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.

Melioidosis 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 melioidosis 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 antigen-based serological tests or vaccines are available for B. pseudomallei infections. Definitive diagnosis of melioidosis still depends on the isolation and identification of B. pseudomallei from blood, sputum, pus, swabs, and other clinical specimens. However, the number of B. pseudomallei organisms in clinical specimens collected from nonbacteremic patients is often low compared to those in infections caused by Staphylococcus aureus, enterobacteria, and anaerobes. Furthermore, laboratory identification is often delayed because many laboratory personnel are unfamiliar with the bacterium, and B. pseudomallei may be misclassified as “Pseudomonas sp.” (14). Therefore, serological tests have been investigated to show the antibody response of patients in the diagnosis of melioidosis. These include indirect hemagglutination assay (IHA), complement fixation assay, indirect immunofluorescent assay, and enzyme-linked immunosorbent assay (ELISA); IHA and ELISA have been the most commonly used methods (4, 17, 23, 24). However, IHA is observer biased, and a 16-fold variation in serological titer is not uncommon due to the variability of the lipopolysaccharide antigen coating on red cells. Recently, it has been shown that ELISA using lipopolysaccharide is superior to IHA in both sensitivity and specificity for the diagnosis of melioidosis (17). However, no antibody detection kit based on recombinant antigens of B. pseudomallei is commercially available at present. Antibody detection tests using recombinant antigens are easier to standardize and may offer higher sensitivity, specificity, and reproducibility. As for vaccine, although it has been suggested that flagellin protein, endotoxin-derived O-polysaccharide antigens, capsular polysaccharide, attenuated strains of B. pseudomallei, and recombinant culture of Francisella tularensis carrying a plasmid with fragments of B. pseudomallei chromosome may be good candidates (5, 12), none of these have been proved to be clinically useful for the prevention of melioidosis.

In this study, we report the cloning of the groEL gene, which encodes an immunogenic protein of B. pseudomallei. DNA sequence analysis reveals that the B. pseudomallei groEL gene has an open reading frame encoding 546 amino acid residues. Basic Local Alignment Search Tool (BLAST) analysis shows that the putative protein is highly homologous to the GroEL proteins of other bacteria. Finally, our results show that patients with melioidosis develop high levels of specific antibody against GroEL, suggesting that GroEL may represent a good target for the development of vaccines for melioidosis.

The B. pseudomallei strain used was isolated from the blood culture of a patient suffering from acute septicemic melioidosis in 1998. The bacterium was grown on blood agar plates at 37°C to obtain single bacterial colonies, which were then cultured in Trypticase soy broth at 37°C for 24 h. Total genomic DNA was obtained from 100 ml of culture of B. pseudomallei according to the standard protocol (1). The genomic DNA was partially digested with Sau3A (Boehringer
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 (Stratagene, 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 0.6 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 in SOLR cells, yielding pBK-CMV plasmids containing the inserts.

Overnight cultures of SOLR cells with pBK-CMV and SOLR cells with pBK-CMV-GroEL were induced with 0.1 mM isoprropyl-ß-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, Hercules, Calif.). The blot was incubated with sera from three patients with culture-documented melioidosis, two patients with GroEL (lanes 1 and 3) and SOLR cells with pBK-CMV-GroEL (lanes 2 and 4) electrophoresed 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

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'-GGATTCCTTATGATCCTA TGCCCATG-3', and LPW144, 5'-CCGGAGATCCGATGGC AGCTAAGACGT-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), deoxyribonucleoside triphosphates, and 1.0 U of Taq 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...
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*.
Stenotrophomonas maltophilia bacteremia, one patient with Acinetobacter baumannii 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 (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 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 isocoding 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 identify 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 important for its neutralizing and complement activation activities. Whether GroEL is a good T-cell target remains to be tested, as cellular immune response would be equally important for defense against melioidosis, especially in patients with the chronic granulomatous types of presentations that resemble tuberculosis.

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

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**REFERENCES**


