REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA) FOR DETECTION OF GENETIC ELEMENTS ASSOCIATED WITH INCREASED INCIDENCE OF SEROGROUP C *NEISSERIA MENINGITIDIS* INFECTION

by

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B.S. in Biology, Indiana University of Pennsylvania, 2003

Submitted to the Graduate Faculty of

Department of Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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April 7, 2006

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Previous studies have demonstrated that the increased incidence of invasive disease caused by serogroup C *Neisseria meningitidis* in the United States during the 1990s was attributed primarily to strains belonging to the ST11 clonal complex. Subcapsular genotyping of a subset of isolates from Maryland identified distinct "early" and "late" clones defined by antigenic shift at the FetA outer membrane protein. Representational difference analysis (RDA) was used to identify additional genetic differences that may have contributed to the emergence of the late clone. A collection of serogroup C isolates representative of the early and late clone was subjected to pulsed field gel electrophoresis (PFGE) to determine genetic relatedness among the isolates and to identify a candidate tester/driver pair for RDA. *Rsa*I-digested tester genomic DNA (late clone) was ligated to specific adaptors followed by two rounds of subtractive hybridization with *Rsa*I-digested driver genomic DNA (early clone). PCR amplification of subtracted tester DNA with adaptor specific primers generated at least three late clone-specific bands that were absent from the early clone. These products were cloned and sequenced and confirmed by Southern blotting with tester and driver digoxigenin-labeled genomic DNA probes to be tester specific. A BLAST search of late clone-specific sequences identified homology to either IS1301 or pJS-B plasmid *N. meningitidis* sequences. PCR with primers specific to either IS1301 or pJS-B plasmid sequences amplified these elements from late clone isolates but not from early clone isolates. Thus, RDA successfully identified two unique genetic elements present in an emergent *N. meningitidis* serogroup C ST-11 clone that had undergone antigenic

shift at FetA. Further investigation is required to determine the potential role of these elements in clonal emergence and *N. meningitidis* pathogenesis. The public health significance of this project stems from increased incidence of meningococcal disease being a major concern: morbidity and mortality increase, outbreaks produce panic and disruption in communities, public health agencies must respond for control and prevention, and mass immunization and antibiotic prophylaxis are often required.

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PREFACE

I would like to thank Dr. Lee Harrison, my thesis advisor, for his continued guidance and support on this project as well as Dr. Yuan Chang and Dr. Jeremy Martinson for being part of my committee. I appreciate all of the time and expertise that you have lent to this project. I would also like to thank Dr. Jane Marsh for her daily guidance in the laboratory and always being available to answer my never-ending questions. A very special thank you to Alicia Price, my "partner in crime", for our daily conversations, the many hours we spent in lab together, and the tremendous support you have given me throughout graduate school. Lastly, I would like to thank my family and friends for being behind me every step of the way and always having faith in me to take the next step forward.

1.0 INTRODUCTION

1.1 EPIDEMIOLOGY OF MENINGOCOCCAL DISEASE

Neisseria meningitidis is a major cause of bacterial septicemia and meningitis worldwide. This gram-negative, capsulated bacterium colonizes mucosal surfaces of the nasopharynx and is transmitted through direct contact with large droplet respiratory secretions from patients or asymptomatic carriers (1, 2). *N. meningitidis* is an obligate human pathogen causing meningeal infection similar to other forms of acute purulent meningitis and is characterized by sudden onset of fever, headache, and stiffness of neck sometimes accompanied by nausea, vomiting, photophobia, and an altered mental status. In the case of meningococcal sepsis, noted in 5-20% of patients, purpuric rash, hypotension, acute adrenal hemorrhage, and multi-organ failure can occur. The overall case fatality rate has remained stable over the past 20 years, at 9-12%, with a rate up to 40% among patients with meningococcal sepsis (3). *N. meningitidis* has been classified into five major pathogenic serogroups (A, B, C, Y, and W135) based on chemical composition of distinctive capsular polysaccharides (2). There are currently two licensed meningococcal vaccines in the U.S.; both cover serogroups A, C, Y, and W135. The polysaccharide-based vaccine licensed in 1982 has immunologic limitations while the protein-conjugate vaccine licensed in 2005 for 11-55 year olds is now recommended for routine use in adolescents (1).

There are substantial fluctuations in meningococcal disease incidence, outbreaks, and epidemics. Each year, an estimated 1,400-2,800 cases of meningococcal disease occur in the U.S., a rate of around 0.5-1.0/100,000 population (1). The rates of disease are highest among infants although rates drop after infancy then increase during adolescence and early adulthood. Serogroups A, B, and C account for most cases of meningococcal disease throughout the world with serogroups B and C responsible for the majority of cases in the Americas and Europe and serogroups A and C predominating throughout Asia and Africa (3).

In the U.S., there was an increased incidence of disease observed in the 1990s that was accompanied by a dramatic surge in meningococcal outbreaks, mostly caused by serogroup C strains. By the mid-1990s, the incidence of meningococcal infection was nearly 3-fold higher than earlier in the decade followed by a subsequent decline in the late 1990s and early 2000s. There were also changes in serogroup distribution during the 1990s with an increased incidence of serogoup Y and C infections (4, 5).

The substantial epidemiologic fluctuations of meningococcal disease are dramatic and poorly understood. Increased incidence of meningococcal disease is a major public health concern: morbidity and mortality increase, outbreaks produce panic and disruption in communities, public health agencies must respond for control and prevention, and mass immunization and antibiotic prophylaxis are often required. Changes in serogroup distribution are also important because the meningococcal vaccines licensed in the U.S. are serogroup-specific and do not provide protection against serogroup B, which is responsible for around a third of all meningococcal disease in the U.S. (1, 2). One instance of meningococcal capsular switching from serogroup C to W-135 in a W-135 vaccine naïve population occurred during the Hajj pilgrimage in Mecca, Saudi Arabia causing extensive public health impacts due to lack of

immunological protection (3). The genetic mechanisms for these changes in incidence and serogroup distribution are not known and will be explored further in this paper among serogroup C isolates.

1.2 SEROGROUP C ANTIGENIC SHIFT

Multi-locus sequence typing (MLST) is one application used to study the molecular epidemiology of *N. meningitidis* by identifying major clonal complexes that are associated with meningococcal disease worldwide (6). MLST defines an isolate's genetic background based on the DNA sequence of 7 housekeeping genes not under selective pressure. The MLST sequence type (ST) is defined by the combination of alleles at all 7 of these genes. An ST complex is defined as a set of STs that have identity at 5 or more of the 7 housekeeping genes. MLST is a highly objective genotyping method and is portable for global strain comparisons. While MLST is a useful genotyping tool for global epidemiology, it is not sufficiently discriminatory for outbreak detection or investigations of clonal fluctuations within ST complexes (6). Sequencing of outer membrane proteins (OMPs) provides an assessment of bacterial antigenic properties as these proteins are under selective pressure and therefore can provide information regarding the emergence of meningococcal clones within ST complexes (4).

In a previous study conducted by Harrison, *et al*., the increased incidence of invasive serogroup C meningococcal disease in Maryland during 1992-2001 was identified by population-based surveillance (4). Molecular characterization of 97 *N. meningitidis* serogroup C isolates was performed by MLST. Subcapsular genotype was determined by sequence typing of the genes encoding 3 OMPs: *por*A variable regions (VRs) 1 and 2, *por*B, and *fet*A VR. MLST

revealed that the majority of serogroup C isolates (92%) belonged to the ST11 complex. It was also noted that ST11 complex isolates with the 2:P1.5,2:F.1-30 (*por*B protein class:P1.*por*A VR1 allele, *por*A VR2 allele:F.*fet*A allele) subcapsular genotype predominated early in the 1990s (early OMP profile) while isolates with the 2:P1.5,2:F.3-6 subcapsular genotype predominated late in the 1990s (late OMP profile) (Figure 1). There was complete homology between amino acid sequences examined for PorA and PorB proteins from the early and late clones. In contrast, early and late clone deduced FetA VR sequences differed by 23 amino acids (Table 1). These results indicated that the increased incidence of serogroup C meningococcal disease in Maryland during the 1990s was associated with antigenic shift at the *fet*A locus (4).

The genetic recombination event at *fet*A resulted in antigenic shift that may be responsible for the emergence of the serogroup C ST11 complex late clone in Maryland during the late 1990s. This genetic event may be associated with the introduction or deletion of genetic elements that may have provided a selective advantage and contributed to the late clone emergence. Representational difference analysis (RDA) can be used to identify the genetic changes associated with OMP antigenic shift in serogroup C isolates and to help examine the molecular epidemiology of clonal complexes associated with meningococcal disease.

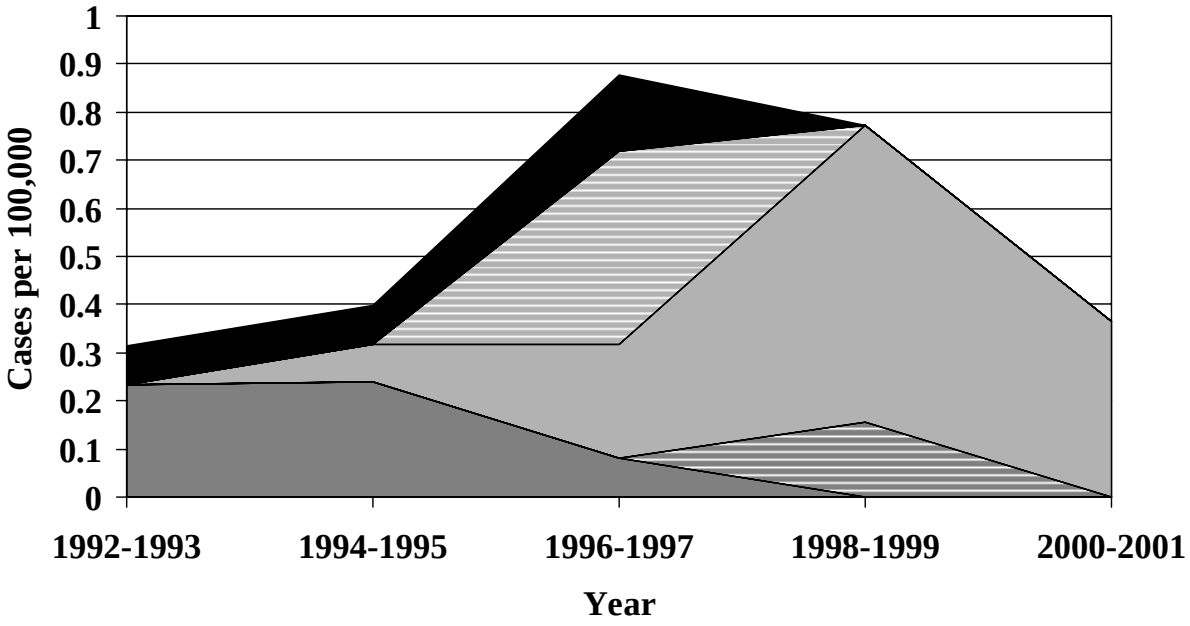

Figure 1: Average annual incidence of serogroup C infection among 15-24 year olds in Maryland, 1992-2001, by OMP sequence profile.
The dark gray area represents infection caused by isolates bearing the early OMP profile (ST11, 2:P1.5,2:F.1-30), the light gray area represents infection causes by isolates bearing the late OMP profile (ST11, 2:P1.5,2:F.3-6), and the black area represents infection caused by isolates with other OMP profiles. The striped areas represent the subset of early and late strains with *por*A deletions (4).

Table 1: Aligned deduced peptide sequences for PorA variable region (VR) 1 and VR2, FetA VR, and PorB loops V and VII, ST11 complex serogroup C meningococcal isolates, Maryland, 1992-2001.

	PorA			
Strain	**Variable Region 1**		**Variable Region 2**	
Serogroup C	**Allele**	**Deduced Sequence**	**Allele**	**Deduced Sequence**
Early	5	PLQNIQPQVTKR	2	HFVQQTPKSQPTLVP
Late	5	PLQNIQPQVTKR	2	HFVQQTPKSQPTLVP
	PorB			
	Loop V		**Loop VII**	
	Allele	**Deduced Sequence**	**Deduced Sequence**	
Early	2-2	KYADLNTDAERVAVNTANAHPVKDY	KVNGVKDANYQYDQ	
Late	2-2	KYADLNTDAERVAVNTANAHPVKDY	KVNGVKDANYQYDQ	
	FetA			
	Allele	**Deduced Sequence**		
Early	1-30	GEFKI-----KD-KSGATQAEKQ-KNRDDENIVKAYRLT		
Late	3-6	SQFSIPKTEKKDGKDVAKSSEQQTKDRKDETIVHSYKLS		

Some of the isolates were *por*A nontypable. For PorB, the predominant alleles are shown. Amino acid differences are shown in red (4).

1.3 REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA)

Representational difference analysis (RDA) is a method used to detect regions of DNA that differ between two closely related genomes that may be involved in genomic evolution. RDA was developed as a tool in eukaryotic organisms to identify genetic polymorphisms in human neoplasia and is built upon subtractive hybridization techniques (7, 8). This technique has also been used to discover the presence of human herpesvirus 8 in the tissues of patients with Kaposi's sarcoma (9).

RDA is a method in which one DNA population (driver) is hybridized in excess against a second population (tester), to remove common sequences, thereby enriching for "target" sequences unique to the tester population. RDA combines a subtractive hybridization approach with positive selection of target sequences by taking advantage of the second-order kinetics of DNA reannealing (8). This approach can be used as a tool for the identification of both differences in gene content and in gene expression (10). For example, Tinsley, *et al*. used RDA to investigate the differences between *N. meningitidis* and *Neisseria gonorrhoeae* pathogenic species to determine the genetic basis for their varying pathogenicities (11). RDA has also been used to identify sequences that are specific to *N. meningitidis* hyper-virulent lineage III clone that has caused increased incidence of meningococcal disease in many countries in Europe and in New Zealand since the 1980s (12). Based on these studies, RDA was selected as a method to identify genetic polymorphisms among *N. meningitidis* serogroup C isolates that underwent antigenic shift at *fet*A.

2.0 SPECIFIC AIM

The increased incidence of serogroup C meningococcal disease in Maryland during the 1990s was associated with the emergence of an ST11 clone that had undergone antigenic shift at the *fet*A gene locus. The specific aim of this project is to identify genetic differences between *N. meningitidis* serogroup C ST11 early and late clones that may have contributed to this emergence by representational difference analysis (RDA).

3.0 MATERIALS AND METHODS

3.1 STUDY ISOLATES

The *N. meningitidis* serogroup C isolates for this study were obtained through active, laboratory- and population-based surveillance for meningococcal infection from January 1, 1992-December 31, 2001 as part of the Maryland Active Bacterial Core Surveillance (ABCs) component of the multi-state Emerging Infections Program Network. A case was defined as isolation of *N. meningitidis* from a usually sterile site (e.g., blood, cerebrospinal fluid) in Maryland as described (13). Meningococcal serogrouping, serosubtyping, and MLST and OMP gene sequence analysis were performed as previously described (4). Out of 97 *N. meningitidis* serogroup C isolates, 89 isolates belonged to the ST11 clonal complex. Of these 89 ST11 complex isolates, 31 had the early clone subcapsular genotype (2:P1.5,2:F.1-30) and 39 had the late clone subcapsular genotype (2:P1.5,2:F.3-6). 13 of the 70 total early/late clone isolates had to be eliminated from the study group because they were not *por*A typable (4). 34 total early/late clone isolates were finally chosen for further analysis by PFGE because they differed in subcapsular genotype only at the *fet*A locus (OMP profile: ST11; *por*B 2-2; *por*A 5-2; *fet*A 1-30 or 3-6). 16 of the 34 chosen study isolates were from the early clone while the remaining 18 isolates were from the late clone.

3.2 PFGE

N. meningitidis isolates were streaked from frozen glycerol stocks onto chocolate agar plates (Becton, Dickinson, and Company, Sparks, MD) and incubated overnight at 37°C with 5% CO_2. The next day, an isolated colony was selected for subculture onto chocolate agar plate under the same growth conditions as previously described. Bacteria were suspended in 2 ml 1X TE Buffer (1.0M Tris-HCl, 0.1M EDTA, Sigma, St. Louis, MO) and the bacterial concentration was adjusted to 3-5% transmission using a Vitek Special DR100 colorimeter (Hach Company, Loveland, CO). A 200 µl volume of the bacterial suspension was mixed with 200 µl melted 2% SeaPlaque® agarose (BioWhittaker Molecular Applications, Rockland, ME) and added to reusable plug molds. Digestion of plugs was performed by soaking plugs in ESP buffer (final concentration, 1% sarkosyl [Fisher Scientific, Pittsburgh, PA], 1 mg/ml Proteinase K [Sigma], 0.43M EDTA) in a 50°C waterbath overnight. The plugs were washed 4 times in 1X TE Buffer (Sigma) in a 37°C waterbath for 30 minutes each. Plugs were cut into thirds and incubated with the restriction enzyme *Nhe*I (New England Biolabs, Ipswich, MA) in a 37°C waterbath overnight. Plugs were inserted into a 1% SeaKem® Gold (Cambrex Bio Science Rockland, Inc., Rockland, ME) agarose gel prepared with 0.5X Tris-borate-EDTA buffer (Sigma). Gels were run on a CHEF-DR® III apparatus (BioRad Laboratories, Hercules, CA) at 14°C for 18 hours (initial pulse time 1 sec., final pulse time 30 sec.) then 8 hours (initial pulse time 5 sec., final pulse time 9 sec.). Gels were stained in ethidium bromide (final concentration, 2 µg/ml, Sigma), destained in fresh distilled water, and digitally photographed on a BioRad GelDoc 2000. Gel analysis and banding pattern interpretation was performed with BioNumerics software (Applied Maths Inc., Austin, TX). Cluster analysis was based on unweighted pair grouping (UPGMA)

and the Dice similarity coefficient while Tenover, *et al.* criteria for genetic relatedness was used for PFGE banding pattern interpretation (14).

3.3 RDA

The extraction of high-quality chromosomal DNA from tester and driver isolates was performed using a phenol-chloroform extraction method previously described (10). The BD PCR-Select™ Bacterial Genome Subtraction Kit (BD Biosciences, Palo Alto, CA) used for RDA in this study (Figure 2) is based on the suppression subtractive hybridization method and selectively amplifies differentially expressed sequences present in tester DNA but absent from driver DNA (15). *E. coli* genomic DNA was spiked with *Hae* III-digested φX174 DNA and used in parallel to the *N. meningitidis* experimental isolates as a positive control for the following RDA procedure.

Tester and driver genomic DNA (2 µg each) was digested with *Rsa*I restriction enzyme (10 units/µl) and 10X restriction buffer (included in the subtraction kit) and incubated overnight at 37°C. Tester DNA was divided into 2 reaction tubes containing T4 DNA ligase (400 units/µl) and 5X DNA ligation buffer (250 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTT, 0.25 mg/ml BSA), both provided in the kit. 2 µl of either Adaptor 1 or Adaptor 2R (Table 2) was added to the ligation mixtures and incubated overnight at 16°C. 1 µl of 0.2M EDTA (Sigma) was added to stop the reaction and the tubes were heated at 72°C for 5 min. to inactivate the ligase. To verify adaptor ligation efficiency, PCR primer 1 (Table 2), which is specific to adaptor 1 and 2R sequences, was used in combination with *N. meningitidis ctr*A forward primer (Table 2) to amplify the fragment that spans the adaptor and *N. meningitidis* tester DNA junction. A similar adaptor ligation efficiency PCR was performed on positive control *E. coli* tester DNA using PCR

primer 1 and 23S RNA primers (Table 2) that were provided in the subtraction kit. The Clontech Advantage cDNA PCR kit (BD Biosciences) was used with the following PCR conditions: 72°C for 2 min. and 94°C for 30 sec. followed by 23 cycles of 94°C for 10 sec., 65°C for 30 sec., and 68°C for 1 min.

In the first round of subtractive hybridization, 2 µl of *Rsa*I-digested driver DNA was added to each of the 2 tester DNA ligation reactions (Adaptor 1 and 2R) for both experimental and positive control samples. This DNA mixture was denatured at 98°C for 1.5 min. and allowed to hybridize by incubating at 63°C for 1.5 hours. In the second round of subtractive hybridization, the two hybridizations with different adaptor ligations (from the first round) were mixed together and 1 µl of fresh, denatured driver DNA (incubated at 98°C for 1.5 min.) was added. This DNA mixture was allowed to hybridize overnight at 63°C. 200 µl dilution buffer (20 mM HEPES-HCl, 2 M NaCl, 0.2 mM EDTA) provided in the subtraction kit was added to the hybridization mixture and incubated at 63°C for 7 min.

Primary amplification of *N. meningitidis* tester-specific sequences was performed on 1 µl of the subtracted DNA reaction in a final reaction volume of 25 µl including 19.5 µl sterile water, 2.5 µl 10X PCR buffer, 0.5 µl dNTP mix (10 mM), 1.0 µl adaptor-specific PCR Primer 1 (10 µM), 0.5 µl 50X BD Advantage cDNA Polymerase Mix (BD Biosciences). PCR primer 1 anneals to sequences common to both adaptor 1 and adaptor 2R, thus, the primary amplification reaction amplifies all sequences harboring any adaptor combination (Figure 2). Unsubtracted *Rsa*I-digested, adaptor-ligated *N. meningitidis* tester DNA was amplified in parallel as a control. In addition, the primary amplification reaction was performed on subtracted and unsubtracted *E. coli* DNA spiked with *Hae* III-digested φX174 DNA and the PCR Control Subtracted DNA provided in the genome subtraction kit as controls for the subtractive hybridization reactions and

the PCR reaction. The primary PCR conditions were as follows: 72°C for 2 min. then 25 cycles of 94°C for 30 sec., 66°C for 30 sec., and 72°C for 1.5 min. The primary PCR products were diluted 1:40 in sterile water.

Both undiluted and diluted primary PCR products were used as DNA template in secondary PCR amplifications. The 25 µl secondary PCR reaction included 18.5 µl sterile water, 2.5 µl 10X PCR buffer, 0.5 µl dNTP mix (10 mM), 1.0 µl adaptor-specific nested primers 1 and 2R (10 µM) (Table 2), 0.5 µl 50X BD Advantage cDNA Polymerase Mix (BD Biosciences), and 1.0 µl diluted or undiluted primary PCR product. Nested PCR conditions were as follows: 12 cycles of 94°C for 30 sec., 68°C for 30 sec., and 72°C for 1.5 min.

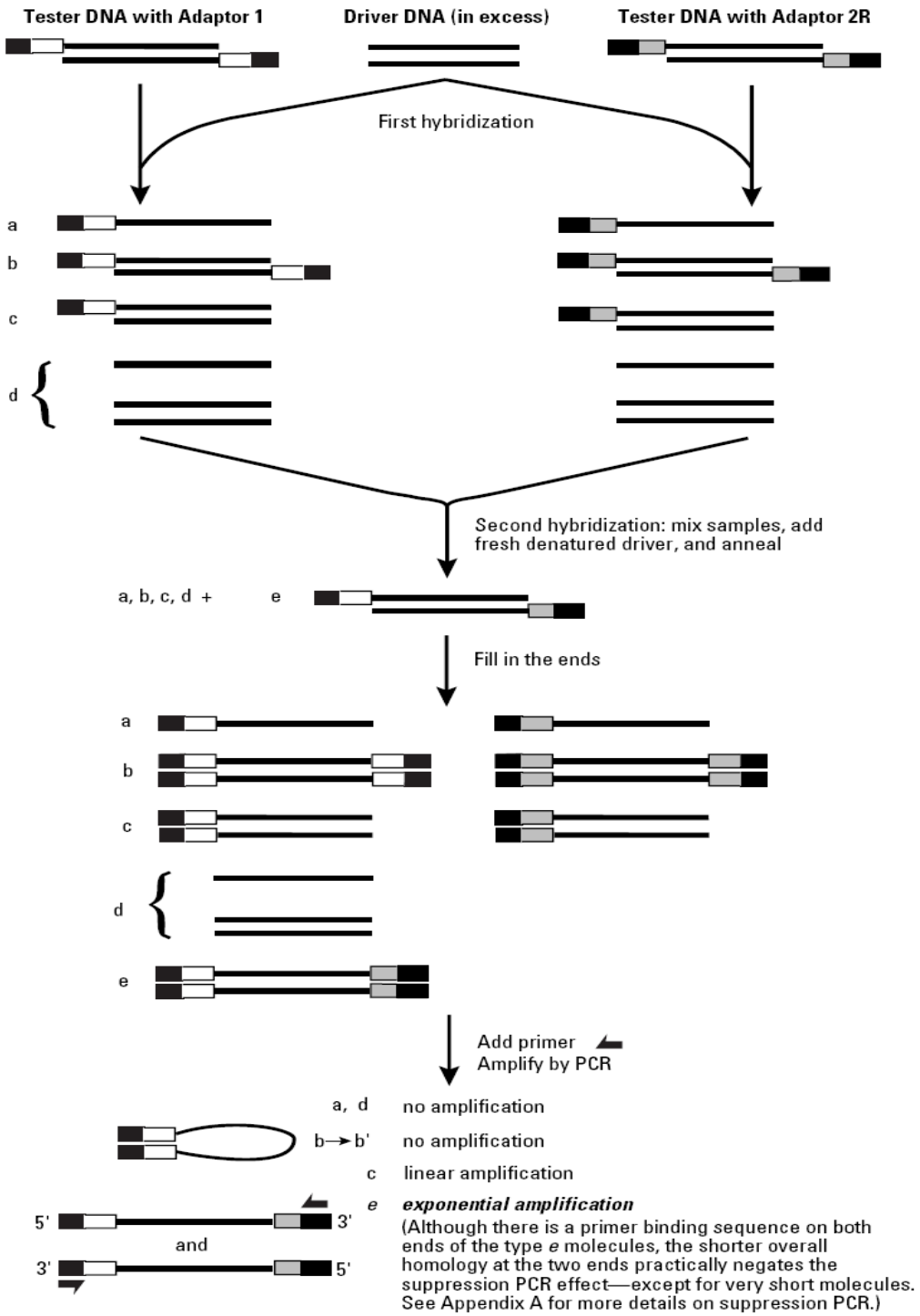

Figure 2: Schematic diagram of BD PCR-Select bacterial genome subtraction kit.
Type *e* molecules are formed only if the sequence is present in the tester DNA, but absent in the driver DNA. Solid lines represent the *Rsa*I-digested DNAs. Solid boxes represent Adaptor 1 and 2R DNA sequences that are homologous to PCR Primer 1. Open boxes represent unique Adaptor 1 DNA sequences and are homologous to Nested Primer 1. Shaded boxes represent unique Adaptor 2R and are homologous to Nested Primer 2R sequence (16).

Table 2: Sequences of the BD PCR-Select adaptors and PCR primers used for RDA.

Adaptor or Primer	Sequence
Adaptor 1	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
Adaptor 2R	CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT
PCR Primer 1	CTAATACGACTCACTATAGGGC
Nested Primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested Primer 2R	AGCGTGGTCGCGGCCGAGGT
*ctr*A Forward	TGTGTTCCGCTATACGCCATT
*ctr*A Reverse	GCCATATTCACACGATATACC
23S RNA Forward	CTACCTTAGGACCGTTATAGTTAC
23S RNA Reverse	GAAGGAACTAGGCAAAATGGTGCC

All primers and adaptors except *ctr*A forward and reverse primers were provided in the Clontech Bacterial Genome Subtraction kit (16).

3.4 CLONING OF RDA-GENERATED FRAGMENTS

3.4.1 Cloning of RDA Secondary PCR Product

A 10 µl aliquot of the final RDA tester-specific secondary PCR product was added to 1 µl of pCR®-XL-TOPO® cloning vector (Invitrogen, Carlsbad, CA) and incubated at room temperature for 5 min. Either 1 µl, 2 µl, or 4 µl of the cloning reaction was transformed into One Shot® TOP10 *E. coli* electrocompetent cells (Invitrogen). *E. coli* cells were electroporated with a BioRad MicroPulser™ and 450 µl of S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose, Invitrogen) was immediately added to cells. The bacterial solution was shaken at 37°C for 1 hour to allow expression of the antibiotic resistance genes. 50 µl, 150 µl, and a resuspended cell pellet in 150 µl S.O.C. medium were each streaked on 50 µg/ml kanamycin (Sigma) Luria-Bertani (LB) agar plates and incubated overnight at 37°C.

A total of 184 colonies were selected at random, sub-cultured onto kanamycin LB/agar plates, and incubated overnight at 37°C. Of the 184 colonies, 132 were screened for the insertion of an RDA-generated fragment by colony PCR with M13 primers (M13 forward, 5'-GTAAAACGACGGCCAG-3' and M13 reverse, 5'-CAGGAAACAGCTATGAC-3') corresponding to the pCR-XL-TOPO vector. Briefly, isolated colonies were resuspended in 50 µl dH_2O and 1 µl of this bacterial suspension was added to a 50 µl AmpliTaq Gold® PCR reaction (Applied Biosystems, Foster City, CA) for amplification. PCR conditions were as follows: 95°C for 5 min. then 30 cycles of 95°C for 1 min., 55°C for 1 min., and 72°C for 1 min followed by 72°C for 7 min. Resulting PCR products were electrophoresed on 1% agarose (Sigma) and ethidium bromide gels with 200 ng of 100 bp (Invitrogen) or φX174/*Hae* III ladder (Invitrogen) to determine the size of the cloned fragments. Gel images were digitally captured on a BioRad GelDoc 2000.

3.4.2 Cloning of RDA Gel-Purified PCR Bands

A 50 µl reaction of RDA tester-specific secondary PCR product was divided into 5 aliquots (10 µl/lane) and run on a 1% agarose/ethidium bromide gel. 4 prominent bands of sizes 1200, 1000, 900, and 850 bp (Bands 1, 2, 3, and 4) were excised from each lane with a scalpel for gel purification with the QIAquick gel extraction kit (Qiagen, Valencia, CA) using the manufacturer's protocol. Duplicate samples were pooled, vacuum-dried, and resuspended in 5 µl of sterile water in order to maximize DNA concentrations. 4 µl of the gel-purified product from each of the 4 bands was added to 1 µl of TOPO TA Cloning® vector (Invitrogen) and incubated for 30 min. at room temperature. 2 µl of the cloning reaction was transformed into One Shot® TOP10 *E. coli* electrocompetent cells (Invitrogen). *E. coli* cells were electroporated and 250 µl

of S.O.C. medium (Invitrogen) was immediately added to cells. The bacterial solution was shaken at 37°C for 1 hour to allow expression of the antibiotic resistance genes. 50 µl and 150 µl of the bacterial solution were each streaked on kanamycin LB/agar plates and incubated overnight at 37°C.

A total of 80 colonies from the Band 1 transformation, 87 from Band 2, 57 from Band 3, and 87 colonies from Band 4 were selected at random, sub-cultured onto kanamycin LB/agar plates, and incubated overnight at 37°C. These colonies were then screened for the insertion of a RDA-generated fragment into the TOPO TA vector using M13 PCR primers corresponding to the vector. Briefly, isolated colonies were resuspended in 50 µl dH_2O and 1 µl of this bacterial suspension was added to a 50 µl AmpliTaq Gold® PCR reaction (Applied Biosystems) for amplification. PCR conditions were as follows: 95°C for 5 min. then 30 cycles of 95°C for 1 min., 55°C for 1 min., and 72°C for 1 min followed by 72°C for 7 min. Resulting PCR products were electrophoresed on 1% agarose/ethidium bromide gels to determine the size of the cloned fragments as previously described.

3.4.3 DNA Sequencing

DNA sequencing was performed on PCR products using M13 forward and reverse primers with the ABI PRISM® BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). PCR products were prepared for sequencing by precipitation with 20% polyethylene glycol (PEG, Sigma) in 2.5M NaCl for 1 hour at room temperature. The precipitated product was centrifuged for 1 hour at 2750 x *g* and washed with 150 µl 70% ethanol twice. The cleaned PCR product was resuspended in 15 µl dH_2O and 1 µl of the PCR product was used as template in the cycle sequencing reactions. Sequencing conditions were as follows: 30 cycles of 96°C for 10 sec.,

50°C for 5 sec., and 60°C for 2 min. Sequence analysis was performed with DNAstar SeqMan and MegAlign software (DNAstar, Madison, WI). Resulting DNA Sequences were screened for homologies with known sequence entries at the National Center for Biotechnology Information database (Bethesda, MD) by BLASTN analysis (17).

3.5 SOUTHERN HYBRIDIZATION

3.5.1 Labeling of Tester and Driver DNA Probes

The DIG High Prime random-primed DNA labeling and detection kit (Roche, Mannheim, Germany) was used for digoxigenin labeling of tester and driver genomic DNA and subsequent detection of tester specific hybrids by Southern Blotting. 1 µg of purified tester and driver genomic DNA was digested with *Eco*R I restriction enzyme (10 U/µl, Invitrogen) and 10X REact®3 buffer (Invitrogen) and incubated overnight at 37°C. After restriction digest clean-up by phenol-chloroform precipitation, the DNA pellet was resuspended in 16 µl sterile water and added to 4 µl DIG-High Prime (Roche). Roche DIG-High Prime is a labeling mixture containing optimal concentrations of random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme, and buffer components for the efficient random primed labeling of DNA. The probe was incubated overnight at 37°C then heated to 65°C for 10 min. Probe yield was determined by blotting serial dilutions of resulting DIG-labeled tester or driver genomic DNA on a positively-charged nylon membrane (Roche) and subjecting the membrane to immunologic detection with anti-digoxigenin-alkaline phosphatase conjugate (Roche) and CSPD (chemiluminescent substrate for alkaline phosphatase, Roche) followed by exposure of X-ray film for 1 – 2 hours (Kodak, Rochester, NY). The relative intensities of the dilution series were compared to serial dilutions

of the DIG-labeled control DNA (pBR328 DNA, 5 μg/ml) to estimate probe yields. The resulting DIG labeled tester and driver genomic DNA probes were diluted to approximately 25 ng/ml in DIG Easy Hyb buffer (Roche) for subsequent Southern Blot analysis.

3.5.2 Southern Blot

The RDA-generated cloned PCR products representing potential tester-specific sequences were denatured at 96°C for 5 min. and immediately placed on an ice bath. 1 μl of PCR product was blotted in duplicate on 2 separate positively-charged nylon membranes designated for tester and driver probes. Membranes were allowed to dry completely then placed on Whatman® filter paper #3 (Whatman International Ltd., Maidstone, England) soaked with 2X AccuGENE® SSC (3.0M NaCl and 0.3M sodium citrate, Cambrex Bio Science Rockland, Inc.). Each membrane was placed face-down on a UV transilluminator (GelDoc 2000, BioRad) for approximately 4 min. to crosslink the dotted RDA-generated PCR products. Prehybridization was performed by placing each membrane in a sealed plastic bag with DIG Easy Hyb buffer (Roche) and incubating at 42°C in a rotating waterbath for 30 min. Hybridization solutions for each DIG labeled probe was made by denaturing the probes in boiling water for 5 min. then immediately adding the denatured probe to 4 ml of DIG Easy Hyb buffer (25 ng random-primed labeled probe/1 ml DIG Easy Hyb buffer). The prehybridization buffer was poured off each membrane and hybridization buffer containing either tester or driver DIG-labeled probes was added. The membranes were incubated overnight in a 42°C rotating waterbath. The next day, the hybridized membranes were washed twice for 5 min. at room temperature in 200 ml low stringency buffer (2X SSC, 0.1% SDS) and twice for 15 min. at 65°C in 200 ml high stringency buffer (0.5X SSC, 0.1% SDS) to eliminate non-specific probe.

Immunologic detection of bound probes was performed according to the manufacturer's instructions (Roche). Briefly, the hybridized membranes were rinsed in 100 ml 1X washing buffer (0.1M Maleic acid, 0.15M NaCl, 0.3% [v/v] Tween 20) for 2 min. followed by incubation in 100 ml blocking solution (supplied in kit) for 30 min. at room temperature. The membranes were incubated for 30 min. in 20 ml anti-digoxigenin-AP solution (1:10,000 dilution), rinsed 2 times in 100 ml 1X washing buffer for 15 min. and equilibrated 2-5 min. in 20 ml 1X detection buffer (0.1M Tris-HCl, 0.1M NaCl). Approximately 1 ml of ready-to-use CSPD was added until membranes were evenly soaked and incubated for 5 min. at room temperature. Membranes were sealed in plastic bags and placed in a 37°C waterbath for 10 min. to enhance the luminescence reaction. The blots were exposed to X-ray film for 1-3 hours at room temperature and film was developed to reveal detection.

3.6 GENETIC ELEMENT PCR

3.6.1 DNA Extractions

DNA extractions were performed on 16 early clone and 18 late clone serogroup C ST11 isolates including RDA tester (MD 1400) and driver (MD1097) isolates using a boil preparation method. *N. meningitidis* isolates were streaked from frozen glycerol stocks onto chocolate agar plates (Becton, Dickinson, and Company) and incubated overnight at 37°C with 5% CO_2. The next day, a thick suspension of organisms was placed in 0.5 ml of BioWhittaker™ phosphate buffer saline (Cambrex Bio Science, Inc., Walkersville, MD), boiled for 20 min., centrifuged, and the supernatant was kept for use. Plasmid preparations were also performed on RDA tester

(MD1400) and driver (MD1097) isolates using the Wizard® Plus Minipreps DNA purification system by following the manufacturer's protocol (Promega, Madison, WI).

3.6.2 pJS-B Plasmid PCR

Primers were designed based on the published pJS-B plasmid sequence (18) (Genbank accession no. AJ277475) using the DNAstar Primer Select software and are listed in Table 3. 1 µl of template DNA from phenol-chloroform, plasmid, or boil preparations of 34 total early and late clone isolates was added to a 25 µl AmpliTaq Gold® PCR reaction (Applied Biosystems) for amplification. Each 25 µl PCR reaction included 16.2 µl sterile water, 2.5 µl 10X buffer, 2.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP mix (10 mM), 1.0 µl forward and reverse primers (10 µM), and 0.5 µl AmpliTaq Gold polymerase (5 U/µl). PCR conditions were as follows: 95°C for 5 min. then 35 cycles of 95°C for 1 min., 54°C for 1 min., and 72°C for 1 min. followed by 72°C for 7 min.

3.6.3 IS1301 PCR

Primers were designed based on published insertion sequence IS1301 data (19) (Genbank accession no. Z49092) and are listed in Table 3. 1 µl of phenol-chloroform or boil prep template DNA from 34 total early and late clone isolates was added to a 25 µl AmpliTaq Gold® PCR reaction (Applied Biosystems) for amplification. Each 25 µl PCR reaction included 16.2 µl sterile water, 2.5 µl 10X buffer, 2.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP mix (10 mM), 1.0 µl forward and reverse primers (10 µM), and 0.5 µl AmpliTaq Gold polymerase (5 U/µl). PCR conditions were as follows: 95°C for 5 min. then 35 cycles of 95°C for 1 min., 54°C for 1 min., and 72°C for 1 min. followed by 72°C for 7 min.

LMK56-2 forward and reverse primers were created using the LMK56 RDA-generated fragment sequence that had partial homology to IS1301 (Table 3, Figure 12). 1 µl of phenol-chloroform or boil prep template DNA from 34 total early and late clone isolates was added to a 25 µl AmpliTaq Gold® PCR reaction (Applied Biosystems) for amplification. Each 25 µl PCR reaction included 16.2 µl sterile water, 2.5 µl 10X buffer, 2.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP mix (10 mM), 1.0 µl forward and reverse primers (10 µM), and 0.5 µl AmpliTaq Gold polymerase (5 U/µl). PCR conditions were as follows: 95°C for 5 min. then 35 cycles of 95°C for 1 min., 54°C for 1 min., and 72°C for 1 min. followed by 72°C for 7 min.

Table 3: PCR primers for detection of genetic elements generated by RDA.

Primer Set	Sequence	Product Size (bp)	Anneal. Temp
pJS-B F pJS-B R	5'-GTGCAAATGGGTCTAAAAATGAAC-3' 5'-AAAAACGCCGCTTGTATCT-3'	1051	54°C
IS1301 F IS1301 R	5'-AGAACCGCAATAACTGACAACATA -3' 5'-GCGATAAAATACTGGGCTTCTT -3'	484	54°C
LMK56-2 F LMK56-2 R	5'-CATTCTCAGCCGCGTATTTCTTCA-3' 5'-CTAGTAACGGCCGCCAGTGTGCT-3'	363	54°C

4.0 RESULTS

4.1 SELECTION OF RDA TESTER AND DRIVER

Thirty-four *N. meningitidis* serogroup C ST11 early/late clone isolates were chosen for analysis by PFGE because they differed in subcapsular genotype only at the *fet*A locus (OMP profile: ST11; *por*B 2-2; *por*A 5-2; *fet*A 1-30 or 3-6). PFGE was performed to determine genetic relatedness among the isolates and to identify a candidate tester/driver pair for RDA. With a few exceptions, the majority of the isolates clustered into either early or late clonal groups (Figure 3) which correlates with the subcapsular OMP genotyping data defined by *por*A, *por*B and *fet*A. The early clonal group was further subdivided into 2 clusters by PFGE. In order to maximize the number of tester-specific sequences generated by RDA, tester/driver pairs with substantial band differences by PFGE were chosen for RDA. Based on the number of band differences and the percent similarities within the PFGE dendrogram, isolate MD1097 was chosen as the representative early clone isolate and MD1400 as the representative late clone isolate for RDA. These isolates exhibit a 10-band difference by PFGE (Figure 4). It is possible that the PFGE defined genetic differences may be due to recombination events involved in the observed emergence of the late clone which may be detected by RDA.

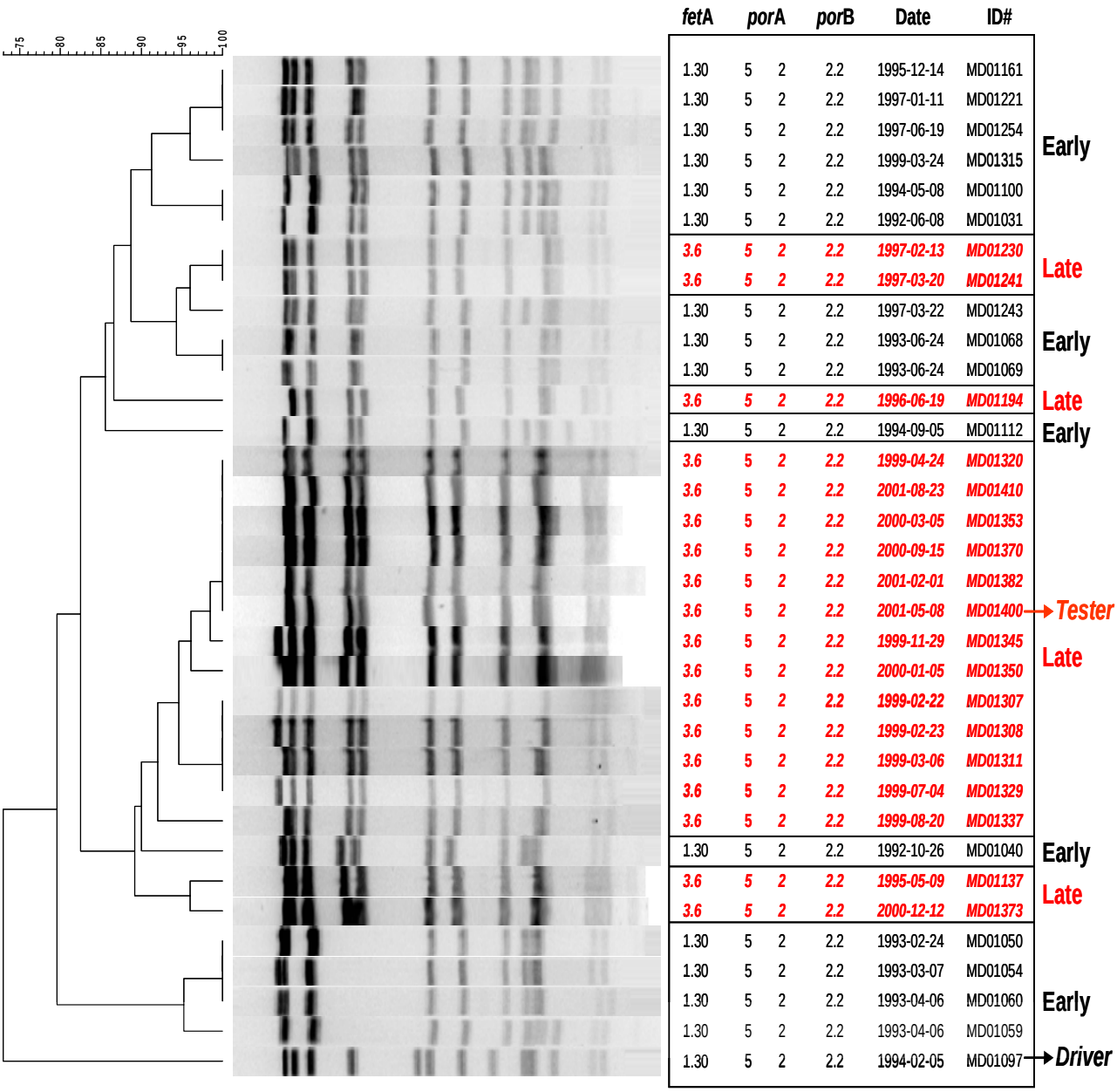

Figure 3: PFGE clustering of serogroup C, ST11, *por*B 2-2, *por*A 5-2 isolates into early and late clones.
Early clone isolates are *fet*A 1-30 and late clone isolates (*red and italicized*) are *fet*A 3-6. The isolates selected for Tester (MD1400) and Driver (MD1097) for RDA are denoted.

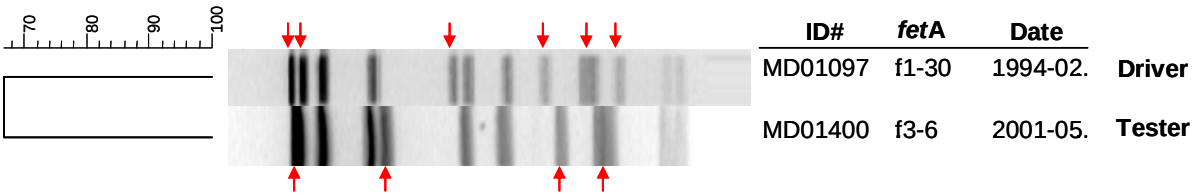

Figure 4: PFGE comparison of driver (early clone) and tester (late clone) used for RDA.
Isolate MD1097 was chosen as driver and isolate MD1400 was chosen as tester for RDA. The red arrows denote the 10 band differences between tester and driver isolates by PFGE.

4.2 RDA ADAPTOR LIGATION EFFICIENCY ANALYSIS

Successful RDA is dependent upon efficient ligation of adaptor sequences to tester genomic DNA for subsequent PCR amplification reactions. To determine the ligation efficiency of adaptors 1 and 2R to the *Rsa*I-digested tester DNA, PCR amplification was performed with a primer specific to the *N. meningitidis ctr*A gene and the adaptor-specific PCR primer 1 (Table 2) to amplify the *Rsa*I fragment containing the *ctr*A gene. A virtual restriction enzyme digest was performed to verify that the *ctr*A gene does not contain a *Rsa*I site and would therefore be a suitable control for adaptor ligation efficiency. Adaptors 1 and 2R were successfully ligated to *N. meningitidis* tester DNA as determined by adaptor-specific PCR with primer 1 and the *ctr*A forward primer (Figure 5, lanes A and B). *ctr*A gene-specific primers (*ctr*A forward and reverse), used as a positive control, amplified the expected size fragment (115 bp) from ligated tester DNA (Figure 5, lanes D and E). In addition, adaptor ligation efficiency on *E. coli Rsa*I-digested genomic DNA was confirmed. To analyze the ligation of control *E. coli* DNA, primers specific to the 23S rRNA gene were used in combination with adaptor-specific PCR primer 1. For *Rsa*I-digested *E. coli* DNA with ligated adaptors, 23S RNA forward primer and PCR primer 1 amplified the expected 374 bp region while 23S RNA forward and reverse primers amplified the expected 270 bp region (data not shown). These experiments demonstrated that the ligation of the RDA adaptor sequences to *Rsa*I-digested tester and control genomic DNA was successful.

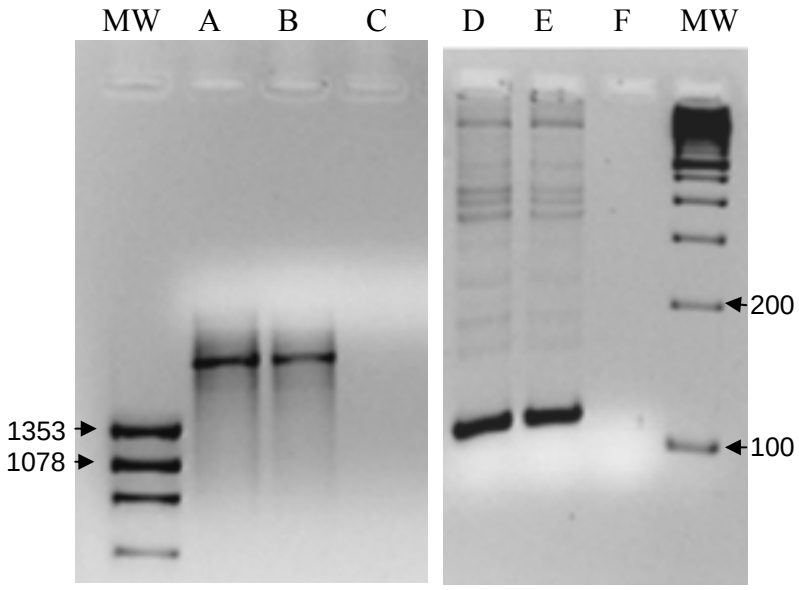

Figure 5: RDA ligation efficiency analysis by PCR amplification.
*N. meningitidis Rsa*I-digested tester DNA with ligated adaptors 1 or 2R was amplified with adaptor-specific primers (PCR primer 1 and *ctr*A forward) (lanes A-C) or gene-specific primers (*ctr*A forward and reverse) (lanes D-F) and run on a 1% agarose/ethidium bromide gel. Lanes: A, tester with adaptor 1; B, tester with adaptor 2R; C, water; D, tester with adaptor 1; E, tester with adaptor 2R; F, water. Molecular weight (MW) markers listed are in base pairs (bp). φX174 RF DNA/*Hae* III ladder is on the left side of the figure and a 100-bp ladder is on the right side.

4.3 RDA-GENERATED TESTER SPECIFIC SEQUENCES

After being ligated to adaptors 1 and 2R, subtracted *N. meningitidis* tester DNA (late clone) underwent two rounds of subtractive hybridization with driver DNA (early clone). Primary PCR amplification with adaptor-specific PCR primer 1 was used to exponentially amplify only ds DNAs with adaptor sequences on each end (Figure 2). Diluted (1:40) and undiluted primary PCR products were used as DNA template for a secondary PCR amplification with adaptor-specific nested primers 1 and 2R to further reduce background and enrich for tester-specific sequences. The secondary PCR amplification generated at least 3 late clone-specific bands that were absent from the early clone as defined by RDA (Figure 6, lanes A & B). Unsubtracted *Rsa*I-digested, adaptor-ligated *N. meningitidis* tester DNA amplified in parallel generated an

expected smear of PCR product representative of the many adaptor-ligated *Rsa*I fragments from the unsubtracted sample (Figure 6, lanes C & D). In addition, the secondary amplification reactions were performed on subtracted and unsubtracted control *E. coli* tester DNA containing 1 copy of *Hae* III-digested φX174 DNA per *E. coli* genome. PCR amplification of the subtracted *E. coli* tester DNA with adaptor specific primers generated major bands that corresponded to the φX174/*Hae* III fragments on the molecular weight ladder (Figure 6, lanes E & F) while the unsubtracted *E. coli* DNA appeared as an expected smear (Figure 6, lanes G & H). The PCR Control Subtracted DNA from the genome subtraction kit provided a positive PCR control containing a successfully subtracted mixture of *Hae* III-digested φX174 DNA fragments (Figure 6, lanes I & J). Thus, PCR amplification with primers specific to the ligated adaptors 1 and 2R successfully amplified at least 3 *Rsa*I fragments from the subtracted *N. meningitidis* late clone tester DNA which may represent late clone specific DNA.

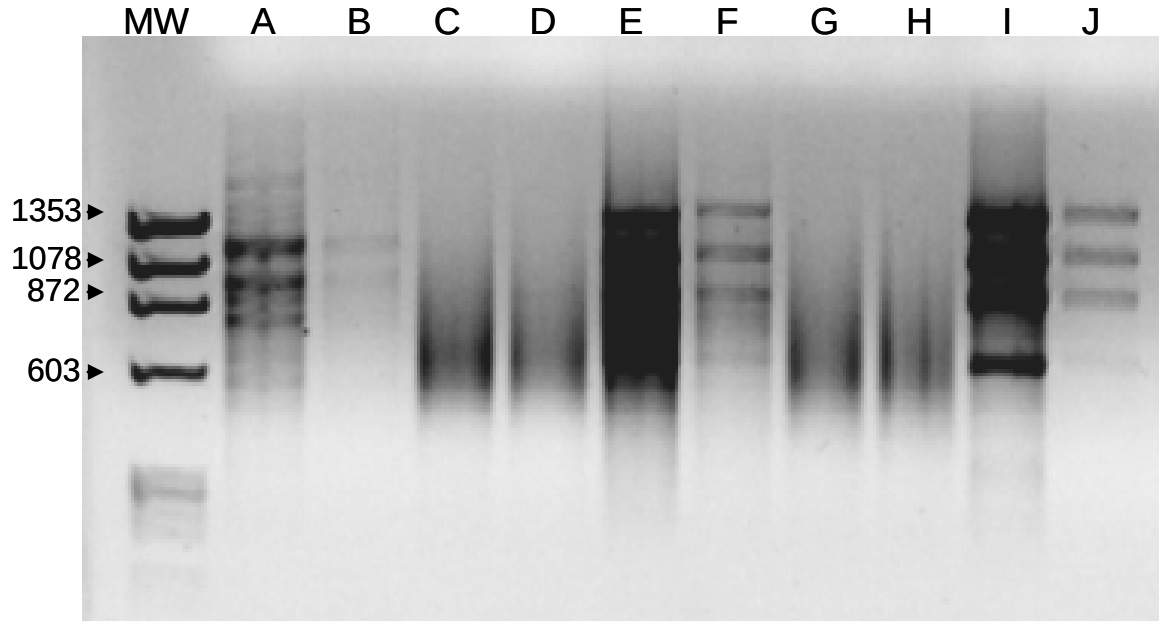

Figure 6: Nested PCR amplification of subtracted and unsubtracted tester DNA.
Lanes: A & B, *N. meningitidis* subtracted tester DNA undiluted and diluted; C & D, *N. meningitidis* unsubtracted tester DNA undiluted and diluted; E & F, *E. coli* subtracted tester DNA containing *Hae* III-digested φX174 DNA undiluted and diluted; G & H, *E. coli* unsubtracted tester DNA containing *Hae* III-digested φX174 DNA undiluted and diluted; I & J, PCR control subtracted DNA containing *Hae* III-digested φX174 DNA undiluted and diluted. Molecular weight (MW) markers of φX174 RF DNA/*Hae* III ladder are listed in base pairs (bp).

4.4 CLONING OF RDA GENERATED TESTER PCR PRODUCTS

The *N. meningitidis* tester PCR products generated by RDA were cloned in 2 separate experiments. In the first experiment, the entire tester-specific RDA secondary PCR reaction was cloned into pCR®-XL-TOPO®. One hundred eighty-four transformants were randomly selected and sub-cultured onto kanamycin LB/agar plates. Of these transformants, 132 colonies grew and were screened for fragment insertion using M13 primers. PCR with the M13 primers identified 119 M13 PCR products with varying fragment insert sizes. Figure 7 shows 40 of these M13 PCR products run on a 1% agarose/ethidium bromide gel displaying fragment sizes ranging from 400-2000 base pairs. These PCR products were further screened by Southern blotting with tester and driver digoxigenin-labeled probes and DNA sequenced to determine if these sequences were tester specific which is described in the next section.

For the second cloning experiment, a 50 µl reaction of RDA tester-specific secondary PCR product was divided into 5 aliquots (10 µl/lane) and run on a 1% agarose/ethidium bromide gel (Figure 8). The 4 most prominent bands of sizes 1200, 1000, 900, and 850 bp (Bands 1, 2, 3, and 4) were excised, gel-purified, concentrated, and cloned into a TOPO TA Cloning® vector (Figure 9). A total of 80 colonies from the Band 1 transformation, 87 from Band 2, 57 from Band 3, and 87 colonies from Band 4 were selected at random, sub-cultured onto kanamycin LB/agar plates, and screened by colony PCR for fragment insertion using M13 primers. One colony from the Band 1 transformation, no colonies from the Band 2 transformation, 5 colonies from the Band 3 transformation, and 1 colony from the Band 4 transformation were positive for fragment insertion into the vector. These 7 total M13 PCR products were DNA sequenced to determine their identity by BLAST analysis.

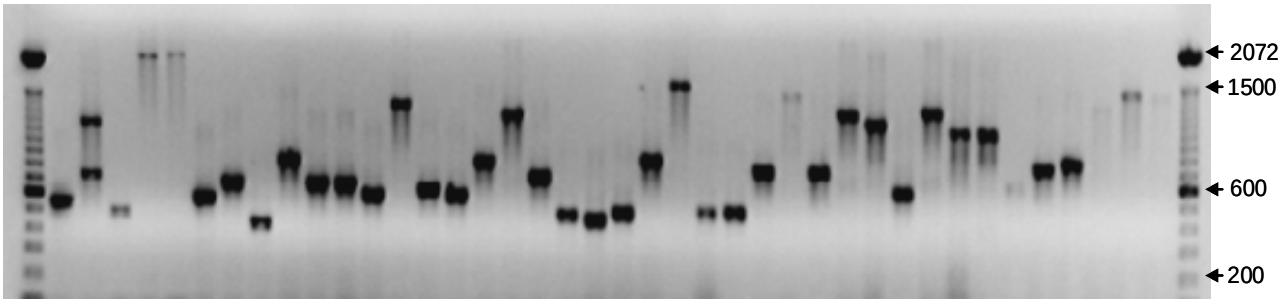

Figure 7: PCR amplification of RDA-subtracted fragments with M13 primers.
The entire tester-specific RDA secondary PCR reaction was cloned into pCR®-XL-TOPO® vector and transformants were screened for fragment insert by PCR with M13 primers. Forty M13 PCR products were run on a 1% agarose/ethidium bromide gel. Molecular weight (MW) markers of a 100 bp ladder are listed in base pairs (bp).

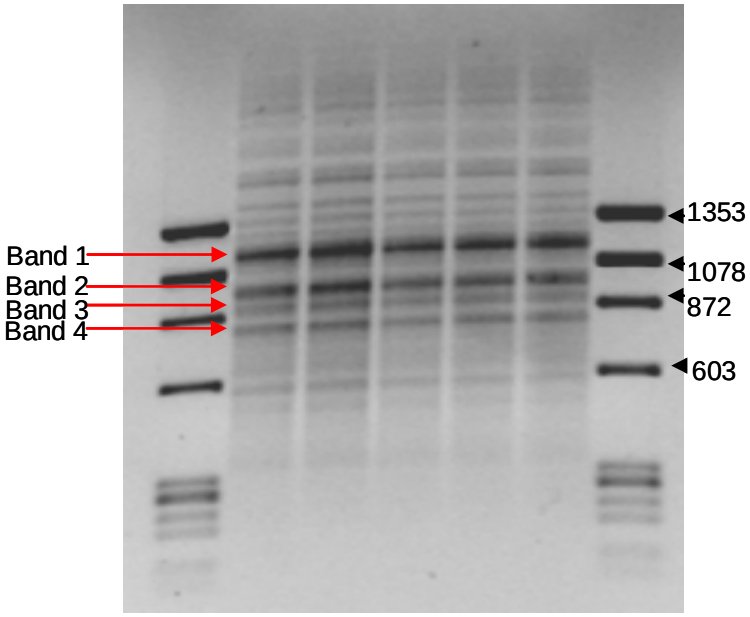

Figure 8: Preparative gel of secondary PCR products from *N. meningitidis* subtracted tester DNA.
A 50 µl reaction of RDA tester-specific secondary PCR product was divided into 5 aliquots (10 µl/lane) and run on a 1% agarose/ethidium bromide gel. 4 prominent bands of sizes 1200, 1000, 900, and 850 bp (Bands 1, 2, 3, and 4) were excised, gel-purified, duplicates pooled together, vacuum-dried, and cloned into a TOPO TA Cloning® vector. Molecular weight (MW) markers of φX174 RF DNA/*Hae* III ladder are listed in base pairs (bp).

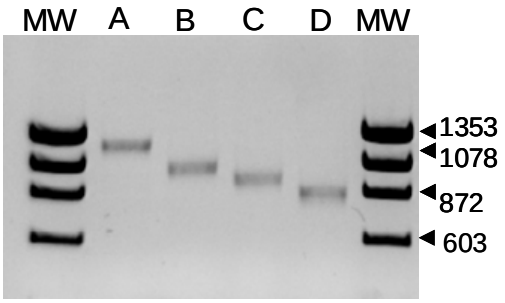

Figure 9: Gel-purified secondary PCR products from *N. meningitidis* subtracted tester DNA.
4 prominent bands from the 5 lanes in Figure 7 were excised and gel-purified using the Qiagen QIAquick gel extraction kit then duplicate samples were pooled together. 10 µl of gel-purified pooled products for Bands 1-4 was run on a 1% agarose/ethidium bromide gel. Lanes: A, Band 1; B, Band 2; C, Band 3; D, Band 4. Molecular weight (MW) markers of φX174 RF DNA/*Hae* III ladder are listed in base pairs (bp).

4.5 SOUTHERN BLOT ANALYSIS

After cloning the entire RDA secondary PCR reaction into a pCR®-XL-TOPO® cloning vector, 119 M13 PCR products with fragment insert were screened by Southern blotting with tester and driver digoxigenin-labeled probes to determine if the inserted fragments were tester-specific. A 1 µl aliquot of each M13 PCR product was blotted in duplicate on 2 separate positively-charged nylon membranes for hybridization with either tester or driver probes. Of the 119 M13 PCR products, 17 (14.3%) were more reactive to the tester DIG-labeled probe in comparison to the driver DIG-labeled probe. Figure 10 shows a representative Southern blot of 58 secondary PCR products blotted in duplicate with either tester or driver DIG-labeled genomic DNA probes. Some background hybridization from the driver probe was observed for all the M13 PCR products and was most likely due to non-specific hybridization from the *Eco*R I-digested DIG labeled genomic DNA. The percentage of clones in the subtracted library corresponding to tester-specific DNA by Southern blotting is typically >50% for the Clontech Bacterial Genome Subtraction kit as stated by the user's manual (16). Therefore, the RDA experiment for the *N. meningitidis* ST11 serogroup C late clone was not optimal.

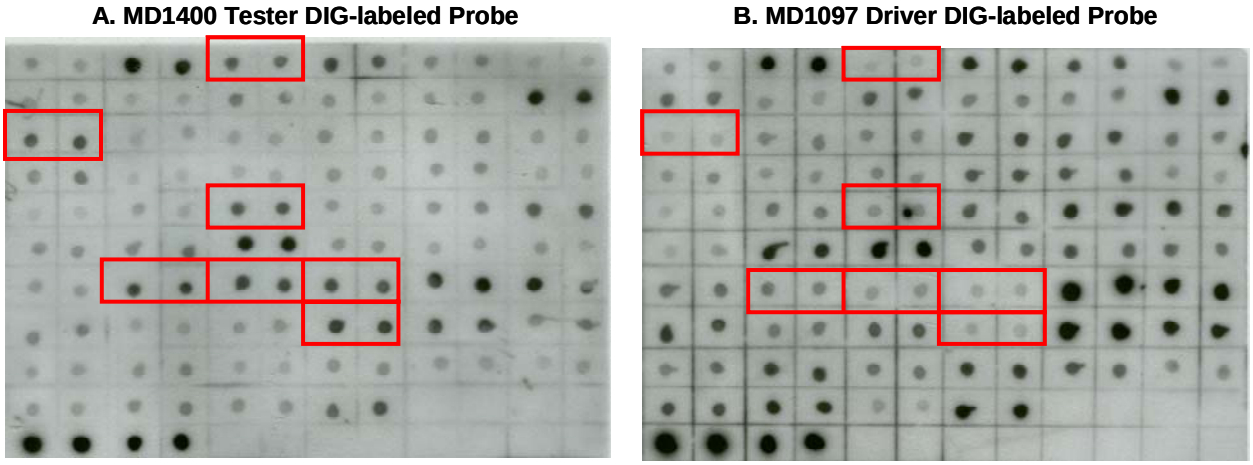

Figure 10: Southern blots of 58 secondary PCR products with either tester or driver DIG-labeled genomic DNA probes.
1 µl of M13 PCR product was blotted in duplicate on 2 separate positively-charged nylon membranes designated for tester and driver digoxigenin-labeled probes. The red boxes outline 7 M13 PCR products that were more specific to the tester probe in comparison to the driver probe.

4.6 HOMOLOGIES OF RDA-SUBTRACTED TESTER-SPECIFIC SEQUENCES

The 17 RDA-subtracted tester-specific M13 PCR products identified by Southern blot were sequenced and a BLAST search was performed on the resulting sequences to find homologies (Table 4). The sequenced insert fragments varied in size from 500 to >1500 bp and all 17 of tester-specific sequences were homologous to either *N. meningitidis* IS1301 insertion sequence (Genbank accession no. Z49092) or *N. meningitidis* pJS-B plasmid sequence (Genbank accession no. AJ277475) with percent homologies ranging from 92.4% to 100%. The 7.2 kb pJS-B plasmid has 8 open reading frames (ORFs) and was first described in *N. meningitidis* clones of the electrophoretic type (ET)-37 complex and the A4 cluster (18). A virtual map of pJS-B plasmid was created using published sequence and DNAstar MegAlign and SeqBuilder software (Figure 11). The 9 RDA-generated fragments aligned at different locations on pJS-B plasmid. Eight of the cloned fragments corresponded to the predicted *Rsa*I restriction fragments for pJS-B

and spanned the 1000-4000 bp area of the plasmid. Some of these predicted *Rsa*I fragments were cloned multiple times and corresponded to the predicted *Rsa*I restriction enzyme pattern for pJS-B (e.g. LMK 14 and 60, or LMK 40, 59 and 121, Figure 11). One RDA-generated fragment (LMK70, 809 bp) demonstrated 92% homology to 800 nucleotides in the 6000 bp region of the plasmid. This fragment did not have corresponding *Rsa*I restriction sites predicted on the pJS-B plasmid map (Figure 11, LMK70). This RDA experiment did not generate clones specific to the *Rsa*I fragments between 4000 bp and 4500bp or between 4500 and 1000 bp on the pJS-B plasmid.

The insertion sequence element, IS1301, is present in multiple copies scattered over the meningococcal genome and is known to be involved in *N. meningitidis* genomic recombination (19). There were 8 RDA-generated fragments that showed homology to IS1301. The majority of the fragments had homology to the 180-844 bp region of this element and corresponded to the predicted *Rsa*I restriction fragments for IS1301 (Figure 12). There were 3 overlapping fragments (LMK56, 132, 141, Figure 12) that extended 585 bp past the end of IS1301. This 585 bp region had no known homology to any other sequence in the BLAST database. The 5' ends of the LMK 132 and 141 fragments corresponded to the predicted *Rsa*I restriction sites in IS1301. While the LMK56 fragment showed approximately 200 bp homology to the 3' end of the IS1301 element, there was no predicted *Rsa*I site corresponding to the start of the LMK56 sequence.

To determine potential locations of IS1301 in the serogroup C genome, a search of the unannotated serogroup C FAM18 genomic sequence was performed (http://www.sanger.ac.uk/). The 844 bp sequence corresponding to IS1301 (accession no. Z49092) was not found in the FAM18 genome. This result indicates that the IS1301 element identified by RDA on the serogroup C ST11 late clone is not present in all serogroup C strains.

In order to determine the identity of the 585 bp region of unknown homology from LMK fragments 56, 132 and 141, we searched the FAM18 genome with this sequence. A 500 bp fragment with 99% nucleotide identity was identified at ~890 kb region of the serogroup C FAM18 genome. BLAST analysis of 5kb flanking regions of this region of the FAM18 genome did not identify any DNA homologies. Thus, RDA identified a unique IS1301 element from the ST11 serogroup C late clone whose 3' flank is homologous to a 500 bp region of unknown function in the serogroup C FAM18 genome.

All 7 M13 PCR products obtained from the second cloning experiment with the 4 gel-purified RDA bands produced ~700 bp band when gel electrophoresed. After being DNA sequenced, it was noted that all 7 sequences aligned with each other, were 415 bp in length, and were homologous to *N. meningitidis* serogroup A, strain Z2491 complete genome, segment 6/7 (Genbank accession no. AL162757). After locating the homologous region of the RDA-generated fragments (26-403 bp) on the Z2491 genome, it was also noted that this area was 97.9% homologous to a partial region of the nusA gene or N utilization substance protein A (159837-159460 bp). NusA is a transcription elongation factor that binds to RNA polymerase and RNA, stimulates rho independent termination by promoting hairpin formation, and also interacts with N protein and prevents hairpin formation thereby preventing termination (Genbank Gene ID no. 907527).

Table 4: Top homology hits for tester-specific sequences.

Top Blast Hit	Colony ID	M13 PCR Band (bp)	Sequenced Insert Fragment (bp)	% Homology
N. meningitidis **IS1301**	LMK3	500	235	99.5%
insertion sequence	LMK50	1000	754	97.1%
Genbank Acc. No.	LMK56	1300	938	94.9%
Z49092	LMK132	1300	986	100%
	LMK140	>1500	1157	99.7%
	LMK141	>1500	1179	99.7%
	LMK180	500	235	99.5%
	LMK186	500	236	100%
N. meningitidis	LMK14	1300	1100	99.5%
ORF 1-8 from	LMK40	1400	1169	100%
pJS-B plasmid	LMK59	1400	719	100%
Genbank Acc. No.	LMK60	1400	1111	99.5%
AJ277475	LMK70	>1500	809	92.4%
	LMK121	1500	1180	99.8%
	LMK122	800	350	99.7%
	LMK151	1200	803	99.9%
	LMK169	600	350	99.4%

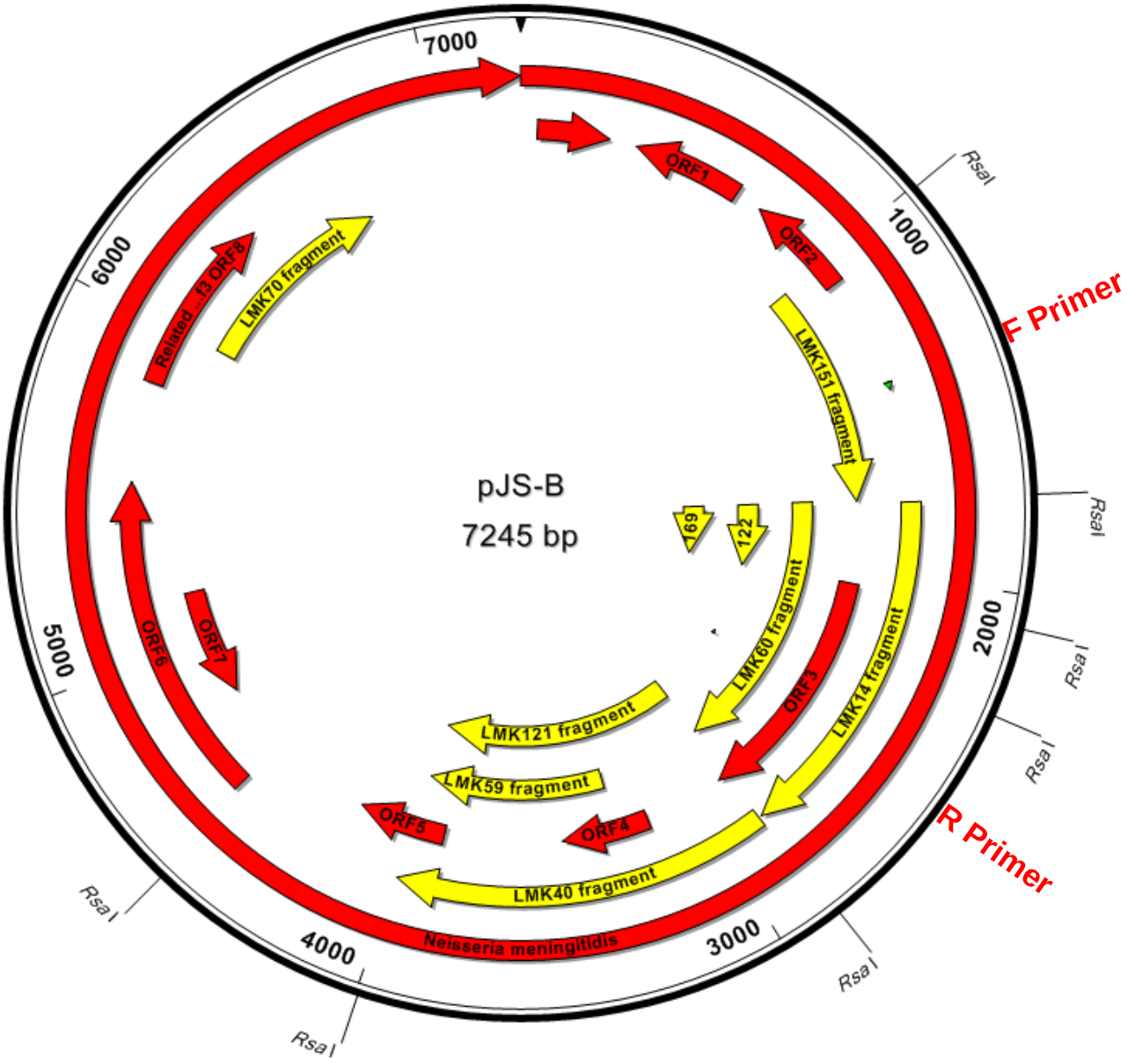

Figure 11: pJS-B plasmid map with aligned RDA-generated fragments and *Rsa*I restriction sites.
Map was created using DNA sequence from Genbank accession number AJ277475 and DNAstar MegAlign and SeqBuilder software (18). pJS-B F and R primers (Table 3) were generated to detect pJS-B plasmid sequences in *N. meningitidis* serogroup C ST11 early and late clone isolates by PCR.

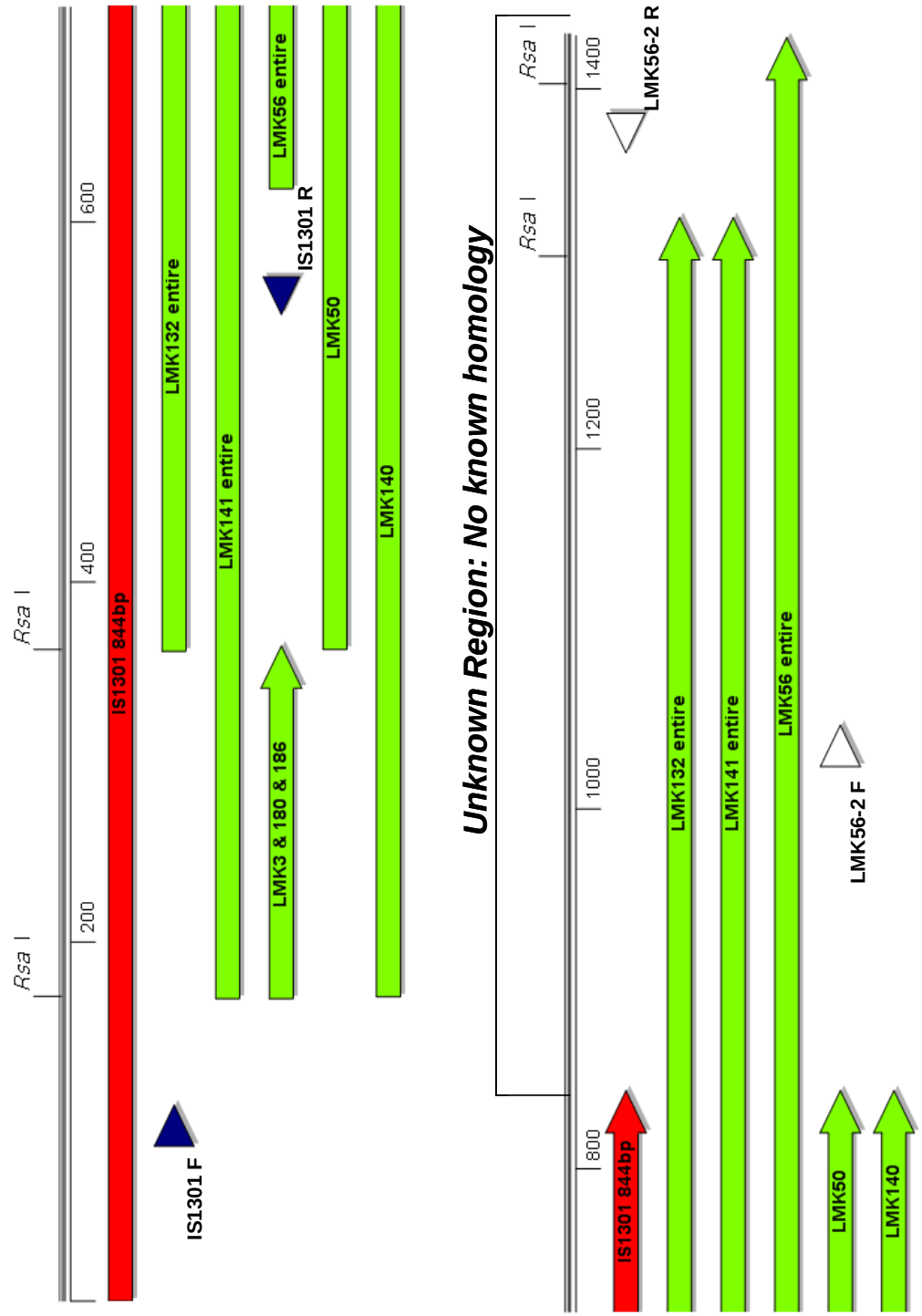

Figure 12: IS1301 insertion sequence map with aligned RDA-generated fragments and *Rsa*I restriction sites. Map was created using DNA sequence from Genbank accession number Z49092 and DNAstar MegAlign and SeqBuilder software (19). Short arrows denote forward and reverse primer pairs generated to detect IS1301 sequences and the 585 bp extended region of unknown homology in *N. meningitidis* serogroup C ST11 early and late clone isolates by PCR.

4.7 PCR DETECTION OF pJS-B PLASMID

pJS-B plasmid PCR primers (Table 3) were generated to determine whether the pJS-B plasmid sequences were tester-specific by PCR amplification on *N. meningitidis* late clone isolates. These primers were specific to a 1051 bp region spanning nucleotides 1400-2450 on the pJS-B published sequence (Figure 11, F Primer, R Primer). Genomic and plasmid DNA prepared from the tester strain (MD1400) amplified a 1051 bp band corresponding to the predicted pJS-B product. In contrast, no PCR product was obtained from the corresponding DNA preparations from the driver strain (MD1097) (Figure 13). The same pJS-B primers were also used to amplify boil prep DNA from *N. meningitidis* serogroup C ST11 early and late clone isolates (34 total including RDA tester and driver) to determine if pJS-B plasmid sequences were specific to the late clone. pJS-B plasmid specific products were amplified from 17 out of 18 (94%) late clone isolates. In contrast, none of the early clone isolates amplified pJS-B plasmid sequences (Figure 14). These data indicate that pJS-B is specific to strains of the late clone lineage.

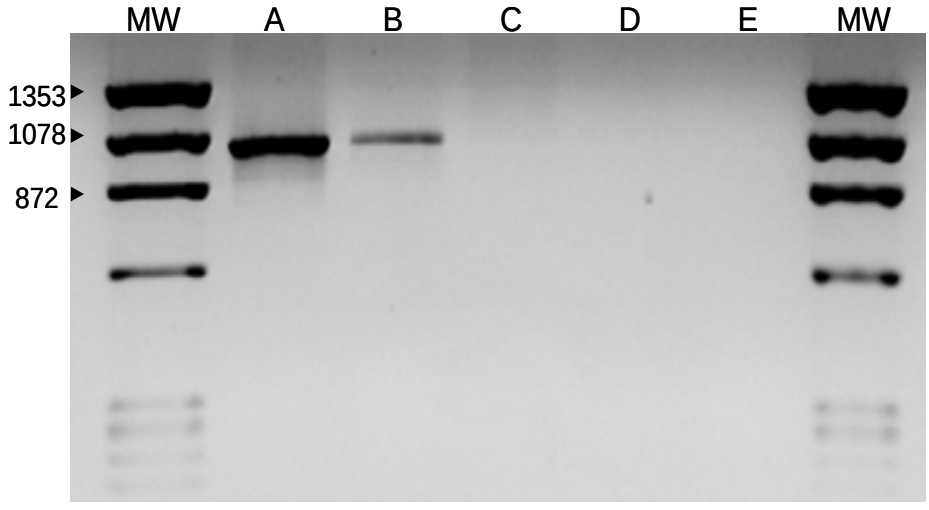

Figure 13: PCR amplification of RDA tester and driver DNA with pJS-B primers.
Lanes: A, MD 1400 (RDA tester) phenol-chloroform preparation; B, MD1400 (RDA tester) plasmid preparation; C, MD1097 (RDA driver) phenol-chloroform preparation; D, MD1097 (RDA driver) plasmid preparation; E, water. Molecular weight (MW) markers of φX174 RF DNA/*Hae* III ladder are listed in base pairs (bp).

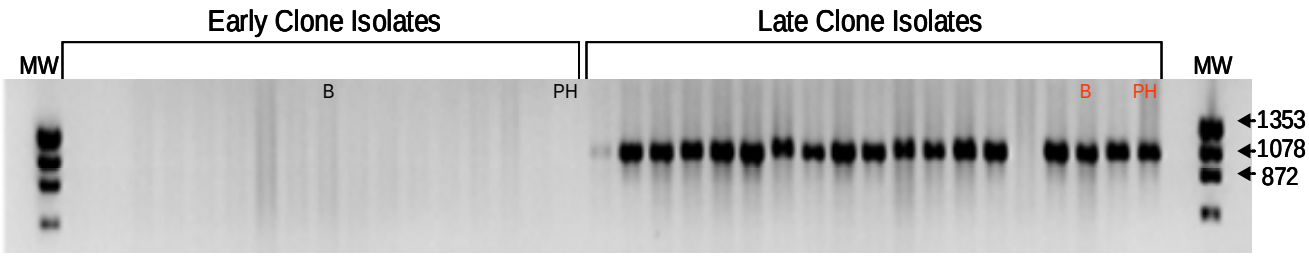

Figure 14: PCR amplification of *N. meningitidis* serogroup C ST11 early and late clone isolates with pJS-B primers.
16 early clone and 18 late clone boil preparations including RDA driver (early) and tester (late) and phenol-chloroform preparations of driver and tester were amplified with pJS-B primers and the products were run on a 1% agarose/ethidium bromide gel. Boil preparations (B) and phenol-chloroform preparations (PH) of driver DNA (early) are denoted while the same preparations for tester DNA (late) are denoted in red (B and PH). Molecular weight (MW) markers of φX174 RF DNA/*Hae* III ladder are listed in base pairs (bp).

4.8 PCR DETECTION OF IS1301

IS1301 PCR was performed to determine whether IS1301 was specific to DNA from the tester strain and other late clone isolates. The IS1301 primers are specific to a 484 bp region spanning nucleotides 87-570 bp on the IS1301 published sequence (Figure 12, IS1301-F, IS1301-R). IS1301 specific sequences were amplified from 18 out of 18 (100%) late clone isolates including the RDA tester isolate (MD1400). In contrast, none of the early clone isolates or the RDA driver isolate (MD1097) were IS1301 positive by PCR (Figure 15). These data indicate that the IS1301 element identified by RDA is specific to the *N. meningitidis* serogroup C ST11 isolates of the late clone lineage.

To determine whether the 585 bp region of unknown homology was specific to the late clone, primers specific to the LMK56 sequences were designed (Figure 12, LMK56-2F, LMK56-2R). This primer set was unsuccessful in amplifying the expected 363 bp fragment from all 34 early and late clone isolates including boil and phenol-chloroform preparations of RDA tester (MD1400) and driver (MD1097). Instead, a 200 bp band or no band at all was observed when

the LMK56-2 PCR products were run on a 1% agarose/ethidium bromide gel (Figure 16). As a result, it could not be determined if the 585 bp region of unknown homology was specific to the late clone isolates. The optimization of PCR conditions and/or the creation of new primer sets spanning this region are needed to further explore this result.

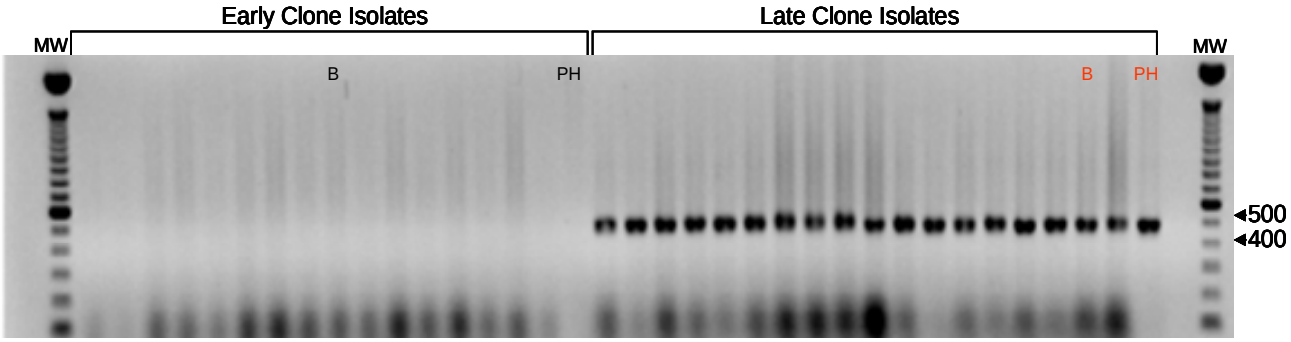

Figure 15: PCR amplification of *N. meningitidis* serogroup C ST11 early and late clone isolates with IS1301 primers.
16 early clone and 18 late clone boil preparations including RDA driver (early) and tester (late) and phenol-chloroform preparations of driver and tester were amplified with IS1301 primers and the products were run on a 1% agarose/ethidium bromide gel. Boil preparations (B) and phenol-chloroform preparations (PH) of driver DNA (early) are denoted while the same preparations for tester DNA (late) are denoted in red (B and PH). Molecular weight (MW) markers of a 100 bp ladder are listed in base pairs (bp).

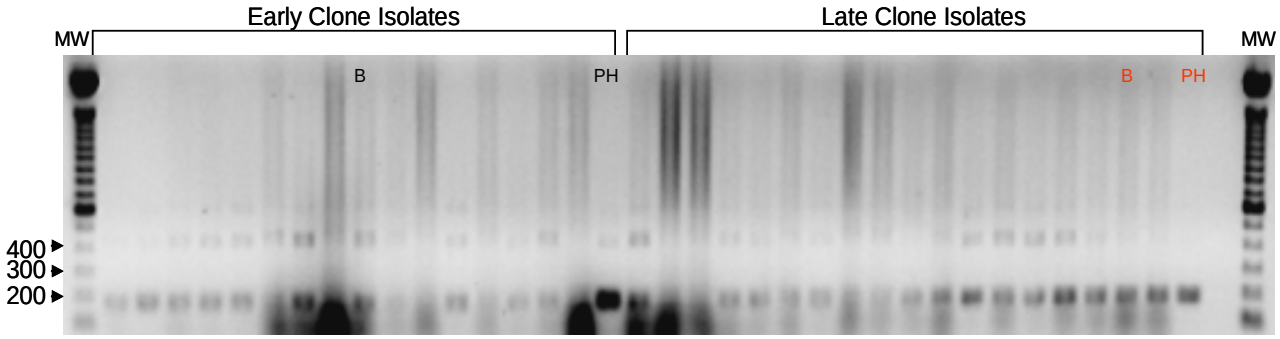

Figure 16: PCR amplification of *N. meningitidis* serogroup C ST11 early and late clone isolates with LMK56-2 primers.
16 early clone and 18 late clone boil preparations including RDA driver (early) and tester (late) and phenol-chloroform preparations of driver and tester were amplified with LMK56-2 primers and the products were run on a 1% agarose/ethidium bromide gel. Boil preparations (B) and phenol-chloroform preparations (PH) of driver DNA (early) are denoted while the same preparations for tester DNA (late) are denoted in red (B and PH). Molecular weight (MW) markers of a 100 bp ladder are listed in base pairs (bp).

5.0 DISCUSSION

In this study, RDA was successful in identifying two unique genetic elements, pJS-B plasmid and IS1301, that were present in an emergent *N. meningitidis* serogroup C ST11 clone that had undergone antigenic shift at FetA. In addition, RDA identified a partial region of the nusA gene and a 585 bp fragment with homology to a region of unknown function on the serogroup C FAM18 genome. PFGE identified clusters of temporally distinct isolates designated early and late clones which correlated with subcapsular OMP genotyping data defined by *por*A, *por*B, and *fet*A. Candidate tester and driver isolates were chosen for RDA based on PFGE band differences in order to maximize the number of tester-specific sequences generated by RDA. *Rsa*I-digested tester genomic DNA (late clone) was successfully ligated to specific adaptors followed by two rounds of subtractive hybridization with *Rsa*I-digested driver genomic DNA (early clone). PCR amplification of subtracted tester DNA with adaptor specific primers generated at least three late clone-specific bands that were absent from the early clone. After cloning these products, Southern blotting with tester and driver DIG-labeled probes confirmed a percentage to be tester-specific. Tester-specific products were DNA sequenced and a BLAST search revealed that all late-clone specific sequences were homologous to either pJS-B plasmid or IS1301 sequences. PCR with primers specific to either IS1301 or pJS-B plasmid sequences amplified these elements from late clone isolates but not from early clone isolates therefore these elements may play a role in the emergence of the serogroup C ST11 late clone in Maryland during the 1990s.

The pJS-B plasmid was originally identified by RDA comparing *N. meningitidis* ET-37 (analogous to ST11 complex defined by MLST) and ET-5 complexes (analogous to ST32 complex defined by MLST) (18). Both ET-37 and ET-5 complexes include a number of different serogroups. In Europe, serogroup B disease is caused mainly by ET-5 whereas serogroup C disease is caused by the ET-37 complex and the A4 cluster (18). The pJS-B plasmid was identified in most ET-37 complex strains (88%) and in many of the A4 cluster strains (58.3%) that were tested. Among the ET-37 complex strains negative for pJS-B was strain FAM18 from serogroup C, which is currently undergoing genomic annotation at the Sanger Centre (18). An ET-37 complex variant was known to cause increased incidence of serogroup C disease in various regions of the world during the 1990s while the A4 clone-complex has been associated with global epidemics and hyperendemic waves of disease (20). In this same study, Southern Blot analysis demonstrated that the pJS-B occurred as a plasmid and as a chromosomal integration in isolates of the ET-37 complex (18). In the current study, the location of pJS-B could not be determined based on the PCR and Southern blots since pure plasmid and genomic DNA preparations could not be assured. Future experiments with Southern blots similar to those described by Claus *et al.* are necessary to determine if pJS-B is integrated into the late clone genome. In addition, these experiments will enable cloning of the pJS-B insertion site. Interestingly, the LMK70 fragment does not correspond to any predicted *Rsa*I restriction sites on the pJS-B plasmid suggesting that this 800 bp plasmid fragment may represent a chromosomal insertion site (Figure 11). It is possible that the RDA fragments that do not correspond to *Rsa*I sites on the published sequence contain *Rsa*I recognition sites (5'-GTAC-3') at the ends of the fragments requiring further analysis. In addition, no clones were identified that corresponded to the predicted *Rsa*I restriction fragment between ~4000 and ~ 4500bp on the

pJS-B plasmid map. These discrepancies are suggestive of genomic recombination but further studies will be required to investigate these regions as potential integration sites. Future experiments also include generating primers to the 3.5 kb region (4500 -1000 bp) of the pJS-B plasmid that does not contain an *Rsa*I site to determine if the entire pJS-B sequence is present in late clone isolates. In addition, the epidemiology of late clone isolate MD1373 needs to be examined because it is the only late clone isolate that did not amplify pJS-B sequences.

The role of pJS-B to *N. meningitidis* virulence is not known. Claus, *et al.* were unable to classify pJS-B plasmid type and were unclear whether it contributes to pathogenicity because no homology to virulence genes could be found by comparison with public databases. Furthermore, not all pathogenic ET-37 strains carry the pJS-B plasmid, indicating that it is not required for virulence (18).

IS1301 has been classified as a member of the IS5-family, group IS427 of insertion sequences and was first described as occurring in 12 copies in *N. meningitidis* serogroup B strain B1940 (21). IS1301 consists of 844 bp and includes two overlapping open reading frames, which are flanked by inverted repeats of 19 bp. It exhibits site-specificity for the target sequence 5'-AYTAG-3', and its insertion results in duplication of the central AT (22). Through insertion/excision, IS1301 has been shown to promote a reversible inactivation of an essential *N. meningitidis* sialic acid biosynthesis gene, *sia*A (or *syn*A), that regulates both capsule expression and endogenous LOS (lipooligosaccharide) sialylation (19). This is important because the bacterium's penetration of the mucosal barrier may require a down-regulation or off-switching of capsule expression, enabling passage through the mucosal epithelium, followed by a re-expression of the cell surface sialic acids to resist the host's immune defenses (19). IS1301 has also been found to cause insertional inactivation of *N. meningitidis* PorA expression. PorA is a

major antigen of the meningococcus and target for bactericidal antibodies making it a useful immunogen for vaccines (23).

In a study examining the distribution of IS1301 in clonal lineages of *N. meningitidis*, IS1301 was detected in only 13-20% of serogroup A, B, and C strains, which are the serogroups causing more than 90% of the disease worldwide, but in 46-89% of serogroup W135, 29E, X, and Y strains. More importantly, IS1301 was found in only 3% of serogroup C ET-37 complex strains which by MLST are analogous to the serogroup C ST11 early and late clone isolates that were the basis of this study (22). These data correlate with the observation that IS1301 was absent from the serogroup C FAM18 genome and all serogroup C ST11 early clone isolates in this study. Because IS1301 occurs most frequently in meningococcal serogoups other than A, B, and C, it is interesting that we found 100% of serogroup C ST11 late clone isolates to be positive for IS1301. By Southern blotting and hybridization of a macrorestriction assay of the *N. meningitidis* serogroup C late clone genome with an IS1301-specific probe, the copy number of IS1301 could be determined as described by Hilse *et al.* (21). The genomic restriction fragments that hybridized to IS1301 sequence could then be cloned and sequenced to determine the different target site specificities for this element. Previous studies have described IS1301 insertion within *sia*A, the first gene of the capsule biosynthesis pathway, while one insertion site was located upstream of *frp*C, a gene encoding an iron-regulated protein in *N. meningitidis* (21).

In order to examine the role of IS1301 in the emergent *N. meningitidis* serogroup C ST11 late clone, the insertion site of this element would need to be located, which is beyond the scope of this project. Genome insertion location is important in determining IS1301's effect on gene expression therefore shedding light on a possible role in *N. meningitidis* pathogenesis. Virtual mapping of the RDA-generated LMK56 fragment on the serogroup C FAM18 genome helped to

approximate the insertion location of IS1301 at ~890 kb region. A BLAST search determined the capsular synthesis genes (e.g. *ctr*A, *syn*A, *syn*B, *syn*C) in FAM18 to be located at ~68 kb region. Therefore, it is assumed that IS1301 does not play a role in the inactivation of *syn*A gene as described because the probable insertion site is distant from *syn*A and other capsular synthesis genes. PCR could not determine if the 585 bp fragment with homology to a region of unknown function on the serogroup C FAM18 genome is specific to the late clone lineage. Southern blotting of early and late clone isolates with a LMK56 DIG-labeled probe could help determine late clone specificity of this region.

RDA also identified a partial region of the *N. meningitidis* nusA gene after cloning 4 gel-purified RDA bands of size 1200, 1000, 900 and 850 bp. All colonies from these individually cloned bands produced ~700 bp band when amplified with M13 PCR primers and the same 415 bp sequence after removing TOPO TA vector sequence. We expected inserted fragments of varying size with homologies to different locations spanning the nusA gene because RDA bands of varying size were cloned. Future experiments should include performing plasmid mini-prep extractions on transformed colonies, cutting with *Eco*R I restriction enzyme, and separating the digest by gel electrophoresis. The resulting bands should correspond to the size of the cloned RDA band determining if the cloning experiment was successful.

Future experiments for this project include using early and late clones in an animal model to test for virulence to provide a more definitive answer regarding the role of RDA-generated elements in *N. meningitidis* pathogenesis. In addition, Southern blotting and cloning can be performed to determine pJS-B and IS1301 copy number and insertion locations to gain insight on their possible regulation of gene expression and/or antigenic variation. Finally, early and late clones for this study were defined based on antigenic shift at the *fet*A gene locus although *fet*A

differences were not identified by RDA. This is probably due to the lack of *Rsa*I restriction sites in the *fet*A 1-30 and 3-6 alleles making it difficult for RDA to identify fragments in the *fet*A region.

Mobile genetic elements, like plasmids and insertion sequences, are found in many different bacterial species in which they play a role in genetic rearrangement and genomic plasticity. The transposition of insertion elements is known to cause a number of effects, including insertions, deletions, and the fusion of two genetic elements, resulting either in silent mutations, in knockout of gene expression, or in the regulation of downstream-located genes (21). In this study, RDA identified two genetic elements, pJS-B plasmid and IS1301, that may play a role in the emergence of *N. meningitidis* serogroup C ST-11 late clone in Maryland during the 1990s. In addition, RDA identified a partial region of the nusA gene and a 585 bp fragment with homology to a region of the serogroup C FAM18 genome. Genetic rearrangements with these elements could help create bacterial diversity and may have assisted the emergent late clone in preadapting to environmental changes and evading the host's immune response. Additional studies are needed to examine the role of these genetic elements, if any, in *N. meningitidis* pathogenesis and their association with increases in serogroup C meningococcal disease incidence.

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