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Use of a Promoter Trap System in *Bacillus anthracis* and *Bacillus subtilis* for the Development of Recombinant Protective Antigen-Based Vaccines

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We have recently reported *Bacillus anthracis* attenuated live vaccine strains efficiently expressing recombinant protective antigen (rPA) and have shown a direct correlation between the level of rPA secreted by these cells and efficacy (S. Cohen, I. Mendelson, Z. Altboum, D. Kobiler, E. Elhanany, T. Bino, M. Leitner, I. Inbar, H. Rosenberg, Y. Gozes, R. Barak, M. Fisher, C. Kronman, B. Velan, and A. Shafferman, Infect. Immun. 68:4549-4558, 2000). To isolate more potent *Bacillus* promoters for a further increase in the production of rPA, we developed a promoter trap system based on various *gfp* reporter genes adapted for use in both *Bacillus subtilis* and *B. anthracis* backgrounds. Accordingly, a *B. anthracis* library of 6,000 clones harboring plasmids with chromosomal *B. anthracis* DNA fragments inserted upstream from *gfpuv* was constructed. Based on fluorescence intensity, 57 clones carrying potentially strong promoters were identified, some of which were DNA sequenced. The most potent *B. anthracis* promoter identified (P*ntr*; 271 bp) was 500 times more potent than the native *pagA* promoter and 70 times more potent than the α-amylase promoter (P*amy*). This very potent promoter was tested along with the other promoters (which are three, six, and eight times more potent than P*amy*) for the ability to drive expression of rPA in either *B. subtilis* or *B. anthracis*. The number of cell-associated pre-PA molecules in *B. anthracis* was found to correlate well with the strength of the promoter. However, there appeared to be an upper limit to the amount of mature PA secreted into the medium, which did not exceed that driven by P*amy*. Furthermore, the rPA constructs fused to the very potent promoters proved to be deleterious to the bacterial hosts and consequently led to genetic instability of the PA expression plasmid. Immunization with attenuated *B. anthracis* expressing rPA under the control of promoters more potent than P*amy* was less efficient in eliciting anti-PA antibodies than that attained with P*amy*. The results are consistent with the notion that overexpression of PA leads to severe secretion stress and have practical implications for the design of second-generation rPA-based vaccines.

Bacillus anthracis, the etiological agent of anthrax, a fatal disease affecting livestock and humans, has recently gained much attention as a potential warfare and bioterrorism threat. The combined effects of a poly-D-glutamic acid capsule, encoded by a native plasmid, pXO2 (16, 27), and two binary secreted toxins, lethal toxin and edema toxin, encoded by a second native plasmid, pXO1 (29, 31; reviewed in reference 26), contribute to the pathogenesis of *B. anthracis*. Both toxins possess a common receptor-binding component, protective antigen (PA), which interacts either with the lethal factor (in the case of lethal toxin) or the edema factor (in the case of edema toxin) to mediate their entry into the cytosol, where they perform their detrimental activities. PA has a major and essential role in the induction of immunity and protection against the disease. Vaccination with PA alone can induce protective immunity (22). A direct relationship exists between the amount of PA administered to experimental animals and the intensity of the humoral immune response elicited against PA, as well as protection against a subsequent challenge with *B. anthracis* (11, 34, 41, 43, 44). Anthrax vaccines licensed for human use are composed of cell-free PA (15, 17, 32, 42). The use of cell-free vaccines entails a prolonged vaccination schedule, and they require repeated boosting (7). Attempts to create a vaccine that provides higher levels of protection have focused on incorporation of additional bacterial constituents, either by the addition of inactivated spores to PA (10) or by generation of live, attenuated bacterial strains expressing recombinant PA (rPA) (3, 11).

Using a nontoxinogenic unencapsulated *B. anthracis* platform, we have developed two recombinant vaccine candidates in which the PA gene is expressed by a high-copy-number plasmid under the regulation of two different promoters: the native endogenous promoter of the PA gene, P*pag*, and the heterologous potent α-amylase promoter of *Bacillus amyloliquefaciens*, P*amy* (11). The latter was found to be 5 to 10 times more efficient in promoting PA expression. It was observed that the protective efficacies of the different recombinant strains in guinea pigs were correlated with the level of PA production in vitro. The establishment of an improved PA-expressing *B. anthracis* strain by the use of a very potent pro-

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TABLE 1. Plasmids and strains

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
pUB110	Km^r in bacilli; *Staphylococcus aureus* origin of replication	Sigma
pGEM-3Z	Ap^r in *E. coli*	Promega
pGFP	Source of wt *gfp* reporter gene	Clontech
pGFPuv	Source of *gfpuv* reporter gene	Clontech
pEGFP	Source of *egfp* reporter gene	Clontech
pXX_{MCS-5}	*E. coli-Bacillus* shuttle vector; composed of an altered fusion of pGEM-3Z/pUB110 without promoter or reporter gene; Ap^r Km^r	Laboratory stock
$pGwt_{MCS-5}$	Promoterless wt *gfp* gene from pGFPwt; inserted in pXX_{MCS-5}	This work
$pGuv_{MCS-5}$	Promoterless *gfpuv* gene from pGFPuv; inserted in pXX_{MCS-5}	This work
pGe_{MCS-5}	Promoterless *egfp* gene from pEGFP; inserted in pXX_{MCS-5}	This work
pPA20-α	Source for P*amy* promoter and *pagA* gene	11
$pGwt_{amy}$	P*amy* promoter fused to wt *gfp* gene cloned in pXX_{MCS-5}	This work
$pGwt_{tms}$	P*tms* promoter fused to wt *gfp* gene cloned in pXX_{MCS-5}	This work
$pGwt_{43}$	P*43* promoter region fused to wt *gfp* gene cloned in pXX_{MCS-5}	This work
$pGuv_{amy}$	P*amy* promoter fused to *gfpuv* gene cloned in pXX_{MCS-5}	This work
$pGuv_{43}$	P*43* promoter region fused to *gfpuv* gene cloned in pXX_{MCS-5}	This work
$pGuv_{sapS}$	$Psap_{short}$ promoter region fused to *gfpuv* gene cloned in pXX_{MCS-5}	This work
$pGuv_{sapL}$	$Psap_{long}$ promoter region fused to *gfpuv* gene cloned in pXX_{MCS-5}	This work
pGe_{43}	P*43* promoter region fused to *egfp* gene cloned in pXX_{MCS-5}	This work
pA_{MCS-5}	Promoterless *pagA* gene from pPA20-α; inserted in pXX_{MCS-5}	This work
pA_{pag}	Formerly named pAαUB; P*pag* promoter region fused to *pagA* gene cloned in pGEM-3Z/pUB110 fusion vector; Ap^r Km^r	11
pA_{amy}	Formerly named pαAUB; P*amy* promoter fused to *pagA* gene cloned in pGEM-3Z/pUB110 fusion vector; Ap^r Km^r	11
pA_{43}	P*43* promoter region fused to *pagA* gene cloned in pXX_{MCS-5}	This work
pA_{sapS}	$Psap_{short}$ promoter region fused to *pagA* gene cloned in pXX_{MCS-5}	This work
pA_{sapL}	$Psap_{long}$ promoter region fused to *pagA* gene cloned in pXX_{MCS-5}	This work
pA_{ntr}	P*ntr* promoter region of library clone L-19 fused to *pagA* gene cloned in pXX_{MCS-5}	This work
B. anthracis Δ14185	$pXO1^-$ $pXO2^-$ strain; a derivative of ATCC 14185	11
B. subtilis WB600	Δ*nprE* Δ*aprE* Δ*epr* Δ*bpf* Δ*mpr* Δ*nprB*	48
E. coli		
DH5α	*recA1*	Clontech
GM2929	*dam*13::Tn*9* (Cm^r) *dcm*-6	New England Biolabs

moter, therefore, represents a challenge for the development of a more efficient anthrax vaccine.

Bacillus subtilis has been proposed as a possible host for production of rPA (1, 11, 21, 38). While many well-established promoters are widely used for expression in *B. subtilis*, few promoters have been tested for driving recombinant-protein expression in *B. anthracis*. In the present study, we carried out a systematic investigation of the identification and use of a variety of promoters in *B. anthracis*. Efficient *B. anthracis* promoters were identified via screening of a chromosomal-DNA library of *B. anthracis* fused to the fluorescent biotracer green fluorescent protein (GFP). Selected promoters were studied for the ability to drive expression of rPA, and a complex relationship between promoter strength and the efficacy of PA production was observed. The implications of driving rPA expression from very potent promoters to generate cell-free or live attenuated vaccines are demonstrated and discussed.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains are listed in Table 1. *Escherichia coli* DH5α and GM2929 were grown in Luria-Bertani (LB) medium (Difco Laboratories) containing 100 μg of ampicillin (Sigma)/ml. Recombinant strains of *B. subtilis* WB600 and *B. anthracis* Δ14185 were grown in modified FAG medium (3.3% tryptone, 2% yeast extract, 0.74% NaCl, 0.4% KH_2PO_4, 0.8% Na_2HPO_4, 2% glycerol, pH 8) supplemented with 25 μg of kanamycin (Sigma)/ml. Overnight culture (30°C) innoculi were diluted in fresh medium to an A_{550} of 0.01 to 0.05, and bacterial propagation was carried out at 37°C under vigorous agitation. Sporulations of *B. anthracis* were carried out at 34°C in Schaeffer's sporulation broth for 72 h. The resulting spores (observed microscopically) were pelleted and washed twice with sterile water. The spores were then submitted to heat shock for 20 min and kept at −70°C.

Recombinant DNA and genetic manipulations. Enzymes were purchased from New England Biolabs or Promega and were used as recommended by the suppliers. For PCR amplifications, *Taq* (Promega) or Expand (Boehringer Mannheim GmbH) DNA polymerase was used. *E. coli* strains were transformed by the method of Hanahan (18). *B. subtilis* and *B. anthracis* were electroporated as described previously (9) with either DNA ligation mixtures or plasmid DNAs that were isolated from *E. coli* GM2929. This strain is defective in DNA methylation systems; therefore, DNA generated in this bacterium is suitable for manipulations in *B. anthracis*, in which methylated DNA is severely restricted. DNA fragments were purified using the Qiagen QIAquick PCR purification kit. Plasmid DNA was extracted from *E. coli* with the Wizard Plus Maxiprep or SV Miniprep kits (Promega). *B. subtilis* and *B. anthracis* cells were treated with lysozyme (L-7651; Sigma) for 30 min at 37°C prior to plasmid extraction. DNA sequences were determined with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer) run on an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Oligonucleotides were constructed using the Applied Biosystems 392 DNA-RNA synthesizer.

Construction of plasmids. Vectors pGFP, pGFPuv, and pEGFP, used as sources for the wild-type (wt) *gfp*, *gfpuv*, and *egfp* genes, respectively, were purchased from Clontech. The *gfp* reporter genes were cloned in an *E. coli-Bacillus* shuttle vector, pXX_{MCS-5}, shown in Fig. 1, to produce $pGwt_{MCS-5}$, $pGuv_{MCS-5}$, and pGe_{MCS-5}. The parental vector, pXX_{MCS-5}, is a product of ligation between pGEM-3Z and pUB110 containing the following adjustments: (i) modification of the pGEM-3Z multiple cloning site (MCS), (ii) deletion of a 521-bp *Aat*II-*Sma*I fragment, (iii) elimination of the *Bgl*II site in the pUB110 fragment, (iv) deletion of an 800-bp *Hin*dIII-*Afl*II fragment of pUB110 and

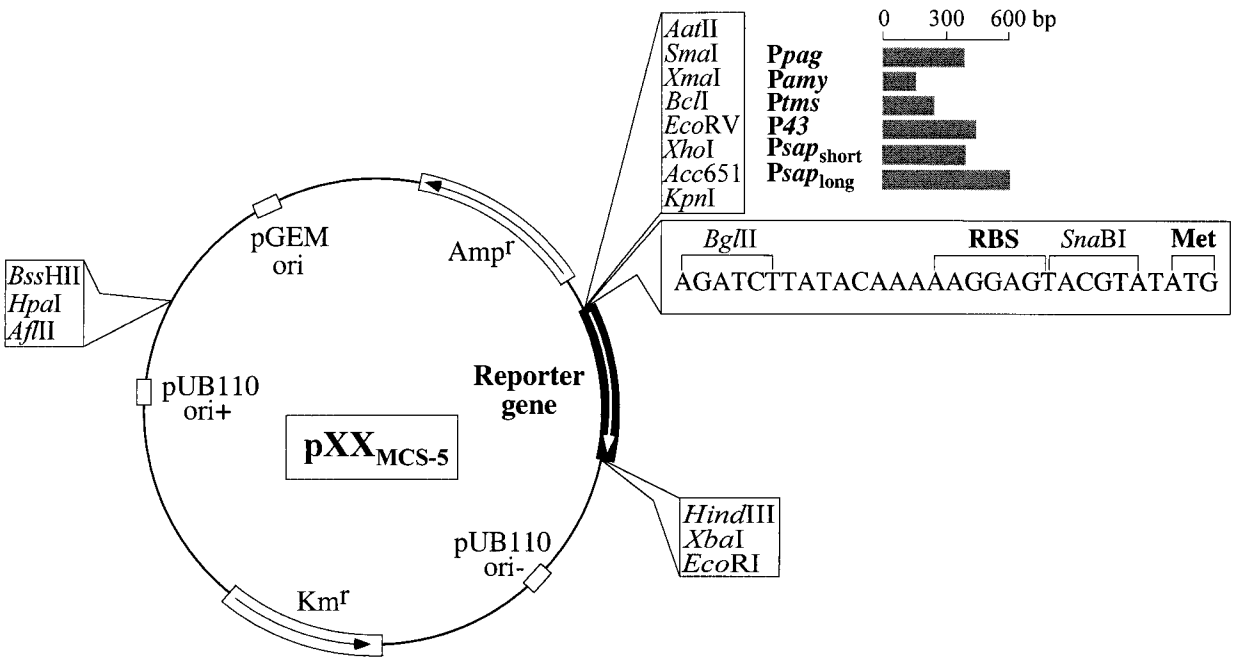


FIG. 1. Schematic representation of the promoter-trapping vector used in the present study. pXX_{MCS-5} is a versatile plasmid conveniently allowing cloning of either promoter cassettes or reporter genes. This plasmid was used for (i) evaluation of various promoters and reporter genes, (ii) construction and screening of a genomic *B. anthracis* library, and (iii) expression of the *pagA* gene. Restriction sites for insertion of the various promoters and different reporter genes, as well as the respective promoter cassettes, are indicated. The linker DNA sequence between the promoter and the reporter gene insertion sites contains the RBS.

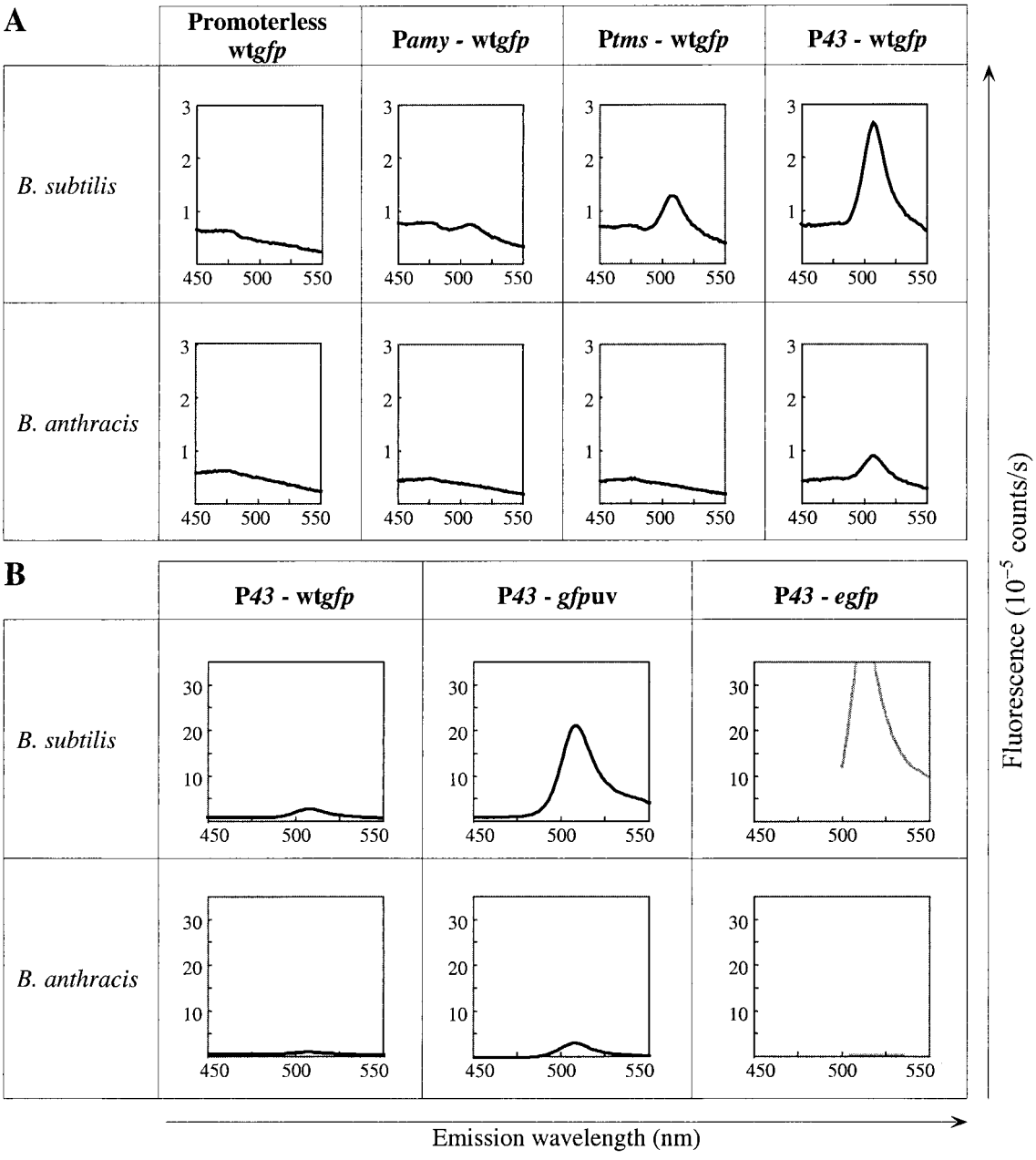
addition of an MCS, and (v) elimination of the *Sna*BI site in the pUB110 fragment. The cloning was performed by ligation of PCR-amplified genes (PCR primers, 5′-ATACGATACGTAATGAGTAAAGGAGAAGAACTTTTC-3′, containing an *Sna*BI site [underlined], and 5′-ATACGATCTAGATTATTTGT AGAGCTCATCCATGC-3′, containing an *Xba*I site [underlined]) into the unique *Sna*BI-*Xba*I sites in the vector. Various promoter cassettes (except for the PA gene and the α-amylase gene promoter fragments, which were constructed as described by Cohen et al. [11]) were cloned within the unique *Eco*RV and *Bgl*II restriction sites, following amplification by PCR of the relevant DNA chromosomal fragment. The following primers (upstream and downstream) were used: P*tms*, 5′-ATACGAGATATCCGAATTCGAAGAAGCTGGAGCTTC-3′ and 5′-ATACGAAGATCTCCATTTATTGGCCTCCAATATCCC-3′; P*43*, 5′-ATA CGAGATATCTGTCGACGTGCATGCAGGCCG-3′ and 5′-ATACGAAGAT CTTATAATGGTACCGCTATCAC-3′; Psap_{short}, 5′-ATACGAGATATCGTC GACGTTAGATGTTGTAATTGG-3′ and 5′-ATACGAAGATCTGTAATAC TAGTTCCATAAT-3′; and Psap_{long}, 5′-ATACGAGATATCGTCGACAAGCT TGTAAACTTATTCT-3′ and 5′-ATACGAAGATCTGTAATACTAGTTCCA TAAT-3′. Note that all the upstream primers have an *Eco*RV restriction site (underlined) at their 5′ termini and the downstream primers have a *Bgl*II site (underlined). The resulting plasmids are listed in Table 1. Vector $pGuv_{MCS-5}$ was also used for construction of the *B. anthracis* Δ14185 promoter library, as described below.

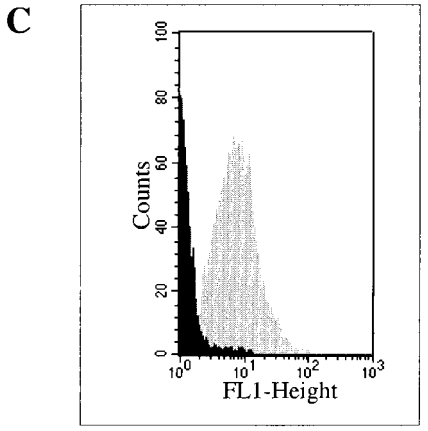
The putative promoter region in the chromosomal fragment of the library isolate L-51 was recloned following PCR amplification: upstream primer (inferred from the genomic sequence and containing an *Eco*RV site [underlined]), 5′-ACACAGATATCGCACTTATCATAAAAAAGAAATATGAACG-3′, and downstream primer (containing a *Bgl*II site [underlined]), 5′-ACACAAGATCT GATAATGAATTTTCGCCCTTG-3′ (see Fig. 5 for the chromosomal organization of this clone).

The *pagA* gene was cloned, replacing the *gfpuv* gene, downstream of the various promoter cassettes. Each vector DNA, containing the *gfpuv* gene with the relevant promoter, was extracted from *E. coli* GM2929. The *gfpuv* gene was excised from the plasmids by digestion with *Bgl*II and *Xba*I (which is blocked by *dam* methylation). The DNA fragment containing the *pagA* gene along with the ribosome-binding site (RBS) was PCR amplified from plasmid pPA20-α (described elsewhere [11]) using the following primers: 5′-GGTGGTGTCGACGT GCATGCAGGCCG-3′ and 5′-ACACATCTAGAAAAA AAGCTTCCTACAA ACAATCTCAAAGGATTATG-3′. The PCR product was digested with *Bgl*II and *Xba*I and ligated to each predigested vector. The resulting plasmids are listed in Table 1.

Analysis of GFP fluorescence. Fluorescent colonies expressing either the wt *gfp* or *gfpuv* gene on solid agar were visualized at a wavelength of 366 nm on a UV illuminator (Reprostar II; CAMAG). Images were captured using an MPEG-MOVIEHQ DSC-S70 compact digital camera (Sony). Formaldehyde-fixed bacterial samples were viewed by a fluorescence microscope (Zeiss) (equipped with an HBO 50-W mercury short-arc photo optic lamp; OSRAM). Quantification of fluorescence in culture samples of selected clones was performed with a SPECTRAFLUOR PLUS (TECAN) fluorimetric microplate reader, using the appropriate filters for excitation, 410 ± 5 (for wt GFP and GFPuv) or 485 ± 5 (for enhanced GFP [EGFP]) nm, and emission of 510 ± 10 nm. The fluorescence spectrum analysis of all GFP derivatives was carried out with a Photon Technology International (C-71) fluorimeter. Culture samples were washed once and diluted to an A_{550} of 1.0 with phosphate-buffered saline (PBS) prior to analysis. Flow cytometry analysis was performed with a FACSCalibur machine (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a 15-mW argon laser as the excitation light source. Fluorescence (530 ± 15 nm), side scatter, and forward-scatter data were collected using logarithmic amplifiers. A mixture of nonfluorescing ($pGuv_{MCS-5}$) and fluorescing ($pGuv_{43}$) bacterial cultures was analyzed into non-GFP-expressing and GFP-expressing fractions.

Construction and initial screening of a *B. anthracis* Δ14185 genomic library. Chromosomal DNA from *B. anthracis* Δ14185 was isolated by a modification of the Marmur method, in which additional steps for lysis of gram-positive bacteria were introduced (23). Cells were grown in 1 liter of FAG medium to an A_{550} of 15, washed three times with saline-EDTA, suspended in 100 ml of saline-EDTA (1:5), and treated with 10 mg of lysozyme/ml for 1 h at 37°C. A full *Sau*3AI digest of purified chromosomal DNA was ligated to nonmethylated *Bgl*II-digested $pGuv_{MCS-5}$ (extracted from *E. coli* GM2929). The ligation mixture was introduced either into *E. coli* DH5α, to define the library quality (the proportion of clones containing inserts and the average size of inserts), or directly into *B. anthracis* Δ14185 to screen for *gfpuv* expression. For library construction, the *B. anthracis* competent cells were prepared at a 5× concentration compared to transformation of ligated vector extracted from an *E. coli* host system. Thus, the electroporation efficiency of the ligated mixture introduced directly into *B. anthracis* Δ14185 was 1.5×10^3 transformants per μg of vector DNA. For estima-


A
Promoterless wtgfp
Pamy - wtgfp
Ptms - wtgfp
P43 - wtgfp
B. subtilis
B. anthracis
B
P43 - wtgfp
P43 - gfpuv
P43 - egfp
B. subtilis
B. anthracis
Fluorescence (10^-5 counts/s)
Emission wavelength (nm)


C
Counts
FL1-Height

tion of the average insert size, minipreparations of plasmid DNA were run on agarose gels following digestion with *Xho*I. Twenty transformants were taken from each of three individual ligations, and the average size was calculated as the reciprocal geometric mean. For initial screening of the promoter library, *B. anthracis* Δ14185 transformants were plated directly on LB agar containing 5 μg of kanamycin/ml. Each electroporation mixture containing 20 ng of vector DNA and 100 ng of chromosomal digest produced 300 to 400 colonies. The agar plates were inspected by UV light (366 nm), and the fluorescent colonies were isolated.

Characterization of the chromosomal inserts in selected GFP-expressing clones. Plasmid DNA isolated from *B. anthracis* served as the template for sequencing reactions, using primers flanking the MCS: upstream primer, 5′-AA ACCAAAGACGTCCCCGGGAATGATCATGATATCG-3′, and downstream primer, 5′-GGGACAACTCCAGTGAAAAGTTCTT-3′. Each sequencing reaction generated 300 to 500 bases of readable sequence that served as a homology tag for the identification of the isolated inserts. The genomic sequences were analyzed with BLASTN and BLASTX software for nucleotide or protein sequence similarity and searched for open reading frames using the ORF Finder program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), in the February 2001 version of the *B. anthracis* Ames chromosomal sequence database drawn with permission from The Institute for Genomic Research, Rockville, Md. (http://www.tigr.org). Putative RBSs were identified by the optimal *Bacillus* RBS sequence (2). For further genetic manipulations, minipreparations of plasmid DNA extracted from *B. anthracis* cells were introduced into *E. coli* DH5α. Following purification, a PCR and restriction analysis was performed for validation of insert sizes, and the relevant inserts were digested and purified from gels for further subcloning procedures.

Immunoblot analysis of GFP. For detection of intracellular GFPuv, cell lysates were prepared as follows: cell pellets, originating from 1-ml cultures, were washed with 0.125 M Tris buffer (pH 6.8) and suspended in the same buffer to an A_{550} of 10.0. Samples were sonicated using a VibraCell sonicator in a 64-mm-diameter glass cup horn (Sonics & Materials), mixed with sample buffer, and resolved on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) gels. Immunoblotting was performed on Hybond-C pure membranes with commercial anti-GFP antibodies (Clontech) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma).

Preparation of rPA samples. *B. anthracis* and *B. subtilis* culture samples were removed during growth and centrifuged. Cell pellets were collected for evaluation of cell-associated PA, and the supernatants, containing secreted PA, were filtered through Acrodisc filters (0.2-μm-pore-size filters; Gelman Science). Cell-associated PA was determined in cell pellets suspended to an A_{550} of 10.0 in PBS buffer and lysed as described above for GFP detection. Samples were stored at −70°C until they were used.

Anti-PA antibodies. Polyclonal anti-PA antibodies were generated in rabbits (designated R-2) and mice (designated M-6) injected with highly purified native PA prepared as described previously (33). B-11 is a polyclonal antiserum raised against a polypeptide chimera of PA (amino acids 453 to 512) and β-galactosidase, prepared as described previously (11). AI-10 is an antiserum prepared in mice injected with a synthetic peptide derived from the amino-terminal sequence of the mature PA (EVKQENRL) and coupled to keyhole limpet hemocyanin through a cysteine residue at its carboxy terminus (using an Imject Maleimide Activated mcKLH kit; Pierce). Antiserum AI-10 recognizes only the mature, and not the preprotein, form of PA.

Quantitation of rPA. Capture enzyme-linked immunosorbent assay (ELISA) for quantification of secreted rPA in culture supernatants was carried out with the antisera R-2 and M-6 (see above). rPA protein, purified from a *B. subtilis* recombinant strain harboring pA_{amy}, of a previously determined concentration was used as a standard. For immunoblot analysis of intracellular rPA, either B-11 or AI-10 was applied. The cytotoxicity of rPA for cells (in conjunction with a constant amount of lethal factor) was evaluated by a macrophage lysis assay with J774A.1 murine cells as detailed previously (11).

Colony stability assay. The plasmid stability assay was performed essentially as described previously (9). Bacteria from selective LB agar (supplemented with 25 μg of kanamycin/ml) were used to inoculate growth in selective liquid FAG medium. At mid-log phase, the cells were diluted, plated on nonselective LB agar, and incubated at 37°C for the growth of well-separated colonies. Then, a mixture of colonies was diluted and plated again. Four hundred colonies from LB agar were analyzed for antibiotic resistance by toothpicking them onto selective plates.

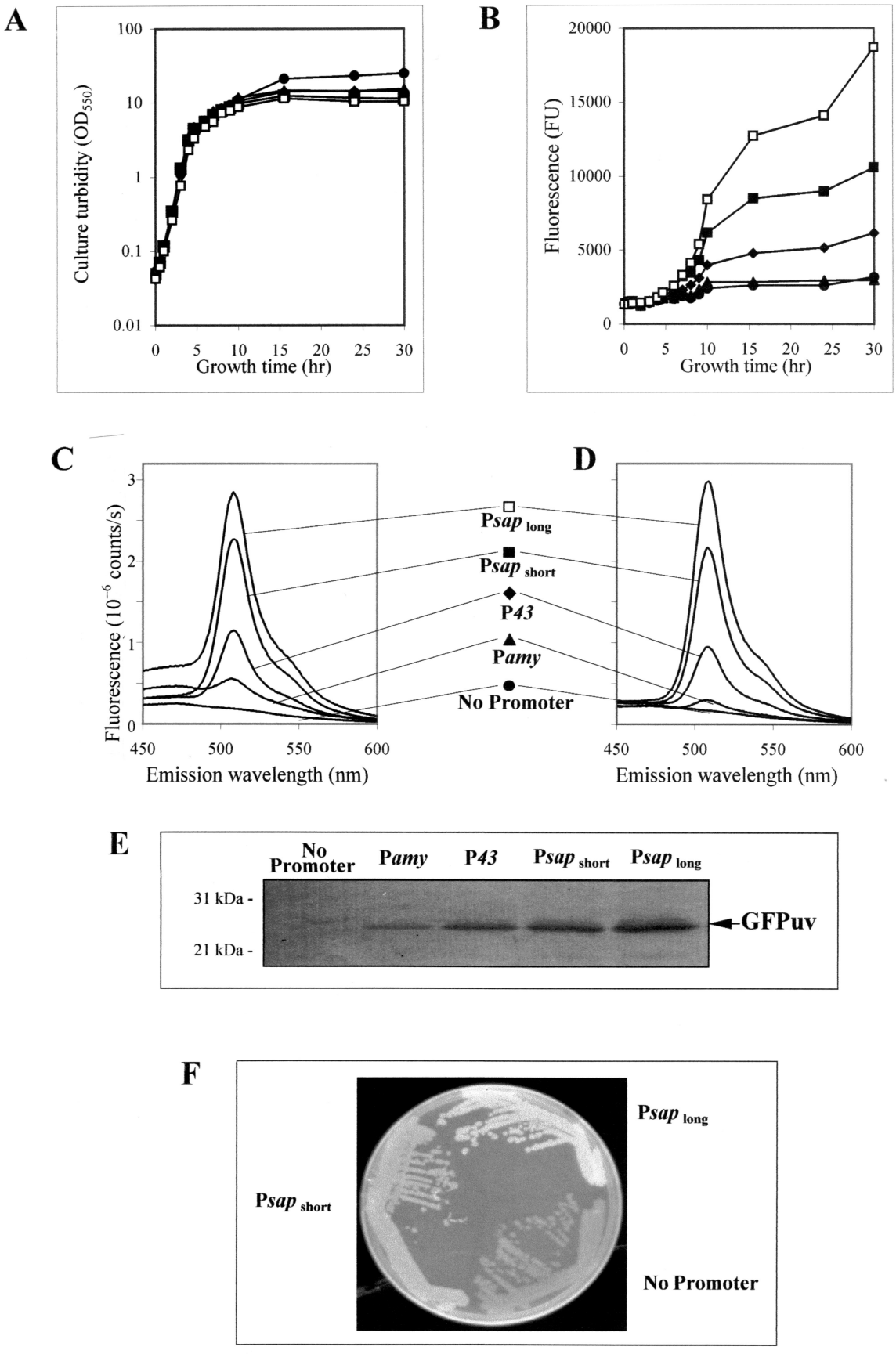
Immunization with *B. anthracis* harboring the various rPA promoter cassettes. Female Hartley guinea pigs (Charles River Laboratories, Sulzfeld, Germany) weighing 220 to 250 g were inoculated subcutaneously with a single dose of 10^6 spores of *B. anthracis* recombinant strains. Animal care and use were in accordance with the National Research Council 1996 Guide for the Care and Use of Laboratory Animals and approved by the local authorities. Six weeks following inoculation of the spores, serum samples were collected for determination of anti-core (representing a crude soluble material of vegetative Δ14185 cells) and anti-PA antibodies by ELISA as described previously (11). Antibody titers were calculated as reciprocal geometric means.

RESULTS

Development of a *B. subtilis*- and *B. anthracis*-specific GFP reporter system for promoter isolation and characterization. In order to evaluate and quantify the abilities of various promoters to drive expression of heterologous genes in *B. anthracis*, we constructed an *E. coli-Bacillus* shuttle vector, pGwt-$_{MCS-5}$, based on altered pGEM-3Z/pUB110 plasmids and carrying a wt *gfp* reporter gene. Initially, a DNA cassette of the α-amylase promoter (P*amy*) containing the RBS (from the pA_{amy} plasmid) and the wt *gfp* reporter gene were ligated to generate plasmid $pGwt_{amy}$. Subsequently, the P*amy* promoter was replaced by two additional well-defined *B. subtilis* promoters. The first promoter, P*tms*, is a moderately potent vegetative promoter (30). *tms*, now known as the *gcaD* gene, is part of a tricistronic operon involved in nucleotide synthesis (19). The second promoter, P*43*, is comprised of two overlapping RNA polymerase σ factor recognition sites, σ^A and σ^B. P*43* is considered to be a strong promoter and was shown to efficiently express the chloramphenicol acetyltransferase reporter gene (46). The vectors carrying the various promoter cassettes were electroporated simultaneously into the *B. subtilis* WB600 and *B. anthracis* Δ14185 strains. GFP expression was evaluated by scanning the fluorescence emitted by the resulting clones (Fig. 2A). In *B. subtilis*, substantial fluorescence could be detected for all three promoters under study; their potency ratios were P*43* > P*tms* > P*amy*. In *B. anthracis*, however, only a weak signal was detected for P*43*-driven expression (Fig. 2A), and no fluorescence signal could be visualized from the other two strains carrying the P*tms* and P*amy* promoters.

The fluorescence of the wt GFP in *B. anthracis* did not meet the required threshold of fluorescence detection for evaluation of promoter potencies. Therefore, two additional versions of

FIG. 2. Fluorescence emitted by *B. anthracis* Δ14185 and *B. subtilis* WB600 expressing different GFP reporter genes from various promoters. Strains expressing the three versions of the *gfp* gene in conjunction with the indicated promoter cassettes were cultured in FAG liquid media. GFP expression was monitored from a bacterial culture drawn at an A_{550} of 5.0 by scanning the emission between 450 and 550 nm at excitations of 410 nm for wt GFP and GFPuv and 488 nm for EGFP. (A) Emission scans of *B. subtilis* and *B. anthracis* cultures expressing wt *gfp*, driven by the P*amy*, P*tms*, and P*43* promoters. (B) Emission scans of cultures expressing the wt *gfp*, *gfpuv*, and *egfp* genes driven by the P*43* promoter. Note the lack of EGFP fluorescence (driven by the P*43* promoter) in *B. anthracis* as opposed to the high fluorescence level in *B. subtilis.* (C) Fluorescence-activated cell sorter-based detection of fluorescent *B. anthracis* vegetative cells expressing *gfpuv* from the P*43* promoter (shaded curve) versus nonfluorescent cells (solid curve). FL1-Height, fluorescence intensity per cell; counts, normalized fractions of cells.


A
Culture turbidity (OD_{550})
100
10
1
0.1
0.01
0 5 10 15 20 25 30
Growth time (hr)
B
Fluorescence (FU)
20000
15000
10000
5000
0
0 5 10 15 20 25 30
Growth time (hr)
C
Fluorescence (10^{-6} counts/s)
3
2
1
0
450 500 550 600
Emission wavelength (nm)
Psap long
Psap short
P43
Pamy
No Promoter
D
450 500 550 600
Emission wavelength (nm)
E
No Promoter
Pamy
P43
Psap short
Psap long
31 kDa -
21 kDa -
GFPuv
F
Psap short
Psap long
No Promoter

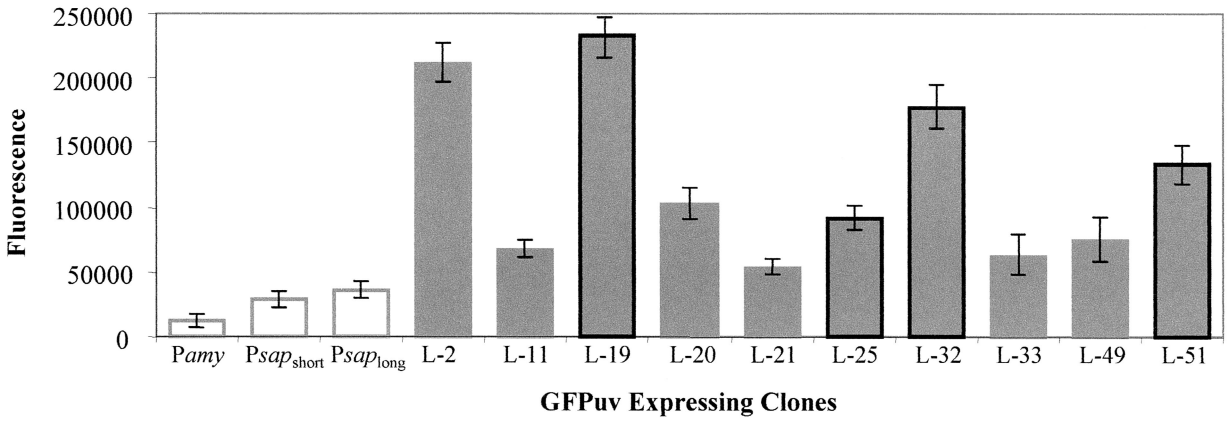
the *gfp* reporter gene were tested, *gfpuv* and *egfp*, known for their enhanced fluorescence intensities and rates of fluorophore formation (4, 40). The *B. anthracis* clone harboring the *egfp* gene under the control of the P*43* promoter did not exhibit any detectable fluorescence (Fig. 2B). However, the same plasmid generated a high signal in *B. subtilis* (Fig. 2B). It is worth noting that the same gene was used successfully as a reporter for in vitro expression in *Bacillus cereus*, a phylogenetically close relative of *B. anthracis* (13). The *gfpuv* expression, driven by the P*43* promoter, generated a very intense signal in *B. subtilis* but also a significant signal in *B. anthracis*, sufficient to be used for a promoter-screening system. Furthermore, it generated a detectable level of fluorescence at 488 nm (12) that allowed detection by fluorescence-activated cell sorter (Fig. 2C). Since *gfpuv* permits detection of fluorescence in both *B. subtilis* and *B. anthracis*, upon irradiation with either UV or red-shifted light, it was selected as the promoter trap system of choice.

For further assessment of the dynamic range of *gfpuv* fluorescence in *B. anthracis*, other promoter cassettes, derived from the *B. anthracis sap* (for surface antigen protein; an S-layer protein [14]) promoter, were tested. The S-layer proteins are often the most abundant proteins in bacterial cells; their promoters are considered to be among the strongest known in nature, their potency being manifested particularly during fast exponential growth (36). The *B. anthracis sap* promoter was cloned upstream of the *gfpuv* reporter gene in two versions (see Materials and Methods). The first version contained the DNA sequence bordered by the −35 RNA polymerase recognition site and the RBS sequence and is referred to as Psap_{short}. In the second version, Psap_{long}, an additional 200-bp DNA fragment upstream of the −35 site is present. The *B. anthracis* cells harboring the various promoter-*gfp* cassettes exhibited identical growth curves (Fig. 3A) and displayed significant differences in the levels of GFPuv expression (Fig. 3B). The high intracellular levels of GFPuv did not appear to have any deleterious effect on either the growth rate or the GFP yield per cell. The relative intensities of the promoter battery were evaluated according to the different levels of fluorescence and were found to be Psap_{long} > Psap_{short} > P*43* > P*amy*. The relative fluorescence did not change along the growth curve (Fig. 3C and D). The amount of intracellular GFPuv expressed by the *B. anthracis* cells was in good correlation with the level of fluorescence, as attested directly by Western blot analysis of the intracellular protein product and by inspecting the level of fluorescence of colonies grown on solid media (Fig. 3E and F). The same constructs were also tested in the *B. subtilis* WB600 background, and a similar relative order of promoter potencies was found (except that P*43* was more potent than Psap_{short} in this background) (data not shown). We concluded from all these studies that the promoter trap system, relying on GFPuv-driven fluorescence, provides a direct correlation between gene expression and promoter activity in a linear manner along a wide range of intensities.

Screening of a *B. anthracis* promoter library and characterization of selected clones. A *B. anthracis* plasmid genomic library was constructed from *Sau*3AI-digested *B. anthracis* DNA fragments (see Materials and Methods), using the *gfpuv* promoter trap system. The hybrid plasmids were used to transform *E. coli* DH5α and *B. anthracis* Δ14185 cells. Clone analyses performed on *E. coli* confirmed that 85 to 90% of the library transformants contained chromosomal inserts, and the average insert size represented in the library was 770 ± 10 bp (the *B. anthracis* genome size is 5.2 Mbp). Therefore, we anticipated that by screening 6,000 clones, a sufficiently high number of promoter inserts might be identified in a nonredundant, although nonexhaustive, manner. Out of ~6,000 *B. anthracis* colonies screened by UV light, 61 generated levels of fluorescence higher than that of the strain harboring $pGuv_{sapL}$ (to identify very potent promoters, the level of $pGuv_{sapL}$ served as the threshold intensity level for selection). Four of the 61 chosen transformants were lost during the following plate streaking, and the fluorescence intensities of the remaining 57 isolates were compared quantitatively. Ten different isolates, which were highly fluorescent on solid media and appeared uniform with respect to morphology and fluorescence intensity, were picked for further examination. Following sporulation, the GFPuv expression of these clones was followed during growth in liquid media, and DNA inserts were analyzed by PCR (Fig. 4). The similar growth rates of the wide range of GFP-expressing clones suggest that overexpression of GFP is indeed nontoxic to the cells.

Four clones, L-19, L-25, L-32, and L-51, the most fluorescent of the 10 isolated clones, were selected for further characterization. Clone L-2 was not selected because of an inherent instability problem (as reflected by the PCR results [Fig. 4]). Following DNA sequencing and bioinformatic analysis against the draft genomic sequence of *B. anthracis* (see Materials and Methods), we identified each of the *B. anthracis* inserts, as summarized in Fig. 5. All four clones contain hypothetical promoters and RBS sequences in the correct orientation for promoting transcription of the reporter *gfpuv* gene. Clone L-19 contains a noncoding promoter region and 42 bp from the 5′ end of the coding sequence of a gene belonging to a classical nitroreductase gene family. L-25 originates from the ligation of two separate genomic regions. Both L-32 and L-51 contain a noncoding region with putative bidirectional promoters. The putative promoter region of clone L-51 (309 bp) was excised and recloned upstream of *gfpuv* (see Materials and Methods for details). The cloned fragment drove expression of GFPuv; however, the recloned, shortened fragment showed lower pro-

FIG. 3. Measurements of GFPuv expression driven by a series of promoters in *B. anthracis* Δ14185 cells. (A and B) Cultures were monitored during growth for optical density at 550 nm (OD_{550}) (A) and whole-cell fluorescence emission (510 nm) following excitation at 410 nm (B). FU, fluorescence units. *B. anthracis* strains expressed *gfpuv* from the following promoter cassettes: ●, no promoter; ▲, P*amy*; ♦, P*43*; ■, Psap_{short}; and □, Psap_{long}. (C and D) Specific fluorescence spectra measured during logarithmic (C) and stationary (D) phases. For measurement of specific activities, whole-cell samples were washed in PBS and resuspended to equal densities (A_{550} = 1.0), and emission scans were inspected in the range of 450 to 600 nm. (E) Amounts of intracellular GFP were also determined by Western blot analysis (with anti-GFP specific antibodies) of *B. anthracis* bacterial extracts obtained from the culture samples 7 h after initiation of growth. (F) Direct UV-mediated visualization of fluorescence emitted by cells expressing the *gfpuv* gene with the indicated promoters.



Growth rate (hr^{-1})	1.2	1.2	1.2	0.8	1.2	1.1	1.2	1.2	1.1	1.1	1.2	1.2	1.2
Insert size (bp)				ND	800	250	ND	800	1800	800	600	1700	1900

FIG. 4. Comparison of GFPuv fluorescence emitted by selected individual *B. anthracis* library isolates. Fluorescence analysis of cultures from 10 clones, which exhibited high fluorescence, compared to cultures expressing *gfpuv* under the control of the P*amy*, Psap_{short}, and Psap_{long} promoters is depicted by the bar diagrams. All measurements were determined in cultures of identical densities. The growth rates (determined by growth curves similar to those shown in Fig. 3) and insert size (determined by PCR analysis) are indicated in the table. Four library isolates, L-19, L-25, L-32, and L-51, indicated by bold-frame histograms, were selected for sequence analysis. ND, not detected. The error bars indicate standard deviations.

moter activity than the parental library isolate (data not shown). Of the four clones, we selected the nitroreductase promoter of clone L-19 (P*ntr*) for further study, because it demonstrated the highest level of *gfpuv* expression and it had the smallest DNA insert (only 271 bp).

***Bacillus anthracis* Δ14185 rPA expression driven by various promoters.** Six different promoters were used to drive expression of PA: P*pag* (the PA native promoter), P*amy*, P*43*, Psap_{short}, Psap_{long}, and P*ntr* (from vectors pA_{pag}, pA_{amy}, pA_{43}, pA_{sapS}, pA_{sapL}, and pA_{ntr}, respectively). It has been reported

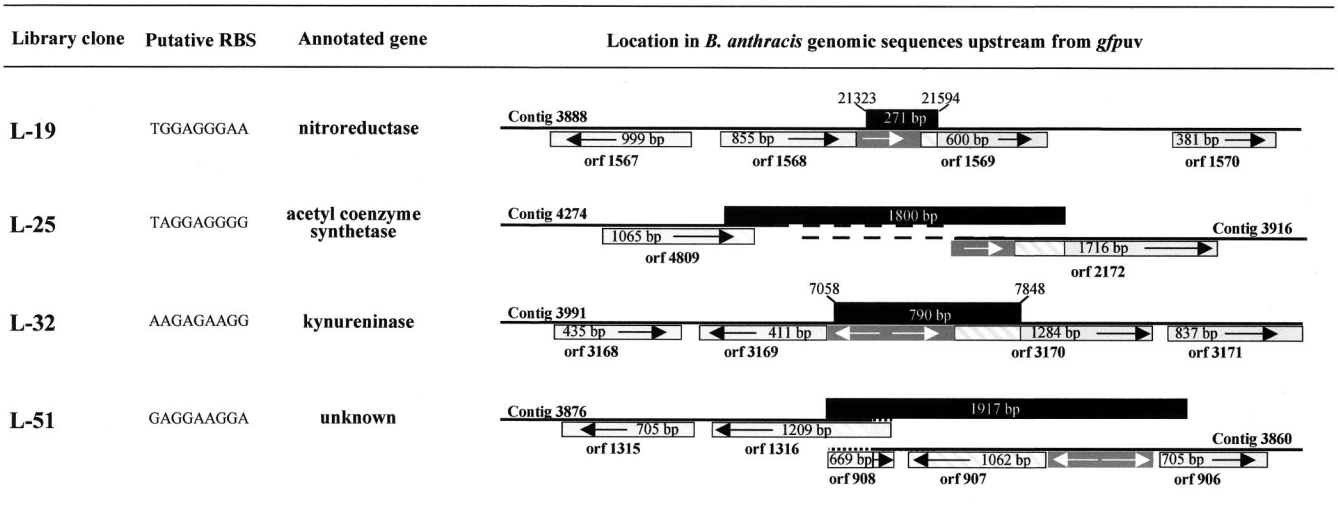


FIG. 5. Chromosomal gene arrangement and promoter locations of the insert fragments from the four selected library clones. The numbers of contigs and open reading frames (orfs) are according to the February 2001 version of the *B. anthracis* Ames draft genome (The Institute for Genomic Research). The putative RBSs contain the core sequence with flanking nucleotides on both sides, with the highest sequence complementary to the 3′ end of the *B. subtilis* 16S rRNA (2). The solid boxes represent the various *B. anthracis* DNA fragment inserts; hatched boxes represent the region not sequenced in clone L-25. Clones L-25 and L-51 originate from ligation of two chromosomal fragments. For clones L-19 and L-32, the first and last base pairs are indicated according to the contig sequence. The darkly shaded rectangles represent promoter noncoding regions, and transcription directions are marked by open arrows.

TABLE 2. Comparison of relative potencies of various promoters in *B. anthracis* background

Promoter	Strain of origin	Relative potency in *B. anthracis*[a]
P*pag*	*B. anthracis*	1
P*amy*	*B. amyloliquefaciens*	7
P*43*	*B. subtilis*	20
Psap_{short}	*B. anthracis*	40
Psap_{long}	*B. anthracis*	55
P*ntr*	*B. anthracis*	500

[a] The potencies of all promoters (except the native P*pag*) were determined by intensities of fluorescence in quantitative assays (see Materials and Methods). Actual values for computation were derived from Fig. 3 and 4 (stationary-phase cultures). The ratio between P*pag* and P*amy* was determined by PA expression.

that the P*amy* promoter is seven times more potent than the native P*pag* promoter in expressing PA, both in vitro and in vivo (11). The potencies of the other four promoters, P*43*, Psap_{short}, Psap_{long}, and P*ntr*, were determined by GFPuv fluorescence to be 3, 6, 8, and 70 times higher than that of P*amy*, respectively (Table 2). While the cell-associated levels of PA were apparently consistent with the order of promoter strengths (Fig. 6B, top), the secreted-PA levels were identical for all the tested strains carrying promoters stronger than P*amy* (Fig. 6A). A close examination of the cell-associated PA by blot analysis using a set of two antibodies (anti-AI-10 and anti-B-11) that can differentiate between the mature and the preprotein forms of PA demonstrates that promoter strength correlates with the preprotein PA levels (before the cleavage of signal peptide [Fig. 6B, top]). In contrast, the cell-associated levels of mature PA are similar for all constructs (Fig. 6B, bottom), exhibiting the same phenotype as secreted PA. The secreted-PA levels were quantitated by capture ELISA, and by the biological cytotoxic assay of the macrophage-like cell line J774A.1 as well. In accordance with the SDS-PAGE analysis (Fig. 6A), the ELISAs also demonstrate that equal amounts of PA are found in the supernatants of all the strains expressing

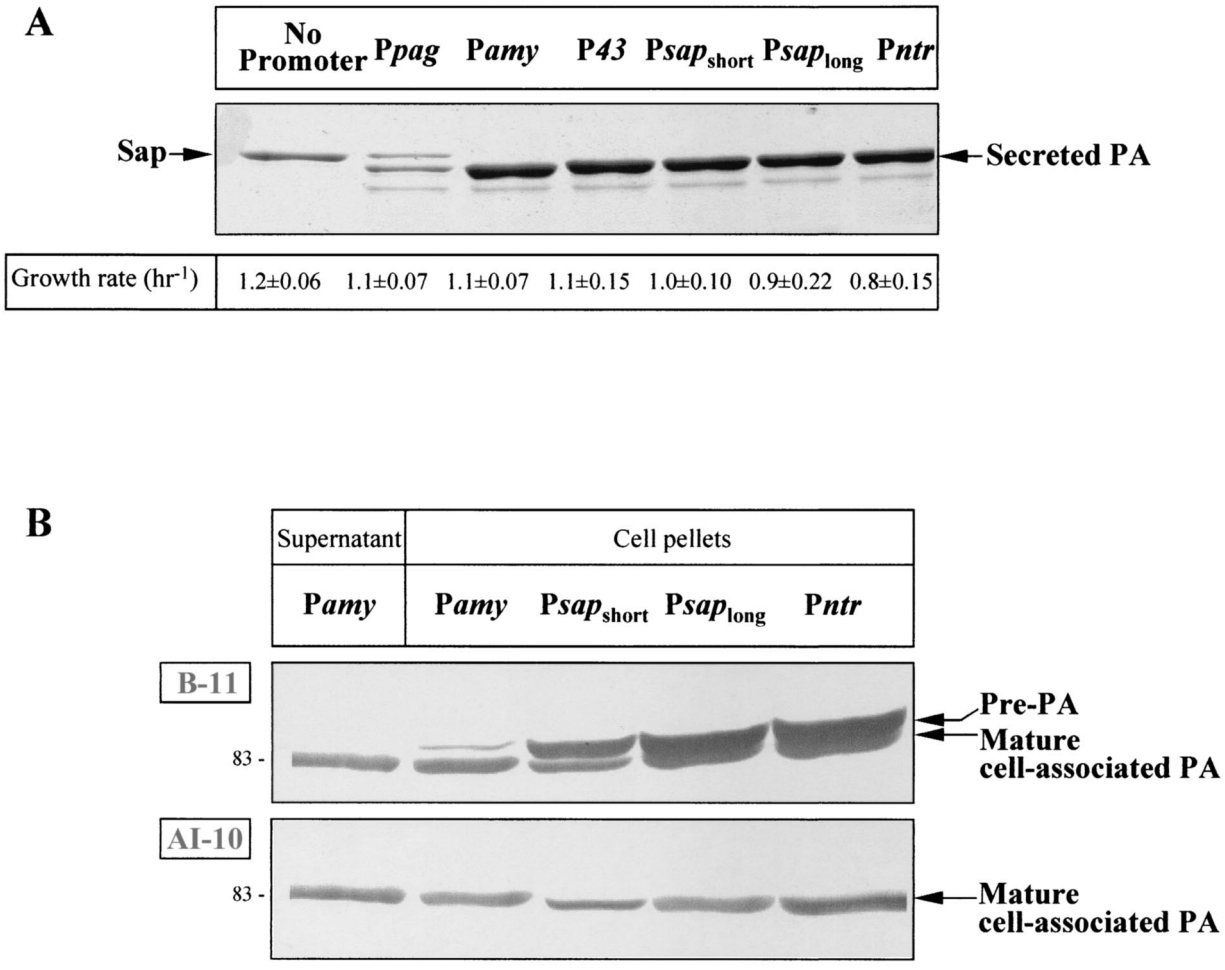


FIG. 6. Analysis of recombinant PA expression in *B. anthracis* by various promoters. (A) SDS-PAGE of extracellular samples taken from liquid cultures at identical cell densities. Migration of the mature secreted PA (83 kDa) is indicated. The upper protein band (94 kDa), which appears only in culture supernatants of strains harboring pA_{MCS-5} (no promoter) and pA_{pag}, represents the S-layer protein Sap, as determined by specific antibodies (not shown). The growth rates are averaged from five independent experiments carried out for each clone. (B) Western blots of cell lysates probed with two anti-PA antibodies targeted to both premature and mature forms of PA (B-11) and to the mature PA form only (AI-10) (see Materials and Methods).

PA from promoters stronger than P*amy*. However, the biological cytotoxic assay, performed on J774A.1 cells, suggests that the specific activity of the PA is somewhat diminished, by about 50 to 70% compared to P*amy*-driven expression, when expression is driven by the strong promoters P*43*, Psap_{short}, Psap_{long}, and P*ntr* (data not shown). DNA sequencing performed on the entire *pagA* gene and the promoter region in all four different expression plasmids did not reveal any mismatches with the known wt sequences. In addition, it could be seen from the electrophoretic mobilities of the various secreted rPA preparations (Fig. 6A) that the recombinant products have molecular weights that are indistinguishable from that of the native protein. We note, however, that the growth rate of *B. anthracis* is strongly affected by the level of expression of PA (Fig. 6A). It appears that high levels of cell-associated PA are deleterious to the bacterium. This is consistent with a few additional findings: (i) very low yields of genetic transformation (up to 3 orders of magnitude) for pA_{sapL} and pA_{ntr}; (ii) manifestation of very small colonies by strains harboring pA_{43}, pA_{sapS}, pA_{sapL}, and pA_{ntr} on solid media; and (iii) development of revertant clones, which form large colonies that do not produce PA. The observations described above imply enhanced genetic instability of the vectors carrying the most potent promoters. To test genetic instability, a simple experiment which directly scores the ability of the bacteria to retain PA-expressing vectors in the absence of selective pressure was carried out (see Materials and Methods). For strains harboring $pA_{MCS\text{-}5}$, pA_{pag}, pA_{amy}, pA_{43}, pA_{sapS}, pA_{sapL}, and pA_{ntr}, the percentages of kanamycin-resistant colonies following colony growth on nonselective agar were 100 ± 1, 100 ± 1, 96 ± 2, 94 ± 4, 61 ± 4, 1 ± 0.5, and 0, respectively. The results clearly demonstrate a direct correlation between the potency of the promoter driving *pagA* expression and genetic (segregational) instability. Furthermore, when cultures of *B. anthracis* harboring the P*ntr-pagA* cassette were propagated at constant logarithmic phase under selective medium for 35 generations and plated on kanamycin-containing plates, we found that 90% of the colonies had DNA rearrangements (deletions) in the *pagA* gene (data not shown). The other strains carrying the less potent promoters did not show any alterations under these conditions.

PA expression in *B. subtilis*. It was reported previously (11) that rPA may be expressed efficiently in *B. subtilis* under the control of the P*pag* and the P*amy* promoters. It was observed that PA expression in *B. subtilis* exhibited a pattern similar to that obtained in *B. anthracis*, where PA production was proportional to the potency of the promoter used. The results reported above, suggesting a limitation in the levels of PA expression in a *B. anthracis* background, prompted us to examine expression of the various promoter-*pagA* cassettes in the *B. subtilis* background. The conclusion from a series of such studies is that *B. subtilis* is actually more susceptible to the toxic effects of a high level of PA than *B. anthracis*, as detailed below.

rPA was secreted by pA_{amy} in *B. subtilis* during growth in liquid culture, as observed before (Fig. 7A), but PA production was accompanied by an altered growth rate (a reduction from 1.4 h^{-1} for the parent WB600 strain to 0.8 h^{-1}) (Fig. 7B). Inspection of the cell-associated compartment of the P*amy-pagA B. subtilis* cells revealed that most of the protein is in the premature high-molecular-weight form (Fig. 7C). We note that

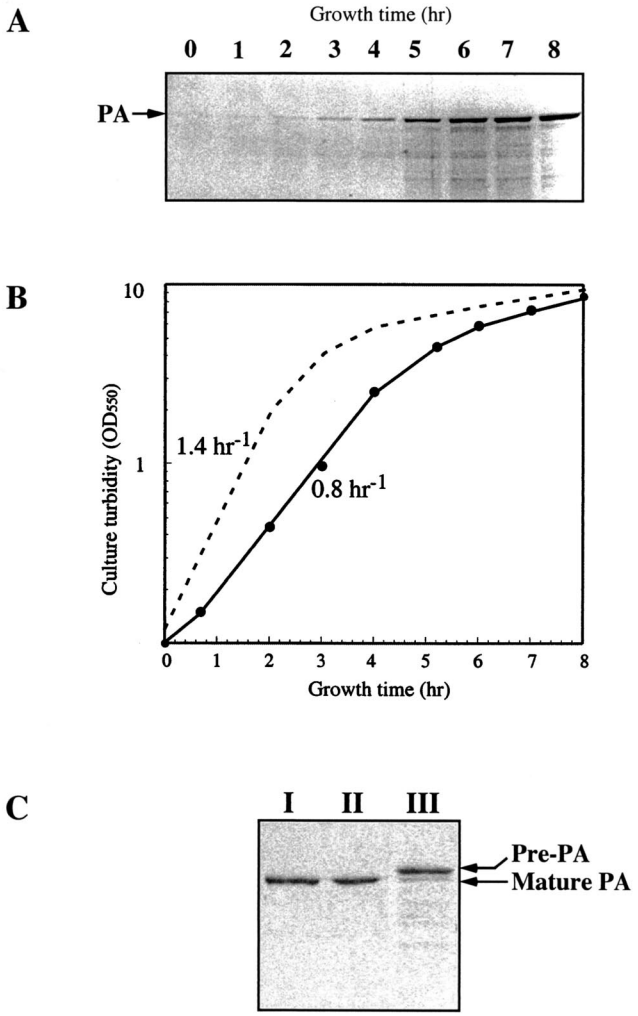


FIG. 7. Expression of rPA driven by P*amy* in *B. subtilis* WB600. (A) SDS-PAGE of extracellular samples collected at the indicated growth times. (B) Growth curves of *B. subtilis* WB600 host strain (dashed line) and recombinant strain harboring pA_{amy} (solid circles). (C) Western blot analysis with anti-PA antibodies (B-11) of control mature *B. anthracis* rPA (I), secreted (II), or cell-associated (III) rPA collected from 8-h cell cultures of a *B. subtilis* strain harboring pA_{amy}.

in *B. anthracis* (Fig. 6), the same construct had very little effect, if any, on the growth rate or production of mature PA. Finally, when the pA_{43}, pA_{sapS}, pA_{sapL}, and pA_{ntr} vectors were electroporated into *B. subtilis*, no transformants could be obtained in three independent electroporation experiments. These results suggest that *B. subtilis* cells displayed enhanced susceptibility to PA expression compared to *B. anthracis*.

Immunization of guinea pigs with *B. anthracis* strains carrying the various promoter-*pagA* cassettes. The level of anti-PA antibodies present in the circulation of vaccinated animals was shown to be proportional to the amount of PA expressed by the inoculated bacteria (11). We have shown (Fig. 6A) that the use of promoters more potent than the α-amylase promoter does not increase the level of secreted rPA but results in an accumulation of cell-associated rPA. It was therefore interesting to determine the immune response following

TABLE 3. Immunization of guinea pigs with *B. anthracis* strains harboring various promoter-*pagA* cassettes

Plasmid[a]	In vitro rPA production (μg/ml)[b]	ELISA titer[c]	
		Anti-PA	Anti-core
pA_{pag}	19 ± 3	15 ± 2	190 ± 5
pA_{amy}	120 ± 10	950 ± 5	190 ± 5
pA_{sapS}	130 ± 30	250 ± 5	200 ± 5
pA_{sapL}	130 ± 40	100 ± 5	540 ± 10
Saline	0	<10	<10

[a] Guinea pigs were inoculated subcutaneously with a single dose of 10^6 spores of *B. anthracis* recombinant strains carrying the indicated plasmids.

[b] Mean values (determined by ELISA of PA in culture supernatant at an A_{550} of 7 to 8) ± the standard error of five experiments.

[c] Values represent antibody titers (geometric mean titers) ± the standard deviation from 10 animals determined 6 weeks postinoculation of spores.

inoculation with *B. anthracis* strains expressing different amounts of cell-bound PA. Guinea pigs were inoculated with one dose of 10^6 spores harboring pA_{pag}, pA_{amy}, $pA_{sap\mathrm{S}}$, or $pA_{sap\mathrm{L}}$. Antibody titers were determined 6 weeks following injection of the spores. The anti-core antibodies (*B. anthracis* Δ14185 specific) were essentially similar in all groups tested (Table 3), as would be expected from the fact that all of the animals were inoculated with identical numbers of spores. A completely different picture emerges from the anti-PA antibody assay. While, as expected, an increase was observed in the anti-PA antibody titers in animals injected with the pA_{amy} strain compared to the titers in the animals injected with the pA_{pag} strain, the level of the anti-PA antibodies decreased in animals injected with the strains bearing promoters stronger than P*amy* (Table 3). This appears to be consistent with in vitro results suggesting increased plasmid instability in the absence of selective pressure.

DISCUSSION

A multivalent vaccine, efficient in the prophylaxis of more than one bioterrorism threat agent, could rely on an attenuated bacterium expressing a variety of antigens. For this purpose, *B. anthracis* is an attractive vehicle. It has a highly developed genetic system, which allows a variety of genetic manipulations, and an elevated DNA transformation capability. It has an efficient secretory mechanism that allows the presentation of self or foreign antigens to the host immunological system. And, most important, *B. anthracis* can be introduced as an attenuated strain cured of the major factors of virulence but still retaining a substantial ability to elicit protective immunity against anthrax.

One of the objectives of the study presented here was to identify promoters more potent than the constitutive α-amylase promoter for expression of the *B. anthracis* PA in *B. anthracis* and *B. subtilis* (11). Such promoters could be useful for in vivo expression of various antigens in a live attenuated vaccine or as sources for biotechnological production of rPA for soluble-antigen vaccine. To this end, a general promoter identification and quantification trap system suitable both for *B. anthracis* and *B. subtilis* expression was developed. Comparison of the three GFP-based reporter genes, wt *gfp*, *gfpuv*, and *egfp*, studied here indicated that *gfpuv* is most suitable as the reporter gene in *B. subtilis* and *B. anthracis*, since it provides the widest dynamic range of detection in both backgrounds. In *B. anthracis*, neither the wt GFP nor the red-shifted EGFP allowed detection when expression was driven by the relatively potent P*amy* and P*43* promoters (Fig. 2).

The *B. subtilis* promoter, P*43*, which is active in the vegetative and postexponential state, was found to be effective in *B. anthracis* and to drive GFPuv expression three times higher than the α-amylase promoter (Table 2). The *B. anthracis sap* promoter, belonging to the family of promoters driving the expression of S-layer proteins, was studied recently (28); however, its actual potency was not reported. Here, we show that the *B. anthracis sap* promoter is eight times more potent than the P*amy* promoter and at least twice as potent as *B. subtilis* P*43* in promoting *gfp* expression. Furthermore, the 200-bp region upstream from the σ^A subunit RNA polymerase recognition site of the *sap* promoter was found to enhance promoter activity, indicating a putative binding site for a hypothetical activator. The deletion of this 200-bp region led to a 25% decrease in expression of *gfp*. This putative regulatory element is also recognized by the *B. subtilis* transcription machinery. The rank orders of promoter potencies in *B. anthracis* and *B. subtilis* were found to be similar, except that in *B. subtilis*, P*43* appeared more potent than Psap_{short}.

In search of *B. anthracis* promoters stronger than Psap_{long}, we applied a shotgun cloning technique by creating a library of *B. anthracis* genomic DNA in the GFPuv promoter trap system. Screening of 6,000 clones led to the identification of a very potent promoter region on a 271-bp fragment (clone L-19) (Fig. 4 and 5). Based on its location upstream from the nitroreductase gene, it was designated P*ntr*. P*ntr* was found to be 10 times more potent than the very strong Psap_{long} promoter and 70 times more efficient than P*amy* in promoting GFPuv expression in *B. anthracis* (Table 2). Similar results for promoter potencies were obtained in *B. subtilis*.

Unlike expression of GFP, several observations suggest that attempts to drive high levels of PA expression in *B. anthracis* are deleterious to the cells. (i) Yields of genetic transformation of *B. anthracis* cells with the most potent *B. anthracis* promoters, Psap_{long} and P*ntr*, fused to *pagA* are very low. In *B. subtilis*, no transformants could be obtained with plasmids bearing these strong promoters. We note that the same promoters fused to the *gfpuv* gene had no effect on the transformation efficiency of either *B. anthracis* or *B. subtilis*. (ii) The strain harboring the less potent *pagA* promoter exhibited higher growth rates and larger colonies. (iii) In the absence of antibiotic selective pressure, plasmids carrying the most potent promoter cassettes, Psap_{long}-*pagA* and P*ntr-pagA*, are very unstable. (iv) Under antibiotic selective pressure, the P*ntr-pagA* cassette undergoes genetic rearrangements, and most of the resulting cells do not produce PA.

We believe that the toxic effect due to high levels of rPA expression probably does not stem from an inherent toxicity of the PA molecule but more likely is a consequence of severe secretion stress. Cases where the secretion capacity of a strain has been exceeded due to high expression levels are well documented in *B. subtilis* (5, 25). PA by itself can be processed and secreted when it is expressed at quite high levels (0.5 pg/cell) without any obvious effect on the cells. However, there appears to be an upper limit to the amount of the mature PA that can be detected in the supernatants of bacteria carrying promoters

equal in strength to or more potent than P*amy* (Fig. 6A). On the other hand, the amount of cell-associated unprocessed PA (pre-PA) increases proportionally to the strength of the promoter. The large accumulation of pre-PA may be another manifestation of the limitation of the secretion process. Usually, unstable intermediate forms of premature secreted proteins cannot be found in cells, and their occurrence indicates a secretion complication (35).

Apart from the discrepancy between secretion levels and the amounts of cell-associated accumulation of the PA transient intermediate, two other observations strongly suggest that protein secretion is the limiting factor in efficient PA production from the most potent promoter. The first is that, concomitantly with the increase in production levels of rPA protein, there appears to be a decrease in the amount of the Sap protein in the extracellular medium (compare Fig. 6A, lanes 1, 2, and 3). The second observation is related to the reduction in the specific activity of the secreted rPA in the supernatant of cells expressing PA from the very strong promoters. Both observations are consistent with the failure of some components belonging to the secretion apparatus, such as inaccurate processing by specific peptidases or misfolding of the mature protein during release from the cells due to a shortage of PrsA (6, 20, 37, 39, 45).

It is interesting that with respect to PA, *B. subtilis* appears to be more susceptible to this secretion stress than *B. anthracis*. This is suggested by the various observations mentioned above and the increased rate of plasmid loss, as well as by the effect on the growth rate, which is very significant even in a *B. subtilis* strain harboring the P*amy-pagA* cassette (Fig. 7). Furthermore, production of PA above the level driven by the P*amy* promoter is lethal to *B. subtilis*, whereas *B. anthracis* can tolerate much higher levels of protein production. The balance among genetic stability, secretion stress, and production of mature and active PA makes the P*amy-pagA* cassette the optimal candidate for inclusion in attenuated *B. anthracis* vaccine. This was indeed established by the comparative immunization experiment, where the relevant strains containing the strong-promoter–*pagA* cassettes were evaluated in vivo (Table 3). Nevertheless, if, as suggested, the high level of PA expression introduces secretion stress, it is possible to use genetic manipulations, such as overexpression of the limiting factors involved in maturation and folding (8, 24, 47), and thus allow the generation of a stable vaccine strain or *B. anthracis* cells that could express and effectively secrete even higher levels of PA.

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