Multiple inverted DNA repeats of Bacteroides fragilis that control polysaccharide antigenic variation are similar to the hin region inverted repeats of Salmonella typhimurium


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Multiple inverted DNA repeats of *Bacteroides fragilis* that control polysaccharide antigenic variation are similar to the hin region inverted repeats of *Salmonella typhimurium*

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The important opportunistic pathogen *Bacteroides fragilis* is a strictly anaerobic Gram-negative bacterium and a member of the normal resident human gastrointestinal microbiota. Our earlier studies indicated that there is considerable within-strain variation in polysaccharide expression, as detected by mAb labelling. Analysis of the genome sequence has revealed multiple invertible DNA regions, designated fragilis invertible (fin) regions, seven of which are upstream of polysaccharide biosynthesis loci and are approximately 226 bp in size. Using orientation-specific PCR primers and sequence analysis with populations enriched for one antigenic type, two of these invertible regions were assigned to heteropolymeric polysaccharides with different sizes of repeating units, as determined by PAGE pattern. The implication of these findings is that inversion of the fin regions switches biosynthesis of these polysaccharides off and on. The invertible regions are bound by inverted repeats of 30 or 32 bp with striking similarity to the *Salmonella typhimurium* H flagellar antigen inversion cross-over (hix) recombination sites of the invertible hin region. It has been demonstrated that a plasmid-encoded Hin invertase homologue (FinB), present in *B. fragilis* NCTC 9343, binds specifically to the invertible regions and the recombination sites have been designated as fragilis inversion cross-over (fix) sites.

INTRODUCTION

*Bacteroides fragilis* is the Gram-negative strictly anaerobic bacterium most frequently isolated from clinical infection, including intra-abdominal, vaginal, pilonidal, perianal and brain abscesses. It is also the most common cause of anaerobic bacteremia, with a potential mortality of up to 19% (Redondo et al., 1995). The major source of these infections is the normal resident colonic microbiota where *Bacteroides* spp. outnumber facultatively anaerobic bacteria such as *Escherichia coli* by a factor of at least $10^2$–$10^3$ (Drasar & Duerden, 1991).

A number of factors may contribute to the virulence of *B. fragilis*; however, extracellular polysaccharides (PSs) are considered to play a key role (Patrick, 2002). Intra-strain phase variation, defined as whether a given characteristic is present or not (Saunders, 1986), is evident with respect to encapsulating surface structures (Babb & Cummins, 1978; Patrick & Reid, 1983). A large capsule (LC) and small capsule (SC) are visible by light microscopy. By electron microscopy, an encapsulating electron-dense layer (EDL) is visible adjacent to the outer membrane on bacteria non-capsulate by light microscopy (Patrick et al., 1986). Expression of the different capsular types is heritable; populations can be enriched by subculture from different interfaces of Percoll step density gradients (Patrick & Reid, 1983). In addition, antigenic variation of individual capsular types can be demonstrated using mAb labelling; variable proportions of bacterial cells within these populations label with individual mAbs (Lutton et al., 1991). The LC and EDL phases have shared epitopes; however, the SC is antigenically different (Reid et al., 1985; Lutton et al., 1991). mAb labelling indicates that there are potentially six different high-molecular-mass polysaccharides (HMMPS)
associated with both the EDL and LC. One additional HMMPs is associated with the SC. Antigenic variation has been observed in clinical isolates from a variety of anatomical sites and different geographical locations, and also in bacteria grown in an in vivo model of peritoneal infection (Patrick et al., 1995a, b). Immunoblotting of these antigenically variable PSs after PAGE reveals patterns characteristic of heteropolymeric PSs with repeating subunits. Ladders with two different sizes of steps have been observed (Lutton et al., 1991), one with a ladder pattern suggesting

Table 1. Oligonucleotide primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tbody>
<tr>
<td>PS1</td>
<td>ccagattatatctcgtgataat</td>
<td>attatcagataaatctcgga</td>
<td>tagaaagagcgcaccccgtga</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>gtaaaaaggtatataagtaa</td>
<td>acgctctcgtacgacacc</td>
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<tr>
<td>PS7</td>
<td>agatatctcatctgataataa</td>
<td>taattctcagtagatattct</td>
<td>agaagcaccacacagcagct</td>
</tr>
</tbody>
</table>

Fig. 1. Alignment of the B. fragilis NCTC 9343 invertible regions of DNA upstream of PS biosynthesis loci 1–7. Note inverted repeats at the right and left ends (arrows) and the B. fragilis consensus promoter sequence (box).
that it may be similar to the O-antigen of smooth Gram-negative bacteria identified by some workers (Poxton & Brown, 1986; Delahoueke et al., 1995), but not others (Lindberg et al., 1990; Comstock et al., 1999).

If an antigenically mixed broth culture is spread onto agar plates and the resulting colonies examined microscopically after immunofluorescence labelling, 90 % or more of the bacteria carry a given epitope in some colonies and 10 % or less in others. The proportion of bacteria expressing a given epitope within a colony is maintained on subculture of the colony into broth culture (Patrick et al., 1999).

The possible mechanism underlying this complex variation was entirely unknown until the B. fragilis whole-genome sequencing project being carried out at the Sanger Wellcome Institute UK (http://www.sanger.ac.uk/Projects/B_fragilis/) revealed the presence of multiple regions of DNA with inverted repeat elements at either end that appear to be present in alternative orientations, within the bulk DNA supplied for sequencing. The DNA was extracted from a defined population, enriched for the EDL/non-capsulate bacteria carry a given epitope in some colonies and 10 % or less in others. The proportion of bacteria expressing a given epitope within a colony is maintained on subculture of the colony into broth culture (Patrick et al., 1999).

DNA sequencing and PCR amplification. Genomic sequence data were produced by the Bacteroides fragilis Sequencing Group at the Wellcome Trust Sanger Institute. These data are available from http://www.sanger.ac.uk/Projects/B_fragilis/. The complete sequence and annotation of the genome will be described elsewhere.

DNA was extracted from populations enriched for one capsular phase and antigenic type, as determined by microscopy, using Qiagen Genomic-tip 100G in accordance with the manufacturer's instructions. DNA concentration was measured spectrophotometrically and diluted accordingly to 1 μg ml−1. Oligonucleotide primer pairs (Table 1) were synthesized by Life Technologies. The PCR mixture used was 45 μl ABgene Reddymix containing 1·5 mM MgCl2, 200 μl each dNTP, 0·025 units Thermoprime Plus DNA Polymerase; 75 mM Tris/HCl (pH 8·6), 20 mM (NH4)2SO4 and 0·01 % (v/v) Tween 20. To the PCR mix described above, 2·5 μl each primer (20 μM) and 2·5 μl template (1 μg ml−1) were added. Primer pairs were either A and C or B and C (Table 1). PCR amplification was performed for 25 cycles of 94°C for 1 min 30 s, 50°C for 1 min 30 s and 72°C for 1 min 30 s using an MJ Research PTC-200 Peltier Thermal Cycler. Samples were then cooled to 4°C and retained at this temperature until collected. After amplification, 5 μl of the amplified product was electrophoresed through a 1 % (w/v) agarose gel in 1 x Tris-acetate-EDTA. PCR product bands were detected by ethidium bromide staining, visualized by UV light (Transilluminator TFX-35M; Gibco-BRL) and photographed using a Kodak DC290 Zoom Digital Camera.

**METHODS**

**Bacterial strains and culture methods.** B. fragilis NCTC 9343 (National Collection of Type Cultures, Colindale Avenue, London, UK), representative of the original strain deposited in the culture collection in 1955 and originally isolated from an abdominal abscess, was used. Identity was confirmed by partial sequencing of 16S rDNA. Bacteria were grown in defined medium (DM) broth or on DM plates (van Tassell & Wilkins, 1978) in an anaerobic cabinet [MACS Anaerobic Workstation; Don Whitley (80 % N2, 10 % CO2, 10 % H2)]. Percoll density gradient enrichment was used to obtain regions that either were non-capsulate by light microscopy and produced an LC detectable by light microscopy, as described previously (Patrick & Larkin, 1993). Populations enriched for individual epitopes were obtained by picking individual colonies from DM agar plates and subculturing in DM broth (Patrick et al., 1999). The proportion of bacteria labelled within a population was monitored by double immunofluorescence labelling with mAbs and polyclonal antisera as detailed previously (Patrick & Larkin, 1993). Images were viewed with a Leitz Ortholux fluorescence microscope, a Nikon DMX1200 digital camera and Lucia GF software. PAGE and immunoblotting were carried out as described previously (Lutton et al., 1991).

**DNA inversion in Bacteroides fragilis**

![Image](http://mic.sgmjournals.org)
fitted to a Kodak EDAS290 Gel Imaging Hood. Gel images were analysed using Kodak ID Image Analysis Software Version 3.5 MAC USB. 16S rDNA primers were included as an internal standard. PCR products were purified using the MinElute PCR Purification Kit (Qiagen), according to the manufacturer’s instructions. The nucleotide sequences of purified PCR products were determined using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and halfBD Terminator Sequencing Reagent (Sigma) using an ABI Prism 3100 Genetic Analyser (Applied Biosystems), according to the manufacturer’s instructions.

**Protein purification and gel retardation.** DNA fragments corresponding to coding sequences of the finA and finB genes, generated by PCR using Pfu polymerase, were cloned into pMAL-c2 (NEB) to produce translational fusions with the maltose-binding protein (MBP) encoded by *malE*. *E. coli* strain DH5α was transformed with

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**Fig. 3.** Immunofluorescence micrographs of mAb QUBF5-enriched population of non-capsulate *B. fragilis* NCTC 9343 labelled with mAb QUBF5 (a), rabbit anti-*B. fragilis* polyclonal antiserum (b; same field of view as in a), mAb QUBF6 (c) and rabbit anti-*B. fragilis* polyclonal antiserum (d; same field of view as in c). The secondary antibodies are anti-mouse FITC and anti-rabbit TRITC conjugates.
plasmids that expressed the invertase-MBP fusions, grown to an OD$_{600}$ of 0.4 and induced with 1 mM IPTG for 1 h. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF), before addition of lysozyme (1 mg ml$^{-1}$) and sonication to produce cell lysates. After centrifugation to remove cell debris, the invertase-MBP fusion proteins were purified by affinity chromatography on amylose resin, according to the manufacturer’s instructions (NEB).

Gel retardation methods were carried out as detailed by Blakely et al. (1993). DNA for gel retardation analysis of the entire invertible fin1 region was generated by PCR with Pfu polymerase, using primers that flanked the fixL and fixR sequences shown in Fig. 1, followed by radiolabelling using [$\gamma$-32P]ATP and T4 polynucleotide kinase. Binding reactions (10 µl) containing approximately 0.03 pmol radiolabelled DNA were performed in 100 mM NaCl, 20 mM Tris/HCl, pH 8, 1 mM EDTA, 10% glycerol and 100 µg poly-dIdC ml$^{-1}$ at 37°C for 10 min before electrophoresis through a pre-run, non-denaturing 4% polyacrylamide gel. Binding gels were dried and then visualized by autoradiography.

RESULTS AND DISCUSSION

An alignment of the invertible regions upstream of the PS biosynthesis loci in $B. fragilis$ is presented in Fig. 1. At either end of these invertible regions are AT-rich sequences containing imperfect dyad symmetry spanning 30–32 bp (inverted repeat regions). The recently proposed consensus $B. fragilis$ promoter regions (Bayley et al., 2000) are conserved and overlap the repeat region by 2 bp.

The inverted repeat regions, designated fragilis inversion cross-over (fixL and fixR) sites (Fig. 2) bear striking similarity to recombination sites found at the ends of some enteric bacterial invertible DNA sequences, for example, the 995 bp hin region within the chromosome of $S. typhimurium$, the invertible regions of phage Mu (Gin system), the $E. coli$ K-12 e14 element (Pin system) and phage P1 (Gin system) (van de Putte & Goosen, 1992; Fig. 2). In $S. typhimurium$, the reversible expression of two antigenically different flagella, H1 and H2 (Lederberg & Lino, 1956) is controlled by inversion of the hin region which carries a promoter and is upstream of the H2 gene, and a repressor for H1. In Mu and P1 phage DNA inversion alters the tail fibres, thereby changing the phage host range (Plasterk et al., 1983; Hiestand-Nauer & Iida, 1983). There is also some similarity with the $Moraxella bovis$ invertible region which is within the Q/I (formerly $\beta$ and $\alpha$) pilus gene (Fulks et al., 1990), the inversion of which mediates pilus phase and antigenic variation. There is no apparent relationship with the 9 bp repeat of the 314 bp invertible region that varies expression of type 1 fimbriae in $E. coli$ which is mediated by the tyrosine family recombinases FimBE (Abraham et al., 1985).

To test the hypothesis that orientation of these regions, with respect to the downstream PS biosynthesis operons, controls variation of different PSs associated with sub-populations that are definable using mAbs, a series of experiments were carried out using ‘orientation-specific’ PCR primers. Primer pairs (Table 1) were designed such that the PCR product would bridge the right end of the invertible region. A product from primer pair A and C would be obtained when the proposed $B. fragilis$ consensus promoter (Bayley et al., 2000) within the invertible region (Fig. 1) was in the correct orientation with respect to the PS biosynthesis operon (‘on’ position); a product from primer pair B and C would be expected when in the opposite orientation (‘off’ position). Populations enriched for reactivity with mAbs QUBF5, 6 and 7 by immunofluorescence microscopy were prepared; a QUBF5-enriched population is illustrated in Fig. 3. Each of these mAbs reacts with antigenically variable epitopes associated with both the LC and non-capsulate/EDL phases. After PAGE and immunoblotting, these mAbs label HMMPS with associated ladder patterns characteristic of heteropolymeric PS with repeating units (Lutton et al., 1991). QUBF7 reacts with PS C (Coyne et al., 2000) which lacks an invertible region upstream of the biosynthesis locus. mAb QUBF5 reacts with an HMMPS with a larger ladder step-size pattern after PAGE and immunoblotting than QUBF6 and 7 (Lutton et al., 1991) (Fig. 4). Primer pairs 1A/C and 5A/C yielded a product with populations enriched for mAbs QUBF6 and 5, respectively (Fig. 5). None of the other A/C primer pairs yielded a similar product for these populations. As the enriched populations contain low

![Fig. 4. Immunoblots of proteinase K extracts from $B. fragilis$ NCTC 9943 after PAGE reacted with mAbs QUBF5 (lane 1), QUBF6 (lane 2) and QUBF7 (lane 3).](http://mic.sgmjournals.org)
proportions of bacteria positive for other epitopes (Fig. 5), PCR product was sometimes obtained in the ‘on’ position from populations enriched for another epitope. For example, where 7–8% of the population enriched for reactivity to mAb QUBF7 also reacted with mAb QUBF6, some PCR product was obtained from primer pairs 1A/C, although this was considerably less than the product from primers 1B/C (Fig. 5). The antigenic variation of the Bf5 HMMPS could explain the conflicting literature concerning the presence or absence of an ‘O-antigen’ in *B. fragilis* (Patrick, 2002).

Sequencing of the PCR products confirmed the sequence of the right end of the invertible region and showed that the inner half-sites of the fixR inverted repeats (i.e. the half-site that ‘flips’ during recombination within the inverting DNA) were in opposite orientations in the ‘on’ and ‘off’ positions and confirmed that site-specific recombination had occurred. This pattern of PCR product was observed for both non-capsulate/EDL and LC phases enriched for the different mAb epitopes. This suggests that the phase change associated with LC formation is not controlled by these loci. A minimum of three separate populations enriched for each phenotype was examined. Comparison of the sequence data indicates that mAbs QUBF5 and 6

```plaintext
1. fixR  TGTGCTATATTAAACGaaCGTTTTTTGAAACA
PSR  ACGAACGTTTTTGAAACA
2. fixR  TGTGTTATAAAACGaaCGTTTTTTGAAACA
PSA  ACGAACGTTTTTGAAACA
3. fixR  TGTTCAATTAAACGaaCGTTTTTTGAAACA
PSD  ACGAACGTTTTTGAAACA
4. fixR  TGTTCAATTAAACGaaCGTTTTTTGAAACA
PSB  ACGAACGTTTTTGAAACA
5. fixR  TGTTCAATTAAACGaaCGTTTTTTGAAACA
PSO  ACGAACGTTTTTGAAACA
6. fixR  TGTTCAATTAAACGaaCGTTTTTTGAAACA
PSP  ACGAACGTTTTTGAAACA
7. fixR  TGTTCAATTAAACGaaCGTTTTTTGAAACA
PSG  ACGAACGTTTTTGAAACA
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**Fig. 5.** PCR products, obtained from *B. fragilis* NCTC 9343 non-capsulate/EDL phase populations enriched for QUBF5, QUBF6 or QUBF7 antigenic phenotype, using primer pairs for PS biosynthesis loci 1 and 5 designed to detect fragilis invertible (fin) regions in the ‘on’ or ‘off’ position with respect to PS expression. Note products in the ‘on’ position for primer pairs for locus 1 and QUBF6-enriched, and locus 5 and QUBF5-enriched (arrows). Lanes 1 and 24, standards starting from the bottom at 100 bp with 100 bp increments. The numbers underneath each lane show the proportion (percentage enriched) of populations labelled with mAbs QUBF5, QUBF6 and QUBF7 as determined by immunofluorescence microscopy.

**Fig. 6.** Alignment of top-strand right-hand of fix1–7 with 19 bp invertible regions (PS A–H) proposed by Krinos et al. (2001 supplementary data Table 1, available on-line). Central dinucleotides of fixR are in lower case.
correspond with PS E and PS D designated by Krinos et al. (2001) who suggested that the invertible repeat associated with the control of PS expression was 19 bp in length and of identical sequence in four of the seven regions (Krinos et al., 2001 supplementary data Table 1, available on-line). The position of the 19 bp repeat relative to the suggested 30 bp repeat is illustrated in Fig. 6. There is a variable gap between the 19 bp repeat element and the consensus promoter (Bayley et al., 2000) sequence situated within the invertible region, whereas the fixL repeat consistently overlaps the consensus promoter (Fig. 1). The asymmetry in the PS1 fix sites would result in the generation of different sequences if the DNA was cut in the 19 bp region rather than the 30 bp palindromic sequence. Examination of the sequences of the PCR product of PS 1 revealed a sequence consistent with the DNA inversion occurring as a result of a double-stranded DNA cut in the centre of the 30 bp (not illustrated). These sequence data were obtained with two replicate enriched populations on two separate occasions.

*S. typhimurium* H flagellar antigen invertase (Hin)-mediated DNA inversion occurs within a topologically defined synapse and proceeds by a concerted pair of double-stranded staggered cuts, at the central dinucleotides of each recombination site, catalysed by nucleophilic attacks mediated by the active site serine from each of the four monomers within the synapse (Heichman et al., 1991). Binding specificity for Hin resides in 4 amino acid residues, invariant amongst the enteric invertases, that interact with base pairs through both the major and minor grooves of the recombination half-site (Feng et al., 1994). Important base pair interactions mediated by helix 3, within the helix–turn–helix structural motif, through the major groove, include Ser174 with A10 and Arg178 with G9. Hin recognition is highly sensitive to alterations at either of these base pairs (Hughes et al., 1992). These bases are highly conserved within the left half-sites of all proposed fix recombination sites, but are more variable in the right half-sites (Fig. 2a). Interactions of Hin with the minor groove of hixL involve Arg140 with A –6, Gly139 with T 5, Lys 187 with T –12 and Asn190 with T –10 and A 10. All these base pairs are conserved within the putative fix recombination sites. This suggested that the fix DNA sequences would interact with recombinases related to the enteric invertase family. Two strong candidates for invertases have been located in *B. fragilis* NCTC 9343. Fragilis invertase (Fin)A is similar to *E. coli* transposon Tn21 resolvase TnpR (30·6 % identity in 196 aa) and FinB is homologous to *S. typhimurium* Hin (46·6 % identity in 191 aa). An alignment of FinA, FinB and Hin amino acid sequences is presented in Fig. 7. In addition to these, there are 21 proteins belonging to the ‘phage integrase’ family, which includes the recombinases xerCD and fimB/E, (Kulasekara & Blomfield, 1999; Burns et al., 2000), two...
Further members of the resolvase family and one protein with weak similarity to the Moraxella pilin inverting gene piv. FinB is encoded on a B. fragilis NCTC 9343 36·5 kbp closed circular plasmid (unpublished).

To investigate the hypothesis that the observed inversions might be mediated by a member of the enteric resolvase/invertase family, we partially purified both FinA and FinB as N-terminal fusions to the MBP. A 263 bp DNA fragment that corresponded to the invertible fin1 region was then used to assess the binding specificity of each invertase to the recombination sites. Fig. 8 demonstrates that increasing concentrations of FinB-MBP gave rise initially to a single protein/DNA complex (complex 1) that was eventually converted to a slower migrating complex (complex 2). Based on the documented attributes of the serine family of site-specific recombinases to either form dimers in solution or bind cooperatively to DNA as dimers (Spaeny-Dekking et al., 1995; Blake et al., 1995), one simple interpretation of the binding pattern (Fig. 8) is that a dimer of FinB-MBP was bound at either fix1L or fix1R to generate complex 1 and that at higher protein concentrations both recombination sites were occupied to give rise to complex 2. The relative amounts of the two bands, under these conditions, suggest that interactions between these complexes are not cooperative. Further analysis of the binding specificities and stoichiometry of FinB present in higher order complexes such as complex 2, however, is required to distinguish between dimer binding or formation of synaptic intermediates. FinB-MBP is also able to bind to a single recombination site derived from the fix5 locus, while the MBP fusion to FinA did not show binding to either invertible fix1 or fix5 region (data not shown).

Together these data show that expression of a significant number of PS biosynthetic operons in B. fragilis is directly regulated by a DNA site-specific recombination inversion that appears to involve a newly identified member of the resolvase/invertase family. Whether the observed level of reversible variation of PS expression reflects the ability of B. fragilis to colonize the hostile environment of the human host as an opportunistic pathogen, as a member of the normal intestinal microbiota or both, remains to be determined. Variation of the surface PSs in B. fragilis undoubtedly occurs during natural infection by B. fragilis. Direct examination of pus drained from abdominal, perianal, ischiorectal, pilonidal, vaginal, Bartholin’s, diverticular, colostomy, groin and neoplasm abscesses as well as blood culture bottles and labelled with six different PS-specific mAbs, revealed diverse labelling patterns. No single PS or group of PSs could be related to infection at one particular site (Patrick et al., 1995b).

The evolutionary origin of the B. fragilis recombination sites remains open to speculation, although it has been suggested that the Hin system in Salmonella arose as a result of integration of a Mu-like phage (van de Putte & Goosen, 1992). The Bacteroidetes diverged early, in evolutionary terms, from other eubacteria, well before the divergence of the Gram-positive bacteria from the phylum Proteobacteria which contains the majority of Gram-negatives such as the enteric bacteria, E. coli and the pseudomonads (Woese, 1987). Given the relatively limited number of other examples of reversible variation in pathogenic bacteria arising by DNA inversion, it may be that the high number of invertible sequences in B. fragilis reflects this early evolutionary divergence. The presence of the invertase gene, which may be responsible for controlling variable expression of multiple chromosomal loci, on a plasmid also raises a number of interesting evolutionary questions. For example, did the plasmid introduce the invertase gene or did it acquire the gene as a mechanism to ensure maintenance?

In conclusion, the putative recombination sites involved in the unprecedented level of DNA inversion observed in B. fragilis show striking similarity to those of the S. typhimurium Hin and related systems and appear to involve a plasmid-borne Hin-like invertase, FinB, which we have demonstrated binds to the invertible regions. We therefore propose that the invertible regions in B. fragilis are designated fin regions and the inverted repeats fixL or fixR.

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**Fig. 8.** Autoradiogram of *in vitro* binding between B. fragilis FinB-MBP and DNA, corresponding to the invertible fix1 region, analysed by gel retardation. Radiolabelled fix1 DNA was incubated with increasing concentrations of FinB-MBP. Lanes: 1, no FinB-MBP; 2–6, increasing concentrations of FinB-MBP.
ACKNOWLEDGEMENTS

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