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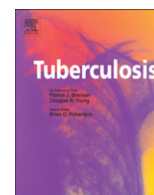

MECHANISMS OF PATHOGENESIS

Subtractive screening with the *Mycobacterium tuberculosis* surface protein phage display library

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SUMMARY

Surface proteins consist of secreted and membrane proteins and play a central role in the interaction of the pathogen with its environment, especially in the pathogenicity of *Mycobacterium tuberculosis* (MTB). Research on surface proteins in MTB has focused on 2D electrophoresis of culture filtrate proteins (CFP), extraction of transmembrane proteins with detergent and predicting their properties with a range of available algorithms. However, functional analysis of these secretomes is possible only if many proteins are expressed and purified individually, which limits a large number of studies to the function of the proteome. Here, we utilized a phage display system to construct a whole genomic surface protein phage display library of MTB, which can complete direct selection, identification, expression, purification and functional research of surface proteins of MTB. With this system we made a new serological approach involving iterative subtraction screening. Cross-reactivity of antibodies was reduced by preadsorption of the surface protein phage display library with the sera of healthy BCG-vaccinated individuals prior to studying their reactivity against the sera of tuberculosis (TB) patients. As a result six antigens were identified, three of which have not previously been reported as diagnosis antigens. The surface protein phage display library shows great promise in the study of MTB.

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1. Introduction

To this day, TB remains one of the leading causes of mortality. Additionally, about one-third of the world population has latent TB and 10% of them develop the disease during their lifetime.[1] Owing to the complex pathogenic mechanism of MTB, there have not been significant breakthroughs in the study of a vaccine and diagnosis of TB. Surface proteins consisting of secreted and transmembrane proteins play a central role in MTB pathogenesis, as they are used by the bacteria for nutrient uptake, adherence to host proteins and modulation of the host immune response.[2] Surface proteins also allow MTB to target and proliferate inside immune cells such as macrophages and dendritic cells.[3] Understanding the interaction between surface proteins of MTB and the host cell is therefore likely to help us better understand the pathogenesis of MTB.

The study of surface proteins of MTB has been limited to 2D electrophoresis of culture filtrate proteins (CFP), extraction of the transmembrane proteins with detergent and predicting their properties with a range of available algorithms.[4–7] They may miss many proteins of interest when expressed at low abundance.[8] Obtaining complete genome sequences of bacterial strains in order to identify their secretomes is inefficient because the secretome is typically comprises only 10–30% of the total number of the open reading frames, and the identified proteins are always well-known antigens that have been previously reported.[9] However, much of the immunological activities of surface proteins of MTB remain unknown. Functional analysis of these secretomes is possible only if every protein is expressed and purified individually, which is complicated by the fact that the secretome proteins are hard to express and purify.[10] All these limit a large-scale study on the function of the proteome. We need a new expression system with higher expression efficiency and also with convenience for functional analysis.

Phage display technology has been widely used because of its ability to link the genotype and the phenotype.[11] Filamentous phage virion proteins are themselves secreted proteins, translocated from cytoplasm via the Sec-dependent pathway and anchored in the cytoplasmic membrane prior to assembly into virions.[12] Therefore, the secreted proteins to be displayed would be

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targeted and folded in the cellular compartment in which they normally reside. Significant efforts have been made to improve the phage display system. Jankovic et al.[13] reported the construction of a new phage display system for displaying the surface proteins of Gram-Positive Bacteria, and this has provided a good platform for studying the surface proteins of MTB. In this study, we used this new system to construct a MTB surface protein phage display library with 840 clones. After sequence analysis of 200 randomly selected clones, there were 140 clones with signal sequence, comprising 47 unique ORFs predicted to encode secretome proteins, which was more efficient than other expression systems.[14] When extrapolated to the 840 clones, there are about 188 surface proteins, which is similar to the number of secreted proteins with signal peptides predicted by bioinformatic analysis.[15]

There will be many potential uses of this library, such as to study the reactivity of antibodies from TB patients to the surface proteins in our library. Also we can analyze the interactions between this surface proteins library and host cells, which can help in our understanding of the nature and function of the interaction between host cells and MTB surface proteins.[16]

Detection of circulating antibody in patients with active TB dates back to 1898.[17] Approximately 90% of TB patients produce antibodies against MTB proteins. Humoral responses in tuberculosis have been studied for several decades, primarily for the purpose of developing serodiagnostic assays. Consequently, numerous antigens have been identified, purified, and tested for serodiagnostic utility.[18] The sensitivity of assays that use these antigens have been reported to range from 16% to 80%, depending on the smear status of the patients and the patient populations studied.[19] The reactivity of sera from normal healthy individuals to antigens in MTB has been a major hindrance in the direct analysis of humoral immune responses in tuberculosis patients.[20] Furthermore, much attention has been focused on screening multiple antigens and multi-epitopes specific for MTB to develop novel serodiagnostic tools for TB.[21]

In our research, with this library, we took an iterative subtraction screening method toward serological study, the major objective of this strategy was to enrich for clones with high specificity to the sera from TB patients. We sought to subtract the surface proteins by first panning against sera from healthy subjects with a history of BCG vaccination to remove all nonspecific cross-reactive clones. This negative selection step was followed by a positive selection step to enrich clones that are specific to TB sera. After a multi-step panning procedure, we identified six antigens, of which three were previously reported and three others were identified for the first time as diagnostic antigens. Sera from infected individuals specifically recognized all six antigens.

Our study suggests that surface protein phage display library subtractive screening may be a powerful tool to identify candidate diagnosis antigens of MTB disease from the BCG vaccination subject. The raw MTB secretome phage display library pool, obtained after the selection step, could be screened using well-established phage display library screening protocols, and could reveal potentially useful information for the study of pathogenicty and diagnosis of TB, and for vaccine development.

2. Materials and methods

2.1. Bacterial strains, plasmid and helper phage

MTB H37Rv (ATCC27294) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China and was cultured in the Middlebrook 7H9 with ADC supplement. *Escherichia coli* (*E. coli*) strain TG1, *E. coli* strain K1976, helper phage VCSM13d3 and phagemid vector pDJ01 were kindly donated by Dr Dragana Jankovic (Massey University).

Active TB patients were diagnosed by skin test reactivity $\geq$10 mm, Sputum positive, as well as by clinical and radiological findings. Serum samples were collected from the Infectious Disease Hospital of Jilin Province, PR China. The highest relative risk of a positive PPD occurred among patients who received BCG vaccination after infancy and within 15 years of the PPD testing.[22] Therefore the BCG control subjects were children about 10 years old who had been vaccinated with bacillus Calmette–Guérin (BCG) around birth, with skin test reactivity about 5–9 mm, and healthy neonates without BCG vaccination who have no BCG antibody. We collected serum from these children at the Jilin Children's Hospital in Jilin Province of China. The children and their parents had not suffered from TB. The Hospital Institutional Review Board approved the study; the children's parents also gave consent.

2.2. Construction of the MTB whole genome library

We cultured the MTB in Middlebrook 7H9 with ADC supplement, and then extracted the genomic DNA by the alkaline lysis method.[23] The whole genome library was constructed from sonicated sheared MTB DNA and ligated into the phagemid vector pDJ01. The fragments obtained varied in size between 0.3 and 1.5 kb. Blunt phosphorylated ends were achieved by treatment with T4 DNA polymerase (NEB) and OptiKinase™ (Promega). Sepharose CL-4B 200 (Sigma) was used to eliminate fragments below 0.3 kb. The pDJ01 was digested with the SmaI enzyme (TAKARA) and dephosphorylated with shrimp alkaline phosphatase (Promega). Approximately 6 μg of the genomic fragments was ligated to 2 μg of the vector pDJ01 using T4 ligase (NEB). The ligated mixture was transformed into *E. coli* TG1 cell by electroporation and amplified overnight at 37 °C with aeration to construct the MTB whole genome library.

2.3. Direct selection of the surface protein phage display library

The construction of the surface protein phage display library was according to the method described by Jankovic as following.[13] A 1 ml aliquot of the overnight culture containing the whole genome library was used to inoculate 25 ml of 2xYT- Cm. The exponentially growing culture (OD600 $\approx$ 0.2) was infected with helper phage VCSM13d3 (MOI = 100) for 1 h and cells were then harvested by centrifugation at 3200$\times g$ for 10 min. Infected cells were incubated for 4 h at 37 °C with 2xYT-Cm-Kpn. The host cells were pelleted by centrifugation 10,000$\times g$ for 20 min and phagemid particles were collected in the supernatant. Extracted phagemid particles were precipitated using 5% (w/v) PEG, 0.5 M NaCl for 1 h at 4 °C, then centrifugation 10,000$\times g$ for 20 min and phagemid particles were collected in the precipitate and resuspended in TN buffer. PIII protein is required for the formation of the stabilizing cap structure at the terminus of the virion; hence, the broken-off pIII-deficient virions (proteins that have no signal sequence) are structurally unstable and are easily disassembled by sarcosyl, to which the pIII-containing virions are resistant.[24] To eliminate defective phagemid particles, the precipitate was treated with sarcosyl at a final concentration of 0.1% (w/v). The ssDNA released from defective phagemid particles was removed by DNase I (100 $\mu g\ ml^{-1}$) in the presence of 5 mM $MgCl_2$, and then inactivated by EDTA (20 mM). The remaining sarcosyl-resistant virions were the surface proteins phage display library of MTB. For sequence analysis of this library, the ssDNA was then extracted from sarcosyl-resistant virions by incubating phagemid particles at 70 °C for 10 min in the presence of 1.2% (w/v) SDS. Further purification of the ssDNA was carried out using a plasmid mini prep kit. To amplify the surface protein library, the ssDNA was then transformed in *E. coli* strain TG1 and poured to 2xYT-Cm plates incubated overnight at 37 °C.

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