. aeruginosa* is grown in an iron-limited medium (2, 27). Recent studies proved that iron affects toxin A production at the transcriptional level (4, 7, 18).

Hedstrom et al. (11) isolated a toxin A positive regulatory gene which increases toxin A production in different *P. aeruginosa* strains. Later studies provided evidence that this gene (*regA*) positively regulates toxin A production in *trans* at the transcriptional level (4, 42). Hindahl et al. (13), through subcloning and complementation analysis, localized the *regA* gene to a 1.9-kilobase (kb) *PstI-XhoI* fragment. DNA sequence analysis of the 1.9-kb *PstI-XhoI* fragment revealed the presence of a 777-base-pair (bp) major open reading frame (12). These and other studies suggested that the *regA* gene codes for a *trans*-acting regulatory protein (13, 42).

In this study, using the pT7 expression vector, we provided evidence that the *regA* gene product is a 29-kDa protein which is localized to the inner membrane of *Escherichia coli*. Synthesis of the RegA protein in *E. coli* requires both an *E. coli* promoter and a ribosome-binding site. We also showed that RegA protein when synthesized in *E. coli* lacks the ability to bind to the upstream region of *toxA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 (*hsd-20 recA13 ara-14 proA2 lac-41 galK2 mtl-1 xyl-5 supE44 rpsL2*) (21) was used as a host for all initial cloning experiments. For the expression of the pT7-7 vector, *E. coli* K38 (HfrC λ) containing plasmid pGP1-2 was used (38). Plasmid pGP1-2 carries the T7 RNA polymerase gene (38). The expression vector pT7-7 was derived from vector pT7-5 (19, 34, 38). Plasmid pT7-7 contains the T7 RNA polymerase promoter Φ10 and the translation start site for the T7 gene protein 10 in front of the multiple cloning site (Stan Tabor, Harvard Medical School, personal communication). The transcription of the T7 promoter in both pT7-5 and pT7-7 vectors is in the opposite orientation to the β-lactamase gene.

Plasmid constructions. Plasmid pMH220 (13) was used as a source of the *regA* gene. Recombinant plasmid pMH220 carries the biologically active *regA* gene on a 1.3-kb *AvaI-PstI* fragment cloned into pUC18 (13). A 777-bp open reading frame region was localized within this fragment. For suitable cloning in the pT7-7 vector, the *AvaI-PstI* fragment was removed from pMH220 as an *AvaI-HindIII* fragment (using the *HindIII* site of the pUC18 vector) (Fig. 1) and cloned into the *SalI-HindIII* sites of pT7-7. This generated plasmid pAH1 (Fig. 1).

Two in-frame deletion derivatives, pAH2 and pAH3, were generated from pAH1. To construct pAH2, plasmid pAH1 was digested with *NcoI* and *EcoRI* enzymes and the recessed ends were filled in with the *E. coli* DNA polymerase I (Klenow fragment) (21) and religated (Fig. 1). In plasmid pAH2, the 40-bp upstream region of the *regA* gene and the region coding for the first 29 amino acids were deleted. Therefore, the fusion protein encoded by pAH2 would contain the last 230 amino acids of the RegA protein plus the first 4 amino acids encoded by the pT7-7 vector (Table 1). No change was made in the amino acid residues of RegA protein in the fusion protein encoded by pAH2 (Table 1). Plasmid pAH3 was generated by completely digesting pAH1 with *BamHI*, filling in the recessed ends, and religating (Fig. 1). In plasmid pAH3, the upstream region of the *regA* gene and the region coding for the first 111 amino acids were deleted. Thus, the fusion protein encoded by pAH3 contains the last 148 carboxy-terminal amino acids of the RegA protein plus the first 9 amino acids encoded by pT7-7. The arginine residue (111) of the RegA protein was substituted with serine in the fusion protein encoded by pAH3 (Table 1).

Expression of *regA* gene in pT7-7 vector. Analysis of the

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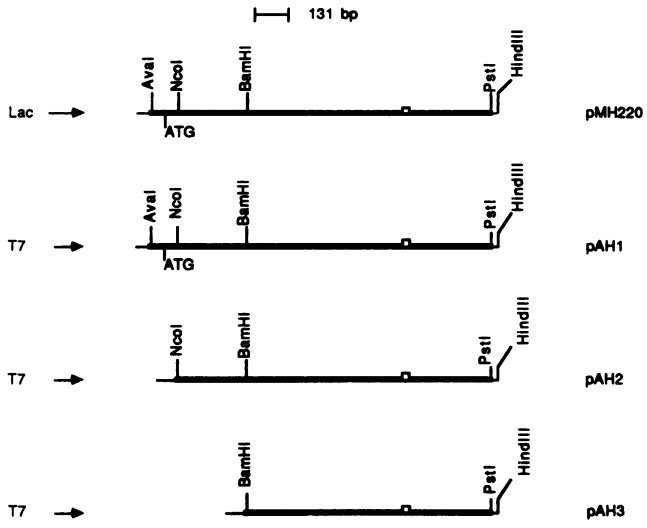

FIG. 1. Restriction map of recombinant plasmids containing *regA* gene. Arrows indicate the direction of transcription of the *lac* promoter in pMH220 and the direction of transcription of the T7 promoter in pAH1, pAH2, and pAH3. Thick lines indicate the 1.3-kb *Ava*I-*Hin*dIII fragment of the *P. aeruginosa* PA103 chromosomal DNA containing the *regA* gene (see text). □, Position of the translation stop codon of the *regA* gene.

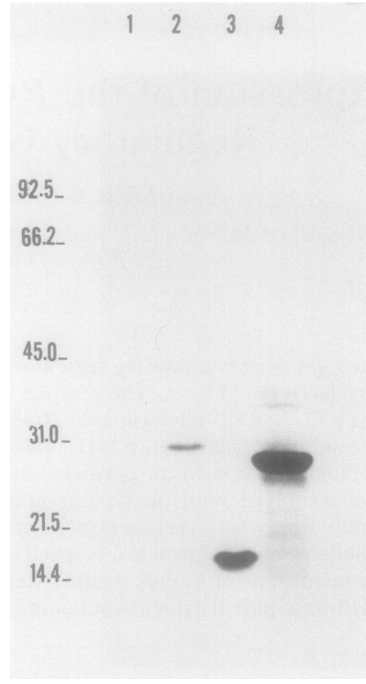

FIG. 2. Translational products of *regA* gene and its deletion derivatives in *E. coli* K38(pGP1-2). Cells containing recombinant plasmids were grown and labeled with [^{3}H]leucine as described previously (38). Protein products were analyzed in 10 to 20% SDS-polyacrylamide gels and by autoradiography. Lanes: 1, K38(pGP1-2)(pT7-7) (vector alone, negative control); 2, K38(pGP1-2)(pAH1); 3, K38(pGP1-2)(pAH3); 4, K38(pGP1-2)(pAH2). The sizes of the molecular mass standards (in kilodaltons) are shown on the left side of the autoradiogram.

translational product of the cloned *regA* gene in pT7-7 was done as described previously (38). K38(pGP1-2) containing the recombinant plasmids was grown at 30°C to an optical density at 590 nm of 0.5 in L broth (22) containing ampicillin and kanamycin (50 μg/ml). A 250-μl sample of the culture was pelleted, washed with M-9 medium (22), and resuspended in 1 ml of M-9 medium containing all amino acids but leucine. After 2 h of growth at 30°C, the culture was shifted to 42°C for 10 min, rifampin was added (200 μg/ml), and the incubation continued for an additional 15 min. The culture was then shifted back to 30°C for 20 min and was pulsed with 50 μCi of [^{3}H]leucine (50 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 10 min. We used [^{3}H]leucine instead of [^{35}S]methionine because the predicted amino acid sequence of the RegA protein contains only one methionine residue (12). Cells were recovered by centrifugation, solubilized in 50 μl of digestion buffer (60 mM Tris hydrochloride [pH 6.8], 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) at 100°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis with 10 to 20% gradient gels (31). After electrophoresis, the gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-AR film.

Cell fractionation and membrane separation. K38(pGP1-2) carrying recombinant plasmids was grown to an optical density at 590 nm of 1.5 at 30°C in M-9 medium containing all amino acids but leucine. Ampicillin and kanamycin were added to the medium at a concentration of 50 μg/ml. The culture was shifted to 42°C for 15 min, rifampin was added to a final concentration of 200 μg/ml, and the incubation continued for an additional 10 min. The culture was then shifted to 37°C for 2 h, pulsed with 50 μCi of [^{3}H]leucine per ml (50 Ci/mmol) for 20 min, and harvested. Cells were fractionated by the cold osmotic shock procedure (15). After removal of the periplasmic fractions, shocked cells were lysed by passing them twice through French pressure cell (SLM Instruments, Inc., American Instruments Co., Urbana, Ill.) at 10,000 lb/in^2. The inner and outer membranes were separated on a 50 to 70% (stepwise) sucrose gradient as described previously (10). At the end of the gradient centrifugation, bands representing inner and outer membranes were removed from the gradient tubes by suction from above and washed several times in distilled water. Membrane fractions were analyzed on 10 to 20% SDS-polyacrylamide gradient gels (31).

DNA binding experiments. The DNA probe used in this study was the 410-bp *Eco*RV-*Nru*I fragment containing the upstream region of *toxA*. Plasmid pMS151 was used as a source of the DNA probe. Plasmid pMS151 is a recombinant plasmid which contains a 2.4-kb *Eco*RV-*Eco*RI fragment of *P. aeruginosa* PAK DNA (carrying intact *toxA*) cloned into the *Sma*I-*Eco*RI sites of pUC18 (19) (Stephen Lory, University of Washington, Seattle, personal communication) (see Fig. 5A). To examine toxin A production by pMS151, we subcloned the plasmid into the *P. aeruginosa* vector PKT230 and the recombinant plasmid was introduced into the *P. aeruginosa* hypotoxigenic mutant PAO-T1 (26). Toxin A production by pMS151 in PAO-T1 was efficiently regulated by the level of iron in the growth medium (A. Hamood and B. Iglewski, unpublished data). This showed that this 410-bp *Eco*RV-*Nru*I fragment contains all the necessary *toxA* sequences required for the iron regulation of toxin A production in *P. aeruginosa*. The 410-bp fragment was removed as a *Pst*I-*Nru*I fragment (using the *Pst*I site in the multiple cloning region of pUC18) (see Fig. 5A) and was end labeled with [γ-^{32}P]ATP (>3,000 Ci/mmol) with T4 polynucleotide kinase as described by Maniatis et al. (21). The unincorporated label was removed with Nensorb 20 Cartridges (Dupont, NEN Research Products, Boston, Mass.). K38(pGP1-

TABLE 1. Structure of the fusion proteins encoded by pAH2 and pAH3 plasmids in *E. coli* K38(pGP1-2)[a]

Plasmid	Amino acid and nucleotide sequences of fusion regions								Change of amino acids in fusion proteins
	Met	Ala	Arg	Ile	His	Gly	Ile	Tyr	
pAH2	A-T-G	G-C-T	A-G-A	A-T-T ↕	C-A-T	G-G-C	A-T-C	T-A-T	No change
					30	31	32	33	
	Arg	Ala	Arg	Gly	Ser	Ile	Leu	Ala	
pAH3	C-G-C	G-C-C	C-G-G	G-G-A	T-C- ↕ G	A-T-C	C-T-G	G-C-C	Arginine (111) → serine
						112	113	114	

[a] Vertical arrows indicate the junction region of the pT7-7 vector and the *regA* gene in each plasmid. Numbers below amino acids indicate the position of these amino acids in the deduced amino acid sequence of RegA protein.

2) containing recombinant plasmids was grown according to the same protocol we used for the expression of the *regA* gene in the pT7-7 vector, except that the labeling of cells with [^{3}H]leucine was omitted. The membrane fractions and the lysate were prepared as described above.

The membrane fractions and the lysate of K38(pGP1-2) containing recombinant plasmids were dialyzed against three changes of the DNA binding buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 10 mM KCl, 0.1 mM dithiothreitol, 5% glycerol, 50 μg of bovine serum albumin per ml), divided into 50-μl aliquots, and stored at −70°C. The DNA binding experiments were done as described by Fried and Crothers (5). The DNA-binding activity was determined by the reduction in the electrophoretic mobility of the labeled DNA fragment. The end-labeled *Pst*I-*Nru*I fragment was incubated with 30 to 40 μg of protein of the inner membrane fractions and lysates of K38(pGP1-2) containing recombinant plasmids in the DNA binding buffer for 30 min at 22°C. Immediately after that, the samples were run on 5% polyacrylamide gels in 1× TBE buffer (0.089 M Tris [pH 8.0], 0.089 M boric acid, 0.002 M EDTA). At the end of the run, the gels were dried and exposed to Kodak X-AR films. As a positive control, the 410-bp probe was incubated with purified *P. aeruginosa* RNA polymerase (a kind gift from A. Kroppinski, Queen's University, Kingston, Ontario, Canada).

RESULTS AND DISCUSSION

Expression of *regA* gene. We were not successful in our previous attempts to detect the product of the *regA* gene using an *E. coli* minicell system or an in vitro cell-free transcription-translation system (cell-free lysate). We detected no translational product when we tried to express the *regA* gene from the T7 promoter by cloning the 1.3-kb *Ava*I-*Hin*dIII fragment containing the *regA* gene into the pT7-5 expression vector (19, 34; data not shown). We also examined the expression of the *regA* gene in *E. coli* using the pT7-7 expression vector. Plasmid pT7-7 is the same as pT7-5, but in addition to the T7 RNA polymerase promoter Φ10, pT7-7 contains the translation start site for the T7 gene protein 10 (Fig. 3) (Stanly Tabor, personal communication). The 1.3-kb *Ava*I-*Hin*dIII fragment was cloned into the *Sal*I-*Hin*dIII sites of the pT7-7 vector, generating plasmid pAH1 (Fig. 1). Plasmid pAH1 produced a 29-kDa protein (Fig. 2). The molecular weight of this protein correlated closely with the predicted molecular weight of the RegA protein (based on nucleotide sequence analysis). The 1.3-kb *Ava*I-*Hin*dIII fragment carried in pAH1 contains a 777-bp open reading frame, which codes theoretically for a 28.824-kDa protein (12). To confirm the cloning of the *regA* gene in pT7-7 (owing to the presence of a 1-bp mismatch between the *Ava*I site of *regA* and the *Sal*I site of pT7-7), we sequenced the pT7-*regA* junction region in pAH1. Nucleotide sequence analysis of the pT7-*regA* junction region in pAH1 revealed the formation of an open reading frame between the multiple cloning area of pT7-7 and the upstream region of the *regA* gene (Fig. 3). This open reading frame, which codes for a 20-amino-acid peptide, terminated at the TAG codon 11 bp upstream of the RegA protein initiation codon (ATG) (Fig. 3). Thus, the *E. coli* ribosome would

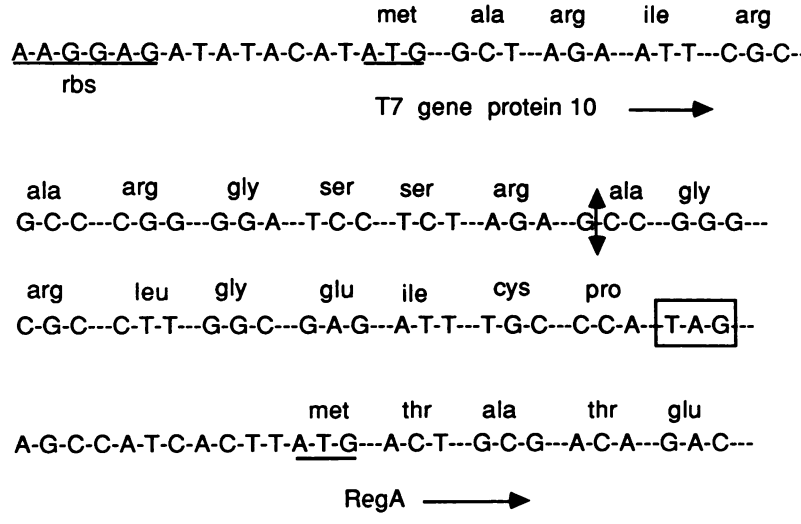

FIG. 3. Nucleotide sequence of the junction region of the pT7-7 vector and *regA* gene in plasmid pAH1. Vertical arrow indicates the point of the junction. The TAG termination codon is boxed.

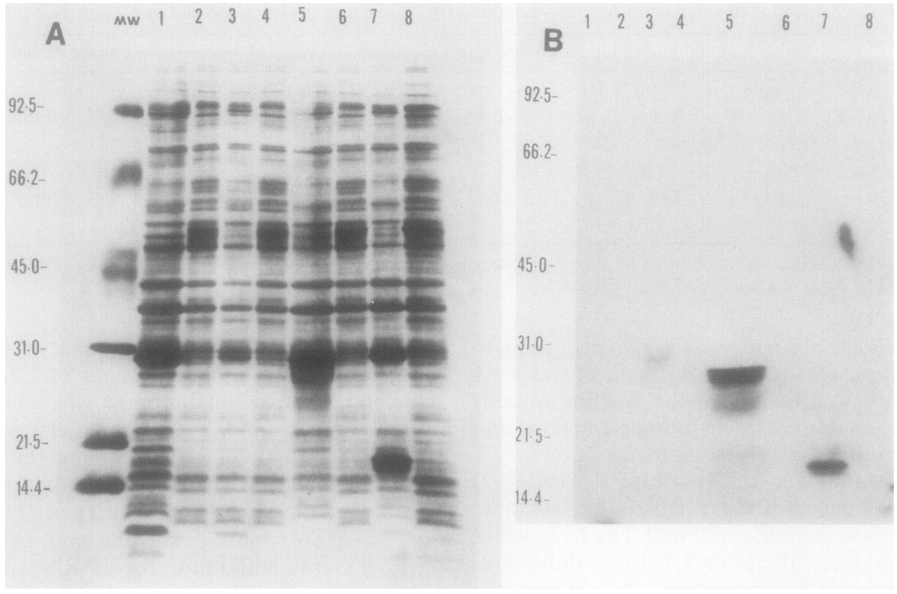

FIG. 4. Localization of RegA protein (encoded by pAH1) and the fusion proteins (encoded by pAH2 and pAH3) in the membranes of *E. coli* K38(pGP1-2). Cells were grown and labeled with [^{3}H]leucine as described in the text. After fractionation, the membranes were separated on a 50 to 70% sucrose gradient. The inner and outer membrane fractions were analyzed with 10 to 20% SDS-polyacrylamide gradient gels. (A) Coomassie blue-stained gel. Lanes: 1, K38(pGP1-2)(pT7-7), inner membrane (negative control); 2, K38(pGP1-2)(pT7-7), outer membrane (negative control); 3, K38(pGP1-2)(pAH1), inner membrane; 4, K38(pGP1-2)(pAH1), outer membrane; 5, K38(pGP1-2)(pAH2), inner membrane; 6, K38(pGP1-2)(pAH2), outer membrane; 7, K38(pGP1-2)(pAH3), inner membrane; 8, K38(pGP1-2)(pAH3), outer membrane. (B) Autoradiogram of the gel shown in panel A. The sizes of the molecular mass standards (in kilodaltons) are shown on the left side of the gel and the autoradiogram. MW, Molecular weight.

initiate the translation of the 20-amino-acid peptide (using the T7 gene protein 10 translation start site), terminate its translation at the UAG codon, and reinitiate the translation of the RegA protein with the *regA* AUG. Translational reinitiation usually occurs if a good initiation codon or a Shine-Dalgarno domain or both are available within 10 or so nucleotides on either side of the termination codon (3, 6, 25). The low amount of detected RegA protein (Fig. 2, lane 2) suggests that the translational reinitiation at the *regA* initiation codon occurred at a low frequency. Also, the absence of a detectable product with the pT7-5 vector indicated that the presence of the *regA* gene translational start site is not enough for its translation in *E. coli*. Deletion plasmids pAH2 and pAH3 (Fig. 1) produced 26- and 18-kDa fusion proteins, respectively (Fig. 2, lanes 3 and 4). The molecular masses of these proteins correlated well with their predicted molecular masses (26.85 kDa for the fusion protein encoded by pAH2 and 18.35 kDa for the fusion protein encoded by pAH3).

Our results suggested that the transcription of the *regA* gene is not sufficient for its translation in *E. coli*. The *P. aeruginosa* toxin A gene as well as the phospholipase C gene, which were not expressed in *E. coli* from their own promoters (8, 39), were efficiently expressed from the T7 promoter in the pT7-5 vector (19, 34). Lory et al. (19) suggested that the lack of the expression of *toxA* in *E. coli* is due to the failure of the *E. coli* transcriptional machinery to transcribe its mRNA. The −10 and −35 promoter sequences of *toxA* and *regA* showed no homology to those of the *E. coli* promoters (8, 12). Thus, it appears that the expression of the *P. aeruginosa* genes in *E. coli* falls in three categories. (i) The first is genes that are expressed (i.e., their promoters are recognized by the *E. coli* transcriptional system and their mRNAs are translated). This includes the *recA* gene, the elastase structural gene, and the pilin gene (28, 33, 37). (ii) The second is genes that require the presence of an *E. coli*-recognizable promoter to direct the synthesis of their mRNAs. Once transcribed, their mRNAs are efficiently translated. Toxin A and phospholipase C genes belong to this category (19, 34). (iii) The third category is genes that require both an exogenous promoter to synthesize their mRNA and a translational initiation site to help translate the mRNA. Our data showed that the *regA* gene is an example of these genes.

Localization of RegA protein in *E. coli*. The hydropathic plot of Kyte and Doolittle (16) identified certain periodic regions of hydrophobicity within the RegA protein (13). However, the amino acid sequence of the amino terminus of the RegA protein showed no resemblance to the regular signal sequences found in several procaryotic periplasmic and outer membrane proteins (9, 29). Upon fractionation of K38(pGP1-2) containing pAH1, pAH2, or pAH3, most of the RegA protein and its deletion derivatives were associated with the membrane fraction (data not shown). Very few of these proteins were detectable in the cytoplasmic fraction, and none was detected in the periplasmic fraction. When the membranes of K38(pGP1-2) containing recombinant plasmids were separated on sucrose gradients, both RegA protein and its fusion derivatives were localized exclusively to the cytoplasmic membrane (Fig. 4). It is possible that the membrane location of the fusion proteins encoded by plasmids pAH2 and pAH3 is an artifact caused by the overproduction of these proteins. However, the intact RegA protein (encoded by plasmid pAH1), which was not overproduced (Fig. 2, lane 2), was also localized to the membrane (Fig. 4B, lane 3). Previous studies showed that some procaryotic regulatory proteins contain membrane-spanning domains (1, 23). An example is the ToxR protein, which positively regulates toxin production in *Vibrio cholerae*. ToxR protein was localized to the inner membrane of *E. coli* (23). ToxR protein contains a 20-amino-acid internal segment which is

very hydrophobic (16 of 20 amino acids are hydrophobic) (23). Miller et al. (23) suggested that this hydrophobic segment helps insert ToxR protein into the *E. coli* membrane bilayer. The most significant hydrophobic region of the RegA protein is a 15-amino-acid segment (residues 124 to 138). This segment contains a stretch of seven hydrophobic residues. The segment is also included in the fusion proteins encoded by plasmids pAH2 and pAH3. These data suggested that the carboxy-terminal region of the RegA protein (residues 124 to 138) is involved in its localization to the membranes. Recently, Zimniak et al. (43) synthesized RegA protein in *E. coli* and used that protein to produce RegA-specific antisera. Using RegA antisera in immunoblotting experiments, they localized RegA protein to the membranes of *P. aeruginosa* PA103 (43). The localization of RegA into the membranes of *E. coli* and *P. aeruginosa* indicates that RegA is a membrane-associated protein.

DNA-binding activity of RegA protein. The transcriptional activation function of RegA protein could be accomplished through binding to certain sequences in the upstream region of *toxA*. Residues 140 to 160 of RegA protein contain the helix-turn-helix motif commonly found in DNA-binding proteins (30, 32). Alignment of amino acids 140 to 160 of the RegA protein with the DNA-binding domains of 10 other regulatory proteins (λ Cro, λ repressor, LacR, GalR, Cap, FNR, Trp repressor, AraC, LexR, and 434 Cro proteins) (30) showed that it contains two of the three highly conserved residues (Ala-145 and Val-155) (data not shown). However, in the same region, RegA protein has arginine (residue 149) instead of the highly conserved glycine. The whole region (residues 140 to 160) showed no strong homology to the DNA-binding domains of other proteins (the highest degree of homology, 30%, was with λ Cro protein).

Using the DNA gel retardation assay system (5), we examined the DNA-binding activity of the RegA protein and the two fusion proteins. The DNA used was the end-labeled 410-bp *Pst*I-*Nru*I fragment containing the *toxA* promoter region. The migration of the labeled DNA was not affected when mixed with the inner membrane fractions or the lysates of K38(pGP1-2) containing pAH1, pAH2, or pAH3 (Fig. 5, lanes 3 to 5). The mixture of purified *P. aeruginosa* RNA polymerase and the 410-bp *toxA* probe showed clear bands of DNA binding (Fig. 5, lane 6). This indicated that the conditions of the DNA binding reaction (including the salt concentration of the DNA binding buffer to which the DNA-binding activity is very sensitive) were proper. Thus, it appears that neither the intact RegA protein nor its deletion derivatives when synthesized in *E. coli* have binding activity to the upstream region of *toxA*. The same results were obtained when the membrane fractions and lysates of K38(pGP1-2) containing recombinant plasmids were examined for binding activity to the *toxA* upstream region by a filter binding assay (36) (data not shown). The presence of RegA protein in *E. coli* membrane could hinder the binding of RegA protein to *toxA* DNA. However, clear cell lysates of K38 containing recombinant plasmids also showed no binding activity. It is also possible that the RegA protein requires a second protein to stabilize it as a DNA-binding protein.

We searched the predicted sequence of the RegA protein for homology with five other positive procaryotic regulatory proteins (ToxR [23], VirG [40], PhoM [1], OmpR [24], and PhoB [20]), using the algorithm of Smith and Waterman (35). RegA protein throughout its entire length showed no significant homology to any of these proteins (data not shown). Miller et al. (23) reported that residues 29 to 127 of ToxR protein shared significant homology with the carboxy-terminal regions of the VirG, PhoM, OmpR, and PhoB proteins. When we compared the deduced amino acid sequence of RegA protein with these regions, no common homologous region was detected. However, residues 58 to 157 of RegA showed some homology to the carboxy-terminal region of the VirG (14%) and OmpR (20%) proteins (data not shown).

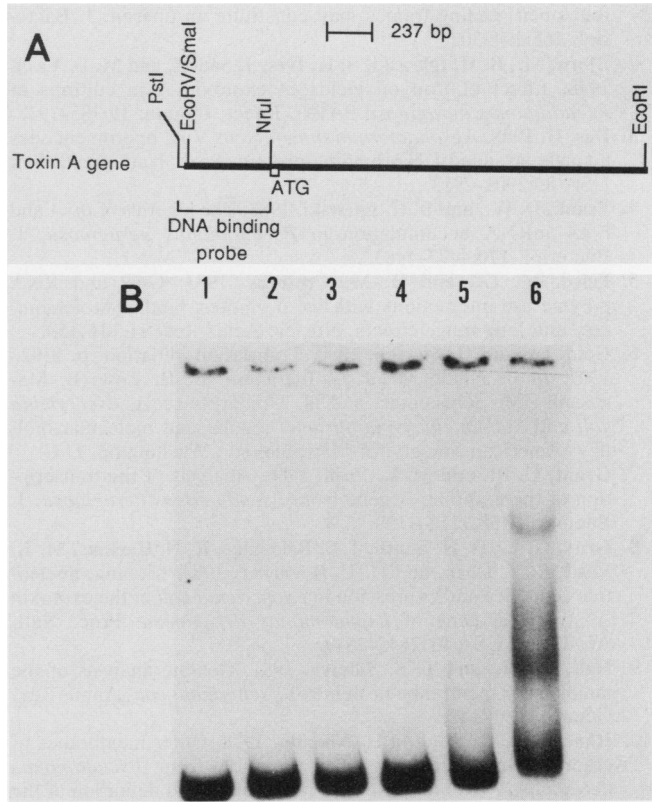

FIG. 5. DNA-binding activity of the membranes of *E. coli* K38(pGP1-2) with the upstream region of the *toxA* gene. Cells were grown and their membranes were prepared as described in the text. The end-labeled 410-bp *Pst*I-*Nru*I fragment was mixed with 30 to 40 μg of the membrane fractions of K38(pGP1-2) containing recombinant plasmids, and the mixtures were run on acrylamide gels as described in the text. (A) Restriction map of the recombinant plasmid (pMS151) containing the *toxA* gene, indicating the region used as a DNA-binding probe. (B) Autoradiogram of the DNA binding gel. The 410-bp probe was incubated with DNA binding buffer only (negative control) (lane 1), K38(pGP1-2)(pT7-7) membranes (lane 2), K38(pGP1-2)(pAH1) membranes (lane 3), K38(pGP1-2)(pAH2) membranes (lane 4), K38(pGP1-2)(pAH3) membranes (lane 5), and purified *P. aeruginosa* RNA polymerase (positive control) (lane 6).

The RegA protein can activate *toxA* transcription through other indirect mechanisms (other than binding to the *toxA* DNA). This may involve the activation of yet another intracellular regulatory factor. Alternatively, RegA protein might bind to the *P. aeruginosa* RNA polymerase.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI25669 from the National Institutes of Health.

We thank Stanly Tabor (Harvard Medical School) for the pT7 plasmid vectors and Stephen Lory (University of Washington, Seattle) for plasmid pMS151.

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