Loss-of-function mutations in the mtr efflux system of Neisseria gonorrhoeae

Wendy L. Veal, Ansley Yellen, Jacqueline T. Balthazar, Wubin Pan, Brian G. Spratt and William M. Shafer

INTRODUCTION

Following the introduction of penicillin to treat gonorrhoea, strains of Neisseria gonorrhoeae expressing clinically significant levels of penicillin resistance emerged. Gonococcal clinical isolates displaying hypersusceptibility to penicillin and structurally diverse hydrophobic agents (HAS) such as drugs, dyes, detergents and host-derived compounds (fatty acids and bile salts) also arose at nearly the same time as penicillin-resistant strains appeared. In one particular study, conducted in North Carolina in 1978, antibiotic-hypersusceptible strains represented upwards of 15% of all gonococcal clinical isolates (Eisenstein & Sparling, 1978). The antibiotic-hypersusceptibility profiles of these clinical isolates were remarkably similar to a set of laboratory-derived mutants and transformants that contained mutations in different loci termed env (envelope) (Sarubbi et al., 1975). On the basis of recombination studies (Eisenstein & Sparling, 1978; Shafer et al., 1984), these antibiotic-hypersusceptible clinical isolates appeared to contain mutations in one of three different env loci, but most env mutations appeared to map at or near the env-2 locus of the laboratory-derived HA-hypersusceptible strain BR87. Although Env^- mutants and transformant strains were found to be hypersusceptible to structurally diverse HAS, many also contained a phenotypically suppressed mutation, now known to be in the mtrR gene (Hagman et al., 1995), which could confer HA resistance when introduced into wild-type strain FA19 (Maness & Sparling, 1973; Sarubbi et al., 1975; Shafer et al., 1984). The biochemical basis for the Env^- property of such mutants has not been resolved, although earlier studies suggested that such strains had decreased amounts of a 52 kDa outer-membrane protein and decreased cross-linking of their peptidoglycan (Guymon et al., 1978). These biochemical changes in the cell envelope chemistry of Env^- strains were postulated to be responsible for their enhanced permeability to HAS.

The mtr locus in N. gonorrhoeae (Maness & Sparling, 1973) encodes the production of a membrane fusion protein, MtrC, that is responsible for HAS hypersusceptibility. This operon is composed of the mtrR gene, which encodes a transcriptional repressor (MtrR), and a three-gene complex (mtrCDE), which encodes cell envelope proteins (MtrC-MtrD-MtrE) that form an energy-dependent efflux pump. HA-hypersusceptible strains are often isolated from patients, but the genetic basis for such hypersusceptibility was heretofore unknown. The genetic basis of HA hypersusceptibility in laboratory-derived strains BR54 and BR87 was studied to learn if this trait could be linked to mutations in the mtr operon. Mutations in the mtrR gene of these strains that could be phenotypically suppressed by mutations in their mtrC or mtrD genes were identified. Thus, small deletions (4-10 bp) in the mtrC or mtrD genes of strains BR87 and BR54 that would result in the production of truncated efflux pump proteins that serve as a membrane fusion protein (MtrC) or transporter of HAS (MtrD) were found to be responsible for their HA-hypersusceptible property.

Keywords: gonococci, efflux pump, antibiotic hypersusceptibility
Table 1. Properties of HA resistant and susceptible isogenic strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>MIC (μg ml⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ery</td>
</tr>
<tr>
<td>FA19</td>
<td>Wild-type</td>
<td>P. F. Sparling</td>
<td>0 25</td>
</tr>
<tr>
<td>FA140</td>
<td>penA penB mtrR-140</td>
<td>P. F. Sparling</td>
<td>2 00</td>
</tr>
<tr>
<td>BR54</td>
<td>penA penB mtrR-140 mtrD-54 (env-3)†</td>
<td>P. F. Sparling</td>
<td>0 06</td>
</tr>
<tr>
<td>BR87</td>
<td>penA penB mtrR-87 mtrC-87 (env-2)†</td>
<td>P. F. Sparling</td>
<td>0 06</td>
</tr>
<tr>
<td>WV1</td>
<td>As BR54 but mtrD⁺</td>
<td>FA19× BR54</td>
<td>2 00</td>
</tr>
<tr>
<td>WV2</td>
<td>As BR54 but mtrD⁺</td>
<td>Spontaneous mutant</td>
<td></td>
</tr>
<tr>
<td>WV3</td>
<td>As BR54 but mtrD⁺</td>
<td>WV2× BR54</td>
<td>1 00</td>
</tr>
<tr>
<td>AY1</td>
<td>As BR87 but mtrC⁺</td>
<td>FA19× BR87</td>
<td>1 00</td>
</tr>
</tbody>
</table>

* Ery, erythromycin; PenG, penicillin G.
† Former designations (Sarubbi et al., 1975) for the mtrD-54 (env-3) and mtrC-87 (env-2) genes of strains BR54 and BR87, respectively, are in parentheses.
‡ Transformant strain WV1 was obtained using the 964 bp PCR product from the mtrD gene of strain FA19 (Fig. 1), transformant WV3 was obtained using the same 964 bp PCR product from WV2 and transformant AY1 was obtained using the PCR-amplified mtrC gene from strain FA19.

1973; Guymon & Sparling, 1975) has been recently characterized (Pan & Spratt, 1994; Hagman et al., 1995) and consists of the mtrR gene, positioned 250 bp upstream and divergently transcribed from a three-gene (mtrCDE) complex (Fig. 1) encoding cell envelope proteins that form an energy-dependent efflux pump (Lucas et al., 1995). This efflux pump, not altered cell envelope permeability (Guymon & Sparling, 1975), appears to be responsible for the HA-resistant property exhibited by certain strains of gonococci (Pan & Spratt, 1994; Shafer et al., 1995). Moreover, the mtr efflux system is crucial for expression of maximal levels of chromosomally mediated penicillin resistance that results from mutations at unlinked genes (Sparling et al., 1975).

The MtrR protein appears to be a transcriptional repressor and performs a critical role in regulating transcription of the mtrCDE gene complex (Hagman et al., 1995). Thus, mutations within the mtrR-coding region or a single base pair deletion in a 13 bp inverted repeat in its promoter enhance mtrCDE gene expression leading to elevated HA resistance (Hagman et al., 1995). However, overexpression of the mtrCDE gene complex has a physiological consequence since it is known (Eisenstein & Sparling, 1978) that transformant strains bearing mtrR mutations display a slower growth rate than that of the wild-type parental strain. A possible selective advantage in vivo, in the absence of HAs (or penicillin), for strains bearing env mutations is that the reduced growth rate imparted by mutations in mtrR can be reversed to wild-type levels by a co-resident mutation in env-2 or env-3 (Eisenstein & Sparling, 1978).

Based on the deduced amino acid sequences of the MtrC–MtrD–MtrE proteins, it is clear that the mtr efflux pump in gonococci is similar to the MexA–MexB–OprM pump of Pseudomonas aeruginosa and the AcrA/E–AcrB/F–ToIC systems of Escherichia coli (reviewed by Nikaido, 1994, 1996; Paulsen et al., 1996). The gonococcal MtrC protein belongs to the membrane fusion protein (MFP) family (Saier et al., 1994) that links the cytoplasmic-membrane MtrD transporter protein, which belongs (Hagman et al., 1997) to the resistance/nodulation/division protein family (Saier et al., 1994), to the MtrE outer-membrane protein. The MtrE protein probably forms an outer-membrane channel that facilitates export of HAs (Delahay et al., 1997), in a manner proposed for the TolC and OprM outer-membrane proteins of E. coli and P. aeruginosa, respectively (Nikaido, 1996). As the mtrCDE-encoded efflux pump performs a predominant role in determining levels of gonococcal resistance to HAs, including toxic fatty acids and bile salts that bathe certain mucosal surfaces (Morse et al., 1982), we analysed whether the Env⁻ strains studied previously by Sarubbi et al. (1975) contain mutations in their mtrCDE gene complex. We now report that these Env⁻ strains contain small deletions in their mtrC or mtrD genes and that these mutations are responsible for their HA-hypersusceptible trait.

METHODS

Strains of N. gonorrhoeae employed and growth conditions. Strain FA19 and a panel of isogenic transformant strains (Table 1) were kindly provided by P. F. Sparling (University of North Carolina, Chapel Hill, NC, USA). HA hypersusceptibility was defined when the MIC of erythromycin was <0.12 μg ml⁻¹ and that of Triton X-100 (TX-100) was <62 μg ml⁻¹. All strains were propagated on GCB agar (Difco) containing glucose and iron supplements (Shafer et al., 1984) with incubation at 37 °C under 3:8% (v/v) CO₂.

PCR amplification and DNA sequencing. Chromosomal DNA was prepared by the method of McAllister & Stephens (1993) and used in PCR amplification reactions as described pre-
Piliated gonococci were transformed with bulk chromosomal DNA or PCR products prepared from strain FA19. Spontaneous mutants were selected by plating suspensions onto media supplemented with erythromycin, penicillin G, and TX-100. The transformants were purified by agarose gel electrophoresis to exclude transformation due to the input DNA. Erythromycin-resistant transformants of strains BR54 and BR87 were selected using erythromycin at 500 µg ml⁻¹. Transformants were scored for levels of high-level HA resistance to wild-type strain FA19 in the mid-1970s (Sarubbi et al., 1975). In support of this hypothesis, we previously found that strains BR54 and BR87 were postulated to occur at different sites because they recombine frequently, yielding HA-resistant strains. Spontaneous HA-resistant mutants were selected by plating suspensions onto GCB agar containing erythromycin (0-12 or 0-25 µg ml⁻¹) or TX-100 (500 µg ml⁻¹).

**SDS-PAGE and Western blotting.** Whole-cell lysates of gonococcal strains were prepared as described previously (Hagman et al., 1995), while total cell envelope proteins were prepared as described by Clark et al. (1987). Proteins were separated by SDS-PAGE (Laemmli, 1970) and electroblotted to nylon membranes (Hagman et al., 1995). MtrE was detected using an anti-MtrE mouse antiserum (kindly provided by C. Ison, St Mary’s Hospital Medical School, London, UK) and goat antimouse IgG conjugated to alkaline phosphatase.

### Results and Discussion

**Presence of phenotypically suppressed mtrR mutations in HA-hypersusceptible transformant strains**

HA-hypersusceptible strains BR54 (*env-3*) and BR87 (*env-2*) were generated in P. F. Sparling’s laboratory in the mid-1970s (Sarubbi et al., 1975) using donor DNA from HA-hypersusceptible strains FA32 (for BR54) or FA47 (for BR87) to transform HA-resistant strain FA140. The *env-3* and *env-2* mutations in strains BR54 and BR87 were postulated to occur at different sites because they recombine frequently, yielding HA-resistant transformants (Sarubbi et al., 1975; Eisenstein & Sparling, 1978; Shafer et al., 1984). Sarubbi et al. (1975) found that strains BR54 and BR87 could also donate high-level HA resistance to wild-type strain FA19 in these transformation experiments, suggesting that they also contain a phenotypically suppressed mutation in their *mtrR* gene (Pan & Spratt, 1994). In support of this hypothesis, we previously found that the *mtrR* gene of strain BR87 differed from that of strain FA19 by a missense mutation at codon 45 (GGC to GAC), which resulted in the replacement of the glycine residue with an aspartic acid at position 45, which is within the helix-turn-helix region of the MtrR efflux pump.

### Table 2. Oligonucleotides used to amplify *mtrRCDE* genes

<table>
<thead>
<tr>
<th>Gene amplified</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Site*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mtrR</em></td>
<td>KH9#3</td>
<td>5'-GACGACAGTGGCAATGCAACG-3'</td>
<td>47 nt downstream of <em>mtrC</em> start; C strand of <em>mtrR</em></td>
</tr>
<tr>
<td></td>
<td>CEL#1</td>
<td>5'-GACAATGTTGAGTGGCAGATTAGG-3'</td>
<td>141 nt downstream of <em>mtrR</em> stop; NC strand of <em>mtrR</em></td>
</tr>
<tr>
<td><em>mtrC</em></td>
<td>KH9#1</td>
<td>5'-GTGCGAGATACCTGCGAGCACC-3'</td>
<td>174 nt downstream of <em>mtrR</em> start; C strand of <em>mtrC</em></td>
</tr>
<tr>
<td><em>mtrD</em></td>
<td>KH9#4</td>
<td>5'-CATCACTTGTGGCGAGCACC-3'</td>
<td>702 nt downstream of <em>mtrD</em> start; NC strand of <em>mtrC</em></td>
</tr>
<tr>
<td><em>mtrD</em> &amp; <em>mtrE</em></td>
<td>mtrD#1</td>
<td>5'-CGGCACTGAGGCAAATGCAACG-3'</td>
<td>10 nt upstream of <em>mtrC</em> start; C strand of <em>mtrD</em></td>
</tr>
<tr>
<td><em>mtrD</em> &amp; <em>mtrE</em></td>
<td>mtrD#10</td>
<td>5'-AGCATCACTTGGCGAGCACC-3'</td>
<td>120 nt downstream of <em>mtrD</em> start; NC strand of <em>mtrD</em></td>
</tr>
<tr>
<td><em>mtrD</em></td>
<td>mtrD#7</td>
<td>5'-ATATACAGGGGAACCACGCCC-3'</td>
<td>2028 nt downstream of <em>mtrD</em> start; C strand of <em>mtrD</em></td>
</tr>
<tr>
<td><em>mtrE</em></td>
<td>mtrE#2</td>
<td>5'-CCTTTGCATTGTCTGCCTGCAC-3'</td>
<td>43 nt downstream of <em>mtrE</em> start; C strand of <em>mtrE</em></td>
</tr>
<tr>
<td><em>mtrE</em></td>
<td>mtrE#3</td>
<td>5'-CGGTGTTGGGTATCCGCTGCAC-3'</td>
<td>5 nt upstream of <em>mtrE</em> stop; NC strand of <em>mtrE</em></td>
</tr>
</tbody>
</table>

*Sites of annealing of oligonucleotides at nucleotide positions are shown upstream or downstream from translational start or stop codons for *mtrRCDE* genes that are on coding (C) or non-coding (NC) strands.
repressor (Pan & Spratt, 1994). This radical amino acid substitution abrogates binding of the MtrR repressor to its target DNA sequence, the overlapping, divergent promoters used for mtrR and mtrCDE expression (Lucas et al., 1997). Importantly, when introduced into strain FA19 by transformation, this missense mutation provided for increased HA resistance (Hagman et al., 1995).

To determine the nature of the phenotypically suppressed mtrR gene in strain BR54, we sequenced its mtrR gene and that of HA-resistant parental strain FA19. Using oligonucleotide primers previously employed (Table 2; Shafer et al., 1995) to amplify the mtrR gene, we determined that the mtrR gene from strains BR54 and FA19 also contained the missense mutation in codon 45 present in BR87 (Fig. 1) but, unlike BR87, they also contained a single base pair deletion in the 13 bp inverted repeat localized within the mtrR promoter (data not presented). The regions of the mtr operon of strain FA19 capable of transforming strains BR87 and BR54 for HA resistance are shown in the boxed regions on their respective lines. The darkened box within the mtrD gene of strain BR54 shows the region (nucleotide positions 2929-2993) mapped by transformation that contained the 10 bp deletion responsible for HA hypersusceptibility in this strain.

**Strains BR87 and BR54 contain small deletions in their mtrC and mtrD genes, respectively**

Since the HA-susceptibility profiles of strains BR54 and BR87 (Sarubbi et al., 1975 and Table 1) resembled those of mutants of strain FA19 that contained insertionally inactivated mtrCDE genes (Hagman et al., 1995, 1997; Delahay et al., 1997), we hypothesized that the env-2 and enu-3 mutations might reside within the mtrCDE gene complex. To test this hypothesis, the mtrC, mtrD and mtrE genes from wild-type strain FA19 were amplified by PCR and used to transform strains BR54 and BR87 for HA resistance.

With strain BR87 (env-2) as the recipient, only the mtrC-encoding sequence (obtained with oligonucleotide primers KH9#1 and mtrD#2; Table 2) from strain FA19 exhibited transforming activity (Fig. 1). A representative transformant (strain AY1) displayed enhanced resistance to TX-100 and a twofold increase in penicillin resistance. DNA sequencing of the mtrC gene from strains BR87, FA19 and AY1 was undertaken to identify the mutation(s) that might be responsible for HA hypersusceptibility in BR87. The results showed that compared to the mtrC gene from strain FA19 (GenBank accession no. U14993; Hagman et al., 1995), the mtrC gene of strain BR87 contained a 4 bp deletion between nucleotide positions 349 and 352 and single nucleotide changes, but not the 4 bp deletion, were also present in parental strain FA140 (data not presented). The 4 bp deletion represents, on the mtrC-coding strand, the last four nucleotides of a six dinucleotide (GC) repeat (341-GCGCGCGCGCG-352; underline shows deletion in BR87) and generates a new stop codon (Fig. 2). Due to this frameshift, the MtrC protein produced by
Overlapping regions of the mtrD gene from strain FA19 and BR54 responsible for its HA-hypersusceptible property, PCR products for transformation. With these different strains, the transforming activity of the mtrD gene from transformant strain AY1 (Table 1) was determined and the results showed that the 4 bp deletion (Fig. 2) and each of the six point mutations described above had been repaired so as to resemble the wild-type sequence. Since the MtrC lipoprotein is a member of the MFP family (Saier et al., 1994), it is likely that the mutD gene of strains FA19, FA140 and WV1, strain BR54 contained a 10 bp deletion (5'-CGTAACCGGG-3') and nucleotide positions 2736-2745 in the mtrD gene of strain FA19), while WV1 had this deletion repaired (Fig. 3). The 10 bp deletion would result in the production of an MtrD protein that would diverge from the wild-type sequence at amino acid position 912. Moreover, due to the introduction of a new stop codon (TGA; nucleotides 2825-2827 in the mtrD gene of strain FA19), the MtrD protein produced by strain BR54 would contain 938 amino acids (molecular mass of 99,935 Da) compared to the 1067 amino acid MtrD protein produced by strain FA19 (molecular mass of 113,928 Da). Importantly, a topology model for the MtrD protein of strain FA19 has been deduced (Hagman et al., 1997) and it predicts the existence of 12 transmembrane domains, seven cytoplasmic loops and six periplasmic loops. From this model, the MtrD protein produced by strain BR54 would lack the last three C-terminal transmembrane domains as well as two periplasmic and cytoplasmic loops with the last 26 amino acids being located in the cytoplasm (M. Nilles, personal communication).

Isolation of spontaneous HA-resistant mutants

To identify mutations that might reverse the effects of the small deletions in the mtrC and mutD genes in BR87 and BR54, respectively, spontaneous HA-resistant mutants of strains BR54 and BR87 were sought. Erythromycin-resistant mutants of strain BR54 arose at a low frequency (4 x 10^-6) but such mutants of BR87 could not be recovered (frequency < 10^-14) despite repeated efforts. A spontaneous erythromycin-resistant mutant (WV2) of BR54 displayed high-level resistance to TX-100 (MIC > 16,000 μg/ml) but resistance to erythromycin and penicillin was twofold lower than that of FA140 or WV1 (Table 1). The DNA sequence of the mtrD sequence could be narrowed to a 964 bp fragment (obtained with oligonucleotide primers mutD#10 and mutD#7; Table 2) located near the 3'-end of the gene (shown as nucleotide positions 2029-2993 in Fig. 1). A representative transformant (WV1) was as HA resistant as the immediate parental strain of BR54 (strain FA140; Table 1).

A mutation in the mtrD gene from strain BR54 was identified by sequencing the aforementioned PCR product obtained from strains FA19, BR54 and transformant strain WV1. From this analysis it was determined that compared to strains FA19, FA140 and WV1, strain BR54 contained a 10 bp deletion (5'-CGTAACCGGG-3') and nucleotide positions 2736-2745 in the mtrD gene of strain FA19), while WV1 had this deletion repaired (Fig. 3). The 10 bp deletion would result in the production of an MtrD protein that would diverge from the wild-type sequence at amino acid position 912. Moreover, due to the introduction of a new stop codon (TGA; nucleotides 2825-2827 in the mtrD gene of strain FA19), the MtrD protein produced by strain BR54 would contain 938 amino acids (molecular mass of 99,935 Da) compared to the 1067 amino acid MtrD protein produced by strain FA19 (molecular mass of 113,928 Da). Importantly, a topology model for the MtrD protein of strain FA19 has been deduced (Hagman et al., 1997) and it predicts the existence of 12 transmembrane domains, seven cytoplasmic loops and six periplasmic loops. From this model, the MtrD protein produced by strain BR54 would lack the last three C-terminal transmembrane domains as well as two periplasmic and cytoplasmic loops with the last 26 amino acids being located in the cytoplasm (M. Nilles, personal communication).
mtrD gene from strain WV2 that could be amplified by PCR with primers mtrD#10 and mtrD#7 was determined. The results showed that an additional 2 bp had been deleted in the mtrD sequence of strain WV2 (shown in Fig. 3 with asterisks above the BR54 sequence). This 2 bp deletion in strain WV2 would restore the correct reading frame for the mtrD gene but would also result in a loss of four amino acids (Val-Thr-Gly-Arg) prior to returning to the wild-type amino acid sequence (Fig. 3). The Thr-Gly-Arg sequence represents the first three amino acids of the fifth periplasmic loop predicted to impact expression of the mtrD gene and not loss or diminished expression of mtrE.

We also confirmed that HA-hypersusceptible strains BR54 and BR87 contain phenotypically suppressed mutations in their mtrR gene, which is consistent with the earlier hypothesis of Sarubbi et al. (1975). The mutations occur within the mtrR-coding sequence that determines the amino acid composition of the helix-turn-helix domain of MtrR or in a 13 bp inverted repeat sequence that lies between the −10 and −35 regions of the mtrR promoter. When transferred to wild-type strain FA19, any of these mutations are sufficient to increase HA resistance (Hagman et al., 1995; Shafer et al., 1995). We have evaluated two recent HA-hypersusceptible clinical isolates because the original report of Eisenstein & Sparling (1978) suggested that such strains would have both phenotypically suppressed mutations in mtrR and mutations similar to the mtrC mutation in strain BR87. We found that these two recent clinical isolates contained a single missense mutation in their mtrR-coding region that mapped to codon 40 (GCC to ACC), causing a replacement of alanine with threonine in the helix-turn-helix region of the MtrR repressor; this replacement is sufficient to mediate increased HA resistance in gonococci (Shafer et al., 1995). Surprisingly, however, the genetic lesion for HA hypersusceptibility in these strains appears not to map within the mtrCDE complex (data not presented). Accordingly, these recent clinical isolates, as opposed to those studied in the 1970s (Eisenstein & Sparling, 1978), could have mutations that affect alternative efflux pumps or permeability systems or unlinked control systems that modulate mtr efflux activity. Nevertheless, the presence of phenotypically suppressed mtrR mutations in these strains and the presence of an unlinked mutation(s) that results in hypersusceptibility of gonococci to HAs is consistent with the earlier hypothesis (Shafer et al., 1984) that mtrR mutations are first selected when gonococci are introduced into an environment bathed in HAs (such as the rectum). Subsequent secondary mutations that reverse the effect of mtrR mutations (HA resistance and slower growth rate) could arise when gonococci enter other sites that may be hostile for those slower growing gonococci harbouring mtrR mutations. The genetic basis for HA hypersusceptibility in these clinical isolates is now under investigation.

ACKNOWLEDGEMENTS

This work was supported by NIH grant AI-21150 (W. M. S.) and funds from the VA Medical Research Service (W. M. S.). W. M. S. is the recipient of an Associate Career Scientist Award from the VA Medical Research Service. W. V. was supported by NIH training grant T32 AI-07470. Work in the laboratory of B. G. S. was supported by the Medical Research Council and Wellcome Trust. W. P. acknowledges the support from the Sir Run Run Shaw Scholarship Fund.
We thank L. Pucko for help in manuscript preparation, M. Nilles for help in understanding the topology of MtrD, the staff of the Emory University Microchemical Facility for preparing oligonucleotides, the DNA sequencing core facility for automatic DNA sequencing services, and D. Stephens for critical evaluation of the manuscript prior to submission. W. M. S. acknowledges the encouragement of P. F. Sparling for pursuing these studies.

REFERENCES


Shafer, W. M., Balthazar, J. T., Hagman, K. E. & Morse, S. A. (1995). Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of Neisseria gonorrhoeae that are resistant to faecal lipids. Microbiology 141, 907–911.


Received 30 September 1997; accepted 19 November 1997.