groEL Encodes a Highly Antigenic Protein in *Burkholderia pseudomallei*

PATRICK C. Y. WOO, PATRICIA K. L. LEUNG, SAMSON S. Y. WONG, PAK-LEUNG HO, AND KWOK-YUNG YUEN

Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, and HKU-Pasteur Research Centre, Hong Kong, China

Received 26 October 2000/Returned for modification 12 March 2001/Accepted 24 April 2001

Meliodosis is a serious human disease, endemic in Southeast Asia, caused by the bacterium *Burkholderia pseudomallei*. *B. pseudomallei* is a natural saprophyte that can be isolated from soil, stagnant streams, rice paddies, and ponds, which are the major natural reservoirs of the bacteria (13). Although meliodosis is endemic in Southeast Asia, human infections have occurred throughout the world between 20° north and south latitudes (10). *B. pseudomallei* is very different from other nonfermentative gram-negative bacteria in terms of the spectrum of disease that it can cause. Illness can be manifested as an acute, subacute, or chronic process. Moreover, the incubation period of melioidosis can vary from 2 days to 26 years (15).

No recombinant protein is available for serodiagnosis of melioidosis. In this study, we report the cloning of the *groEL* gene, which encodes an immunogenic protein of *Burkholderia pseudomallei*. Bidirectional DNA sequencing of *groEL* revealed that the gene contained a single open reading frame encoding 546 amino acid residues with a predicted molecular mass of 57.1 kDa. Basic Local Alignment Search Tool analysis showed that the putative protein encoded by *groEL* is homologous to the chaperonins encoded by the *groEL* genes of other bacteria. It has 98% amino acid identity with the GroEL of *Burkholderia cepacia*, 98% amino acid identity with the GroEL of *Burkholderia vietnamiensis*, and 82% amino acid identity with the GroEL of *Bordetella pertussis*. Furthermore, it was observed that patients with melioidosis develop a strong antibody response against GroEL, suggesting that the recombinant protein and its monoclonal antibody may be useful for serodiagnosis in patients with melioidosis and that the protein may represent a good cell surface target for host humoral immunity. Further studies in these directions would be warranted.

Methods

1. GroEL Encodes a Highly Antigenic Protein in *Burkholderia pseudomallei*.

   The *groEL* gene, which encodes an immunogenic protein of *Burkholderia pseudomallei*, Bidirectional DNA sequencing of *groEL* revealed that the gene contained a single open reading frame encoding 546 amino acid residues with a predicted molecular mass of 57.1 kDa. Basic Local Alignment Search Tool analysis showed that the putative protein encoded by *groEL* is homologous to the chaperonins encoded by the *groEL* genes of other bacteria. It has 98% amino acid identity with the GroEL of *Burkholderia cepacia*, 98% amino acid identity with the GroEL of *Burkholderia vietnamiensis*, and 82% amino acid identity with the GroEL of *Bordetella pertussis*. Furthermore, it was observed that patients with melioidosis develop a strong antibody response against GroEL, suggesting that the recombinant protein and its monoclonal antibody may be useful for serodiagnosis in patients with melioidosis and that the protein may represent a good cell surface target for host humoral immunity. Further studies in these directions would be warranted.

2. No recombinant protein is available for serodiagnosis of melioidosis.

   In this study, we report the cloning of the *groEL* gene, which encodes an immunogenic protein of *Burkholderia pseudomallei*. Bidirectional DNA sequencing of *groEL* revealed that the gene contained a single open reading frame encoding 546 amino acid residues with a predicted molecular mass of 57.1 kDa. Basic Local Alignment Search Tool analysis showed that the putative protein encoded by *groEL* is homologous to the chaperonins encoded by the *groEL* genes of other bacteria. It has 98% amino acid identity with the GroEL of *Burkholderia cepacia*, 98% amino acid identity with the GroEL of *Burkholderia vietnamiensis*, and 82% amino acid identity with the GroEL of *Bordetella pertussis*. Furthermore, it was observed that patients with melioidosis develop a strong antibody response against GroEL, suggesting that the recombinant protein and its monoclonal antibody may be useful for serodiagnosis in patients with melioidosis and that the protein may represent a good cell surface target for host humoral immunity. Further studies in these directions would be warranted.

3. Methods

   Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The methods included the isolation and identification of *B. pseudomallei* from clinical specimens, including blood, sputum, pus, swabs, and other clinical samples. The serodiagnosis of melioidosis involved the use of recombinant antigens, which are easier to standardize and may offer higher sensitivity, specificity, and reproducibility. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.

4. Results

   Results for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The results included the isolation and identification of *B. pseudomallei* from clinical specimens, including blood, sputum, pus, swabs, and other clinical samples. The serodiagnosis of melioidosis involved the use of recombinant antigens, which are easier to standardize and may offer higher sensitivity, specificity, and reproducibility. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.

5. Discussion

   Discussion for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The discussion included the importance of recombinant antigens in the serodiagnosis of melioidosis and the potential benefits of using recombinant antigens for the development of vaccines for melioidosis. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.

6. Conclusion

   Conclusion for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The conclusion included the importance of recombinant antigens in the serodiagnosis of melioidosis and the potential benefits of using recombinant antigens for the development of vaccines for melioidosis. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.

7. References

   References for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The references included studies on the isolation, identification, and serodiagnosis of melioidosis and the use of recombinant antigens in the development of vaccines for melioidosis. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.

8. Acknowledgments

   Acknowledgments for the isolation, identification, and serodiagnosis of melioidosis were described in detail. The acknowledgments included the support provided by the sponsors and the assistance of the staff involved in the isolation, identification, and serodiagnosis of melioidosis. Methods for the isolation, identification, and serodiagnosis of melioidosis were described in detail.
mannheim, Mannheim, Germany). The partial digest with fragments of 1.5 to 6 kb were then ligated to the BamHI site of the vector provided by the ZAP Express vector kit (Strategene, La Jolla, Calif.), and a phage expression library was constructed according to the manufacturer’s instructions. The library had at least 1 million independent phage plaques, with more than 95% containing inserts of an average size of 2.3 kb, as checked by restriction enzyme digestion of 100 clones with SalI and XhoI (Boehringer Mannheim).

Approximately 50,000 plaques of this library were screened with serum obtained from a patient with culture-documented melioidosis according to the manufacturer’s instructions. Briefly, the library was plated on NZY plates at 5,000 PFU per plate with 600 µl of XL1-Blue cells at an optical density at 600 nm of 0.5 and 6.5 ml of NZY top agar. The plates were incubated at 42°C for 6 h and at 37°C for 4 h. The proteins were transferred to nitrocellulose membranes. After being blocked with 3% bovine serum albumin (BSA) and 7% skim milk in phosphate-buffered saline (PBS), the membranes were incubated with serum obtained from a patient with culture-documented melioidosis at a 1:2,000 dilution at 25°C for 1 h. After being washed with 3% BSA in PBS three times, the membranes were incubated with rabbit anti-human antibody conjugated with horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, Calif.) at 1:5,000 dilution at 25°C for 1 h. After being washed with 3% BSA in PBS three times, antigen-antibody interaction was detected with the ECL fluorescence kit (Amersham Life Science, Little Chalfont, United Kingdom). Twenty-four positive phage clones were isolated, and their DNA inserts were excised with ExAssist helper phage (Boehringer Mannheim).

Overnight cultures of SOLR cells with pBK-CMV and SOLR cells with pBK-CMV-GroEL were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The cells were centrifuged at 13,000 rpm for 5 min and resuspended in PBS with 1% (vol/vol) Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride. The cells were sonicated three times (10 s each time). Twenty-five microliters of the cell extracts obtained were electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Bio-Rad). The blot was incubated with 1:2,000 dilution of the serum used for library screening and serum of a normal blood donor using 5% skim milk in PBS with 0.1% (vol/vol) Tween 20 as the blocking buffer, and antigen-antibody interaction was detected with the ECL fluorescence kit.

DNA sequencing was carried out by using vector primers of pBK-CMV (T3 and T7) and synthetic primers designed from the sequencing data of the first and second rounds of the sequencing reaction (LPW124, 5′-CGGCAAGGAAGGCCTGAT-3′; LPW134, 5′-CGCACGCAATCGAAGAA-3′; LPW136, 5′-GATCGCGCAGCTGCTGC-3′; and LPW125, 5′-GCTACACGTCGGACT-3′). Bidirectional DNA sequencing was performed with an ABI automatic sequencer (Perkin-Elmer, Norwalk, Conn.) according to the manufacturers’ instructions (21). The DNA sequence was analyzed by BLAST search with the National Center for Biotechnology Information server at the National Library of Medicine (Bethesda, Md.). The searches were performed at both the protein and DNA levels. Phylogenetic-tree construction was performed by the Clustal method with MegAlign 4.00 (DNASTar Inc., Madison, Wis.).

To produce a fusion plasmid for protein purification, the sequence coding for amino acid residues 1 to 546 of GroEL was amplified by PCR using the pBK-CMV-GroEL plasmid as a template. The pBK-CMV-GroEL plasmid was amplified with 0.5 µM primers (LPW127, 5′-GGAATTCCTTACATGCTCA TGCCCATG-3′, and LPW144, 5′-CGGGATCCGATGGC AGCTAAAGACGT-3′) (Gibco BRL, Gaithersburg, Md.). The PCR mixture (50 µl) contained pBK-CMV-GroEL, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl2, and 0.01% gelatin), 200 µM (each), deoxynucleoside triphosphates, and 1.0 U of Tag polymerase (Boehringer Mannheim). The mixtures were amplified in 40 cycles of 94°C for 1 min, 50°C for 1 min, and 68°C for 2 min and a final extension at 68°C for 10 min in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). The amplified fragment was cloned into the BamHI and EcoRI sites of expression vector pGEX-5X-3 in frame and downstream of the glutathione S-transferase (GST) coding sequence. The GST-GroEL fusion protein was expressed and purified with the GST gene fusion system (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions (3). Approximately 2.5 mg of highly purified GST-GroEL fusion protein was routinely obtained from 1 liter of Escherichia coli carrying the fusion plasmid.

Highly purified GST-GroEL fusion protein samples were run on an SDS–10% polyacrylamide gel (35 µg per lane) and electroblotted onto a nitrocellulose membrane (Bio-Rad). The blot was incubated with a 1:2,000 dilution of sera from three patients with culture-documented melioidosis, two patients with Pseudomonas aeruginosa bacteremia, two patients with B. pseudomallei bacteremia, two patients with culture-documented melioidosis, and two patients with culture-documented melioidosis (lane 4) but not with the serum of a healthy blood donor (lane 2).

![FIG. 1. Western blot analysis of GroEL of B. pseudomallei. Shown are cell extracts of overnight cultures of SOLR cells with pBK-CMV (lanes 1 and 3) and SOLR cells with pBK-CMV-GroEL (lanes 2 and 4) electrophoresed on an SDS–10% polyacrylamide gel. Antigen-antibody interaction was detected with the serum of a patient with melioidosis (lane 4) but not with the serum of a healthy blood donor (lane 2).](image)
FIG. 2. Phylogenetic trees based on known bacterial GroEL amino acid sequences (a), nucleotide sequences (b), and their corresponding 16S rRNA gene sequences (c) illustrating the position of B. pseudomallei.
Serum of one patient with Acinetobacter baumannii bacteremia, one patient with Stenotrophomonas maltophilia bacteremia, or healthy blood donors, and antigen-antibody interaction was detected as described above. All sera were collected from patients during acute illness.

About 50,000 independent phage plaques were screened with the serum obtained from a patient with melioidosis. Twenty-four positive plaques were selected, purified, and converted into plasmids. When induced with isopropyl-β-D-thiogalactopyranoside, 2 of the 24 isolates produced protein bands of about 57 kDa that were recognized by the serum from a patient with melioidosis on a Western blot (Fig. 1).

Bidirectional DNA sequencing of the insert revealed that the DNA contained a single open reading frame of 1,638 bp, encoding 546 amino acid residues with a predicted molecular mass of 57.1 kDa.

BLAST analysis was performed to search for homologs that might suggest potential biological functions. It revealed that the putative protein encoded by the gene is homologous to the GroEL proteins of other bacteria (Fig. 2a). It has 98% amino acid identity with the GroEL of Burkholderia cepacia (GenBank accession no. AF104907), 98% amino acid identity with the GroEL of Burkholderia vietnamiensis (GenBank accession no. AF104908), and 82% amino acid identity with the GroEL of Bordetella pertussis (GenBank accession no. U12277). The gene was named groEL of B. pseudomallei.

Strong antigen-antibody interaction was detected with the sera of three patients with melioidosis, none of whom had a past history of Burkholderia infection (Fig. 3, lanes 1, 2, and 5). Weaker antigen-antibody interaction was detected with the sera of one patient with P. aeruginosa bacteremia (lane 4) and one patient with S. maltophilia bacteremia (lane 8). No antigen-antibody interaction was detected with the sera of one patient with A. baumannii bacteremia (Fig. 3, lane 6), one patient with P. aeruginosa bacteremia (Fig. 3, lane 3), one patient with S. maltophilia bacteremia (Fig. 3, lane 7), and all five healthy blood donors (Fig. 3, lanes 9 to 13).

Chaperonins are large protein complexes that assist protein folding in vivo. Although protein folding is often regarded as a spontaneous, thermodynamically stable process in vitro, the folding of polypeptide chains in vivo is often faced with adverse conditions, with high protein concentration and temperature that favor strong intermolecular hydrophobic interactions, leading to protein misfolding and aggregation. Therefore, chaperonins are essential to assist this last step of the information transfer pathway from genes to functional proteins (7, 9). Chaperonins have been identified in all three domains of life: Bacteria, Archaea, and Eukarya (including cytosolic chaperonins, as well as chaperonins in endosymbiotically derived organelles, such as mitochondria and chloroplasts) (6, 8). By comparing their nucleotide and amino acid sequences, chaperonins are classified into two groups: group I, which includes members from bacteria (GroEL), mitochondria (Hsp60), and chloroplasts (Rubisco binding protein), and group II, which includes members from archaea and the cytosol of eukaryotes (20, 22).

The amino acid sequence of GroEL of B. pseudomallei resembles those of other gram-negative bacteria, especially the nonfermentative gram-negative bacteria, and other Burkholderia species. It is interesting to note that the phylogenetic tree based on the amino acid sequences of the GroEL proteins in various bacteria (Fig. 2a) resembles the phylogenetic tree of the 16S rRNA gene sequences of the corresponding bacteria (Fig. 2c) more than the tree based on the nucleotide sequences of the groEL genes of the bacteria (Fig. 2b). From Fig. 2, it can be observed that the nucleotide sequence of groEL of Neisseria flavescens is more distantly related to those of the Burkholderia species and B. pertussis, but the corresponding amino acid sequences and 16S rRNA gene sequences of these bacteria are very closely related. Furthermore, the amino acid sequences and 16S rRNA gene sequences of groEL of Leptospira interrogans and Porphyromonas gingivalis are very distantly related to the other bacteria, but the corresponding nucleotide sequences of these two bacteria are more closely related to the other species in the phylogenetic trees. We speculate that the divergence of the tree based on groEL nucleotide sequences could...
be a result of codon usage bias of the various bacteria during evolution, which is due to a purifying selection governed by the relative abundance of the isoaccepting tRNA, as well as a biased mutation pressure due to the different GC contents, in the various bacteria (11, 18).

The cloning of groEL may have direct implications for laboratory diagnosis of B. pseudomallei infections. Since cystic fibrosis is very rare in southeast Asia, B. cepacia (whose GroEL showed 98% amino acid identity with that of B. pseudomallei) is not commonly found. Although cross-reactivity was shown in the sera of patients with P. aeruginosa and S. maltophilia bacteria, the intensities of the bands were much lower than that in the sera of patients with melioidosis. As clinical diagnosis of melioidosis is often difficult because most patients present with fever without localizing signs and the number of bacteria in clinical specimens obtained from nonbacteremic patients is often low and the organism is often misidentified, the presence of a high level of antibody response in the absence of bacteremia due to other organisms and specific clinical features may suggest the diagnosis of melioidosis. An ELISA using purified GroEL should be further evaluated in a prospective clinical study for the serodiagnosis of melioidosis. In such a study, more sera from patients with P. aeruginosa, S. maltophilia, other Burkholderia species, and B. pertussis infections should be included.

Besides laboratory diagnosis, the GroEL protein can be used for immunization in those patients at high risk of developing B. pseudomallei infections. Chaperonins are immunodominant proteins in microbial infections, and GroEL has been investigated for use in vaccination against bacterial infections such as tuberculosis, brucellosis, and yersiniosis (2, 16, 19). From our results, the GroEL of B. pseudomallei was further shown to be closely associated with humoral immunity. Since B. pseudomallei is acquired by inhalation of infectious droplets, immunization could be administered through the mucosal route to stimulate the production of secretory immunoglobulin A, which is closely associated with humoral immunity. Since B. pseudomallei is not commonly found. Although cross-reactivity was shown in the sera of patients with P. aeruginosa, S. maltophilia, other Burkholderia species, and B. pertussis infections should be included.

Nucleotide sequence accession number. The nucleotide sequence of the groEL gene of B. pseudomallei has been deposited with GenBank under accession no. AF287633.

This work was partly supported by the Committee of Research and Conference Grants, The University of Hong Kong.

REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, and K. Struhl (ed.). 1998. Current protocols in molecular biology, p. 2.4.1–2.4.2. John Wiley & Sons, Inc., New York, N. Y.

2. Bae, J. E., and T. E. Toth. 2000. Cloning and kinetics of expression of Brucella abortus heat shock proteins by baculovirus recombinants. Vet. Microbiol. 75:199–204.

3. Cao, L., C. M. Chan, C. Lee, S. S. Wong, and K. Y. Yuen. 1998. MPI encodes an abundant and highly antigenic cell wall mannoprotein in the pathogenic fungus Penicillium marneffei. Infect. Immun. 66:986–973.

4. Charoenwong, P., P. Lumbiganon, and S. Puupermponsiri. 1992. The prevalence of the indirect hemagglutination test for melioidosis in children in an endemic area. Southeast Asian J. Trop. Med. Public Health 23:698–701.

5. Charuchaimontri, C., Y. Suputtamongkol, C. Nilakul, W. Chaowagul, P. Chetchoitsakd, N. Leritapatnasuwan, S. Intaranongpai, P. J. Brett, and D. E. Woods. 1999. Antilipopolysaccharide II: an antibody protective against fatal melioidosis. Clin. Infect. Dis. 29:813–818.

6. Ellis, R. J. 1996. The chaperonins. Academic Press, San Diego, Calif.

7. Fayet, O., T. Ziegelhofer, and C. Georgopoulos. 1989. The GroES and GroEL heat shock gene products of Escherichia coli are essential for bacterial growth at all temperatures. J. Bacteriol. 171:1379–1385.

8. Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. Nature 381:571–579.

9. Horwich, A. L., K. B. Low, W. A. Fenton, I. N. Hirshfield, and K. Furtak. 1993. Folding in vivo of bacterial cytoplasmic proteins: role of GroEL. Cell 74:909–917.

10. Howe, C., A. Sampath, and M. Spotnitz. 1971. The pseudomallei group: a review. J. Infect. Dis. 24:598.

11. Ikemura, T. 1981. Correlation between the abundance of Escherichia coli transfer RNAs and the occurrence of the respective codons in its protein genes. J. Mol. Biol. 146:1–21.

12. Iliukhin, V. I., N. N. Kislichkin, L. K. Merinova, L. A. Riapis, I. I. Denisov, S. M. Farber, and O. I. Kislichkina. 1999. The outlook for the development of live vaccines for the prevention of melioidosis. Zh. Mikrobiol. Epidemiol. Immunobiol. 3:52–55.

13. Kanapluin, P., N. Thirawattanasak, Y. Suputtamongkol, P. Naigowit, D. A. Dance, M. D. Smith, and N. J. White. 1993. Serology and carriage of Pseudomonas pseudomallei: prospective study in 1000 hospitalized children in northeast Thailand. J. Infect. Dis. 167:230–233.

14. Leelarasamee, A., and S. Bovornkiti. 1989. Melioidosis: review and update. Rev. Infect. Dis. 11:413–425.

15. Mays, E. E., and E. A. Rickets. 1975. Melioidosis: recrudescence associated with bronchogenic carcinoma twenty-six years following initial geographic exposure. Chest 68:261.

16. Noll, A., and I. B. Autenrieth. 1998. Yersinia-hsp60-reactive T cells are efficiently stimulated by peptides of 12 and 13 amino acid residues in a MHC class II (1-Ab)-restricted manner. Clin. Exp. Immunol. 105:231–237.

17. Petkanjanapong, V., P. Naigowit, E. Kondo, and K. Kanai. 1992. Use of endotoxin antigens in enzyme-linked immunosorbent assay for the diagnosis of P. pseudomallei infections (melioidosis). Asian Pac. J. Allergy Immunol. 10:145–150.

18. Sueoka, N. 1982. On the genetic basis of variation and heterogeneity of DNA base composition. Proc. Natl. Acad. Sci. USA 48:582–592.

19. Tacon, R. E., M. J. Colston, S. Ragno, E. Stavropoulos, D. Gregory, and D. B. Lowrie. 1996. Vaccination against tuberculosis by DNA injection. Nat. Med. 2:888–892.

20. Trent, J. D., E. Nimmesgern, J. S. Wall, F. U. Hartl, and A. Horwich. 1991. A molecular chaperone from a thermophilic archaeabacterium is related to the eukaryotic protein-1 complex polypeptide-1. Nature 354:490–493.

21. Woo, P. C. Y., P. K. L. Leung, H. W. Tsui, and K. Y. Yuen. 2001. Cloning and characterization of malE in Burkholderia pseudomallei. J. Med. Microbiol. 50:330–338.

22. Yaffe, M. B., G. W. Farry, D. Miklos, A. L. Horwich, M. L. Sternlicht, and H. Sternlicht. 1992. TCP1 complex is a molecular chaperone in tubulin biogenesis. Nature 358:245–248.

23. Yap, E. H., Y. C. Chan, T. Y. Ti, T. W. Thong, A. L. Tan, M. Yeo, L. C. Ho, and M. Singh. 1991. Serodiagnosis of melioidosis in Singapore by the indirect haemagglutination test. Singapore Med. J. 32:211–213.

24. Zysk, G. W., D. Splettstoesser, and H. Neubauer. 2000. A review on melioidosis with special respect on molecular and immunological diagnostic techniques. Clin. Lab. 46:119–130.