Abstract

In an effort to develop genetically engineered *Brucella abortus* (BA) vaccines, the genes encoding heat shock proteins (HSPs) GroEL, GroES, and HtrA were cloned and expressed in the BAC-TO-BAC Baculovirus System, and the kinetics of protein expression were analyzed using various insect cell lines in suspension cultures, different cell densities in suspension cultures, multiplicities of infection and recombinant virus replication times. *Trichoplusia ni* cells expressed only BA HtrA, but *Spodoptera frugiperda* (**Sf9**) cells expressed all three recombinant proteins. The best GroEL expression was achieved by infecting $2 \times 10^6$ **Sf9** cells/ml with an MOI 10 of recombinant virus and harvesting the cells after 96 h of virus replication. GroES and HtrA were best expressed when infecting $2 \times 10^6$ **Sf9** cells/ml with an MOI 1 of recombinant viruses and harvesting the cells after 120 h of virus replications. Under these conditions BA recombinant HSPs were expressed as follows: GroEL at 16% of the total cellular proteins (105 $\mu$g/ml concentration); GroES 2% (15.25 $\mu$g/ml); and HtrA 8% (84.48 $\mu$g/ml). This is the first report of cloning and expression of BA genes in the baculovirus system. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Brucella abortus*; Heat shock proteins; Baculovirus; Cloning; Kinetics of expression

1. Introduction

Keeping in mind their safety, subunit vaccines containing only the immunogenic components from a particular pathogen are potential alternatives to live vaccines. The
selection of specific antigens that induce Th1 subset responses against intracellular pathogens, such as *Brucella abortus* (BA), is important (Mosmann and Coffman, 1989). Heat shock proteins (HSPs) are major antigens (Shinnick et al., 1988), inducing humoral and cell-mediated immune responses against a variety of bacterial pathogens (Kaufmann, 1990). Thus, analysis of BA HSPs, as candidates for subunit vaccines, is warranted and to generate recombinants of such proteins we elected to use an *Autographa californica* baculovirus (BV) cloning and expression system. Although eukaryotic and viral genes have been expressed in BV (Miller, 1988; Young, 1988; Makoff et al., 1989), it was necessary to explore the factors that may influence the expression of BA proteins in this system. In this paper, we report the generation of BV recombinant viruses for BA GroEL, GroES and HtrA HSPs, and the analysis of the conditions optimal for expression of recombinant proteins.

2. Materials and methods

2.1. Plasmids, insect cells, generation of baculovirus recombinants for *Brucella abortus* genes

Plasmid pMBgroELS containing the BA *groEL* and *groES* genes, and plasmid pBA32 containing the BA *htrA* gene were obtained from Dr. G. Schurig, VA-MD Regional College of Veterinary Medicine (VMRCVM). *Sf9* (Gibco-BRL), *Sf21* (kindly provided by Dr. D.E. Lynn, USDA/ARS) and *T. ni* (Gibco-BRL) insect cells were propagated in *Sf*900II-serum free media (Gibco-BRL) at 28°C. Competent *E. coli* DH10BAC cells, containing Bacmid (baculovirus shuttle vector plasmid) and helper plasmid, were used to generate recombinant Bacmids according to manufacturer’s (BAC-TO-BAC Baculovirus Expression System, Gibco-BRL) instructions. The 2.1 Kb *groEL*, 0.4 Kb *groES* and 1.9 Kb *htrA* genomic fragments were excised from their respective plasmids by appropriate restriction enzymes. Inserting the fragments into the pFASTBAC1 donor plasmid yielded the 6.9 kb *groEL*, 5.2 kb *groES* and 6.7 kb *htrA* recombinants, respectively. The pFASTBAC recombinants were transformed into competent *E. coli* DH10BAC cells, and the gene of interest was transposed into Bacmid through lacZ gene disruption. White colonies, containing the recombinant Bacmids were selected on Luria agar, containing ampicillin, kanamycin, gentamycin, tetracycline, bluo-gal, and IPTG. Recombinant Bacmids were purified, digested with restriction enzymes, and genomic inserts were demonstrated by Southern blotting (Southern, 1975) using digoxygenin-labeled specific DNA probes. *Sf9* cells were transfected with recombinant Bacmids using CELLFECTIN reagent (Gibco-BRL) (Inumaru and Yamada, 1991). A mixture of recombinant Bacmids and CELLFECTIN diluted in *Sf*-900II-serum free medium without antibiotics was laid over the washed *Sf9* cells in the 6-well plates. The cells were incubated for 5 h at 28°C, rinsed, and incubated for another 72 h. Media were harvested, centrifuged, and the virus containing supernatant was titrated. As the plaque assay did not work, infective titers were determined in 96-well plates, seeded with *Sf9* cells. Uptake of trypan blue by dead cells was used to determine end-points in calculating the median tissue culture dose (TCID<sub>50</sub>) per ml (Lynn, 1992).
were converted to plaque forming units (pfu/ml) by using the formula: pfu/ml = TCID_{50}/ml × 0.69 (Summer and Smith, 1987). The infective titer of recombinant baculovirus stocks, which were grown in adherent or suspension cultures of Sf9 cells were compared.

2.2. Identification and analysis of kinetics of recombinant protein expression

Proteins were identified in Western blots of the lysates of recombinant virus-inoculated Sf9 cells. Sf9 cell-adsorbed goat BA RB51 polyclonal antibody (G. Schurig, VMRCVM); mouse monoclonal antibody to GroEL (M. Stevenson, IA State U.); goat monospecific antibody to GroES and mouse monospecific antibody to HtrA (G. Schurig), were used as primary antibodies. Appropriate species specific immunoglobulins, conjugated to horseradish peroxidase, were used as secondary antibodies. We analyzed the effect of several factors in various combinations on the level of protein expression. The factors were: Sf9 or T. ni cells in suspension cultures; cell densities of 1 × 10^6 or 2 × 10^6 cells/ml, multiplicity of infection (MOI) of 1, 5, or 10, and recombinant virus replication times of 24, 48, 72, 96, or 120 h. Total protein concentration in recombinant protein expressing cell extracts was measured by the Bradford dye-binding procedure at 595 nm in a spectrophotometer (Perseptive Biosystems, Framingham, MA). An identical preparation of each cell extract was run parallel on duplicate SDS-PAGE gels. One gel was stained by Coomassie blue and scanned by a densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA) to visualize and quantify the stained bands. The other gel was analyzed by Western blotting. Lanes with the strongest bands representing particular recombinants proteins were selected for densitometry analysis, having confirmed the identity (Western blot) and the molecular weight (Coomassie-gel, Western blot) of the protein. Based on the total protein concentration and on the proportion of the recombinant protein band in the total protein content, the concentration (% of total protein) and the quantity (µg/ml) of each recombinant protein were calculated.

3. Results and discussion

Heat shock proteins are required for intracellular survival of bacteria. They are immunogenic, eliciting both cellular and humoral immune responses in infected hosts. GroEL and GroES induce protective immunity against pathogens, such as *Mycobacterium tuberculosis* (Silva et al., 1994) and *Helicobacter pylori* (Ferrero et al., 1995), respectively. Oxidative killing is the primary mechanism by which host phagocytes eliminate intracellular Brucellae (Enright, 1990; Baldwin and Winter, 1994), thus BA HtrA may contribute to the survival of this agent in host phagosomes. Heat shock proteins such as chick HSP90 (Binart et al., 1995), desiccation stress protein (dsp28) from the beetle *Tenebrio molitor* (Graham et al., 1996), and *M. tuberculosis* chaperonin 10 (Akins et al., 1994) have been successfully expressed in BV system. We speculated that the three BA HSPs may be capable of inducing cell-mediated and protective immune responses, and it would be worthwhile to explore their immunogenicity and their suitability as components of a genetically engineered BA subunit vaccine.
Subcloning BA HSP genes into baculovirus vector, generation and identification of recombinant Bacmids in Southern blots were accomplished (data not shown). To determine the infectivity of recombinant BVs, we explored several factors for the plaque assay: cell lines (Sf9, Sf21, T. ni), cell-densities (1 × 10^7, 2 × 10^6, 1 × 10^6, 7.5 × 10^5 cells/well), viral dilutions (10^{-1}–10^{-9}), virus replication times (4–9 days), percentages of agarose gels (0.75%, 1.5%), the presence or absence of serum in the culture medium, and staining dead cells with trypan blue or staining live cells with neutral red. No plaques were observed under any conditions. We have not explored and have no explanation for our difficulties in using the plaque assay. Titration of the recombinant viruses by endpoint TCID_{50} (Lynn, 1992) was consistent and reliable. The infective titers of the recombinant viruses generated in suspension cultures were higher than those generated in adherent cultures (Table 1). The 60 kDa GroEL, 10 kDa GroES, and 60 kDa HtrA recombinant proteins were demonstrated by Western blots (Fig. 1). The small size difference between BA RB51 GroES and recombinant GroES may be due to lack of complete glycosylation of the recombinant. The HtrA recombinant shows the characteristic double-band appearance that was also noticed in vaccinia virus recombinants of this protein (Toth, unpublished data). T. ni cells expressed only HtrA, but Sf9 cells expressed all three recombinant HSPs. Recombinant GroEL protein was expressed at the highest level when 2 × 10^6 Sf9 cells/ml were infected with an MOI of 10 and harvested after 96 h of recombinant BV replication. Recombinant GroES and HtrA proteins were expressed the best when 2 × 10^6 Sf9 cells/ml were infected with an MOI of 1 and harvested after 120 h of replication. Under optimal conditions GroEL, GroES, and HtrA recombinant proteins were expressed as 16% (105 µg/ml), 2% (15.25 µg/ml), and 8% (84.48 µg/ml), respectively, of the total cellular proteins. Our study is the first expressing BA HSPs in the BV system. Because of the ease of cultivation, higher cell viability, resistance to shear stress, and expression of all three recombinant proteins, Sf9 cells were found more suitable than T. ni cells for production of proteins by recombinant baculoviruses.

In a previous study (O’Reilly et al., 1992), levels of heterologous proteins expressed in a baculovirus system approached up to 25% of the total cellular protein, ranging from 10 to 100 mg per 10^9 cells. In our studies, the levels of recombinant HSPs expressed under optimal conditions were as follows: GroEL, GroES, and HtrA reaching 53, 8, and 42 mg, respectively, per 10^9 cells. These values represent average expression levels for GroEL and HtrA, and below average for GroES in terms of the expected baculovirus recombinant protein yields.

### Table 1

<table>
<thead>
<tr>
<th>Recombinants virus</th>
<th>Suspension cultures</th>
<th>Adherent cultures</th>
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<tbody>
<tr>
<td>GroEL</td>
<td>5×10^9</td>
<td>1.23×10^8</td>
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<tr>
<td>GroES</td>
<td>2.95×10^9</td>
<td>0.84×10^8</td>
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<tr>
<td>HtrA</td>
<td>3.23×10^8</td>
<td>0.83×10^8</td>
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References


