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Transcriptional Regulation of the Protective Antigen Gene of *Bacillus anthracis*

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Bicarbonate is required for production of the major virulence factors, the toxins and capsule, of *Bacillus anthracis*. In this study we examined the basis for stimulation of production of protective antigen (PA), a central component of the two anthrax toxins encoded by plasmid pXO1. RNA prepared from *B. anthracis* grown in media with and without added bicarbonate was probed for PA mRNA. Data showed that bicarbonate was required for increased transcription of the PA gene (*pag*) in minimal medium. Transcription of *pag* was low in rich medium and could not be stimulated by the addition of bicarbonate. To characterize further the factors required for transcriptional regulation of *pag*, the promoter region of *pag* was fused to the chloramphenicol acetyltransferase gene (*cat-86*) of vector pPL703 and transformed by electroporation into pXO1$^+$ (Tox$^+$) and pXO1$^-$ (Tox$^-$) strains of *B. anthracis*. Analysis of chloramphenicol acetyltransferase produced by the *pag–cat-86* fusion in each of these backgrounds confirmed the results obtained by hybridization. Data obtained with this fusion also revealed that the large toxin plasmid, pXO1, found in virulent strains of *B. anthracis*, was required for stimulation of transcription of *pag* by bicarbonate. This result suggests the existence of a *trans*-acting factor that is involved in the activation of *pag* transcription.

The major virulence factors of *Bacillus anthracis*, the causative organism of anthrax, are a poly-D-glutamic acid capsule and two toxins. These virulence determinants are plasmid encoded. The genes involved in capsule synthesis reside on a 90-kilobase (kb) plasmid, pXO2 (9, 34). The three toxin genes, *cya* (edema factor), *lef* (lethal factor), and *pag* (protective antigen), encoding the proteins that make up the two holotoxins, are located on the 174-kb plasmid pXO1 (20). The genes encoding the three toxin components have been cloned, and *pag* and *cya* have been sequenced (22, 27, 28, 33, 35, 36). Although the three toxin proteins are encoded by separate genes, they interact in a manner similar to the A-B enzyme-binding structure characteristic of a number of well-studied toxins (for a review, see reference 19). Protective antigen (PA), analogous to the B chain, provides a receptor-binding activity. Studies done in vitro have shown that, after binding to a eucaryotic cell in tissue culture, PA is cleaved at a single site, exposing a binding site for the other toxin components (16). Edema factor, a calmodulin-dependent adenylate cyclase, in combination with PA, causes an increase in intracellular cyclic AMP (14). Lethal factor, in combination with PA, is lethal to susceptible animals. An enzymatic activity has not yet been associated with lethal factor.

Elaboration of both the capsule and all three toxin components of *B. anthracis* in vitro are influenced by medium composition (6, 18, 32). Toxin yields are low in most rich media, whereas cells grown in certain minimal media produce toxin, but only if bicarbonate is added to the medium or if the culture is grown under CO_2. Production of several other bacterial virulence factors, including cholera toxin (10, 30) and the toxic shock syndrome toxin 1 of *Staphylococcus aureus* (11), is stimulated by CO_2.

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In this report, we demonstrate that PA production was stimulated at the transcriptional level by bicarbonate, as determined both by dot blot analysis of RNA prepared from *B. anthracis* and by fusion of the *pag* promoter region to the *cat-86* gene of plasmid pPL703 (23), which encodes the easily measured enzyme chloramphenicol acetyltransferase (CAT). Additionally, analysis of the promoter fusions in *B. anthracis* indicated that there is a segment of pXO1 DNA, possibly encoding a *trans*-acting factor, that is required for the bicarbonate-mediated stimulation of *pag* transcription.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. anthracis* Weybridge UM23-1(pXO1) (Tox$^+$ Cap$^-$ Ura$^-$ Str^r) and UM23C1-1 (Tox$^-$ Cap$^-$ Ura$^-$ Str^r) were obtained from C. Thorne. *Bacillus subtilis* DB104 (*his nprR2 nprE18 ΔaprA3*) (12) was obtained from R. Doi. All plasmids used are diagrammed in Fig. 1. Phagemid p201, a gift from D. Robertson, consists of vector pTZ18R (United States Biochemical Corp., Cleveland, Ohio), a pUC18 vector that contains the intragenic region of bacteriophage f1 and contains a 4.3-kb *Xba*I-*Bam*HI fragment of pXO1 encoding the entire PA gene. Plasmid pPL703 (23), a promoter-cloning vector containing a promoterless CAT gene designed for use in gram-positive bacteria, was obtained from P. Lovett. Construction of pFD1, a plasmid containing a *pag–cat-86* fusion, is described below.

Media and culture conditions. *B. anthracis* was grown routinely in 50 ml of either brain heart infusion broth (BHI) or R minimal medium containing 0.1 M Tris hydrochloride (pH 8.0) (15, 26) in tightly closed, screw-cap 250-ml Erlenmeyer flasks. Cultures were incubated at 37°C with slow shaking (80 rpm). Minimal medium was supplemented with 40 μg of uracil per ml. Sodium bicarbonate and horse serum were added to give final concentrations of 0.8 and 5%, respectively. Streptomycin, when added, was used at a concentration of 500 μg/ml. Strains carrying pPL703 or

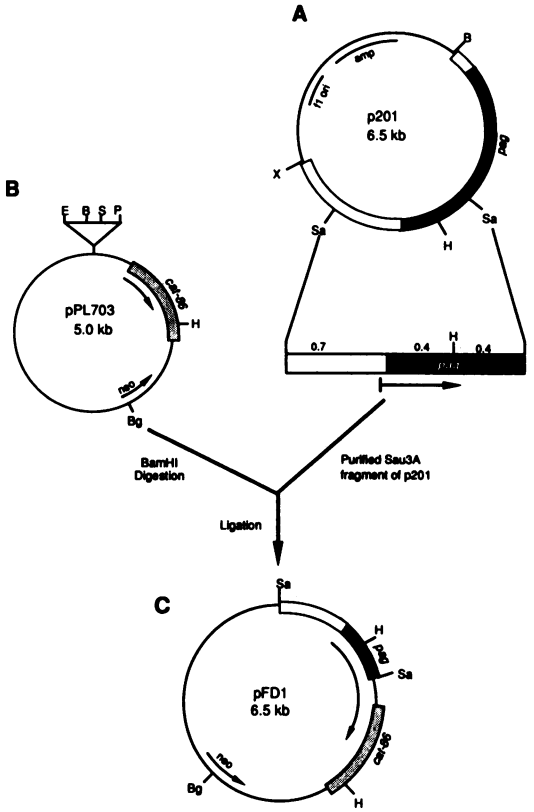

FIG. 1. Construction of a *pag–cat-86* fusion in vector pPL703. (A) Plasmid p201, which contains a 4.3-kb fragment of pXO1 DNA (open bar) including the entire PA gene, *pag* (solid bar). Beneath the circular map of p201 in a linear diagram of the 1.5-kb *Sau*3A fragment that contains the *pag* promoter region and about 800 base pairs of the PA-coding region. This fragment also contains about 700 base pairs of DNA upstream from the *pag* promoter (36). The approximate start and direction of transcription from the *pag* promoter is indicated by the arrow. (B) Promoter-cloning vector pPL703 containing a neomycin resistance gene and the promoterless *cat-86* gene (stippled bar). The arrows indicate gene orientations. (C) Recombinant plasmid pFD1 was generated by ligating the 1.5-kb *Sau*3A fragment from p201 into the *Bam*HI site of pPL703, which placed the *cat-86* gene under the control of the *pag* promoter. Transcription of *cat-86* started at the *pag* promoter and continued into the *cat-86* coding region, as indicated by the arrow. Restriction site abbreviations: X, *Xba*I; B, *Bam*HI; Sa, *Sau*3A; H, *Hin*dIII; S, *Sal*I; P, *Pst*I; E, *Eco*RI; Bg, *Bgl*III.

pFD1 were grown routinely in the presence of 50 μg of kanamycin per ml to maintain the plasmids.

RNA extraction. Total RNA was isolated by hot phenol extraction from cells that had been lysed with sodium dodecyl sulfate (SDS) by a method modified from that of Battisti et al. (2). Briefly, *B. anthracis* UM23-1 or UM23C1-1 was grown to an A_{660} of 0.8, and 25-ml portions were added to 10 ml of frozen, crushed E killing buffer (0.04 M Tris-acetate [pH 7.9], 0.002 M tetrasodium EDTA, 15% sucrose, 0.02 M sodium azide) and centrifuged at 3,000 × *g* for 5 min at room temperature. The pellets were washed with 1/10 volume of ice-cold E killing buffer. The cell pellets were suspended in 0.5 ml of E buffer (E killing buffer without sodium azide) and lysed by the addition of 1 ml of lysis buffer (0.05 M Tris hydrochloride [pH 7.5], 15% sucrose, 3% SDS) followed by incubation at 60°C for 30 min. Nucleic acids were extracted for 5 min at 65°C by the addition of an equal volume of phenol saturated with lysis buffer lacking SDS (equilibrated to 65°C). An equal volume of chloroform-isoamyl alcohol (24:1) was added, and the samples were incubated on ice for 5 min. RNA samples were centrifuged to separate the phases, and the aqueous phase was extracted with hot phenol two more times. After ether extraction and ethanol precipitation, the RNA samples were treated with DNase I, as described by Ambulos et al. (1) and stored at −70°C.

Labeling of a probe specific for PA mRNA. A probe was labeled by primer extension on a template of single-stranded DNA prepared from phagemid p201. The template DNA was isolated by using a modification of the protocol described in the United States Biochemical Corp. product literature for pTZ18R. One hundred milliliters of cells was incubated overnight in L broth containing 50 μg of kanamycin per ml in the presence of M13K07 helper phage. Bacteriophage present in the supernatant from 50 ml of the overnight culture was precipitated on ice for 1 h by the addition of 10 ml of cold 25% polyethylene glycol–2.5 M NaCl. Bacteriophage was pelleted by centrifugation at 10,000 rpm for 10 min in a Sorvall HB-4 rotor. The pellet was suspended in 10 ml of TE (10 mM Tris hydrochloride [pH 7.4], 1 mM disodium EDTA) and centrifuged at 10,000 rpm for 10 min in an HB-4 rotor to remove debris. The supernatant was reprecipitated on ice for 30 min by the addition of 1 ml of 25% polyethylene glycol–2.5 M NaCl. After centrifugation at 10,000 rpm for 10 min in an HB-4 rotor, the pellet was suspended in 50 μl of TE plus 0.1% SDS and heated at 55°C for 10 min. The lysate was diluted with 200 μl of TE and extracted with an equal volume of TE-saturated phenol. The aqueous layer was removed and extracted once with phenol-chloroform (1:1), once with chloroform-isoamyl alcohol (24:1), and three times with water-saturated ether. After ethanol precipitation, the p201 DNA pellet was suspended in 40 μl of TE. A radioactive complement to PA mRNA was synthesized from the p201 single-stranded DNA as described by Ambulos et al. (1). Synthesis of the complementary strand was initiated with an 18-nucleotide oligonucleotide primer with the sequence 5′-ATATATGTTAGTTGCGTT-3′ and which hybridizes to a DNA sequence 485 bases upstream of the carboxy terminus of *pag* (36).

Hybridization conditions. RNA samples (0.625 to 2.5 μg) were applied to nitrocellulose filters by using a slot blot manifold (Schleicher & Schuell, Inc., Keene, N.H.) in accordance with the directions of the manufacturer. Blots were air dried and heated at 80°C in a vacuum oven. Prehybridization was for 30 min in hybridization buffer N, containing 0.2 M Tris hydrochloride buffer (pH 7.4), 10% dextran sulfate, 50% (vol/vol) formamide, 1× Denhardt solution (2% polyvinylpyrrolidone, 2% bovine serum albumin, 2% Ficoll 400), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 2 mg of salmon sperm DNA per ml (4). Hybridization to labeled probe (10^6 cpm in 20 ml of hybridization buffer N) was carried out overnight at 42°C. The blots were washed four times at room temperature with 2× SSC–0.1% SDS and twice at 55°C in 1× SSC–0.1% SDS. Autoradiography was at room temperature for 4 h or at −70°C overnight.

Construction of a *pag–cat-86* fusion. The construction of an operon fusion between *pag* and the promoterless CAT gene (*cat-86*) in vector pPL703 is diagrammed in Fig. 1. Phagemid p201 was digested with *Sau*3A, and the fragments were separated on a 0.8% agarose gel. A 1.5-kb fragment that contained the promoter region of *pag* was excised from the gel and purified by treatment with Gene Clean (Bio101, La Jolla, Calif.). The vector plasmid, pPL703, was digested with

*Bam*HI and ligated to the 1.5-kb *Sau*3A fragment, and the chimeric DNA was transformed into cells of *B. subtilis* DB104 that had been made competent by the method of Dubnau and Davidoff-Abelson (5). Transformants were plated on BHI agar plates containing 10 μg of chloramphenicol per ml. Plasmid DNA was prepared from chloramphenicol-resistant transformants and digested with *Hin*dIII to determine the orientation of the 1.5-kb insert. A plasmid containing an insert of the correct size and in the correct orientation was designated pFD1.

Electroporation of *B. anthracis*. Plasmid pFD1 was introduced into *B. anthracis* Weybridge UM23-1 (Tox^+) and UM23C1-1 (Tox^-) by electroporation by the procedure of G. Dunny (personal communication). Briefly, an overnight culture of *B. anthracis* was diluted 1:20 into BYGT (19% BHI, 0.5% yeast extract, 2% glucose, 0.1 M Tris [pH 8]) and incubated with shaking for 1 h at 37°C. Cells were harvested by centrifugation, washed in one-third volume of ice-cold electroporation buffer (0.625 M sucrose, 1 mM $MgCl_2$ [pH 4.0, filter sterilized]), and resuspended in 1/10 the original culture volume of electroporation buffer. After a 30-min incubation on ice, 0.8 ml of cells was mixed with 1 μg of pFD1 DNA and electroporated with a Gene Pulser (Bio-Rad Laboratories, Rockville Center, N.Y.) set at 2500 V and 25 μF capacitance. The cells were diluted into 2.2 ml of BYGT, incubated at 37°C for 1 h with shaking, and plated onto BHI agar plates containing 100 μg of kanamycin per ml to select for the neomycin-kanamycin resistance marker on pFD1.

Toxin and CAT assays. PA in culture supernatants was measured by an antibody-capture enzyme-linked immunosorbent assay (ELISA) system (35). Cultures to be assayed for CAT activity were grown in medium containing 2 μg of chloramphenicol per ml (23). Growth of the cultures was measured with a Klett-Summerson colorimeter. Samples (10 ml) were removed, centrifuged at 15,000 rpm in an SS34 rotor, and washed with 1 ml of 50 mM Tris hydrochloride (pH 7.8)–30 μM dithiothreitol. Cell pellets were resuspended in 200 μl of 50 mM Tris hydrochloride (pH 7.8)–30 μM dithiothreitol, and cell extracts were prepared by sonication. CAT activity was measured by the colorimetric assay described by Shaw (29).

RESULTS

Correlation between *pag* mRNA levels and extracellular PA protein. Cultures were grown, and PA protein in the culture supernatant and PA mRNA in cell extracts were measured. The relative amounts of PA mRNA produced by UM23-1 cultures grown in BHI, R medium, R medium plus 0.8% bicarbonate (RC medium), or RC medium plus 5% horse serum (RCH medium) are shown in Fig. 2. There was a good correlation between the amount of PA protein detected in culture supernatants and the amount of PA mRNA (Fig. 3); however, there was less correlation when horse serum was added. PA mRNA was not detected in cultures of nontoxigenic *B. anthracis* Weybridge UM23C1-1 (data not shown), demonstrating the specificity of the DNA probe, nor was PA mRNA detected at high levels in BHI-grown cultures (final pH, 8.0). In most experiments, toxigenic cultures grown in R medium in the presence of bicarbonate (final pH, 7.7 to 8.2) contained 10-fold more PA mRNA than cultures grown in the absence of bicarbonate (final pH, 7.5 to 7.7), although the induction ratio was somewhat variable, ranging from 10 to 40-fold.

Early experiments in production of anthrax toxin in vitro involved adding horse serum to the growth medium (6).

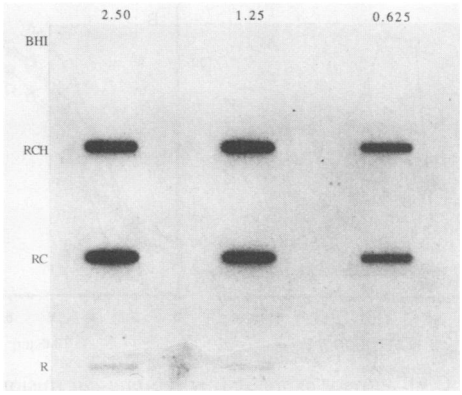

FIG. 2. Analysis of PA mRNA by slot blot hybridization. Cells were grown in either R medium (R), R medium containing 0.8% sodium bicarbonate (RC), RC plus 5% horse serum (RCH), or BHI, and samples were removed for RNA extraction when the cultures reached an A_{660} of 0.8. The samples, containing 2.5, 1.25, and 0.625 μg of total RNA, were blotted onto nitrocellulose and hybridized to a probe specific for PA mRNA. The data shown represent five experiments.

Further experiments showed that one of the factors in serum necessary for antigen production is bicarbonate (7). The addition of horse serum (5%) to RC medium routinely resulted in a two- to threefold increase in the amount of PA detectable by ELISA in the culture supernatant, but in the presence of bicarbonate, the serum did not have a measurable effect on the amount of PA mRNA (Fig. 3). In experiments for which results are not shown, however, we found

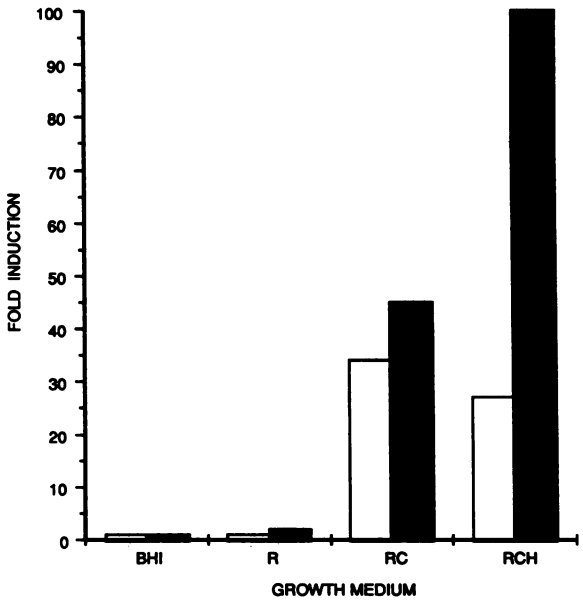

FIG. 3. Correlation of PA mRNA levels with secreted PA protein. Designations of media are the same as those in Fig. 2. PA mRNA prepared as for Fig. 2 was quantitated by densitometry. The concentration of PA protein in culture supernatants was measured by ELISA (done in duplicate). PA protein and mRNA levels of BHI-grown cells were used as a baseline to calculate the fold increase in expression. Open bars represent PA mRNA, and solid bars represent PA protein. The concentrations (micrograms per milliliter) of PA (micrograms per milliliter)) in culture supernatants were as follows: BHI, 0.08; R, 0.16; RC, 7.2; and RCH, 17.6.

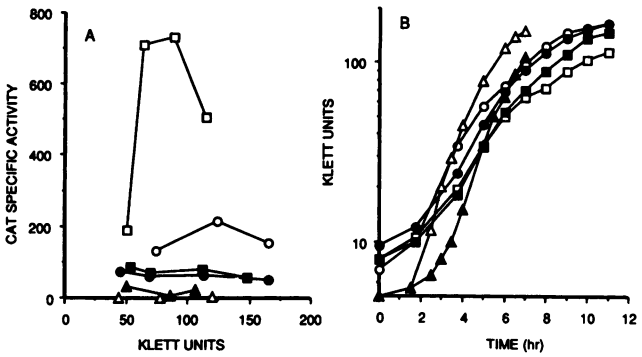

FIG. 4. CAT activity expressed by the *pag-cat* (fusion plasmid (pFD1) in *B. anthracis* strains with or without toxin plasmid pXO1 and grown in media with or without bicarbonate. (A) CAT activity of UM23-1 Tox$^+$(pFD1) grown in R (○), RC (□), or BHI (△) and of UM23C1-1 Tox$^-$(pFD1) grown in R (●), RC (■), or BHI (▲). CAT-specific activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of protein. Assays were done in duplicate, and the data presented represent three separate experiments. (B) Growth curves of the cultures described above. Symbols are the same as in panel A, and the media designations are as for Fig. 2.

that horse serum can partially replace bicarbonate in stimulating *pag* transcription but that maximal levels of toxin production could be achieved only by adding sodium bicarbonate. We have not examined this further since we believe that the stimulation of toxin production in the presence of horse serum is primarily an effect of bicarbonate.

Analysis of a *pag-cat-86* fusion in pXO1$^+$ and pXO1$^-$ strains of *B. anthracis*. To further characterize the factors involved in the stimulation of *pag* transcription by bicarbonate, the promoter region of *pag* was fused to the CAT gene (*cat-86*) of vector pPL703 (Fig. 1). Plasmid pPL703 contains a selectable neomycin-kanamycin resistance gene in addition to the coding region of *cat-86*. Preceding the *cat-86* coding region are a multiple cloning site; translational stop signals (in every reading frame), which effectively block translation initiating within the cloned promoter-containing fragment; and a ribosome-binding site that functions in *B. subtilis* (23). The *pag–cat-86* operon fusion was constructed by ligating a 1.5-kb *Sau*3A DNA fragment from p201 into the *Bam*HI site of pPL703. The *Sau*3A fragment contains the putative *pag* promoter and 650 base pairs of upstream DNA. Also included in this fragment are two regions of dyad symmetry, one of which encompasses the −10 and −35 regions of the *pag* promoter (36). The resulting chimeric plasmid, pFD1, was isolated from DB104(pFD1) and transformed by electroporation into *B. anthracis* Weybridge UM23-1 (Tox$^+$) and UM23C1-1 (Tox$^-$). All of the kanamycin-resistant transformants analyzed were resistant to at least 10μg of chloramphenicol per ml and contained a plasmid that comigrated on an agarose gel with pFD1. All pFD1 transformants of UM23-1 retained the toxin plasmid pXO1.

Expression of the *pag–cat-86* fusion was measured by growing *B. anthracis* UM23-1 Tox$^+$(pFD1) and UM23C1-1 Tox$^-$(pFD1) in BHI, R medium, and R medium plus 0.8% sodium bicarbonate and by assaying extracts for CAT activity. Growth curves and CAT assay data are given in Fig. 4. Cells of UM23-1 Tox$^+$(pFD1) grown in R medium plus 0.8% sodium bicarbonate exhibited a sixfold increase in CAT activity during the late-log/early-stationary phase compared with cells grown in the absence of bicarbonate (Fig. 4A and B). This stimulation of CAT expression by bicarbonate was not, however, observed with UM23C1-1 (Tox$^-$), indicating that toxin plasmid pXO1 was required for stimulation of *pag* transcription by bicarbonate. Additionally, CAT levels in cultures of UM23-1 Tox$^+$(pFD1) grown in R medium were elevated two- to threefold over those in cultures of UM23C1-1 Tox$^-$(pFD1) grown in R medium. This slight stimulation was presumably due to low levels of CO_2 produced from glucose fermentation in the microaerophilic cultures. It has been shown previously that the small amount of CO_2 produced from the fermentation of glucose and maintained in solution (at neutral pH or above) as bicarbonate ion could stimulate toxin production to a small degree (24, 25). In addition, production of toxin is dependent on the pH of the medium, being decreased at low pH (below 6.5), and we found that this effect is also exerted at the level of transcription (data not shown). This pH effect presumably results from the decreased bicarbonate ion concentration at pH values approaching the pK of carbonic acid (pH 6.4). CAT activity was uniformly low in BHI-grown cultures of both UM23-1 Tox$^+$(pFD1) and UM23C1-1 Tox$^-$(pFD1). Addition of bicarbonate to BHI-grown cultures did not increase the level of CAT activity (data not shown). The data obtained with the *pag–cat-86* fusion, therefore, confirm the results obtained by slot blot hybridization, i.e., that the increased PA production in cultures grown in the presence of bicarbonate was due to increased levels of PA mRNA. Additionally, the failure of bicarbonate to stimulate CAT activity from the *pag–cat-86* fusion in a pXO1$^-$ strain of *B. anthracis* implies that a region or regions of pXO1 are necessary for transcriptional activation of *pag* by bicarbonate in minimal medium.

A second *pag–cat-86* fusion (pFD2) was constructed which contains an additional 1 kb of DNA-sequence upstream of the *pag* promoter, including a cryptic open reading frame encoding 192 amino acids (36). *B. anthracis* strains containing pFD2 gave CAT-specific activities which were virtually identical to those presented for pFD1 (Fig. 4) and also required the presence of pXO1 for increased expression of the CAT fusion in cultures grown in RC medium (data not shown). This result indicates that the cryptic open reading frame does not encode the information necessary for transcriptional activation of *pag* by bicarbonate.

DISCUSSION

Prior to the work described here, several explanations had been offered to explain the enhancement of toxin production by bicarbonate. Puziss and Howard (24) presented evidence that bicarbonate increases the permeability of *B. anthracis*, releasing toxin that otherwise accumulates intracellularly. Work by Strange and Thorne (31) showed that secreted toxin is degraded by proteases present in the culture supernatant, especially at pH values below 7. Those experiments showed that bicarbonate helped to maintain the pH above 7 and thereby protected the toxin. Other buffers (Tris, etc.) that maintained an elevated pH did not yield toxin equal to that produced in bicarbonate-supplemented media, suggesting a specific effect of bicarbonate. These experiments have been confirmed in this laboratory (15; S. H. Leppla unpublished results). In work not detailed here, we confirmed that toxin is more stable in culture supernatants at pH values above 7. However, comparison by ELISA of PA in culture supernatants and in the corresponding cell pellets has not revealed accumulation of toxin within *B. anthracis* cells grown under any condition, and we question whether the effects on permeability described above do occur. The effect of bicar-

bonate does not appear to be due to elevated osmotic strength or a high level of sodium ion, based on our observations that toxin production was not stimulated under these conditions in the absence of bicarbonate and the fact that CO_2 was also effective in stimulating toxin production. The work described in this study shows that much of the effect of bicarbonate on toxin production resulted from an increase in the steady-state levels of PA mRNA. Although we could not rule out that this increase in PA mRNA was due to increased message stability, preliminary experiments designed to address this possibility indicate that this is not the case (unpublished data). Additionally, the changes in expression of the *pag–cat-86* fusion in response to bicarbonate are consistent with the idea that the elevated levels of PA mRNA are due to an increase in transcription initiating at the *pag* promoter.

The observation that maximal expression of the CAT fusion in *B. anthracis* grown in bicarbonate-containing medium required DNA sequences from pXO1, in addition to those contained in the 1.5-kb *Sau*3A fragment of pFD1, suggests the existence of a *trans*-acting regulatory factor. However, since these experiments were conducted with recombination-proficient strains of *B. anthracis*, we could not rule out the possibility of homologous recombination between pFD1 and pXO1 and could not, therefore, rule out the possibility that the positive regulatory factor acts in *cis*. If the DNA sequences on pXO1 that are required for transcriptional activation of *pag* are *cis* acting, however, they must be located more than 1.7 kb upstream of *pag* and be separated from *pag* by the DNA sequence encoding the cryptic open reading frame.

Coordinate regulation of virulence factors of pathogenic bacteria in response to environmental stimuli appears to be a common theme (for a review, see reference 21). In the well-studied case of *Bordetella pertussis*, the products of the regulatory genes *vir* and *mod* interact in a manner that is characteristic of a two-component sensor-transducer regulatory system, and this interaction results in the activation of some genes, the so-called *vir*-activated genes, which include a number of virulence factors, and in the repression of other, *vir*-repressed genes, whose functions have not been characterized (13). Coordinate regulation of virulence factors may occur in *B. anthracis* also, as evidenced by the requirement for bicarbonate for production of edema factor, lethal factor, and the capsule, as well as for production of PA. Preliminary evidence indicates that the steady-state level of edema factor mRNA was elevated in bicarbonate-containing cultures (unpublished data). Other factors in *B. anthracis* may also be influenced by bicarbonate. Sporulation appears to be inhibited by the presence of bicarbonate (unpublished observation), and lysozyme sensitivity in some strains is increased (3, 8, 37). Whether the enhanced production of these factors (and the inhibition of sporulation) by bicarbonate requires the same factors as those involved in the transcriptional stimulation of *pag* remains to be determined. It appears, however, that the regulation of virulence determinants in *B. anthracis* may well involve a number of different genetic loci. Recent data of Makino et al. (17) indicate that there is a regulatory gene located on pXO2 that is involved in the CO_2-mediated stimulation of capsule synthesis. This gene appears to stimulate capsule synthesis in a *trans* configuration in *B. anthracis*. We have not yet examined the possibility that the *trans* activator of the capsule genes may also act as a *trans* activator of the PA gene. Use of gene fusions, such as the *pag–cat-86* construction described here, however, can be used to address this possibility and will facilitate the identification and cloning of any additional regulatory loci. Work currently in progress, involving the construction of gene fusions in vivo, as described by Youngman et al. (38), will aid in the identification of other genes whose transcription may be affected by bicarbonate and which may be coordinately regulated with *pag*.

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