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Autogenous Regulation of the *Bacillus anthracis pag* Operon

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Protective antigen (PA) is an important component of the edema and lethal toxins produced by *Bacillus anthracis*. PA is essential for binding the toxins to the target cell receptor and for facilitating translocation of the enzymatic toxin components, edema factor and lethal factor, across the target cell membrane. The structural gene for PA, *pagA* (previously known as *pag*), is located on the 182-kb virulence plasmid pXO1 at a locus distinct from the edema factor and lethal factor genes. Here we show that a 300-bp gene located downstream of *pagA* is cotranscribed with *pagA* and represses expression of the operon. We have designated this gene *pagR* (for protective antigen repressor). Two *pagA* mRNA transcripts were detected in cells producing PA: a short, 2.7-kb transcript corresponding to the *pagA* gene, and a longer, 4.2-kb transcript representing a bicistronic message derived from *pagA* and *pagR*. The 3′ end of the short transcript mapped adjacent to an inverted repeat sequence, suggesting that the sequence can act as a transcription terminator. Attenuation of termination at this site results in transcription of *pagR*. A *pagR* mutant exhibited increased steady-state levels of *pagA* mRNA, indicating that *pagR* negatively controls expression of the operon. Autogenous control of the operon may involve *atxA*, a *trans*-acting positive regulator of *pagA*. The steady-state level of *atxA* mRNA was also increased in the *pagR* mutant. The mutant phenotype was complemented by addition of *pagR* in *trans* on a multicopy plasmid.

Bacillus anthracis is the etiological agent of anthrax, a disease that affects all mammals, including humans. Key virulence factors of the bacterium include the proteins that comprise the edema and lethal toxins. These binary toxins are composed of distinct enzymatic proteins and a common protein that mediates entry into target cells. Protective antigen (PA) and edema factor (EF) comprise edema toxin, while PA plus lethal factor (LF) comprise lethal toxin. PA binds to a specific receptor on target cells and following endocytosis of the toxin-receptor complex, facilitates translocation of EF and LF across the cell membrane such that the enzymatic proteins can contact their cytosolic substrates (15, 19, 34).

The genes encoding the toxin proteins, *pagA* (previously known as *pag*) (which encodes PA), *cya* (which encodes EF), and *lef* (which encodes LF), are located noncontiguously within a 30-kb region of the 182-kb plasmid, pXO1 (12, 17, 22, 23, 29, 32). As might be expected due to the pivotal role of PA in intoxication, the *pagA* gene is highly expressed. When *B. anthracis* is cultured under optimal conditions, culture supernatants contain up to 20 mg of PA per liter, while LF and EF are present at 5 and 1 mg per liter, respectively (16). Analyses of toxin gene expression employing reporter gene fusions suggest that the steady-state level of mRNA of *pagA* is 4-fold higher than that of *lef* and 14-fold higher than that of *cya* (25).

Two host-related cues, CO_2-bicarbonate and temperature, are important signals for expression of all three toxin genes. During in vitro growth, optimal expression of *pagA*, *cya*, and *lef* is achieved when *B. anthracis* is cultured in buffered R medium, a defined medium containing glucose, salts, and all amino acids, or CA medium, a medium containing Casamino Acids and glucose (16, 21, 27). Toxin synthesis is greatest when cultures are incubated in elevated (5% or greater) atmospheric CO_2 or when bicarbonate is added to culture medium in a closed vessel. Toxin synthesis is also increased when cultures are incubated at 37°C compared to when they are incubated at 28°C. CO_2-bicarbonate- and temperature-controlled gene expression is at the level of transcription (2, 4, 12, 25, 31).

The *atxA* gene, located within the toxin gene region of pXO1, positively controls transcription of all three toxin genes and at least one gene required for capsule synthesis, *capB*, which is located on the 93-kb plasmid pXO2 (9, 12, 29, 30). Under all growth conditions tested, *atxA* is essential for transcription from the unique start sites of *cya* and *lef* and for transcription from the major start site, P1, of the *pagA* gene (7, 12). The mechanism by which *atxA* activates expression of virulence genes and the relationship(s) between host-related cues and *atxA* function are not known.

In an effort to identify regulatory genes that act downstream of *atxA* to activate toxin gene expression, we screened for CO_2-enhanced *atxA*-dependent loci on pXO1. We created random transcriptional *lacZ* fusions using transposon Tn*917*-pLTV3 and tested insertion mutants for CO_2-enhanced *atxA*-dependent β-galactosidase activity. In addition to mutants harboring transposon insertions in the toxin genes, our screen yielded a number of mutants harboring apparent CO_2-enhanced *atxA*-regulated fusions at distinct loci on pXO1 (10). Here we characterize a 300-bp open reading frame (ORF), *pagR*, identified in our screen. We show that the *pagR* gene is cotranscribed with the toxin gene *pagA* and that a *pagR* mutant shows increased levels of *pagA* and *atxA* transcripts.

MATERIALS AND METHODS

Strains, media, and growth conditions. *Escherichia coli* JM109 was used as a host for cloning. *B. anthracis* strains and their relevant characteristics are listed in Table 1. All *B. anthracis* strains are derivatives of the Weybridge strain, a noncapsulated toxigenic isolate originally obtained from the Microbiological Research Establishment, Porton Down, England. For DNA extractions, the *B. anthracis* strains were grown in brain heart infusion medium (Difco, Detroit, Mich.) containing 10% horse serum. For electroporation experiments, *B. anthra-*

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant characteristic(s)[a]	Source and/or reference
Plasmids		
B. anthracis		
pXO1	Tox^+	26
E. coli		
pBSIIKS$^+$	Ap^r	Stratagene
pG$^+$host5	Em^r	Appligene
pGEM-T	Ap^r	Promega
pSL301	Ap^r	Invitrogen
pUC18::Ω*km*-2	pUC18 carrying the Ω*km*-2 cassette; Ap^r Km^r	18
pUTE40	3.0-kb *Hpa*I-*Bam*HI pXO1 fragment, containing *pagA*, in pMK4	12
pUTE41	3.0-kb *Hpa*I-*Bam*HI pXO1 fragment, containing *pagA*, in pBSIIKS$^+$	This work
pUTE103	4.3-kb *Pst*I-*Xba*I pXO1 fragment containing *pagR* and flanking sequences in pG$^+$host5	This work
pUTE308	*Bam*HI-*Kpn*I PCR product containing 0.9 kb upstream of *pagR* and the 5′ end of *pagR* in pSL301	This work
pUTE331	1.1-kb PCR product containing the 3′ end of *pagA*, the *pagA* attenuator, and the 5′ end of *pagR* in pGEM-T	This work
pUTE333	2.6-kb *Bam*HI-*Sac*I fragment from pUTE103, containing *pagR*, in pUTE41	This work
Bifunctional		
pUTE29	Ap^r in *E. coli*, Tc^r in *B. anthracis*	12
pUTE34	2.7-kb *Sna*BI-*Eco*RI pXO1 fragment containing *atxA* in pUTE29	12
pUTE314	4.0-kb *Sac*I-*Pst*I pXO1 fragment containing *pagR* and flanking sequences in pUTE29	This work
pUTE315	Ω*km*-2 cloned into *Bam*HI site between *pagA* and *pagR* in pUTE314	This work
pUTE334	5.6-kb *Sac*I-*Kpn*I fragment from pUTE333, containing *pagA* and *pagR*, in pUTE29	This work
pUTE366	pUTE334 containing +1 frameshift within *pagR*	This work
Strains		
B. anthracis		
UM44[b]	Tox^+ Ind^-	26
UT53	*atxA*-Null derivative of UM44, *atxA* is replaced by Ω*km*-2; Ind^- Km^r	7
UT62	P1 start site of *pagA* disrupted with the Ω*km*-2, derivative of UM44; Ind^- Km^r	7
UT82	Tn*917*-LTV3 insertion mutant of UM44; Cm^r MLS^r, CO_2-*atxA*-regulated *lacZ* expression	10
UT119	*pagR* Mutant derivative of UM44, Ω*km*-2 inserted at *Bam*HI site between *pagA* and *pagR*; Ind^- Km^r	This work
E. coli		
GM1684	F′ F-*lacI*^{q}ΔM15 *pro*$^+$/*dam-4* Δ(*lac-pro*)*X111 thi-1 glnV44* (*relA1*)	R. Kolter
JM109	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	35
BMH 71-18	*thi supE* Δ(*lac-proAB*) [*mutS*::Tn*10*][F′ *proAB lacI*^{q}*Z*ΔM15]	Clontech

[a] Abbreviations: Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Ind, indole; Km^r, kanamycin resistant; MLS^r, macrolide resistant; Tc^r, tetracycline resistant; Tox, anthrax toxin proteins.
[b] UM44 was derived from the Weybridge strain.

cis strains were grown in brain heart infusion medium containing 0.5% glycerol. For RNA extractions, *B. anthracis* strains were grown in CA broth (27) buffered with 100 mM HEPES (pH 8.0). CA medium contained 0.8% sodium bicarbonate for cultures incubated in 5% CO_2.

All antibiotics were purchased from Sigma (St. Louis, Mo.) or Fisher Scientific (Pittsburgh, Pa.) and were added to media at the following concentrations when appropriate: for *E. coli*, ampicillin, 100 μg/ml; kanamycin, 20 μg/ml, and tetracycline, 10 μg/ml; for *B. anthracis*, erythromycin, 1 μg/ml; lincomycin, 25 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 5 μg/ml.

Plasmid and strain constructions. Plasmid DNA was extracted from *B. anthracis* by the method of Green et al. (8). Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures (1). *B. anthracis* was electroporated with plasmid DNA from *E. coli* GM1684, as described previously (12). Restriction enzymes, T4 ligase, and *Taq* polymerase were purchased from Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.).

Plasmids and their relevant characteristics are shown in Table 1. Plasmid pUTE308 contains DNA downstream of *pagA*, including the 5′ end of *pagR*. To construct pUTE308, a 946-bp PCR product was generated by using oligonucleotides 5′-GTAAGAAATACAAGGAGAGTATG-3′ and 5′-CGCATAGGA**GG TAC**CATTGTTTTT-3′. The former oligonucleotide is complementary to a region 62 to 85 bp downstream of the translational stop of *pagA* and just upstream of a *Bam*HI site (see Fig. 1). The latter oligonucleotide is complementary to a region 62 to 86 bp downstream of the predicted translational start of *pagR* and contains an engineered *Kpn*I site (shown in boldface type). The PCR product was digested with *Bam*HI and *Kpn*I and ligated into *Bam*HI-*Kpn*I-digested pSL301.

Plasmid pUTE331 contains DNA from the 3′ end of *pagA* through the 5′ end of *pagR*. To construct pUTE331, a 1.1-kb PCR product was generated with oligonucleotides 5′-GGGGATACTTAGTACCAACGGG-3′ and 5′-GTTCAGCA TCATCTTCTAAACTC-3′. The former oligonucleotide is complementary to a region 45 to 68 bp upstream of the translational stop codon of *pagA*. The latter oligonucleotide is complementary to a region 36 to 58 bp downstream of the predicted translational start of *pagR*. The PCR product was ligated into pGEM-T.

Plasmid pUTE334 carries *pagA* and *pagR*. It was created by first ligating a *Bam*HI-*Sac*I fragment (containing *pagR*) from pUTE103 into *Bam*HI-*Sac*I-digested pUTE41 (containing *pagA*), resulting in pUTE333. The 5.6-kb *Sac*I-*Kpn*I fragment from pUTE333 was ligated into *Sac*I-*Kpn*I-digested pUTE29, resulting in pUTE334. The frameshift mutation within *pagR* (pUTE366) was generated by using the Transformer Site-Directed Mutagenesis kit from Clontech (Palo Alto, Calif.). The oligonucleotide, 5′-GACAGTATTTGTACGATCATAAAATTG-3′, was used as a mutagenic primer to add a C residue (underlined) 15 bases downstream of the ATG start codon of *pagR*. The frameshift mutation was confirmed by DNA sequencing.

UT119 carries the Ω*km*-2 element inserted in the *Bam*HI site between *pagA* and *pagR* (see Fig. 1). Insertion of the Ω*km*-2 element into the *B. anthracis* genome was accomplished as described previously (7). To create UT119, pUTE315 (Table 1) was electroporated into UM44 with selection for kanamycin resistance. UT119 was isolated following a screen for tetracycline-sensitive kanamycin-resistant mutants. The location of the Ω*km*-2 element was confirmed by using PCR.

RNA analysis. Methods for RNA extraction, primer extension reactions, and RNase T2 protection assays have been described (12, 28). RNA was extracted

from *B. anthracis* cultures grown to late log phase (optical density at 600 nm of 0.8 to 1.0) in CA medium. RNA was quantified spectrophotometrically and by visualization on 1.2% formaldehyde gels. Oligonucleotides used for *atxA* and *pagA* primer extensions were described previously (6). Oligonucleotides were labeled with [γ-^{32}P]ATP (6,000 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.), hybridized to 20 μg of RNA, and extended by using avian myeloblastosis virus reverse transcriptase (Promega). The 5′ ends of the *atxA* and *pagA* genes were sequenced by the dideoxy-chain termination method (1), using the appropriate primers and a Sequenase version 2.0 DNA sequencing kit purchased from United States Biochemical Corp. (Cleveland, Ohio). The [α-^{35}S]dATP (>1,000 Ci/mmol) for sequencing was purchased from Amersham Corp. Primer extension and sequencing reaction mixtures were subjected to electrophoresis on 6% polyacrylamide and 42% urea gels. Primer extension products were quantified by using a Packard Instant Imager.

Antisense RNA probes (riboprobes) 1 and 2 (for illustrations see Fig. 3A) were used for RNase T2 protection assays. RNA polymerases and RNase T2 were purchased from Promega. To make riboprobe 1, pUTE331 was linearized with *Nco*I and antisense RNA was generated by using Sp6 RNA polymerase and [α-^{32}P]CTP (>800 Ci/mmol) (Amersham). To make riboprobe 2, pUTE308 was linearized with *Bam*HI and antisense RNA was generated by using T7 RNA polymerase and [α-^{32}P]CTP. Riboprobes were hybridized to 20 μg of *B. anthracis* RNA prior to digestion with RNase T2.

For Northern hybridizations, biotinylated DNA probes complementary to the internal DNA sequences of *pagA* and *pagR* were created by using a Phototope kit (New England Biolabs). A 1.7-kb *Xmn*I-*Hin*cII fragment from pUTE40 was biotinylated to make the *pagA* probe. The *pagR* probe was made from a 242-bp PCR product generated by the following oligonucleotides: 5′-GAGTTTAGAA GATGATGCTGAAC-3′ and 5′-TAATAATCCCTTCAACTTTTGG-3′. The oligonucleotide primers were complementary to regions 35 to 58 and 264 to 286 bp downstream of the predicted translational start codon of *pagR*. Twenty micrograms of RNA was run on a 1.2% agarose gel (containing formaldehyde) and capillary blotted to Maximum Strength Nytran Plus membranes (Schleicher & Schuell, Keene, N.H.). Membranes were hybridized with probes overnight at 45°C. Blots were developed by using a Phototope-Star detection kit (New England Biolabs) and exposed to film.

Immunoblotting. Relative levels of PA produced by parent and mutant strains were determined by assaying cell lysates for protein that reacted with polyclonal anti-PA antiserum. PA in culture supernatants is subject to varying levels of degradation by proteases secreted by *B. anthracis*. Therefore, for monitoring small differences in PA levels, more-reproducible data can be obtained by examining cell lysates rather than culture supernatants. *B. anthracis* cultures were grown in 25 ml of CA medium to an optical density at 600 nm of 0.8 to 1.0. Cells were collected on cellulose acetate membranes (pore size, 0.45 μm) (Nalge, Rochester, N.Y.), resuspended in 1 ml of buffer (1% sodium dodecyl sulfate, 200 mM dithiothreitol, 28 mM Tris HCl, 22 mM Tris OH, 2 mM phenylmethylsulfonyl fluoride), and passed through a French press minicell three times at 20,000 lb/in^2. The soluble fraction was obtained following centrifugation at 16,000 $\times$ *g* for 5 min, and protein concentrations were determined by using a Bio-Rad protein assay reagent (Bio-Rad, Hercules, Calif.). Samples (5 μg) were subjected to electrophoresis on sodium dodecyl sulfate–7.5% polyacrylamide gels.

Following electrophoresis, proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were reacted with rabbit anti-PA serum (diluted 1:6,000 in TBS-T (20 mM Tris OH, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]) containing 5% milk for 2 h at room temperature. Membranes were washed in TBS-T and finally reacted with donkey anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (1:4,000 in TBS-T with 5% milk) for 1 h at room temperature. Cross-reactive material was visualized on autoradiographs by using an ECL immunodetection kit purchased from Amersham (Little Chalfont, Buckinghamshire, England).

Nucleotide sequence accession number. The complete nucleotide sequence of the *pagR* gene and flanking regions has been deposited in the GenBank database under accession no. AF031382.

RESULTS

Identification of *pagR*. In experiments reported previously, we used the transposon Tn*917*-pLTV3 to identify *B. anthracis* promoters that exhibited increased expression during growth in elevated CO_2 and were *atxA*-dependent (10). Tn*917*-LTV3 carries a promoterless *lacZ* gene at one end and generates a transcriptional fusion when inserted downstream of an active promoter (3). In this investigation, we studied one Tn*917*-pLTV3 insertion mutant that showed CO_2- and *atxA*-dependent β-galactosidase activity.

Restriction analysis and Southern hybridization experiments revealed that the mutant UT82 contained a Tn*917*-pLTV3 insertion in the approximately 2.8-kb region between the *pagA* and *lef* genes on pXO1 (data not shown). We cloned and sequenced this intergenic region from the parent strain. To determine the exact insertion site and orientation of Tn*917*-pLTV3 in UT82, we cloned and sequenced the *B. anthracis* DNA flanking the 5′ end of the transposon insertion. As shown in Fig. 1, sequence analysis indicated that the transposon insertion was 1,339 nucleotides (nt) downstream of *pagA*. A 300-nt ORF was noted between *pagA* and the Tn*917*-LTV3 insertion. This ORF lies in the same orientation as the *pagA* gene and the promoterless *lacZ* gene associated with the transposon. The 3′ end of the ORF maps to 122 bp upstream of the transposon insertion. For reasons made evident below, we have designated the downstream ORF *pagR* (for protective antigen repressor). A potential ribosome binding site beginning 12 bp upstream of the translational start codon of *pagR* was noted.

The *pagR* gene is predicted to encode a 99-amino-acid protein with sequence similarity to two proteins that regulate transcription in other species. The predicted *pagR* gene product is 26% identical and 52% similar to CadC (13.5 kDa), a predicted regulator of cadmium resistance in *Listeria monocytogenes* (14). The putative PagR protein is 33% identical and 57% similar to NolR (13.3 kDa), a *trans*-acting negative regulator of the *nod* regulon of *Rhizobium meliloti* (13). The NolR protein has been shown to bind specifically to promoter regions of several *nod* genes. The sequence similarity of PagR to both NolR and CadC is found throughout the predicted amino acid sequences of the proteins. Analysis of the PagR amino acid sequence by using the motifs program from the University of Wisconsin Genetics Computer Group (GCG) software package identified a potential tyrosine kinase phosphorylation site in the carboxy-terminal region of the protein (Fig. 1).

***pagR* is cotranscribed with *pagA*.** Northern hybridization experiments were performed to detect mRNA corresponding to the *pagR* gene. Biotinylated DNA probes corresponding to sequences internal to *pagR* and *pagA* were hybridized to RNA isolated from the parental strain, UM44, and mutants UT62 and UT119. Results are shown in Fig. 2. Hybridization of the *pagR* probe with UM44 RNA revealed a single band representing a 4.2-kb transcript, while hybridization of UM44 RNA with the *pagA* probe resulted in two bands corresponding to 4.2- and 2.7-kb transcripts. Mutations in UT62 and UT119 are shown schematically in Fig. 2C. UT62 carries an Ω*km*-2 insertion that prevents transcription from the major transcription start site of *pagA* (12). RNA isolated from UT62 did not hybridize to the *pagR* or *pagA* probes. UT119 is a mutant containing an Ω*km*-2 insertion in a *Bam*HI restriction site (Fig. 1) between the *pagA* and *pagR* genes. No transcripts were detected when UT119 RNA was probed with *pagR*. When the *pagA* probe was hybridized to RNA from UT119, only the smaller, 2.7-kb transcript was detected. Taken together, these results indicate that transcription from the *pagA* promoter results in a monocistronic transcript corresponding to *pagA* and a bicistronic transcript corresponding to *pagA* and *pagR* (see Fig. 2C).

The size of the monocistronic transcript is indicative of termination between the *pagA* and *pagR* genes. Welkos et al. (33) proposed that a nucleotide sequence beginning 43 bp downstream of the *pagA* coding sequence represents a transcription terminator (Fig. 1 and 3A). The sequence could facilitate formation of a hairpin structure with a 19-bp stem and a calculated free energy of -22.2 kcal/mol. We performed RNase T2 protection assays to determine if the 3′ end of the short transcript mapped near this site. A riboprobe extending from the center of *pagR* to 114 bp upstream of the inverted repeat sequence (riboprobe 1, Fig. 3A) was hybridized to RNA from UM44 and subsequently digested with RNase T2. Two small, protected fragments were observed (Fig. 3B, lane 1). The sizes

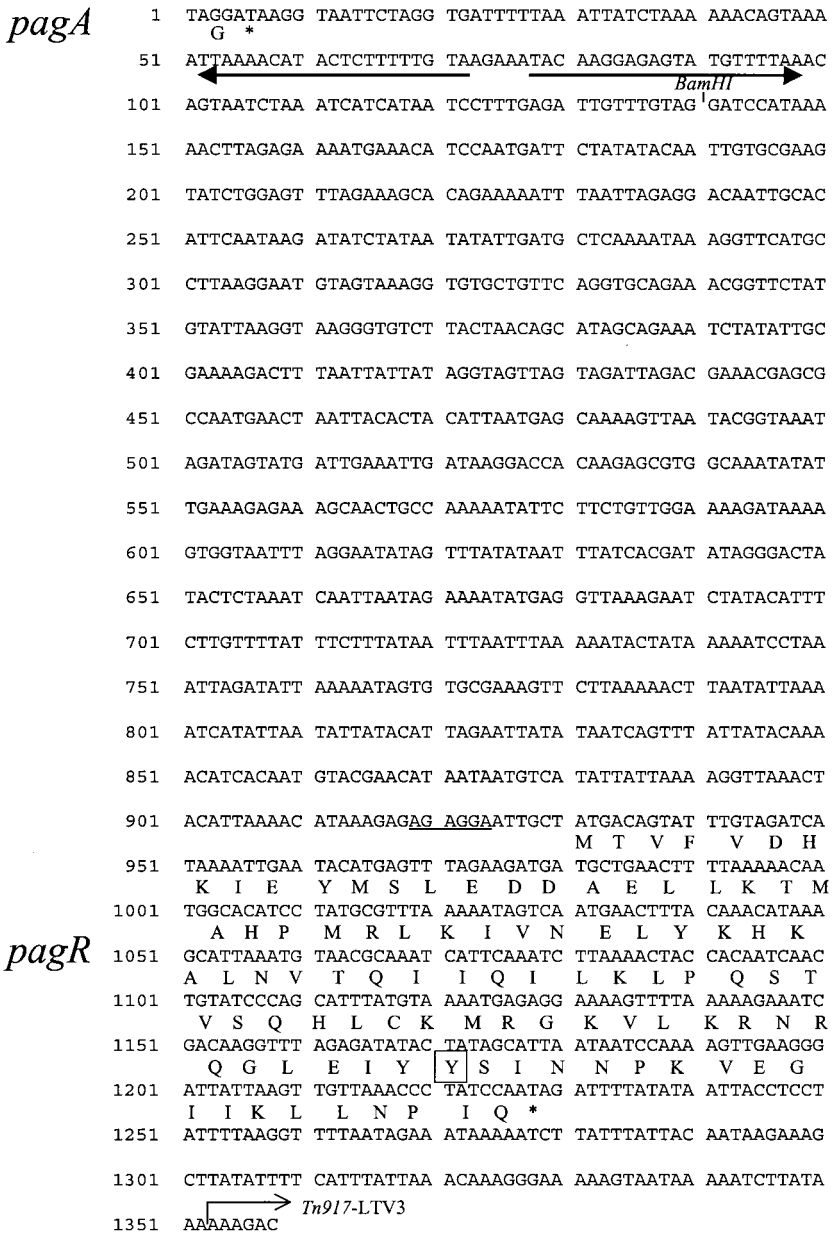

FIG. 1. pXO1 DNA sequence extending from the 3′ end of *pagA* through *pagR*. Predicted amino acid sequences are indicated under the *pagA* and *pagR* DNA sequences. Straight arrows indicate an inverted repeat sequence, located downstream of *pagA*, that functions as a weak transcription terminator. A potential ribosome binding site upstream of *pagR* is underlined. A tyrosine residue predicted to be a tyrosine kinase phosphorylation site is boxed. Locations of a *Bam*HI site (the site of the Ω*km*-2 insertion in UT119) and a Tn*917*-LTV3 insertion in UT82 are as indicated.

of the fragments, 162 and 167 nt, indicate that they are *pagA* transcripts with 3′ ends mapping 3 and 8 bp downstream of the inverted repeat sequence. As expected, the full-length probe was also protected from digestion with RNase T2 due to hybridization with the bicistronic (*pagA-pagR*) transcript (not shown in the figure). These results indicate that termination occurs at the predicted site but is incomplete. The inverted repeat sequence can therefore be considered an attenuator.

Transcription of *pagA* from the major start site, P1, is dependent upon the *trans*-acting regulatory gene, *atxA* (12). In addition, steady-state levels of *pagA* mRNA are increased when cells are grown in elevated levels of CO_2-bicarbonate compared to growth in air (2, 12, 25). RNase T2 protection assays were performed to confirm that control of *pagR* transcription was similar to that of the *pagA* gene. A riboprobe extending from within *pagR* to a *Bam*HI restriction site located between the *pagA* and *pagR* genes (riboprobe 2, Fig. 3A) was hybridized to RNA isolated from UM44, UT53, UT53 (pUTE34), and UT119 and subsequently digested with RNase T2. Results are shown in Fig. 3C. All RNase-protected hybridization products were of similar size, indicating complete protection of the probe, as expected. The comparison of the intensities of bands obtained by using RNA from UM44 cultured in 5% CO_2 and RNA from UM44 cultured in air confirmed that the *pagR* transcript is CO_2-regulated. No transcript was detected by using RNA isolated from the *atxA*-null mutant,

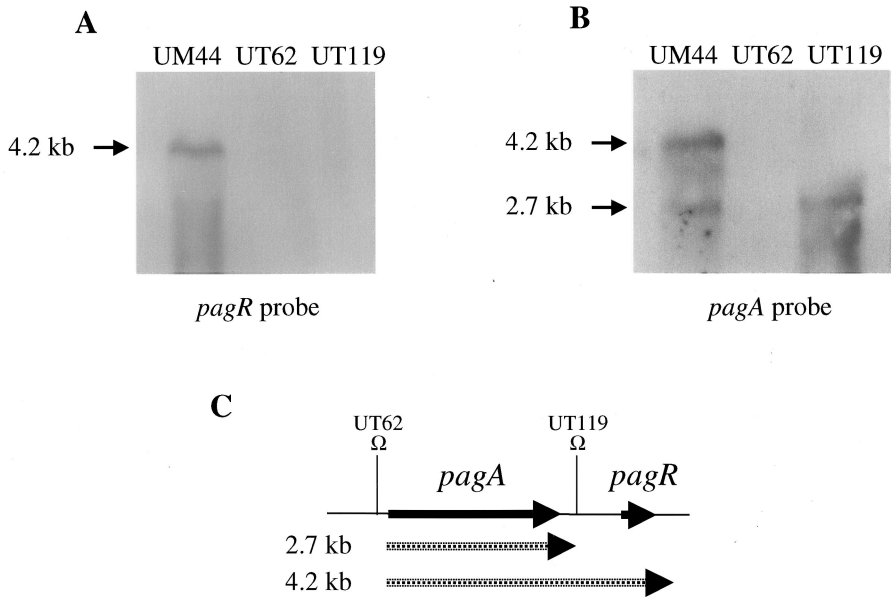

FIG. 2. Northern blot detection of *pagR* and *pagA* mRNA. RNA samples (20 μg) from cells grown at 37°C in 5% CO_2 were probed with biotinylated DNA probes corresponding to sequences internal to *pagR* (A) and *pagA* (B). Transcript sizes are indicated. (C) The relative locations of the *pagA* and *pagR* mRNA transcripts and the Ω*km*-2 insertions in UT62 and UT119 are shown.

UT53. *pagR* transcript was detected in RNA isolated from UT53 carrying *atxA* in *trans* on pUTE34, although the amount was lower than the wild-type level. It has been shown previously that the steady-state level of *pagA* transcripts in strains overexpressing *atxA* in *trans* is significantly lower than that detected in the UM44 parent strain (6). No *pagR* transcript was detected from UT119, confirming that there are no *pagR* transcripts that initiate between *pagA* and *pagR*. Thus, *pagR* is cotranscribed with *pagA* and is expressed in a manner similar to *pagA*.

***pagR* represses *pagA* expression.** Considering that *pagR* is cotranscribed with *pagA* and that the predicted PagR protein has sequence similarity to regulators of transcription, we hypothesized that *pagR* affects expression of *pagA* in a feedback

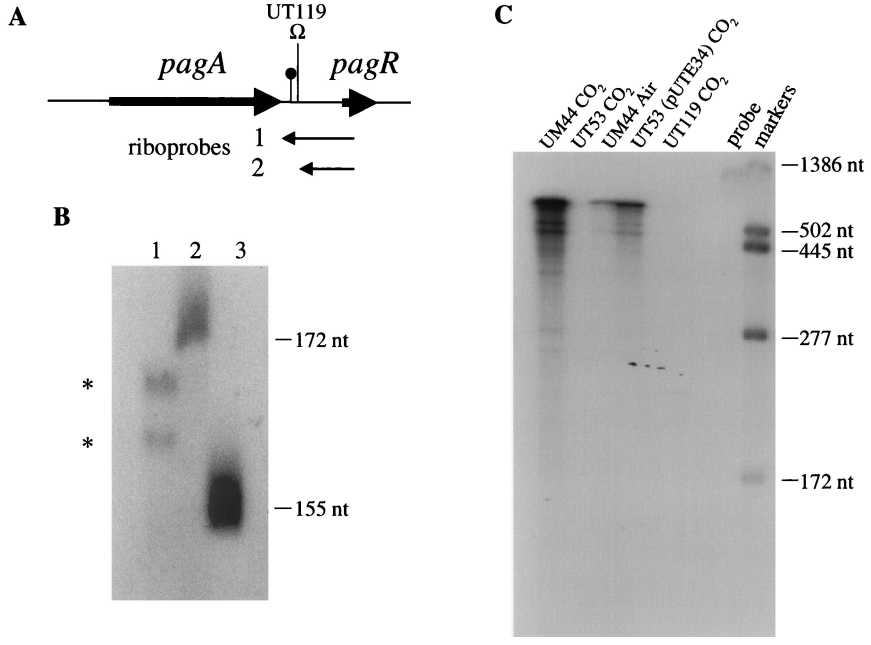

FIG. 3. Mapping the 3′ ends of the monocistronic *pagA* transcript and determining the effects of CO_2 and *atxA* on *pagR* expression. RNase T2 protection assays were performed with 20 μg of RNA. (A) Locations of antisense riboprobes, the Ω*km*-2 insertion in UT119, and the transcription attenuator are shown. (B) Lane 1: riboprobe 1 was hybridized to RNA from UM44 grown in 5% CO_2. RNase T2-protected fragments are indicated. Lanes 2 and 3: nucleotide size markers, as indicated. (C) Riboprobe 2 was hybridized to RNA from cells grown in 5% CO_2 or in air, as indicated. RNase T2-protected fragments are shown. Size markers are as indicated. The probe is 1,167 nt (including 222 nt of vector-derived sequences).

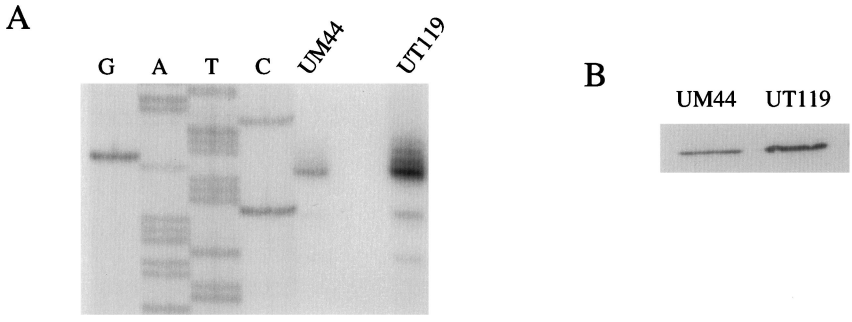

FIG. 4. Regulation of *pagA* by *pagR*. Cultures for isolation of RNA and protein were incubated in 5% CO_2. Strains are as indicated. (A) Primer extension experiments were performed with 20 μg of RNA. The end-labeled primer was complementary to a 33-bp sequence located 32 nt downstream of the first nucleotide of the *pagA* translational start site. Lanes G, A, T, and C correspond to the dideoxy sequencing reaction carried out with the same oligonucleotide primer. (B) Western blot showing PA detected in cell lysates.

control loop. Primer extension experiments were performed to measure steady-state levels of *pagA* transcripts in UM44 and UT119 (*pagR*) grown at 37°C in 5% CO_2. Results of a representative experiment are shown in Fig. 4A. In repeated experiments, the level of *pagA* transcript was 2.5- to 7.0-fold higher in UT119 than in UM44, indicating that *pagR* represses *pagA* expression. It is not clear why the level of repression varied in different experiments. Identical RNA samples used to measure expression of *atxA* (see below) did not exhibit such variation. Western blottings were performed to determine if the increase in *pagA* expression in the *pagR* mutant also led to an increase in PA protein level (Fig. 4B). Levels of PAs from UT119 lysates were 2.0-fold higher than those from UM44 lysates.

***pagR* represses *atxA* expression.** The *pagA* gene is positively regulated in *trans* by *atxA* (12, 29). We tested for *pagR*-controlled expression of *atxA*. Results of primer extension experiments are shown in Fig. 5. The steady-state level of *atxA* mRNA was increased twofold in UT119 (*pagR*) compared to that in UM44, indicating that *pagR* represses *atxA* at a low level.

UT119 contains an Ω*km*-2 insertion between the *pagA* and *pagR* genes and does not express *pagR*. To confirm that the UT119 phenotype was due to the lack of *pagR* expression, we attempted to complement UT119 with a plasmid containing *pagR* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P*spac* (36). After induction with IPTG, the *pagR* transcript in UT119 containing the recombinant plasmid was barely detectable, suggesting that the truncated transcript was unstable. Therefore, we tested for complementation of the UT119 phenotype using two different constructs. Plasmid pUTE334 contains a 5.6-kb pXO1 fragment containing *pagA* and *pagR*. Plasmid pUTE366 contains the same pXO1 fragment with a frameshift mutation within *pagR*. As shown in Fig. 5, pUTE334 complemented the mutation in UT119, restoring *atxA* expression to the level observed in the parent strain, UM44. UT119 was not complemented by pUTE366. These results show that the UT119 phenotype is due to disruption of *pagR* expression.

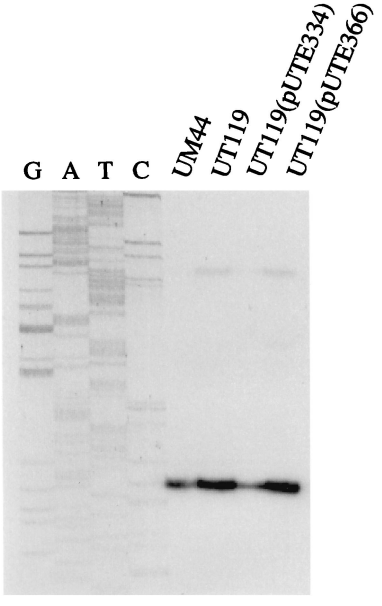

FIG. 5. Regulation of *atxA* by *pagR*. Primer extensions were performed as described in the legend for Fig. 4. The end-labeled primer was complementary to a 27-bp sequence located 55 bp downstream of the first nucleotide of the translational start site of the *atxA* gene. Strains are as indicated.

DISCUSSION

In this study, we determined that a previously unknown gene, *pagR*, is cotranscribed with the toxin gene *pagA* (previously known as *pag*) and negatively controls expression of the operon. Incomplete transcription termination near the 3′ end of an inverted repeat sequence downstream of *pagA* results in mono- and bicistronic transcripts containing *pagA* mRNA. There is no evidence that attenuation at this site is regulated. Expression of *pagR* appears to mimic *pagA* expression. In light of our results, we refer to the structural gene for PA as *pagA*. Thus, the *pag* operon is composed of *pagA* and *pagR*.

The predicted amino acid sequence of the putative PagR protein is similar to those of regulatory proteins NolR from *R. meliloti* (13) and CadC from *L. monocytogenes* (14). NolR and CadC contain helix-turn-helix motifs and are predicted to be DNA-binding proteins. If the PagR protein binds DNA specifically, the PagR target may be within the promoter region of *pagA* or within the promoter region of some other gene that affects *pagA* expression.

A *pagR* mutant shows elevated expression of *pagA* and *atxA*, a *trans*-acting positive regulator of *pagA*. Yet it is unlikely that increased *pagA* expression in a *pagR* mutant is attributed simply to increased AtxA levels. Results of previous studies indicate that AtxA levels are not limiting for expression of *pagA*. Strains carrying multiple copies of the *pagA* promoter synthe-

size normal levels of PA (24). Furthermore, a 10-fold increase in *atxA* expression has a negative effect on *pagA* expression, resulting in a 60 to 70% decrease in PA levels (6). Thus, PagR may control expression of the *pag* operon independent of AtxA or in conjunction with AtxA and some other regulatory protein(s).

Our data indicate that *pagR* functions to limit *pagA* expression under culture conditions which are optimal for synthesis of protective antigen. In experiments not presented here, we tested virulence of the *pagR* mutant in a mouse model for anthrax. Subcutaneous inoculation of mice with high doses of spores of the toxigenic noncapsulated Sterne strain results in a lethal disease (20). In this model, the 50% lethal dose for the *pagR* mutant was the same as that for the parent strain (11). This result indicates that increased PA synthesis by the *pagR* strain does not increase virulence in the mouse model. It is also possible that *pagR* function during infection differs from that observed during culture of *B. anthracis* in vitro.

The *pagA* gene is the only toxin gene that is known to be transcribed in an operon. The 5′ ends of transcripts corresponding to the *cya* and *lef* genes have been mapped (7); however, the sizes of the *cya* and *lef* transcripts have not been determined. Sequence analysis of regions downstream of *cya* and *lef* does not indicate that there are putative transcription regulators adjacent to these genes. A 332-bp ORF lies 286 bp downstream of *cya* and in the same orientation (accession no. AF003936). The predicted protein product of this ORF has sequence similarity to the purine salvage pathway enzyme adenine phosphoribosyltransferase of many organisms. A 303-bp ORF is located 1.4-kb downstream of *lef* and in the same orientation (accession no. AF031382). The predicted protein product of this ORF has no homology to any sequences in GenBank.

The *pagA* gene is coordinately expressed with the other toxin genes, *cya* and *lef*, in response to the same signals, i.e., CO_2-bicarbonate and temperature, and the same activator, *atxA*. It is possible that *pagR* also regulates the *cya* and *lef* genes. Cataldi et al. (5) examined EF activity and LF protein level in the culture supernatant of a *B. anthracis* strain harboring an erythromycin resistance cassette in the *pagA* gene. EF activity was increased twofold, while LF protein level was unchanged, compared to that in the strain without the cassette (5). If the insertion in the *pagA* mutant had a polar effect on *pagR* expression, it is possible that the observed increase in EF activity was due to increased expression of *cya* in the absence of *pagR* expression. In future experiments we will address the mechanism for *pagR*-mediated repression and determine whether or not *pagR* also regulates the other toxin genes.

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