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# Genetic characterization of a new variant within the ET-37 complex of *Neisseria meningitidis* associated with outbreaks in various parts of the world

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## SUMMARY

A new variant within the electrophoretic type (ET)-37 complex of *Neisseria meningitidis*, ET-15, first detected in Canada in 1986, has been associated with severe clinical infections and high mortality rates in several European countries, Israel and Australia. To ascertain the genetic and epidemiological relationships of ET-15 strains from different geographical areas, 72 ET-15 isolates from 10 countries were compared to 13 isolates representing other clones of the ET-37 complex. The 85 strains were analysed by pulsed-field gel electrophoresis (PFGE) using 2 restriction endonucleases and Southern hybridization with 10 genetic markers. Four ET-15 strains and 4 other strains of the ET-37 complex were further examined using an additional restriction enzyme and a total of 18 genetic markers. PFGE fingerprints of the ET-15 strains were closely related. Strains within each country were even more closely related, suggesting single introductions of the clone. Physical mapping of genes in ET-15 and other strains of the ET-37 complex demonstrated that large genetic rearrangements of the genome have occurred in association with the appearance of the ET-15 variant.

## INTRODUCTION

During the early 1990s outbreaks of serogroup C meningococcal disease occurred in Canada [1, 2]. As a result of the public health emergency that developed, mass vaccination campaigns were undertaken [2]. Multilocus enzyme electrophoresis, which has been used extensively for genotyping of *Neisseria meningitidis* [3, 4], revealed that the strains responsible for these outbreaks belonged to the ET-37 complex, but were characterized by a rarely occurring allele at the fumarase locus [1]. This new variant of ET-37 complex was designated ET-15. Retrospective analysis of disease causing strains in Canada showed that the ET-15 variant first appeared in 1986 and then spread throughout the whole country. In 1997, over 80% of serogroup C disease in Canada was associated with ET-15 [F. E. Ashton, unpublished data]. ET-15 was subsequently associated with meningococcal disease in parts of the United States [5, 6], Israel [7], the Czech Republic [8], Iceland [7], Finland [7], Norway [7], England [9] and Australia [10, 11]. Disease caused by the ET-15 variant commonly affects the 15–19 years age group, in contrast to the usual pattern of higher rates of meningococcal disease in age groups under 4 years. Morbidity and mortality are greater than they have been previously seen with other strains of the ET-37 complex [2].

Apart from alterations in the fumarase housekeeping gene, which has allowed differentiation of this new strain, little is known about other genetic events associated with the occurrence of this clone, which, apparently, has increased virulence. The aims of this

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Table 1. *Characteristics of the 85 N. meningitidis isolates of the ET-37 complex*

| Strain number | Country of origin | Phenotype | Year of isolation | *Spe*I* | *Nhe*I* |
|---|---|---|---|---|---|
| ET-15 isolates | | | | | |
| 88135 | Canada | C:2a:P1.5,2 | 1988 | A | B |
| 2111 | Australia | C:2a:P1.5 | 1996 | A | AH |
| 90232 | Canada | C:2a:P1.5,2 | 1990 | A | D |
| 88080 | Canada | C:2a:P1.2 | 1988 | A | B |
| M702/91 | Iceland | C:2a:NST† | 1991 | A | C |
| 94N369 | Australia | C:2a:P1.5 | 1994 | B | H |
| 94N266 | Australia | C:2a:P1.5 | 1994 | C | H |
| 95N668 | Australia | C:2a:P1.5 | 1995 | C | J |
| 31515 | Finland | C:2a:P1.2 | 1994 | C | Z |
| 311671 | England | C:2a:P1.5,2 | 1995 | C | R |
| 11/94 | Norway | C:2a:P1.5 | 1994 | D | C |
| 86/94 | Norway | C:2a:P1.5,2 | 1994 | E | G |
| 934286 | England | C:2a:P1.5 | 1993 | F | N |
| 31648 | Finland | C:2a:P1.5 | 1994 | G | AB |
| 76486 | Finland | C:2a:P1.5 | 1995 | G | AE |
| 2/93 | Czech Republic | C:2a:P1.5,2 | 1993 | H | V |
| 23/93 | Czech Republic | C:2a:P1.5,2 | 1993 | H | V |
| 186/93 | Czech Republic | C:2a:P1.2 | 1993 | H | V |
| 259/93 | Czech Republic | C:2a:P1.2 | 1993 | H | V |
| 284/93 | Czech Republic | C:2a:P1.2 | 1993 | H | V |
| 287/93 | Czech Republic | C:2a:P1.2 | 1993 | H | V |
| 343/93 | Czech Republic | C:2a:P1.2 | 1993 | H | V |
| 344/93 | Czech Republic | C:2a:P1.5,2 | 1993 | H | V |
| 393/93 | Czech Republic | C:2a:P1.5,2 | 1993 | H | V |
| 490/93 | Czech Republic | C:2a:P1.2 | 1993 | H | W |
| 17 | Finland | C:2a:P1.5 | 1992 | I | Y |
| 58 | Finland | C:2a:P1.2 | 1994 | I | Z |
| 61 | Finland | C:2a:P1.2 | 1994 | I | Z |
| 67 | Finland | C:2a:NST | 1994 | I | AA |
| AK20 | Greece | C:2a:P1.5,2 | 1989 | J | T |
| 76375 | Finland | C:2a:P1.5 | 1995 | K | AD |
| 92001 | Canada | C:2a:P1.5,2 | 1992 | L | C |
| 310894 | England | C:2a:P1.5,2 | 1995 | M | P |
| M929 | Israel | C:2a:P1.2 | 1993 | N | G |
| 81/94 | Norway | C:2a:P1.5,2 | 1994 | O | C |
| 97N005 | Australia | C:2a:P1.5 | 1997 | P | L |
| 54602 | Australia | C:2a:P1.5 | 1996 | P | L |
| 93487 | Canada | C:2a:P1.2 | 1993 | P | B |
| 50060 | Australia | C:2a:P1.5 | 1996 | P | L |
| 50447 | Australia | C:2a:P1.5 | 1996 | P | L |
| 92029 | Canada | C:2a:P1.5,2 | 1992 | P | F |
| 57463 | Australia | C:2a:P1.5 | 1996 | Q | K |
| DM016 | Australia | C:2a:P1.5 | 1996 | R | L |
| 311268 | England | C:2a:P1.5 | 1995 | S | N |
| M877 | Israel | C:NT‡:P1.5,2 | 1993 | T | G |
| M881 | Israel | C:NT:P1.5,2 | 1993 | T | G |
| M970 | Israel | C:NT:P1.5,2 | 1994 | T | G |
| M837 | Israel | C:2a:P1.2 | 1992 | U | G |
| M894 | Israel | C:2a:P1.5,2 | 1993 | U | G |
| M951 | Israel | C:NT:P1.5,2 | 1993 | U | U |
| M999 | Israel | C:NT:P1.5,2 | 1994 | U | G |

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Table 1. (*cont.*)

| Strain number | Country of origin | Phenotype | Year of isolation | *Spe*I* | *Nhe*I* |
|---|---|---|---|---|---|
| 31941 | Finland | C:2a:P1.5 | 1994 | V | AC |
| 76365 | Finland | C:2a:P1.5 | 1995 | V | AC |
| 88170 | Canada | C:2a:P1.2 | 1988 | W | C |
| 91482 | Canada | C:2a:P1.5,2 | 1991 | X | E |
| 92048 | Canada | C:2a:P1.2 | 1992 | Y | B |
| 251033 | England | C:2a:P1.5 | 1995 | Z | M |
| 310396 | England | C:2a:P1.5,2 | 1995 | AA | O |
| 92201 | Canada | C:NT:P1.5,2 | 1992 | AB | AG |
| 91297 | Canada | C:2a:NST | 1991 | AB | C |
| G144/93 | Iceland | C:2a:NST | 1993 | AC | C |
| 11050775 | Iceland | C:2a:NST | 1993 | AD | C |
| 95N477 | Australia | B:2a:P1.2 | 1995 | AE | I |
| 311340 | England | C:2a:NST | 1995 | AF | X |
| 311375 | England | C:2a:NST | 1995 | AG | Q |
| 313112 | England | C:2a:P1.5,2 | 1995 | AH | S |
| 313223 | England | C:2a:P1.5,2 | 1995 | AH | S |
| 901636 | Netherlands | C.2a:P1.5,2 | 1990 | AI | R |
| 87052 | Canada | C:2a:P1.5,2 | 1987 | AJ | A |
| 89036 | Canada | C:2a:P1.5,2 | 1989 | AJ | AF |
| 89486 | Canada | C:2a:NST | 1989 | AK | B |
| 89631 | Canada | C:2a:P1.5,2 | 1989 | AL | A |
| Other ET-37 complex isolates | | | | | |
| 393 | Austria | C:2a:P1.5,2 | 1991 | BA | BG |
| 4575 | Australia | C:2a:P1.5,2 | 1989 | BA | BG |
| 93-01 | Australia | C:2a:P1.5,2 | 1993 | BA | BG |
| 93-45 | Australia | C:2a:P1.5,2 | 1993 | BB | BI |
| M710 | Israel | B:2a:P1.5 | 1991 | BC | BH |
| 24850 | South Africa | W135:2a:P1.5,2 | 1996 | BD | BK |
| PB8 | Norway | C:2a:P1.2 | 1986 | BE | BD |
| 8732 | Chile | B:2a:P1.10 | 1985 | BF | BC |
| 53/94 | Norway | C:NT:P1.5,2 | 1993 | BG | BJ |
| 88/19730 | Scotland | C:2a:P1.2 | 1988 | BH | BE |
| 500 | Italy | C:2a:P1.5,2 | 1984 | BI | BB |
| 88048 | Canada | C:2a:NST | 1988 | BJ | BF |
| M55 | USA | B:2a:NST | 1917 | BK | BA |

* Distinct patterns obtained with *Spe*I or *Nhe*I are designated by a letter or combination of letters.
† NST, non serosubtypable.
‡ NT, non serotypable.

study were to ascertain the genetic and epidemiological relationships of ET-15 to other members of the ET-37 complex and to assess the genetic events associated with the occurrence of this virulent clone. We used a combination of pulsed-field gel electrophoresis (PFGE) and Southern hybridization to construct physical maps of ET-15 strains isolated in different countries and of other strains of ET-37 complex. The results of this analysis demonstrated that, additional to the change in the fumarase locus, large genetic rearrangements have occurred within the ET-37 complex in association with the appearance of ET-15.

## MATERIALS AND METHODS

### Bacterial isolates

The 85 strains studied are listed in Table 1. They were chosen on the basis of their multilocus enzyme genotype among the isolates of *N. meningitidis* referred to the WHO Collaborating Centre for Reference and Research on Meningococci, Oslo. All 85 strains were identified as belonging to the ET-37 complex by allelic variation at 14 enzyme loci [4, 12]; 72 of these 85 strains presented the allele 2 at the fumarase locus, identified in Canada as a marker for the ET-15 variant. Sequencing of a fragment of the

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*fumC* gene in 20 of these strains with allele 2 recently revealed a G to A mutation in position 640, characteristic for ET-15 [13].

The 85 strains of the ET-37 complex examined in this study were recovered from the following countries: Australia, Canada, the Czech Republic, England, Finland, Iceland, Greece, Israel, the Netherlands, Norway, USA, Scotland, Italy, Austria and South Africa. Strains H44/76 and B1940, serogroup B meningococci for which physical maps of the genomes have been previously produced [14, 15], were included as controls.

### Serogrouping, serotyping and serosubtyping

Serogrouping was performed using capsular polysaccharide specific monoclonal antibodies from the National Institute of Biological Standards and Control, Potter's Bar, UK (NIBSC), with a dot–blot method [11, 16] or by slide agglutination with polyclonal antisera (Wellcome, UK). Serotypes and serosubtypes were determined using a dot–blot technique [11, 16] with monoclonal antibodies obtained from NIBSC. The antibodies recognized epitopes on the class 2 or 3 outer membrane protein specific for serotypes: 1, 2a, 2b, 4, 14 and 15, and on the class 1 outer membrane protein specific for serosubtypes: P1.1, P1.2, P1.3, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15 and P1.16.

### PFGE

PFGE was performed as described previously [11]. The agarose plugs of all 85 strains were digested with 20 units of *Spe*I and 30 units of *Nhe*I (New England Biolabs, USA) in 180 $\mu$l of the restriction buffers supplied with the enzymes, at 37 °C for 16 h. In addition, chromosomal DNA of a subset of 8 strains, 4 representing ET-15 (87052, 259/93, 94N369, M894) and 4 other ET-37 complex strains (M55, PB8, 500 and 393) was digested with 30 units of *Bgl*II (New England Biolabs, USA), using the restriction buffer supplied with the enzyme at 37 °C for 16 h.

One-sixth of a plug was loaded onto a 1·2% SeaKem (FMC, USA) agarose gel and PFGE was performed with either a CHEF DRII (Biorad, USA) or the Gene Navigator (Pharmacia Biotech, Sweden) apparatus. The running buffer was 0·5 Tris borate EDTA (0·089 M Tris, 0·089 M borate and 0·045 M EDTA, pH 8·0) with a chiller temperature of 10 °C. Switch times were 5–30 s for *Spe*I and *Nhe*I with the CHEF DRII and 1–40 s with Gene Navigator. The total run time was 22 h for both pieces of equipment. Switch time was 1·0–17·5 s for *Bgl*II with a run time of 24 h. The gradient used was 6 v/cm. Phage lambda concatemers, including a Lambda/*Hin*dIII digestion (New England Biolabs, USA), were used as size standards. The resultant restriction fragments were visualized by ethidium bromide staining (0·5 $\mu$g/ml) and photographed under UV transillumination.

### Data analysis

The PFGE patterns were analysed using the computer programme GelCompar version 4.0 (Applied Maths, Belgium) and a dendrogram was generated using the unweighted pairwise group method of arithmetic averages (UPGMA) clustering technique.

### PCR amplification and probe preparation

The following gene products were prepared for use as probes to analyse the genomic DNA of the 85 strains: *ctrA*, *dhps*, *iga*, *opa*, *penA*, *pilE*, *pilQ*, *porA*, *porB* and *recA*. PCR to amplify these genes were performed on chromosomal DNA from strain H44/76, except for *ctrA* for which another serogroup B strain was used. The following primer pairs were employed: for *ctrA*, 5′-ATGCGGTGGCTGCGGTAGGT-3′ and 5′-CCGGCGAGAACACAAACGACAAG-3′ [17]; *dhps* NM7, 5′-TTGGCAGGCAGGACGGTTTGTA-3′ and NM10, 5′-CCGCCGCCACGCTGCCGTGT-3′ [18]; i*ga* IgA1, 5′-TCAGAAGCAGCATTGGTCAGAGACGATG-3′ and IgA2, 5′-GCCTGTTAACAAGAAACGGTTTTG-3′ [19]; *opa* 5′-GCAAGTGAAGACAGGCGCAGCCCG-3′ and 5′-ATCGATGCTGTGTCTGACGTGTCC-3′ [20]; *penA* penA1, 5′-GCCTGTGTGCCGGAATCG-3′ and penA2, 5′-TCGTGAATTCGGGGATATAACTGCGGACGTC-3′ [21]; *pilE* 5′-CCCTTATCGAGCTGATGATTGTGATTG-3′ and 5′-GCTGGCATGACTTGAATCGTGGCAGG-3′ [22]; *pilQ* 5′-CGAATCGGACGATACCGTGTCCGCCC-3′ and 5′-CGGCAAAGCCCAATGCCGCCAATT-3′ [23]; *porA* 730, 5′-AAACTTACCGCCCTCGTA-3′ and 733, 5′-TTAGAATTTGTGGCGCAAACCGAC-3′ [24]; *porB* 907, 5′-TGAAAAAATCCCTGGCCCTGAC-3′ and 733, 5′-TTAGAATTTGTGGCGCAAACCGAC-3′ [25]; *recA* 5′-TCAGACGACAAAAGCAAAGCCC-3′

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Table 2. *SpeI fragments hybridizing with the various probes in a selection of ET-15 strains (87052, 259/93, 94N369, M894) and other strains of the ET-37 complex (500, PB8, 393, M55). The number indicates to which restriction fragment each probe hybridized (1 being the largest fragment)*

| Strain | *ctrA* | *siaD* | *pilE* | *porB* | *pilQ* | *iga* | *pgm* | *adk* | *abcZ* | *pdhC* | *gdh* | *porA* | *recA* | *fumC* | *dhps* | *penA* | *aroE* | *opa* | *opa* | *opa* | *opa* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 87052 | 1 | 1 | 1 | 8 | 4 | 4 | 5 | 5 | 15 | 1 | 16 | 14 | 7 | 7 | 6 | 3 | 3 | 3 | 6 | 7 | 18 |
| 259/93 | 1 | 1 | 1 | 7 | 3 | 4 | 4 | 4 | 16 | 1 | 14 | 15 | 6 | 6 | 5 | 2 | 2 | 2 | 5 | 6 | 19 |
| 94N369 | 1 | 1 | 1 | 8 | 4 | 4 | 4 | 4 | 14 | 2 | 16 | 13 | 7 | 7 | 6 | 3 | 3 | 3 | 6 | 7 | 17 |
| M894 | 1 | 1 | 1 | 9 | 4 | 4 | 4 | 4 | 16 | 1 | 17 | 15 | 18 | 7 | 6 | 3 | 3 | 3 | 6 | 7 | 19 |
| 500 | 2 | 2 | 2 | 8 | 4 | 5 | 4 | 4 | 15 | 2 | 16 | 13 | 7 | 7 | 6 | 1 | 7 | 1 | 6 | 7 | 20 |
| PB8 | 1 | 1 | 1 | 8 | 4 | 3 | 3 | 3 | 16 | 1 | 17 | 15 | 7 | 7 | 7 | 5 | 9 | 5 | 6 | 7 | 18 |
| 393 | 1 | 1 | 1 | 9 | 2 | 3 | 3 | 3 | 13 | 1 | 14 | 12 | 8 | 8 | 7 | 4 | 7 | 4 | 6 | 7 | 21 |
| M55 | 7 | 14 | 14 | 13 | 20 | 1 | 1 | 1 | 20 | 4 | 15 | 15 | 10 | 9 | 9 | 3 | 3 | 3 | 9 | 10 | 20 |

and 5′-CGGGTCGTTCGCCGTCGGTTTCG-3′ [26]. Reactions were carried out in 50 $\mu$l volumes containing 1 × buffer (1·5 mM $MgCl_2$, 0·1 M Tris HCl pH 8·3, 0·5 M KCl, 0·1% gelatin), 1 unit of Ampli Taq polymerase (Perkin–Elmer, USA), 200 $\mu$M (each) dNTPs, 1 $\mu$l each primer (50 pmoles). For *ctrA* the cycling conditions were 40 cycles of 94 °C 25 s, 58 °C 30 s and 72 °C 30 s. Cycling conditions for the other probes were for 30 cycles, as follows: *dhps*, *penA*, *porA* and *porB*: 94 °C 1 min, 60 °C 1 min, 72 °C 1 min 30 s; *opa*, *pilE* and *recA*: 95 °C 30 s, 68 °C 1 min, 72 °C 1 min 30 s; i*ga*: 95 °C 1 min, 55 °C 1 min, 72 °C 1 min; and *pilQ*: 94 °C 30 s, 65 °C 1 min, 72 °C 30 s.

To provide further confirmation of the relative positions of the DNA fragments, chromosomal DNA of the 8 strains (4 ET-15, 87052, 259/93, 94N369, M894 and 4 other ET-37 complex strains, M55, PB8, 500 and 393) digested with the three restriction enzymes *Spe*I, *Nhe*I, and *Bgl*II was hybridized with 8 additional probes specific for the *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, *pgm* and *siaD* genes (Tables 2–4). PCR products for *abcZ*, *adk*, *aroE*, *fumC gdh*, *pdhC* and *pgm* were made using DNA from an ET-37 strain, and primers and cycling conditions as previously described [27, 28]. The *siaD* probes were prepared by R. Porritt (South Western Area Pathology Service, Liverpool, Australia) using the primers; *siaD*B, 5′-CTCTCACCCTCAACCCAATGTC-3′ and 5′-TGT-CGGCGGAATAGTAAT-3′, and *siaD*C, 5′-GCAC-ATTCAGGCGGGATTAG-3′ and 5′-TCTCTTGT-TGGGCTGTATGGTGTA-3′ [17]. The cycling conditions were 40 cycles of 94 °C 25 s, 55 °C 40 s and 72 °C 60 s. The DNA sources were a systemic serogroup C strain for the *siaD*C PCR and a systemic serogroup B strain for *siaD*B.

The PCR products were visualized on a 0·7% agarose gel using ethidium bromide and purified using TE100 Clontech spin columns (Clontech USA). The purified products were labelled using the ECL Direct System (Amersham, UK) according to the manufacturer's instructions.

### DNA transfer and hybridization

After depurination for 30 min, denaturation for 20 min and neutralization for 20 min, the DNA fragments from the PFGE were transferred for 1 h onto Hybond $N^+$ membranes (Amersham, UK) using the Vacugene XL system (Pharmacia Biotech, Sweden). The blots were then air-dried for 30 min and UV nicked for 5 min.

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Table 3. *Nhe*I *fragments hybridizing with the various probes in a selection of ET-15 strains* (*87052, 259/93, 94N369, M894*) *and other strains of the ET-37 complex* (*500, PB8, 393, M55*). *The number indicates to which restriction fragment each probe hybridized* (*1 being the largest fragment*)

| Strain | *ctrA* | *siaD* | *pilE* | *porB* | *pilQ* | *iga* | *pgm* | *adk* | *abcZ* | *pdhC* | *gdh* | *porA* | *recA* | *fumC* | *dhps* | *penA* | *aroE* | *opa* | *opa* | *opa* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 87052 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 8 | 1 | 4 | 11 | 3 | 3 | 5 | 6 | 3 | 3 | 6 | 11 |
| 259/93 | 10 | 10 | 1 | 1 | 2 | 1 | 1 | 1 | 8 | 4 | 4 | 11 | 3 | 3 | 5 | 6 | 3 | 3 | 6 | 11 |
| 94N369 | 1,12 | 12 | 1 | 1 | 2 | 1 | 1 | 1 | 8 | 4 | 4 | 11 | 3 | 3 | 5 | 6 | 3 | 3 | 6 | 11 |
| M894 | 1 | ND* | 1 | 1 | 3 | 1 | ND | ND | ND | ND | ND | 9 | 4 | ND | 6 | 7 | ND | 4 | 7 | 9 |
| 500 | 8 | ND | 1 | 1 | 3 | 1 | ND | ND | ND | ND | ND | 4 | 4 | ND | 6 | 7 | ND | 4 | 7 | 9 |
| PB8 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 7 | 1 | 4 | 9 | 3 | 3 | 5 | 6 | 2 | 3 | 6 | 9 |
| 393 | 1,8 | 8 | 1 | 1 | 1 | 1 | 1 | 1 | 7 | 4 | 4 | 9 | 3 | 3 | 5 | 6 | 2 | 3 | 6 | 9 |
| M55 | 1 | 1 | 1 | 1 | 2 | 4 | 4 | 4 | 8 | 4 | 11 | 11 | 5 | 5 | 6 | 3 | 1 | 3 | 5 | 11 |

* ND, not determined.

Table 4. *Bgl*II *fragments hybridizing with the various probes in a selection of ET-15 strains* (*87052, 259/93, 94N369, M894*) *and other strains of the ET-37 complex* (*500, PB8, 393, M55*). *The number indicates to which restriction fragment each probe hybridized* (*1 being the largest fragment*)

| Strain | *ctrA* | *siaD* | *pilE* | *porB* | *pilQ* | *iga* | *pgm* | *adk* | *abcZ* | *pdhC* | *gdh* | *porA* | *recA* | *fumC* | *dhps* | *penA* | *aroE* | *opa* | *opa* | *opa* | *opa* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 87052 | 17 | 17 | 1 | 1 | 16 | 14 | 7 | 7 | 12 | 3 | 3 | 16 | 9 | 9 | 8 | 10 | 10 | 4 | 9 | 16 | 21 |
| 259/93 | 15 | 15 | 1 | 1 | 14 | 13 | 6 | 6 | 11 | 4 | 4 | 14 | 8 | 8 | 7 | 9 | 9 | 3 | 8 | 14 | 18 |
| 94N369 | 17 | 17 | 1 | 1 | 20 | 14 | 7 | 7 | 11 | 3 | 3 | 16 | 8 | 8 | 6 | 9 | 9 | 4 | 8 | 16 | 22 |
| M894 | 14 | 14 | 1 | 1 | 13 | 12 | 6 | 6 | 10 | 3 | 3 | 13 | 8 | 8 | 7 | 9 | 9 | 2 | 7 | 13 | 19 |
| 500 | 13 | 13 | 1 | 1 | 2 | 12 | 6 | 6 | 12 | 3 | 3 | 6 | 6 | 6 | 5 | 9 | 9 | 4 | 6 | 17 | 23 |
| PB8 | 16 | 16 | 1 | 1 | 1 | 14 | 8 | 8 | 14 | 3 | 3 | 8 | 8 | 8 | 6 | 10 | 10 | 4 | 8 | 19 | 24 |
| 393 | 15 | 15 | 1 | 1 | 2 | 13 | 8 | 8 | 13 | 4 | 4 | 8 | 8 | 8 | 6 | 9 | 9 | 4 | 8 | 18 | 24 |
| M55 | 1 | 1 | 1 | 1 | 11 | 13 | 6 | 6 | 13 | 3 | 3 | 7 | 7 | 7 | 5 | 9 | 9 | 4 | 7 | 15 | 21 |

Hybridizations using the labelled probes were performed according to the manufacturer's instructions for the ECL Direct System. Briefly, hybridization was carried out at 42 °C and stringency washing was for 10 min at 55 °C followed by 2 × 5 min washes in 0·1 × SSC with 0·4% SDS. The blot was exposed to Hyperfilm (Amersham, UK) for between 10 and 30 min. The film was then developed according to standard development protocols. Undigested lambda was labelled according to the manufacturer's instructions and was used in hybridization reactions in order to estimate the size and location of the fragment to which the probe hybridized on each blot.

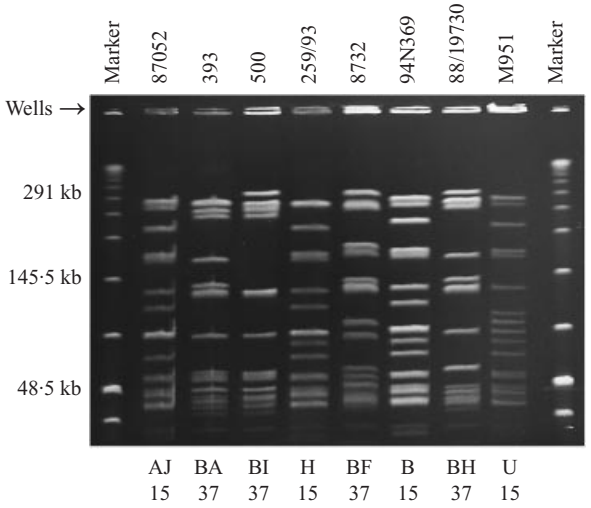


**Fig. 1.** *Spe*I digestion of representative ET-15 and ET-37 complex meningococci. The strain number is indicated at the top of the wells and the *Spe*I pattern (as per Table 1) and ET are shown at the bottom each lane. From left to right: lambda ladder, 87052, 393, 500, 259/93, 8732, 94N369, 88/19730, M951 and lambda ladder.

## RESULTS

### Restriction endonuclease analysis

PFGE of the whole genomic DNA from the 85 strains was performed using the infrequent cutting restriction enzymes *Spe*I and *Nhe*I. These restriction enzymes were chosen because they cleave the meningococcal chromosome into 10–20 fragments and have been used previously for epidemiological studies and to make physical maps of the meningococcal genome [5, 11, 29–31]. *Spe*I digestion of the chromosome of the ET-37 complex strains yielded 15–22 fragments (ranging in size from 339 to 16 kb) and *Nhe*I digestion yielded 12–15 fragments (ranging in size from 436 to 16 kb). The existence, in all digestions, of one or more additional fragments under 20 kb, could not be excluded. The genomic sizes for the 85 strains were estimated from the sum of the fragment sizes for each enzyme and were between 1·9 Mb and 2·3 Mb. This is in accordance with the genome sizes of *N. meningitidis* previously published [14, 15, 32].

There were 38 patterns obtained with *Spe*I (designated by letters A to AL in Table 1) and 34 with *Nhe*I (designated by letters A to AH in Table 1) among the 72 ET-15 isolates recovered from patients in 10 countries between 1987 and 1997. The 13 other isolates of the ET-37 complex exhibited 11 patterns with both *Spe*I and *Nhe*I (designated by letters BA to BK in Table 1). None of these 11 patterns was found among the ET-15 strains. Thus, a total of 49 *Spe*I patterns and 45 *Nhe*I patterns were distinguished among 85 isolates of the ET-37 complex examined (Table 1).

Figure 1 shows the PFGE fingerprints obtained with *Spe*I for 4 ET-15 and 4 other ET-37 complex isolates. The figure illustrates the differences between the fingerprints obtained for the non ET-15/ET-37 complex strains and those of the ET-15 strains. While numerous PFGE patterns were seen among the ET-15 isolates, overall a high proportion of fragments of similar size was obtained.

The relationships among the fingerprints for the 85 strains, based on the *Spe*I restriction analysis, are shown by the dendrogram generated by cluster analysis using the GelCompar software (Fig. 2). All ET-15 strains were included into a large cluster, distinct from the remaining ET-37 complex strains (marked by an asterisk in Fig. 2). The fingerprints of strains recovered within a same country were usually closely related. Some stability over time of the fingerprint was noted. For example, the ET-15 strains recovered from patients in Australia over a 3-year period were very similar although isolated from geographically distant regions.

The ET-15 strains recovered from outbreaks in three regions of the Czech Republic in 1993 were the most homogeneous, with 9 of 10 isolates being indistinguishable both by *Spe*I and *Nhe*I digestions. The 8 Israeli strains showed four distinct *Spe*I and *Nhe*I patterns combinations, while strains from the remaining countries were more heterogeneous. No ET-15 strains isolated in different countries had identical *Spe*I and *Nhe*I pattern combinations. However, *Spe*I pattern P was found in isolates from

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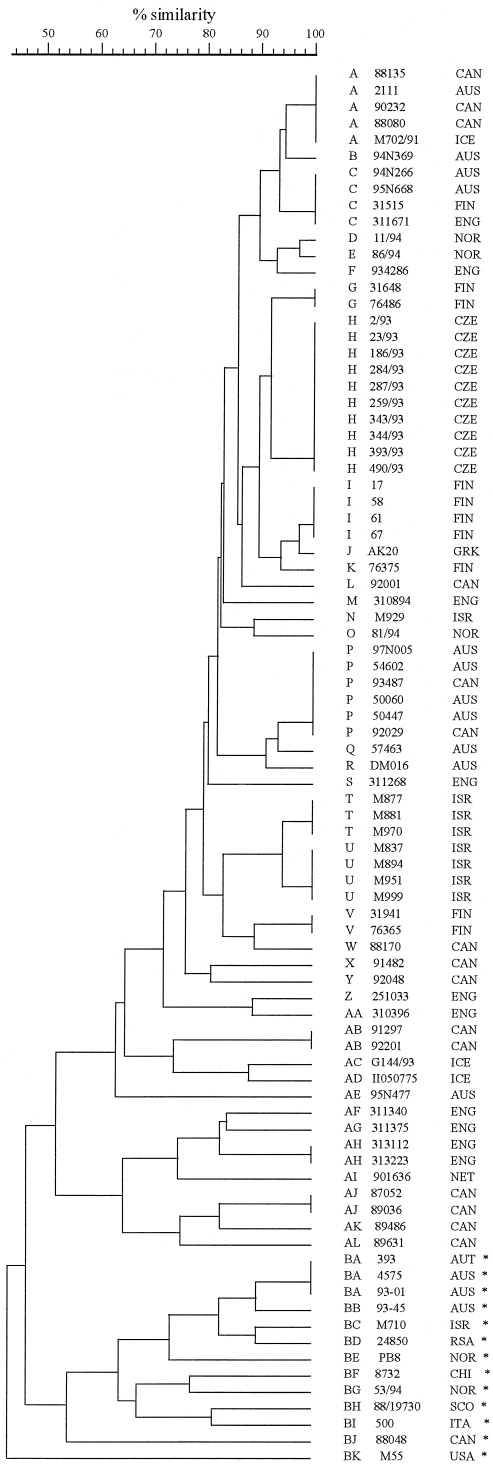


**Fig. 2.** Dendrogram generated by UPGMA cluster analysis of the *Spe*I fingerprints obtained for ET-37 complex isolates (indicated by *) and strains of the ET-15 variant. Fingerprint similarities were generated using the Dice coefficient and a band optimization setting of 0·8. CAN, Canada; AUS, Australia; ICE, Iceland; FIN, Finland; ENG, England; NOR, Norway; CZE, Czech Republic; GRK, Greece; ISR, Israel; NET, Netherlands; AUT, Austria; RSA, Republic of South Africa; CHI, Chile; SCO, Scotland; ITA, Italy; USA, United States of America.

Australia and Canada, *Spe*I pattern A in isolates from Australia, Canada and Iceland, and *Spe*I pattern C was represented by strains from Australia, England and Finland. Two *Nhe*I patterns were seen in strains from different countries: pattern C was found in Canada, Iceland and Norway, and pattern G in Israel and Norway. The only serogroup B ET-15 variant, 95N477, included in this study, had a distinct fingerprint pattern, but many bands were in common with those of the serogroup C, ET-15 fingerprints.

The fingerprints of some of the non-ET-15 strains of the ET-37 complex were similar to one another, even when isolated in different years and geographic regions. Two non ET-15 variant/ET-37 complex strains from Australia and Austria were indistinguishable with both *Spe*I and *Nhe*I.

## Hybridization studies

In all 85 strains, the *opa* probe hybridized to 3 and 4 different fragments of the chromosome digested with *Nhe*I and *Spe*I, respectively. This suggests the presence of 4 copies of *opa* in strains of the ET-37 complex, which is in agreement with the finding of Hobbs and colleagues [33]. All the remaining probes hybridized to a single restriction fragment suggesting single copies of the genes. B1940 and/or H44/76 were run in parallel with the ET-37 complex isolates to estimate the size of the hybridization fragments in conjunction with the ECL labelled phage lambda ladder. The 10 probes used in the hybridization studies of all 85 strains usually hybridized to *Spe*I fragments of similar size for all the ET-15 strains, including the serogroup B isolate, independently of the geographical source of the strains. Briefly, *iga* and *pilQ* hybridized to the same fragment for all but six strains; *pilE* and *ctrA* were usually on the same fragment and *dhps*, *penA* and *recA* were associated each with one *opa* gene. One exception was seen for 7 of the 8 isolates from Israel that showed hybridization of *recA* with a small 16 kb fragment instead of the large 121 kb fragment associated with one *opa* gene, as in all other ET-15 isolates. No such overall association was seen in the genome of the other strains of the ET-37 complex. For example, *pilE* and *ctrA* were associated on the same *Spe*I fragment in only 8 of the 13 strains and *iga* and *pilQ* were on the same fragment in only 2 strains.

There was less clear distinction between the hybridization patterns of the ET-15 strains versus the other strains of the ET-37 complex obtained with the *Nhe*I digestions. The probes for *iga*, *porB* and *pilE*

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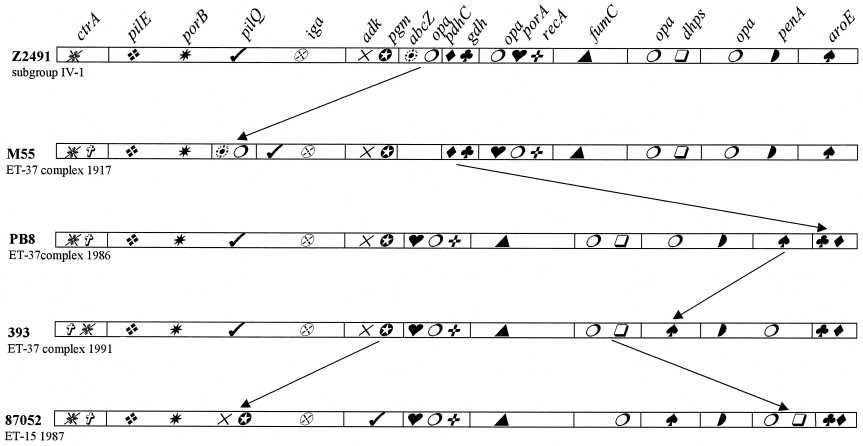


**Fig. 3.** Chromosome organization in ET-15 strain 87052 compared to that of three other ET-37 complex strains, M55, PB8 and 393, and to that of the reference strain Z2491 (constructed using data from Dempsey et al. [32], Maiden et al. [27] and Parkhill et al. [46]. Arrows indicate the reassortments of genetic markers.

hybridized to the same fragment in all but four non ET-15 strains and *penA* and *recA* were associated each with one *opa* gene, respectively, in all but one strain.

To provide confirmation of the gene positions indicated by the hybridization results with the *Spe*I and *Nhe*I digestions, 4 ET-15 strains (87052, 259/93, 94N369 and M894 isolated in Canada, the Czech Republic, Australia and Israel, respectively) and 4 other strains of the ET-37 complex (M55, 393, PB8 and 500 isolated in USA, Austria, Norway and Italy, respectively) were examined using an additional restriction enzyme, *Bgl*II. Restriction of the genomic DNA with *Bgl*II produced 20–30 fragments (ranging in size from 388 to 9 kb). All 8 strains showed a distinct fingerprint pattern, but the patterns obtained for the 4 ET-15 strains were very similar (results not shown).

Hybridization for these strains was performed using a total of 18 probes, the original 10 ones plus the *siaD* probe and the probes for the 7 loci used in the multilocus sequence typing (MLST) scheme [27, 28]. The fragments obtained with the three restriction enzymes were numbered in relation to their size (decreasing order). The results of the hybridization of the probes with the restriction fragments are presented in Tables 2–4, for *Spe*I, *Nhe*I and *Bgl*II, respectively. The number listed under each gene in Tables 2–4 indicates the restriction fragment to which the corresponding probe hybridized.

Four copies of *opa* were found that hybridized to *Bgl*II fragments of approximately the same sizes (ranging from 145 to 16 kb) in all 8 strains. Hybridization with the *porB* and *pilE* probes occurred with the largest *Bgl*II fragment (388 to 169 kb) in all 8 strains (Table 4). In these 8 strains, the *gdh* and *pdhC* probes hybridized to the same 133 kb fragment, the *adk* and *pgm* probes hybridized a 94 kb fragment, the *fumC* and *recA* probes hybridized to the same size fragment as one copy of *opa* (85 kb for the 4 ET-15 strains and 97 kb for the other ET-37 complex strains), and the *penA* and *aroE* probes were located on a 73 kb fragment. The *siaD*B or *siaD*C and *ctrA* probes hybridized to the same fragment in all 8 strains, either with the largest fragment in M55 or with fragments between 35 and 60 kb for the remaining 7 strains.

However, a number of differences were observed between the hybridization patterns of the 4 ET-15 strains and the 4 strains belonging to other clones of the ET-37 complex. In the other ET-37 complex strains, the *porA* probe hybridized to the same fragment 97 kb fragment as the *fumC* and *recA* probes, while, in ET-15, it bound to a 40 kb fragment with another copy of *opa* and, in 3 of the 4 strains, together with *pilQ*. In the non-ET-15 strains, the *pilQ* probe hybridized with the larger fragments of strains 500, PB8 and 393 and with a fragment approximately 70 kb for M55. The *iga* and *abcZ* probes bound to different fragments in the ET-15 strains, but on the same one in all 4 other ET-37 complex strains.

*Nhe*I and *Spe*I hybridizations showed less clear association of the probes. *Nhe*I blots of strains M894 and 500 were not examined using the *siaD* probe and the 7 genes of the MLST scheme. The *pgm* and *adk* genes were associated on the same *Spe*I and *Nhe*I fragments in all strains tested. *porB* and *pilQ* were located on the largest *Nhe*I fragment in all 8 strains, which usually also bound *iga*, *pgm* and *adk*, except for M55. The *fumC* and *recA* probes hybridized to the

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same *Nhe*I fragment for the 6 strains tested and to the same *Spe*I fragment for 87052, 259/93, 94N369, 500, PB8 and 393. The *aroE* probe hybridized to the same *Spe*I fragment as *penA* and one copy of *opa* in the ET-15 strains, but not in the other strains.

### Comparison of the chromosomal maps of ET-37 complex strains

The hybridization results were used to generate partial macrorestriction maps for the strains for which all markers were used in the hybridization experiments. These partial maps were deduced by determining how the fragments from different digests overlapped and then were ordered into a macrorestriction map of the chromosome. The overall chromosomal organizations of the three ET-15 strains were very similar. Minor variations in the probe associations could be explained solely by the occurrence of additional restriction sites from one strain to another. For example, the difference in *Spe*I hybrization patterns with *pilQ*, *iga*, *pgm* and *adk* in 259/93 *vs*. 87052 could result from an additional *Spe*I restriction site in 259/93 between *iga* and *pilQ*, while in 94N369 *vs*. 87052 an additional *Spe*I restriction site between *iga* and *pgm* must be invoked.

Linear representations of the maps of one ET-15 strain (87052) and three other ET-37 complex strains are shown in Figure 3, together with a linear representation of the chromosome of Z2491 [32], where the MLST genes have also been placed [27]. Symbols denote the various markers that have been mapped in this study and arrows have been placed to indicate the movement of genetic markers relative to Z2491 and M55. No detail of the orientation of the gene loci examined or their relative order, when hybridized to the same fragment, was determined. The hybridization results obtained with *abcZ* and one copy of *opa* did not allow placement on the maps of the ET-15 strains, PB8 and 393. However, in strain M55, the *abcZ* probe hybridized to a *Spe*I fragment of the same size (30 kb) as a copy of *opa* and *pilQ*, and in the other ET-37 complex strains the *abcZ* probe hybridized to the same *Bgl*II fragment as the *iga* probe.

M55, an ET-37 complex strain isolated in 1917, had a similar arrangement of the genes in its chromosome as that of Z2491, except for the position of the *abcZ* marker. The two other ET-37 complex strains, PB8 and 393, differed in the position of the *aroE* probe which was located on the same *Spe*I fragment as *dhps* in strain 393. When compared to M55, both PB8 and 393 had *gdh* and *pdhC* located at proximity of the *ctrA* and *siaD* genes instead of being adjacent to the *porA* gene.

The map of the ET-15 strain, 87052, was most similar to that of 393. However, rearrangement involving the *adk*/*pgm* region must be invoked to explain the difference in the relative position of the genes between the two strains. The mapping also indicated rearrangement of the *dhps* gene, which was located after *fumC* in other ET-37 complex meningococci, but found to have translocated to associate with the *gdh*/*pdhC* genes. These rearrangements are indicated by arrows in Fig. 3.

## DISCUSSION

This study was prompted by the detection, in the early 1990s, of a new variant within the ET-37 complex of *N. meningitidis*, followed in the subsequent years by outbreaks of meningococcal disease in many parts of the world caused by similar organisms. The identification of the geographical spread of this new clone was based solely on a single gene marker, the allele 2 at the fumarase locus. To further examine the genetic relationships between ET-15 isolates, as well as their connection to other strains of the ET-37 complex, we undertook a detailed study using a collection of strains from diverse origins.

The ET-37 complex of *N. meningitidis* represents a group of closely related clones that has been associated with local outbreaks of disease and epidemics for a long time and has reached a global distribution [7]. The first isolate of the ET-37 complex identified, M55, recovered in 1917, was included in this study. Considerable genetic diversity has been found overall among strains of meningococci, as a result of high rate of recombination via DNA transformation [34]. However, certain genotypes, such as clones of the ET-37 complex, may be recognized over many decades because their rate of spread is sufficiently high to reduce the effects of recombination.

Using rare cutting restriction endonucleases and PFGE, we confirmed the results of multilocus enzyme electrophoresis and provided further evidence that the outbreaks traced to ET-15 in various parts of the world in the past decade were epidemiologically related, and were the consequence of intercontinental spread of a new, distinct, clone of the ET-37 complex.

Similar PFGE fingerprints after digestion with *Spe*I and *Nhe*I of a few ET-15 isolates from Canada, the Czech Republic and Germany has been shown previously [35, 36]. In addition to demonstrating the overall similarity of the ET-15 strains, our study revealed a greater similarity of the PFGE patterns of the ET-15 strains isolated within individual countries, as well as some pattern stability over time. These data suggest single introductions of the clone within each country and then, spread through the local population. Achtman and co-authors have shown that, in serogroup A meningococci, a reduction of the genetic diversity occurs during epidemic spread, as a consequence of reduction of the bacterial population size during dissemination over geographical areas. This mechanism has been referred to as sequential bottlenecking [37–39] and may result in progressive removal of the genetic variation generated by recombination [39]. This phenomenon may also explain the uniformity observed in PFGE fingerprinting of the ET-15 strains isolated from diverse geographic areas, over a period of 10 years. Similarly, Wang and colleagues observed that, despite the overall diversity of the complex, for example in their class 5 outer membrane proteins, ET-37 complex isolates from any one outbreak were homogeneous [4]. Kriz and colleagues have recently examined the polymorphism of *pil*A and *pil*D genes in a collection of 138 strains assigned to ET-15 recovered in the Czech Republic between 1993 and 1997. They found that variants of the original ET-15 clone started to appear after its introduction in the Czech Republic, affecting the capsule and the *pilA* gene [40].

The acquisition of allele 2 at the fumarase locus, which was recently shown to result from a single base mutation at the *fumC* locus [13], represents a unique genetic event that enabled the identification of this new epidemiologically significant and especially virulent clone. No *Spe*I fingerprints obtained for the 72 ET-15 strains in this study were shared with those of the 13 other ET-37 complex strains and many *Spe*I fragments conserved among the 72 ET-15 strains were not found in *Spe*I fingerprints of the other ET-37 complex strains. Thus, *Spe*I fingerprints may be useful as a means of identifying ET-15 strains from other strains belonging to the ET-37 complex, for laboratories without facilities for multilocus enzyme electrophoresis or DNA sequencing.

Acquisition of a different allele at a housekeeping gene, however, is unlikely to have an effect on virulence or other properties of a bacterial clone resulting in such a spread as that observed with ET-15. In an attempt to identify other genetic events related to the origin of the ET-15 clone, macrorestriction maps of the chromosome of ET-15 and other clones of the ET-37 complex were therefore generated.

We identified several rearrangements that have occurred in the genome of clones of the ET-37 complex. Overall, all non ET-15 strains presented heterogeneous macrorestriction maps using 10 probes. For the 3 non ET-15 strains analysed in detail using 18 markers, at least one genetic rearrangement must have occurred between each pair to explain the sequence of the probes on their chromosome. All ET-15 strains had similar chromosome arrangement, but again to explain the association of probes on the different fragments in the ET-15 clone in comparison to the other ET-37 complex strains, gene shuffling had be invoked.

Chromosome maps of one serogroup A and two serogroup B meningococcal strains, as well as that of the closely related species *N. gonorrhoeae*, have been published [14, 15, 32]. When comparing the gonococcal and the meningococcal genome, Dempsey and colleagues showed that large regions of the chromosome had been rearranged or inverted [32]. The map of strain Z2491 revealed a region of approximately 500 kb where several genes were translocated or inverted in orientation. Similarly, Gäher and colleagues compared the chromosome of the serogroup B meningococcus, B1940, to that of *N. gonorrhoeae* FA1090 and MSN11-N198 and observed that while some degree of conservation was evident, complex rearrangements or several independent transpositions must have occurred between these two genomes [15]. The gene movements that were found to have occurred between these meningococcal and gonococcal strains could only be explained by significant chromosomal rearrangements. Frøholm and colleagues also found evidence of gene rearrangements among 30 strains belonging to the ET-5 complex occurring over a 30-year period of global dissemination [14]. Thus, it appears that genetic rearrangement is a frequently occurring mechanism in meningococci, leading to diversification of clonally related strains. The rearrangements found between the ET-15 variant and other ET-37 complex chromosomes could only have arisen due to extensive crossing-overs as suggested to explain the inversions found between both Z2491 and B1940 and the gonococcal strains [15, 32]. Frequent recombinational

events have been previously evidenced in the ET-37 complex, involving rearrangement among the *opa* genes [33].

One serogroup B ET-15 variant from 1995 was included in this study and this strain had the same chromosomal arrangement as the serogroup C, ET-15 strains. Increased detection of serogroup B ET-15 meningococci has been reported in both Canada and the Czech Republic. Our data support that the appearance of these serogroup B ET-15 strains result from homologous recombination between serogroup B and C capsule genes, followed by selection through the pressure associated with mass vaccination campaigns [41].

Rearrangements occur in bacterial chromosomes by either homologous recombination or site-specific recombination [42]. Some of these rearrangements are quite stable with low reversion rates similar to point mutation rates while others are quite readily reversible. Rearrangements may play an important role in evading the host immune responses [42]. We are unable to surmise the selection advantage that the rearrangements identified here would confer to the ET-15 variant. However, epidemiological data suggest a reduced herd immunity directed against this strain, as the ET-15 variant is associated with a much higher attack rate in the 15–24 years old, cases frequently occur as clusters, and a higher morbidity than previously is seen [1, 2, 6, 8, 11, 43–45].

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