Involvement of the gonococcal MtrE protein in the resistance of *Neisseria gonorrhoeae* to toxic hydrophobic agents

R. M. Delahay,¹ B. D. Robertson,¹ J. T. Balthazar,² W. M. Shafer^{2,3} and C. A. Ison¹

Author for correspondence: C. A. Ison. Tel: +44 171 594 3965. Fax: +44 171 262 6299.
e-mail: c.ison@ic.ac.uk

¹ Department of Medical Microbiology, Imperial College School of Medicine at St Mary's, Norfolk Place, London W2 1PG, UK

² Laboratories of Microbial Pathogenesis, Medical Research Service, VA Medical Center (Atlanta), Decatur, GA 30033, USA

³ Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA

Low-level resistance of *Neisseria gonorrhoeae* to toxic hydrophobic agents (HAs), including some antibiotics, is chromosomally mediated via the multiple transferable resistance (*mtr*) efflux system. The gene encoding the 48·3 kDa outer-membrane protein MtrE, which is associated with the *mtr* phenotype, was identified and is homologous to export-associated outer-membrane proteins, including the OprM (formerly OprK) lipoprotein of *Pseudomonas aeruginosa*. Insertional inactivation of the *mtrE* gene in *N. gonorrhoeae* strain FA19 resulted in the loss of the outer-membrane protein, with concomitant hypersusceptibility of the mutant strain to a range of HAs. The properties of this mutant confirmed the role of MtrE in multidrug resistance mediated by an active efflux mechanism. Secondary structure predictions for MtrE indicated a largely hydrophilic protein with a single α -helical transmembrane region. A transposon-like element, similar to that found downstream of the region containing the promoters for *mtrR* and *mtrC* in *Neisseria meningitidis*, was identified 63 bp downstream of the *mtrE* gene.

Keywords: *Neisseria gonorrhoeae*, efflux, hydrophobic agents, antimicrobial resistance, MtrE

INTRODUCTION

The ability of Gram-negative bacteria to resist the toxic action of a variety of structurally diverse hydrophobic agents (HAs), including antimicrobials, appears to be due to synergism between the impermeability of the outer membrane and active efflux mechanisms (Nikaido, 1994). The multiple transferable resistance or *mtr* phenotype in *Neisseria gonorrhoeae* is associated with increased resistance to inhibitory HAs, and recent studies have determined that the *mtr* locus encodes an active efflux mechanism (Hagman *et al.*, 1995; Lucas *et al.*, 1995). The prevalence of *mtr* strains in rectal isolates, compared to the relative HA sensitivity of strains isolated from other sites, has led to the proposal that the resistant phenotype confers a survival advantage on gonococci in environments which contain high concentrations of inhibitory detergent-like agents, including bile salts, steroids and fatty acids (Morse *et al.*, 1982;

Shafer *et al.*, 1995). Molecular studies on the *mtr* locus have shown that the resistant phenotype results from mutations in the coding (Pan & Spratt, 1994) and promoter (Hagman *et al.*, 1995; Hagman & Shafer, 1995) region of one gene in the *mtr* operon, *mtrR*.

mtrR encodes a putative transcriptional repressor (Hagman *et al.*, 1995) and is located upstream and divergent from the single *mtrCDE* transcriptional unit encoding the structural components of the efflux system. By virtue of homology to components of the *acrRAB* and *acrSEF* systems of *Escherichia coli* and the *mexABoprM* system of *Pseudomonas aeruginosa*, which are important in determining resistance to multiple antibiotics (Ma *et al.*, 1993; Poole *et al.*, 1993), it appears that *mtrD* encodes an inner-membrane transporter of the resistance/nodulation/cell division (RND) family (Saier *et al.*, 1994), and that *mtrC* encodes the associated periplasmic-spanning membrane fusion protein. *mtrE* is proposed to encode an outer-membrane protein completing a system likely to translocate HAs across both membranes in a single step (Nikaido, 1994).

We report here the cloning of the *mtrE* gene, and show that the derived MtrE protein is homologous to the

Abbreviation: HA, hydrophobic agent.

The GenBank/EMBL accession number for the *mtrE* nucleotide sequence reported in this paper is X95635.

outer-membrane protein OprM of *P. aeruginosa*, and to members of the outer-membrane factor (OMF) family (Fath & Kolter, 1993), which are associated with single-substrate transporters.

METHODS

Bacterial strains and growth conditions. The isogenic, non- β -lactamase producing *N. gonorrhoeae* strains FA19 (wild-type, HA^S) and FA136 (*penA*, *mtrR*-171, HA^R) were described by Sparling *et al.* (1975). Strain KH15 (*mtrR*-171, HA^R) was constructed by transformation of piliated (P⁺) FA19 with PCR-amplified DNA corresponding to the *mtrR* region of strain FA171 (*mtrR*-171, HA^R) (Hagman *et al.*, 1995). Strains FA136 and KH15 both contain a single base pair deletion in the 13 bp inverted repeat located within the *mtrR* promoter region. Gonococci were routinely grown as P⁺ colonies on GC agar base (Difco) containing 1% (w/v) IsoVitaleX (Becton Dickinson) at 36 °C, under 5% (v/v) CO₂. *E. coli* strains XL1-Blue MRF and SOLR were obtained from Stratagene and grown on LB agar, or in LB broth at 37 °C with shaking. Media contained either 12.5 µg tetracycline ml⁻¹, 50 µg kanamycin ml⁻¹ or 100 µg ampicillin ml⁻¹, as appropriate.

Production of polyclonal antisera against gonococcal outer-membrane proteins. Outer-membrane proteins were isolated from overnight plate cultures of FA136 by Sarkosyl extraction (Guymon *et al.*, 1978). Proteins were separated by SDS-PAGE, transferred to nitrocellulose and stained with Ponceau S. Protein bands of 48–52 kDa were excised, destained and dissolved in 250 µl acetone in the barrel of a glass Luer-lock syringe. Following evaporation of the acetone, the proteins were redissolved in 250 µl PBS (0.137 M sodium chloride, 0.0027 M potassium chloride, 0.015 M potassium dihydrogen phosphate, 0.08 M disodium hydrogen phosphate), then mixed *in situ* with 250 µl Freund's complete adjuvant (Difco). One hundred microlitres of suspension was used to immunize each of three female Balb/c mice intraperitoneally. Subsequent boosts were delivered intraperitoneally in Freund's incomplete adjuvant (Difco) at 2 week intervals. Immune responses were monitored by Western blots. The antiserum was enriched for antibody specific for the 48–52 kDa proteins by mixing 20 excised and blocked [5% (w/v) nonfat dried milk in PBS] nitrocellulose strips carrying the proteins with 10 µl antisera for 3 h at 37 °C. Strips were subsequently washed and absorbed antibody dissociated by the addition of 0.5 ml 100 mM glycine pH 2.5; the supernatant was then immediately neutralized with 25 µl 1 M Tris/HCl pH 8.0. The semi-purified antisera was also absorbed against whole *E. coli* XL1-Blue MRF cells.

Construction and immunoscreening of a lambda ZapII expression library of strain FA136. Chromosomal DNA for library construction was extracted from strain FA136 by the method of Stern *et al.* (1984), partially digested with 2 U *EcoRI* µg⁻¹, and fragments of 3–10 kb isolated on a 5–25% (w/v) sucrose gradient. DNA fragments were ligated to alkaline-phosphatase-treated lambda ZapII/*EcoRI* arms (Stratagene) and packaged into phage heads using Gigapack II Gold packaging extracts (Stratagene) according to the manufacturer's instructions. Both the ratio of recombinants to non-recombinants (45:1), and the titre of the library (1.35 × 10⁷ p.f.u. ml⁻¹) were favourable for immunological screening using the partially affinity-purified antiserum as probe. Positive plaques were purified and maintained as plasmid DNA following *in vivo* excision. All the positive clones analysed had an insert of 6.3 kb.

Cloning and sequencing of *mtrE*. Limited sequence data from

the 5'-terminus of the *mtrE* gene has been reported (Hagman *et al.* 1995). Forward and reverse primers (*mtrE*-F, 5'-CCTTTGCATTGTCTGCCTGCAC-3', and its inverse complement, *mtrE*-R) were designed from this information and used in conjunction with M13 forward and reverse primers, which anneal to the pBluescript vector, to confirm the presence of *mtrE* within the 6.3 kb insert in plasmid pBSGC2. Cycling conditions for PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 3 min; 30 cycles) were optimized for amplification of a 3 kb fragment, based on the assumption that *mtrE* would be of a size similar to *oprM* (Poole *et al.*, 1993). Products were only obtained with primer pairs *mtrE*-F/M13-F and *mtrE*-R/M13-R. Cycle sequencing was performed using kits from Toyobo and Cambridge Biosciences, and primer walking. The sequence was digitized using a digital gel reader and the MacVector program, and sequences were aligned using the AssemblyLIGN program (all IBI).

Homology searches of the GenBank/EMBL and SWISS-PROT databases employed FASTA and TFASTA search programs from the GCG package (Dereux *et al.*, 1984) available from the Human Genome Mapping Project, Cambridge. Representations of secondary structure were generated with GCG and MacVector software, using the statistical algorithms of Chou & Fasman (1978) and Garnier *et al.* (1978). Multiple sequence alignment was achieved with the GCG programs PILEUP and PRETTYBOX. Hydrophobicity plots were derived from the algorithm of Kyte & Doolittle (1982). Further predictions of secondary structure were attempted using the SAPS (statistical analysis of protein sequences) program of Brendel *et al.* (1992) and the PHD (profile network from Heidelberg) neural network, PredictProtein described by Rost & Sander (1993), both available via the World Wide Web.

Insertional inactivation of *mtrE*. The complete *mtrE* gene was subcloned as a 2131 bp *HincII*/*SmaI* fragment from pBSGC2 into pBluescript to create pBSGC2-8. A null mutant, termed pBSGC2-8K, was constructed by replacing an internal 392 bp *StyI* fragment with an 850 bp *aphA*-3 non-polar kanamycin resistance cassette which lacks a promoter and transcriptional terminator (Ménard *et al.*, 1993). This cassette and any downstream genes in the *mtr* operon will be transcribed from the *mtr* promoter. Correct insertion of the cassette was confirmed by sequencing and Southern blotting (data not shown).

Piliated HA^R gonococcal recipient strain KH15 (1 × 10⁸ c.f.u. ml⁻¹) was transformed with 0.5 µg ml⁻¹ *BsaI*-linearized pBSGC2-8K in GCB broth (Difco) containing defined supplements I and II (Shafer *et al.*, 1984), 2 mM MgCl₂ and 0.043% (w/v) sodium bicarbonate at 36 °C for 30 min. One hundred microlitres of the transformation mix was plated on GC agar plates for 5 h at 36 °C under 5% CO₂, then overlaid with top agar containing 50 µg kanamycin ml⁻¹. Incubation was continued for a further 24–48 h. The Kan^R transformant RD1 was isolated and used for MIC determination. Homologous recombination and allelic replacement was confirmed by Southern blotting using the *mtrE* sequence as probe (data not shown); Western blotting experiments demonstrated the loss of a protein of a molecular mass corresponding to MtrE (data not shown).

RESULTS

Isolation and analysis of a clone expressing immunoreactive protein

Polyclonal antiserum was raised against proteins (48–52 kDa) associated with the *mtr* phenotype in the

Table 1. Homology of MtrE with outer-membrane proteins associated with efflux/transport systems

Organism	Outer membrane component	Identity (%) and amino acid overlap*	Accession number†
<i>Pseudomonas aeruginosa</i>	OprM	40.9 in 465 aa overlap	L23839
<i>Burkholderia cepacia</i>	OpcM	31.5 in 467 aa overlap	U38944
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	NodT	27.2 in 459 aa overlap	P24145
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	NodT	27.4 in 464 aa overlap	P15727
<i>Bordetella pertussis</i>	CyaE	24.0 in 459 aa overlap	P11092
<i>Pseudomonas aeruginosa</i>	AprF	20.9 in 398 aa overlap	Q03027
<i>Erwinia chrysanthemi</i>	PrtF	14.9 in 377 aa overlap	P23598
<i>Alcaligenes eutrophus</i>	CzcC	20.3 in 301 aa overlap	P13509
<i>Burkholderia cepacia</i>	FusA	25.8 in 229 aa overlap	P24126
<i>Escherichia coli</i>	TolC	22.6 in 186 aa overlap	P02930

* Identity is expressed as a percentage of the total amino acid overlap.

† Numbers given for *P. aeruginosa* and *B. cepacia* refer to the GenBank/EMBL database, those for all other organisms to the SWISS-PROT database.

HA-resistant gonococcal strain FA136 (*penA*, *mtr-171*). The antibody was affinity-purified and used to screen a lambda ZapII expression library made from the same strain. An immunoreactive clone, pBSGC2, containing a 6.3 kb insert was isolated. pBSGC2 directed the synthesis of a recombinant protein of approximately 50 kDa, which reacted with the polyclonal antibody by immunoblotting (data not shown).

Sequence data from the 5'-terminus of the *mtrE* gene has been reported (Hagman *et al.*, 1995) and was used to design primers for sequencing. This revealed the complete *mtrE* ORF 52 bp downstream of the *mtrD* TAA stop codon. This intergenic region contained a single copy of the gonococcal uptake sequence (Goodman & Scocca, 1988) and a putative Shine-Dalgarno ribosome-binding site. No consensus promoter sequences were identified, but a putative transcriptional terminator consisting of a further copy of the uptake sequence and an inverted repeat was identified contiguous with the *mtrE* stop codon. This strongly suggests that *mtrE* is the last gene in the operon.

Abadi *et al.* (1996) recently analysed the *mtrR* region in the meningococcus. It is 97% identical at the nucleic acid level to the corresponding gonococcal gene, but contains a 157 bp insertion. This insertion is also found 62 bp downstream of the gonococcal *mtrE*, in the form of an inverted repeat. The gonococcal element is 86% identical to the meningococcal unit. These sequences are homologous to the transposon-like elements described by Correia *et al.* (1988), multiple copies of which are known to occur in the genomes of pathogenic *Neisseria* spp.

Homology of MtrE to other export proteins

The *mtrE* gene encodes a 467 amino acid protein of 50.4 kDa with a 21 amino acid signal peptide, which includes a signal peptidase II cleavage site (residues 18–21), indicating post-translational modification of the prolipoprotein (Hayashi & Wu, 1990). The recombinant protein from *E. coli* could be labelled with [³H]palmitate whereas cells containing the non-recombinant vector demonstrated no such labelling of a corresponding protein (data not shown), indicating that MtrE is a lipoprotein.

A search of the SWISS-PROT database identified a number of outer-membrane proteins associated with transport or efflux systems that are similar to MtrE (Table 1). Alignment of these protein sequences (Fig. 1) revealed two consensus motifs, consisting largely of hydrophobic non-polar residues, located between residues 339 to 344, and 421 to 427 of MtrE. They may be represented as follows:

(i) (Leu/Ile/Val) Xxx (Leu/Val/Ile) Pro (Ile/Leu) (Phe/Leu/Tyr)

(ii) Leu Xxx (Leu/Val) Leu Xxx Ala (Gln/Glu) (Arg/Gln/Ser)

In both cases, the first residue listed in brackets is the most frequently encountered, and Xxx represents any residue. Neither motif appears in the TolC sequence. The first motif occurs in the remaining nine of the outer-membrane proteins in Table 1, plus MtrE. The second motif is modified at two residues in the OpcM polypeptide.

Table 2. Susceptibilities of gonococcal strains to a range of inhibitory HAs

Strain	MIC ($\mu\text{g ml}^{-1}$) of:*				
	TX-100	Ery	PA	CaA	ChA
FA19 (wild-type)	100	0.25	200	300	250
KH15 (<i>mtrR</i> -171)†	> 16000	2.0	800	> 500	> 500
RD1 (<i>mtrE</i> :: <i>aphA</i> -3)	2.0	0.06	50	100	100

* TX-100, Triton X-100; Ery, erythromycin; PA, palmitic acid; CaA, capric acid; ChA, cholic acid.

† Strain KH15 contains a single base pair deletion in the 13 bp inverted repeat located in the *mtrR* promoter region.

Characterization of an *mtrE* null mutant

To establish the importance of MtrE in the HA^R phenotype mediated by the *mtrCDE* complex, the *mtrE* gene was inactivated as described in Methods. The effect of *mtrE* inactivation in the Kan^R transformant strain RD1 (*mtrE*::*aphA*-3) was established by the hyper-susceptibility it demonstrated to a panel of HAs (Table 2), including the fatty acids palmitic acid and capric acid, and the bile salt, cholic acid. The loss of an individual efflux protein is apparently sufficient to cause a complete collapse of the *mtr* system, with consequent loss of the resistant phenotype. No significant change in MIC was demonstrated between wild-type, HA^R and *mtrE* mutant strains to the hydrophilic fluoroquinolone ciprofloxacin.

DISCUSSION

We have cloned the gene for the outer-membrane component of the *mtr* efflux system, *mtrE*. It is the last gene in the *mtrRCDE* operon (Fig. 2) and encodes a putative lipoprotein of 48.3 kDa. The hydrophilic nature of the protein, identification of a signal sequence typical of exported proteins, and the homology it demonstrates

to other established outer-membrane proteins, are all compatible with its expected location. However, preliminary secondary structure analysis of the MtrE polypeptide failed to unequivocally identify β -sheet structures which might be expected for a protein associated with the outer-membrane bilayer. In the case of porins, β -sheet structure predominates and the formation of a β -barrel has been suggested, both to determine the ultimate conformation of the protein in the membrane and to facilitate export to its final location (Nikaido & Saier, 1992). Aside from a consistently strong indication of a single transmembrane α -helix (residues 325–347), the membrane conformation of the polypeptide could not be elucidated on the basis of the available predictive algorithms.

MtrE demonstrates homology to a number of outer-membrane proteins associated with transport/efflux systems. These include the OprM and OpcM lipoproteins associated with multidrug-resistance (MDR) efflux systems in *P. aeruginosa* and *Burkholderia cepacia*, respectively (Poole *et al.*, 1993; Burns *et al.*, 1996), and the NodT export proteins of *Rhizobium* spp. (Surin *et al.*, 1990; Economou *et al.*, 1990).

Limited homology has been established with the outer

Fig. 2. Organization of the *mtrCDE* region of *N. gonorrhoeae* (a) and the corresponding region in *Neisseria meningitidis* (b). Transposon-like elements are orientated and labelled according to the nomenclature of Correia *et al.* (1988). The elements are flanked by inverted repeats of a 26 bp sequence, designated left (L) and right (R). The element located downstream of the recently described promoter region of the *mtrR* and *mtrC* genes of *N. meningitidis* (Abadi *et al.*, 1996) is inverted with respect to the *N. gonorrhoeae* sequence, and displaces *mtrC* from its promoter.

membrane transporters of the outer-membrane factor (OMF) family (Fath & Kolter, 1993), which are predominantly associated with the transport of single physiological metabolites. Possibly these homologies reflect the broader substrate specificity of the MDR-associated outer-membrane proteins within a family of proteins likely to adopt similar structures in the outer membrane. The identification of conserved motifs within the polypeptide sequence of these proteins, consisting largely of hydrophobic residues, may be of relevance in this respect. The absence of these signatures within the TolC sequence is intriguing however, since the TolC protein is known to function in association with several export systems in a similar capacity to other OMF proteins (Fath & Kolter, 1993; Fralick & Burns-Keliher, 1994; Wang *et al.*, 1991).

Analogous to a strain carrying an *mtrC* null mutation (Hagman *et al.*, 1995), loss of MtrE by insertional inactivation is sufficient to cause a collapse of the entire efflux system, indicating the requirement for all three structural proteins (MtrC, MtrD and MtrE) in the efflux process. The hypersensitivity to a variety of HAs demonstrated by these mutants argues in favour of an essential role for the efflux system in gonococcal survival, and highlights the importance of this mechanism in addition to the physical permeability barrier presented by the outer membrane. We cannot be sure that membrane disorganization resulting from the loss of MtrE does not impact upon any other membrane systems, or indeed that loss of MtrE does not have more pleiotropic effects similar to those demonstrated by the loss of the *E. coli* TolC lipoprotein (Morona *et al.*, 1983). However, no changes in total cell-envelope proteins were observed in either Coomassie-Blue-stained SDS-PAGE gels or Western blots probed with a polyclonal antiserum raised against whole cells of strain FA136 (data not shown).

The location of a 155 bp transposon-like element 62 bp downstream of the *mtrE* stop codon is notable (Fig. 2). The repeated unit, containing highly conserved 26 bp inverted repeats at either end of the sequence, was originally reported by Correia *et al.* (1988). The repetitive sequence was indigenous to the pathogenic *Neisseria*, occurring in at least 20 locations in the genome. The sequence from *N. meningitidis* recently reported by Abadi *et al.* (1996) contains a similar element downstream of the region containing the promoters for *mtrR* and *mtrC*. This region, spanning *mtrR*, its promoter region and the 5' end of *mtrC*, was otherwise 97.2% identical to the gonococcal sequence. The relevance of these elements has not been determined; however, in the meningococcus it could potentially function in a regulatory capacity by displacing the structural *mtrC* gene from its transcriptional promoter (W. M. Shafer, unpublished observations). The elements are potentially equipped with their own promoters (Correia *et al.*, 1988), and could direct transcription of the structural efflux genes independently, thereby relieving repression due to MtrR. The element associated with the meningococcal *mtrR* is

inverted with respect to the element downstream of *mtrE* in the gonococcus (Fig. 2). It is presently unknown if a similarly inverted sequence is present downstream of the meningococcal *mtrE* gene. Should this be the case, then transcription of the structural genes *mtrCDE* could be further modulated with respect to repression by *mtrR* by recombinational events involving the two elements.

ACKNOWLEDGEMENTS

R.M.D. gratefully acknowledges the support and encouragement of Ivor Brown and Martin Gill. This work was supported by grants from the Joint Standing Research Committee, St Mary's Hospital (C.A.I.) and NIH grant AI-21150 (W.M.S.). B.D.R. is in receipt of a Research Career Development Fellowship from the Wellcome Trust. W.M.S. is the recipient of an Associate Career Scientist Award from the VA Medical Research Service.

REFERENCES

- Abadi, F. J. R., Carter, P. E., Cash, P. & Pennington, T. H. (1996). Rifampin resistance in *Neisseria meningitidis* due to alterations in membrane permeability. *Antimicrob Agents Chemother* **40**, 646–651.
- Brendel, V., Bucher, P., Nourbakhsh, I. R., Blaisdell, E. & Karlin, S. (1992). Methods and algorithms for statistical analysis of protein sequences. *Proc Natl Acad Sci USA* **89**, 2002–2006.
- Burns, J. L., Wadsworth, C. D., Barry, J. J. & Goodall, C. P. (1996). Nucleotide sequence analysis of a gene from *Burkholderia (Pseudomonas) cepacia* encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. *Antimicrob Agents Chemother* **40**, 307–313.
- Chou, P. Y. & Fasman, G. D. (1978). Empirical prediction of protein conformation. *Annu Rev Biochem* **47**, 251–276.
- Correia, F. F., Inouye, S. & Inouye, M. (1988). A family of small repeated elements with some transposon-like properties in the genome of *Neisseria gonorrhoeae*. *J Biol Chem* **263**, 12194–12198.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Economou, A., Hamilton, W. D. O., Johnston, A. W. B. & Downie, J. A. (1990). The *Rhizobium* nodulation gene *nodO* encodes a Ca²⁺-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J* **9**, 349–354.
- Fath, M. J. & Kolter, R. (1993). ABC transporters: bacterial exporters. *Microbiol Rev* **57**, 995–1017.
- Fralick, J. A. & Burns-Keliher, L. L. (1994). Additive effect of *tolC* and *rfa* mutations on the hydrophobic barrier of the outer membrane of *Escherichia coli* K-12. *J Bacteriol* **176**, 6404–6406.
- Garnier, J., Osguthorpe, D. J. & Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol* **120**, 97–120.
- Goodman, S. D. & Socca, J. J. (1988). Identification and arrangement of the DNA sequence recognised in specific transformation of *Neisseria gonorrhoeae*. *Proc Natl Acad Sci USA* **85**, 6982–6986.
- Guymon, L. F., Walstad, D. L. & Sparling, P. F. (1978). Cell envelope alterations in antibiotic-sensitive and -resistant strains of *Neisseria gonorrhoeae*. *J Bacteriol* **136**, 391–401.
- Hagman, K. E. & Shafer, W. M. (1995). Transcriptional control of

- the *mtr* efflux system of *Neisseria gonorrhoeae*. *J Bacteriol* 177, 4162–4165.
- Hagman, K. E., Pan, W., Spratt, B. G., Balthazar, J. T., Judd, R. C. & Shafer, W. S. (1995). Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. *Microbiology* 141, 611–622.
- Hayashi, S. & Wu, H. C. (1990). Lipoproteins in bacteria. *J Bioenerg Biomembr* 22, 451–471.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157, 105–132.
- Lucas, C. E., Hagman, K. E., Levin, J. C., Stein, D. C. & Shafer, W. M. (1995). Importance of lipooligosaccharide structure in determining gonococcal resistance to hydrophobic antimicrobial agents resulting from the *mtr* efflux system. *Mol Microbiol* 16, 1001–1009.
- Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. (1993). Molecular cloning and characterisation of *acrA* and *acrE* genes of *Escherichia coli*. *J Bacteriol* 175, 6299–6313.
- Ménard, R., Sansonetti, P. J. & Parsot, C. (1993). Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* 175, 5899–5906.
- Morona, R., Manning, P. A. & Reeves, P. (1983). Identification and characterization of the TolC protein, an outer membrane protein from *Escherichia coli*. *J Bacteriol* 153, 693–699.
- Morse, S. A., Lysko, P. G., McFarland, L., Knapp, J. S., Sandstrom, E., Critchlow, C. & Holmes, K. K. (1982). Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. *Infect Immun* 37, 432–438.
- Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264, 382–387.
- Nikaido, H. & Saier, M. H., Jr (1992). Transport proteins in bacteria: common themes in their design. *Science* 258, 936–942.
- Pan, W. & Spratt, B. G. (1994). Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. *Mol Microbiol* 11, 769–775.
- Poole, K., Krebs, K., McNally, C. & Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol* 175, 7363–7372.
- Rost, B. & Sander, C. (1993). Prediction of protein secondary structure at better than 70 % accuracy. *J Mol Biol* 232, 584–599.
- Saier, M. H., Jr, Tam, R., Reizer, A. & Reizer, J. (1994). Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol Microbiol* 11, 841–847.
- Shafer, W. M., Guymon, L. F., Lind, I. & Sparling, P. F. (1984). Identification of an envelope mutation (*env-10*) resulting in increased antibiotic susceptibility and pyocin resistance in a clinical isolate of *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 25, 767–769.
- 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.
- Sparling, P. F., Sarubbi, F. A., Jr & Blackman, E. (1975). Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. *J Bacteriol* 124, 740–749.
- Stern, A., Nickel, P., Meyer, T. F. & So, M. (1984). Opacity determinants of *Neisseria gonorrhoeae*: gene expression and chromosomal linkage to the gonococcal pilus gene. *Cell* 37, 447–456.
- Surin, B. P., Watson, J. M., Hamilton, W. D., Economou, A. & Downie, J. A. (1990). Molecular characterisation of the nodulation gene, *nodT*, from two biovars of *Rhizobium leguminosarum*. *Mol Microbiol* 4, 245–252.
- Wang, R. C., Seror, S. J., Blight, M., Pratt, J. M., Broome-Smith, J. K. & Holland, I. B. (1991). Analysis of the membrane organization of an *Escherichia coli* protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins. *J Mol Biol* 217, 441–454.

Received 5 December 1996; revised 20 February 1997; accepted 3 March 1997.