รายงานวิจัยฉบับสมบูรณ์

โครงการ การค้นหายีนที่จำเพาะในเชื้อเลปโตสไปราที่ก่อโรค โดยใช้วิธี

Representational Difference Analysis

โดย

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สัญญาเลขที่ MRG4580028

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Representational Difference Analysis

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สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

การวิจัยเรื่อง การค้นหายีนที่จำเพาะในเชื้อเลปโตสไปราที่ก่อโรค โดยใช้วิธี Represen tational Difference Analysis นี้สำเร็จลุล่วงไปได้ด้วยดีโดยความอนุเคราะห์และช่วยเหลือของ ศ. เกียรติคุณ ดร.สถิตย์ สิริสิงห และ รศ.ดร.ศันสนีย์ ไชยโรจน์ (นักวิจัยที่ปรึกษา ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล) ที่กรุณาให้คำแนะนำตลอดระยะเวลาการทำวิจัย การวิเคราะห์ผลการทดลอง รวมทั้งแนะนำเอกสารทางวิชาการ หนังสือ และวารสารต่างๆ ที่เป็นประโยชน์ต่อการวิจัย

ขอขอบคุณ Dr. Mathieu Picardeau (Institut Pasteur, Paris, France) ที่ให้ความอนุเคราะห์มอบเชื้อ *Leptospira biflexa* serovar patoc strain Patoc I และ The Wellcome Trust-Mahidol University Oxford Tropical Medicine Research Programme (Faculty of Tropical of Medicine Mahidol University) ที่ให้ความอนุเคราะห์มอบเชื้อ *Leptospira interrogans* serovar pyrogenes strain L784 *Leptospira interrogans* serovar autumnalis strain L116 และ *L. interrogans* serovar autumnalis strain L453 ที่แยกได้จากผู้ป่วยโรคเลปโตสไปโรซิส เพื่อใช้ในการวิจัยครั้งนี้

ขอขอบคุณ ศ. ดร. ศกรณ์ มงคลสุข (ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล / ห้องปฏิบัติการเทคโนโลยีชีวภาพ สถาบันวิจัยจุฬาภรณ์) ที่ให้ความอนุเคราะห์ในงานด้าน DNA sequencing ซึ่งมีความสำคัญในงานวิจัยนี้

การวิจัยครั้งนี้ได้รับการสนับสนุนจาก คณบดีคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และได้รับการสนับสนุนเงินทุนวิจัยจาก ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ จากสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) ทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) รวมถึงทุนสนับสนุนอาจารย์ใหม่จากคณะวิทยาศาสตร์ มหาวิยาลัยมหิดล และ จากภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ซึ่งเป็นปัจจัยสำคัญที่ทำให้การวิจัยนี้สามารถดำเนินการและบรรลุความสำเร็จลงได้

บทคัดย่อ

รหัสโครงการ : MRG4580028

ชื่อโครงการ : การค้นหายีนที่จำเพาะในเชื้อเลปโตสไปราที่ก่อโรคโดยวิธี Representational difference analysis

ชื่อนักวิจัยหลัก : ผดุงศรี วิชิวานิเวศน์

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ระยะเวลาโครงการ : 2 ปี 10 เดือน

เชื้อแบคทีเรียพวกสไปโรชีตที่อยู่ใน genus *Leptospira* ประกอบด้วย species ที่ก่อโรค คือ *Leptospira interrogans* (*L. interrogans*) และ species ที่ไม่ก่อโรค คือ *Leptospira* เชื้อเลปโตสไปราที่ก่อโรคเป็นต้นเหตุของโรคเลปโตสไปโรสิส (Leptospirosis) หรือโรคฉี่หนู ซึ่งเป็นโรคที่ติดต่อจากสัตว์สู่คน (zoonosis) ที่พบได้ทั่วโลก สำหรับในประเทศไทย มีรายงานผู้ป่วยโรคเลปโตสไปโรสิสประมาณ 5,000 รายต่อปี อาการของผู้ป่วยที่ติดเชื้อเลปโตสไปรามีทั้งแบบที่ไม่มีอาการรุนแรงเป็นคล้ายไข้หวัด จนถึงแบบที่มีอาการรุนแรงถึงขั้นเสียชีวิตได้ ในการศึกษาครั้งนี้ได้นำเทคนิคการทำ Representational difference analysis เข้ามาใช้ในการค้นหายีนที่จำเพาะต่อเชื้อ *L. interrogans* serovar autumnalis strain L453 ซึ่งเป็นเชื้อที่แยกได้จากผู้ป่วยโรคเลปโตสไปโรสิสที่มีอาการรุนแรง โดยการทำ subtractive hybridization ระหว่างโครโมโซมของเชื้อ L453 กับ *L. biflexa* serovar patoc สายพันธุ์ Patoc I ผลการศึกษาครั้งนี้ได้สร้าง library ของ subtractive clone ที่มี insert ของ RDA product อยู่จำนวน 65 clone ในการนี้จากการทำ Southern blot hybridization analysis พบว่า insert DNA fragments ของ 5 subtractive clone ที่เลือกนำมาทำการทดสอบเป็น DNA fragment ที่จำเพาะในสายพันธุ์ L453 และไม่พบใน *L. biflexa* ผลการทำ DNA sequence analysis พบว่า fragment ที่ได้มี sequence similarity กับ putative lipoprotein, the outer membrane proteins ใน Rhs family protein, methyl malonyl-CoA mutase, acyl-CoA thioesterase และ putative propionyl-CoA carboxylase ของ *L. interrogans* serovar copenhageni และ serovar lai สำหรับ putative lipoprotein และ outer membrane protein ใน Rhs family protein เป็นโปรตีนที่อาจจะเกี่ยวข้องในการก่อโรค ส่วน methyl malonyl-CoA mutase, acyl-CoA thioesterase และ putative propionyl-CoA carboxylase เป็นเอนไซม์ที่เกี่ยวข้องใน lipid metabolism สรุปได้ว่าเทคนิค RDA สามารถนำมาใช้ในการ identify ยีนที่จำเพาะในเชื้อเลปโตสไปราสายพันธุ์หนึ่งที่ไม่ปรากฏอยู่ในเชื้ออีกสายพันธุ์ได้ การศึกษาขั้นต่อไปถึงบทบาทของโปรตีนเหล่านี้ อาจทำให้ได้องค์ความรู้ใหม่ที่จะช่วยเพิ่มความรู้ ความเข้าใจในเรื่องของกลไกการทำให้เกิดโรคเลปโตสไปโรสิสอันจะเป็นประโยชน์ต่อการป้องกัน หรือรักษาโรคได้ในอนาคต

คำหลัก: RDA, *Leptospira*, subtractive hybridization

ABSTRACT

Project Code : MRG4580028

Project Title : Identification of genomic differences in pathogenic and non-pathogenic *Leptospira* using representational difference analysis

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Project Period : 2 years 10 months

Spirochetes in the genus *Leptospira* are divided into two species, the pathogenic *Leptospira interrogans* (*L. interrogans*) and the free-living nonpathogenic *L. biflexa*. Pathogenic leptospires are the causative agent of leptospirosis, a worldwide zoonotic disease that is now regarded as one of the major emerging infectious diseases. In Thailand, there are about 5,000 cases of leptospirosis per year. Clinical manifestations of leptospirosis are varied ranging from a mild flu-like illness to a severe syndrome of multi-organ infection with a high mortality rate. In this study, the technique of representational difference analysis (RDA) has been employed to identify genomic differences between *L. interrogans* serovar autumnalis strain L453, isolated from a Thai patient with severe leptospirosis, and the non virulent strain, *L. biflexa* serovar patoc strain Patoc I. DNA sequences present only in the virulent strain, L453, were achieved through subtractive hybridization of genomic DNA of L453 with *L. biflexa*. A library of 65 subtractive clones containing the amplified difference products was obtained. Southern blot hybridization analyses, using the insert difference products from 5 randomly chosen clones as a probe, indicated that all inserts are unique to L453. DNA sequence analysis of those inserts showed sequence similarities to a putative lipoprotein, the outer membrane protein encoded by the *rhs* gene family, methyl malonyl-CoA mutase, acyl-CoA thioesterase and putative propionyl-CoA carboxylase of *L. interrogans* serovars copenhageni and lai. The putative lipoprotein and the outer membrane protein encoded by the *rhs* gene family are considered as potential virulence factors, whereas methyl malonyl-CoA mutase, acyl-CoA thioesterase and putative propionyl-CoA carboxylase are involved in lipid metabolism. Therefore, RDA proved effective for the identification of genomic regions unique to *L. interrogans* strain L453. Functional analysis of these gene products may provide an insight to the differences in pathogenicity in the L453 strain that causes severe disease.

Key words: RDA, *Leptospira*, subtractive hybridization

เนื้อหางานวิจัย

BACKGROUND AND SIGNIFICANCE

Spirochetes are a group of helically shaped bacteria that include both free-living and parasitic species, some of which are pathogenic to humans and animals. These organisms are classified in the Class *Spirochaetes* in the order *Spirochaetales* which contains the families; *Spirochaetaceae*, *Serpulinaceae* and *Leptospiraceae* (42). The family *Spirochaetaceae* contains two genera of medical importance; *Borrelia*, the causative agent of Lyme disease/relapsing fever and *Treponema*, the causative agent of syphilis (26). The family of *Leptospiraceae* is divided into three genera: *Leptospira, Leptonema* and *Turneria* (formerly *L. parva*) (14). *Leptospira* is the only member of this family that causes disease. Leptospirosis is a ubiquitous zoonosis of global importance. The genus *Leptospira* is serologically classified into two species, the pathogenic *L. interrogans* and the saprophytic *L. biflexa*. Both species are further divided into serovars based on results of cross-agglutinin adsorption tests (CAAT). Antigenically related serovars are grouped into serogroups. Presently, more than 60 serovars of *L. biflexa* have been found and over 200 serovars comprising 24 serogroups of *L. interrogans* have been recorded (4). Recently, a molecular approach has been used to classify *Leptospira* based on DNA relatedness that has identified 16 genomospecies (excluding *L. parva*): *L. interrogans*, *L. santarosai*, *L. borgpetersenii*, *L. kirschneri, L. noguchii, L. weilii, L. inadai*, *L. fainei*, *L. biflexa, L. meyeri, L. alexanderi, L. wolbachii*, and genomospecies 1, 3, 4, 5 (7). While the genotypic classification is taxonomically consistent, it is incompatible with the older system of serogroups and serovars that has been used for many years (31). Therefore, strains of *Leptospira* are still generally referred to by serovar.

The severity of leptospirosis ranges from subclinical illness to a severe, potentially fatal syndrome of multi-organ involvement resulting in clinical manifestations such as jaundice, renal failure and hemorrhage. The incubation period is usually about 5-14 days (ranges from 2-26 days) (55). The clinical presentation is biphasic with an acute, septicemic or leptospiremic phase lasting about a week, followed by an immune or leptospiruric phase that is characterized by the production of antibodies and the excretion of leptospires in the urine. During the acute phase, leptospires can be recovered from blood and cerebrospinal fluid (CSF). The immune phase is usually accompanied by the disappearance of leptospires in the blood (31). There are two

forms of the disease: anicteric and icteric leptospirosis. Icteric leptospirosis, also known as Weil's disease, is a severe form that occurs in about 5 -10% of leptospirosis patients and is characterized by jaundice that is not associated with hapatocellular necrosis and azotaemia. Severely jaundiced patients exhibit renal failure, haemorrhages and cardiovascular collapse that contribute to a 5-15 % mortality rate (31, 55). The more common form, anicteric leptospirosis, is a self-limited febrile illness that is found in about 90% of cases of *Leptospira* infection. The illness usually starts after an average incubation period of 10 days with headache, fever and severe muscular pain, particularly in the thighs, calves, lumbosacral region and abdomen (55). In some studies, it has been reported that the majority of leptospirosis patients display conjunctival suffusion (31). Acute leptospirosis in animals is manifested by listlessness, loss of appetite, ruffled fur, red eyes, fever and signs of potential hemorrhage and jaundice. Infection in domestic livestock can lead to abortion, stillbirth, infertility, decreased milk production, and death (14).

Leptospirosis occurs worldwide in both developed and developing countries. The infection is more common in the tropics than in temperate regions because the environmental conditions are more favorable for transmission (4). An outbreak of leptospirosis is usually associated with flooding or a recreational event which increases the possibility of human exposure to leptospire-contaminated water and soil (46). For example, two outbreaks in 2000 were associated with heavy flooding in Thailand and the EcoChallenge Sabah 2000 competition in Borneo, Malaysia (31). In the United States, 74 of 639 athletes were infected with *Leptospira* in the 1998 Wisconsin and Illinois triathlons (38). In Thailand, according to the preliminary report from Center of Epidemiological Information, Bureau of Epidemiology, ministry of Public Health in 2004, there were at least 3,096 reported cases of leptospirosis and 47 patients died from the disease. A variety of wild, peridomestic and domestic animals such as rodents, livestock and dogs can become reservoir hosts of the infection. Leptospires may persist and proliferate in immunologically privileged organs of animals with chronic infections in regions such as the brain, the anterior chamber of the eye, the genital tract and, particularly, the proximal renal tubule. Renal carriers may shed the organisms in urine for their lifetime without symptoms. Humans are accidental hosts in the transmission cycle. Transmission to humans is usually through either direct or indirect contact with infected animals, contaminated urine or urine-contaminated water or moist soil (13). Excreted leptospires can survive as free-living organisms for months in a moist and

relatively neutral to alkaline (pH 6.2 -8) environment at temperatures exceeding 22°C. The organisms cannot survive in dry and acidic environments. It is believed that the organisms enter through abrasions or breaches of the surface integument such as those that can occur during prolonged immersion of the skin in water. The organisms are also able to enter through the mucosal surfaces of the eye, mouth and nasopharynx (14). Human infection is mostly acquired via occupational and recreational exposures. Occupational risk groups include butchers, veterinarians, rice field workers, sugar cane cutters, dairy-farmers, miners, sewer workers, soldiers. Recreational exposures are associated with wading, white water rafting, canoeing, swimming and kayaking (31).

Leptospires are thin, tightly coiled, highly motile, chemoheterotrophic, Gram-negative bacteria with pointed ends, one or both of which are usually bent or hooked (Fig. 1, Arrows). The size of leptospires is approximately 0.1 μm in diameter and 6-20 μm in length and the organisms can pass through 0.2 μm pore-size filters (55). Dark-field or phase-contrast microscopy is required for direct visualization of unstained leptospires because the organisms stain poorly. The organisms utilize long-chain fatty alcohols as carbon and energy sources and ammonium salts for their nitrogen requirement. They cannot use carbohydrates or amino acids as major sources of energy (27). Leptospires are obligately aerobic bacteria with an optimum growth temperature between 28 and 30°C.

Spirochetes have a typical double membrane structure consisting of a cytoplasmic membrane and a cell wall. The cytoplasmic membrane of spirochetes is closely associated with the peptidoglycan layer, followed by the periplasmic space located between the peptidoglycan-cytoplasmic membrane complex and the outer membrane sheath (Fig. 2). Unlike the outer membrane of other Gram-negative bacteria, the outer membrane sheath of spirochetes appears to be fluid and labile (18). Leptospiral lipopolysaccharide (LPS) has similar constituents to that of other gram-negative bacteria. However, it was shown that LPS extracted from *L. interrogans* serovar canicola had lower endotoxic activity than that extracted from *E. coli* (25). In addition, leptospiral LPS activates macrophages via CD14 and the Toll-like receptor 2 (TLR2) rather than TLR4, a central component for recognition of Gram-negative LPS (56). Leptospires possess two periplasmic flagella or axial filaments that reside in the periplasmic space (Fig. 2). Each flagellum is attached subterminally and extends toward the center of the cell without overlapping (27). Rotation of the two periplasmic flagella in opposite directions (i.e. clockwise and counter-clockwise) allows the cell to move in a

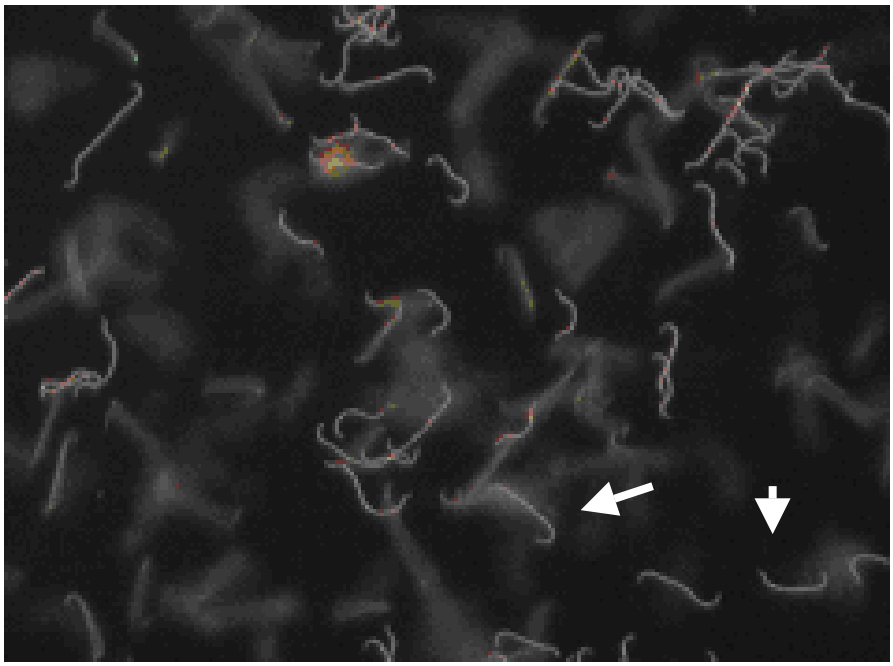


Fig. 1. Photomigraph of leptospires under dark field microscope. Arrows indicate ends of the cells which which are usually bent or hooked.

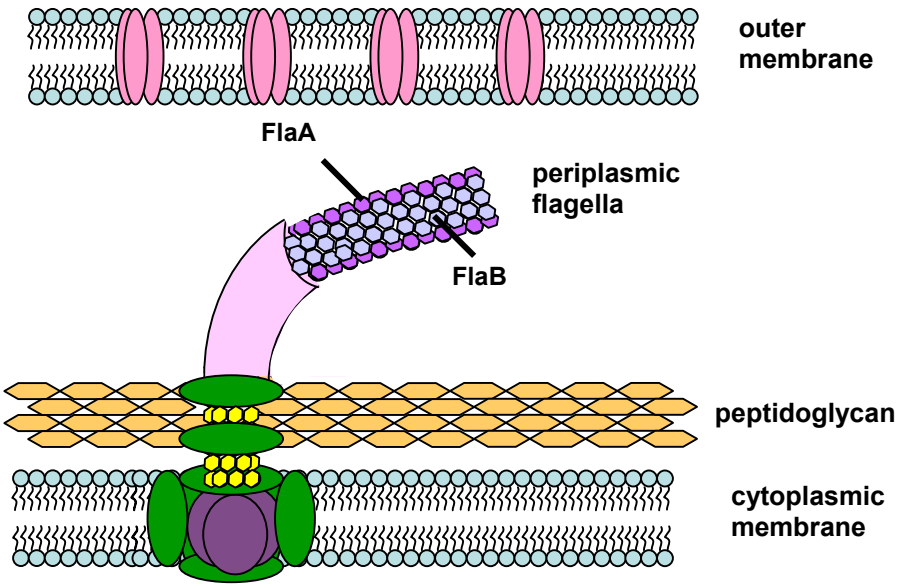


Fig. 2. The structure of periplasmic flagella of spirochetes.

run mode. Non-translational motility occurs when both flagella rotate in the same direction (32).

The leptospiral genome is much larger than the 1.1 and 1.5 Mb genomes of *Treponema pallidum* and *Borrelia burgdorferi*, respectively. This reflects the ability of leptospires to live both as free living organisms and as pathogens inside animal hosts (4). The genome consists of two circular chromosomes; the 4.4 Mb CI and the 350 kb CII replicons (61). The CII chromosome contain some essential genes required for growth and viability such as *metF*, encoding methylene tetrahydrofolate reductase that is involved in methionine biosynthesis (6), *asd*, encoding aspartate β-semialdehyde dehydrogenase that is involved in amino acid and cell wall syntheses and *ndh*, encoding NADH dehydrogenase and clusters of genes involved in the *de novo* biosynthesis of haem (47). Leptospires do not possess any linear or circular plasmids (61). Insertion elements such as IS*1500*, IS*1501*, IS*1502* and IS*1503* were found to be distributed throughout the genomes of *L. interrogans* serovars lai and copenhageni (41). Many genes encoding proteins with structural similarity to eukaryal and archaeal proteins, such as *paf*AH (encoding the mammalian platelet-activating factor acetyl hydrolase), *vwa* (encoding von Williebrand factor type A domains) and *pon* (encoding paraoxonase), have also been identified in the genomes of both serovars (47). This suggested the possibility of lateral gene transfer as one contributing factor in the evolution process involved in the emergence of pathogenic leptospires (47).

The existence of genetic exchange mechanisms between *Leptospira* have not been demonstrated and genetic tools for this organism have only recently been developed (60). In *L. biflexa* serovar patoc strain Patoc I it was shown that the LE1 temperate bacteriophage can infect and replicate in the prophage state as a circular plasmid (48). This has led to the construction of a *L. biflexa-Escherichia coli* (*E. coli*) shuttle vector using the origin of replication of the LE1 bacteriophage and the kanamycin resistance gene from *Enterococcus faecalis* (48). Subsequent gene replacement techniques have been established in the saprophytic strains *L. biflexa* and *L. meyeri* (3, 44) and just recently, pathogenic *L. interrogans* mutants have been generated by random insertional mutagenesis using the *Himar 1 mariner* transposon (5). The existance of these new techniques for genetic analysis in *Leptospira* provides a basis for future molecular studies of the physiology and pathogenic mechanisms in this organism. Since leptospires are facultative parasites that can live as free-living organisms for months prior to infection of mammalian hosts, the organisms must have

the ability to regulate gene expression in response to changes in the environment. To date, it is not known how leptospires regulate gene expression in response to environmental cues. Whole genome analysis of *L. interrogans* serovars lai and copenhageni revealed that both serovars possess sigma-70, sigma-28, sigma-54 homologs, as well as, 11 extra cytoplasmic function (ECF) sigma factors, 9 anti-sigma factors and 14 putative two-component sensor histidine kinase-response regulator pairs that probably act to control gene expression. Previous studies showed that leptospiral outer membrane proteins (OMPs); LipL32 (19), LipL41 (2), LipL45 (36), the porin OmpL1 (2) and the immunoglobulin-like repeat proteins, LigA and LigB (35) were expressed during mammalian infection, whereas the OMP, LipL36 was downregulated (2, 21). Moreover, it has been shown that many OMPs such as Q1p42 (39), LipL36 (40), pL50 (10), pL24 (10) were regulated in response to temperature, iron availability or growth phase.

Most pathogenic bacteria use virulence factors such as adhesins, invasins, protein secretion systems and/or toxins to facilitate bacterial entry, colonization and survival in host cells. Genes encoding these virulence factors may be located in a particular region within a prokaryotic genome called pathogenicity islands (PAIs). A PAI comprises a large genomic region of 10-200 kilobases present only in the genome of pathogenic strains but absent from the genome of non-pathogenic members of the same or related species (22). Many Gram negative and Gram positive pathogens, such as *Shigella*, *Yersinia*, *Salmonella*, *Listeria ivanovii* and *Staphylococcus aureus*, possess PAIs on their genomes or plasmids. However, PAIs are not found in a number of pathogens including *Mycobacterium*, *Chlamydia*, and spirochetes (49).

Pathogenic mechanisms of leptospirosis are thought to be the results of direct effects by the organism in conjunction with host immune response to infection. Up until now, little is known about pathogenesis of this disease. Motility and the ability of leptospires to swim through viscous media may be crucial for initial infection and dissemination of spirochetes from the entry site to sites of end-organ damage such as lungs, liver, kidneys, eyes, and brain (4). It has been shown that virulent *Leptospira* strains rather than avirulent strains, exhibited chemotaxis towards haemoglobin (59). Analysis of genome sequence of *L. interrogans* serovar lai showed at least 50 of the 4768 predicted genes related to motility and found 12 methyl-accepting chemotaxis proteins (47). Leptospires possess other potential virulence factors aside from motility and chemotaxis that may facilitate the migration of the organism through host tissues.

Leptospiral invasion may be mediated by secretion of enzymes that degrade host cell membrane such as sphingomyelinase C (*sphA*) (50), and haemolysin (*sphH*) (29). These enzymes were shown to be cytotoxic pore-forming proteins on several mammalian cells (30). Several leptospiral OMPs and lipoproteins such as LipL21 (11), LipL32 (19) have also been suggested as potentially significant factors that play a significant role in pathogenesis. Comparative genomic analysis of *L. interrogans* serovar lai and copenhageni revealed two potential afimbrial adhesins that may play a role in host cell attachment and invasion (41). The first candidate, consisting of three paralogous genes (*ligA*, *ligB*, and *ligC*), contains a bacterial immunoglobulin-like (Big) domain similar to that of *E. coli* intimin and *Yersinia pseudotuberculosis* invasin. It was shown that *ligA* and *ligB* were expressed only in low-passage, virulent strains but not in culture attenuated, high passage strains of *L. interrogans* and *L. kirschneri* (34). Another candidate is a group of putative integrin alpha-like proteins, LIC12259, LIC10021, and LIC13101 in *L. interrogans* serovar copenhageni and LA1499, LA0022, and LA388 in serovar lai (41).

The mechanisms by which pathogenic leptospires penetrate, disseminate, persist and cause disease in the host are still unclear. Many leptospiral factors are thought to be involved in these processes. Previous studies showed that genes encoding some of the prospective virulence factors, including *sphH* (29) and *lipL21* (11), are present only in pathogenic leptospires but absent in nonpathogenic strains. Thus, it is of interest to identify genomic differences between pathogenic and nonpathogenic leptospires. This would allow the exploration of novel genes that may play essential roles in pathogenic mechanisms of leptospirosis such as genes involved in the survival of pathogenic leptospires in the host and the escape mechanisms from host immune response. Further studies of the functions of these novel gene products may lead to a better understanding of the pathogenesis of this infectious disease.

To differentiate between two closely related genomes, several techniques currently exist. One such technique is representational difference analysis (RDA), a PCR-based subtractive hybridization, recently developed for identification of genomic DNA present only in one genome but absent from another. This technique, originally described by Lisitsyn *et al.* (33) in 1993, was adapted for comparison of bacterial genomic DNA by Tinsley and Nassif in 1996 (53). In RDA, the genomic DNA sequences sought out are called “testers” and the genomic DNA sequences subtracted from the testers are referred to as “drivers”. Subtractive hybridization is carried out in a reaction

containing excess amount of the driver acting as a competitor for self-reannealing of the tester. Only tester DNA fragments are ligated to 5'- nonphosphorylated 24-base oligonucleotide and 12-base oligonucleotide adaptor pair. A short 12-base oligonucleotide is not covalently attached to the tester DNA fragment and dissociates from the 24-base oligonucleotide primer during temperature elevation. After subtractive hybridization, selective amplification of unique tester homoduplexes is achieved through addition of the 24-base oligonucleotide primer, thereby, enriching for sequences unique to the tester DNA (Fig. 3). This method proved successful in the analysis of genetic differences between the two closely related pathogenic *Neisseria*; *N. meningitidis* and *N. gonorrhoeae.* The results of the RDA analysis showed the identification of three distinct chromosomal regions found only in *N. meningitidis* (53). Later, this method was used to identify DNA specific for pathogenic *Neisseria* species as compared to commensal *N. lactamica* (43). Furthermore, RDA exposed genomic differences between *Vibrio cholerae* O1 and O139 serogroups and between the O1 classical and O1 El-Tor biotypes (8). Another recent application of RDA identified genomic differences between *Pseudomonas aeruginosa* strains PA14 and PAO1 (9). The results of this study revealed that *pilC, pilA,* and *uvrD* in strain PA14 differed from their counterparts in strain PAO1 and a homolog of *Yersinia ybtQ* was identified in strain PA14 but not in strain PAO1. Thus, this technique proved effective for the identification of genomic regions unique to virulent strains. The RDA technique requires only a small quantity of genomic DNA and is relatively rapid and simple to perform. In this study, the technique of RDA will be employed to identify genomic differences between *L. interrogans* serovar autumnalis strain L453, isolated from Thai patient with severe leptospirosis and the saprophytic strain, *L. biflexa* serovar patoc strain Patoc I. Libraries of DNA sequences present only in the virulent strain are achieved through the subtractive hybridization of the chromosomal DNA of the virulent strain with that of the free living non-pathogenic strain. We hope that some DNA sequences in the libraries may contain sequences that are responsible for the differences in pathogenicity in the strain that causes severe disease, *Leptospira interrogans* serovar autumnalis.

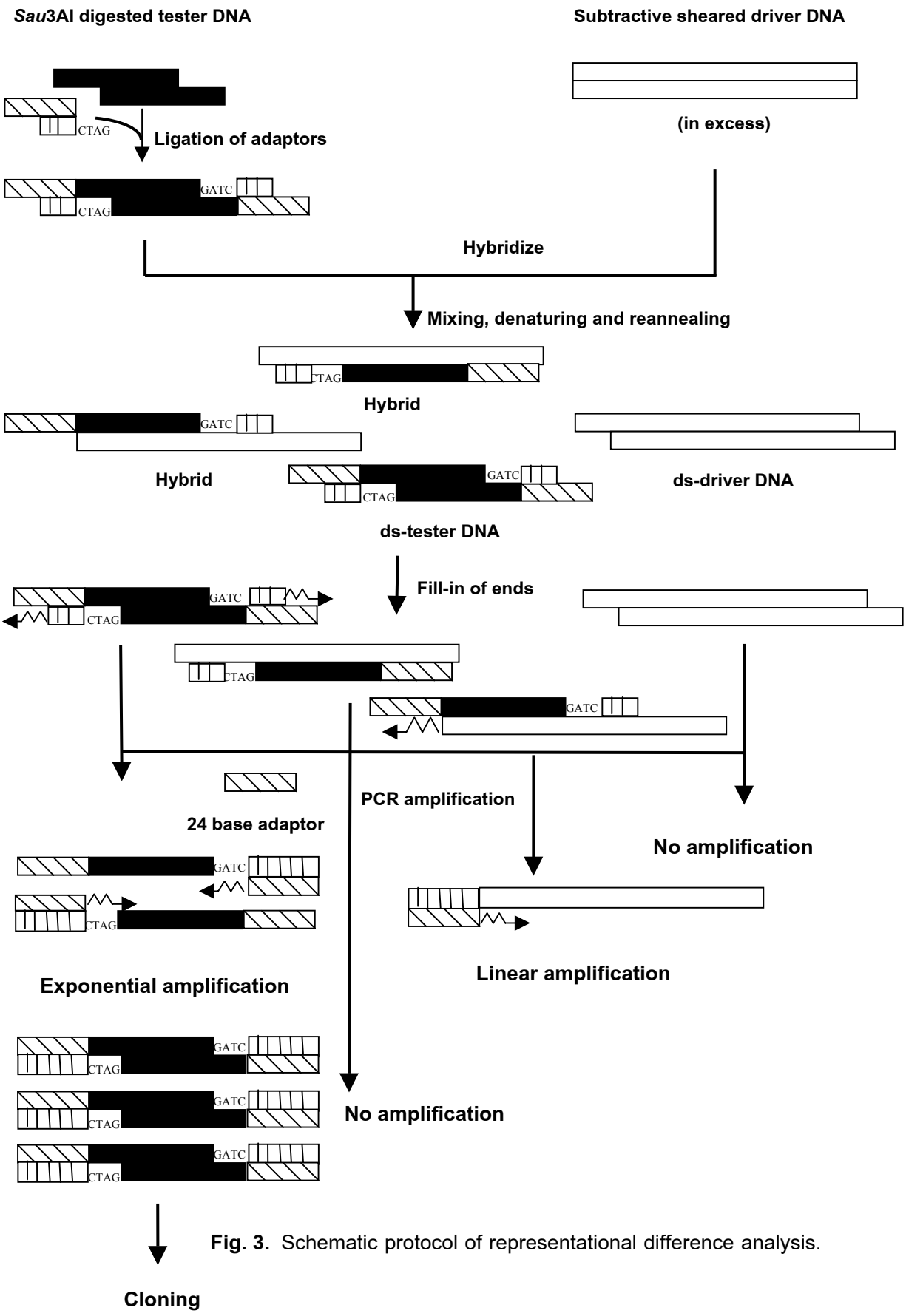


Fig. 3. Schematic protocol of representational difference analysis.

MATERIALS AND METHODS

Bacterial strains and plasmids

Pathogenic leptospires used in the RDA experiment were obtained as a gift from the Wellcome Trust Research Unit, Faculty of Tropical Medicine, Mahidol University. *L. interrogans* serovar autumnalis strain L453 and L116 were isolated from Thai patients with severe and mild symptoms of leptospirosis, respectively. *L. interrogans* serovar pyrogenes strain L784 was also a clinical isolate from Thai patient. The saprophytic *L. biflexa* serovar patoc strain Patoc I and *L. meyeri* serovar semaranga strain Veldrat were kindly provided by Dr. Mathieu Picardeau at Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, Paris, France.

Escherichia coli (*E. coli*) strain JM109 (58) was used as a host for transforming recombinant plasmids.

pDrive vector (Qiagen, Hilden, Germany) was used to clone PCR fragments.

Media and growth conditions

Leptospires were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) base medium (Difco, Sparks, MD, USA) supplemented with 1% BSA and 1.25% tween 80 (28) under aerobic conditions at 30^{o}C. For pathogenic strain, 2% rabbit serum will be added to the EMJH medium. Cultures were checked for the presence of contaminating bacteria after 7 days, prior to subculturing or chromosomal DNA extraction.

E. coli was grown on Luria-Bertani (LB) medium at 37^{o}C. Antibiotic concentrations were 100 μg/ml for ampicillin and 50 μg/ml for kanamycin.

DNA manipulations

Standard protocols were used for routine DNA techniques such as plasmid preparation, restriction enzyme digestion, DNA ligation, agarose gel electrophoresis and bacterial transformation (1).

Chromosomal DNA extraction

Chromosomal DNA extraction was performed according to a protocol described by Grimberg et al. (17). Approximately 100 ml cultures of pathogenic and saprophytic leptospires grown at late log phase was centrifuged at 12,000 × g for 30

minutes at 4ºC. The cell pellet was washed once with 1 ml TNE solution (10 mM Tris pH 8, 10mM NaCl, 10 mM EDTA) and transferred to a 1.5 ml eppendorf tube. After centrifugation, the pellet was resuspended in 270 μl TNE solution (10 mM Tris, pH 8, 10 mM NaCl, 10 mM EDTA), prior to the addition of 1% sodium dodecyl sulfate. Then, 30 μl of 5 mg/ml lysozyme and 10 μl of 10 mg/ml RNase A were added to the cell lysate and the mixture was incubated at 37°C for 30 minutes. After the addition of 12 μl of 25mg/ml proteinase K, the mixture was further incubated at 65°C for 3 hours. The mixture was extracted once with an equal volume of phenol equilibrated with Tris buffer pH 8, twice with phenol-chloroform, and twice with chloroform. The mixture was then ethanol precipitated and centrifuged for 10 minutes at 16,000 × g. The DNA pellet was redissolved in an appropriate volume of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

Representational difference analysis

Representational difference analysis was performed according to a protocol described by Choi et al. (9) and Perrin et al. (43) with modifications (Fig. 3).

- Tester DNA preparation

In this study, the tester DNA was the genomic DNA of *L. interrogans* serovar autumnalis strain L453. Approximately 3 μg of the tester DNA was partially digested with 0.1 U of *Sau3*AI at 37°C for 45 minutes and the *Sau3*AI (Peomega, Inc., Madison, WI, USA) was inactivated at 65°C for 15 minutes. Fresh *Sau3*AI (0.1 U) was then added to the digestion reaction and the reaction was then incubated at 37°C for additional 15 minutes. The *Sau3*AI digested tester DNA was ligated with 2-5 nmol of either the JSau adaptor pair (JSau12 and JSau24) or the RSau adaptor pair (RSau12 and RSau24) at 16°C for 18 hours.

The sequences of the two oligonucleotide adaptor pairs were:

JSau adaptor pair: JSau12……5'-**GATC**TGTTCATG-3'

JSau24……3'-ACAAGTACCTATCAGCTGCAGCCA-5'

RSau adaptor pair: RSau12…….5'-**GATC**TGCGGTGA-3'

RSau24……....3'-ACGCCACTCTCCGACCTCTCACGA-5'

The unincorporated adaptor pair was removed from the ligation reaction using PCR purification kit (Qiagen, Hilden, Germany). Prior to the ligation, the JSau12 and JSau24 primers or the RSau12 and RSau24 primers were annealed by heating at 95°C and slow cooling to 4°C.

- Driver DNA preparation

The subtracting driver genomic DNA of *L. biflexa* serovar patoc strain Patoc I was sheared by repeated passage through a 29x1/2 gauge hypodermic needle to give fragments ranging from about 3 to 10 kb. The sheared DNA fragments were then phenol purified and ethanol precipitated.

- Subtractive hybridization and kinetic enrichment

Three rounds of subtraction and kinetic enrichment were performed. In the first round of subtractive hybridization, a 100-fold excess of sheared *L. biflexa* DNA fragments (~20-30 μg) was added into 400 ng of *Sau3*AI digested, Jsau adaptor-linked L453 DNA fragments. The DNA mixture was ethanol precipitated and redissolved in 8 μl of EE buffer (10 mM *N*-[2-hydroxyethyl] piperazine-*N'*-[3-propanesulfonic acid], 1mM EDTA [pH 8.0]). The DNA mixture was overlayered with 30 μl of mineral oil to prevent evaporation and the DNA was denatured by boiling for 5 minutes. The DNA mixture was put in the 45°C water bath and 2 μl of 5 M NaCl was subsequently added into the mixture. Subtractive hybridization was carried out at 50°C for 24 hours. After 24 hours incubation, the reaction mixture was diluted 10-fold with 50°C preheated EE buffer-NaCl and immediately placed on ice. A portion of the diluted subtraction mixture (10 μl) was added to 40 μl of PCR reaction mix in the presence of 4 mM $MgCl_2$, 0.125 mM of each dNTPs, 2.5 U of *Taq* polymerase (Peomega, Inc., Madison, WI, USA). The PCR reaction mix was incubated at 72°C for 10 minutes to fill in the complementary strand to the ligated 24-base primer (JSau24). Kinetic enrichment was achieved through PCR amplification of the filled-in reaction mixture in the presence of the appropriate 24-base primer (JSau24). The amplification reaction contained 10 μl of the filled-in reaction mixture, 2 mM $MgCl_2$, 0.5 mM of each dNTPs, 6 μM of JSau24 primer and 2.5 U of *Taq* polymerase. After denaturation for 5 minutes at 95°C, the mixture was amplified for 10 cycles of 1 minute at 95°C and 3 minutes at 72°C, followed by a final extension at 72°C for 10 minutes. Single-stranded DNA molecules present after 10 cycles of amplification was digested with 2 U of mung bean nuclease (Peomega, Inc., Madison, WI, USA) at 37 °C for 30 minutes. After inactivation of the enzyme by heating at 95°C for 5 minutes, the reaction mixture was further amplified for 30 cycles under the same conditions described above. The amplified DNA fragments were analyzed on a 2% agarose gel. The JSau adaptors were cleaved from the first-round difference products with *Sau*3AI and the *Sau*3AI digested products were purified using PCR purification kit in order to remove the cleaved JSau adaptors. The purified *Sau*3AI digested difference products

from the first round subtraction were then ligated with 2 nmol a different adaptor pair, RSau12 and RSau24. After the un-ligated RSau adaptor pair was removed using PCR purification kit, the Rsau adaptor-linked difference products from the first round subtraction were used as the tester DNA in the second round of subtractive hybridization. The third round of subtractive hybridization was carried out using the Jsau adaptor-linked difference products from the second round subtraction as the tester DNA. Finally, the third-round difference products were cloned into pDrive U-A cloning vector and transformed into *E. coli* JM109 competent cells by electroporation. Cells containing the insert DNA fragments were screened on LB agar plates containing 20 μg/ml X-gal, 0.1 mM IPTG and 50 μg/ml kanamycin. The insert difference products were released from the pDrive vector by digestion with *Eco*RI.

Southern-blot hybridization

Prior to agarose gel electrophoresis, 3 μg of leptospiral chromosomal DNA was digested with an appropriate restriction enzyme. The digested chromosomal DNA was then transferred to Hybond-N^+ *Leptospira* genomic DNA will be subjected to electrophores on 0.8% agarose gels and capillary transferred onto Hybond-N^+ membranes (Amersham, Pisscataway, NJ, USA) using the alkaline blotting technique described by Ausubel et al. (1). Pool difference products from the third round subtraction and the difference products from the selected clones of transformants were labeled with [α-^{32}P] dCTP using Rediprime II DNA labeling kit (Amersham, Pisscataway, NJ, USA) and used as probes for Southern blots. Hybridizations were carried out according to a protocol described by a manufacturer (Amersham, Pisscataway, NJ, USA). The blots were prehybridized in a hybridization buffer [5x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS] for 2 hours at 45^{o}C, prior to the addition of the probe. The hybridization reaction was carried out for overnight at 45^{o}C. The blots were washed at 50^{o}C for 5 minutes twice with 2x SSC, 0.1% SDS, for 10 minutes twice with 1x SSC, 0.1% SDS, for 5 minutes twice with 0.2x SSC, 0.1% SDS, and finally for 5 minutes twice with 0.1x SSC, 0.1% SDS.

DNA sequencing and analysis

DNA sequences of the difference products from the selected colonies of transformants that are unique to the tester DNA were determined using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with

either M13 forward or M13 reverse primers. The sequences were analyzed for homologies with previously-published sequences in the database of GenBank using the BLASTN and BLASTX programs from the National Center for Biotechnology Information (NCBI).

RESULTS

Construction of a library of DNA fragments of *L. interrogans* serovar autumnalis strain L453 not found in the genome of *L. biflexa* serovar patoc strain Patoc I.

The genomic DNA of *L. interrogans* serovar autumnalis strain L453 (driver) was partially digested with 0.1 U of *Sau3*AI at 37°C for 45 mintues (Fig. 4, lane 1). After heat inactivation of the enzyme, the chromosome was redigested with additional 0.1 U of *Sau3*AI for 15 minutes (Fig. 4, lane 2). After ethanol precipitation, approximately 1 μg of the *Sau3*AI digested tester DNA (Fig. 5, lane 1) was ligated with 5 nmol of the JSau adaptor pair (JSau12 and JSau24). The unincorporated adaptor pair was removed from the *Sau3*AI digested tester DNA prior to subtractive hybridization with an excess of sheared chromosomal DNA fragments from *L. biflexa* serovar patoc strain Patoc I (Fig. 5, lane 2). Three rounds of subtractive hybridization followed by PCR amplification in the presence of the 24-base primer were performed to kinetically-enriched the L453 DNA fragments absent from or having no significant homology with the *L. biflexa* DNA fragments. The amplified difference products from the first, second and third round of subtraction were between 150 and 350 bp (Fig. 6, lanes 1-3). During the third round subtraction/PCR amplification, the amplified difference products were not obtained, if either the Jsau24 primer (Fig. 6, lane 4) or the template (Fig. 6, lane 5) was omitted from the PCR reactions.

To confirm that the amplified difference products from the third round of subtraction were specific to L453 chromosomal DNA, the pool of third-round difference products were used to probe the genomic DNA of *L. interrogans* strain L453 and *L. biflexa*. The hybridization temperature was carried out at 45°C with low stringency wash conditions. The results from Southern blot analysis showed that the probe was exclusively hybridized to the *Hin*dIII digested L453 genomic DNA (Fig. 7, lane 2) but not the *Hin*dIII digested genomic DNA of *L. biflexa* (Fig. 7, lane 1). This result suggested that the third-round difference products were specific to the tester strain. The pool of third-round difference products were then cloned into pDrive and a library of 65 subtractive clones, denoted PA 1-65, were obtained. Sizes of the insert fragments were in the range of 100-200 bp. The *Eco*RI digested DNA fragments of the plasmids isolated from representative clones PA 13-36 were shown in Fig. 8.

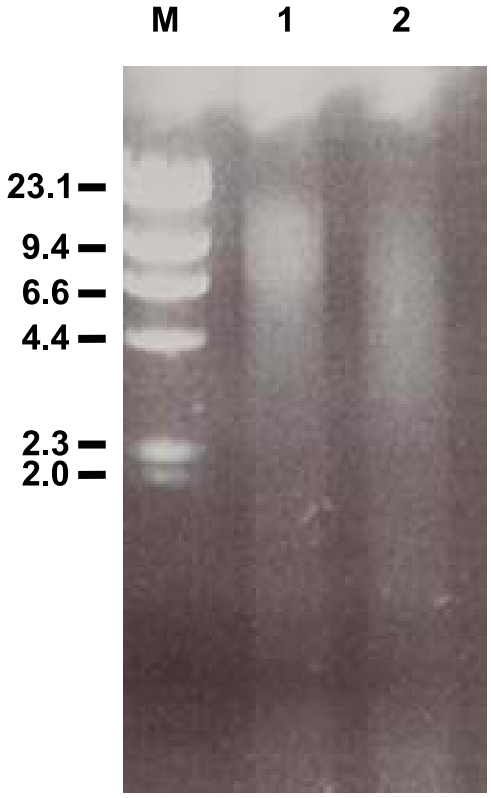


Fig. 4. Agarose gel electrophoresis (1% gel) of *Sau*3AI partially digested chromosomal DNA of *L. interrogans* serovar autumnalis strain L453. The L453 chromosomal DNA was partially digested with 0.1 U *Sau*3AI at 37°C for 45 minutes (lane 1) and for additional 15 minutes with fresh *Sau*3AI (0.1 U) (lane 2). Lane M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the left.

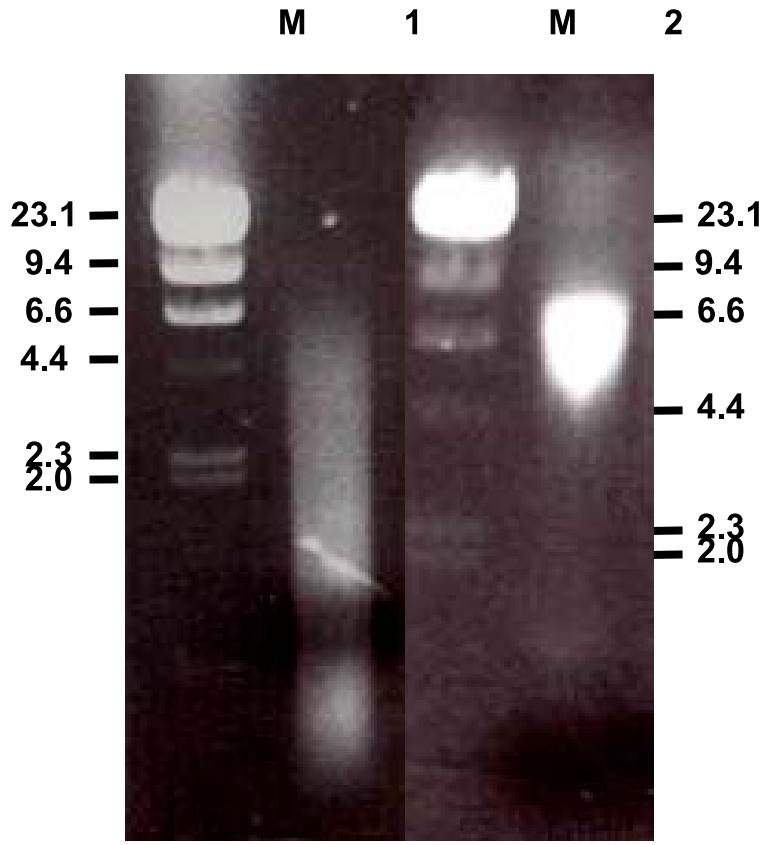


Fig. 5. Agarose gel electrophoresis (1% gel) of *Sau*3AI partially digested chromosomal DNA of *L. interrogans* serovar autumnalis strain L453 (lane 1) and sheared chromosomal DNA of *L. biflexa* (lane 2). Lanes M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated on both sides.

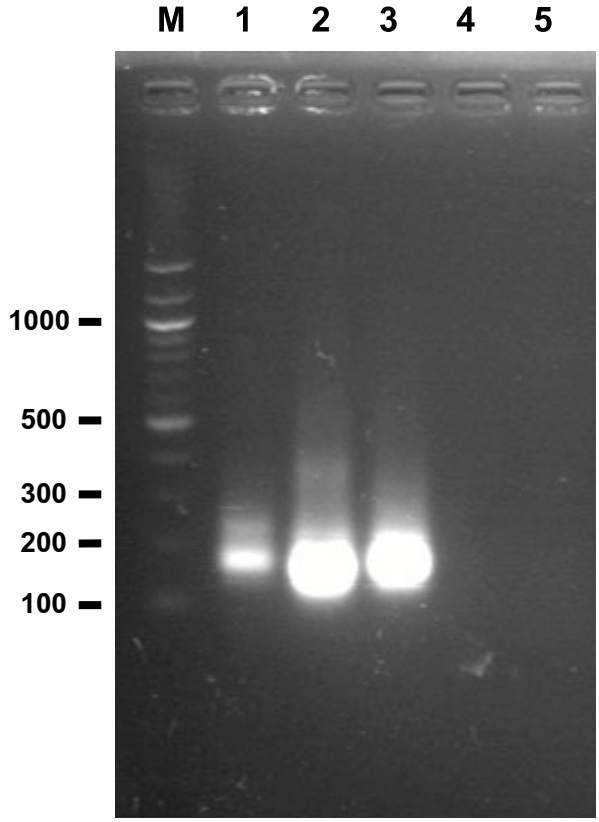


Fig. 6. Agarose gel electrophoresis (2% gel) of amplified difference products from subtractive hybridization/PCR amplification between chromosomal DNA of *L. interrogans* serovar autumnalis strain L453 (tester) and *L. biflexa* (driver). The amplified difference products after the first-round (lane 1), second-round (lane 2) and third-round (lane 3) of subtraction are shown. Lanes 4 and 5 contained 18 μl of the PCR amplification reactions in the absence of the JSau24 primer and the template, respectively. Lane M contained 100 bp DNA ladder. Sizes (bp) are indicated to the left.

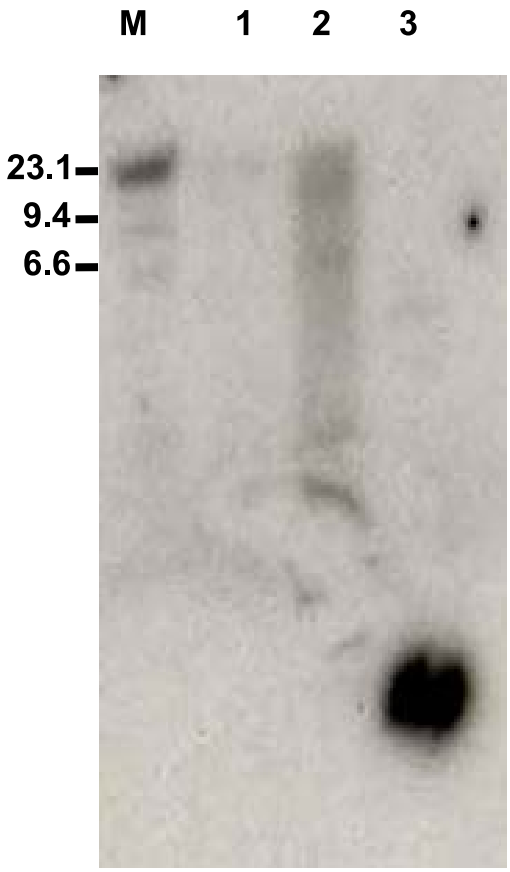


Fig. 7. Southern blot hybridization analysis using the pool of third-round difference products to probe the *Hin*dIII-digested chromosomal DNA of *L. biflexa* (lane 1) and *L. interrogans* strain L453 (lane 2). Each lane contained 1 μg of the chromosomal DNA. Lane 3 contained pool of the third-round difference products (positive control). Lane M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the left.

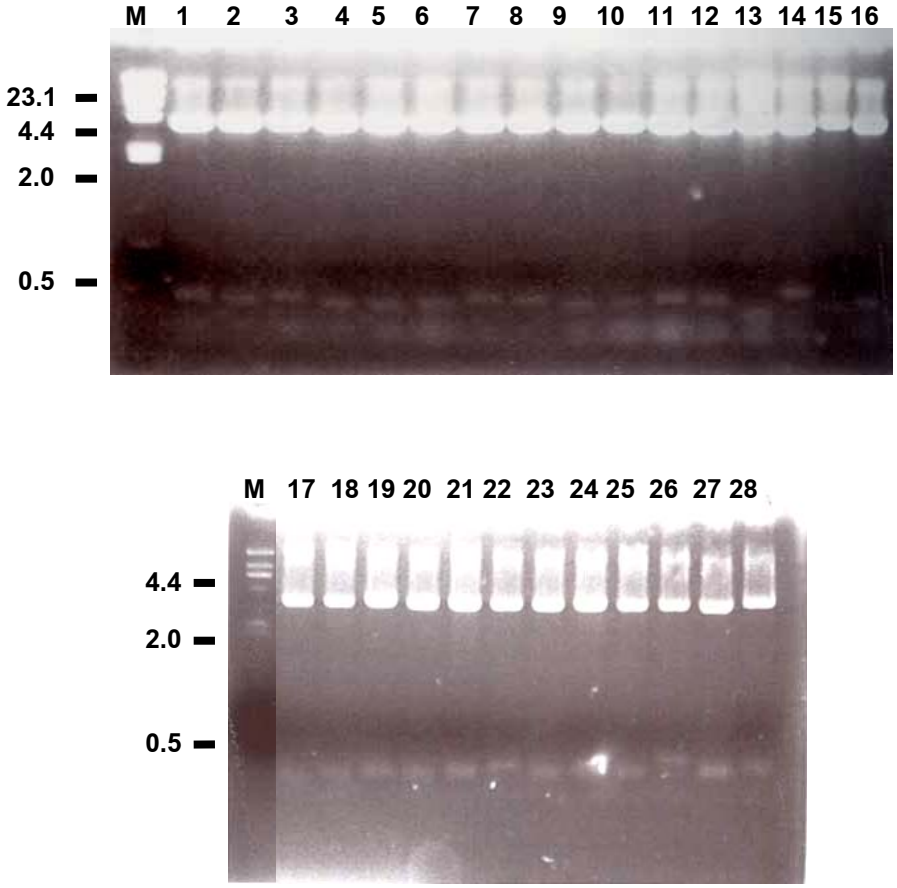


Fig. 8. Agarose gel electrophoresis (1.2% gel) of the *Eco*RI digested DNA fragments of the plasmids isolated from representative clones PA13-36 (lane 1-28). Lanes M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the left.

DNA sequence analysis of the randomly selected third round difference products.

The *Eco*RI insert DNA fragments from the representative clones PA11, PA12, PA19, PA21 and PA24 were used to generate a probe for Southern blot hybridization analysis. The results showed that all inserts were present L453 and absent in *L. biflexa* (Fig. 9, 10, 11, 13, compare lanes 2 and 1; Fig. 12, compare lanes 5 and 3). Moreover, a strong band of approximately 9.4 kb was also observed in the lanes containing the *Hin*dIII digested genomic DNA from *L. interrogans* serovar autumnalis strain L116 and *L. interrogans* serovar pyrogenes strain L784 hybridized with the *Eco*RI insert from PA21 (Fig. 12, lanes 4 and 1, respectively). There was little or no signal in the lanes containing genomic DNA from non-pathogenic leptospires, *L. meyeri* and *L. biflexa* (Fig. 12, lanes 2 and 3, respectively). These results suggested that PA21 was specific not only to L453 but also to the other pathogenic *Leptospira* strains, L116 and L784. Alignment of the nucleotide and deduced amino acid sequences from clones PA11, PA12, PA19, PA21 and PA24 revealed high sequence similarities to a putative lipoprotein (Fig. 14), the outer membrane proteins encoded by the *rhs* gene family (Fig. 15), methyl malonyl-CoA mutase (Fig. 16), acyl-CoA thioesterase (Fig. 17) and putative propionyl-CoA carboxylase (Fig. 18) of *L. interrogans* serovars copenhageni and lai, respectively.

Southern blot hybridization analysis has not yet been performed to verify whether clones PA32, PA33, PA36, PA38, PA40 and PA43 were specific to L453. DNA sequence analysis of these clones revealed that PA32 and PA43 had nucleotide and predicted amino acid sequence similarities to DNA/pantothenate metabolism flavoprotein of *L. interrogans* serovars copenhageni and lai (Fig. 19). The nucleotide and amino acid sequences of PA36 and PA40 were similar to the ribosomal protein S3 of *L. interrogans* serovars copenhageni and lai (Fig. 20). The nucleotide and deduced amino acid sequences of PA33 and PA38 showed sequence similarities to ATP synthase F_1 α subunit (Fig. 21) and RNA polymerase β subunit (Fig. 22) of *L. interrogans* serovars copenhageni and lai. DNA sequences of PA24, PA35, PA39 and PA41 were similar (Fig. 18). The results from BLASTX search were summarized in Table 1.

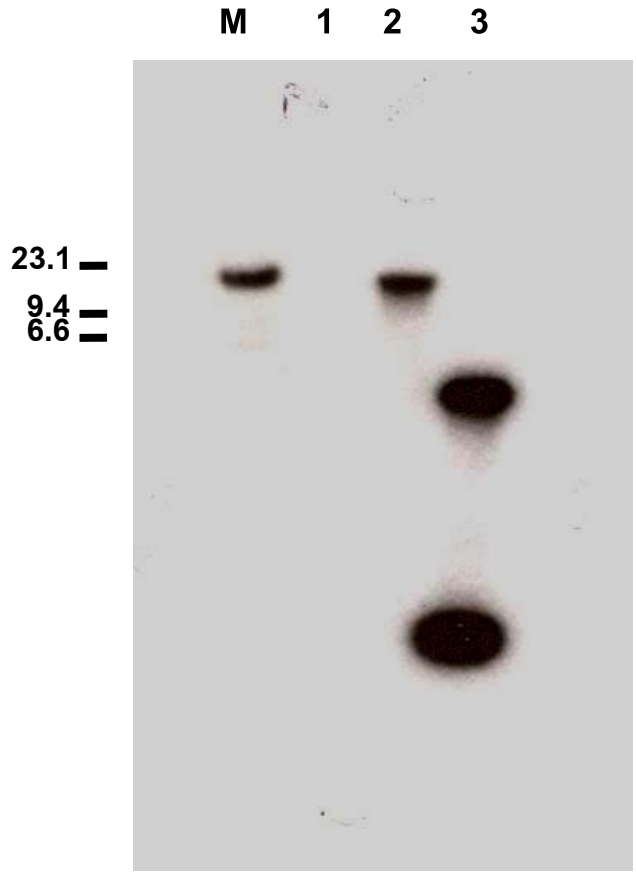


Fig. 9. Southern blot hybridization analysis using the *Eco*RI insert fragment of the plasmid from PA11 to probe the *Hin*dIII-digested chromosomal DNA of *L. biflexa* (lane 1) and *L. interrogans* strain L453 (lane 2). Each lane contained 1 μg of the chromosomal DNA. Lane 3 contained the *Eco*RI digested fragments of the plasmid from PA11 (positive control). Lane M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the left.

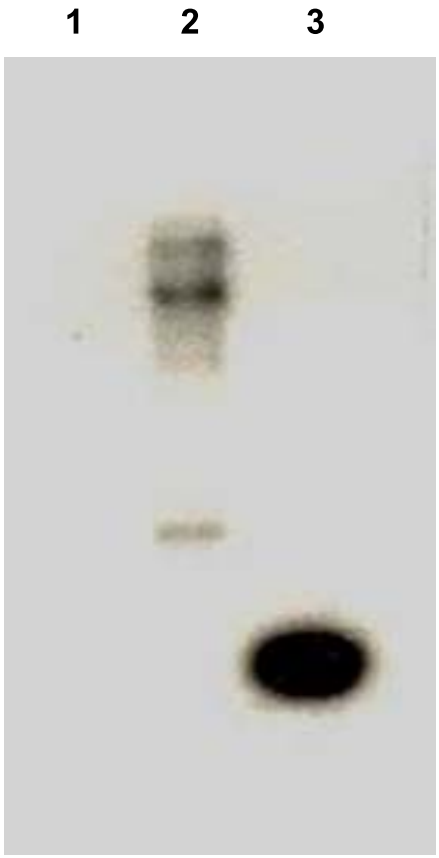


Fig. 10. Southern blot hybridization analysis using the *Eco*RI insert fragment of the plasmid from PA12 to probe the *Hin*dIII-digested chromosomal DNA of *L. biflexa* (lane 1) and *L. interrogans* strain L453 (lane 2). Each lane contained 1 μg of the chromosomal DNA. Lane 3 contained pool of the third-round difference products (positive control).

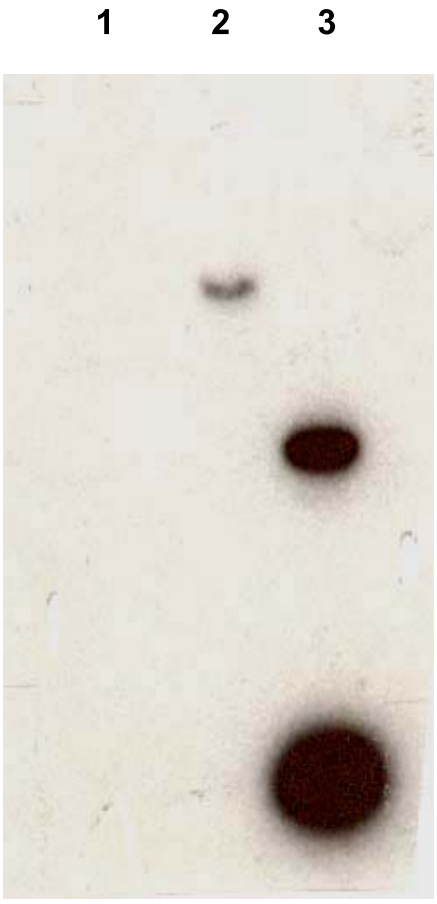


Fig. 11. Southern blot hybridization analysis using the *Eco*RI insert fragment of the plasmid from PA19 to probe the *Hin*dIII-digested chromosomal DNA of *L. biflexa* (lane 1) and *L. interrogans* strain L453 (lane 2). Each lane contained 1 μg of the chromosomal DNA. Lane 3 contained the *Eco*RI digested fragments of the plasmid from PA19 (positive control).

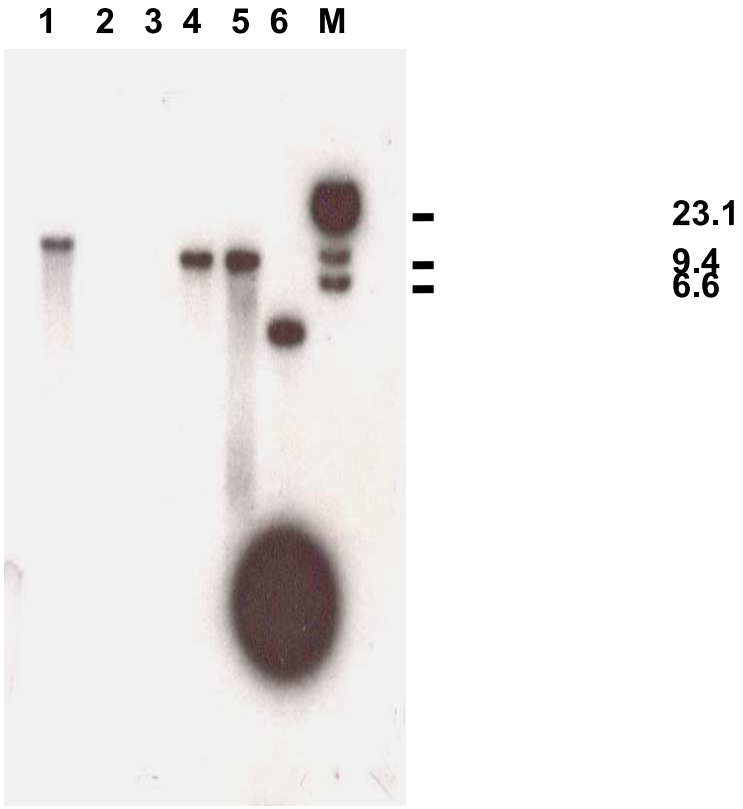


Fig. 12. Southern blot hybridization analysis using the *Eco*RI insert fragment of the plasmid from PA21 to probe the *Hin*dIII-digested chromosomal DNA of *L. interrogans* serovar pyrogenes strain L784 (lane 1), *L. meyeri* (lane 2), *L. biflexa* (lane 3), *L. interrogans* serovar autumnalis strain L116 (lane 4) and *L. interrogans* serovar autumnalis strain L453 (lane 5). Each lane contained 1 μg of the chromosomal DNA. Lane 6 contained the *Eco*RI digested fragments of the plasmid from PA21 (positive control). Lane M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the right.

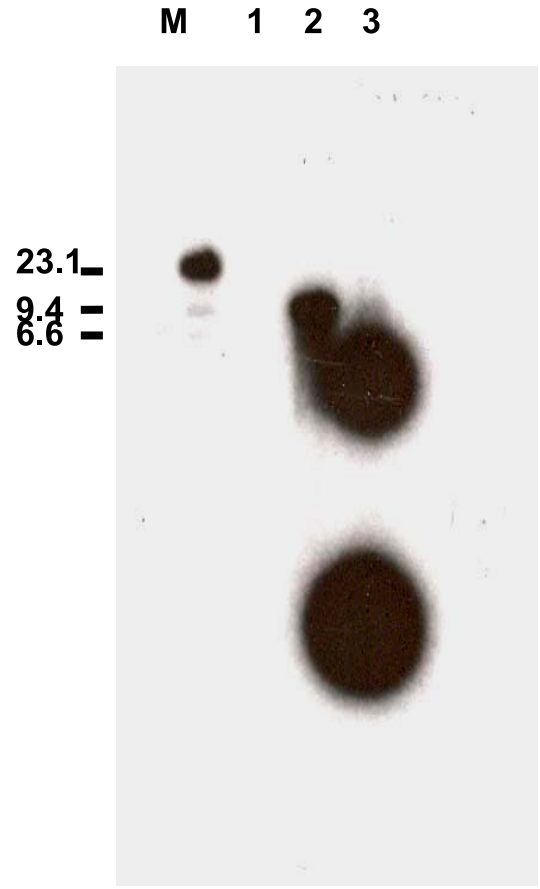
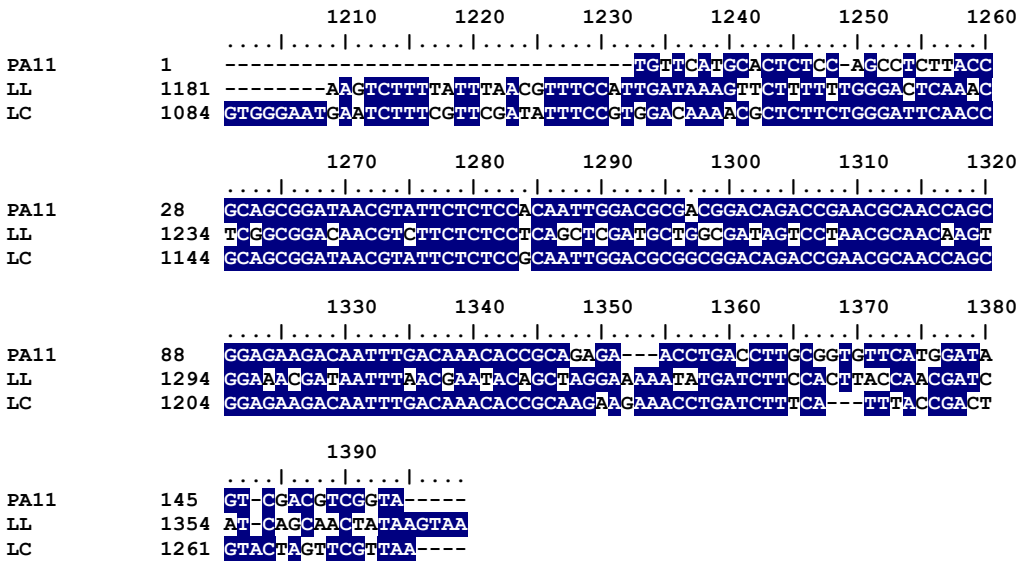


Fig. 13. Southern blot hybridization analysis using the *Eco*RI insert fragment of the plasmid from PA24 to probe the *Hin*dIII-digested chromosomal DNA of *L. biflexa* (lane 1) and *L. interrogans* strain L453 (lane 2). Each lane contained 1 μg of the chromosomal DNA. Lane 3 contained the *Eco*RI digested fragments of the plasmid from PA24 (positive control). Lane M contained λ *Hin*dIII markers. Sizes (kb) of the λ *Hin*dIII fragments are indicated to the left.

A.



B.

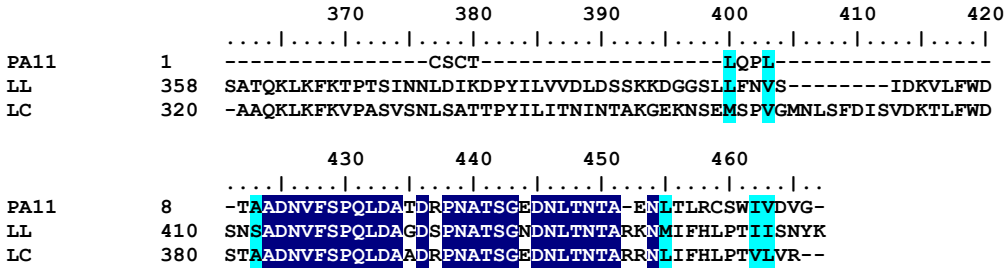
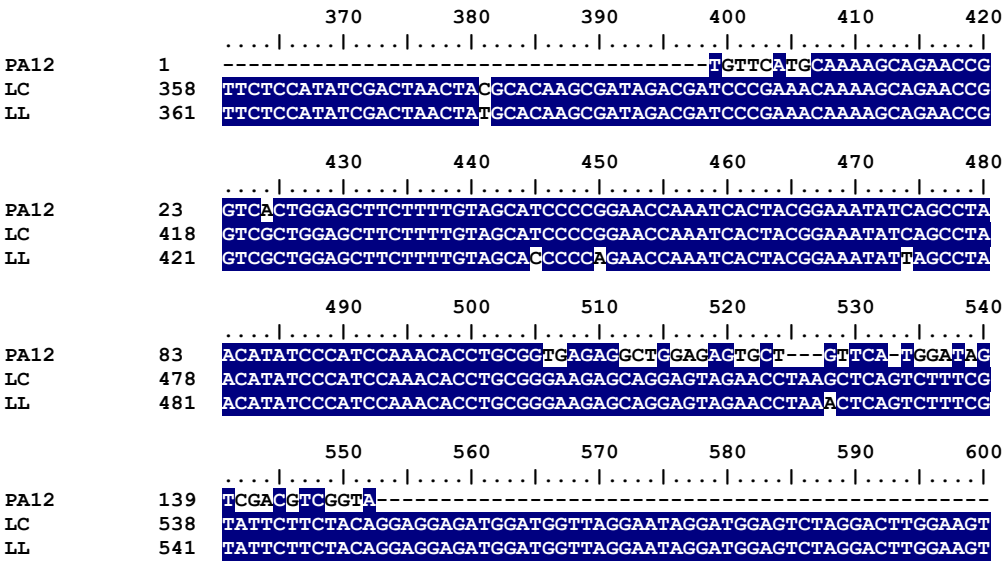


Fig. 14. Alignment of nucleotide (A) and predicted amino acid (B) sequences of putative lipoprotein homologs from *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_000629.1) and *L. interrogans* serovar lai (LL) strain 56601 (AAN49963.1) to PA11.

A.



B.

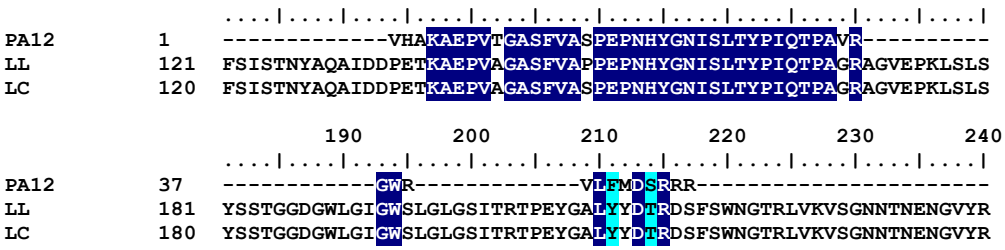
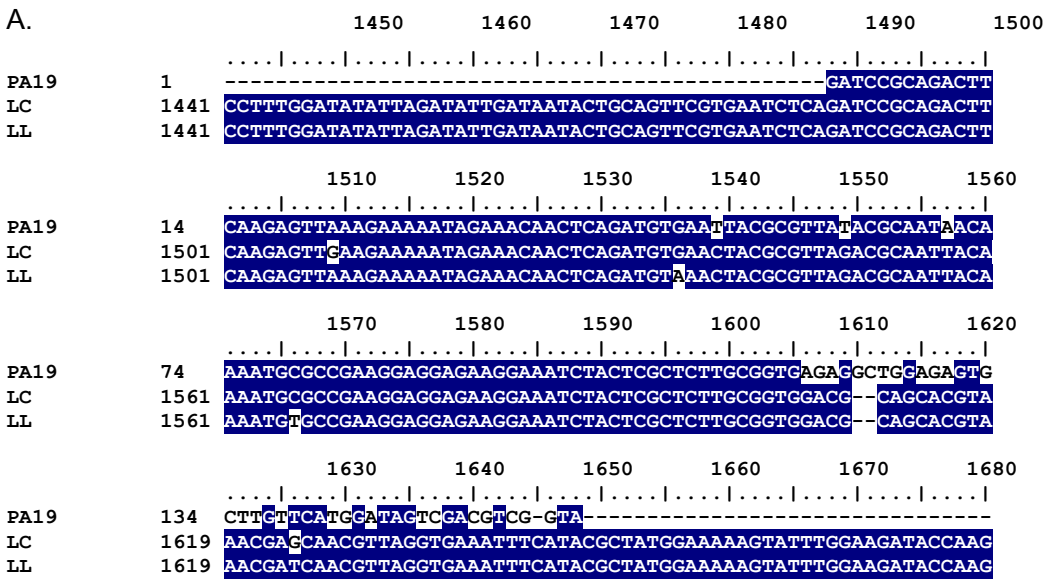


Fig. 15. Alignment of nucleotide (A) and predicted amino acid (B) sequences of Rhs cytoplasmic membrane protein homologs from *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_001982.1, AAS70619.1) and *L. interrogans* serovar lai (LL) strain 56601 (AAN48964.1) to PA12.

A.



B.

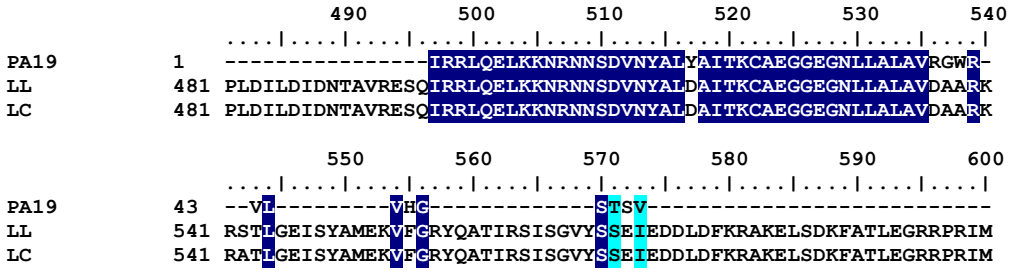
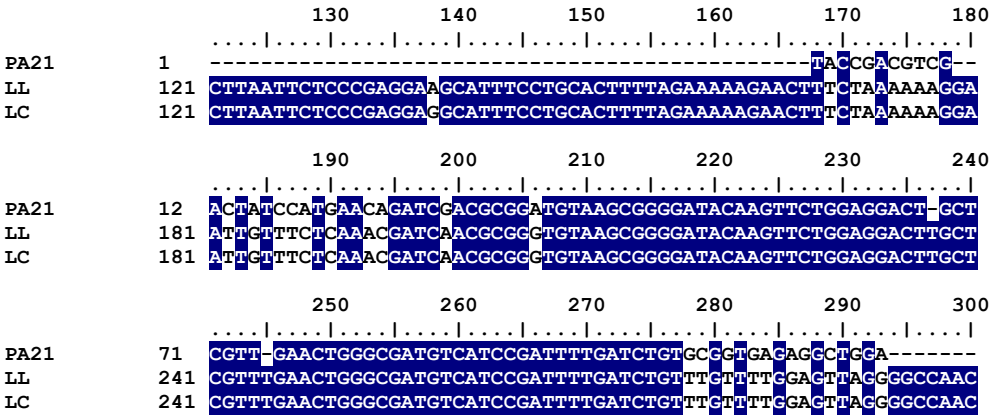


Fig. 16. Alignment of nucleotide (A) and predicted amino acid (B) sequences of methylmalonyl-CoA mutase homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN51833.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_003598.1) to PA19.

A.



B.

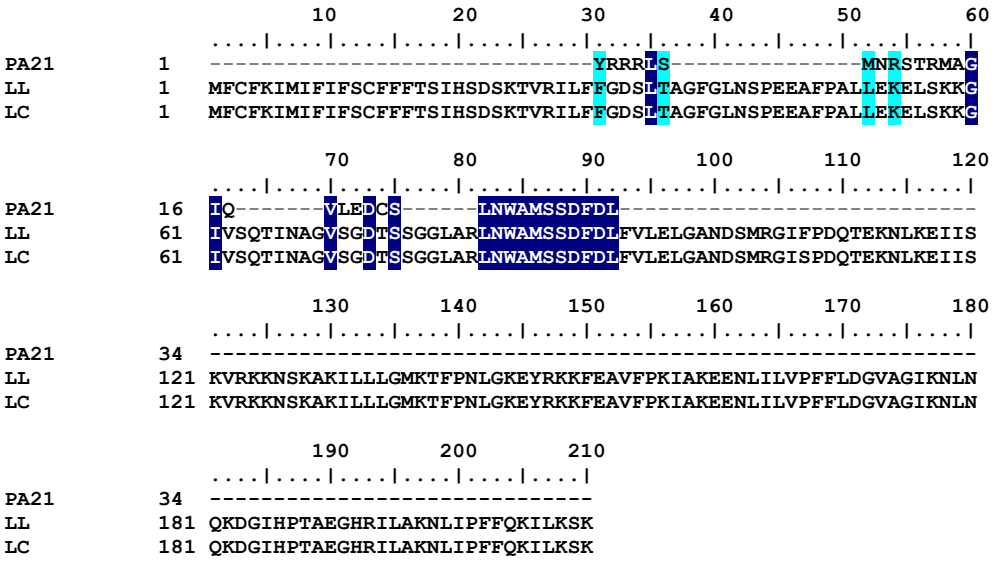
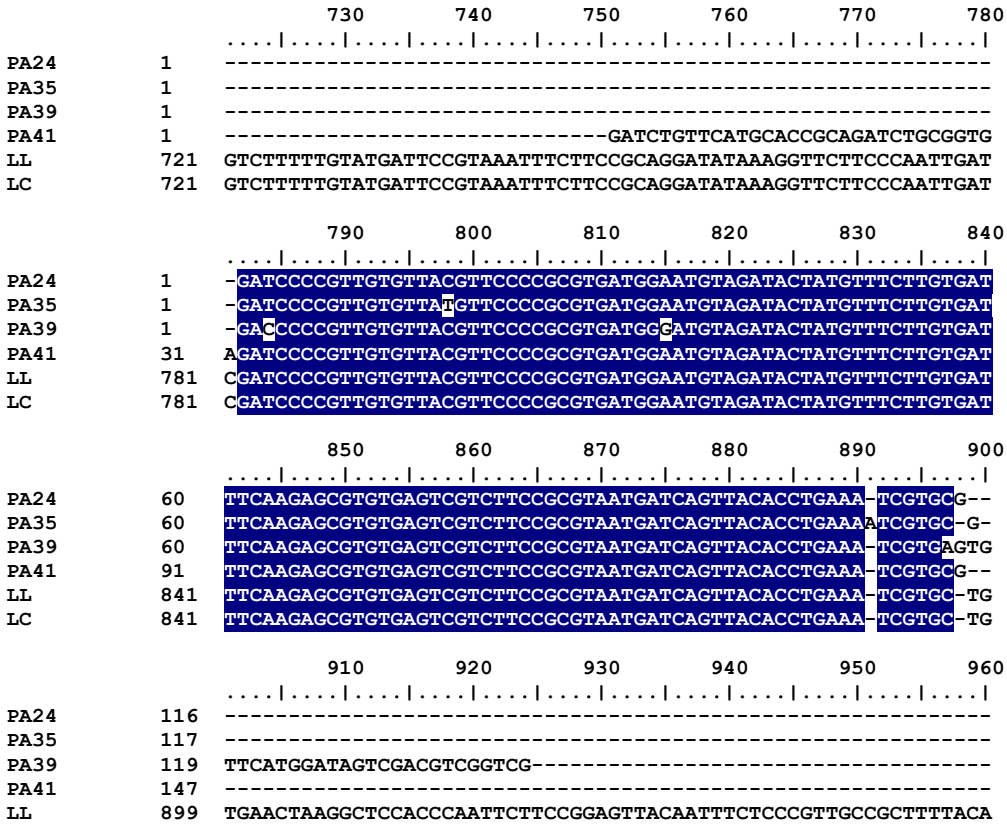


Fig. 17. Alignment of nucleotide (A) and predicted amino acid (B) sequences of acyl-CoA thioesterase homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN49760.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_001377.1) to PA21.

A.



B.

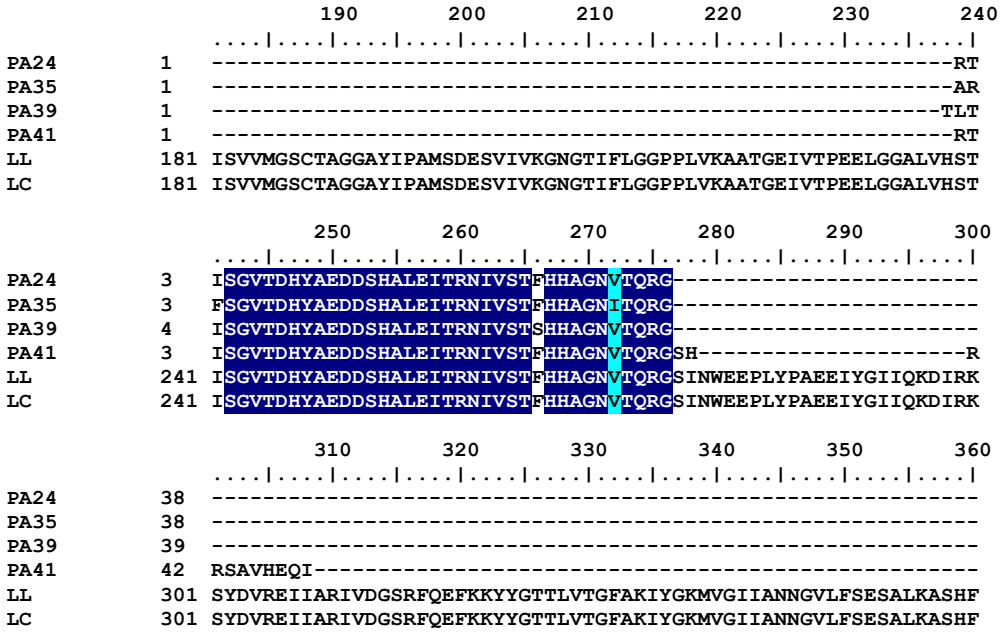
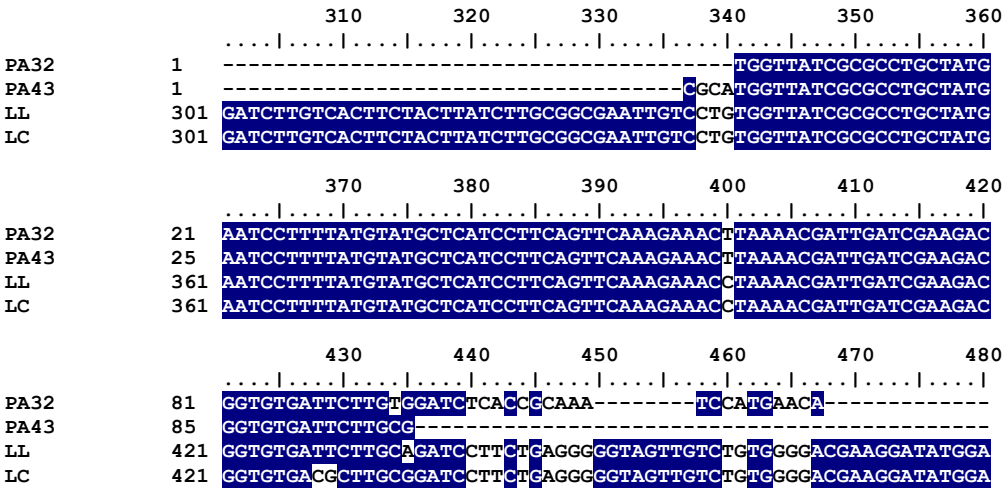


Fig. 18. Alignment of nucleotide (A) and predicted amino acid (B) sequences of propionyl-CoA carboxylase homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN51001.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_000428.1) to PA24, PA35, PA39 and PA41.

A.



B.

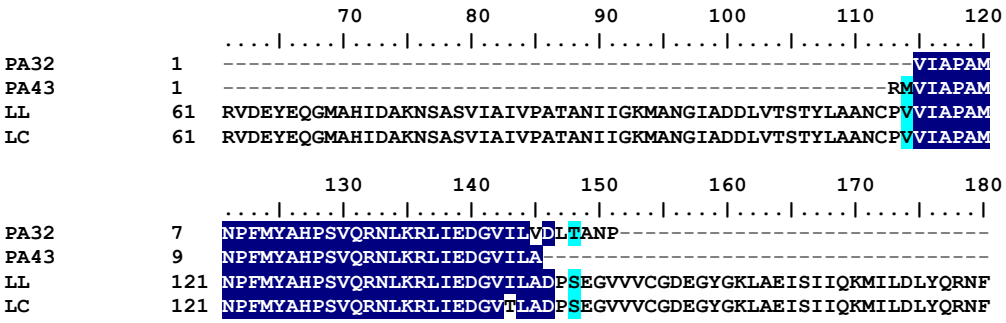
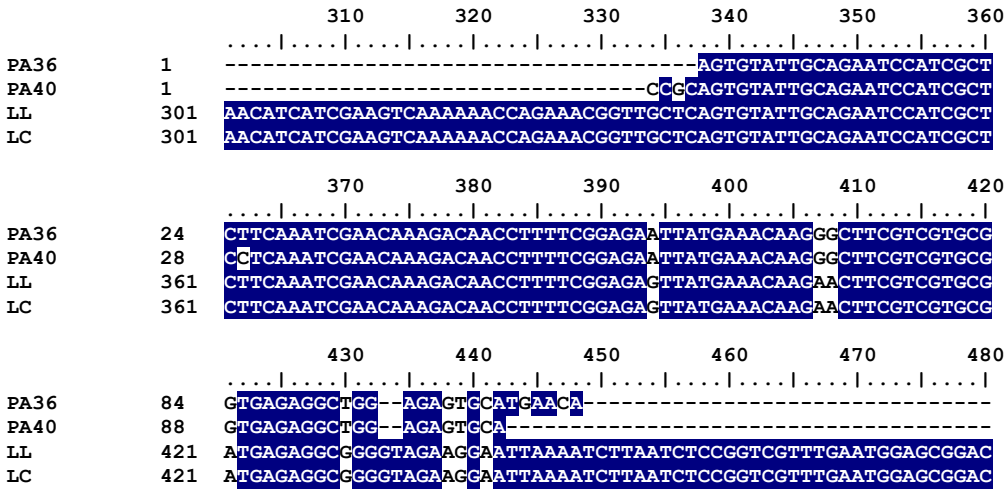


Fig. 19. Alignment of nucleotide (A) and predicted amino acid (B) sequences of DNA/pantothenate metabolism flavoprotein (DFP) homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN51134.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_003050.1) to PA32 and PA43.

A.



B.

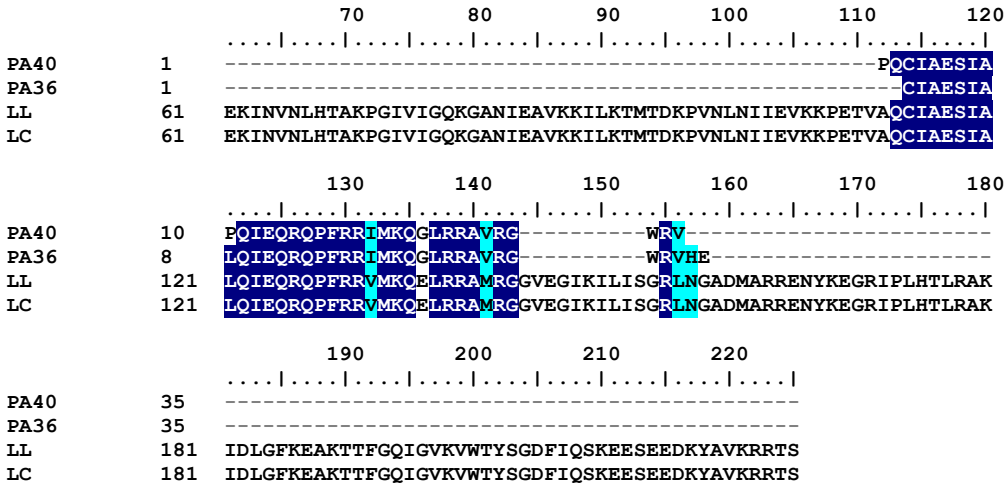
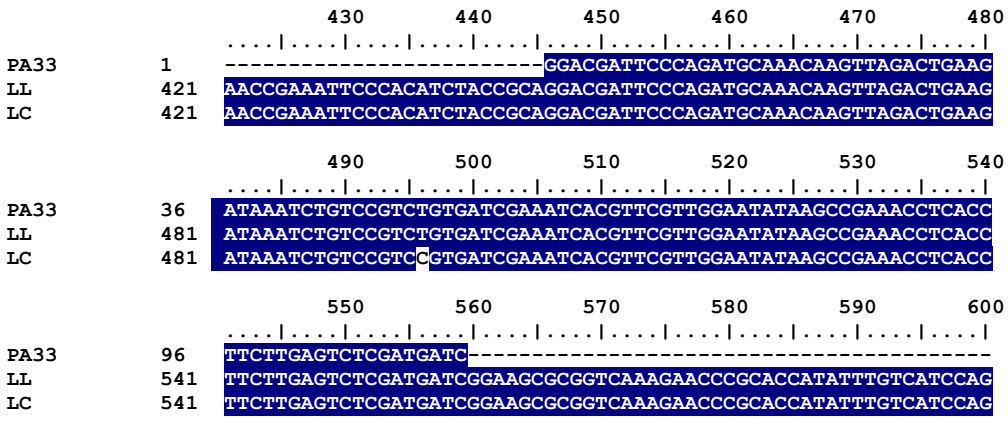


Fig. 20. Alignment of nucleotide (A) and predicted amino acid (B) sequences of ribosomal protein S3 homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN47944.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (AAS71420.1) to PA36 and PA40.

A.



B.

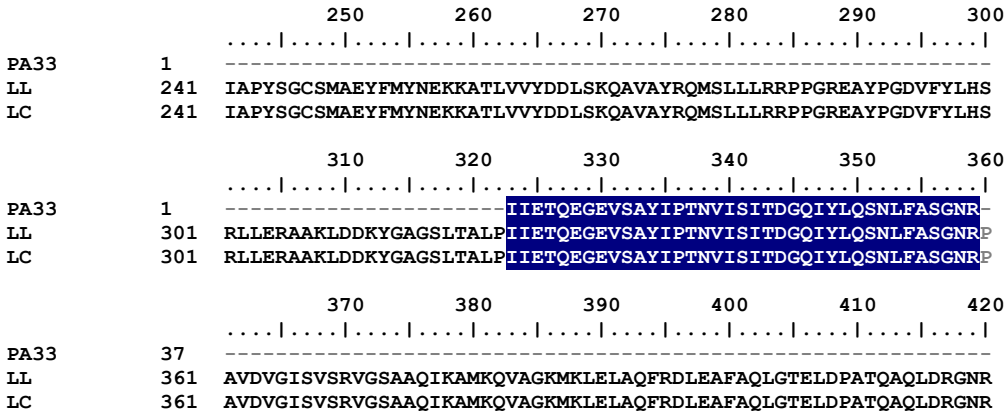
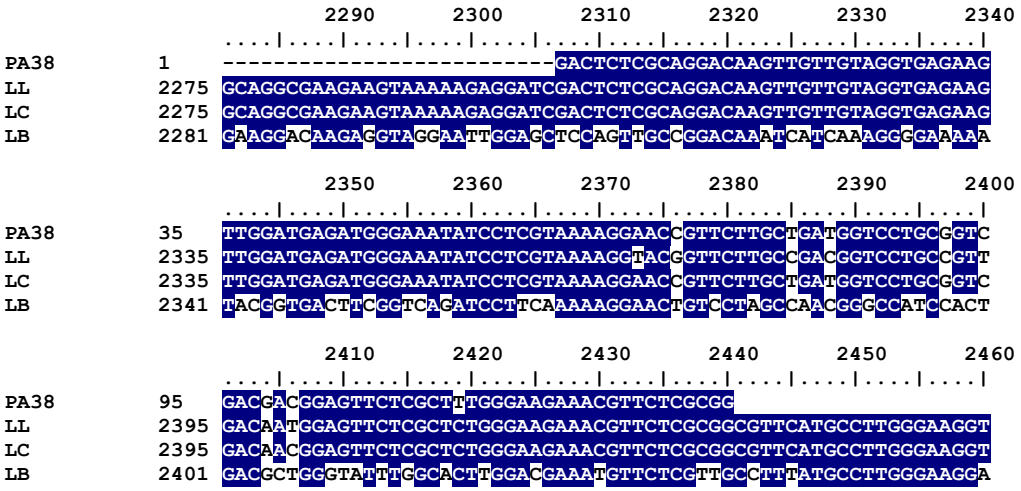


Fig. 21. Alignment of nucleotide (A) and predicted amino acid (B) sequences of ATP synthase F_1, α subunit homologs from *L. interrogans* serovar lai (LL) strain 56601 (AAN49978.1) and *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (AAS69847.1) to PA33.

A.



B.

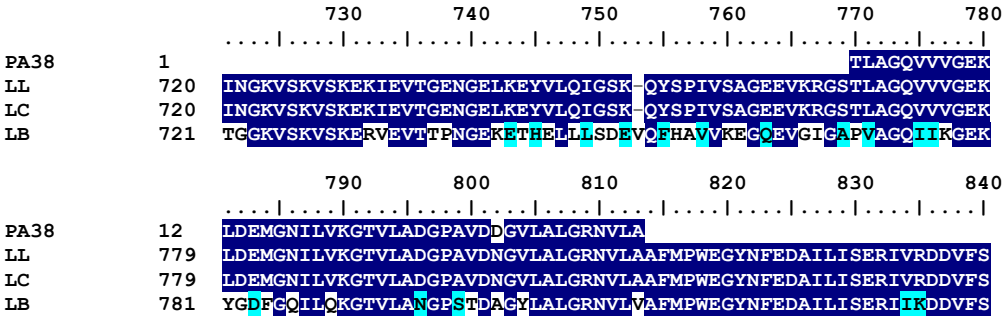


Fig 22. Alignment of nucleotide (A) and predicted amino acid (B) sequences of RNA polymerase beta subunit from *L. interrogans* serovar lai (LL) strain 56601 (AAN50618.1), *L. interrogans* serovar copenhageni (LC) strain Fiocruz L1-130 (YP_000733.1), and *Leptospira biflexa* serovar patoc (LB) (AAF73184.1) to PA38.

Table 1. Summary of BLASTX search of *L. interrogans* strain L453 specific clones

Clone	Insert size (bp)	Homology with BLASTX	Amino acid identity (%)	Accession Number
PA11	157	putative lipoprotein of *L. interrogans* serovar copenhageni	96	YP_000629.1
PA12	136	outer membrane protein (*rhs* gene family) of *L. interrogans* serovar copenhageni	98	AE_017295.1
PA19	160	methylmalonyl-CoA mutase of *L. interrogans* serovar lai	97	AAN51833.1
PA21	122	acyl-CoA thioesterase of *L. interrogans* serovar copenhageni	66	YP_001377.1
PA24	116			
PA35	117			
PA39	142	putative propionyl-CoA carboxylase of *L. interrogans* serovar lai	100	AAN51001.1
PA41	147			
PA32	119			
PA43	399	DNA / pantothenate metabolism flavoprotein of *L. interrogans* serovar lai	96	AAN51134.1
PA33	114	ATP synthase F_1 α subunit of *L. interrogans* serovar lai	100	AAN49978.1
PA36	109			
PA40	107	ribosomal protein S3 of *L. interrogans* serovar lai	87	AAN47944.1
PA38	134	RNA polymerase β subunit of *L. interrogans* serovar lai	97	AAN50618.1

DISCUSSION

After entering into the bloodstream, virulent leptospires grow in the bloodstream and tissues, whereas nonpathogenic and avirulent leptospires are rapidly eliminated by reticulo-endothelial phagocytosis (14). Therefore, virulent leptospires must adapt to the new environment inside the host and evade the host immune response in order to survive and proliferate within host tissues. At present, little is known about the mechanisms virulent bacteria use to cause disease. The bacteria may acquire virulence traits that allow the organisms to invade, colonize and survive in the host. Previous studies showed that pathogenic leptospires possess genes that are absent in nonpathogenic strains. Some of these genes, including *sphH* (29, 30) and *lipL21* (11), may contribute to pathogenesis of leptospirosis. In this study, the RDA method has been exploited to identify genetic differences between *L. interrogans* serovar autumnalis strain L453, isolated from a Thai patient with severe leptospirosis and the saprophytic strain, *L. biflexa* serovar patoc strain Patoc I. A subtractive hybridization library of 65 clones was obtained from this study. Of these, 5 clones; PA11, PA12, PA19, PA21 and PA24, were randomly selected for Southern blot hybridization analysis to confirm that the cloned DNA inserts were not present in the genome of *L. biflexa* (Figs. 9-13, compare lanes 2 and 1). Further analysis showed that PA21 is present in 3 strains of pathogenic leptospires, *L. interrogans* serovar pyrogenes strain L784 (Fig. 12, lane 1), *L. interrogans* serovar autumnalis strain L116 (Fig. 12, lane 4). PA21 was not present in two strains of nonpathogenic leptospires, *L. biflexa* (Fig. 12, lane 3) and *L. meyeri* (Fig. 12, lane 2). Analysis of the DNA sequences of the L453 genomic clones; PA11, PA12, PA21, PA19, PA21, revealed that clones PA11 and PA12 had sequence similarity to potential virulence factors, whereas clones PA19, PA21 and PA24 had sequence similarity to genes involved in lipid metabolism.

Surface-exposed lipoproteins are believed to play essential roles in the interactions of spirochetes with their environments. For example, the proteins may be used to facilitate bacterial attachment to host cells or to avoid host immune response via antigenic variation (18). It was shown that the leptospiral lipoproteins, LipL32 (19) and LipL41 (2) were expressed in pathogenic leptospires during mammalian infection. Immunization of hamsters with a combination of LipL41 and the transmembrane porin, OmpL1, provide synergistic immunoprotection (20). Comparative genomics of *L. interrogans* serovars copenhageni and lai showed that there are 7 putative genes encoding lipoproteins that are unique to either serovar. This discrepancy may reflect the

adaptation of each serovar to different animal hosts (41). The nucleotide sequence from PA11 was more similar to a putative lipoprotein of *L. interrogans* serovar copenhageni than that of *L. interrogans* serovar lai (Fig. 14a). The predicted amino acid sequence of PA11 had about 96% identity to the putative lipoprotein from both serovars copenhageni and lai (Fig. 14b). Genes encoding lipoproteins are considered as one of the genes potentially involved in pathogenesis and virulence (41). Thus, functional study of the putative lipoprotein specific to L453 could be useful in understanding the mechanism of host-pathogen interaction and for future development of vaccines.

The *Rhs* elements are accessory genetic elements that are widely distributed among *E. coli* population. These elements consist of a family of large composite genetic elements, *RhsA* through *RhsH* (54). One common feature of this element is a 3.7-kb, GC-rich core with a single open reading frame. The predicted protein from the *Rhs* core sequence contain 28 repetitions of a peptide motif, xxGxxRYxYDxxGRL(I or T)xxxx (15) and this peptide motif was later termed YD-repeats (37). The expression of this predicted protein has never been detected during routine cultivation of *E. coli* strain K-12 (24). The sequence of the predicted core protein is similar to the sequence of a *Bacillus subtilis* wall-associated protein (WAPA) (16). The N-terminal part of WAPA is associated with the wall and it was suggested that the free YD repeats may be involved in host interactions (16). In addition, it has been shown that the more distantly related YD-repeats of *Clostridium difficile* toxin A and *Streptococcus mutans* glucosyltransferases bind to carbohydrates (12). These led to the speculation that the *Rhs* products are attached to the cell surface and may aid in host interactions via carbohydrate interaction (24). The YD-repeats of teneurin-1, a chicken homologue of the *Drosophila* pair-rule gene ten-m, bind to heparin (37). Another common feature of *Rhs* elements is the presence of insertion sequences (ISs) (54) such as a mobile genetic element *vgrG* (51). Recent evidence suggested that the components of *Rhs* elements, VgrG may be responsible for the acquisition of the phospholipase gene in *Pseudomonas aeruginosa* (57) and the actin cross-linking domain (ACD) of RTX toxin in *Vibrio cholerae* (51). PA12 had sequence similarity to the *L. interrogans* serovars lai and copenhageni outer membrane protein of the *rhs* gene family. Thus, this protein may be involved in host interactions or the acquisition of virulence factors of pathogenic leptospires.

PA19 encoded putative methylmalonyl-CoA mutase (Fig. 16) which catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. Clones PA24, PA35, PA39

and PA41 encoded putative propionyl-CoA carboxylase (Fig. 18), catalyzing the carboxylation of propionyl-CoA to methylmalonyl-CoA. Both enzymes, methylmalonyl-CoA mutase and propionyl-CoA carboxylase, are involved in the oxidative carboxylation of acetyl-CoA to succinyl-CoA through the 3-hydroxypropionate pathway (47). At present, it is not known whether these enzymes are not present in *L. biflexa* or the sequences of the *L. biflexa* enzymes are diverged from those of *L. interrogans*. Ping et al. (45) demonstrated that the gene encoding methylmalonyl-CoA decaboxylase is present in *L. interrogans* serovar lai, but absent in *L. biflexa* serovar monvalerio. Interestingly, this enzyme catalyzes the reverse reaction of propionyl-CoA carboxylase through the decarboxylation of methylmalonyl-CoA to propionyl-CoA (23). PA21 encoded putative acyl-CoA thioesterase (Fig. 17) which hydrolyzes fatty acyl-CoAs, basic unit of lipid synthesis, yielding free fatty acid and CoASH (52).

Southern blot hybridization analysis has not yet been performed to determine whether clones, PA32, PA43, PA33, PA36, PA40 and PA38, are L453 specific. Both PA32 and PA43 encoded DNA/pentothenate metabolism flavoprotein. This result was in agreement with earlier work by Ping et al. (45) who reported that gene encoding pantothenate metabolism flavoprotein-like protein is specific to *L. interrogans* serovar lai (45). The coding sequences of pantothenate metabolism flavoprotein-like protein and DNA/pentothenate metabolism flavoprotein in the GenBank database of *L. interrogans* serovar lai genome are 4303-4989 and 5006-5548 on the minus strand, respectively. This suggested that the two genes may be transcribed as an operon. One clone spanned the portion of RNA polymerase β subunit (*rpoB*). While this gene is present in both *L. biflexa* and pathogenic *L. interrogans* serovars lai and copenhageni, the region spanned by clone PA38 diverged significantly from the sequence of *L. biflexa rpoB* (Fig. 22). This demonstrated the utility of the RDA method in identifying unique sequences from L453 genomic DNA. Thus, RDA is proven to be useful in the identification of genomic differences between two closely related bacterial strains.

FUTURE WORK

- Screening more clones in the library to identify virulence specific genes.
- Functional study of the putative lipoprotein and the outer membrane protein of the *rhs* gene family.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. การเสนอผลงานในที่ประชุมวิชาการนานาชาติ The 8th Asian Conference on Transcription (ACT 8) ที่จัดขึ้นที่สถาบันวิจัยจุฬาภรณ์ หลักสี่ กรุงเทพมหานคร เมื่อวันที่ 16-19 พฤศจิกายน 2004
2. การผลิตบัณฑิตปริญญาโท นางสาว สิริมา มิ่งมงคลชัย
3. กำลังเขียนผลงานเพื่อส่งตีพิมพ์ในวารสารวิชาการนานาชาติ

ชื่อเรื่อง: Identification of genomic differences between pathogenic and non-pathogenic *Leptosira* using representational difference analysis

ชื่อวารสารที่จะตีพิมพ์: The American journal of tropical medicine and hygiene