รายงานวิจัยฉบับสมบูรณ์

โครงการ การเปลี่ยนแปลงโครงสร้างของไลโปพอลิแซ็กคาไรด์ ของเชื้อก่อโรคเมลิออยโดสิสในพยาธิวิทยากำเนิดของโรค

โดย นางสาว ดวงจิต กนิษฐานนท์

กันยายน 2548

สัญญาเลขที่ MRG4780203

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ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล

มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
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(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

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Project Title (ชื่อโครงการ): Surface remodeling of *Burkholderia pseudomallei* lipopolysaccharide: an implication in pathogenesis of infection

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Abstract

Burkholderia pseudomallei (Bps) is a Gram-negative bacterium causing melioidosis, a disease endemic in northeastern region of Thailand. Lipopolysaccharide (LPS) is a major component of bacterial cell wall and lipid A part is a bioactive part of LPS in stimulating host inflammatory response. Modification of lipid A structure can lead to an alteration in bacterial virulence and host response. This study aimed to determine lipid A profiles of virulent Bps compared to lipid A of *B. thailandensis* (Bth), an avirulent counterpart. In addition, lipid A profiles of bacteria grown in low and high magnesium conditions were determined. Bacteria were grown in LB media, or in N-minimal media supplemented with 8 μM (a condition mimicking intracellular condition), or 1 mM of $MgCl_2$. LPS was isolated from bacteria by hot phenol-water extraction or Tri-reagent extraction methods and was converted into lipid A by mild acid hydrolysis. Lipid A profiles were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in negative ion mode. There was no difference in lipid A patterns between Bps (strain K96243) and Bth (strain K95019). Lipid A profiles of bacteria grown in low or high Mg concentration were also similar, suggesting that bacteria may not alter their lipid A structure when they are inside the infected cells. However, strain S95019, although belongs to Bth species, was isolated from a patient and may not represent a truly avirulent bacterium. Therefore, more isolates of Bps were further included in the study. There seems to be a possible correlation between lipid A patterns and the sources of bacteria when lipid A from more strains were examined. In conclusion, Bps and Bth showed variety in lipid A profiles, which possibly correlate with the source of bacteria. Further study of more lipid A profiles from bacteria isolated from different sources, and their biological characteristics such as the ability to stimulate host immune response or the sensitivity to antimicrobial peptides may unravel the significance of lipid A structures in terms of bacterial virulence and pathogenesis.

Keywords: *Burkholderia pseudomallei*, *Burkholderia thailandensis*, melioidosis, lipopolysaccharide, lipid A

บทคัดย่อ

เชื้อแบคทีเรีย *Burkholderia pseudomallei* เป็นเชื้อชนิดกรัมลบที่ก่อโรคเมลิออยโดสิสซึ่งเป็นโรคที่พบมากในภาคตะวันออกเฉียงเหนือของประเทศไทย สารไลโปโพลิแซ็กคาไรด์เป็นโครงสร้างหลักของผนังเซลล์และมีส่วน ไลปิดเอ ที่เป็นส่วนที่สามารถกระตุ้นระบบภูมิคุ้มกันของร่างกายได้ การดัดแปลงโครงสร้างของไลปิดเอสามารถเปลี่ยนแปลงความรุนแรงในการก่อโรคของเชื้อและการตอบสนองทางระบบภูมิคุ้มกันของร่างกายได้ การศึกษานี้มุ่งจะดูแบบแผนของไลปิดเอที่แยกได้จากเชื้อก่อโรคเมลิออยโดสิส เปรียบเทียบกับไลปิดเอของเชื้อไม่ก่อโรค *Burkholderia thailandensis* นอกจากนี้จะเปรียบเทียบแบบแผนของไลปิดเอที่แยกได้จากเชื้อที่เลี้ยงในสารอาหารที่มีความเข้มข้นของแมกนีเซียมสูงและต่ำ ซึ่งเป็นการเลียนแบบสภาวะภายในเซลล์ที่ติดเชื้อ สารไลโปโพลิแซ็กคาไรด์ถูกแยกจากเซลล์โดยวิธี hot phenol-water extraction หรือ Tri-reagent extraction และทำการย่อยด้วยกรดอ่อนๆเพื่อให้ได้ส่วนของไลปิดเอ การดูแบบแผนของไลปิดเอทำโดยวิธี matrix-assisted laser desorption/ionization time-of-flight mass spectrometry จากการศึกษาเชื้อสองสายพันธุ์คือ K96243 ซึ่งเป็นตัวแทนของสายพันธุ์ก่อโรค และสายพันธุ์ S95019 ซึ่งเป็นตัวแทนของสายพันธุ์ไม่ก่อโรค พบว่าแบบแผนของไลปิดเอของสองสายพันธุ์นี้ไม่แตกต่างกัน รวมทั้งไม่พบความแตกต่างเมื่อเชื้อถูกเลี้ยงในความเข้มข้นของแมกนีเซียมต่างๆ เนื่องจากเชื้อสายพันธุ์ S95019 นั้นแม้จะถูกจำแนกให้เป็นเชื้อไม่ก่อโรคแต่ก็เป็นเชื้อที่แยกได้จากคนไข้ จึงอาจไม่ได้เป็นตัวแทนของเชื้อไม่ก่อโรคอย่างแท้จริง จึงได้ทำการศึกษาแบบแผนของไลปิดเอในเชื้อจำนวนมากขึ้น เมื่อได้ทำการศึกษาแบบแผนของไลปิดเอจากเชื้อที่แยกได้จากแหล่งต่างๆ ได้พบว่ามีแนวโน้มที่จะมีความสัมพันธ์ระหว่างแบบแผนของไลปิดเอกับแหล่งและสายพันธุ์ของเชื้อ และอาจส่งผลถึงความสามารถในการก่อโรคของเชื้อ การศึกษาเพิ่มเติมเกี่ยวกับแบบแผนของไลปิดเอ รวมถึงการศึกษาในส่วนของคุณสมบัติทางชีวภาพ เช่น การกระตุ้นระบบภูมิคุ้มกันของร่างกายและความไวต่อสาร antimicrobial peptides อาจแนะถึงความสำคัญของโครงสร้างของไลปิดเอกับความสามารถในการก่อโรคและการก่อพยาธิสภาพได้

คำหลัก: เชื้อก่อโรคเมลิออยโดสิส สารไลโปโพลิแซ็กคาไรด์ ไลปิดเอ

เนื้อหางานวิจัย

Introduction

Burkholderia pseudomallei (Bps) is a Gram-negative bacterium causing melioidosis in humans. Clinical spectrum of melioidosis ranges from asymptomatic infection, localized skin infection, pulmonary infection, disseminated infection or fatal septicemic infection following exposure to contaminated soil or water. The disease is endemic in northeastern Thailand and northern Australia (1-2). Patients with melioidosis often have underlying condition that compromises host immune system such as diabetes mellitus. This organism was classified as category B organism by Center of Disease Control and Prevention (CDC) (http://www.bt.cdc.gov/agent/agentlist.asp), and has a potential threat for biological terrorism. *Burkholderia thailandensis* (Bth) is a closely related bacterial species but is avirulent and found mainly in environment. Bth is classified as biosafety level 1 organism by ATCC.

Lipopolysaccharide (LPS) is a potent inducer of host inflammatory response due to its ability to stimulate host Toll-like receptor 4 (TLR4), or TLR2 in some bacteria, signaling system. Lipid A, a biological active component of LPS, is the part that interact with molecules host uses to detect the presence of LPS and then induce host inflammatory response. Modifications of LPS/lipid A by bacteria can lead to bacterial evasion of host immune system and are important for bacterial pathogenesis (3-4). It was previously shown that Bps LPS could stimulate host immune response by TLR2 pathway (5). However, an MD-2 molecule, a component associated with TLR4 and required for LPS recognition by TLR4 (6), was omitted in this study. Another study using both *in vitro* (cell culture) and *in vivo* (knock-out mice) models demonstrated that Bps LPS could also activate host inflammatory response through TLR4 system (7). Since the structure of LPS can affect the ability of the molecule to be recognized by TLR, therefore, it is interesting to extensively study the structure of lipid A of *B. pseudomallei* and *B. thailandensis* and see if there is any correlation in the biological activity of lipid A such as stimulation of host inflammatory response or the role in pathogenesis.

Materials and Methods

Bacterial strains

Bps strains K96243, K95025, K95027, K95028, K95030, and Bth strain S95019 were kindly provided by Professor Tararaj Dharakul (Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University). Bth strain E264 was provided by Dr. Robert K Ernst (University of Maryland-Baltimore).

LPS preparation and lipid A analysis

Bps and Bth were cultured in Luria Bertani (LB) media supplemented with 1 mM $MgCl_2$ at 37ºC for overnight. Hot phenol-water extraction was conducted for large scale LPS extraction (4). An overnight culture was centrifuged at 5,000 rpm for 10 min to collect bacterial cell pellet. Bacterial pellet was resuspended in deionized water and 90% phenol was added. The suspension was then incubated at 65ºC for 1 hr, cooled down on ice for 5 min and centrifuged at 3,000 rpm for 15 min at room temperature. Aqueous phase was collected and the extraction was repeated once. Pooled aqueous phase was dialyzed in large volume of deionized water at 4ºC for at least 24 hrs with frequent changes of water. After dialysis, the aqueous phase was centrifuged again to remove any precipitates, then frozen and lyophilized. After lyophilization, LPS was then digested with RNase A, DNase and proteinase K to remove any impurities. The digestion reaction was extracted once with water-saturated phenol and dialyzed in deionized water at 4ºC for another 24 hrs. After dialysis, LPS was frozen, lyophilized and stored at room temperature for further analysis.

For Tri-reagent extraction (small scale LPS extraction), approximately 10 ml culture was extracted with 1 ml of Tri-reagent solution (Molecular Research Center, Cincinnati, OH) and the mixture was incubated at room temperature for 15 min. Chloroform (200 ul) was added and the mixture was incubated for another 15 min before centrifugation. Aqueous phase was collected and the culture was re-extracted for three more times. Pooled aqueous phase containing LPS was frozen and

lyophilized. LPS from both extraction methods were converted into lipid A by mild acid hydrolysis in 1% sodium dodecyl sulfate at pH 4.5 for 1 hr at 100ºC. Lipid A was washed with acid ethanol (200 ul of 4 N HCl in 95% ethanol) once, then in 95% ethanol twice. The washing process was repeated for additional 2 rounds. The final product of lipid A was mixed with 5-chloro-2-mercaptobenzothiazole and analyzed on Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Incorporated).

Results

Lipid A profiles of Bps K96243 and Bth S95019 reference strains

LPS were isolated from K96243 and S95019 grown in LB medium at 37ºC. Lipid A were derived and analyzed by MALDI-TOF MS. There was no difference in patterns of lipid A between these two strains (Figure 1).

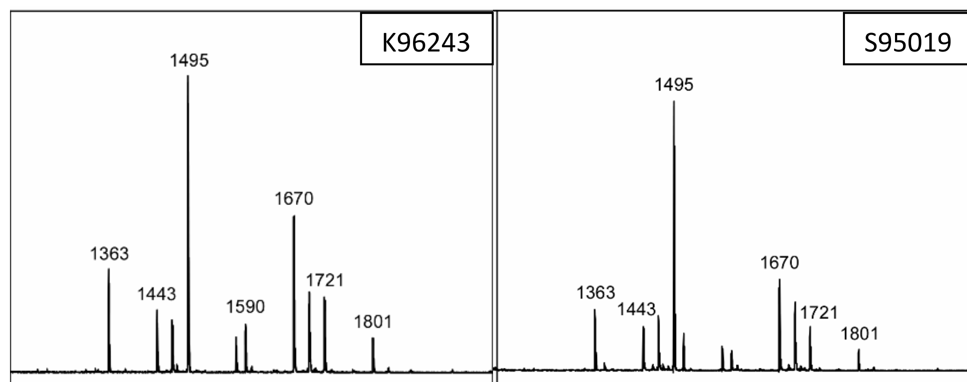

Figure 1 Lipid A spectra of K96243 (Bps) and S95019 (Bth)

Lipid A profiles of clinical isolates of Bps

LPS were isolated from more strains of Bps isolated from patients in Khon Kaen province and lipid A patterns were studied. Mass spectrum analysis demonstrated that lipid A profiles of Bps isolated from clinical specimens showed similar pattern (Figure 2).

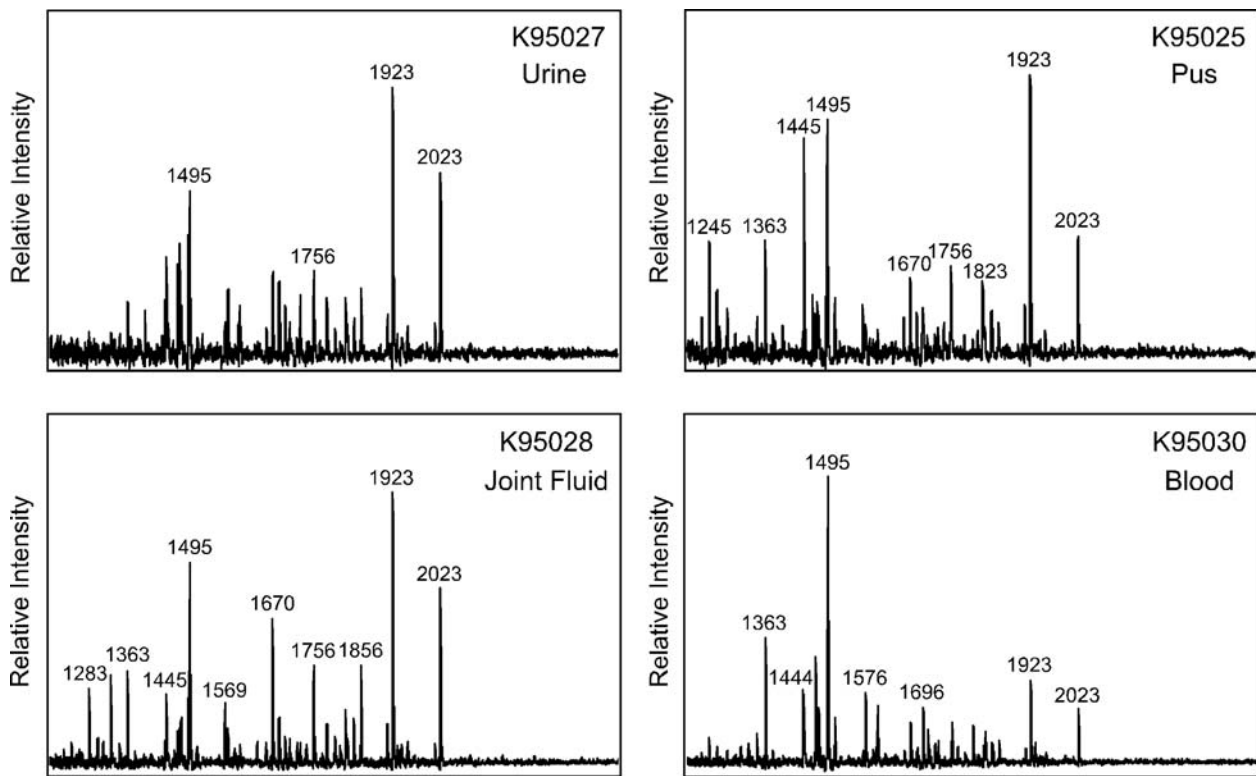

Figure 2 Lipid A spectra from clinical isolates of *B. pseudomallei*

Lipid A profile of Bth grown at different Mg concentration

Bth strain E264 were grown in N-minimal media supplemented with 8 uM (low Mg) and 1 mM (high Mg) and lipid A profiles were analyzed. There was no difference in lipid A patterns when bacteria were grown in different Mg concentrations (data not shown).

Discussion

Lipid A is an immunological active part of LPS that can stimulate pro-inflammatory response in host. Modification of lipid A such as acylation, deacylation, dephosphorylation, or carbohydrate modification will alter the way host senses and responds to bacteria (8). The lipid A structures of *B. caryophylli* and *B. cepacia* complex have been reported (9-11), but the exact chemical structure of *B. pseudomallei* has not yet been extensively identified. We preliminarily studied lipid A patterns of Bps and Bth isolated from clinical sources and found closely similar patterns of lipid A. However, our unpublished result on lipid A patterns isolated from limited number of environmental strains of Bps and Bth showed that the lipid A profiles of environmental Bps were, to some extent, much similar to those of clinical Bps, and were different from lipid A profiles of environmental Bth (unpublished data). This may be important for the ability of bacteria to adapt themselves to survive in environment, or to host environment in order to cause disease in host, or to evade host immune response. It has been demonstrated that *Pseudomonas aeroginosa* isolated from patients with cystic fibrosis adapted themselves to the environment in the lungs by modifications of lipid A, such as the addition of sugar (aminoarabinose) or palmitate (12). The cystic fibrosis-specific lipid A modifications led to bacterial resistance to host killing by antimicrobial peptides and increase in pro-inflammation stimulation. Therefore, it is interesting to study lipid A patterns in more strains of Bps and Bth isolated from different sources (clinical specimens from different sources or forms of disease, or from environment at different geographical regions). And if the correlation between lipid A pattern and source of bacteria can be established, it will be interesting to investigate the

biological properties of different lipid A patterns particularly in the potential effect in bacterial virulence/pathogenesis and alternation of host immune response.

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Non clonal diversity of Lipid A of *Burkholderia pseudomallei* and *Burkholderia thailandensis* isolated from clinical samples and environment in Thailand (manuscript in preparation)

Collaboration between investigator and researchers at University of Maryland-Baltimore and University of Washington Seattle

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A *Francisella* Mutant in Lipid A Carbohydrate Modification Elicits Protective Immunity

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