Plasmid stability and antibiotic resistance of *Neisseria gonorrhoea* during glucose-limited continuous culture

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Summary. Clinical isolates of *Neisseria gonorrhoeae* harbouring resistance (R) plasmids of mol. wts 4.4×10^6 (Asian) or 3.2×10^6 (African) were grown in prolonged glucose-limited continuous culture to determine the segregation efficiency of each type of plasmid and their expression of penicillinase activity in the absence of antibiotic selective pressure. One strain contained the African plasmid and cryptic and conjugative plasmids, which were all retained after 96 generations in the chemostat. By contrast, the other strain lost all plasmids after 100 generations. Both strains showed increased sensitivity to a range of antibiotics, particularly to the penicillins. Loss of penicillinase activity at minimal inhibitory concentration decreased was confirmed for both strains by assaying the enzyme spectrophotometrically. Activity decreased with the number of generations and none was detectable at the time of complete plasmid loss. This decrease was apparently due to individual bacteria ceasing to produce enzyme rather than a gradual decline in production by the whole population. The sensitivities to a broad range of antibiotics also generally increased during glucose-limited growth, but one strain became more resistant to clindamycin and the other to tetracycline.

Introduction

Penicillin resistance of *Neisseria gonorrhoeae* is largely attributable to the production of a periplasmic TEM-1 type β lactamase which diffuses across the outer membrane (Bergstrom *et al.*, 1978). Its synthesis is encoded by one of two different resistance (R) plasmids: one of mol. wt $(3.2\text{--}3.4) \times 10^6$ associated with strains originating in Africa, and one of mol. wt $(4.4\text{--}4.7) \times 10^6$ associated with strains of Asian origin (Cannon and Sparling, 1984). Both types share 40–50% of the 3.2×10^6 -mol. wt transposon Tn2 insert and are closely related to penicillin-resistance plasmids found in *Haemophilus* spp. Most gonococci also harbour a cryptic plasmid, mol. wt 2.6×10^6 , of unknown function (Aalen and Gundersen, 1985) and some strains contain a conjugative plasmid, mol. wt 24.5×10^6 , that can mobilise the transfer of the R plasmids (Sox *et al.*, 1978). Retention of plasmids in a population depends on the segregation efficiency as mother cells divide into daughters. This

can be enhanced by the selective pressure of antibiotic treatment. Nevertheless, β -lactamase production persists *in vivo* in the absence of antibiotic therapy and is widely established in gonococci throughout the world.

Plasmid stability has been investigated frequently by continued sub-culture of micro-organisms on agar media or during repeated cycles of batch growth in liquid media. However, Primrose *et al.* (1982) have eloquently argued why the factors influencing the segregation efficiency of plasmids might be elucidated more successfully by growing bacteria in the precise environments imposed during continuous culture. This alternative approach has been concentrated on plasmids of industrial interest—typically, plasmids which are good interspecies vectors and contain cloned foreign prokaryotic or eukaryotic DNA (Harris and Emtage, 1986). The hosts employed are usually well defined laboratory strains such as *Escherichia coli* or *Bacillus subtilis*. By contrast, the segregation efficiency of plasmids of clinical interest harboured by pathogens has been little studied, despite the fact that these plasmids encode for potential virulence determinants such as toxin production, resistance to host defence mechanisms, invasive factors and antibiotic resistance (Elwell and Shi-

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pley, 1980; Dougan and Fairweather, 1985; Hardman and Gowland, 1985).

An important attribute of continuous culture is that it facilitates the study of low-nutrient environments which might be encountered in the host, together with the precise control of other related parameters. It may, therefore, provide an environmentally related model (a microcosm) in which *in vivo* behaviour of bacteria can be simulated (Bull, 1980). We have, therefore, chosen to investigate the stability and expression of the different gonococcal R plasmids during growth in continuous culture in the absence of antibiotic selection, to gain insight into their persistent retention *in vivo*. To facilitate meaningful comparison, the plasmid compositions of the clinical isolates investigated were the relatively common set of mol. wts (10^6) 2.6, 4.5 and 24.5 and the less typical but directly analogous set of mol. wts (10^6) 2.6, 3.2 and 24.5.

Materials and methods

Strains and maintenance

Clinical isolates of penicillin-resistant gonococci were kindly provided by Mr K. A. Witt (CAMR, Porton Down) and were confirmed to harbour the African (strain KW1) or Asian (strain KW2) R plasmids by agarose gel electrophoresis (see later). Bacteria were stored in liquid nitrogen and revived on pre-warmed, gonococcal clear typing agar (GCCT; Swanson, 1978) in a candle jar at 37°C to assess the presence of pili and outer-membrane proteins II. Pilation results in small, domed, highlighted (when viewed with transmitted light) colonies whereas non-piliated cells produce large, flat, non-highlighted colonies (Jephcott *et al.*, 1971). The presence of proteins II creates colonies which are opaque and granular whereas their absence results in transparent colonies (Swanson, 1978). The pilated, transparent variants were isolated for investigation.

Laboratory strains of *Escherichia coli* harbouring plasmids pBR322 or pDS1109 or *E. coli* strain V517 harbouring eight defined plasmid species (Macrina *et al.*, 1978) were kindly supplied by Dr C. Duggleby (CAMR, Porton Down) as freeze-dried isolates and were revived as required.

Media and growth

Gonococci were grown in continuous culture in the defined medium of Manchee *et al.* (1980) with the glucose and cystine concentrations modified to 1 and 0.1 g/L, respectively, to provide glucose-limited growth (Keevil *et al.*, 1986). The pH of the medium was adjusted to 7.0 with 1M HCl and sterilised by passage through a 0.22- μ m filter (Millipore). Purified isolates were grown as lawns on GCCT for 16 h, harvested with a loop and inoculated directly into the fermenter containing fresh medium. The

apparatus comprised a LH Fermentation 500 Series II modular fermenter system (Stoke Poges, Bucks) and was stirred magnetically. The dilution rate was 0.1 h^{-1} (equivalent to a mean generation time of 6.9 h) and excess culture was removed via an overhead weir. The pH was maintained automatically at 7.0 ± 0.05 with 2M NaOH. The dissolved oxygen tension was maintained at 20% air saturation by feedback from an immersed galvanic oxygen electrode (Uniprobe, Cardiff) to the stirrer speed controller. Filtered air was introduced over the surface of the culture at 200 ml/min via the medium input port to sweep aerosol out and prevent grow-back into the medium line. All parts of the fermenter with which the medium and culture would be in contact were made of glass, polypropylene or autoclavable nylon, because the presence of trace elements in stainless steel has been shown to affect the growth of several pathogens (Novotny and Cownley, 1979; Aranha *et al.*, 1982). The culture was, therefore, maintained at 37°C with an external infra-red lamp rather than the usual stainless steel heating element.

Plasmid extraction

Plasmids were extracted from gonococci or from standard strains of *E. coli* containing defined plasmids by a modification of the method of Holmes and Quigley (1981). Briefly, strains were grown as lawns on GCCT agar for 24 h and a small loopful was suspended in 0.1 ml of 50 mM Tris-HCl buffer, pH 8.0, containing sucrose 8% w/v, Triton X-100 5% w/v and 50 mM EDTA (STET buffer). Alternatively, samples were obtained directly from continuous cultures and 1.5 ml pipetted into a 2-ml Eppendorf microcentrifuge tube. This was centrifuged for 1 min at 12 000 rpm in an MSE Microcentaur benchtop centrifuge. The pelleted bacteria were resuspended in 0.1 ml of STET buffer and all samples were treated with 8 μ l of freshly prepared lysozyme solution (10 mg/ml) for 10 min at room temperature. Samples were rapidly boiled for 40 s to precipitate contaminating chromosomal DNA. The cell debris was removed by centrifugation at 12 000 rpm for 10 min and the supernate carefully transferred to a similar centrifuge tube. An equal volume of isopropanol was added to the supernate which was incubated at -20°C to precipitate the DNA. After 1 h the samples were centrifuged at 12 000 rpm for 5 min, the supernate was carefully removed and the DNA pellet resuspended in 70 μ l of ethanol. The DNA was pelleted by centrifugation at 12 000 rpm in the microcentrifuge and the ethanol was carefully removed. The remaining DNA was allowed to dry in air before being dissolved in 5 μ l of 40 mM Tris-acetate buffer, pH 8.0 containing 1 mM disodium EDTA (electrophoresis buffer).

The plasmids were identified by adding 5 volumes of plasmid solution to 1 volume of bromophenol blue 0.25% w/v in sucrose 40% w/v solution and electrophoresing 5 μ l through horizontal agarose (0.8% w/v) gels (10 $\times$ 7 cm) immersed in electrophoresis buffer. The gel was run at 100 mA for 30 min or until the dye had run 3–5 cm

into the gel. The plasmids were visualised by immersing the gels in ethidium bromide 0.5 µg/ml solution in the dark at 4°C for 10 min before viewing under UV light at 300 nm. The gels were photographed with Polaroid Type 667 landpack film and a Kodak 22A Wratten filter.

Antibiotic resistance

Penicillin resistance was monitored initially by serially diluting samples obtained from the chemostat in pre-warmed GCCT medium and culturing on GCCT agar with or without a supplement of penicillin G (Sigma) 30 mg/L, as described previously (Primrose *et al.*, 1982). The difference between the numbers of colony forming units (cfu) growing on triplicate plates of both media indicated the percentage of the population which was penicillin resistant. Differences in growth on the penicillin plates were not consequences of inoculum size because a range of dilutions was investigated. Moreover, continuous cultures were checked regularly for purity by subculturing undiluted samples (up to 10^{10} bacteria/ml) on to GCCT agar in the presence or absence of penicillin. Good growth was evident on penicillin agar at the start of continuous culture experiments but none was detectable with this high concentration of cells after prolonged glucose limitation. This was confirmed by picking 100 colonies from the agar medium lacking penicillin and subculturing on to agar medium containing penicillin. Percentages of survivors identical with those observed by the first method were noted.

Alternatively, continuous culture samples were diluted to 10^5 bacteria/ml in pre-warmed GCCT medium in Sensititre® plates (Gibco) to determine the minimal inhibitory concentration (MIC) for a range of antibiotics. The plates were incubated in a candle jar at 37°C for 24 h and examined for the presence or absence of turbidity, indicating growth. The cut-off point at which no growth was detectable in each row of wells indicated the MIC. No growth occurred in the negative wells on further incubation. Subculture of samples from the turbid wells on GCCT agar for 24 h at 37°C confirmed the presence of viable gonococci which were not contaminated with other species. A decrease in the penicillin MIC was confirmed by absence of growth on GCCT agar containing penicillin at a concentration of only 1 mg/L whereas penicillin-resistant gonococci grew on media containing penicillin 30 mg/L.

Penicillinase activity

β-Lactamase production was assessed by two methods. (1) Colonies grown on agar plates for 24 h at 37°C were flooded with the chromogenic cephalosporin, nitrocefin (Glaxo), at a concentration of 0.5 mg/ml and the proportion that turned red was noted. These proportions were identical to the percentage of the population able to grow on penicillin agar (described earlier). (2) The specific penicillinase activity was measured by incubating 50 µl of the nitrocefin solution with 3 ml of culture (diluted in phosphate-buffered saline, pH 7.0, to 5 µg of cells (dry wt/ml) at room temperature and following the rate of

change in absorbance. The millimolar extinction coefficient for nitrocefin at 486 nm was 21.1 (O'Callaghan *et al.*, 1972).

Results

Plasmid content

The extraction procedure of Holmes and Quigley (1981) gave good recoveries of plasmids from the standard *E. coli* strains and from clinical isolates of *N. gonorrhoeae* grown on agar or in continuous culture. Comparison with the *E. coli* plasmids indicated that the penicillin-resistant strain of *N. gonorrhoeae*, KW1, harboured an African-type R plasmid of mol. wt 3.5×10^6 , together with the cryptic plasmid of mol. wt 2.6×10^6 and a high-mol. wt plasmid of approximately 24.5×10^6 which was probably the conjugative plasmid (data not shown). By contrast, penicillin-resistant strain KW2 harboured an Asian-type R plasmid of mol. wt 4.8×10^6 , together with the cryptic plasmid of mol. wt 2.6×10^6 and the conjugative plasmid of mol. wt 24.5×10^6 . Other penicillin-sensitive clinical isolates tested lacked the R plasmids. However, the interpretation of the number of plasmids present in different strains of bacteria must always be regarded with some caution. The presence of multiple plasmid bands can be confusing because, depending on the extraction procedure, a single plasmid might occur in the open circular or closed circular forms; the latter migrates the faster. This possibility was investigated by electrophoresing the plasmids in one dimension and irradiating them with short wavelength light in the presence of ethidium bromide, before electrophoresing in the second dimension (Hintermann *et al.*, 1981). This procedure converts all the closed circular material to the open circular form so that if any bands co-migrate in the second dimension they are probably the same plasmid with different original configurations. No evidence of co-migration was obtained for any of the major gonococcal plasmid bands resolved in this study.

Segregation of plasmids in continuous culture

Continuous cultures of strain KW1 retained the African, cryptic and conjugative plasmids during prolonged glucose limitation. These plasmids were still detectable in gonococci after 96 generations. In contrast, strain KW2 apparently lost its conjugative plasmid after only 30 generations, although the Asian and cryptic plasmids were still detectable at that stage. However, no plasmids were detected after 100 generations.

Antibiotic sensitivity

Both gonococcal strains were initially able to grow on agar containing penicillin 30 mg/L. However, they became progressively incapable of growth on medium supplemented with penicillin G at concentrations of either 1 or 30 mg/L after prolonged continuous culture with glucose limitation. This apparent increase in sensitivity to penicillin was confirmed by the Sensititre micro-dilution procedure for MIC determinations. The sensitivities of both strains to a range of antibiotics increased, particularly to the family of penicillins (table). The least inhibitory of these was oxacillin. Cephalosporin sensitivity also increased, and cefoxitin remained the most inhibitory of this class of antibiotics. Interestingly, whilst sensitivities to a broad range of antibiotics generally increased, strain KW1 became more resistant to clindamycin whereas strain KW2 became more resistant to tetracycline and the MIC for chloramphenicol remained unchanged.

Penicillinase production

Spectrophotometric assay showed that penicillinase activity of continuous cultures decreased slowly during the first 80 generations (fig. 1). Thereafter, the activity decreased rapidly until none was detectable by 100 generations, the approximate time of complete plasmid loss.

Gonococci recovered on agar media were exposed to an overlay of nitrocefin to determine the proportion of colonies expressing penicillinase activity. The results indicated that the decreased penicillinase production by cultures was due to individual bacteria ceasing production (fig. 2). At the commencement of glucose-limited continuous culture, all the bacteria expressed penicillinase activity. However, the numbers expressing penicillinase decreased gradually for approximately 70 generations, after which they declined sharply. This was particularly so for strain KW2 harbouring the Asian R plasmid. The ability of individual colonies to produce penicillinase was reflected in a similar proportion of the populations able to grow on penicillin agar at various times after recovery from prolonged glucose-limited continuous culture. No recovered gonococcal colonies reacted with the nitrocefin by 100 generations.

Discussion

Although isolates of *N. gonorrhoeae* were found to be resistant to penicillin as early as 1943 (Parker,

Table. Variations in the antibiotic sensitivity of *N. gonorrhoeae* harbouring the African or Asian R plasmids during prolonged glucose-limited growth in continuous culture

Antibiotic	MIC (mg/L) after 45, 110 and 140 generations of strains harbouring the given plasmid			
	African		Asian	
	45	110	45	140
Penicillin	32	0.5	32	0.5
Carbenicillin	32	0.5	128	0.5
Cephalexin	8	4	8	4
Cefoxitin	4	2	4	1
Amikacin			4	1
Gentamicin			1	0.25
Erythromycin	1	1	0.5	0.25
Tetracycline			0.25	4
Chloramphenicol	8	4	4	4
Clindamycin	0.5	2	2	0.125
Sulphamethoxazole			64	16

1946), it was not until 1976 that penicillinase production attributable to the presence of R plasmids was observed (Ashford, 1976; Percival *et al.*, 1976). Their incidence in the population has increased markedly since that time, even in areas where penicillin therapy is little used. During the 1976 outbreak in England, three women who denied further intercourse were found to harbour penicillinase-producing gonococci in the cervix 3–5 months after first isolation (Arya *et al.*, 1984). In another woman, penicillinase-producing gonococci were isolated after she had given birth and she claimed not to have had intercourse for 8 months. This suggests that plasmids expressing penicillinase are more stable *in vivo* than was suggested by *in vitro* studies in the absence of selective pressure exerted by penicillin (Roberts *et al.*, 1977; Sparling, 1979).

The failure to contain the spread of penicillinase-producing gonococci has raised the possibility that non-pathogenic, indigenous bacterial species could serve as alternative reservoirs for the R plasmids. However, the host range for conjugation is narrow, even within the genus *Neisseria*, and the conjugal plasmid is not stably maintained (Genco *et al.*, 1984). By contrast, the results presented here suggest that the segregation efficiency of plasmids and their retention in the population might instead depend on individual strains and their growth environment. For example, in the Asian strain grown in continu-

Fig. 1. Penicillinase activity of *N. gonorrhoeae* strains KW1 (○) and KW2 (●) during prolonged glucose-limited continuous culture. Samples of culture were obtained directly from the chemostat and assayed spectrophotometrically for the ability to hydrolyse nitrocefin.

Fig. 2. Percentage of *N. gonorrhoeae* KW1 (○) and KW2 (●) colonies producing penicillinase on agar medium flooded with nitrocefin solution after prolonged glucose-limited continuous culture.

ous culture the presence of the conjugative plasmid is not sufficient to stabilise its own retention, let alone that of the R plasmid. Not only is the conjugative plasmid lost rapidly but also many more generations of growth are required before complete loss of the Asian plasmid occurs. Of note also is the loss of the cryptic plasmid. This is present in almost all gonococci and, therefore, was formerly believed to be efficiently segregated (Cannon and Sparling, 1984). However, increasing numbers of clinical isolates are now being found to lack this plasmid (C.A. Ison, personal communication) casting some doubt on its stable maintenance within a population and on the importance of its function to the cell. Glucose limitation is only one of several nutrient limitations which gonococci might experience *in vivo* (Keevil *et al.*, 1986). Other limitations which are currently being investigated might actually encourage the persistence of gonococcal plasmids, possibly explaining the apparent ubiquity of the enigmatic cryptic plasmid in nature.

Our chemostat data might also help explain the relative proportions of Asian and African strains found in an epidemiological study of penicillinase-producing gonococci in the Netherlands (van Klingeren *et al.*, 1983). The incidence of Asian strains was consistently low, irrespective of the presence or absence of the conjugative plasmid. Glucose-limited environments might be encountered frequently by gonococci growing *in vivo*, possibly enhancing the persistence of the African plasmid but not of the Asian plasmid. Interestingly, however, whilst all the plasmids of the African strain KW2 were retained during prolonged glucose-limited growth, penicillin resistance was lost. This might suggest that either the penicillinase gene was not expressed correctly or that the enzyme was unable to reach the target antibiotic molecules, perhaps due to changes in the cell envelope

preventing secretion (Cannon and Sparling, 1984). The latter possibility seems unlikely because the transport across the cell envelope of the majority of the antibiotics used for the MIC studies was apparently unimpaired. Interestingly, however, the MIC for clindamycin increased 4-fold for the African strain whilst the MIC for tetracycline increased 16-fold for the Asian strain. This increase in tetracycline resistance has also been found for the Asian strains *in vivo* (van Klingeren *et al.*, 1983). The most common mechanism of such resistance involves chromosomal mutations affecting ribosomal function. Clindamycin inhibits peptide-bond formation by binding to 50S ribosomal subunits whereas tetracycline inhibits binding of aminoacyl-tRNA to the A site of the 30S ribosome (Pestka, 1971). Thus, mutations to the relevant ribosomal proteins prevent the binding of the antibiotics. It appears, therefore, that prolonged continuous culture affects the synthesis of ribosomal proteins, even in the absence of strong antibiotic selective pressure which would inhibit their activity. If glucose-limited continuous culture is indeed analogous to potential sites of infection for *N. gonorrhoeae*, the chromosomal mutations observed here might help account for the recent spontaneous appearance of several spectinomycin-resistant gonococci around the world which must have modified 30S ribosomal proteins (Cannon and Sparling, 1984). Thus, the ribosomal mutation hypothesis might account for the very occasional appearance of induced antibiotic resistance in a bacterial population, particularly if the recommended antibiotic therapy in current use enriches for this trait whilst killing the originally dominant sensitive population. Future work will further investigate the effects of environmental stress on the selection of antibiotic-resistant gonococci.

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