PREPARATION OF BACTERIAL CULTURE MEDIA

LB Medium (Luria Bertani medium)
Dissolve 20 gms of LB powder (Hi Media) in double distilled water. Sterilize the media by autoclaving for 20 minutes at 15 lb / sq. in.

LB Agar
Dissolve 35 gms of LB agar powder (Hi Media) in double distilled water. Sterilize the media by autoclaving for 20 minutes at 15 lb / sq. in. Allow LB agar to cool and pour in 90 mm disposable Petri plates (Tarsons) along with appropriate antibiotics and allow to solidify.

ANTIBIOTIC SOLUTION

Ampicillin

100 mg / ml stock solution prepare in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C.

SOLUTIONS FOR PLASMID ISOLATION AND PURIFICATION

Solution I
50 mM Glucose
25 mM Tris. Cl (pH 8.0)
10 mM EDTA
Prepare solution I in batches of 100 ml, autoclave for 20 minutes at 15 lb /sq. in. and store at 4°C.

Solution II
0.2 N NaOH (freshly diluted from 10 N stock)
1 % SDS

Solution III
5M potassium acetate 60 ml
glacial acetic acid 11.5 ml
dd H₂O 28.5 ml

The resulting solution is 3M with respect to potassium and 5 M with respect to acetate. Autoclave at 15 lb / sq. in. for 20 minutes and store at 4°C.

STOCK SOLUTIONS OF COMMONLY USED REAGENTS

1 M Tris
Dissolve 121.1 gms of Tris base in 800 ml of double distilled water and adjust the desired pH (6.8, 7.4, 8.0) with concentrated HCl. Make up the volume to 1 litre and autoclave.
0.5 M EDTA
Add 186.1 gms of disodium EDTA. 2H2O to 800 ml of double distilled water. Stir vigorously on a stirrer, adjust the pH to 8.0 with NaOH (about 20 gms of NaOH pellets), make up the volume to 1 litre and autoclave.

10 % SDS
Dissolve 10 gms of electrophoresis grade SDS in 70 ml of dd H2O heat at 68°C to dissolve and make up the volume to 100 ml.

Ethidium bromide (10 mg / ml)
Dissolve 10 mg of ethidium bromide in 1 ml of dd H2O. Store in a dark bottle at 4°C.

30 % Acrylamide stock
Dissolve 29.2 gms of acrylamide and 0.8 gms of bis - acrylamide in 50 ml of dd H2O. Make up the volume to 100 ml, filter the solution through Whatman no. 1 paper, degas and store in a dark bottle at 4°C.

Calcium chloride (0.1 M)
Dissolve 1.47 gms of CaCl2-2H2O in 100 ml of dd H2O and sterilize by autoclaving.

Sodium phosphate (1 M)

Monobasic
Dissolve 138 gms of NaH2PO4. H2O in 800 ml of dd H2O and make up the volume to 1 litre.

Dibasic
Dissolve 268 gms of Na2HPO4. 7H2O in 700 ml of dd H2O and make up the volume to 1 litre.

Ammonium persulfate (10 %)
To 1 gm of ammonium persulfate add 10 ml of dd H2O. The solution may be stored for several weeks at 4°C or frozen in aliquots at -20 °C.

100 mM Phenyl methyl-sulfonyl fluoride (PMSF)
Dissolve 17.4 mg of PMSF in 1 ml of isopropanol. Divide the solution into aliquots and store at -20°C.

BUFFERS

50 X TAE buffer (Tris-acetate, EDTA)
Dissolve 242 gms of Tris base in 700 ml dd H2O and add 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA pH 8.0. Make up the final volume to 1 litre.

10 X TBE buffer (Tris-borate, EDTA)
Dissolve 108 gms of Tris base, 55 gms of boric acid and 9.3 gms Na2EDTA.H2O in 700 ml dd H2O and make up the final volume to 1 litre.

Phosphate buffered saline (PBS)
Dissolve 8 gms of NaCl, 2 gms of KCl, 1.44 gms of Na₂HPO₄ and 0.24 gms of KH₂PO₄ in 800 ml of ddH₂O. Adjust the pH to 7.4 with HCl. Make up the volume to 1 litre and sterilize by autoclaving at 15 lb / sq. in. for 20 minutes and store at room temperature.

**SDS - PAGE electrophoresis buffer**

Dissolve 3 gms of Tris base, 14.4 gms of glycine and 1 gms of SDS in 1 litre of ddH₂O.

**Protein transfer buffer**

Dissolve 5.8 gms of Tris base, 2.9 gms of glycine and 0.33 gms of SDS in 0.5 litre of ddH₂O. Add 200 ml of ethanol and make up the volume to 1 litre.

**2 X SDS - PAGE sample buffer**

The composition of sample buffer is as follows

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM</td>
<td>Tris.Cl (pH 6.8)</td>
</tr>
<tr>
<td>200 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>4 %</td>
<td>SDS</td>
</tr>
<tr>
<td>0.2 %</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>20 %</td>
<td>Glycerol</td>
</tr>
<tr>
<td>10 %</td>
<td>β-mercaptoethanol</td>
</tr>
</tbody>
</table>

**10 X Restriction enzyme Kpn I buffer (L)**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM</td>
<td>Tris.Cl pH 7.5</td>
</tr>
<tr>
<td>100 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>10 mM</td>
<td>DTT</td>
</tr>
</tbody>
</table>

**10 X Restriction enzyme Bam HI buffer (K)**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM</td>
<td>Tris.Cl pH 8.5</td>
</tr>
<tr>
<td>100 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>10 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>1000 mM</td>
<td>KCl</td>
</tr>
</tbody>
</table>

**10 X Ligation buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM</td>
<td>Tris.Cl pH 7.8</td>
</tr>
<tr>
<td>100 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>100 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>10 mM</td>
<td>ATP</td>
</tr>
</tbody>
</table>

**10 X Amplification buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM</td>
<td>Tris.Cl pH 8.3</td>
</tr>
<tr>
<td>15 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>500 mM</td>
<td>KCl</td>
</tr>
<tr>
<td>0.1 %</td>
<td>gelatin</td>
</tr>
</tbody>
</table>

123
DNA loading dye (6 X)
Dissolve 0.25 gms bromophenol blue, 0.25 gms xylene cyanol and 30 ml of glycerol and make up the volume to 100 by autoclaved ddH₂O.

SDS - PAGE reagents

Composition of resolving gel (12 %) (10 ml)
- 4.0 ml 30 % acrylamide solution
- 2.5 ml 1.5 M Tris.Cl pH 8.8
- 3.3 ml ddH₂O
- 100 µl 10 % SDS
- 100 µl 10 % APS
- 10 µl TEMED

Composition of stacking gel ( %) (5 ml)
- 0.83 ml 30 % acrylamide solution
- 0.68 ml 1.0 M Tris.Cl pH 6.8
- 3.4 ml ddH₂O
- 50 µl 10 % SDS
- 50 µl 10 % APS
- 5 µl TEMED

Staining solution
Dissolve 1 gm of Coomassie blue in 450 ml of methanol. Add 100 ml of glacial acetic acid and make up the volume to 1 litre by double distilled water. Filter through Whatman no.1 and store at room temperature.

Destaining solution
Dissolve methanol : water : acetic acid in the ratio 45 : 45 : 10. Store at room temperature.

Animal tissue culture medium RPMI 1640

Dissolve the following components in 100 ml of double distilled water
- RPMI salt 4.16 gms
- NaHCO₃ 0.8 gms
- HEPES 4.8 gms
- Glutamine 0.12 gms
- Penicillin 0.024 gms
- Streptomycin 0.04 gms
Adjust the pH 7.2 - 7.4 with HCl and make up the volume to 400 ml. Filter sterilize the media and store at 4°C.
Constitutive Expression of Protective Antigen Gene of Bacillus anthracis in Escherichia coli

Vibha Chauhan, Aparna Singh, S. Mohsin Waheed, Samer Singh, and Rakesh Bhatnagar
Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Received March 19, 2001

The fatal bacterial infection caused by inhalation of the Bacillus anthracis spores results from the synthesis of protein toxins—protective antigen (PA), lethal factor (LF), and edema factor (EF)—by the bacterium. PA is the target-cell binding protein and is common to the two effector molecules, LF and EF, which exert their toxic effects once they are translocated to the cytosol by PA. PA is the major component of vaccines against anthrax since it confers protective immunity. The large-scale production of recombinant protein-based anthrax vaccines requires overexpression of the PA protein. We have constitutively expressed the protective antigen protein in E. coli DH5α strain. We have found no increase in degradation of PA when the protein is constitutively expressed and no plasmid instability was observed inside the expressing cells. We have also scaled up the expression by bioprocess optimization using batch culture technique in a fermentor. The protein was purified using metal-chelate affinity chromatography. Approximately 125 mg of recombinant protective antigen (rPA) protein was obtained per liter of batch culture. It was found to be biologically and functionally fully active in comparison to PA protein from Bacillus anthracis. This is the first report of constitutive overexpression of protective antigen gene in E. coli. © 2001 Academic Press

Anthrax is a potentially fatal infection caused by Bacillus anthracis, a gram-positive, spore-forming bacterium (1). Disease incidences are usually seen in herbivores, but the disease also affects humans through the use of infected animal products (2). In recent years, the anthrax toxin has been engineered to deliver foreign molecules to the cytosol as part of therapeutic strategies utilizing the targeting properties of the toxin (3, 4). The study of molecular pathogenesis of the disease also remains of interest as a model experimental system (5). There are two major virulence factors of Bacillus anthracis—a tripartite protein exotoxin encoded on the plasmid pXO1 which causes the bacteria associated with anthrax infection and a poly-glutamic acid capsule encoded on the plasmid pXO2, that helps the bacteria to evade phagocytosis. The three components of the exotoxin are protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa) (6–8). The genes for all the three proteins have been cloned and sequenced. As the name suggests, PA is the most immunogenic of the three and hence serves as a major component of all vaccines against anthrax. None of the proteins is toxic alone. PA, like the other A–B models of bacterial toxins, combines with LF to form lethal toxin (LeTx) and with EF to form edema toxin (EdTx). LF/EF then act as the active or effector molecules. At the gene level, PA gene (pag) expression is positively controlled by atxA gene and negatively by pagR gene (10, 11). As a first step in the intoxication process, PA binds to a ubiquitous cell surface receptor. One or more proteases including a furin-like protease PACE4 is present at the exterior of cells and plays a role in the proteolytic activation of receptor bound PA (12). This cleavage exposes a high affinity site on PA to which LF/EF can bind competitively. PA63 heptamerizes and inserts into the membrane as a pore upon exposure to acidic pH after receptor mediated endocytosis (13, 14). The PA63 oligomer translocates EF/LeTx into the cytosol (15).

EF is a calcium/calmodulin-dependent adenylate cyclase which increases the intracellular cyclic AMP levels resulting in edema (16). Intracellular cAMP (cyclic AMP) regulates the production of several cytokines that modulate edema formation and play important roles in host defense against invading bacteria. LF is a zinc metalloprotease that cleaves the N-terminal of mitogen-activated protein kinases which inactivates them and inhibits the MAP kinase signal transduction pathway (17, 18). A proteasome mediates the toxic process initiated by LF in the cell cytosol (15). This step occurs downstream of cleavage of MAP kinase kinase or other putative substrates by LF. Continuous protein
synthesis and the presence of extracellular calcium are required for lethal toxin activity (19, 20). Sublytic doses of LF cause substantial reduction in the inflammatory response to permit growth and differentiation of bacteria in the first stages of infection (18). Macrophages are uniquely sensitive to anthrax toxin and act as mediators of the shock and death associated with LeTx in vivo (21-23). Once large levels of LeTx are produced within the host, destruction of bacteria by administration of antibiotics is unsuccessful and prior vaccination is the best option (24). Currently available human vaccines in the United States and Europe consist of alum precipitated material from cultures of toxigenic, nonencapsulated strain of *Bacillus anthracis*. Immunization with these vaccines requires several boosters and occasionally causes local pain and edema. Both native and mutant recombinant PA preparations elicit high antibody response. PA protein therefore can be used to develop an effective acellular recombinant vaccine against anthrax (25). The crystal structure of PA has been previously determined (26). Effective utilization of PA is hampered due to its thermolability. Efforts have been made to thermally stabilize PA by using different solvent additives (27).

In this communication, we would like to report the constitutive expression of the PA gene using *E. coli* DH5α strain without compromising on the activity of the protein. We have designed a model system where the recombinant PA gene is under the control of the phage T5 promoter. This promoter can be recognized by any *E. coli* host strain RNA polymerase. There is no requirement of IPTG induction of this promoter and the recombinant protein is expressed constitutively. We have also tried to scale-up the expression and the yield of recombinant PA using high-density batch culture technique.

**MATERIALS AND METHODS**

**Reagents and supplies.** The enzymes and chemicals used for DNA manipulation were purchased from Life Technologies (U.S.A.), Boehringer Mannheim (Germany), Amersham Inc. (U.K), and New England Biolabs (U.S.A.). The oligonucleotides were obtained from Monica Talmo (Critical Technologies for Molecular Medicine, Yale University Medical School, U.S.A.). The PCR was performed on Perkin-Elmer thermal cycler using DNA amplification kit from Perkin-Elmer (U.S.A.). DNA purification kit, gel extraction kit, expression vector pQE30, Ni-NTA agarose was obtained from Qiagen (Germany). Agarose (Sea Kem GTG) was from FMC Corp. (U.S.A.). Cell culture plasticware was obtained from Corning (U.S.A.). Fetal calf serum (FCS), RPMI 1640, Dulbecco’s modified Eagle medium (DMEM), Hanks’ balanced salt solution (HBSS), trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dialkylamino) propyl dimethylammonio-1-propanesulfonic acid (Chaps), isopropyl-thio-beta-D-galactopyranoside (IPTG), bovine serum albumin (BSA), and other chemicals were purchased from Sigma Chemical Co. (U.S.A.). *E. coli* strain DH5α and J774A.1, a macrophage like cell line were obtained from ATCC (American Type Culture Collection; U.S.A.). Media components for bacterial growth were purchased from Hi-Media Laboratories (India). PA purified from *Bacillus anthracis* was obtained as a generous gift from Dr. Stephen H. Leppla (NIDR, NIH, U.S.A.). Silicone antifoam-A concentrate was obtained from Sigma (U.S.A.).

**Plasmid construction.** The PA gene was PCR amplified using the *Bacillus anthracis* plasmid pX01 as template and cloned in pQE30 (construct designated as pMW1) using KpnI and BamHI restriction enzyme sites (28).

**Expression of PA gene from pMW1 in E. coli DH5α cells.** *E. coli* DH5α (ATCC) was the host strain used for the expression studies. Competent cells were transformed with pMW1 plasmid. Preparation and transformation of competent *E. coli* DH5α cells were performed according to procedures described by Maniatis et al. (29). The presence of the plasmid pMW1 was confirmed by minipreparations of plasmid DNA (29). Cells harboring the plasmid were grown on LB media containing 100 μg/ml ampicillin at 37°C, 250 rpm overnight (ON). These ON seed culture cells (5 ml) were diluted in shake flasks containing 100 ml LB medium and incubated at 37°C, 250 rpm and agitated was 60 rpm. The culture was subjected to a 12% SDS–PAGE and also electroblotted. The absorbance of these culture aliquots was also measured simultaneously at 600 nm.

**Protein localization.** For localizing the expressed recombinant protein in the cell, the *E. coli* strain DH5α carrying the recombinant plasmid pMW1 was grown ON at 37°C in 100 ml Luria Broth with the appropriate antibiotics. Cells were harvested by centrifugation at 5000g for 10 min. The cytosol and inclusion bodies were checked for the presence of PA as previously shown (29) followed by SDS–PAGE analysis of different fractions.

**Plasmid stability assay.** The *E. coli* strain DH5α carrying the recombinant plasmid pMW1 was grown ON at 37°C in the presence of the appropriate antibiotics followed by at least ten ON passages/subbactures. 1-ml culture aliquots were collected each day and subjected to a 12% SDS–PAGE. Minipreparations of plasmid DNA were also done.

**Fermentor operation.** A 5-L Biostat B (B Braun Biotech International) fermentor was used for the work. It was equipped with pH, temperature, and dissolved oxygen monitoring and control. The fermentor was interfaced with a personal computer. MFC/swin 2.0 software was used for data acquisition and proper operation of the fermentor. Silicone antifoam-A concentrate from Sigma (U.S.A.) after dilution (10% v/v) in silicone fluid was used as antifoam agent. The medium for culture had the following composition: Tryptone (10.0 g/liter), yeast extract (5.0 g/liter), MgSO4 · 7H2O (50 mM), KH2PO4 (5 g/liter), glucose (0.5% v/w) at pH 7.0. KH2PO4 was autoclaved separately and added aseptically before starting the run. The pH probe was calibrated between pH 7.0 and 9.2 before sterilization by using standard buffers. After sterilization of the fermentor, the pH of the fermentor media was automatically set to pH 7.0 by the addition of 1 N NaOH/1 M HCL and temperature to 37°C. The dissolved oxygen (DO) probe was calibrated by setting the zero value of the dissolved oxygen to zero nanometers (nA) and 100% value was given to the oxygen tension in the medium on aeration with air at an agitation rate of 250 rpm. The fermentor was started in batch phase with a working volume of 2 L. Cells harboring the recombinant plasmid were grown on LB media containing 100 μg/ml ampicillin at 37°C, 250 rpm overnight. 5% (v/v) of the ON seed culture was added to the fermentor aseptically. Following inoculation the DO began to fall. As soon as it decreased below 40% level, the agitation was increased in a cascade mode. Once the maximum rpm values were achieved, the run was continued until the nutrients were exhausted and the OD became constant. The cell density and PA production was measured by collecting samples every hour through a sterile sample port. The culture samples was suitably diluted in normal saline in the range 0.1 < OD < 0.4. The optical density was promptly read at 600 nm.

**Purification of PA protein.** The PA protein was purified using metal-chelate affinity chromatography under denaturing conditions.

Vol. 283, No. 2, 2001  BIOCHEMICAL AND BIOophysical RESEARCH COMMUNICATIONS
as follows. The pellet from 1 liter culture was resuspended in 25 ml of denaturing buffer containing 100 mM sodium phosphate buffer, 300 mM sodium chloride and 8 M urea (pH 8.0). The resuspended pellet was incubated at 37°C for 60 min on a rotary shaker. The lysate was centrifuged for 30 min at room temperature and the supernatant was mixed with 50% Ni-NTA slurry. Slurry was packed into a column and allowed to settle. The flow through was reloaded on the column to allow maximal protein binding. Ni-NTA matrix was washed with 50 ml denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein using 8–0 M urea gradient. The protein was eluted with 250 mM imidazole chloride in elution buffer, 100 mM sodium phosphate of pH 8.0 with 250 mM imidazole and 300 mM sodium chloride. 10 µl of each fraction was analyzed on 12% SDS–PAGE. Fractions containing the protein were collected, pooled, and dialyzed against 10 mM Hepes buffer containing 50 mM NaCl and stored frozen at –70°C in aliquots.

Protein estimation. Protein estimation was done using the protein determination dye (USF) based on the Bradford dye-binding procedure. The assay is based on the color change of Coomassie brilliant blue G-250 dye from red to blue in response to various concentrations of protein. The protein sample was taken in a volume of 800 and 200 µl of the dye was added and the mixture was vortexed. The absorbance was then taken at 595 nm. This was compared with a standard curve of BSA (optical density plotted against varying concentrations of BSA). From the standard curve the amount of protein was determined.

Macrophage lysis assay. rPA protein was assayed for their functional activity in the J774A.1 macrophage lysis assay, as described earlier (19, 20). Various concentrations of rPA protein along with LF (1 mg/ml) was added to the cells. The native PA along with LF was kept as the positive control. After 3 h, cell viability was determined using MTT dye and the resulting precipitate was dissolved in a buffer containing 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl in 90% isopropanol alcohol. Absorption was read at 540 nm and percent viability was determined.

Proteolytic cleavage of PA and in vitro binding to LF in solution. rPA was tested for susceptibility to cleavage by trypsin. The protein (1.0 mg/ml) was incubated with trypsin (1 ng/µg of protein) for 30 min at room temperature in 25 mM Hepes, 1 mM CaCl₂, 0.5 mM EDTA, pH 7.5. The digestion reaction was stopped by adding PMSF to a concentration of 1 mM. Trypsin nicked PA (1.0 mg/ml) was incubated with LF (1.0 mg/ml) and in 25 mM Tris, pH 9.0, containing 2 mg/ml Chaps (3-(3-cholamidopropyl) dimethyl ammonio)propanesulfonic acid) for 15 min at room temperature. Samples were applied to non-denaturing 4.5%polyacrylamide gel.

Iodination of PA. PA was iodinated using chloramine T method of Hunter and Greenwood (1962). PA (50 µg) was mixed gently with 100 µl of 0.1 M sodium phosphate buffer, pH 7.0, containing 5 mg of chloramine-T. 125-I-sodium iodide (1 mCi) was added to the mixture and incubated on ice for 5 min. The reaction was terminated by adding 10 µg of sodium metabisulfite in 0.1 M sodium phosphate buffer pH 7.0. The labeled protein was separated from free iodine by passing the reaction mixture on a gel filtration (Sephadex G-25) column pre-equilibrated with PBS. Nonspecific binding of the iodinated protein was minimized by washing the column with 1% BSA in PBS. Fractions of 0.5 ml were collected and monitored on Gamma counter. The first peak containing the iodinated protein was pooled together. The labeled protein was stored at 4°C.

Binding of PA protein to cell surface receptors. The binding of PA protein to cell surface receptor was carried out in 24 well plates using constant amount of radiiodinated native PA (0.1 mg/ml). J774A.1 cells were washed twice with cold HBSS for 5 min each and then placed on ice. The medium was replaced with cold binding medium (EMEM without sodium bicarbonate containing 1% bovine serum albumin and 25 mM, Hepes, pH 7.4). The cells were incubated with 0.1 mg/ml of iodinated PA and varying concentrations of the recombinant PA protein at 4°C for 3 h and then washed with cold HBSS. The cells were dissolved in 0.1 N NaOH and radioactivity measured in Gamma counter.

RESULTS
Expression of PA Gene from pMW1 in E. coli DH5α Cells

To check the expression of PA gene from the recombinant plasmid pMW1 in E. coli DH5α cells, the bacterial growth curve was plotted and the protein profile was studied at different time points by collecting culture aliquots every hour until 24 h of culture growth. It was observed that PA protein was expressed simultaneously with the culture growth and there was no impairment of culture growth due to constitutive expression of the PA protein. Electrophoresis followed by the Western blot with anti-PA antibodies confirmed the presence of the specific (PA) protein.

Protein Localization

To localize the protein inside the cells, cytosol and inclusion body fractions were prepared and subjected to SDS–PAGE. The expressed recombinant protein was found to be localized inside inclusion bodies in the cell (Fig. 1).

Plasmid Stability Assay

To check the stability of the recombinant plasmid inside the constitutively expressing cells, the culture was subjected to at least 10 O/N passages/subcultures. 1-ml culture aliquots were collected each day and the
protein profile was seen on SDS–PAGE (Fig. 2). No loss/decrease in protein expression was seen from Day 0 to Day 10, implying that the plasmid was stably present inside the cells and expressing the protein to normal levels.

Biomass Scale-up

The carbon and nitrogen in complex media comes from yeast extract and tryptone. It was possible to obtain a final OD₆₀₀ of 17.75 units. There was about three- to fourfold increase in biomass compared to the biomass in the shake flask. The bacterial growth curve was plotted (Fig. 3) and the protein profile was studied at different time points by collecting culture aliquots every hour of the fermentor run (Fig. 4a). E. coli cells yielded an insoluble protein aggregating to form inclusion bodies with an apparent molecular mass of 83 kDa (Fig. 1). Antibodies against PA reacted with recombinant PA in the Western blotting assay (Fig. 4b).

Purification of PA Protein

The PA protein was purified using metal-chelate affinity chromatography under denaturing conditions followed by on-column renaturation of the protein before elution. The purified protective antigen protein fraction obtained was found to be better than 95% pure. It was possible to achieve a yield of ~125 mg of the purified protein from 1 liter culture broth as determined by Bradford's method (Fig. 1).

Macrophage Lysis Assay

Biological activity of rPA (recombinant PA) and nPA (native PA from Bacillus anthracis) was compared by macrophage lysis assay in which rPA and nPA proteins were added at concentrations ranging from 1 ng/ml to 1 µg/ml in combination with LF (1 µg/ml) using MTT dye. Live cells oxidized the dye to formazan crystals and the precipitate was solubilized and absorbance read at 540 nm. It was found that rPA along with LF was fully able to lyse macrophage cells and its biological activity was similar to nPA. The EC₅₀ of rPA and nPA was found to be ~50 ng/ml each (Fig. 5).

Proteolytic Cleavage of PA in Solution and in Vitro Binding to LF Protein

To compare the ability of PA to bind to LF, both rPA and nPA were nicked with trypsin and incubated with LF. PA-LF binding was analyzed on 4.5% polyacrylamide gel. It was observed that rPA like nPA was capable of getting nicked with trypsin, binding to LF and forming PA-LF complex (Fig. 6). The mobility shift of the protein upon PA-LF binding could be visualized on the gel.

Binding of PA Protein to Cell Surface Receptors

Receptor binding of rPA and nPA was compared by radiolabeling the proteins and allowing them to bind to cell surface receptors present on J774A.1 cells. The total radioactivity bound to the cell surface/mg of cell protein was measured. It was found to be 1.20 × 10⁶ cpm/µg in case of nPA and 1.09 × 10⁶ cpm/µg in case of rPA. The total cell protein was found to be 0.95 ± 0.05 mg. Both rPA and nPA were found to bind to the cell surface receptors in comparable amounts with ~7.25 ng of nPA bound/mg cell protein and ~7.72 ng of rPA bound/mg cell protein (Table 1).
DISCUSSION

Several constitutive and inducible expression systems exist for the production of recombinant proteins (30, 31). The PA gene of Bacillus anthracis has also been expressed in both constitutive and inducible expression systems. But constitutive expression systems are limited by low protein expression levels, rapid degradation of the protein inside the expression host cells due to longer exposure to cellular proteases and/or cell death due to toxicity of the recombinant protein (32–34). Most of the inducible expression systems also require promoter induction with expensive non-metabolizable molecules such as isopropyl-thio-β-D-galactopyranoside (IPTG) for protein expression (35). The cost of IPTG limits the usefulness of strong promoters for large scale production of recombinant PA protein. The issue of IPTG toxicity also becomes relevant in case the protein is to be used for therapeutic purposes (36). IPTG induction can only be circumvented by using either lactose as an inducer or by using thermosensitive variants of the lacI repressor protein.

FIG. 4. (a) Electrophoretic analysis of the total cellular proteins of E. coli cells expressing PA. Proteins were separated on 12% SDS-PAGE and stained with Coomassie blue. Lanes A–D, E. coli DH5α cells containing pMW1 plasmid (cultures grown for 3, 6, 9, and 12 h, respectively); Lane E, PA purified from B. anthracis; Lane F, DH5α cells without pMW1; Lane G, molecular weight standard. (b) Western blot of E. coli DH5α cells expressing PA, developed with rabbit polyclonal antibody against PA. Lanes A–D, E. coli DH5α cells containing pMW1 plasmid (cultures grown for 3, 6, 9, and 12 h, respectively); Lane E, PA purified from B. anthracis; Lane F, DH5α cells without pMW1.

FIG. 5. Macrophage lysis assay: Biological activity of PA purified from Bacillus anthracis and E. coli DH5α cells. J774A.1 cells were incubated with varying concentrations of PA alone or in combination with LF (1 µg/ml) for 3 h at 37°C. ▲ PA from Bacillus anthracis, ■ PA from E. coli, ● PA from Bacillus anthracis with LF, ○ PA from E. coli with LF.

FIG. 6. Binding of PA to LF protein in solution: LF (1 µg/ml) was incubated with trypsin-nicked PA (1 µg/ml) and samples were analyzed on a 4.5% nondenaturing polyacrylamide gel. The gel was stained with Coomassie blue. Lane A, nicked PA purified from B. anthracis; Lane B, nicked PA purified from E. coli DH5α cells; Lane C, LF purified from B. anthracis; Lane D, Trypsin-nicked PA from B. anthracis incubated with LF; Lane E, Trypsin-nicked PA from E. coli DH5α cells incubated with LF.
to allow heat induction (37). However, lactose is a metabolizable sugar and has to be added continuously.

In the case of heat inducible expression systems also, host proteases have been observed to get activated as a heat shock stress response leading to an elevation in the rate of degradation of the recombinant protein resulting in low protein yields (38). Also, once the induced cultures reach logarithmic phase, the expression is downregulated and there is no subsequent increase in the expression of protein even if the culture is allowed to grow further. PA has previously been expressed constitutively at very low levels in different bacterial strains, where gene instability and protease activity have led to low levels of recoverable protein, and overexpressed in IPTG inducible systems in E. coli (32–35). The protective antigen and lethal factor genes of Bacillus anthracis have previously been overexpressed as recombinant proteins in our laboratory following IPTG induction (28, 39). The PA gene cloned in pQE30 has been designated as pMW1. The pQE plasmid is derived from the pDS family of plasmids and contains all the elements essential for protein expression and purification from the cloned gene including a strong phage T5 promoter, a 6x-histidine tag, ampicillin resistance gene, and a synthetic ribosome binding site (RBS) along with translational stop codons in all reading frames with two transcriptional terminators (40, 41). The expression is very efficient. This plasmid system can use any E. coli host strain for expression since the phage T5 promoter can be recognized by any E. coli RNA polymerase. We have observed that it is not necessary to use specific host strains such as SG13009, M15, JM109, TG1. The E. coli host strain used in these studies—DH5α (supE44 ΔlacU169 Δ80lacZΔM15) has R17 recA1 endA1 gyrA96 thi-1 relA1—is a recombination deficient suppressing strain used for plating and growing plasmids and cosmids. The Δ80lacZΔM15 permits alpha complementation with N-terminal of β-gal encoded in pUC vectors (29). Since the lacIYA locus is deleted, the strain cannot code for the lac repressor. We have overexpressed the PA gene in this strain since this strain is used for most cloning purposes and is easily available. Tight regulation of a promoter is essential only for the synthesis of proteins that may be detrimental to the host cell (42, 43). Incompletely repressed expression systems have been seen to cause plasmid instability, a decrease in cell growth rate and loss of recombinant protein production (44), so, we checked for all these possibilities when PA is expressed constitutively in DH5α cells. On preparation of the cytosol and insoluble inclusion body fractions, PA was found trapped in inclusion bodies inside the cells. It has been seen that there are two major parameters correlated with inclusion body formation-charge average and turn forming residue fraction of the protein (45). Inclusion bodies also offer several advantages such as isolation of the protein in high purity and concentration, protection from proteases and desirable production of proteins that are lethal to host cells in an inactive form giving higher protein yields (37, 46, 47). Most strains commonly utilized for expression are protease deficient (48) unlike the strain used in our studies. Proteolysis is a selective, highly regulated process that plays an important role in cellular physiology and serves to remove misfolded/aggregated material (49). It is possible that the absence of proteases might result in increased toxicity to the host as a result of accumulation of abnormal proteins (31). The growth profile and plasmid stability studies done on our system indicate that the expression of the PA protein is not toxic to the host cells and also does not result in detrimental cell growth. In the case of constitutive expression of PA from DH5α cells, the PA protein is possibly protected from the host cell proteases due to the formation of inclusion bodies. We propose that since PA is overexpressed and has a tendency to form inclusion bodies, it can be expressed in DH5α or other strains that are not protease deficient and commercially used for cloning purposes without degradation from cytoplasmic proteases and any basal repression of expression. In the case of other proteins not forming inclusion bodies, proteolysis can be minimized by utilizing different strategies including the use of protease inhibitors and optimization of fermentation conditions (50, 51).

Various strategies were tried to scale-up the expression of the recombinant protective antigen by bioprocess optimization of the constitutively expressing cultures using batch culture technique in a 5-liter fermentor. The media, along with a specified carbon source was used for the growth. High cell density cultivation has been one of the most effective ways to increase cell as well as the product yields. The importance of recombinant products for both research and commercial use has inevitably led to a need to increase the volumetric productivity of fermentation processes. Escherichia coli has been widely used as the favorable host for many recombinant DNA products. Recombinant methodologies of E. coli are very well developed. In fermentor cultivation, the attention is focused on increasing the volumetric productivity through an increase in host cell mass. In the conventional batch

<table>
<thead>
<tr>
<th>Protein</th>
<th>CPM</th>
<th>PA (ng)</th>
<th>PA/cell protein (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nPA</td>
<td>82679 ± 1169</td>
<td>6.89</td>
<td>7.25</td>
</tr>
<tr>
<td>rPA</td>
<td>79896 ± 1388</td>
<td>7.34</td>
<td>7.72</td>
</tr>
</tbody>
</table>

* J774A.1 cells were incubated with 1 μg of radiiodinated PA (nPA and rPA) for 3 h at 4°C.

* Protein content of the cells per well was 0.95 ± 0.05 mg as determined by Lowry's method.
process, the production phase is short, due to the deple-
tion of the carbon energy source. Furthermore, the fer-
mentor agitation is controlled such that the dis-
solved oxygen concentration does not become limiting
and the aerobic cultivation operates within the limits of
the system. We tried various C-sources such as glu-
cose, lactose and L-malic acid, glycerol, and galactose.
Glucose leads to the formation of ethanol even in the
presence of sufficient dissolved oxygen (DO) if an ex-
cess of sugar is present in the culture medium. Ethanol
is the main cause of low cell yields. We found glycerol
as the most appropriate C-source in terms of biomass
yields and protein production. We could successfully
cultivate recombinant E. coli to an optical density of
17.75. The protein was purified by a single step of
Ni-NTA chromatography. We could purify ~125 mg of
rPA/1 of the batch culture.

We have demonstrated that high cell densities are
obtainable for this expression system, with concomi-
tant higher recombinant protein expression. Use of
glycerol as an alternate C-source is advantageous.
The present work is an effort to harness the capabilities of
the microorganisms to produce the recombinant pro-
teins for further use in industry, medicine and re-
search, etc. in the most cost effective manner by the
utilization of easily and commonly available resources.
This work would make the large-scale production of PA
protein based anthrax vaccines possible in the near
future. Attempts are being made to further increase
the yields of protective antigen.

ACKNOWLEDGMENT

We thank the Department of Biotechnology, Government of India,
for supporting this work.

REFERENCES

assessments of risk from environments contaminated with an-
fusion proteins for intracellular delivery of macromolecules.
J. Appl. Microbiol. 87, 284.
4. Goetz, T. J., Klimpel, K. R., Leppala, S. H., Keith, J. M., and
Bergofsky, J. A. (1997) Delivery of antigens to the MHC class I
sequence and analysis of the DNA encoding protective antigen of
Bacillus anthracis. Gene 69, 287–300.
expression in Escherichia coli of the lethal factor gene of Bacillus
and expression of the Bacillus anthracis edema factor toxin gene:
A calmodulin-dependent adenylate cyclase. J. Bacteriol. 170,
2263–2266.
lation of the Bacillus anthracis pag operon. J. Bacteriol. 181,
4485–4492.
Identification of a receptor-binding region within domain 4 of
the protective antigen component of anthrax toxin. Infect. Immun.
67, 1860–1865.
11. Sucic, J. F., Moehring, J. M., Iacocino, N. M., Luca, J. W.,
and Moehring, T. J. (1999) Endoprotease PACE4 is CalC-
dependent and temperature-sensitive and can partly rescue the
phenotype of a furin-deficient cell strain. Biochem. J. 339(Pt 3),
639–647.
12. Wesche, J., Elliott, J. L., Falnes, P. O., Olsnes, S., and Collier,
R. J. (1998) Characterization of membrane translocation by an-
anthrax toxin: Insertion and pore formation by protective anti-
67, 3055–3060.
15. Hoover, D. L., Friedlander, A. M., Rogers, L. C., Yoon, I. K.,
differentially regulates lipopolysaccharide-induced monocyte
production of tumor necrosis factor alpha and interleukin-6 by
increasing intracellular cyclic AMP. Infect. Immun. 62, 4432–
4439.
lethal factor contains a zinc metallopeptase consensus sequence
which is required for lethal toxin activity. Mol. Microbiol. 13,
1003–1100.
17. Pulizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Mon-
tecucco, C. (1999) Anthrax lethal factor cleaves MMK3 in mac-
rophages and inhibits the LPS/IFN-gamma-induced release of
NO and TNFalpha. FEBS Lett. 462, 199–204.
is required for expression of anthrax lethal toxin cytotoxicity.
(1999) Calcium is required for the expression of anthrax lethal
toxin activity in the macrophage-like cell line J774A.1. Infect.
Immun. 67, 2107–2114.
201.
21. Friedlander, A. M., Bhatnagar, R., Leppala, S. H., Johnson, L.,
and resistance to anthrax lethal toxin. Infect. Immun. 61, 245–
252.
and Collier, R. J. (1994) Role of macrophage oxidative burst in
nization against anthrax with the purified recombinant protective
25. Petosa, C., Collier, R. J., Klimpel, K. R., Leppala, S. H.,


